Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Paraclinical Sciences

Philosophiae Doctor (PhD)
Thesis 2022:66

Molecular studies of *Yersinia ruckeri* in Norwegian aquaculture

Molekylære studier av *Yersinia ruckeri* i norsk akvakultur

Andreas Riborg

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“There ain’t nothin’ to it, but to do it”

- Ronnie Coleman (athlete, 1964)

Innhold

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Larsnes, August 2022
Andreas Riborg

Abbreviations and acronyms

BA - Blood agar

BIG - Bacterial immunoglobulin

BLAST - Basic Local Alignment Search Tool

BRIG - BLAST ring image generator

BT – Biotype

CC - Clonal complex

CDS - Coding sequence

CIN - Yersinia Selective Agar

Ct - Cycle threshold

ddPCR - digital droplet PCR

DNA - Deoxyribonucleic acid

ERM - Enteric redmouth disease

IP - Intraperitoneal

LAMP - Loop-Mediated Isothermal Amplification

LD_{50} - Dosage lethal to 50% of the population

LPS - Lipopolysaccharide

MALDI-TOF - Matrix-assisted laser desorption ionization time of flight

MGB - Minor groove binder

MLVA - Multiple Loci VNTR Analysis

OMP - Outer membrane protein

ONT - Oxford Nanopore Technologies

PacBio - Pacific Biosciences

PCR - Polymerase chain reaction

Pers. com. – Personal communication

qPCR - Quantitative PCR

RAS - Recirculating aquaculture systems

RNA - Ribonucleic acid

SDS - Sodium dodecyl sulphate

Tn - Transposon

VNTR - Variable Number of Tandem Repeats

WGS - Whole genome sequencing

XLD - Xylose Lysine Deoxycholate Agar

Summary

Yersinia ruckeri is the causative agent of yersinosis in fish, a haemorrhagic septicaemia occurring predominantly in farmed species of salmonids. In Norway, yersiniosis has been regarded as a regionally limited disease of minor concern, associated with sustained low levels of mortality and occasional acute outbreaks in Atlantic salmon hatcheries and smolts newly transferred to sea. This situation has changed in recent years, with an increasing occurrence of acute outbreaks also affecting quite large fish at sea with high mortalities. The situation has now been ameliorated to a significant degree by introduction and broad adoption of yersiniosis vaccines administered by injection. Acute yersiniosis in Norwegian aquaculture is associated with a distinct genetic lineage known as *Y. ruckeri* CC1 which is exclusive to Norway. In the work presented here, qPCR assays specific to *Y. ruckeri* and *Y. ruckeri* CC1 respectively were developed for screening purposes. While the virulent CC1 was identified in acute outbreaks of yersiniosis, samples from the environment were found to be frequently positive for other, putatively avirulent strains *Y. ruckeri*. Shedding of virulent *Y. ruckeri* from subclinically infected fish was found to occur during thermal delousing in the field. This phenomenon was then replicated and confirmed experimentally. Comparative genomic analysis of virulent and avirulent *Y. ruckeri* enabled identification of accessory genetic determinants associated with virulence, one of which, the inverse-autotransporter putative invasin *yrIlm*, correlated fully with the virulent phenotype. Virulent isolates from Norway display duplication of this gene over time, with a single copy in isolates from the 1980s and contemporary isolates carrying two or three copies. The emergence of non-motile strains of *Y. ruckeri* has been associated with vaccine failure and virulence internationally. Only seven non-motile isolates were identified amongst 263 Norwegian isolates collected between 1985 and 2019. Three belong to the currently dominating virulent clone and four from a different virulent lineage which has not been identified since the early 1990s. Thus, while loss of motility was revealed in the dominant virulent lineage in Norway, the low frequency identified indicates that the escalating yersiniosis situation in recent years cannot be associated with an emergence of the non-motile phenotype. Rather, dissemination of the currently dominating virulent clone, and genetic changes in this clone, i.e. *yrIlm* duplication, and likely increasing stresses in the lifecycle of farmed fish seem to be the main contributing factors.

Summary in Norwegian

Yersinia ruckeri forårsaker yersinose hos fisk, en hemorragisk septikemi som opptrer primært i laksefisk i oppdrett. Sykdommen ble påvist i Norge for første gang i 1985 og har siden vært assosiert med kroniske tilstander og tidvis akutte utbrudd i klekkerier for Atlantisk laks, men ansett som et regionalt problem av mindre betydning for norsk akvakultur som helhet. Dette har dog endret seg i senere år med stadig økende forekomst av akutte utbrudd langs norskekysten fra omkring 2010 og fremover, som også har rammet stor fisk i sjøfasen i økende grad. Omkring 2017 ble det tatt i bruk injeksjonsvaksiner mot yersiniose langs store deler av kysten for å forhindre ytterligere eskalering. Akutt yersiniose i norsk akvakultur er assosiert med en bestemt genetisk variant, kjent som *Y. ruckeri* CC1, som forekommer kun i Norge. I arbeidet som presenteres her ble det utviklet qPCR assay for spesifikk deteksjon av henholdsvis *Y. ruckeri* og *Y. ruckeri* CC1 for målrettet screening. Den virulente varianten ble påvist ved akutte utbrudd av yersiniose, men ved screening av miljøprøver ble det funnet at disse ofte inneholdt *Y. ruckeri*, men andre, trolig avirulente stammer. Screening ved termisk avlusing i felt fant utskilling av virulent *Y. ruckeri* i behandlingsvannet. Det samme ble observert i dyreforsøk med subklinisk infisert fisk utsatt for termisk stress. En rekke virulens-assosierte genetiske faktorer ble identifisert ved komparativ genomisk analyse av virulente og avirulente *Y. ruckeri*. En av disse, det invasin-lignende *yrIlm*, korrelerte med en virulent fenotype. I virulente isolater fra Norge er dette genet duplisert over tid, hvor isolater fra 1980-tallet har en enkelt kopi, og isolat fra 2000 og fremover har to eller tre kopier. *Y. ruckeri* er beskrevet som bevegelig, men stadig økende forekomst av ikke-bevegelige isolater som mangler flagell har vært assosiert med virulens og vaksinesvikt internasjonalt. Syv ikke-bevegelige isolater ble funnet blant 263 undersøkte norske isolater. Kun tre av disse tilhører den dominerende virulente varianten. Tap av bevegelighet forekommer dermed også i den virulente varianten i norsk akvakultur, men den lave frekvensen tyder på at økende problemer med yersiniose i senere år ikke kan tilskrives utbredelse av denne ikke-bevegelige fenotypen. Derimot, spredning av den dominerende virulente varianten og andre genetiske endringer i denne, som duplisering av *yrIlm*, samt økt forekomst av stress, hvor ikke-medikamentell avlusing utmerker seg i høy grad, later til å være betydelige bidragsytere til økt omfang og alvorlighetsgrad av akutt yersiniose i i norsk akvakultur i senere år.

List of articles

Paper I:

Biotyping reveals loss of motility in two distinct *Yersinia ruckeri* lineages exclusive to Norwegian aquaculture

Andreas Riborg, Duncan J. Colquhoun, Snorre Gulla.

Published: *Journal of fish diseases*, *45*(5), 641–653. 2022. DOI: 10.1111/jfd.13590

Paper II:

qPCR screening for *Yersinia ruckeri* clonal complex 1 against a background of putatively avirulent strains in Norwegian aquaculture

Andreas Riborg, Snorre Gulla, David Strand, Jannicke Wiik-Nielsen, Anita Rønneseth, Timothy J. Welch, Bjørn Spilsberg, Duncan J. Colquhoun.

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Paper III:

Pan-genome survey of the fish pathogen *Yersinia ruckeri* links accessory- and amplified genes to virulence

Andreas Riborg, Snorre Gulla, Eve Zeyl Fiskebeck, David Ryder, David W. Verner-Jeffreys, Duncan J. Colquhoun, Timothy J. Welch.

Submitted to: PLOS Pathogens, August 2022.

1 Introduction

1.1 Yersiniosis, general introduction

Yersiniosis in fish is a haemorrhagic septicaemia caused by the Gram-negative bacterium *Yersinia ruckeri* (Ewing et al. 1978). The disease is especially prevalent in farmed salmonid fish (McDaniel 1971; Busch 1978) but is also known to affect various other fish species in aquaculture (Liu et al. 2016; Eissa et al. 2008; Manna et al. 2003; Gudmundsdottir et al. 2014) and has been isolated from a wide range of fish species in the wild (Table 1). The disease was first discovered in farmed rainbow trout (*Oncorhynchus mykiss*) in mid-western USA in the 1950s, being described as a bacterial septicaemia accompanied by characteristic subcutaneous haemorrhages in and around the mouth and throat (McDaniel 1971). The descriptive name redmouth disease was given, with the associated unidentified Gram-negative bacterium referred to as the redmouth bacterium (Rucker 1966). The redmouth bacterium was later described as a member of the *Enterobacteriaceae* by Ross, Rucker and Ewing (1966), hence the name enteric redmouth (ERM), which remains widely used as a synonym for yersiniosis. The causative agent was eventually identified as a member of the genus *Yersinia* and named *Y. ruckeri* in 1978 in honour of microbiologist Robert R. Rucker for his dedication in study of the disease (Ewing et al. 1978).

1.2 Reservoirs and transmission

It is generally believed that *Y. ruckeri* may become established in the water source of hatcheries, especially if the water source is populated by wild fish or escaped farmed fish (McDaniel 1971; Dulin et al. 1976; Hjeltnes et al. 2012). A small proportion of asymptomatic carriers in a rainbow trout population may serve as a long-term reservoir, propagated by transfer between fish by direct contact or shedding the bacterium into the water (Busch 1978). Shedding seemingly occurs in cycles (~30 - 40 days) but may also be spontaneously triggered by stress, such as stressful handling events (Busch & Lingg 1975; Hunter, Knittel & Fryer 1980). In farmed Atlantic salmon (*Salmo salar*) outbreaks also occur in seawater, which has led to some speculation as to how this freshwater-associated bacterium finds its way to cages at sea (Willumsen 1989). As asymptomatic carriers have also been

documented for Atlantic salmon (Bruno & Munro 1989; Willumsen 1989), subclinical infection occurring in the freshwater phase is a possible route of transmission in such cases. The presence of *Y. ruckeri* DNA in Chinook salmon (*Oncorhynchus tshawytscha*) eggs and ovarian fluids indicates that some form of vertical transmission may be possible although no evidence exists of the bacterium residing intracellularly in fish eggs, i.e. 'true' vertical transmission (Glenn et al. 2015). In addition to being established in populations of salmonid fish by long-term sub-clinical infection, the bacterium is well adapted for survival in freshwater environments, being able to survive for long periods in purified water (Thorsen et al. 1991), in sediments and sand (Romalde et al. 1994; Bomo et al. 2004), and produces hardy biofilms in freshwater environments (Coquet et al. 2002a; Coquet et al. 2002b).

While the disease occurs primarily in salmonid fish in intensive aquaculture, *Y. ruckeri* has been isolated from a wide range of non-salmonid fish species in aquaculture and in the wild, including some strictly marine species, and on occasion from warm-blooded animals such as marine birds (*Larus marinus*), otter (*Lutra lutra*) and muskrat (*Ondatra zibetica*), usually in close vicinity of ERM outbreaks in fish (species and references are listed in Table 1). *Y. ruckeri* has been isolated from a human on one occasion, recovered from a deep wound shortly after the patient was cut on a stone while paddling in a river (De Keukeleire et al. 2014). While there is no evidence of *Y. ruckeri* causing disease in warm-blooded animals (O'Leary 1977; O'leary, Rohovec & Fryer 1979), they may likely serve as passive vectors for spread (Willumsen 1989). Freshwater invertebrates such as crayfish and snails have been speculated to serve as reservoirs but *Y. ruckeri* have not been isolated from such organisms (Dulin et al. 1976; Wade 2019).

Table 1: Combined and modified tables 2 and 3 from Wade (2019), animal species from which *Yersinia ruckeri* has been isolated.

Common name	Scientific name	Reference
Salmonid species		
Arctic charr	*Salvelinus alpinus*	Willumsen 1989
Atlantic salmon	*Salmo salar*	Sparboe et al. 1986
Brook trout	*Salvelinus fontinalis*	Stevenson & Daly 1982
Brown trout	*Salmo trutta*	Fuhrmann et al. 1984
Cisco	*Coregonus artedii*	Stevenson & Daly 1982
Chinook salmon	*Oncorhynchus tshawytscha*	McDaniel 1971
Coho salmon	*Oncorhynchus kisutch*	Arkoosh et al. 2004
Cutthroat trout	*Oncorhynchus clarkii*	Daly et al. 1986
Dolly varden trout	*Salvelinus malma*	Daly et al. 1986
European whitefish	*Coregonus lavaretus*	Taksdal, Håstein & Onstad 1993
Lake trout	*Salvelinus namaycush*	Daly et al. 1986
Northern whitefish	*Coregonus peled*	Rintamaki et al. 1986
Rainbow trout	*Oncorhynchus mykiss*	Ewing et al. 1978
Sockeye salmon	*Oncorhynchus nerka*	Bullock et al. 1978
Steelhead trout	*Oncorhynchus mykiss*	Daly et al. 1986
Whitefish	*Coregonus muksun*	Rintamaki et al. 1986
Other freshwater or diadromous fish species		
Amur sturgeon	*Acipenser schrencki*	Shaowu et al. 2013
Burbot	*Lota lota*	Dwilow et al. 1987
Bighead carp	*Aristichthys nobilis*	Joh et al. 2010
Carp	*Cyprinus carpio*	Fuhrmann et al. 1984
Channel catfish	*Ictalurus punctataus*	Danley et al. 1999
Emerald shiner	*Notropis atherinoides*	Mitchum 1981
European eel	*Anguilla anguilla*	Fuhrmann et al. 1983
Indian carp	*Labeo* spp.	Ummey et al. 2021
Japanese eel	*Anguilla japonica*	Joh et al. 2010
Fathead minnow	*Pimephales promelas*	Michel et al. 1986
Goldfish	*Carassius auratus*	McArdle & Dooley-Martyn 1985
Nile tilapia	*Oreochromis niloticus*	Eissa et al. 2008
Perch	*Perca fluviatilis*	Valtonen et al. 1992
Roach	*Rutilus rutilis*	Valtonen et al. 1992
Rudd	*Scardinius erytnropthalmus*	Popovic et al. 2001
Siberian sturgeon	*Acipenser baeri*	Vuillaume et al. 1987
Sturgeon	*Acipenser* spp.	Wade 2019
Walleye	*Sander vitreus*	Wade 2019
Marine fish species		
Atlantic cod	*Gadus morhua*	Gudmundsdottir et al. 2014
Corkwing wrasse	*Symphodus melops*	Gulla 2017
Lumpsucker/lumpfish	*Cyclopterus lumpus*	Gulla, Gu & Olsen 2019
Sablefish	*Anoplopoma fimbria*	Wade 2019
Saithe	*Pollachius virens*	Willumsen 1989
Seabass	*Dicentrarchus labrax*	Bullock & Cipriano 1990
Seabream	*Sparus auratus*	Bullock & Cipriano 1990
Turbot	*Scophthalmus maximus*	Bullock & Cipriano 1990
Other animals		
European otter	*Lutra lutra*	Collins, Foster & Ross 1996
European pond turtle	*Emys orbicularis*	Nowakiewicz et al. 2015
Greater black-backed gull	*Larus marinus*	Willumsen 1989
Muskrat	*Ondatra zibetica*	Stevenson & Daly 1982

1.3 History and spread of the disease

ERM was initially a regional problem, affecting farmed rainbow trout in the Hagerman Valley of Idaho and in surrounding states in the Rocky Mountain area of western USA (McDaniel 1971). By 1970 the disease had spread to other areas in western USA, including Alaska, and during the 1970s spread to eastern states in the USA (e.g. Ohio and Tennessee) and Canada (Saskatchewan and British Columbia) (Busch 1978). The spread of ERM has been attributed to transfer of fish from the Hagerman valley area in several instances (Wobeser 1973; Barnes 2011). While the description of transmission in these instances were likely accurate, retrospective identification of isolates from moribund rainbow and brook trout collected in the early 1950s in West Virginia, USA (1952) and in Australia (1959) as *Y. ruckeri*, around the same time as the first isolation in Idaho, indicate a wider geographical range than previously supposed (Bullock, Stuckey & Shotts 1977; De Grandis et al. 1988; Barnes 2011).

In the early 1980s, reports of ERM causing mortality in rainbow trout started to appear in the UK (Roberts 1983; Frerichs & Collins 1984; Frerichs Stewart & Collins 1985) and on mainland Europe, including Germany (Fuhrmann et al. 1983), France (Lesel et al. 1983), Denmark (Dalsgaard et al. 1984) and Switzerland (Meier 1986). Some sources mention the possibility of the disease already being widespread and perhaps having gone under the radar due to being ignored or misdiagnosed (Frerichs, Stewart & Collins 1985; Meier 1986). Others, perhaps most, describe the symptoms and characteristics of the disease as unlike any previously encountered. ERM apparently having replaced furunculosis as the dominant disease in many areas further indicates that dissemination of *Y. ruckeri* in the European rainbow trout industry in the 1980s was quite rapid (Dalsgaard et al. 1984; Giorgetti, Geschia & Sarti 1985; Schlotfeldt et al. 1985). Descriptions of yersiniosis in farmed Atlantic salmon in Norway (Sparboe et al. 1986), Finland (Rintamäki, Valtonen & Frerichs 1986) and Australia (Llewellyn 1980) appeared in the mid-late 1980s and would eventually include other salmon-farming countries such as the UK (Collins, Foster & Ross 1996) and Chile (Bastardo et al. 2011).

The host range of the disease is considered to include all salmonids (the *Salmonidae* family) (McDaniel 1971), including whitefish (*Coregoninae*) (Rintamäki, Valtonen & Frerichs 1986). Yersiniosis has been described in rainbow trout farming from all corners of the world, including South Africa (Bragg & Henton 1986), Peru (Bravo & Kojagura 2004), Iran (Akhlaghi & Yazdi 2008) and India (Sharma et al. 1995), and the disease is currently recognized to be endemic throughout the world wherever there is salmonid aquaculture. In recent years, increasing numbers of non-salmonid species in intensive aquaculture have been recognized as primary hosts, with outbreaks of yersiniosis reported from channel catfish (*Ictalurus punctatus*) in China (Liu et al. 2016), Nile tilapia (*Oreochromis niloticus*) in Egypt (Eissa et al. 2008), and carp (*Labeo* spp.) in India (Manna et al. 2003; Ummey et al. 2021).

1.4 Symptoms and diagnosis

The disease was initially associated with sustained low levels of mortality in rainbow trout hatcheries, but generally manifests in a similar way in other salmonids (Busch 1978; Bullock 1984). While mortalities in such 'chronic conditions' may be as low as 0.5% per month and thus not considered an immediate concern, they will accumulate to significant losses over time if left untreated (McDaniel 1971; Busch 1978). Acute outbreaks with higher mortalities may occur if fish populations are stressed in conjunction with handling, crowding, low levels of dissolved oxygen or other suboptimal environmental conditions, or during outbreaks or treatment of other diseases (Busch 1978; Myhr & Lillehaug 1987). In farmed salmonid species transferred to seawater, outbreaks are also known to occur in conjunction with transfer to saltwater and/or stress that may occur post-transfer (Carson & Wilson 2009). The incubation time is considered to be 5-10 days (at 13-15°C), affected by various environmental factors (Bullock 1984). Severity of the disease seems to correlate with temperature, with many severe outbreaks occurring at >13°C (Rodgers 1991a). Mortalities of 1-5% per week were reported by early case studies, from what were described as acute conditions (Wobeser 1973; Lesel et al. 1983; Frerichs Stewart & Collins 1985; Meier 1986), which may add up to 25 to 75% cumulative mortality over the course of an untreated outbreak (Busch 1978). Such conditions are, however, regarded as subacute in more recent publications, with the acute form of the disease producing similar cumulative losses (30-70%) but over the course of just 4-10 days (Furones, Rodgers & Munn 1993).

Typical early signs include darkening of the skin and behavioural changes such as loss of appetite and lethargic swimming near the surface (Kumar et al. 2015). As the disease progress, fish may develop the characteristic reddening in and around the mouth and along the base of the fins and anus, due to subcutaneous haemorrhaging (Busch 1978; Figure 1). Exophthalmos is commonly observed, as well as haemorrhaging in the eyes (Tobback et al. 2007). The fish develop internal signs of a bacterial haemorrhagic septicaemia with petechial haemorrhage on the surface of organs and tissues (liver, pancreas, pyloric caeca, swim bladder and lateral muscles). Tissue oedema, typically affecting the kidney, liver and spleen is commonly observed (Busch 1978; Tobback et al. 2007). Opaque yellowish fluid consisting of necrotized tissue may be present in the lower intestine (Kumar et al. 2015).

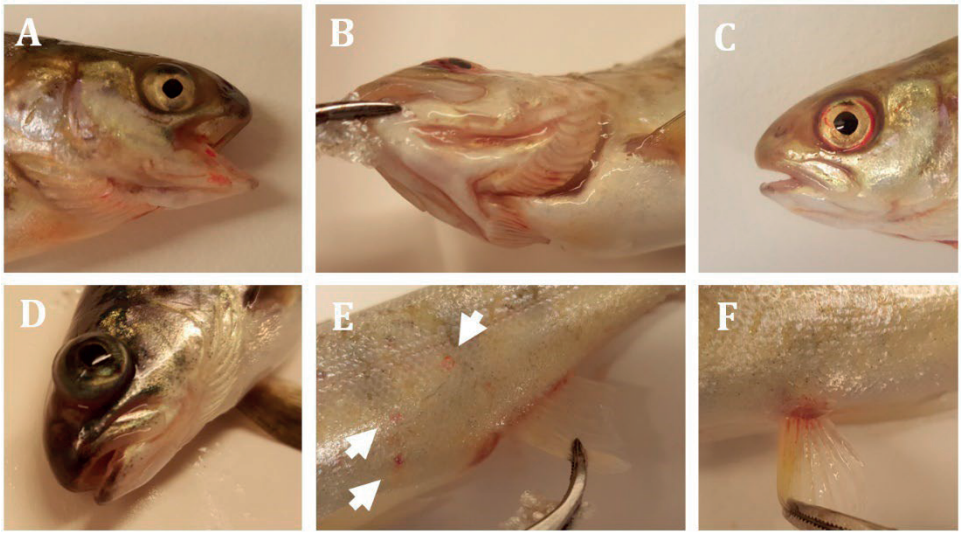

Figure 1: External signs of acute yersiniosis in ~27g Atlantic salmon (*Salmo salar*) juveniles experimentally infected with *Y. ruckeri* CC1 (cohabitation, freshwater), showing reddening/bleeding around the mouth and gills (A and B), bleeding in the eye (C), exophthalmos (D), bleeding in the skin (E, indicated by arrows), anus and base of anal fin (E), and bleeding at base of pelvic fin (F). Photos: Andreas Riborg.

Histopathological examination reveals an acute bacteremia with inflammatory response in virtually all tissues, presence of bacteria in well vascularized tissues, particularly in kidney, spleen, liver, heart and gills (Kumar et al. 2015), and internally in macrophages (Carson & Wilson 2009). Specific pathological changes include degradation of tubular structures in the kidney, and focal necrosis in the liver and spleen (Kumar et al. 2015).

External and internal macroscopic clinical signs and histopathologically visible changes are somewhat general for systemic infections caused by Gram-negative pathogens, and diagnosis may be especially difficult in 'chronic'/'looming' infections with sustained low level mortality. If there is no prior known history of ERM at a site, such mortalities are prone to being ignored as unspecific 'background mortality' as the symptoms may be vague or absent (McDaniel 1971; Barnes 2011). Acute mortality in small fry/fingerlings may occur in the complete absence of external clinical signs (Barnes 2011). In larger fish the hallmark symptom of reddening around the mouth is not always present, making the commonly used descriptive name of the disease somewhat misleading (Frerichs Stewart & Collins 1985). Misdiagnosis may easily occur as symptoms overlap with other bacterial septicaemias, some of which may also cause subcutaneous haemorrhages (e.g. *Edwardsiella tarda*) (Austin & Austin, 2007; Barnes 2011). Diagnosis may also be complicated by coinfection with fungal (usually *Saprolegnia* sp.), viral, or other bacterial diseases (Myhr & Lillehaug 1987; Rintamäki, Valtonen & Frerichs 1986; Taksdal et al. 1993; Collins, Foster & Ross 1996; Bornø & Sviland 2011). A tentative diagnosis can be arrived at based on clinical signs and previous ERM history at the location, although isolation of the bacterium from tissue samples followed by identification by phenotypic, serological and/or molecular examination should be performed for certain diagnosis (Barnes 2011).

Y. ruckeri is readily isolated by direct plating from kidney tissue of lethargic or moribund fish on general purpose agar media e.g. tryptic soy agar or blood agar, on which the organism appears as 1-2mm diameter (24h), smooth, round, translucent, white cream in colour, non-pigmented colonies (Ross, Rucker & Ewing 1966; Furones, Rodgers & Munn 1993). *Y. ruckeri* displays gamma-haemolysis (lack of observable haemolytic activity) on sheep blood agar (O'leary, Rohovec & Fryer 1979) and bovine blood agar (personal observations).

Isolation from fish intestines or from environmental samples is possible but difficult due to competing growth (Rodgers & Hudson 1984; Rodgers 1992; personal observations). While some efforts have been made in development of selective and differential agar media (Waltman & Shotts 1984; Rodgers 1992), of which ribose ornithine deoxycholate (ROD) agar is the most notable, the differential properties rely on traits that are variable in *Y. ruckeri*, i.e. lipase activity and degradation of SDS (Figure 2), and are, therefore, only useful for the isolation of certain phenotypic variants (Furones, Rodgers & Munn 1993; Carson & Wilson 2009).

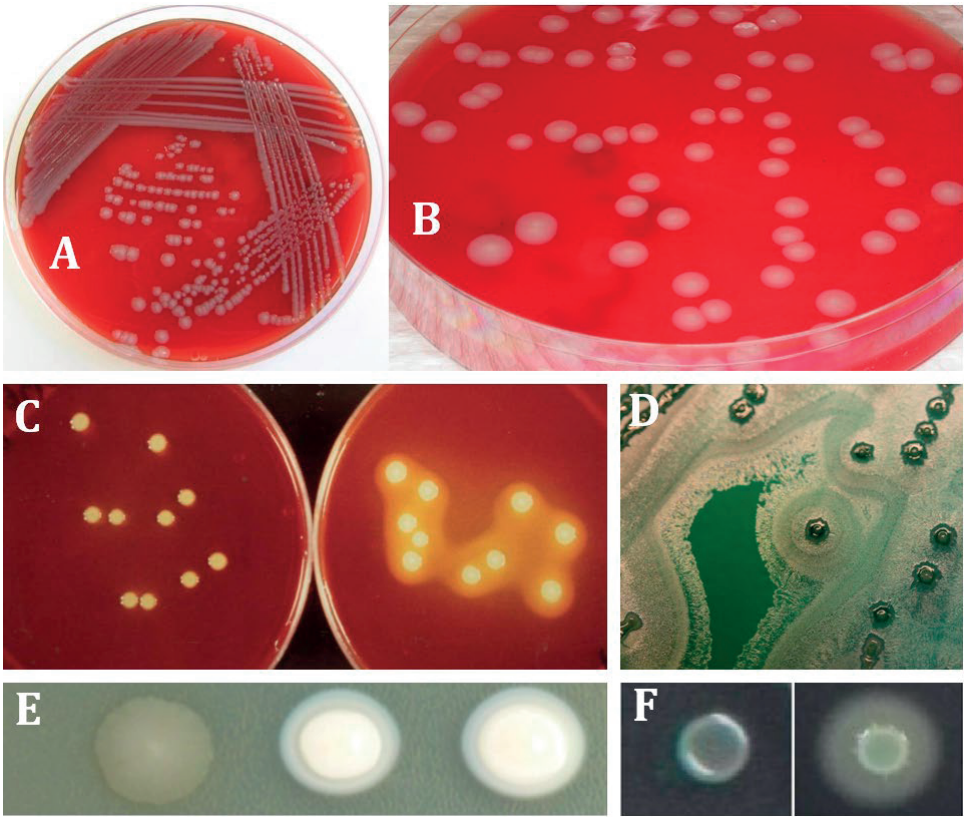

Figure 2: *Y. ruckeri* appearance on sheep blood agar (A; 25°C/48h incubation; image from Carson & Wilson 2009), bovine blood agar (B; 22°C/2h incubation; photo by Andreas Riborg), SDS-degrading (left) and non-degrading (right) strains on ribose ornithine desoxyeholate agar (C; image from Rodgers 1991b), biotype 1 (lipase positive) strain on agar media described by Waltman and Shotts (1984)(D; photo by Eva Jansson/SVA 2019), SDS-degrading (left) and non-degrading (mid and right) strains on SDS-containing tryptic soy agar (E; image from Navais et al. 2014a), biotype 2 (lipase negative; left) and biotype 1 (right) strains on Tween-80/lipase agar (F; image from Jozwick, Graf & Welch 2017), with permission.

Selective and differential media developed for human pathogenic *Yersinia*, such as Yersinia Selective Agar (CIN agar) does not support growth of *Y. ruckeri* (Renaud et al. 2012). However, semi-selective agars containing Sodium Deoxycholate (the selective agent used in the ROD agar in addition to SDS), such as the CIN base (CIN without antibiotics), Xylose Lysine Deoxycholate (XLD) and *Salmonella Shigella* agar may be used. XLD agar notably differentiates *Hafnia alvei* (xylose positive), which may also occasionally cause a haemorrhagic septicaemia in salmonid fish (Gelev et al. 1990; Carson & Wilson 2009), as well as most other *Yersinia* spp. (see Table 2 in Murros-Kontiainen et al. 2011). Salmonella-Shigella Agar differentiates H_2S positive species (notably *Edwardsiella tarda*) and lactose fermenters (Austin & Austin 2012). Bile Esculin Agar has similar selective properties as these agars and offer some value in differentiation from the esculin reaction (personal observations). Further presumptive identification of isolates can be made by various biochemical tests (Table 2; Table 3), however, as a number of other *Enterobacteriaceae* display reaction patterns similar to that of *Y. ruckeri* while also appear similarly on general agar media, serology with appropriate antisera, 16S rDNA sequencing, MALDI-TOF or PCR should be performed for species verification (Barnes 2011).

In the absence of bacterial culture, a yersiniosis diagnosis may be arrived at based on histopathological analysis using immunohistochemistry with appropriate antibodies (FAT, IFAT), ideally from kidney tissue (Barnes 2011; Furones, Rodgers & Munn 1993). Rapid detection of *Y. ruckeri* antigens by FAT on tissue smears (Furones, Rodgers & Munn 1993) or enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies (Austin et al. 1986; Romalde et al. 1995) has seen some use, although detection by PCR has been more widely utilized for rapid detection in recent years (Gibello et al. 1999; LeJeune & Rurangirwa 2000; Temprano et al., 2001; del Cerro, Marquez & Guijarro, 2002; Bastardo, Ravelo & Romalde, 2012; Keeling et al. 2012). PCR also enables rapid detection from a variety of sample materials, such as fish faeces and environmental samples (Ghosh et al. 2018, Shea et al. 2020; Lewin et al. 2020). A protocol for specific detection by Loop-Mediated Isothermal Amplification (LAMP) has also been described (Saleh et al. 2008).

1.5 Prevention and control

If *Y. ruckeri* is not already present in a freshwater aquaculture site, preventive efforts should focus on preventing introduction of *Y. ruckeri* to the environment in and around the site, the water source, and the fish stock, as *Y. ruckeri* seem capable of colonizing any of these niches. The water source is of particular interest as it may serve as a constant source of contamination and thus possibly foil efforts to sanitize within the site itself (Hjeltnes et al. 2012). Sites utilizing either flow-through or recirculation technologies (RAS) may both be affected (Hjeltnes 2014; Bornø & Linaker 2015; Colquhoun 2016; Amlie 2018). Sites with spring/well-water sources seem to be at significantly reduced risk than those utilizing river water although this may relate to the former usually being smaller scale operations (Rodgers 1991a). Outside of the water source, sourcing fish and/or eggs from *Yersinia*-free stocks, and limit transfer of equipment and biological materials is highly advised to prevent introduction (Furones, Rodgers & Munn 1993). At specific sites or in areas where *Y. ruckeri* is already established, a number of preventive measures related to maintenance of good fish welfare and reduction of stress are prescribed to prevent acute utbreaks and/or escalation of subclinical infections. These include strict hygiene and avoiding over-crowding and otherwise stressful situations during procedures such as grading, vaccination, water temperature fluctuations (Rodgers 1991a; Furones, Rodgers & Munn 1993).

In the 1980s, prophylactic use of antibiotics to prevent yersiniosis and other bacterial diseases in aquaculture was recommended and widely practiced. This became increasingly controversial going into the 1990s (Rodgers 1991a; Furones, Rodgers & Munn 1993), and such practices were subsequently banned in most developed countries due to possible development of resistance and residues in the final product (Hernández 2005). Antibiotics are, however still prescribed to treat acute outbreaks (Tobback et al. 2007). A wide range of substances and dosage regimes have been successfully utilized, with oxolinic acid, potentiated sulphonamide and oxytetracycline commonly used (Furones, Rodgers & Munn 1993), and increasingly florfenicol in recent years (Duncan J. Colquhoun pers. com.). Resistance develops readily *in vitro* against these compounds (Rodgers 2001), and both chromosomal mutations and antimicrobial resistance plasmids have been shown to confer resistance (Welch et al. 2007; Shah et al. 2012). While case reports

of resistance to relevant compounds do exist (De Grandis & Stevenson 1985; Gibello et al. 2004; Huang et al. 2014; Shah et al. 2012), they do not seem to be widespread (Welch et al. 2007; Calvez et al. 2014), and it appears that such isolates are generally still sensitive to the remaining first-line compounds.

Development of ERM vaccines started early (Ross & Klontz 1965), and inactivated bacterin-based immersion vaccines against ERM and vibriosis became, in the late 1970s, the first commercially available vaccines for fish (Sommerset et al. 2005; Gudding & Van Muiswinkel 2013). Significant protection was reported for immersion, oral and injection variants of early ERM vaccines, and vaccination quickly became the single most important tool to prevent the disease (Stevenson, Flett & Raymond 1993). Vaccination has not, however, completely eliminated the disease, as acute outbreaks may still occur under poor environmental conditions or high stress (Busch 1983; Rodgers 1991a). In recent years there have been many case-reports of ERM vaccine failure, associated with the emergence of non-motile *Y. ruckeri* strains. Modification of the vaccine by changing the vaccine strain, utilization of autologous/autogenic vaccines or otherwise altering the formulation or administration of the vaccine as intraperitoneal (IP) injection instead of immersion, has proven effective in resolving such issues (Austin et al. 2003; Fouz, Zarza & Amaro, 2006; Arias et al. 2007; Costa et al. 2011; Welch et al. 2011).

In addition to preventing introduction where possible, maintaining favourable, low-stress conditions and vaccination, probiotic use against yersiniosis has generated promising experimental results in recent years. Formulations containing *Bacillus* sp., *Carnobacterium* sp. and *Aeromonas sobria* are seemingly able to induce some degree of protective immunity in the fish (Robertson et al. 2000; Kim & Austin 2006; Brunt et al. 2007; Capkin & Altinok 2009; Abbass, Sharifuzzaman & Austin 2010), and lactic acid bacteria may be able to outcompete *Y. ruckeri* in fish mucus, intestine, and on abiotic surfaces (Balcázar et al. 2008; Sica et al. 2012).

1.6 *Yersinia ruckeri*

Previously known as a member of the Family *Enterobacteriaceae*, *Y. ruckeri* is currently recognized, after recent revision of the *Enterobacterales* order, as a member of the family *Yersiniaceae* together with other members of the genera *Yersinia*, *Serratia*, *Rahnella*, *Rouxiella* and *Ewingella* (Adeolu et al. 2017). *Y. ruckeri* cells are facultative anaerobic, short Gram-negative rods (1.0 x 2.0 to 3.0 µm), with motile strains displaying peritrichously arranged flagella (Ross, Rucker & Ewing 1966). Like other *Yersinia*, *Y. ruckeri* is mesophilic, able to grow well under refrigeration, while also capable of rapid growth at higher temperatures (Percival & Williams 2014). *Yersinia* are well known for temperature-dependant gene regulation, with expression of key virulence factors, flagella and several metabolic systems being subject to strict temperature-dependant control. Temperature is a primary cue for modulating expression of systems relevant for *Yersinia* survival in the environment or in cold- and warm-blooded hosts (Horne & Pruss 2006; Minnich & Rohde 2007). *Y. ruckeri* has an optimum growth temperature of 28°C (O'leary, Rohovec & Fryer 1979), not unlike the human pathogenic *Yersinia* species, but is not well adapted for growth at temperatures typical of warm-blooded hosts (Ewing et al., 1978). Growth is severely inhibited at 37°C for many *Y. ruckeri* strains, a trait associated with a certain large conjugative plasmid commonly present in virulent strains (De Grandis & Stevenson 1982). While other *Yersinia* spp. are also commonly encountered in aquatic environments, *Y. ruckeri* distinguishes itself in being primarily associated with freshwater environments with various species of fish as its primary host organism, while other described *Yersinia* species are generally also associated with terrestial environments and warm-blooded hosts (Robins-Browne 1991; Falcão et al. 2004; Reuter et al. 2014). As such, the utility of temperature-dependant regulation in *Y. ruckeri* seem less obvious, but features such as abolishing expression of flagella at higher temperatures are nonetheless conserved (O'leary, Rohovec & Fryer 1979). *Y. pestis*, the causative agent of the 'Black Death' plague, is non-motile due to mutations in essential flagellar genes (Chain et al. 2004). Such events have also occurred in certain lineages in *Y. ruckeri* rendering such strains permanently non-flagellated (Davies & Frerichs, 1989; Welch et al. 2011). Certain key-virulence factors which expression is upregulated at higher temperatures in human pathogenic *Yersinia*, i.e. the *Yersinia* virulence plasmid (Rohde et al. 1999; Horne & Pruss 2006), are notably absent in *Y. ruckeri* (Guilvout et al. 1988).

Y. ruckeri display a metabolic profile which is notably negative for a large number of relevant differentiating compounds (Ewing et al. 1978; O'leary, Rohovec & Fryer 1979; Table 2). In addition to the well known negative sucrose test (Waltman & Shotts 1984), negative results for urease and esculin are useful to differentiate *Y. ruckeri* from a large number of *Yersiniaceae* and other relevant *Enterobacteriaceae* (Green & Austin 1983; Bullock & Cipriano 1990). The only test in which *Y. ruckeri* distinguishes itself amongst *Yersinia* with a positive reaction seems to be Lysine decarboxylase (Table 2; see also Table 1 in Hurst et al. 2011 where more species are listed). This limited biochemical repertoire relates to a significant reduction in genome size in *Y. ruckeri,* which has resulted in the loss of several carbohydrate utilization pathways. With a genome size of 3.6-3.9 Mbp, *Y. ruckeri* possesses the smallest genome amongst sequenced *Yersinia*, which are otherwise generally in the 4.6-4.8 Mbp range (Chen et al. 2010). While reduced genome size is a common trait amongst obligate intracellular pathogens (Stepkowski & Legocki 2001), this does not apply to *Y. ruckeri*. Rather, adaptation to an aquatic, oligotrophic environment is likely the cause (Barnes et al. 2016), where metabolic flexibility is of less value than in terrestrial environments, and conserving resources is of key importance (Rosinski-Chupin et al. 2013; Reuter et al. 2014).

While initially placed in the *Yersinia* genus, this taxonomic placement was noted as uncertain, with *Yersinia* preferred over *Serratia* due to differences in GC % (Ewing et al. 1978; O'leary, Rohovec & Fryer 1979). Several authors have since called for a revaluation of the taxonomic status due to the numerous traits distinguishing it from other *Yersinia* in terms of phenotype and results from early genetic methods (Green & Austin 1983; Farmer et al. 1985; De Grandis et al. 1988). While *Y. ruckeri* is clearly situated on the *Yersinia* side of a *Yersiniacea* tree (Figure 3), it does indeed display a distinct phylogenetic position relative to other *Yersinia* (Hall et al. 2015). The more recently discovered species *Y. entomophaga* and *Y. nurmii* do however 'close the gap' between *Y. ruckeri* and remaining *Yersinia* species, both in terms of biochemical reactivity (Murros-Kontiainen et al. 2011; Hurst et al. 2011) and whole-genome-based phylogeny where they share a deep branch with *Y. ruckeri* (Reuter et al. 2014; Savin et al. 2019). *Y. nurmii* is also similar to *Y. ruckeri* in terms of genome size at 4.1 Mbp (acc. no. GCF_001112925). No further calls for revision of the taxonomic status of *Y. ruckeri* have been made in the post-WGS era.

Table 2: Notable differentiating phenotypic traits of *Y. ruckeri* and some selected *Yersiniaceae,* and some additional *Enterobacterales* members relevant for salmonid aquaculture according to Austin and Austin (2012).

	Y. ruckeri	*Y. nurmii*	*Y. entomophaga*	*Y. pestis*	*Y. pseudotuberculosis*	*Y. enterocolitica*	*Y. intermedia*	*S. liquefaciens*	*S. marcescens*	*S. plymuthica*	*C. freundii*	*E. tarda*	*H. alvei*
Motility	**v**	+	+	-	+	+	+	+	+	**v**	+	+	+
Urease	-	-	-	-	+	+	+	-	-	-	-	-	-
Esculin	-	-	-	+	+	-	+	+	+	+	-	-	-
Rhamnose	-	-	-	-	+	-	+	-	-	-	+	-	+
Salicin	-	-	-	-	+	-	+	+	+	+	-	-	-
Sucrose	-	+	+	-	-	+	+	+	+	+	+	-	-
Cellobiose	-	+	+	-	-	+	+	+	-	+	**v**	-	+
Melibiose	-	-	+	-	+	-	-	+	-	+	+	-	-
Ornithine	+	+	+	-	-	+	+	+	+	-	-	+	+
Lysine	+	-	-	-	-	-	-	+	+	-	-	+	+
Xylose	-	-	-	+	+	**v**	-	+	-	+	+	-	+
Arabinose	-	-	-	+	+	+	+	+	-	+	-	-	+
H_2S	-	-	-	-	-	-	-	-	-	-	+	+	-

Phenotypic traits according to O'leary, Rohovec and Fryer (1979), Murros-Kontiainen et al. (2011), Hurst et al. (2011), Austin and Austin (2012) and Farmer et al. (1985).
v = relevant inter-strain variability.

Table 3: A selection of *Yersinia ruckeri* phenotypic traits with emphasis on inter-strain variability and temperature-dependant reactions.

Test	Result	Range (°C)
Hemolysis	γ	
Motility	Variable (a)	18, 22, 27
Catalase	+	
Oxidase	-	
Arginine dihydrolase	+	18, 22
Lysine decarboxylase	+	18, 22
Ornithine decarboxylase	+	
Lipase	Variable (a)	9, 18, 22
β-galactosidase/ONPG	Variable (b)	
Casein hydrolysis	Variable (c)	
Voges Proskauer	Variable (d)	
Citrate utilization	Variable (d)	
Gelatin hydrolysis	Variable (d)	27
DNAase	-	positive at 37°C
SDS degradation	Variable (e)	unknown
Arabinose (A)	-	
Fructose (A)	+	
Glucose (A)	+	
Lactose (A)	-	
Maltose (A)	+	
Rhamnose (A)	-	
Ribose (A)	+	
Salicin (A)	-	
Sorbitol (A)	Variable (f)	
Sucrose (A)	-	
Trehalose (A)	+	
Xylose (A)	-	

Temperature-dependant variation is according to tests performed at 9, 18, 22, 27 and 37°C by O'leary, Rohovec and Fryer (1979) (SDS degradation was not tested).

(A) indicate acid produced from utilization of carbohydrate

Mechanisms for variability are as follows:

(a) Biotype (Davies & Frerichs, 1989)

(b) Associated with transposase activity in the *lac* operon (paper III)

(c) May relate to Yrp1 expression (Fernández et al. 2002; Fernández et al. 2003)

(d) Unknown

(e) Presence of the alkyl sulphatase YraS (Furones et al. 1990)

(f) Presence of sorbitol-specific transport/utilization cluster *gut*/*srlD* (Barnes et al. 2016)

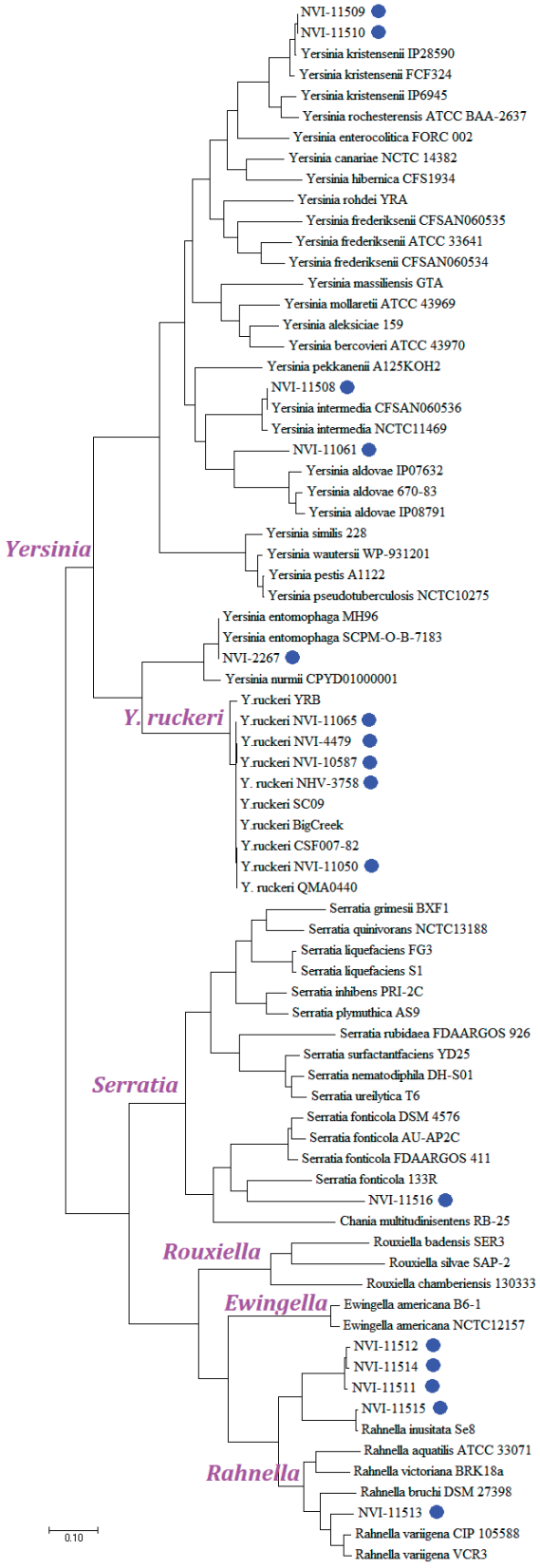

Figure 3: Modified figure S1 from paper II, core-gene *Yersiniaceae* phylogeny. Strains denoted by a blue circle originate from salmonid aquaculture in Norway. NVI-11508 to 11516 were isolated during present work.

1.7 Intraspecific diversity

Serotypes

Several serotypes of *Y. ruckeri* exist (O'Leary et al. 1982) but most isolates are of either serotypes O1 or O2, with O1 being the most common (Bullock, Stuckey & Shotts 1978; Davies 1990). Various serotyping schemes have been developed and used over the years (Romalde et al. 1993). Outside of a distinction between serotype subtypes which is sometimes made (e.g. between serotypes O1a and O1b), typing schemes generally agree on the most frequently occurring serotypes O1 and O2, while differences between schemes relate to the less common serotypes (Davies 1990). A notable exception is the British salmon-pathogenic serotype O8 which may be identical to serotype O1b (Ormsby et al. 2016). Additionally, certain isolates that appear as O5 in older works are designated as O1 in more recent works (e.g. RD154/NVI-344), perhaps due to unspecific reactions which are known to occur between O5 strains and O1 antisera (Davies 1990; Duncan J. Colquhoun pers. com.) and such 'errors' are sometimes reprinted in more recent works (e.g. RD154 in Haig et al. 2010). Amongst the less common serotypes, several strains originally designated as O3 (not regarded as a valid serotype by current schemes), are designated as O7 in more recent works (Davies 1991c).

Serotype O2 isolates have been repeatedly described as less virulent (Bullock et al. 1978; McCarthy & Johnson 1982; Barnes 2011). No such generalisation in terms of virulence can be made regarding serotype O1 as both highly virulent and avirulent strains display this serotype (Verner-Jeffreys et al. 2011). The fundamental genetic difference between O1 and O2 serotypes has been identified as a 20-gene deletion within the O-antigen cluster responsible for producing high-molecular weight lipopolysaccharides. This results in a LPS deficiency, although core lipooligosaccharide is still produced in such strains (Welch & LaPatra 2015; Barnes et al. 2016). Similar LPS-deficient phenotypes are readily obtained by selecting for resistance to a lytic bacteriophage that likely utilizes *Y. ruckeri* LPS as a receptor for entry. Such isolates display attenuated virulence in rainbow trout and sensitivity to rainbow trout serum (Welch 2019). Some naturally occurring serotype O2 strains are also sensitive to rainbow trout serum (Haig et al. 2011; Timothy Welch pers. com.).

Biotypes

While wild-type *Y. ruckeri* display flagellar motility, there has been an increasing dominance of non-flagellated, non-motile isolates recovered from acute outbreaks in rainbow trout worldwide in recent years. Such isolates are, in contrast to motile strains, lipase negative (Figure 4). These observations have resulted in the recognition of two biotypes (BT), with BT1 defined as motile and lipase positive, and BT2 defined as non-motile and lipase negative (Davies & Frerichs 1989). BT2 is sometimes referred to as biotype 'EX5' (Austin, Robertson & Austin 2003; Tinsley et al. 2011; Scott 2012). The genetic basis for BT2 development has been shown to be the result of detrimental mutations in essential flagellar genes, specifically genes in the flagellar type III secretion system (Welch et al. 2011). Secretion of lipase enzyme is then also disabled as it relies on the same secretion system (Young, Schmiel & Miller, 1999; Evenhuis et al. 2009). The presence of several distinct motility-disabling mutations amongst *Y. ruckeri* strains have shown that loss of motility has occurred independently in several geographically distinct areas (Welch et al. 2011).

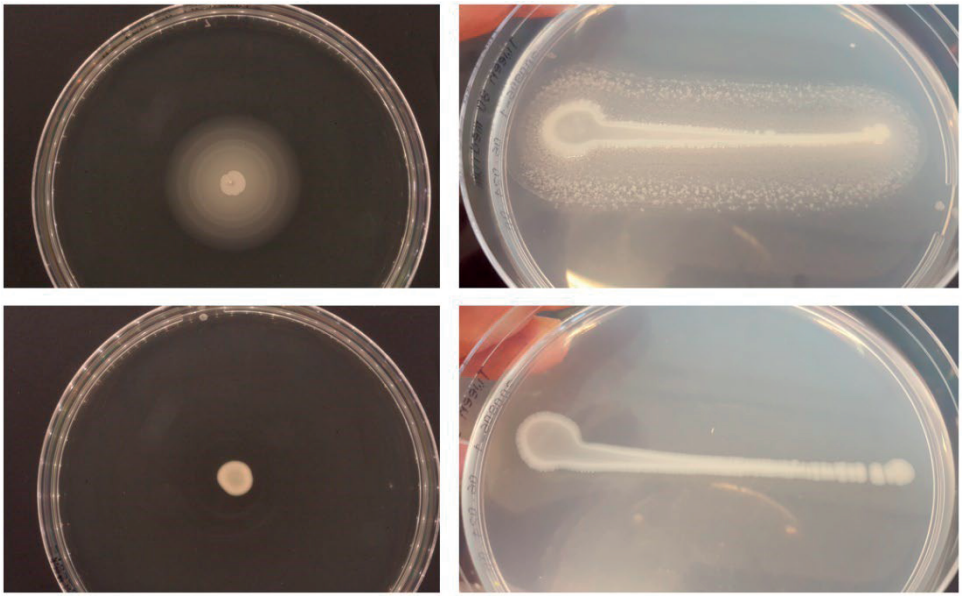

Figure 4: Agar-based biotyping with motility agar (left) and lipase agar (right) as described in paper II, with results for biotype 1 (top, both assays positive) and biotype 2 (bottom, both assays negative). Photos: Andreas Riborg.

As non-motile *Y. ruckeri* isolates have been recovered in several case studies in which immersion-style vaccines failed to provide protection against disease, BT2 development has been suspected as a case of vaccine-escape (Wheeler et al. 2009; Welch et al. 2011; Tinsley et al. 2011; Barnes et al. 2016; Kennedy & Read 2017). Presumably, loss of highly immunogenic flagella could aid the bacterium in establishing infection in an immunized host. However, such an advantage has not been demonstrated experimentally. Bacterial strains of each biotype, utilised in in trials designed to study the effect of biotype on vaccination, also differed in LPS antigen structure and virulence (Tinsley et al. 2011; Deshmukh et al. 2012), rendering the results largely inconclusive regarding the specific role of biotype in vaccine-induced immunity. In these studies, and generally, variance in vaccine-induced protection is seemingly eliminated when the vaccine bacterin is prepared from a strain more similar or identical to the disease-causing strain, combining different vaccine strains, or otherwise improving vaccine formulation or delivery (see chapter 1.4). The flagella do not seem to play a major role in vaccine-induced immunity, which rather relies on O-antigen/LPS (Welch & LaPatra 2016). By replicating naturally occurring BT2-inducing mutations in a virulent BT1 strain, it was shown that biotype does not significantly affect virulence in challenge trials (Jozwick, Graf & Welch 2017). While any specific biological advantage has yet to be identified, the non-motile phenotype has become increasingly common across Europe and USA (Fouz, Zarza & Amaro, 2006; Wheeler et al. 2009; Welch et al. 2011; Gulla et al. 2018), and a slight advantage for non-motile strains relating to immune-evasion is currently assumed (Jozwick, Graf & Welch 2017; Jozwick et al. 2019).

Biochemical variation

Intraspecific phenotypic variation relating to biochemical reaction patterns do exist in *Y. ruckeri*. Perhaps the most well-known is the ability of some strains to utilize sorbitol (O'Leary 1977; O'leary, Rohovec & Fryer 1979; O'Leary et al. 1982), a trait associated with horizontally acquired sorbitol-specific membrane transport and utilization genes in serotype O2 strains (Barnes et al. 2016). Sorbitol utilization has been used to differentiate between strains, as a substitute for serotyping, and hence as an indicator of virulence as serotype O2 is regarded as less virulent than O1 (Cipriano & Pyle 1985). Sorbitol positive isolates of serotype O1 do however exist (Stevenson & Airdrie I984; Rintamäki, Valtonen & Frerichs 1986).

The ability to degrade SDS is present in some serotype O1 strains, including strains associated with severe outbreaks in rainbow trout worldwide. Thus, this ability, related to the presence of an alkyl sulphatase (YraS), has been regarded as a virulence factor (Furones et al. 1990). SDS containing agar media (at 1%) has been utilised as both a selective and differential component (Furones, Gilpin & Munn 1992; Rodgers 1992). Resistance to SDS is inherent to the species (and presumably *Yersiniacea* in general) regardless of SDS degrading phenotype, and the SDS degrading phenotype has since been shown to correlate poorly with virulence (Navais et al. 2014a). Use of SDS as a differential component would thus hide potentially virulent *Y. ruckeri* as background, including those causing disease in Norwegian aquaculture which are invariably SDS-negative (personal observations).

While various other intraspecific biochemical differences have been reported (Table 3), the genetic basis for these are generally not known, although the likely genetic basis for the ONPG negative phenotype was discovered in current study (paper III). The observed/reported phenotype may also vary dependent on the exact test used. The results of e.g. citrate utilization, gelatine hydrolysis, Voges-Proskauer, nitrate reduction and lysine decarboxylase are known to vary between tests and between manufacturers (Davies & Frerichs 1989; Stevenson & Daily 1982; Huang et al. 2015). Many authors have cautioned the use of the API20E system (Biomerieux) in particular, as the results seem to depend heavily on the exact incubation times and temperatures used (API20E is optimized for incubation at 37°C for 24h which is not suitable for *Y. ruckeri*) (Stevenson & Daly 1982; Davies & Frerichs 1989; Furones, Rodgers & Munn 1993). Utilization of amino acids by *Y. ruckeri* notably varies with temperature, occurring at 18 and 22°C but not at the optimum growth temperature (28°C), while the opposite is the case for gelatinase activity (O'leary, Rohovec & Fryer 1979; Table 3).

Proteomic and genetic diversity

Plasmid profiling has demonstrated that plasmids are variably present in *Y. ruckeri*. Virulent strains recovered from rainbow trout usually harbour two plasmids, one of which seem to inhibit growth at 37°C (De Grandis & Stevenson 1982; De Grandis & Stevenson 1985). Some plasmids have been found to confer resistance to antibiotics, although these do not appear to be widely distributed in the species (Welch et al. 2007).

Typing efforts with multilocus isoenzyme electrophoresis revealed limited diversity amongst the varied panel of *Y. ruckeri* isolates investigated, relative to *E. coli* (Schill, Phelps & Pyle 1984). More recent studies utilizing outer-membrane-protein (OMP) profiling were more successful in differentiating isolates (Davies 1991a). The OMP electrophoresis profile represents various proteins, including OmpA, OmpC and the major porin OmpF which seem to display corresponding variation in predicted amino acid sequences (Ormsby et al. 2019; Ormsby & Davies 2021). Combinations of OMP-profiling, serotyping and biotyping have been used in epidemiologic studies. As isolates sharing an OMP-profile may vary in serotype, and vice versa, combining these techniques increase resolution. Biotyping was able to further differentiate between serotype O1 isolates (Davies 1991b; Davies 1991c).

More recently, whole-genome-based studies have demonstrated that a substantial portion of the genetic diversity in *Y. ruckeri* relates to mobile elements such as transposons, prophages and plasmids (Barnes et al. 2016). The most notable differences otherwise are the LPS O-antigen cluster deletion associated with the O2 serotype, and some other large deletions observed in specific lineages or individual isolates. The wide variety of prophages and transposons with payloads that may confer resistance to lytic phages (i.e. restriction modification systems) have been associated with the lack of CRISPR-Cas loci in *Y. ruckeri* (Barnes et al. 2016).

1.8 Phylogeography

In addition to classical phenotype-based methods, a wide variety of molecular typing techniques have been utilized to study the population structure of *Y. ruckeri*, including Pulsed-field Gel Electrophoresis (PFGE), Multi-Locus Sequence Typing (MLST), PCR-based repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC) typing, ribotyping, plasmid profiling, and combinations of such techniques (Bastardo et al. 2015; Calvez et al. 2015; Bastardo et al. 2011; Garcia et al. 1998; Wheeler et al. 2009). Bastardo, Ravelo & Romalde (2012) combined bio- and serotyping, biochemical- (API20E), OMP- and LPS-profiling (silver staining), and REP- and ERIC-PCR-profiling to maximize resolution. Findings from such studies were generally the same; while appreciable diversity does exist within the species, the phenotypic and genetic diversity amongst strains that cause outbreaks of disease in aquaculture seem quite limited.

Some diversity exist between isolates recovered from Atlantic salmon, and these are generally distinct from strains recovered from rainbow trout (Ormsby et al. 2016). Thus, phylogenetic analysis does not support any epidemiological relationship between the strains causing yersiniosis in rainbow trout and Atlantic salmon respectively in Europe, even though they were seemingly disseminated more or less simultaneously during the 1980s (Gulla et al. 2018). Virulent strains recovered from rainbow trout, believed to originate from the Hagerman valley area in the USA and sometimes collectively referred to as 'the Hagerman strain' are, however, highly similar and not differentiated to a degree suitable for epidemiological studies by any of the aforementioned techniques or reasonable combinations thereof. Isolates recovered from rainbow trout globally were found in several studies to consist of a single, or just two nearly identical genotypes (Garcia et al. 1998; Wheeler et al. 2009; Calvez et al. 2015). Using MLST, these two genotypes are separated by a single SNP, with isolates from the USA and the UK generally belonging to one genotype, while isolates from mainland Europe belong to the other (Bastardo, Ravelo & Romalde 2012; Bastardo, Ravelo & Romalde 2015). As both biotypes exist within both these groups, biotyping is able to increase the epidemiological resolution to a degree. Study of the specific causative mutations was able to further subdivide these isolates as those from Nordic countries (Denmark and Finland) and from mainland Europe as each had respective unique BT2-causative mutations, indicating that loss of motility had occurred independently at least twice in this region. The causative mutation was found to be identical between BT2 isolates from the USA and the UK, indicating that exchange of strains between these regions has occurred at least twice; the initial transfer from the USA, and then transfer of the non-motile variant in an unknown direction (Welch et al. 2011). Additional independent occurrences of BT2 also exist in this lineage (Figure 5).

WGS has been shown to be well able to discern between closely related isolates, and has confirmed a high degree of clonality in the *Y. ruckeri* population structure. Isolates form discrete, highly conserved and fairly distantly related clusters (Barnes et al. 2016; Abdel-Glil et al. 2021). Besides WGS, multiple-locus variable number of tandem repeats analysis (MLVA) has demonstrated sufficient resolution to differentiate between strains/isolates within the same lineage (Gulla et al. 2018), and MLVA typing is the foundation for the state of the art understanding of the population structure within *Y. ruckeri* as a species.

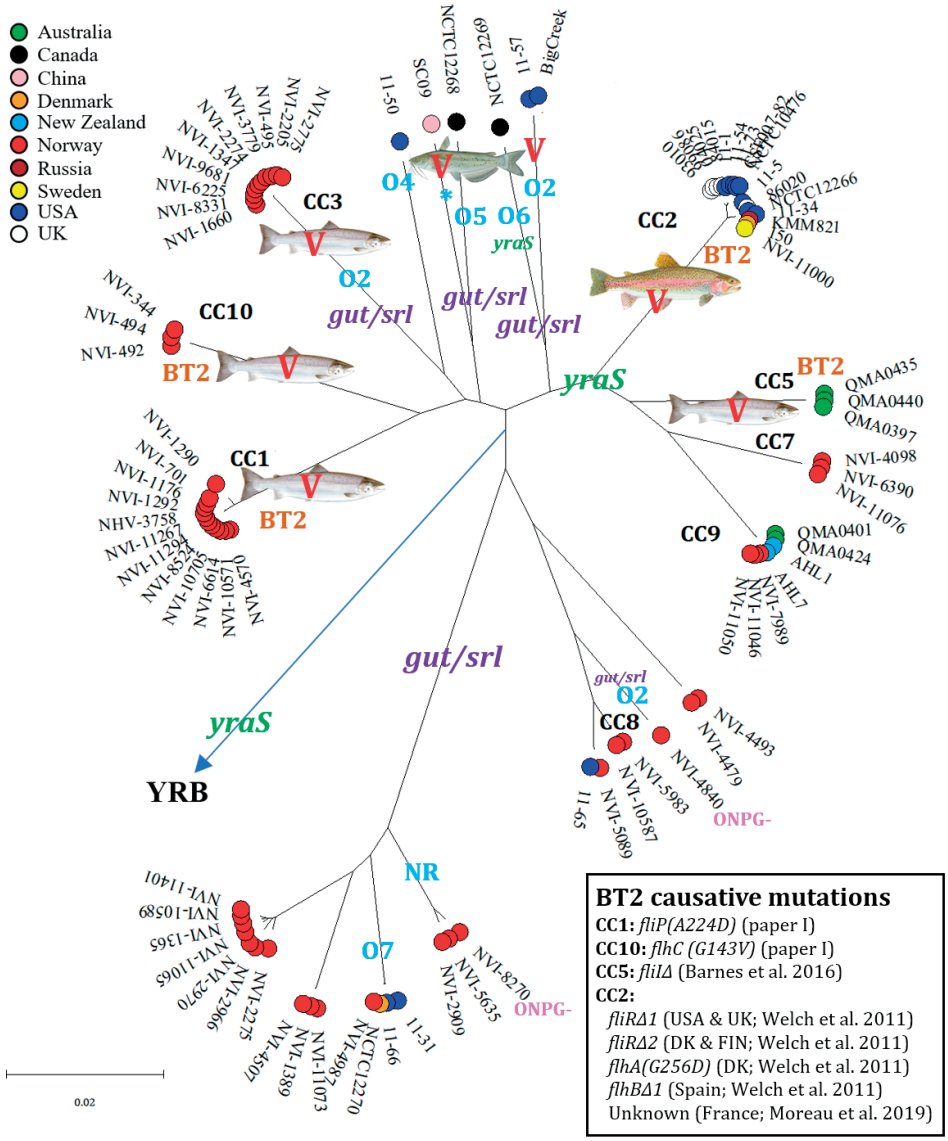

Figure 5: Modified Figure S1 from paper III. Non-O1 serotypes have branches labelled with serotype in blue. NR represents no reaction. SC09 has an unknown non-O1 serotype. Branch labels also indicate presence of sorbitol utilization genes *gut/srl* and SDS-degrading alkyl sulphatase encoding *yraS*. A red 'V' indicates virulent lineage (CC3 is considered mildly virulent). Primary host species for prominent virulent lineages are indicated by images depicting *S. salar*, *O. mykiss* or *I. punctataus* (SC09). Ocurrences of BT2 are indicated. Blue arrow indicates branch to the YRB 'outgroup'. See paper III for more details regarding hosts, phenotypes and genotypes.

Disease in rainbow trout in the USA, UK and mainland Europe is dominated by a single lineage of closely related isolates, clonal complex (CC) 2, which is further subdivided into sub-complexes CC2a, b and c. Independent occurrences of BT2 are present within each sub-complex. The mainland Europe genotype, CC2c, is shared by some older isolates from the USA, suggesting at least three independent exchanges of the CC2 lineage between USA and Europe; two exports from USA, to the UK and mainland Europe respectively, and further exchange of CC2 BT2 between USA and UK. The mainland European sub-lineage is also present in Peru. Isolates from disease in Atlantic salmon are more diverse, with discrete lineages generally displaying a geographically limited distribution in Norway (CC1), the UK (CC4) and Australia (CC5) (Gulla et al. 2018; Figure 6). Salmon pathogenic CC5 isolates do not cluster with any lineages known to be present in North America or in Europe (Gulla et al. 2018), but are closely related to a sequenced isolate from Chile, possibly related to transfer by salmon eggs imported by Chile from Australia between 2005 and 2012 (Barnes et al. 2016). Besides these dominant virulent clones, all of which are serotype O1, a large variety of genotypes exist that are considered less virulent or avirulent, including other serotype O1 and serotype O2 and the various less common serotypes (Barnes et al. 2016; Gulla et al. 2018).

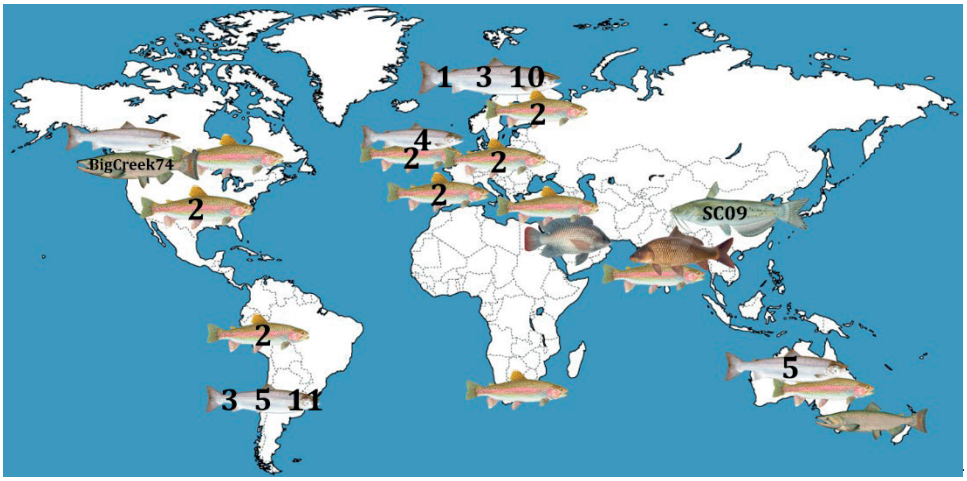

Figure 6: Select occurrences of *Y. ruckeri* in aquaculture globally. Host species are indicated by images depicting salmonids *S. salar*, *O. mykiss* and *O. tshawytscha*, and *O. niloticus* (Egypt), *Labeo* spp. (India) and *I. punctataus* (China). See Table 1 for references. MLVA clonal complexes (1, 2, 3, 4, 5, 10, 11) are indicated according to geography and host species in which they primarily occur (Gulla et al. 2018; paper I; Snorre Gulla pers. com.) and strains BigCreek74 and SC09. MLVA CCs 4 and 11 are not represented in paper III and Figure 5.

1.9 Virulence determinants

As mentioned, there exist strains of *Y. ruckeri* regarded as less virulent or avirulent (Verner-Jeffreys et al. 2011; Barnes et al. 2016). While strains isolated from one host species may be virulent in another, this is not always the case, as virulent strains seem to display a significant degree of host specificity (Haig et al. 2011). However, such differences in virulence are not currently associated with the presence/absence of any specific genetic determinants, outside of the general association between serotypes (Bullock et al. 1978; McCarthy & Johnson 1982) and perhaps the presence of large plasmids (De Grandis & Stevenson 1982; Stave, Cook & Roberson 1987). Plasmid content was initially studied due to the interest in the *Yersinia* virulence plasmid (pYV) as an essential virulence factor in human pathogenic *Yersinia* species (Cornelis et al. 1998). Virulent *Y. ruckeri* strains were found not to harbour pYV (Guilvout et al. 1988), but rather held the large (50-70 Mda, corresponding to 75-105 Kbp at 1500 bp/Mda) *Y. ruckeri*-specific plasmid pYR3, present in the CC2 lineage (Méndez et al. 2009; Nelson et al. 2015), and the very similar pYR4 present in CC1 (Wrobel et al. 2018b). This plasmid has been implicated in virulence as mutants with knockout of various essential components of the conjugative Type IV secretion system displayed moderately reduced LD_{50} values in trout (challenge by IP injection), although these genes were believed to be located in the chromosome at the time (Méndez et al. 2009). However, some virulent strains do not carry plasmids, including the Australian Atlantic salmon pathogenic lineage CC5 (Barnes et al. 2016).

Several *Y. ruckeri* virulence factors represent effectors secreted into the surroundings, and injection of fish with extracellular products from a *Y. ruckeri* culture results in haemorrhage and necrosis at the injection site (Romalde & Toranzo 1993). The YhlA haemolysin is responsible for haemolytic activity *in vitro* (observable only in liquid culture conditions, not on blood agar), presumably also *in vivo*, and YhlA mutants display severely reduced virulence (Fernández, Prieto & Guijarro 2007). This haemolysin is not known from other *Yersinia*, and was presumed acquired from *Serratia* where similar haemolysins are present (Fernández, Prieto & Guijarro 2007), but the most simmilar homologues are present in the more recently described *Yersinia* species *Y. nurmii* and *Y. entomophaga* (personal observations).

The Yrp1 protease is another notable extracellular effector which may hydrolyse a range of tissue-associated proteins (Secades & Guijarro, 1999). Proteolytic activity is severely affected in mutants, as is virulence, and Yrp1 activity may cause leakage of blood through micro-haemorrhages in capillaries, i.e. the primary symptom of the disease (Fernández et al. 2002; Fernández et al. 2003).

As in many other pathogens of vertebrates, *Y. ruckeri* possesses a siderophore ('ruckeribactin') iron acquisition system, and mutants display a 100-fold reduction in LD_{50} (IP injected) (Fernández et al. 2004). The high-affinity zinc transporter ZnuABC has been identified in *Y. ruckeri*, with mutants displaying no growth defects *in vitro* but perform poorly in competitive challenge by immersion (Dahiya & Stevenson 2010a). Several metabolic systems, secreted enzymes and some outer membrane proteins have also been linked to virulence through experimental observations, i.e. reduced or attenuated virulence in mutants, although their specific induvidul contribution towards virulence seems to be related to general nutrient aquisition or is otherwise unknown (Table 4).

The SDS-degrading alkyl sulphatase YraS was once regarded as a virulence factor due to its general association with virulent strains (present in CC2), but many virulent strains also lack it (Navais et al. 2014a), including CC1 (personal observations).

A prominent virulence factor unique to *Y. ruckeri* within the *Yersinia* genus is the T6SS-like Anti-feeding prophage, which is also found in *Serratia* species (Heymann et al. 2013; Hurst et al. 2018). The catalytic domain of the putative toxin component AFP18 disrupts the actin cytoskeleton by depolymerizing actin microfilaments in zebrafish cells (Jank et al. 2015), but no specific studies have been performed to assess whether Anti-feeding prophage is required for *Y. ruckeri* virulence.

Table 4: Modified Table 2 from paper III, distribution of characterised and putative virulence factors in *Yersinia ruckeri*.

Name	Classification	Reference	Distribution
yrp1	Protease	Secades & Guijarro, 1999	Core
yrpAB	Peptidase	Navais et al. 2014b	Core
yhlBA	Haemolysin	Fernández et al. 2007	Core
Yst2	Type II secretion	Liu et al. 2016	Core
AFP	Type VI-like secretion	Jank et al. 2015	Core
Ruckerbactin	Iron acquisition	Fernández et al. 2004	Core
cdsAB	L-cysteine acquisition	Méndez et al. 2010	Core
znuABC	Zinc acquisition	Dahiya & Stevenson 2010a	Core
barA-uvrY	Response regulator	Dahiya & Stevenson 2010b	Core
ompF	OMP	Wang et al. 2018	Core
filamentous hemagglutinin	OMP	Ormsby et al. 2019	Core
Yst1	Type II secretion	Liu et al. 2016	Shell
Ysa	Type III secretion	Gunasena et al. 2004	Shell
yrIlm	IAT-invasin	Wrobel et al. 2018a	Shell
yrInv	IAT-invasin	Wrobel et al. 2018a	Shell
Unnamed invasin	IAT-invasin	Paper III	Shell
yraS	HSF/SDS	Furones et al. 1990	Shell
Biofilm polymer	Secreted polymer	Paper III	Shell
Tc	Toxin complex	Barnes et al. 2016	Shell
VirB/VirD4 T4SS	Type IV secretion	Liu et al. 2020	Shell
Novel invasin	IAT-invasin	Paper III	Cloud
STIR, *tcpA*	Secreted proteins	Liu et al. 2020	Cloud

Core indicates presence in 100%, shell in 15-95%, and cloud in less than 15% of genome assemblies (n=86) in paper III.

A series of papers have recently highlighted the presence of autotransporter-invasin-like genes in *Y. ruckeri* (Wrobel et al.2018a; Wrobel et al. 2020). Such proteins display an intricate mechanism for self-export, with one end of the protein becoming embedded in the outer membrane and facilitating export of the other end of the same protein. The external structure is in some cases unusually large for a single bacterial protein (Leo et al. 2015), and these proteins are sometimes referred to as biofilm-associated proteins due to their involvement in adhesion between cells and surfaces during formation of biofilms (Latasa et al. 2006). In *Yersinia* they are primarily associated with adhesion to host cells (Leo & Skurnik 2011), with perhaps the most well known being YadA, which reside on the pYV plasmid and is essential for *Y. enterocolitica* virulence (Mühlenkamp et al. 2015). Knockout experiments have shown the putative invasins of *Y. ruckeri* to contribute to biofilm formation and virulence (LD_{50}, injected) in greater wax moth (*Galleria mellonella*) larvae (Wrobel et al. 2020).

Another series of publications focused on putative secreted effectors of a chromosomally encoded VirB-type type IV secretion system. These effectors are however exclusively present in strain SC09 associated with disease in channel catfish in China (Liu et al. 2019; Liu et al. 2020).

Some virulence factors quite prominent in other species, including other *Yersinia*, are known to exist in *Y. ruckeri* but have not yet been studied in any detail. These include the Yst1 and Ysa type II and III secretion systems (Gunasena, Komrower & Macintyre, 2003; Liu et al. 2016), both of which are required for full virulence in *Y. enterocolitica* (Haller et al. 2000; Iwobi et al. 2003; Venecia & Young 2005), and Toxin-complex (Tc) genes (Barnes et al. 2016; Cascales et al. 2017) which in other *Yersinia* species are associated with cytotoxic effects in host cells (Tennant et al. 2005; Hares et al. 2008).

Several virulence factors are subject to temperature-dependant regulation, such as YhlA haemolysin, Yrp1 protease, and siderophore-mediated iron acquisition, which are all upregulated *in vivo* at 18°C relative to the optimum growth temperature of 28°C (Fernández, Méndez & Guijarro 2007). Another relevant environmental cue seems to be limited iron availability which induces expression of haemolysin and siderophore synthesis genes (Fernández, Méndez & Guijarro 2007).

While older publications tend to focus on the intestines as both a point of entry and localization during subclinical infections, more recent studies point to the gills as a likely point of entry into the circulatory system. The bacterium is present internally in gill tissues almost immediately post-bath challenge, but specific underlying mechanisms for invasion are currently unknown (Tobback et al. 2009; Ohtani et al. 2015). Like the human pathogenic *Yersinia* species (Pujol & Bliska 2005), *Y. ruckeri* is considered a facultative intracellular pathogen which may survive and intermittently reside within macrophages (Ryckaert et al. 2010), and readily invades various types of fish cells *in vitro* although the role of host cell invasion during infection is not known (Tobback et al. 2010; Menanteau-Ledouble et al. 2018; Menanteau-Ledouble et al. 2020). The specific mechanisms involved in *Y. ruckeri* passing internal host barriers and interaction with host defences are generally not known.

1.10 Yersiniosis in Norway

While yersiniosis is primarily associated with rainbow trout internationally, in Norway the disease occurs almost exclusively in Atlantic salmon with a low number of occurrences in Arctic char (*Salvelinus alpinus*) (Gulla 2017; Gulla, Gu & Olsen 2019). This is notable as Norway is also a prominent producer of rainbow trout, which constitutes ca 6% of the ~1.3 million tonnes total salmonid production per year (Lindland et al. 2019; Baklien 2020). This situation is perhaps similar to that in Australia where yersiniosis also occurs primarily in Atlantic salmon despite rainbow trout and other salmonids also being produced (Barnes et al. 2016), and a standing example of the apparent host-specificity that seems to exist between the various pathogenic *Y. ruckeri* lineages.

The earliest documented instance of ERM in Norway was an outbreak in Atlantic salmon at a marine ongrowing site in 1985, described as a sudden increase in mortality post-handling (Sparboe et al. 1986). The disease was shortly thereafter identified at several locations along the coast, with more than 50 cases registered in 1987 (Figure 7). Although the two most frequently cited Norwegian case studies, Sparboe et al. (1986) and Willumsen (1989), described outbreaks in Atlantic salmon at sea, the norm through the late-1980s, 1990s and 2000s were recurring acute outbreaks in freshwater, in Atlantic salmon hatcheries (Brun & Bornø 2010).

It should be noted that yersiniosis has never been a notifiable disease in Norway. Sustained low level mortalities ('chronic conditions') in freshwater are less likely to be reported/registered, and these are known to having occurred also around the turn of the millennium, even though the statistics do not reflect this. On the other hand, acute outbreaks in large fish at sea are quite notable events for which veterinary services and/or relevant authorities are more likely to be contacted regarding treatment and general support, and as such, the increasing trend in occurrences at sea during the 2010s does indeed reflect conditions in the field (Duncan J. Colquhoun pers. com.).

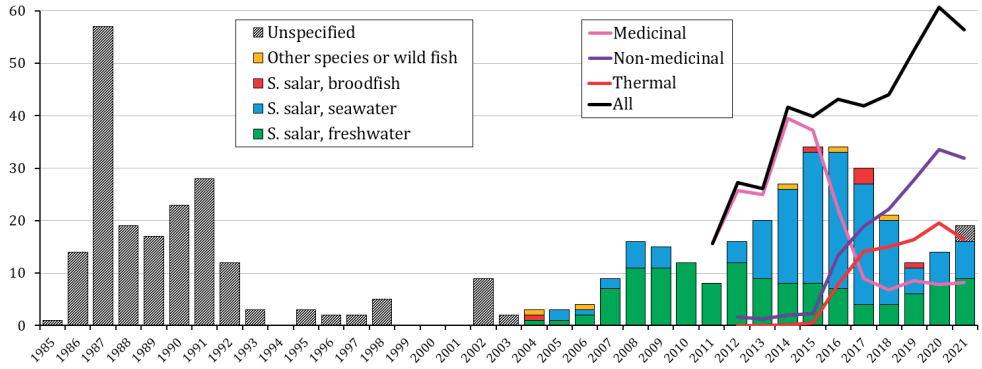

Figure 7: Number of *Salmo salar* aquaculture sites in Norway with detection of *Yersinia ruckeri* per year registered by the Norwegian Veterinary Institute, and cases involving other species in aquaculture (e.g. *Salvelinus alpinus*) or the wild (including *S. salar*). Unspecified cases relate mostly to *S. salar* aquaculture. Number of registered salmon lice treatments per year according to Stige, Qviller and Helgesen (2022) is overlayed. Non-medicinal treatments include thermal. The total number of treatments in 2021 was 4275.

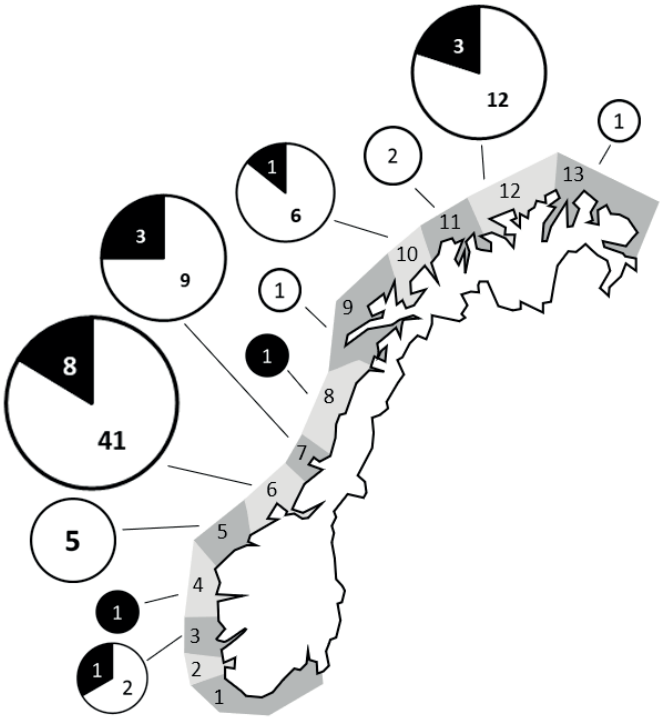

Figure 8: Map showing the geographic distribution (as defined by the aquaculture production zones 1-13) of Norwegian salmon farms with isolation of *Y. ruckeri* CC1 confirmed at NVI from 2012 to 2021, in freshwater (black) and seawater (white). Figure by Snorre Gulla.

Norwegian aquaculture started in the late 1960s, gradually grew to become one of Norway's most economically important industries, and Norway is currently the world's largest producer of salmonids (Lindland et al. 2019; FAO 2022). The 1980s marked a turning point in Norwegian aquaculture, with sustainability of the industry as a whole threatened by ever-increasing disease-associated losses and animal welfare issues, remediated by unacceptable levels of antibiotics use. A number of general zoo-sanitary measures were introduced during this period, including several relating specifically to eggs and juveniles in the freshwater phase, e.g. depth requirement or disinfection of intake water, double-disinfection of eggs and hygiene barriers between age groups, enforced by license requirements on production units (Midtlyng, Grav & Horsberg 2011). Such improvements in husbandry likely played a significant positive role in management of yersiniosis and other diseases, as did the introduction of vaccines against bacterial diseases, primarily vibriosis (*Vibrio anguillarum*), coldwater vibriosis (*Vibrio salmonicida*) and furunculosis (*Aeromonas salmonicida*), resulting in a significant reduction in the use of antibiotics in Norwegian aquaculture during the early 1990s (Grave et al. 1999). *Y. ruckeri* was notably not included in any of the commercially produced multivalent vaccines, perhaps an attest to the relatively modest historical status of the disease in Norway, i.e. as a regionally limited disease of minor concern, generally managed through immersion vaccines at specific hatcheries experiencing recurring problems (Brit Tørud pers. com). This situation changed some time during the early 2010s, when despite efforts to control the disease through vaccination and sanitation efforts, incidents in both freshwater and at sea were steadily increasing. With the frequency of serious outbreaks with high mortalities in large fish at sea increasing dramatically, yersiniosis became one of the most significant bacterial diseases in Norwegian aquaculture (Hjeltnes 2014; Bornø & Linaker 2015; Gulla 2017). Treatment of yersiniosis in a low number of populations of rather large Atlantic salmon at sea in 2017 and 2018 resulted in the total use of antibiotics in Norwegian aquaculture being more than doubled compared to preceding years (Gulla, Gu & Olsen 2019; Lillehaug et al. 2019).

At least some proportion of these outbreaks in large salmon at sea were associated with delousing. Delousing treatment represent a stressful handling event, and experiences from the early-mid 2010s in Norway show that high mortality may follow delousing, although association with any specific bacterial or viral disease were generally unspecific or unknown (Hjeltnes 2014; Bornø & Linaker 2015; Grøtvedt & Jansen 2016). In recent years, non-medicinal delousing has become widely utilized as the salmon louse population became ever increasingly resistant to chemotherapeutics (Roth 2016; Overton et al. 2019). While effective in removing sea lice by physical or thermal treatment, such methods are also considered stressful for the fish, and outbreaks of various diseases may follow such treatments, including yersiniosis specifically (Gulla, Gu & Olsen 2019; Nilsson et al. 2019; Overton et al. 2019; Sviland Walde et al. 2021; Folkedal, Utskot & Nilsson 2021).

Presumably, immunity awarded by immersion vaccines administered in the freshwater phase is not sufficient, or the effect is too short-lived, to reliably prevent outbreaks of yersiniosis at sea when a significant stressor is also added e.g. sea transfer or delousing. Fortunately, trials with administering the immersion vaccine as IP injection proved effective in preventing yersiniosis outbreaks at sea, and this practice had been broadly adopted by hatcheries in the most affected areas in the north and mid-west (Figure 8) by 2018/2019 (Gulla & Olsen 2020). The number of registered outbreaks at sea became significantly reduced post-widespread adoption of IP vaccination, with the disease once again becoming primarily an issue during the freshwater phase of production. Notably, no antibiotics were prescribed to treat ERM in 2019, bringing the use of antibiotics in Norwegian aquaculture back down to the norm from prior years (ca. 250 kg/year) (Gismervik et al. 2020). While the number of incidents seem to be increasing yet again in 2020 and 2021, this may be due to additional surveys performed in recent years, or relaxation in IP vaccination following reduction of outbreaks, and the situation is being carefully monitored (Gulla & Olsen 2022).

2 Current challenges, aims and objectives

In recent years, yersiniosis has become a serious challenge in Norwegian aquaculture. The disease has been increasingly affecting large fish at sea, resulting in substantial economic losses and at times an unfortunate increase in the use of antibiotics. While a full-blown crisis was averted by introduction of widespread IP vaccination during the present study period, the underlying issues seem to persist. Some hatcheries now administer three separate vaccinations per generation, twice by immersion in addition to the IP injection administered when the fish have grown large enough, to prevent outbreaks of this disease. The stresses from handling and otherwise biological impacts on the fish associated with this practice, as well as the costs, are substantial. While effective as a short-term solution, such practices becoming the default regiment for the entire industry would be far from ideal, especially if similar immunization regimes are to be required to handle future bacterial diseases that have yet to emerge. Thus, *Y. ruckeri* still represents a major fish-health issue for Norwegian aquaculture.

Detection and screening

Y. ruckeri is seemingly able to colonise a wide variety of niches relevant to Norwegian aquaculture. Presumably, the pathogen may reside in the water source, in production environments, or be sustained as subclinical infections in fish stocks. Thus, detailed knowledge on the prevalence and mechanisms behind spread and establishment in such niches are essential to prescribe sensible and accurate countermeasures locally, and to prevent further dissemination of the pathogen in Norwegian aquaculture. Such investigations are critically reliant on effective screening tools. As isolation of the organism from the environment is not trivial, direct-PCR strategies are preferred. Existing PCR analyses are known to frequently be positive at sites regarded as yersiniosis-free, likely due to detection of putatively avirulent *Y. ruckeri* strains also inhabiting aquaculture environments. A possible solution is to develop PCR assays that specifically target virulent strains.

Aim 1: Develop and employ PCR assays to investigate the presence of Y. ruckeri clonal complex 1 in Norwegian aquaculture.

Virulence factors

Substantial efforts have gone into investigating specific virulence factors of *Y. ruckeri*. However, outside of plasmid-profiling and general observations relating to serotype, little effort has been made to investigate the genetic basis for the variation in virulence potential between *Y. ruckeri* strains associated with acute outbreaks, and those which are considered less virulent or avirulent. As virulence potential across host species has been demonstrated, the presence of common virulence mechanisms are expected, presumably relating to genetic determinants specific to virulent strains which should be possible to identify through comparative genomics. Revealing such accessory, critical virulence determinants should better our understanding of yersiniosis etiology and may prove useful in terms of differentiation or specific detection of virulent strains. Such knowledge also has potential value in targeted breeding efforts and in development of more efficient vaccination strategies.

Aim 2: Reveal the genetic determinants that differentiate virulent and avirulent Y. ruckeri strains

Biotype 2

The effective management of yersiniosis in Norway through the 1990s and early 2000s should, in hindsight, be considered highly successful for the industry as a whole. While both the previous successes and the current substantial challenges are likely multi-factorial, a contributing factor could potentially be the occurrence of a shift in biotype of the pathogen. While any substantial biological advantage of BT2 has yet to be demonstrated experimentally, one or several slight advantages may be able to tip the scales in favor of the pathogen in intensive salmonid aquaculture. One such mechanism could be a reduction in the efficiency of immersion-style vaccines. BT2 may also relate to advantages yet to be thoroughly tested, like survival and spread in the environment or in secondary hosts. Nonetheless, given the general association of BT2 with increased challenges in management of the disease internationally, with the proportion of non-motile strains ever increasing in North America and the rest of Europe, it is highly relevant to investigate whether such emergences have occurred in Norway.

Aim 3: Investigate the presence and impact of Y. ruckeri biotype 2 in Norway.

3 Summary of articles

Paper I: Biotyping reveals loss of motility in two distinct *Yersinia ruckeri* lineages exclusive to Norwegian aquaculture

Two hundred and sixty-two *Y. ruckeri* isolates originating from Norway between 1985 and 2020 were biotyped. Amongst these was the non-motile isolate described by Sparboe et al. (1986). MLVA typing of this and a number of additional isolates from this era revealed them to belong to a distinct clonal complex, designated CC10. A CC10 sub-cluster consisting of four isolates, recovered from northern Norway between 1985 and 1987, were all non-motile. An additional three non-motile isolates recovered from two different sea-farms in mid-western Norway in 2017, belonged to the currently dominating virulent clone CC1. The causative mutations were identified by WGS in a comparative analysis as non-synonymous SNPs in essential flagellar genes; *flhC* in isolates from the 1980s, and *fliP* in isolates from 2017. As unmistakably motile CC1 isolates would often display negative lipase reactions, the lipase assay was found unreliable in biotyping of Norwegian isolates. Though motile, such isolates also seemed to display reduced motility on motility agar. As a stable, strong lipase reaction and vigorous motility could eventually be induced by subculture, a semi-hereditary regulatory mechanism is indicated. In non-motile isolates, prolonged incubation on motility agar could trigger a biotype reversion by spontaneous mutation in the exact BT2-inducing SNP, which was eventually achieved for both Norwegian BT2 variants and verified by Sanger sequencing. Motility was also restored by complementation with wild-type alleles *in trans*. While BT2 does not seem to have become established in Norwegian aquaculture, this study was able to document BT2 emergence in two virulent *Y. ruckeri* lineages that affects Atlantic salmon, in addition to the previously documented BT2 occurring in Atlantic salmon in Australia (CC5) and the multiple independent occurrences in rainbow trout lineage CC2 across North-America and Europe. Thus, development of *Y. ruckeri* BT2 seems to be a general evolutionary path not only in strains affecting rainbow trout, but also amongst highly virulent strains in salmonid aquaculture in general.

Paper II: qPCR screening for *Yersinia ruckeri* clonal complex 1 against a background of putatively avirulent strains in Norwegian aquaculture

qPCR assays respectively specific for *Y. ruckeri* and *Y. ruckeri* CC1 were developed and employed in screening of a diverse range of environments and sample types relating to Atlantic salmon aquaculture in Norway. Retrospective screening of preserved DNA extracts from environmental samples collected at Atlantic salmon hatcheries in 2017/2018 revealed such samples to be frequently positive for non-CC1 *Y. ruckeri*. These results serve to explain the high frequency of positive PCR screening results at locations that had not experienced yersiniosis-related issues. Additionally, a number of freshwater hatcheries were screened, several of which were found positive for *Y. ruckeri* CC1, although this could be related to use of yersiniosis vaccines in the facility. Such vaccine-related 'false' positives recurred consistently for several weeks post-vaccination, and may presumably occur sporadically for a substantially longer period, which limits the utility of environmental PCR screening of the environment in facilities that frequently employ such vaccines. Thus, this study highlighted issues relating to PCR screening of aquaculture environments that should be considered prior to employing such procedures, or in interpretation of screening results. The assays were further used to document shedding of high concentrations of *Y. ruckeri* CC1 from Atlantic salmon subjected to thermal delousing treatment in the field. This was replicated in a challenge trial by establishing sub-clinical infection in Atlantic salmon in the freshwater phase, with corresponding levels of shedding observed when the fish were subjected to a simulated thermal delousing treatment post-transfer to seawater. This result highlights stressful treatment and handling in general, and thermal delousing operations specifically, as high-risk in regard to biosafety, and a suitable sampling-site for assessment of subclinical *Y. ruckeri* (and possibly also other) infections.

Paper III: Pan-genome survey of the fish pathogen *Yersinia ruckeri* links accessory- and amplified genes to virulence

This study set out to reveal genetic differences between virulent and avirulent *Y. ruckeri* strains, to discover which determinants are responsible for the virulent phenotype. For convenient study of larger functional units such as secretion systems, plasmids, and virulence factors which consist of long and complex repeated sequences, this work was based on complete circular genomes. Sequences were downloaded from NCBI or generated by an Illumina/nanopore hybrid assembly approach, and covered a diverse set of genetic lineages including highly-, moderately- and non-virulent representatives. Discovery of accessory genetic determinants was achieved by stepwise construction of the pan-genome. Accessory elements deemed as relevant due to distribution or classification, and any previously described virulence determinants, were screened *in silico* with BLASTn against a larger panel of *Y. ruckeri* genome sequences (complete and non-complete/draft sequences) to further verify the findings. The distribution of several genetic determinants correlated well, but with some variation, with the virulent phenotype and/or host-specificity. However, only the inverse-autotransporter invasin encoding *yrIlm* was present in all virulent strains, including moderately virulent serotype O2 and those affecting non-salmonids, while absent in all lineages regarded as avirulent. The well-documented highly virulent lineages, clonal complexes 1 and 2, were found to display duplication of the *yrilm* locus. Duplication seems to have evolved over time in CC1, as early isolates display a single copy while isolates from post-2000 display two or three. The genetic basis for O1-LPS deficiency, associated with reduced virulence potential of serotype O2 strains, was found to be a common trait of all non-O1 serotypes. Thus, *yrIlm* seems to be required for virulence in general, while serotype O1 LPS and *yrIlm* duplication are traits associated with particularly *high* virulence. Certain accessory features are overrepresented in virulent lineages and may conceivably contribute positively to virulence, such as a chromosomal type IV secretion system, toxin complex genes, and the large and highly similar plasmids pYR4 and pYR3 uniquely present in CC1 and CC2 respectively.

4 Methodological considerations

4.1 Strains and culture

The NVI strain collection

Bacterial isolates were generally sourced from the NVI strain collection of fish pathogenic bacteria, consisting of historical and contemporary isolates primarily from Norway, collected for diagnostic purposes or research, and a number of reference strains and isolates shared by international research partners. The current primary method for species verification is MALDI-TOF, although early isolates were speciated/serotyped using biochemical-based testing, serology and 16S rDNA sequencing. The *Y. ruckeri* collection is especially well curated, currently consisting of ca 1100 isolates. Detailed metadata are available for many isolates, as well as relevant typing data including serotype, biotype, MLVA and WGS, all of which were continually improved upon during the course of the present study in conjunction with this and other ongoing projects.

Culture and isolation

Y. ruckeri grows well on many general-purpose agars and liquid media, tolerates cryopreservation well and is easy to resuscitate by direct plating. Blood agar (BA) is often used as it provides some differential value between α, β and γ hemolytic colonies. Culture of *Y. ruckeri* is relatively straightforward during diagnostic investigation of clinically affected fish, by direct plating from kidney tissues on general agar media. Kidney smears from severely affected or dead fish may yield mixed culture with various background growth (e.g. *Aeromonas hydrophila*, personal observations), but these are usually easily differentiated on the basis of colony appearance on BA. *Y. ruckeri* in the NVI strain collection originating from Norway have generally been recovered by such direct plating techniques on 5% bovine BA.

In the present study, isolation of putatively avirulent *Y. ruckeri* from the environment was desired, both in relation to PCR screening and comparative genetic analysis. Isolation of the bacterium outside of a clinical context does however present a considerable challenge, especially when working with environmental samples where a multitude of other *Yersiniaceae* species may exist in

large numbers. Many of these display practically identical growth characteristics to *Y. ruckeri*, as well as similar general morphology and metabolic profiles. While some differential agars have been formulated to ease isolation of *Y. ruckeri* they tend to rely on biochemical properties for which *Y. ruckeri* is variable. One such example is the agar described by Waltman and Shotts (1984) which relies on differentiation by sucrose (acid production) and lipase activity (tween). The inherent flaw of relying on a positive lipase reaction to identify *Y. ruckeri* has been obvious ever since the description of BT2 (Figure 2, another photograph of this agar in Balta & Balta 2019). Even in the current situation in Norway, with BT2 being quite rare, the variability in lipase activity on agar displayed by many CC1 isolates represents an additional deterrent from relying on this reaction for species differentiation. SDS has also been used as a differential component, and doubles as a selective component. While *Y. ruckeri* isolates seem equally resistant to SDS, many lineages, including those most relevant in Norway (CC1 and CC3), are negative for the SDS degradation trait and thus appear as background flora on SDS-containing agars such as the ribose ornithine deoxycholate (ROD) agar described by Rodgers (1992) (Figure 2). The usefulness of this agar is thus limited to the isolation of SDS-positive lineages such as CC2 (rainbow trout world-wide) and CC5 (Atlantic salmon in Australia).

Relying on direct plating on BA, a number of isolates that resembled *Y. ruckeri* in physical appearance and a limited number of biochemical tests (sucrose, arabinose, rhamnose, and eventually esculin which was notably quite usefull) were recovered from environmental swab samples, but turned out to be other *Yersinia* spp. or undescribed *Yersiniaceae* (by MALDI-TOF and/or sequencing). Selective and differential components could possibly be employed to exclude or differentiate a number of such non-target species, but further efforts were abandoned as it became increasingly evident that culture would not become suitable for screening purposes notwithstanding significant improvement. Ultimately, isolation was deemed not essential for going forward as some environmental isolates were already available from the NVI strain collection. Isolation from ovarian fluids was less challenging, presumably due to a higher concentration of *Y. ruckeri* relative to problematic background, and a number of such isolates were recovered from sites which regularly screen such material for *Y. ruckeri*. Such aquaculture-related isolates not related to clinical disease, like those recovered from farm environments, are evidently present in aquaculture while genetically distinct from disease-causing

lineages (by MLVA), and thus also generally considered as avirulent. Some isolates in the collection have been recovered from screening of healthy fish, in aquaculture or in the wild, and are as such not associated with clinical yersiniosis even though a species of fish is listed as the biological source. The isolate from seagull described by Willumsen (1989) is a notable exception as it has a clear association with yersiniosis outbreaks that were ongoing in the area at the time, which is confirmed by MLVA placing it in the CC1 lineage.

4.2 Biotyping

Assaying flagella-driven motility

The primary method for assaying flagellar motility is semi-solid 0.3% agar plates. This assay is generally not affected by non-flagellar locomotion such as the pilin-driven twitching motility present in isolates of both biotypes. There are two general precautions to keep in mind with this agar. The first is rather obvious as the agar is not fully solid, requiring plates to be kept upright at all times. The second, which is perhaps less straightforward, is to maintain an appropriate level of humidity to prevent the agar from drying out, while also preventing condensation as non-flagellated isolates may migrate by twitching motility through droplets or low-viscosity areas otherwise on the agar surface. Storage in room temperature and leaving plates unstacked overnight to dry the agar surface proved useful for consistent results. To prevent the plates from drying out during extended incubation, when aiming to induce biotype reversion, the plates were individually sealed with parafilm post-inoculation and placed in a plastic container together with a 100ml beaker of water. Plates were prepared in a laminar flow clean bench to prevent air contamination (moulds) which may appear on agar after prolonged incubation. The initial discovery of biotype reversion following prolonged incubation was incidental. As initial attempts to reproduce these results were foiled by issues relating to contamination and humidity, it seems that some luck was involved, in addition to failure in disposing of the agar plates in a timely fashion. While spontaneous reversion may likely occur under standard assay conditions, such mutants will need some time to multiply and spread, to successfully escape the densely populated colony of non-motile cells to become readily visible. Given the observed durations in successful reversion experiments of >1 week in all cases, 48

hours incubation seems like a sensible maximum duration to prevent misidentification due to spontanious reversion during incubation. This should also leave plenty of time for an initially motile strain to demonstrate unmistakable motility. Moreover, as the migration and proliferation of spontaneous mutants expand from a single point of a non-motile colony, and are guided by quorum sensing to escape the densely populated origin colony, the expanding circular zone of reverted motile bacteria should display an obviously asymmetrical pattern (Figure 9, B) in contrast to the near-perfect circular symmetry displayed by initially motile isolates (Figure 9, A).

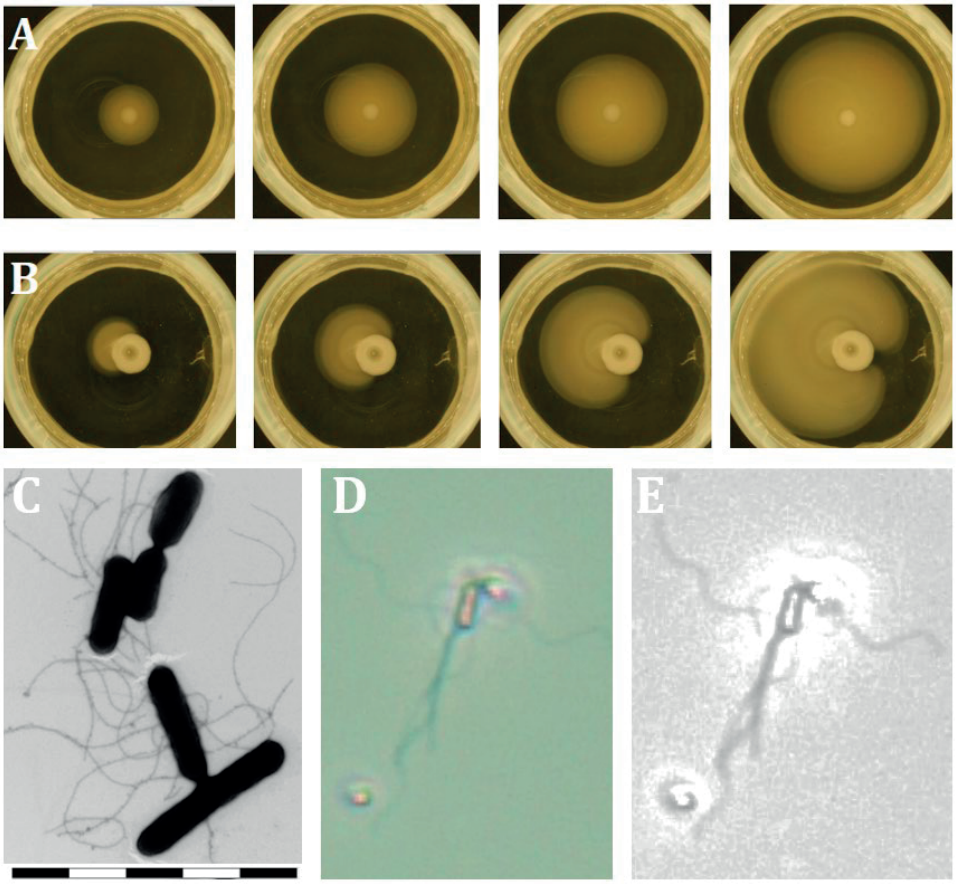

Figure 9: Timelapse photography series (~6 hours intervals) of *Y. ruckeri* biotype 1 (A) and biotype 2 with an emerging biotype reversion mutant (B). Electron microscopy (C, scale bar is 5 µm) and phase-contrast microscopy with a flagella stain as seen through the lens (D) and with adjusted color and contrast (E). Microscopy images show NVI-344 which is excessively flagellated due to plasmid complementation with the *flhDC* operon. (C). Photos: Andreas Riborg (A, B, D, E) and Jannicke Wiik-Nielsen (C).

Several methods are relevant for assaying the presence of intact flagella, such as electron microscopy, fluorescence microscopy (fluorescence *in situ* hybridization with fluorescently labelled anti-flagellin), or conventional microscopy paired with a flagella stain, all of which may reveal the presence of flagella visually. Agglutination with anti-flagellin and protein electrophoresis/blotting are other options although an intact flagella structure is not revealed directly/visually. While flagella are expressed in motile *Y. ruckeri* when cultured under refrigeration, they are not functional at these temperatures (O'leary, Rohovec & Fryer 1979). Conceivably, some mutations could disable flagellar activity at higher temperatures, resulting in a non-motile flagellated phenotype which would not be revealed by any of these techniques. Thus, while such methods are suitable to complement semi-solid agar they should not replace it.

Flagella were initially observed by electron microscopy. While flagella were indeed absent in non-motile isolates, producing consistent images highlighting the presence of flagella in motile strains was challenging due to their frequent detachment and/or breakage, which likely occurred during preparation. With electron microscopy being performed externally and eventually subject to covid restrictions, efforts to optimize were not further prioritized as an alternative method, phase-contrast microscopy with a flagellar stain, was possible to perform in-person and on-site. As flagella are too thin to be visible with a conventional microscope, a stain is used to thicken the flagella by oligomerization of large organic molecules which are then stained, in this case with crystal violet (Heimbrook, Wang & Campbell 1989). The process is, however, critically reliant upon favorable conditions for oligomerization, with no oligomerization is occurring if the prep is too moist, and uncontrolled crystallization occurs when the prep becomes too dry. Hence, the stain is only functional in a zone where conditions are just right, and only for a limited time as the preparation will eventually dry out and crystallize. Thus, when observing BT2 isolates with this technique it is sometimes difficult to know whether the lack of observable flagellated cells is due to the absence of flagella or unfavorable conditions for the stain. Frequent panning between crystallized and liquid zones was employed to alleviate such uncertainty, while comparing development in a BT1 control stained at the same time. Flagella are however unmistakable when they first do appear, and as the procedure involves direct staining of diluted viable cells there were no significant issues relating to damaged or detached flagella with this method.

Assaying lipase activity

Correlation between motility and lipase activity has served as the basis for non-motile strains being referred to as a distinct biotype rather than simply 'non-motile'. The inability to secrete lipase enzyme is however due to the exact same physiological trait, a nonfunctional flagellar type III secretion system (Young, Schmiel & Miller, 1999; Evenhuis et al. 2009). This connection between functional secretion of flagella and lipase is convenient as the lipase assay may serve to indicate specifically the secretion of flagella. As such, disagreement between the lipase and motility assays would indicate that something was amiss. Outside of mutations affecting expression of a functional lipase enzyme, a motile but lipase-negative isolate could indicate non-flagellar motility. The reverse case could result from flagella being assembled but inactive or non-functioning otherwise, meaning a novel mechanism for loss of motility. As such, the lipase assay has been considered reasonable as a control or supplement to the semi-solid agar motility assay. However, in employing the lipase assay for biotyping of isolates belonging to CC1, it was revealed that the lipase assay is not as robust a method. While various culture conditions and subculturing could seemingly modulate the activity of these isolates relating to motility, resulting in relatively minor but noticeable variation in swimming speeds, results with the lipase assay were affected in a qualitative manner, varying between strictly positive or negative between subcultures of the same isolate (unless incubated for e.g. 2 weeks as seen in Figure S1 in paper I). This inconsistency presumably relates to regulatory differences between subcultures, resulting in quantitative differences in lipase secretion, which fluctuates across the detection limit of the lipase assay, or possibly a more or less qualitative difference in secretion which is accurately represented by the assay. While subculturing procedures could be employed to resolve such issues, they would likely carry significant risk for biotype reversion and should therefore not be used as a general preparative step. Varying the incubation times and temperatures affected the general degree of precipitation but the qualitative results were unaffected. The bottom line here is that the lipase assay proved unsuitable for biotyping of isolates belonging to CC1, and biotype results in the present study therefore relied solely on the semi-solid agar motility assay, supported by microscopy for selected isolates.

Complementation analysis

As WGS-based comparative analysis revealed few differences between non-motile and motile variants, confidence was high in identifying the correct causative mutations based solely on sequence data. Pending some form of verification however, the possibility remains for other mutations being partly or fully responsible for the non-motile phenotype observed. A significant source of doubt in this case is the nature of the mutations, being non-synonymous SNPs for which the detrimental effect on protein function is less obvious than the frameshift-inducing indels reported in most other similar studies (Welch et al. 2011). While plasmid complementation or restoring the mutated gene to the wildtype allele are standard means for verifying genotype/phenotype correlations, performing such experiments may seem unnecessary in hindsight as the spontaneous reversion mutants were far simpler means of demonstrating such correlation. Complementation experiments were however already well underway when reversion mutants were observed and fully characterised.

Complementation utilising a plasmid vector *in trans* is generally less difficult than correcting the affected gene by making permanent alterations in the chromosome. While the latter is regarded as a more robust method, due to effects relating to competition from the putative faulty gene/protein and plasmid copy number (as likely observed and discussed in paper I), it was not considered pertinent in order to demonstrate correlation in this case if plasmid-mediated complementation was successful. As the *flhDC* operon is tightly orchestrated, the entire operon was supplied on a fairly low-copynumber plasmid, enough to offset the nonfunctional FlhC while limiting detrimental effects that would otherwise likely occur if a high-copynumber plasmid vector were to be used. Still, complemented cells were indeed excessively flagellated (Figure 9), as expected from increasing the copynumber of the master flagella regulator operon. As the expression of FliP is tightly regulated in concert with a number of other genes relating to the flagellar type III secretion system, utilizing its native promoter could quite possibly result in detrimental polar effects. Therefore, FliP was rather consecutively expressed by a weak promoter (the wildtype *bla* promoter from *E. coli*), which seemed to be just enough to offset the presence of non-functional FliP presumably still expressed from the chromosome.

There was a realistic possibility that complemented isolates actually represented reversion mutants, but with the BT2 phenotype restored post-curing of the plasmid vector in both cases we were able to demonstrate that this was not the case. Although the risk for spontaneous reversion is quite specific to these experiments, the ability to vary complementation in a strictly qualitative manner with relative ease, by the presence or absence of a plasmid, is a specific advantage which plasmid complementation has in general, over making permanent alterations to the chromosome which are as difficult to restore as they are to generate.

4.3 PCR-based detection

As culture-based detection of *Y. ruckeri* is generally not reliable from samples containing problematic background flora (Rodgers & Hudson 1984; Rodgers 1992; discussed above), PCR stands out as the only conventional and reasonable option for *Y. ruckeri* screening of environmental samples. PCR is also suitable for quantification purposes (with 'real-time'/qPCR or digital PCR), and comes with a possibility to target specific genetic determinants present exclusively in a particular lineage and may thus enable lineage-specific screening. Some previously described methods utilize conventional PCR for specific detection of *Y. ruckeri* (LeJeune & Rurangirwa 2000; Gibello et al. 1999, Altinok, Grizzle & Liu 2001; Temprano et al., 2001). This is generally not optimal for large-scale screening due to relying on visualization of the PCR product by agarose gel electrophoresis. Electrophoresis is omitted with qPCR, where the reaction is assessed by measuring fluorescence from a dsDNA-binding fluorescent dye or hydrolyzed fluorescently labelled probe for each cycle of amplification, thus allowing integrated detection of amplification while also enabling relative quantification (Kralik & Ricchi 2017). Probe-based qPCR is generally considered the more specific approach as the probe, much like the primers, relies on specific binding within the target sequence (Holland et al. 1991).

A general requirement for assays based on hydrolysis-probes (i.e. TaqMan-like probes) is a higher melting temperature for the probe than the primers, generally resulting in a quite long probe (Kutyavin et al. 2000). Probe design is subject to sequence requirements much like the primers, with some additional probe-specific requirements, which make probe design quite difficult in some cases where design space is limited (Rodríguez et al. 2015), as is the case with assays targeting 16S

rDNA or the limited stretch of CC1-specific sequence identified in paper II. Minor groove binder (MGB) probe technology does alleviate this to some degree, by significantly lowering the melt-temperature due to a 3'-end covalently bound MGB protein (which doubles as a quencher) and thus allowing for more design freedom for probe-based qPCR assays as the probe may be significantly shorter (Kutyavin et al. 2000).

Digital PCR was considered, specifically droplet digital PCR (ddPCR; Bio-Rad) which was available in the laboratory. Quantification with such systems is very straightforward as an estimation of targets per reaction is directly provided as a result. ddPCR generally has a higher reagent cost per sample (unless trading qPCR triplicates for singular reactions on digital PCR), and seemingly slightly reduced sensitivity (Johnsen et al. 2020; personal observations) which perhaps relates to loss of sample template during preparation (i.e. generating droplets). While analysing some of the sample material in parallel on both systems, it was eventually decided to use qPCR exclusively as ddPCR did not provide any significant advantages to justify its use in this project.

Species-specific assay

As many previously described PCR protocols and screening studies rely on 16S rDNA-based assays (Gibello et al. 1999; LeJeune & Rurangirwa 2000; Gosh et al. 2018), all of which report high specificity and advantages in sensitivity due to targeting a multicopy gene (n=7), such an approach seemed like a natural starting point for an assay specific to *Y. ruckeri* at the species level. However, it soon became evident that a proportion of samples from aquaculture environments, and indeed pure cultures of non-*Y. ruckeri* isolated from such samples, could generate false positives with such assays. This problem persisted even when pairing previously described primers (YER8 and YER10 from Gibello et al. 1999) with a novel probe design (FAM-TAATAGCACTGAACATTGACG-MGB) which binds to the *Y. ruckeri*-specific region immediately downstream of the forward primer. Following sequencing (Sanger) of the 16S rRNA gene from isolates causing such issues, it seems that false positive signals relate to detection of minority alleles, perhaps present in a single copy as judging from the Sanger chromatograms, which seem more similar to *Y. ruckeri* than to the majority, consensus sequence. The conventional PCR/Sanger approach is unfortunately not suitable to reveal the exact

sequence of each allele, and such minority alleles are thus likely not well represented in sequence databases. Another limitation of relying on sequence databases to assess specificity *in silico* is that environmental samples may contain a multitude of undescribed bacterial species that are generally not represented in relevant sequence databases. In addition to advantages regarding sensitivity from multiple copies of the 16S rRNA gene being present per bacterial genome, the popularity of 16S-based assay designs is largely based on historical reasons, being universally utilized for characterisation and identification of bacterial species for several decades. Access to broader sequence data was generally limited until relatively recently. While the 16S rDNA sequence is suitable for both identification and PCR detection of certain bacterial species, the highly conserved nature of these genes will often limit resolution to the genus level or even higher (Janda & Abbott 2007). For *Y. ruckeri* specifically, it seems clear that what were once believed to be *Y. ruckeri*-specific sequences are actually not so.

Nonetheless, any apparent issues relating to specificity were immediately resolved on targeting a *Y. ruckeri*-specific CDS (LuxR family transcriptional regulator; paper II). Although a lack of false positives from PCRs based on specific protein coding genes is by no means guaranteed, the chance of homologues in other species sharing significant sequence similarity at the DNA level is low as these genes generally evolve much quicker than 16S rDNA, except for cases where the gene in question is frequently mobilized between species. The target gene in the present study is not even present in sequenced relatives, further limiting the possibilities for false positives that could otherwise exist if targeting a widely present or housekeeping gene. With this assay, environmental samples were less frequently positive, but difficulties in isolating *Y. ruckeri* from positive samples to verify its presence persisted. A significant difference between this assay and the 16S-based assays, however, was that no amplification occured with any non-*Y. ruckeri* DNA templates tested. Moreover, samples which were positive with a fairly low Ct could be directly genotyped with the *Y. ruckeri*-specific MLVA protocol (Gulla et al. 2018), yielding complete- or near-complete profiles corresponding to known genotypes. No such specific MLVA PCR products could be generated from negative samples, nor from any of the non-*Y. ruckeri* isolates in pure culture.

While efforts at culturing *Y. ruckeri* from environmental swab samples were generally not successful, they were indeed quite useful for PCR development as novel isolates clearly demonstrated that 16S-based assays are not reliable with such sample materials. Indeed, the highly specific assays eventually developed revealed that a number of previous isolation attempts were based on samples that likely did not contain *Y. ruckeri* at all. If this was not the case, efforts in isolation could quite possibly have been more fruitful.

Lineage-specific assay

Most candidate target loci for a lineage-specific assay, i.e. sequences uniquely present in *Y. ruckeri* CC1, are mobile elements such as plasmids and prophage. As such elements are frequently mobilized they are likely to also exist outside of *Y. ruckeri* CC1 even though they do not necessarily appear in public sequence databases. The target chosen in the present study is situated close to a prophage that may have served as a vector for transfer of this gene. The gene is, however, not present in otherwise similar prophages inhabiting the same site in some other *Y. ruckeri* strains (e.g. strain QMA0440, CC5), and the gene itself seems quite rare according to public sequence databases. Nevertheless, to limit the possibility for detection of the same element in other species, the forward primer was set to target a *Y. ruckeri*-specific sequence immediately adjacent to the acquired gene, while the reverse primer and probe bind within the CC1-specific sequence. Highly conserved tRNA sequences, which likely serve as the prophage attachment site, are situated on the opposite side of the phage and are thus not targeted by the assay. With the exception of Ct values close to LOD (Ct >38), the CC1-specific assay never produced positive amplification with samples negative for the *Y. ruckeri* species assay. Moreover, Ct values significantly lower for CC1 than the species assay were never observed. Thus, detection of CC1 was always associated with detection of *Y. ruckeri* in equivalent amounts (or CC1 was lower, as expected if also other lineages were present), which is a strong indication towards the target of the CC1-specific assay not existing amongst other species in these environments. In addition to the results from the field published in paper I, the assay was also used for specific detection of *Y. ruckeri* CC1 from kidney samples collected from acute outbreaks in several instances, and *Y. ruckeri* was indeed isolated and MLVA-genotyped as CC1 in these cases.

Sample collection and processing

Qualitative agreement in terms of PCR-results between swabs and filtering of the same body of water was remarkably consistent in initial field-testing performed at four Atlantic salmon hatcheries in 2018 (data not included in paper II). This was also true within triplicates of swab samples collected from the same tank. As swabbing is generally more affordable, does not require trained personnel nor specialized equipment (Figure 10), and is most convenient in regard to DNA extraction, this form of sampling was preferred when possible. However, swabs are less suitable for quantification, at least in our case as the sampled surface area and amount of captured material is not normalized and may thus vary substantially. Filtering is better suited for quantification as the results are relative to the amount of water filtered, which is routinely measured and noted for each sample. Moreover, where biofilm is not available, or in situations where the concentration of *Y. ruckeri* in the water is of interest and is rapidly changing, filtering is required. This includes samples collected to monitor shedding from fish into the water downstream at a sea farm, during delousing in the field, and in the challenge trial, where the goal was to obtain a snapshot of the current concentration in the water at the time of sampling.

As sampling by swabbing does not require training or equipment other than the swab itself, sampling equipment may be shipped to sites and sampling performed by site staff. Staff at aquaculture sites generally have experience with collecting tissue samples from fish, including samples for PCR screening which is extensively used in Norwegian aquaculture, and e.g. water quality samples.

Several methods were briefly tested for DNA extraction, e.g. different spin-column-based kits including variants and modifications (DNeasy, PowerWater; both Qiagen), CTAB extraction (according to Vrålstad et al. 2009), and the Gentra Puregene Yeast/Bact. Kit (also used for sequencing templates). However, no obvious differences in sensitivity were revealed except that approaches which rely on precipitation of DNA in a tube (CTAB and Gentra) were prone to failure (i.e. no precipitation or discarding the DNA pellet) if the amount of starting material was low. The method thus chosen (DNeasy) does not rely on specialized equipment and is relatively universal such that only minor adjustments are needed between relevant sample types (tissue, swabs and filters), which is both convenient and generally positive in regard to comparability of results between different sample

types. Automated DNA extraction systems would presumably also perform satisfactory in terms of sensitivity, and have advantages in terms of throughput and reproducibility, although these were not considered due to restricted hands-on access, and the scope in terms of number of samples to be processed was initially much lower.

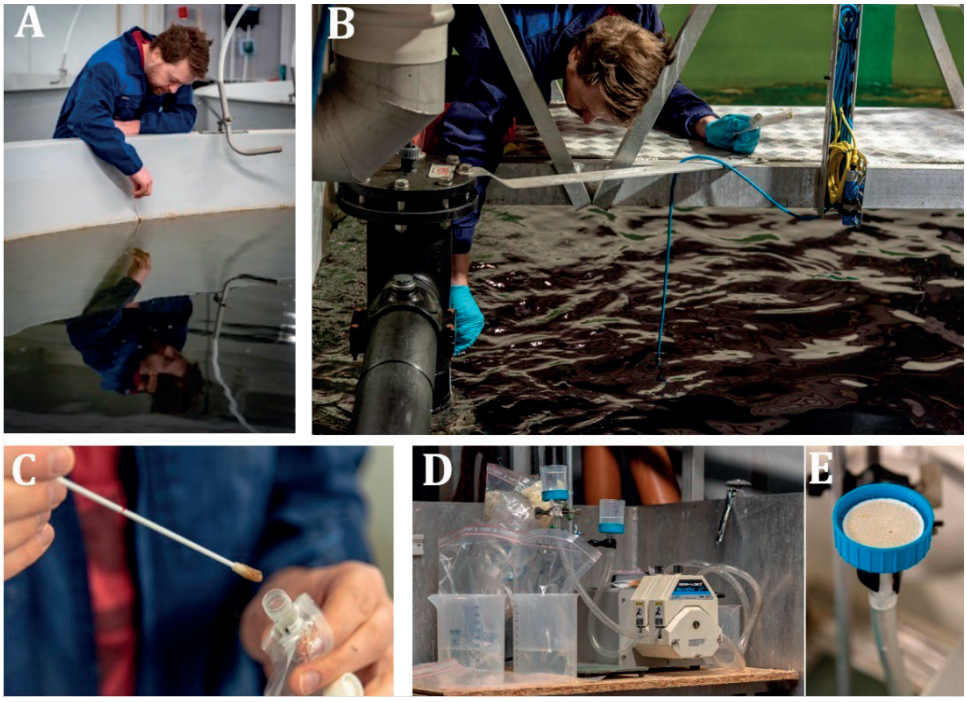

Figure 10: Sampling in the field by swabbing (A, B). Field equipment for swabbing (C) and water filtering (D). Filter paper post-filtering in filtering rig (E). Photos: David Strand.

4.4 Challenge trial

While some individual characteristics of a pathogen relating to virulence may be measured in the laboratory, e.g. growth rates and expression of known virulence factors, a host organism is generally required for overall study of their pathogeneses. Cell cultures or model systems that rely on organisms that presumably do not experience stress and pain on the same level as vertebrates are preferred, although still subject to ethical considerations. As the scope of paper II was to investigate how external stressors in the form of handling and high temperature affects a subclinically infected host, the use of experimental fish was required. IP injection is effective for infecting a fish host and causing symptoms, but

the primary physical barriers to infection are bypassed and the immune system of the fish is caught off-guard by suddenly facing a substantial number of bacteria at a location where they would never initially appear under natural circumstances (Nordmo, Sevatdal & Ramstad 1997). With challenge by immersion, the bacteria must be able to pass the initial physical barriers and the immune system is given a fair chance to respond. Immersion does however not quite reflect field conditions as the fish are immersed in unrealistically high concentrations of the pathogen for a short time. With cohabitation, where pre-infected shedders are kept together with naïve cohabitants, the latter are effectively infected by immersion, but with pathogen concentrations and exposure timespans closer to field conditions. Pathogen concentrations faced by the cohabitants will vary a great deal depending on how the shedders respond, but this will also come into effect in other challenge types as those also develop into a cohabitation-like situation as the disease progresses (Nordmo, Sevatdal & Ramstad 1997). Oral administration is another possible route to administrate the pathogen but has not been widely utilized for challenge trials with *Y. ruckeri*.

4.5 Whole genome sequencing

Technological considerations

Illumina sequencing technology is currently regarded as the 'gold standard' for most general purpose WGS, including sequencing of bacteria, and papers I and II relied on WGS data generated solely with Illumina. However, as with any of the massively parallel technologies, the downstream assembly algorithm relies on patching together overlapping, relatively short reads, and tandem or interspersed sequence repeats exceeding the effective read length will thus result in segmentation of interspersed repeats, truncation of direct repeats, and sometimes misassembly (Kolmogorov et al. 2019). Genetic variability amongst *Y. ruckeri* strains consists largely of mobile elements that frequently contain such repeated sequences, and several relevant virulence factors in *Y. ruckeri* are associated with or contain such repeats. To be able to investigate such challenging sequence features, generation of complete circular genome assemblies was of particular interest for paper III.

Sanger sequencing is generally not a cost-efficient method to close the many gaps resulting from short-read *de novo* assembly. Rather, for this purpose, one of the massively parallel technologies capable of generating relatively inaccurate but extremely long individual sequence reads should be used, currently available from Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (ONT). PacBio sequencing errors are mostly random in nature and can be largely resolved by increasing coverage, allowing this platform to produce fairly accurate *de novo* assemblies as a standalone solution. As a portion of ONT errors relate to consistent misinterpretation of 'difficult' sequences they are not as easily remedied by simply increasing coverage, and ONT should thus in many cases be paired with accurate short-read data such as Illumina in order to generate both complete and highly accurate assemblies. ONT in combination with Illumina is generally a similar priced solution as PacBio alone, however, as Illumina data is often already available, being the standard methodology for general purpose WGS, Illumina and ONT hybrid sequencing constitutes the more affordable option in many cases, including for this project. The ONT MinION sequencer also has the advantage of being extremely compact, affordable and simple to operate, and as such becoming increasingly commonplace in molecular biology labs.

Assembly and annotation

Raw sequence data was processed using conventional methods for base calling, trimming and filtering. SPAdes (Bankevich et al. 2012) and Unicycler (Wick et al. 2017) were used for assembly of Illumina only and Illumina ONT hybrid data respectively, as they are generally regarded as the current 'gold-standard' for assembly of bacterial genomes by each respective approach. While generation of complete genomes was largely straightforward, the *yrIlm* locus sometimes caused issues as additional copies of the locus were sometimes omitted. Such long and complex repeat structures are generally difficult to resolve, and the assembler may find several solutions that although satisfactory from a computational standpoint are not necessarily accurate. Reads connecting two copies may be interpreted as a circular sequence, and additional copies of *yrIlm* did indeed frequently appear in the final assembly as a circular plasmid. While there are also long reads that span the entire junction, and thus display all of the *yrIlm* copies in a single read, this solution is sometimes discarded possibly due to their classification by the software as

chimeric reads (see White et al. 2017 for chimeric reads with ONT) or artifact otherwise. This is unlikely to be the actual case, however, as chimeric reads are sporadic in nature, and alignment of five (or more) individual nanopore reads covering the entire region agreed fully on the configuration in absolutely all cases. The *yrIlm* copy number was further substantiated by inspection of relative coverage over this region from Illumina data (not shown in paper III). While such manual intervention required here in some cases is not unheard of (e.g. Schmid et al. 2018), it is somewhat worrying as such cases may easily go unnoticed. Other direct repeated loci of similar complexity might therefore be more common than it currently seems. Enriching the long-read data to contain a higher proportion of extremely long reads will likely have a positive impact on assembly, which may be accomplished *in vitro* during DNA extraction or library prep, or post-sequencing by filtering base called reads by length (employed to some degree in paper III). Development of novel assemblers or optimizing existing algorithms to account for such complex repeat structures is an ongoing process (Kolmogorov et al. 2019).

Genome annotation was performed by conventional methods, primarily with Prokka (Seeman 2014) which relies on the Prodigal algorithm for *de novo* annotation (Hyatt et al. 2010). Two specific mechanisms resulting in a lack of annotation of virulence-related genes were observed. Firstly, genes split between contigs may be partially annotated but are generally not annotated at all. For a gene such as *yrIlm* this is often the case in short-read assemblies (depending on the algorithm 'solving' the repeats by truncation or segmentation). Secondly, mis-annotation may occur, where an alternative ORF overlapping or nested within a gene of interest receives priority during the annotation process. This may happen with *yrIlm* in complete assemblies, presumably due to the lack of stop-codons in reverse reading frames representing an ORF spanning several thousand base pairs, which is sometimes prioritized over *yrIlm* by the annotation algorithm. Fortunately, it does seem that the more comprehensive annotation pipelines such as RAST and the NCBI annotation pipeline are not as susceptible to such mistakes. To avoid such annotation-related issues, *in silico* discovery and screening was entirely based on DNA sequence.

Downstream analysis

There are no pre-existing databases or frameworks available for *in vitro* or *in silico* evaluation of virulence factors in *Y. ruckeri*, therefore a broad scope investigation was needed to get an overview of the presence and distribution of virulence determinants. To this end, genetic comparison of virulent and avirulent variants of the same species may provide valuable insight (Björkholm et al. 2001; Chen et al. 2004; McClure et al. 2018). Determinants vital to *Y. ruckeri* virulence should be omnipresent amongst virulent strains, while dispensable in avirulent strains unless they also grant benefits outside of the 'virulent niche'.

Due to the lack of a suitable model for discrimination of *de facto* virulent and avirulent strains across the broad ranges of hosts and environments in which the disease occurs, our ability to accurately score virulence for *Y. ruckeri* strains is unfortunately limited (discussed further in chapter 5.3). As a consequence, the data is not well suited for statistical calculations, and neither for a genome-wide-association type approach (Falush 2016; Uffelmann et al. 2021). The highly clonal population structure associated with bacteria may also cause issues with such an approach (Lees & Bentley 2016), and representatives in our dataset regarded as virulent do indeed display a high degree of clonality. However, assessing virulence in a more general sense is possible based on the context of isolation for many *Y. ruckeri* isolates in the NVI strain collection, and for strains documented as virulent in the literature by context or challenge trials conducted with appropriate models (i.e. immersion or cohabitation).

BLASTn is used in multiple steps of the analysis, with each step affected by BLAST search parameters such as cutoffs for nucleotide identity, size and e-value. The visual representation generated with BRIG furthermore relies on its own set of parameters. In particular, features consisting of rather short sequences are prone to being missed at several steps due to poor visibility or cutoffs relating to length (length and e-value). Furthermore, as we are looking strictly at presence/absence, we are oblivious to differences between alleles of genes or systems whose function may be significantly altered due a single non-synonymous SNP (such as the mutations causing the BT2 phenotype in paper I), or interruption by stop-codons or frameshifts from SNP or indels. Differences in copy number, recombination, or interruptions caused by relocating transposases are also generally not revealed

(unless investigated specifically, as was the case for the *yrIlm* locus and in ONPG-negative isolates). Expression analyses have been performed for certain determinants by other workers (e.g. YrIlm, YrInv, Yrp1, HlyA, YrpAB and the ruckerbactin operon, see references in Table 4) but have not been documented for all determinants highlighted in paper III, and any differences in expression between strains are generally not known and are not revealed by our analysis. Lack of absolute correlation between certain virulence factors and virulence could be due to incorrect virulent/avirulent classification of some isolates. This should be taken into particular consideration as we are relying on our impression of virulence according to partially subjective observations from the field. Additionally, virulence factors missing in a subset of virulent lineages may be complemented by other factors. This may e.g. easily be the case for fimbria/pillin genes which are numerous across the chromosome and plasmids and may presumably overlap in function.

5 General discussion

5.1 Biotype and vaccines

The BT2 trait

During infection, bacterial pathogens may need flagella for adhesion, invasion and spread within the host, and functional flagella are critical for virulence in most pathogenic species (Ramos, Rumbo & Sirard 2004; Pallen & Wren 2007). Of particular relevance in aquatic environments is that chemotaxis may be useful for localizing a fish host and a favourable point of attachment or entry (Bordas et al. 1998; O'Toole et al. 1999). However, once the host is successfully colonized, the flagella may become detrimental due to the presence of pattern-recognition (toll-like) receptors in the innate immune system of vertebrates, which may trigger a strong immune response upon recognizing bacterial flagellin (Steiner 2006; Minnich & Rohde 2007). This is also the case for *Y. ruckeri* flagellin in rainbow trout (Wangkahart et al. 2016). Bacterial pathogens employ a number of strategies to circumvent this, which usually includes tight repression of flagella once they are no longer useful during infection (Rossez et al. 2015). *Yersinia* species are known for complete repression of flagella at higher temperatures (Cornelis 1992), and artificial consecutive expression of flagellin in *Y. enterocolitica* does indeed lead to attenuated virulence (Minnich & Rohde, 2007). Some highly specialized pathogenic bacterial species have resorted to dispense with flagella permanently, including *Bacillus anthracis* (Papaparaskevas et al. 2004), *Y. pestis* (Chain et al. 2004) and *Burkholderia mallei* (Song et al. 2010). In some Gram-negative pathogenic species such as *Legionella pneumophila* (Leatham et al. 2005) and *E. coli* (Ren et al. 2006), loss of motility is seen only in certain strains, or may develop during chronic infection as is the case for *Pseudomonas aeruginosa* in cystic fibrosis (Feldman et al. 1998). In such cases, the non-motile trait is associated with increased virulence and/or immune-evasive properties.

Selection for the non-motile trait in *Y. ruckeri* may relate to the temperature dependent aspects of motility expression in *Yersinia* being of little benefit during infection of a cold-blooded host. While depression of flagella in motile *Y. ruckeri* does eventually occur during the infection cycle, this is a quite slow process (Jozwick et al. 2019). Thus, BT2 may relate to a need for more rapid regulatory responses in the modern intensive aquaculture niche. Solving this issue by evolving a more responsive and tight control at lower temperatures may require extensive rewiring of core regulatory pathways, and would presumably require a long time to evolve. While there are signs of a general reduced motility in salmon lineages CC1 and CC5 (Barnes et al. 2016; paper I), this seems to represent a stepping-stone towards BT2 as also members of these reduced motility lineages eventually dispense with flagella permanently.

On motility agar, the steep investment cost of dispensing with flagella is clearly evident. While non-motile cells will eventually succumb to competition from its progeny due to local nutrient depletion and buildup of toxic waste products, motile cells may spread and proliferate throughout the entire agar volume. This disadvantage for non-motile cells is perhaps less profound *in natura* where they may be transported by currents of water or the circulatory system of the host, and non-flagellar locomotion presumably provide some utility in less viscous milieus. However, chemotaxis relies on flagellar locomotion (Faguy & Jarrell 1999) and is therefore unavailable to non-flagellated cells. The flagella are also useful for biofilm formation which is generally regarded as an important mechanism for survival and persistence for this species (Coquet et al. 2002a; Coquet et al. 2002b; Wrobel et al. 2020). Thus, any advantage from BT2 development relating to survival outside of a host seem rather counter-intuitive, and as this trait is known exclusively from virulent strains (not likely due to the lack of assaying avirulent isolates), it is presumably a net-disadvantage outside of the virulent niche.

In light of the fact that no specific advantage of the BT2 phenotype has yet been confirmed experimentally, any connection between vaccination and immune-evasive properties in a more general sense remains a general assumption. The advantage(s) of BT2 may be too subtle or complex to detect, or the experimental systems employed may simply be unsuitable to reveal them. Whatever the advantage may be, it seems to be consistent across salmonid pathogenic strains.

The reason for BT2 not yet being widespread within CC1 in Norway may be due to the existence of an additional bottleneck in Atlantic salmon farming compared to farming of rainbow trout. Onward survival of BT2 strains emerging in sea-farmed salmon will almost certainly rely on transport back to freshwater in order to infect the next generation of salmon hosts. Presumably, this constitutes an unlikely event, and any non-motile cells completing the journey will face competition from already well-established populations of motile variants. BT2 emerging in broodfish may conceivably become established more easily as broodfish populations are routinely brought back to freshwater.

BT2 and vaccination failure –specific correlation?

Cases of apparent *Y. ruckeri* 'vaccine failure' have commonly been attributed to emergence of BT2, although many authors also mention that issues have been resolved by changing the vaccine formulation regarding adjuvant or method of antigen preperation (e.g. Costa et al. 2011), method of delivery (IP injection is mentioned in Welch et al. 2011), or simply preparing the vaccine bacterin with a strain more similar to the one causing disease (Deshmukh et al. 2012). The use of autologous/autogenic vaccines, i.e. bacterin prepared from the exact disease-causing strain, has been successfully employed to resolve such issues (Fouz, Zarza & Amaro 2006). Biotype shift should in itself not affect the LPS structure, and replication of naturally occurring BT2-inducing mutations in a BT1 parent strain did not provide any competitive advantage in a rainbow trout challenge (by immersion) (Jozwick, Graf & Welch 2017), suggesting that virulence also remains unaffected.

As strains utilized in studies comparing vaccines based on different biotypes also differ in both LPS structure and virulence (Tinsley et al. 2011; Deshmukh et al. 2012) there will almost certainly exist additional genetic differences between the strains studied in these cases, meaning that the findings do not necessarily relate to biotype. No experiments are described in scientific literature involving comparison of vaccine-induced immunity between *Y. ruckeri* biotypes where the sole genetic difference is a naturally-occurring BT2-inducing SNP or indel, although NVI-10990 and its 'perfect' reversion mutant (paper I) would be suitable for such experiments. Nonetheless, if the BT2 phenotype enables the pathogen to evade vaccine-induced immunity, vaccines would presumably be less efficient in protecting against such strains on a general basis, but this has so far not been demonstrated. Rather, it

seems that vaccines prepared with bacterins more similar or identical to the local disease-causing strain offer ample protection, irrespective of biotype. This is in general agreement with identification of LPS as the primary contributor to vaccine-induced immunity against *Y. ruckeri* in rainbow trout (as opposed to flagella) (Welch & LaPatra 2016). There are also some indications towards differences in sequence and structure of certain prominent surface-exposed proteins conferring antigenic variation relevant for vaccine-driven immunity (Ormsby & Davies 2021), which are neither related to biotype. Thus, it seems that at least some of the described cases of 'vaccine failure' probably relate primarily to introduction of a new strain of the bacterium which display subtle antigenic differences, presumably a variation in serotype O1-LPS, and/or increased virulence. The BT2 phenotype may as such also offer some advantage towards general fitness within the aquaculture niche, but the contribution of this trait specifically in relation to vaccine-induced immunity appears to be small or inconsequential.

The history and current situation in Norwegian aquaculture may provide some additional insights in this regard. Several changes in yersiniosis vaccine regimes have been made over the years in Norway, in the almost complete absence of BT2 strains. When the disease first emerged in Norway in the late 1980s, a vaccine imported from the USA was employed, but the effect was unsatisfactory, likely due to antigenic differences, but a different vaccine imported from Canada, based on a serotype O1 strain, offered better protection (Brit Tørud pers. com.). In later years, some sites experiencing recurring outbreaks switched to autogenous vaccines, produced with bacterin from the local disease-causing isolate (Brit Tørud pers. com.). Recently, many farms have switched vaccination regiment yet again, from immersion to IP-injected bacterin, the vast majority of which are located in areas where BT2 has not been identified. Thus, there has been a need to improve vaccine regimes in Norway multiple times, regardless of emergence of non-motile strains. Although BT2 development has not been a significant factor in Norwegian aquaculture, Norwegian *Y. ruckeri* has changed in other ways. While the CC1 lineage has dominated in Norwegian aquaculture for several decades, the very first documented case of yersiniosis in Norway (Sparboe et al. 1986) was caused by a different lineage, CC10, which was present in mid- and northern Norway during the late 1980s prior to CC1's dominance from the early 1990s and onwards. While the currently dominating CC1 lineage is highly conserved genetically, some genetic

changes have occurred over the years, including the increased copy number of the likely critical virulence determinant *yrlIm*, as identified in paper III. Plasmid content has also changed over time in this lineage. These changes may have affected the efficiency of vaccines due to antigenic differences or increased fitness in the virulent niche.

Although the pathogen has undoubtedly changed over time, one must also take into account the general increase in production intensity in modern-day aquaculture and how this impacts fish welfare. While non-medicinal delousing techniques have received attention for welfare-related issues in recent years, and perhaps thermal delousing in particular, the increase in yersiniosis incidents at sea seem to precede the widespread use of such methods (Figure 7). However, the more conventional methods also represent prominent handling events, and physical damage and post-treatment mortality is known occur also with such methods in Norwegian aquaculture (Hjeltnes 2014; Bornø & Linaker 2015; Grøtvedt & Jansen 2016). In the period between ca. 2015-2016, some acute yersiniosis episodes reported at sea 'post-handling' may relate to delousing, and some mortality events occurring post-delousing may relate to yersiniosis, although such specific connections were not known/noted at the time, perhaps due to the status of yersiniosis as a non-notifiable disease. In more recent years, several serious outbreaks of yersiniosis in large fish at sea are known to have occurred post-delousing (Gulla & Olsen 2020; Gismervik et al. 2020) and findings in paper II are in general supportive of such stressful treatments having a negative effect in subclinically infected salmon populations. Notably, the annual increase in yersiniosis cases align quite well with the total number of delousing treatments (any method), until a significant proportion of Atlantic salmon populations at sea became IP vaccinated (from ca 2018/2019 and onwards) (Figure 7). In farmed Atlantic salmon in Norway, immersion-style vaccination is considered essential for controlling yersiniosis in freshwater farms experiencing recurrent outbreaks in juvenile fish, although immunity seems relatively short lived. Delivery by IP injection when the fish is large enough offers significantly increased protection compared to immersion (Chettri et al. 2013), and general experiences from the field in Norway indicate that Atlantic salmon vaccinated by injection in the freshwater phase remain fully protected throughout the sea phase, with most outbreaks at sea now generally involving unvaccinated fish (Duncan J. Colquhoun pers. com.).

IP vaccination has also been used successfully in rainbow trout farming in some areas of the USA severely affected by yersiniosis caused by non-motile strains (Welch et al. 2011). Many other BT2-associated cases of 'vaccine failure', e.g. several of those known from Europe, have been resolved by modifying the vaccine strain but still administrating the vaccines by immersion. The prophylactic measures used in Norway are thus notably more advanced than what is currently practiced in many cases involving BT2 internationally, which further indicate that any vaccine-related advantage of BT2 would be of relatively minor siginifcance compared to antigenic differences, increased virulence, and external factors such as increased handling/stress.

While BT2 may not be instrumental in reduced vaccine efficiency, development of the BT2 trait may still relate to vaccination. The use of flagellin-containing whole-cell bacterin vaccines over several decades may have exerted a sustained selection pressure against the flagella of motile variants with subsequent loss of motility as a consequence. In such a scenario, non-motile strains may benefit from a very slight vaccine-related competitive edge, for example in establishing subclinical infection within a vaccinated population, while the efficiency of vaccines in preventing clinical disease may remain largely unaffected. This would certainly be challenging to demonstrate experimentally, but fits well into the general current opinion, or perhaps emerging opinion, of BT2 conferring an as yet unidentified advantage whereby it (possibly) evades the immune system of the fish host. An observation from Norway which seem to counter this view is the isolate recovered from the very first documented case of yersiniosis in Norway in 1985, belonging to the CC10 lineage and notably non-motile. Naturally, no vaccination against yersiniosis was occurring in Norway at the time, and as this lineage is known exclusively from Norway, it is difficult to relate this instance of BT2 to vaccination. Another relevant point is that the perspective of sustained selection pressure against the flagella also works perfectly well in the absence of vaccination. Indeed, adaptation to the highly intensive production style of modern-day aquaculture could be sufficient to explain the emergence of BT2, assuming that BT2 has a general advantage in the virulent niche, while the current availability and susceptibility of hosts in aquaculture allows for a high degree specialization into virulence.

The possibility that the BT2 trait may not be directly/strongly linked to vaccines has been raised previously, and was e.g. discussed in detail in the PhD thesis of Callum J. W. Scott printed in March of 2012 (Scott 2012). While experimental evidence obtained since have all pointed towards such a link being no more than slight, a consensus on the topic will likely not be reached until the specific benefits of BT2 have been demonstrated experimentally. In order to offset the apparent disadvantages of this phenotype, these yet unidentified benefits are presumably substantial, which makes their illusiveness all the more interesting.

5.2 PCR specificity

During the initial phase of the present study, enquiries were made regarding where *Y. ruckeri* could be found in aquaculture environments. While some described *Yersinia* as a hygiene issue best remedied by keeping a clean and sanitized environment, implying that *Y. ruckeri* is residing in the environment, others insisted on *Y. ruckeri* residing inside the fish more or less permanently. Both opinions are well supported in the literature, as is the idea of the pathogen being more or less ubiquitously present throughout affected sites, established in both the fish and the environment. With ever-increasing knowledge of virulence being specific to certain distinct lineages, and coexistence of virulent and avirulent strains, it becomes relevant to investigate whether the seemingly ubiquitous presence throughout affected sites does indeed relate specifically to the virulent strains. If there are distinctions in the distribution of virulent and avirulent strains in aquaculture environments, such knowledge could possibly lead to the development of targeted prevention strategies as an alternative or supplement to vaccination. However, a currently more pressing issue is the possibility of costly countermeasures being employed due to detection of *Y. ruckeri* at sites colonized exclusively by avirulent strains. Moreover, it seems that there have been some issues in Norwegian aquaculture in recent years regarding false positive detection of non-*Y. ruckeri* species utilising what was believed to be *Y. ruckeri*-specific PCR assays. In such cases, countermeasures may have been employed based on detection of strains that do not even belong to the *Y. ruckeri* species. In the period from 2018 to 2020, our laboratory received occasional samples from sites that had experienced conflicting results depending on which lab had performed the analysis. These samples were generally negative with assays described in paper II and the 16S-based assay. At one

point there were even mentions of a novel emerging disease, termed 'untypical yersiniosis' and reportedly caused by *Serratia* spp. which had been isolated from moribund fish, although no further description of the isolates or disease was ever provided. While the isolation of *Serratia* spp. from moribund salmonids is not completely unheard of (Austin & Austin 2012), no known *Serratia* spp. are currently considered clinically relevant for farmed Atlantic salmon in Norway currently, and such isolates are not known to appear as opportunistic in moribund/dead fish with any relevant frequency (Colquhoun 2017). *Serratia* spp. lacking pigment and sucrose activity may be distinguished from *Y. ruckeri* by a positive esculin test (Bullock & Cipriano 1990).

Specificity at the species level

Several PCR assays for specific detection of *Y. ruckeri* target 16S rRNA gene sequences believed to be specific to *Y. ruckeri*. These assays were developed some time ago which means that *Y. ruckeri*'s closest known relatives, i.e. the recently (2011) described *Y. entomophaga* and *Y. nurmii*, were likely not considered during assay design. This is of significance as *Y. entomophaga* has been isolated from aquaculture environments in Norway (Figure 3). Moreover, as we eventually discovered, currently undescribed species may generate signals with such assays. The assays by Bastardo, Ravelo and Romalde (2012) and Keeling et al. (2012) target housekeeping genes, *recA* and glutamine synthetase respectively. These assays were never tested *in vitro* in our lab, but primer-BLAST searches reveal the possibility of false positives with *Serratia* spp. and other *Yersinia* species respectively for these two assays, if the reactions allow for just a slight degree of primer mismatch in non-priming ends. A wide range of *Yersiniaceae* may be isolated from freshwater environments in aquaculture, including as yet undescribed species. Accounting for undiscovered species is generally not possible during PCR design, but avoiding the use of ubiquitous and highly conserved genes should serve to significantly reduce the risk of false positive results. Our assay specific for *Y. ruckeri* at the species level was designed to target a gene which is not known to be present in any other species, a strategy also employed by other recently published protocols (Lewin et al. 2020) and some private diagnostics labs serving the aquaculture industry in Norway (personal communications). It should be noted that the older assays were developed at a time when access to sequence data was still quite limited, and they were indeed

appropriate and well-designed assays for the time. Moreover, the risk of false positives with any of the assays described in the literature should be at a minimum when used with clinical samples from internal tissues, i.e. kidney or spleen, where the prevalence of other *Yersiniaceae* should be non-existent in most cases. Thus, the continued use of such assays should not be controversial if employed in a sensible manner.

Lineage-specific assays

With specificity at the species level regarded as resolved, issues relating to the detection of avirulent *Y. ruckeri* strains still persist. During screening for paper II we found a high proportion of positives with the species-specific assay, while CC1 was absent, in several sites that had never experienced issues with yersiniosis. In this situation, with avirulent variants being widely distributed, the utility of PCR screening in aquaculture environments with a species-specific assay alone is minimal as it is not possible to make any sort of assessment regarding risk of outbreaks or spread of disease-causing strains. Specific detection of *Y. ruckeri* CC1 on the other hand reveals the presence of this specific disease-causing variant. As *Y. ruckeri* isolates belonging to CC1 are nearly identical genetically, with the exception of some historical variability in virulence gene content (paper III), and responsible for all serious outbreaks, the presence of CC1 arguably represent a significant biosecurity risk in the shorter or longer term. While one should be extremely careful in relying solely on PCR in any sort of diagnosis, a seemingly healthy but *Y. ruckeri* CC1-positive population indicates ongoing or imminent subclinical infection, which may quickly develop to acute disease if the fish are subjected to stress or otherwise unfavorable conditions. As such, the CC1-specific assay has a substantially higher potential utility (in Norway specifically) as specific detection of CC1 may warrant preventative measures such as especially careful handling to prevent clinical outbreaks, and isolation and restriction in transport to prevent spread. It is however important to keep in mind that correlation between PCR detection of CC1 and subclinical infection, and further correlation with risk of acute yersiniosis, has not yet been investigated.

Specific yet undesirable detection

A potential issue with molecular-based detection of microbial pathogens concerns detection of non-viable cells e.g. post-disinfection (Rudi et al. 2005; Bonilauri et al. 2016). This 'problem' with its implications for biosecurity became apparent during the present project and involved detection of *Y. ruckeri* CC1 in delousing equipment, post-disinfection but prior to delousing. Viability in itself was not a major concern otherwise as we were primarily interested in the general presence of *Y. ruckeri* in these environments. A related issue is the introduction of a significant amounts of *Y. ruckeri* CC1 DNA to the sampling environment in the form of formalin-killed whole cell bacterin in the yersiniosis vaccine. While detection of the vaccine was in itself not surprising, the magnitude and persistence of the signal in the environment was indeed unexpected. As some aquaculture facilities vaccinate quite frequently, up to three times per generation, screening results became difficult to interpret in a sensible manner in some cases (Figure 11).

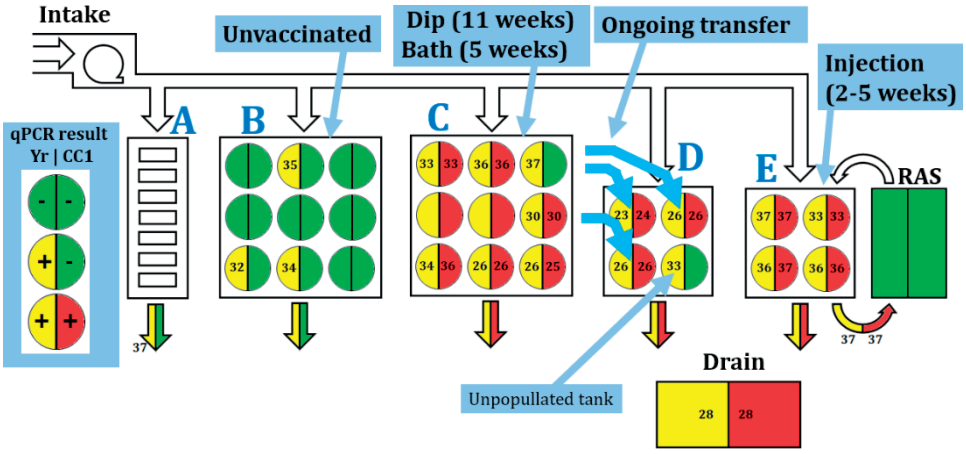

Figure 11: Detailed qPCR screening results from an Atlantic salmon hatchery. Sample points are colored and split in two where green indicate negative, yellow indicates positive with the *Yersinia ruckeri* specific assay, and red indicates positive with the *Y. ruckeri* CC1 specific assay. Ct values are also indicated (averages of three samples, not shown if extreme or qualitative variance). Letters A through E indicate hatching (A), start-feed (B) and further increasingly larger fish and tanks. E use recirculation technology (RAS). Swabs and substrate samples from the RAS system were negative with both assays. Transfer of fish from C to D was ongoing during sampling. Method and time passed since yersiniosis vaccination is indicated as dip or bath (i.e. immersion) or IP injection. Unsampled tanks are not shown.

In the laboratory, verification by culture may be used to discern viable and non-viable cells, but this is difficult for environmental samples as culture of *Y. ruckeri* in the dominating presence of background bacterial flora is not currently very reliable. Alternatively, employing a viable/dead stain technique prior to DNA extraction may render DNA in/from inactivated cells undetectable by PCR, although such techniques are highly labour-intensive, complicated and generally not suitable for complex environmental samples (Dinh Than et al. 2017). Outside of somehow genetically altering target DNA loci in the vaccine strain, an unrealistic and not very sensible endeavour which might also raise GMO-related legislative questions, issues relating to detection of the vaccine in environmental samples will likely persist as long as whole-cell bacterin vaccines are used.

Virulence-based PCR detection

As CC1 is the only virulent *Y. ruckeri* lineage of relevance present in Norway currently, the lineage-specific PCR developed in paper II was effectively functioning as a virulence-specific assay in our studies. However, this assay is not relevant for use elsewhere as yersiniosis is caused by other strains abroad. Conceivably, an assay designed to target critical virulence determinants could be universally applicable internationally. As the high virulence trait seem to be reliant on the presence of two distinct genetic determinants, i.e. the *yrIlm* invasin and serotype O1 LPS, a single, monoplex assay would unfortunately not suffice. An assay targeting serotype O1 would also detect most avirulent strains, and an assay targeting *yrIlm* would also detect some rather low-virulent serotype O2 lineages, such as CC3 in Norway. While such strains are virulent in principle, their detection is (arguably) undesirable in a virulence-specific assay as the threat posed by CC3 is insignificant compared to that of CC1 in the current situation in Norway, and similar situations presumably exist internationally. Specific detection of both serotype O1 and *yrIlm* in a duplex assay would be vulnerable to the presence of e.g. CC3 and a serotype O1 avirulent strain in the same sample, effectively mimicking specific detection of a highly virulent strain such as CC1 or CC2. It is also relevant to keep in mind that the *yrIlm* locus may very well exist in other bacterial species as it is found within a seemingly active complex transposon and thus likely a highly mobile element. Thus, while such a 'virulence-specific' assay could be useful to assess isolates in pure culture for these virulence-related traits, it would not be suitable for PCR screening purposes in aquaculture

environments. As such, development of lineage-specific assays targeting other virulent lineages, such as CC2 and CC5, would be highly relevant to enable similar investigations internationally.

5.3 Assessing virulence

As many previously documented virulence factors are present in all sequenced *Y. ruckeri*, such as the *yhlA*-encoded hemolysin and the *yrp1*-encoded protease associated with the primary symptoms of the disease, strains regarded as avirulent may be able to produce symptoms of yersiniosis under certain circumstances. Currently unpublished and not included in the work presented here, although briefly mentioned in paper II, we performed challenge trials utilizing a cohabitation model which confirmed that such avirulent isolates cause the same symptoms and mortality as CC1 when IP injected, but that they are incapable of horizontal transmission to cohabitant fish. Thus, the virulence-related phenotypic differences between strains regarded as virulent or avirulent seem to relate to the ability to attach to and/or pass the external barriers of a healthy fish host. This is perhaps a somewhat simplistic explanation that does not account for the host-specificity that apparently exist between the various virulent lineages, and conceivably, strains currently percieved as avirulent could be virulent towards other host species. While this cannot be easily ruled out, experiments conducted by Haig et al. (2011), utilizing an appropriate immersion challenge model, was able to demonstrate virulence across host species, although at reduced efficiencies. Moreover, in the UK where yersiniosis affects both rainbow trout and Atlantic salmon, instances of 'rainbow trout -like' isolates causing disease in salmon have on occasion been mentioned in the literature (Ormsby et al. 2016), and these isolates have been confirmed to belong to the rainbow trout-associated lineage CC2 (Gulla et al. 2018), and one such example is included in paper III (Fig 1, strain 86020). Thus, while the perceived virulence of a specific strain is dependent on the host species, there are indeed signs of common mechanisms for virulence amongst the virulent lineages.

With a few notable exceptions, the knowledge of specific mechanisms and associated genetic determinants in relation to *Y. ruckeri* virulence is quite limited. The known presence in *Y. ruckeri*, but lack of study, of several genetic determinants associated with virulence in human pathogenic *Yersinia*, such as Yst1 and Ysa types

II and III secretion systems and Tc genes, is an attest to this. Certain other accessory virulence-related determinants have been studied in some detail, although they are not ubiquitous amongst virulent lineages. A relevant example is the highly similar pYR3 and pYR4 plasmids, present in CC2 and CC1 and highlighted as a virulence contributor by *in vitro* and *in silico* studies respectively in each lineage (Méndez et al. 2009; Wrobel et al. 2018b), while the virulent lineage CC5 does not carry any plasmids. Another relevant example are the putatively secreted proteins which have thus far been identified exclusively in strain SC09 (STIR/TcpA; Liu et al. 2019), but whose contribution to virulence have nonetheless been studied in great detail in this strain. While there are no apparent reasons to question the findings from these studies, these factors may for example provide host-specific benefits, but overall, it seems like most of the accessory determinants studied in detail previously does not directly enable virulence. Thus, other accessory determinants are presumably more relevant to investigate, and the study performed in paper III was essential for identifying which ones.

While acquiring sequence data for comparative genomics study is entirely a practical matter, there was also a need to assess virulence for a large number of isolates. High-throughput lab models for 'virulence characterisation' do exist, but generally rely upon model organisms such as nematodes, wax moth larvae or cell-culture (e.g. Raju et al. 2012; Bernardin Souibgui et al. 2017; Fan et al. 2022), or IP injection of a relatively low number of fish (e.g. Méndez et al. 2009). Injection is generally more reliable and has lower variance than immersion or cohabitation (Nordmo, Sevatdal & Ramstad 1997; Deshmukh et al. 2011) and thus requires fewer experimental fish, generates results quicker and is an overall simpler and more affordable model. High-throughput models, or 'semi-high throughput' with IP injected fish, are however not suitable to study the type of differences we are interested in here, as we expect strains regarded as avirulent in field conditions to demonstrate virulence in such models. Such issues are well known from study of other pathogens, including human pathogenic *Yersinia* where delivery by the oral route may reveal critical differences relating to accessory virulence factors which injection does not (Iwobi et al. 2003).

While development of accurate models for assaying virulence of bacterial pathogens is certainly possible with due effort (e.g. Maury et al. 2016), further complicating things with *Y. ruckeri* is the occurrence of yersiniosis amongst different host species. Indeed, challenge by IP injection is sometimes the model of choice in rainbow trout specifically (with larger fish, >6 months) as mortality using alternative models is generally too low (Deshmukh et al. 2011). Yersiniosis also occurs in non-salmonids where general physiology and mechanisms in immunity may be substantially different. Any sensible benchmarking of virulence reflecting field conditions across host species and situations would thus require quite comprehensive studies. This is a tall order even for a single strain, and hardly realistic for a multitude of isolates. As such, a very general classification of virulence on a per-lineage basis was the only realistic approach for such a broad scope investigation. It is however important to keep in mind that this approach is by no means 'perfect', being especially vulnerable to bias relating to which cases become documented and subjective interpretations of clinical impact, and furthermore which specific isolates are chosen/available for sequencing. The availability in public databases of sequences from isolates regarded as avirulent is especially lacking, and such sequences were thus heavily dominated by isolates from Norway, sequenced by us for this specific project. Furthermore, strains regarded as avirulent based on genetics or context may indeed be virulent towards other host species and/or in different situations, and our dataset included just a single isolate well documented as virulent towards non-salmonid fish (strain SC09; Liu et al. 2019).

Despite potentially substantial sources of error in mis-classification of individual isolates and lack of representation, we were successful at matching this 'general qualitative virulence' trait at the lineage-level with the presence of a specific genetic determinant, i.e. the *yrilm* invasin, the general predicted function of which represents a specific mechanism for attaching to and/or permeating the external barriers of a fish host. Significant differences in the capacity for adhesion to host gill and intestinal mucus between virulent and avirulent strains have been reported previously (Tobback et al. 2010), which indicates that YrIlm may be involved in attachment to these tissue types. The observed amplification of this invasin quite elegantly contributed to an explanation of the seemingly increasing virulence of this lineage over the years in Norway, and presumably also in CC2 internationally, and may indicate that these two lineages are indeed especially well adapted for

virulence. Moreover, the absence of O1-LPS, previously associated with reduced virulence, and extended to all of the non-O1 serotypes (except for O8), is perhaps a simplistic but still quite reasonable explanation as to how some strains which possess the invasin are almost exclusively associated with sporadic and/or less serious cases. Strain SC09, a non-O1 serotype strain pathogenic towards channel catfish (Liu et al. 2016), does however indicate that the O1-LPS connection may not be quite as broadly applicable. It may be the case that O1-LPS is not needed for high virulence in this non-salmonid host species, and/or that perhaps other genetic determinants contribute substantially towards its virulence, such as the type IV secretion-associated factors or plasmids unique to this strain. Thus, the findings from strain SC09 hint at potential limitations in our findings, and looking further into yersiniosis in non-salmonid fish may provide valuable insight in this regard.

6 Concluding remarks

The non-motile BT2 phenotype seems to occur, at least eventually, in any highly virulent *Y. ruckeri* lineage in salmonid aquaculture. BT2 has also been detected in Norway, represented by two novel causative genotypes documented here, although this phenotype is currently not widespread and thus cannot be associated with the recent need to improve vaccination strategies in Norwegian Atlantic salmon aquaculture. PCR screening with *Y. ruckeri* species- and lineage-specific assays revealed a broad presence of avirulent strains in aquaculture environments, and a significant possibility for detecting *Y. ruckeri* DNA from vaccine remnants. This has likely affected the outcome of both screening studies and general diagnostics in the past, and must be taken into account going forward. Several virulence factors highlighted in previous studies seem common to all *Y. ruckeri* strains, virulent and avirulent alike, and thus cannot be responsible for the observed variance in virulence. The high-virulent phenotype in salmonids is strongly associated with a combination of the gene encoding the YrIlm invasin and genes required for serotype O1-LPS synthesis. While global dissemination of virulent lineages has been documented previously, WGS-based identification of several putatively avirulent *Y. ruckeri* lineages across continents in the present study is further indicative of broad dissemination of such strains, indicating that issues relating to detection of virulent/avirulent strains by PCR screening is not limited to aquaculture in Norway.

7 Future perspectives

Identification of the specific advantages conferred by the BT2 phenotype in virulent *Y. ruckeri* lineages should provide further insights into the specific mechanisms utilized by the pathogen to cause disease. Lineage-specific assays should be developed for lineages relevant internationally, i.e. CC2, to enable similar screening-studies abroad and thus further insights into the distribution and ecology of highly virulent *Y. ruckeri* strains. Findings relating to virulence determinants must be verified experimentally, by generation of relevant knockout mutants for employment in challenge trials that reflect field conditions.

8 Reference List

Abbass, A., Sharifuzzaman, S. M., & Austin, B. (2010). Cellular components of probiotics control *Yersinia ruckeri* infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of fish diseases, 33*(1), 31–37. https://doi.org/10.1111/j.1365-2761.2009.01086.x

Abdel-Glil, M. Y., Fischer, U., Steinhagen, D., McCarthy, U., Neubauer, H., & Sprague, L. D. (2021). Phylogenetic Relatedness and Genome Structure of *Yersinia ruckeri* Revealed by Whole Genome Sequencing and a Comparative Analysis. *Frontiers in microbiology, 12*, 782415. https://doi.org/10.3389/fmicb.2021.782415

Akhlaghi, M. & Yazdi, S. (2008). Detection and identification of virulent *Yersinia ruckeri*: the causative agent of enteric redmouth disease in rainbow trout (*Oncorhynchus mykiss*) cultured in Fars province, Iran. *Iranian Journal of Veterinary Research, 9*(4).

Altinok, I., Grizzle, J. M., & Liu, Z. (2001). Detection of *Yersinia ruckeri* in rainbow trout blood by use of the polymerase chain reaction. *Diseases of aquatic organisms*, *44*(1), 29–34. https://doi.org/10.3354/dao044029

Amlie, T. (2018). The yersiniosis epidemic in mid-Norway, do RAS play a role? Nordic RAS Workshop, Oslo, 19.11.2018. Presentation available from https://www.nmbu.no/download/file/fid/35600

Arias, C. R., Olivares-Fuster, O., Hayden, K., Shoemaker, C. A., Grizzle, J. M., & Klesius, P. H. (2007). First report of *Yersinia ruckeri* biotype 2 in the USA. *Journal of Aquatic Animal Health ,19*(1), 35-40.

Austin, B., & Austin, D. A. (2012). *Enterobacteriaceae* Representatives. In Austin, B., & Austin, D. A. (Eds.), *Bacterial Fish Pathogens: Disease in Farmed and Wild Fish. 5th Edition* (pp. 229-275). Published by Chichester: Springer Praxis Press. https://doi.org/10.1007/978-94-007-4884-2 eBook retrieved from https://link.springer.com/content/pdf/10.1007/978-94-007-4884-2.pdf

Austin, B., Bishop, I., Gray, C., Watt, B., & Dawes, J. (1986). Monoclonal antibody-based enzyme-linked immunosorbent assays for the rapid diagnosis of clinical cases of enteric redmouth and furunculosis in fish farms. *Journal of Fish Diseases, 9*(5), 469–474. https://doi.org/10.1111/j.1365-2761.1986.tb01042.x

Austin, D. A., Robertson, P. A., & Austin, B. (2003). Recovery of a new biogroup of *Yersinia ruckeri* from diseased rainbow trout (*Oncorhynchus mykiss*, Walbaum). *Systematic and applied microbiology*, *26*(1), 127–131. https://doi.org/10.1078/072320203322337416

Baklien, A. T. (2020). Statistics Norway. Published online 29th of October 2020. Retrieved from https://www.ssb.no/jord-skog-jakt-og-fiskeri/artikler-og-publikasjoner/nok-et-rekordar-i-oppdrettsnaeringen

Balcázar, J. L., Vendrell, D., de Blas, I., Ruiz-Zarzuela, I., Muzquiz, J. L., & Girones, O. (2008). Characterization of probiotic properties of lactic acid bacteria isolated from intestinal microbiota of fish. *Aquaculture, 278*(1–4), 188-191. https://doi.org/10.1016/j.aquaculture.2008.03.014.

Balta, F., & Dengiz Balta, Z. (2019). Preparation of O-antigen from *Yersinia ruckeri* Serotype O1 and Use in the Slide Agglutination Test. *Anatolian environmental and animal sciences, 4*(3), 480-483. https://doi.org/10.35229/jaes.645416

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., Lesin, V. M., Nikolenko, S. I., Pham, S., Prjibelski, A. D., Pyshkin, A. V., Sirotkin, A. V., Vyahhi, N., Tesler, G., Alekseyev, M. A., & Pevzner, P. A. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of computational biology : a journal of computational molecular cell biology*, *19*(5), 455–477. https://doi.org/10.1089/cmb.2012.0021

Barnes, A. C. (2011). Enteric redmouth disease (ERM) (*Yersinia ruckeri*). In Woo, P. T. K., & Bruno, D. W. (Eds.), Fish diseases and disorders. Volume 3: viral, bacterial and fungal infections (pp. 484-511). Published by CABI. http://dx.doi.org/10.1079/9781845935542.0000

Barnes, A. C., Delamare-Deboutteville, J., Gudkovs, N., Brosnahan, C., Morrison, R., & Carson, J. (2016). Whole genome analysis of *Yersinia ruckeri* isolated over 27 years in Australia and New Zealand reveals geographical endemism over multiple lineages and recent evolution under host selection. *Microbial genomics, 2*(11), e000095. https://doi.org/10.1099/mgen.0.000095

Bastardo, A., Bohle, H., Ravelo, C., Toranzo, A.E., & Romalde, J. R. (2011). Serological and molecular heterogeneity among *Yersinia ruckeri* strains isolated from farmed Atlantic salmon *Salmo salar* in Chile. *Diseases of Aquatic Organisms, 93*(3), 207-14. https://doi.org/10.3354/dao02296

Bastardo, A., Ravelo, C., & Romalde, J. L. (2012). A polyphasic approach to study the intraspecific diversity of *Yersinia ruckeri* strains isolated from recent outbreaks in salmonid culture. *Veterinary microbiology, 160*(1-2), 176–182. https://doi.org/10.1016/j.vetmic.2012.05.024

Bastardo, A., Ravelo, C. & Romalde, J. L. (2012). Highly sensitive detection and quantification of the pathogen Yersinia ruckeri in fish tissues by using real-time PCR. *Applied Microbiology and Biotechnology, 96*, 511–520. https://doi.org/10.1007/s00253-012-4328-1

Bastardo, A., Ravelo, C., & Romalde, J. L. (2015). Phylogeography of *Yersinia ruckeri* reveals effects of past evolutionary events on the current strain distribution and explains variations in the global transmission of enteric redmouth (ERM) disease. *Frontiers in microbiology*, *6*, 1198. https://doi.org/10.3389/fmicb.2015.01198

Bernardin Souibgui, C., Zoropogui, A., Voisin, J., Ribun, S., Vasselon, V., Pujic, P., Rodriguez-Nava, V., Belly, P., Cournoyer, B., & Blaha, D. (2017). Virulence test using nematodes to prescreen *Nocardia* species capable of inducing neurodegeneration and behavioral disorders. *PeerJ, 5*, e3823. https://doi.org/10.7717/peerj.3823

Bent, Z. W., Poorey, K., Brazel, D. M., LaBauve, A. E., Sinha, A., Curtis, D. J., House, S. E., Tew, K. E., Hamblin, R. Y., Williams, K. P., Branda, S. S., Young, G. M., & Meagher, R. J. (2015). Transcriptomic Analysis of *Yersinia enterocolitica* Biovar 1B Infecting Murine Macrophages Reveals New Mechanisms of Extracellular and Intracellular Survival. Infection and immunity, 83(7), 2672–2685. https://doi.org/10.1128/IAI.02922-14

Björkholm, B., Lundin, A., Sillén, A., Guillemin, K., Salama, N., Rubio, C., Gordon, J. I., Falk, P., & Engstrand, L. (2001). Comparison of genetic divergence and fitness between two subclones of Helicobacter pylori. *Infection and immunity*, *69*(12), 7832–7838. https://doi.org/10.1128/IAI.69.12.7832-7838.2001

Bonilauri, P., Bardasi, L., Leonelli, R., Ramini, M., Luppi, A., Giacometti, F., & Merialdi, G. (2016). Detection of Food Hazards in Foods: Comparison of Real Time Polymerase Chain Reaction and Cultural Methods. *Italian journal of food safety*, *5*(1), 5641. https://doi.org/10.4081/ijfs.2016.5641

Bordas, M. A., Balebona, M. C., Rodriguez-Maroto, J. M., Borrego, J. J., & Morinigo, M. A. (1998). Chemotaxis of pathogenic *Vibrio* strains towards mucus surfaces of gilt-head sea bream (*Sparus aurata* L.). *Applied and environmental microbiology*, *64*(4), 1573–1575. https://doi.org/10.1128/AEM.64.4.1573-1575.1998

Bornø, G., & Linaker, L. M. (Eds.) (2014). *Fish Health Report 2014*. Published by the Norwegian Veterinary Institute. Retrieved from https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2015/fish-health-report-2014

Bornø, G. & Sviland, C. (Eds.) (2011). *Fiskehelserapporten 2010* [Fish health report 2010]. In Norwegian. Published by the Norwegian Veterinary Institute. Retrieved from https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2011/fiskehelserapporten-2010

Bragg, R. R., & Henton, M. M. (1986). Isolation of *Yersinia ruckeri* from rainbow trout in South Africa. *Bulletin of the European Association of Fish Pathologists, 6*(1), 5-6.

Bravo, S., & Kojagura, V. (2004). First isolation of *Yersinia ruckeri* from rainbow trout (*Oncorhynchus mykiss*) in Peru. *Bulletin of the European Association of Fish Pathologists 24*(2), 104-108.

Brun, E., & Bornø, G. (2010). Fiskehelsemessige aspekter i forhold til oppdrett av røye (*Salvelinus alpinus*) i merder [Aspects of fish welfair in culture of Arctic char (*Salvelinus alpinus*) in cages]. In Norwegian. Published by the Norwegian Veterinary Institute. Report series 2010, 19. Retrieved from https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2010/fiskehelsemessige-aspekter-i-forhold-til-oppdrett-av-rye-salvelinus-alpinus-i-merder

Brunt, J., Newaj-Fyzul, A., & Austin, B. (2007). The development of probiotics for the control of multiple bacterial diseases of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of fish diseases, 30*(10), 573–579. https://doi.org/10.1111/j.1365-2761.2007.00836.x

Bullock, G. L. & Cipriano, R. C. (1990). Enteric redmouth disease of salmonids. US Fish & Wildlife *Publications, 131*.

Bullock, G. L., Stuckey, H. M., & Shotts, E. B. Jr. (1977). Early records of North American and Australian outbreaks of enteric redmouth disease. *Fish Health News, 6*(2), 96-97.

Bullock, G. L., Stuckey, H. M., & Shotts, E. B. Jr. (1978). Enteric redmouth bacterium: comparison of isolates from different geographic areas. *Journal of fish diseases, 1*(4), 351–356. https://doi.org/10.1111/j.1365-2761.1978.tb00039.x

Busch, R. A. (1978). Enteric redmouth disease (Hagerman strain). *Marine Fisheries Review, 40*(3), 42-51

Busch, R. A., & Lingg, A. (1975). Establishment of an asymptomatic carrier state infection of enteric redmouth disease in rainbow trout. *Journal of the Fisheries Research Board of Canada 32*(12), 2429-2432. https://doi.org/10.1139/f75-279

Calvez, S., Gantelet, H., Blanc, G., Douet, D. G., & Daniel, P. (2014). *Yersinia ruckeri* Biotypes 1 and 2 in France: presence and antibiotic susceptibility. *Diseases of aquatic organisms*, *109*(2), 117–126. https://doi.org/10.3354/dao02725

Capkin, E., & Altinok, I. (2009). Effects of dietary probiotic supplementations on prevention/treatment of yersiniosis disease. *Journal of applied microbiology*, *106*(4), 1147–1153. https://doi.org/10.1111/j.1365-2672.2008.04080.x

Carson, J., & Wilson, T. (2009). Yersiniosis in Fish. *In* Australia and New Zealand Standard Diagnostic Procedure. 1-19 p. available from: https://www.awe.gov.au/sites/default/files/sitecollectiondocuments/animal/ahl/ANZSDP-Yersiniosis.pdf

Cascales, D., Guijarro, J. A., García-Torrico, A. I., & Méndez, J. (2017). Comparative genome analysis reveals important genetic differences among serotype O1 and serotype O2 strains of *Y. ruckeri* and provides insights into host adaptation and virulence. *MicrobiologyOpen*, *6*(4), e00460. https://doi.org/10.1002/mbo3.460

Chain, P. S., Carniel, E., Larimer, F. W., Lamerdin, J., Stoutland, P. O., Regala, W. M., Georgescu, A. M., Vergez, L. M., Land, M. L., Motin, V. L., Brubaker, R. R., Fowler, J., Hinnebusch, J., Marceau, M., Medigue, C., Simonet, M., Chenal-Francisque, V., Souza, B., Dacheux, D., Elliott, J. M., Derbise, A., Hauser, L. J., & Garcia, E. (2004). Insights into the evolution of *Yersinia pestis* through whole-genome comparison with *Yersinia pseudotuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America, 101*(38), 13826–13831. https://doi.org/10.1073/pnas.0404012101

Chen, T., Hosogi, Y., Nishikawa, K., Abbey, K., Fleischmann, R. D., Walling, J., & Duncan, M. J. (2004). Comparative whole-genome analysis of virulent and avirulent strains of *Porphyromonas gingivalis*. *Journal of bacteriology*, *186*(16), 5473–5479. https://doi.org/10.1128/JB.186.16.5473-5479.2004

Del Cerro, A., Marquez, I., & Guijarro, J. A. (2002). Simultaneous detection of *Aeromonas salmonicida*, *Flavobacterium psychrophilum*, and *Yersinia ruckeri*, three major fish pathogens, by multiplex PCR. *Applied and environmental microbiology*, *68*(10), 5177–5180. https://doi.org/10.1128/AEM.68.10.5177-5180.2002

Chen, P. E., Cook, C., Stewart, A. C., Nagarajan, N., Sommer, D. D., Pop, M., Thomason, B., Thomason, M. P., Lentz, S., Nolan, N., Sozhamannan, S., Sulakvelidze, A., Mateczun, A., Du, L., Zwick, M. E., & Read, T. D. (2010). Genomic characterization of the *Yersinia* genus. *Genome biology*, *11*(1), R1. https://doi.org/10.1186/gb-2010-11-1-r1

Chettri, J. K., Deshmukh, S., Holten-Andersen, L., Jafaar, R. M., Dalsgaard, I., & Buchmann, K. (2013). Comparative evaluation of administration methods for a vaccine protecting rainbow trout against *Yersinia ruckeri* O1 biotype 2 infections. *Veterinary immunology and immunopathology*, *154*(1-2), 42–47. https://doi.org/10.1016/j.vetimm.2013.04.001

Cipriano, R. C., & J. B. Pyle. (1985). Development of a culture medium for determination of sorbitol utilization among strains of *Yersinia ruckeri*. *Microbios Letters, 28*:79-82.

Collins, R. O., Foster, G., & Ross, H. M. (1996). Isolation of *Yersinia ruckeri* from an otter and salmonid fish from adjacent freshwater catchments. *Veterinary Record, 139*(7), 169–169. https://doi.org/10.1136/vr.139.7.169

Coquet, L., Cosette, P., Junter, G. A., Beucher, E., Saiter, J. M., & Jouenne, T. (2002a). Adhesion of *Yersinia ruckeri* to fish farm materials: influence of cell and material surface properties. *Colloids and Surfaces B: Biointerfaces*, 26(4), 373-378. https://doi.org/10.1016/S0927-7765(02)00023-1

Coquet, L., Cosette, P., Quillet, L., Petit, F., Junter, G. A., & Jouenne, T. (2002b). Occurrence and phenotypic characterization of *Yersinia ruckeri* strains with biofilm-forming capacity in a rainbow trout farm. *Applied and environmental microbiology*, *68*(2), 470–475. https://doi.org/10.1128/AEM.68.2.470-475.2002

Colquhoun, D. J. (2016). Yersiniosis. In Hjeltnes, B., Walde, C., Bang jensen, B., & Haukaas, A. (Eds.). *The Fish Health Report 2015* (pp. 45-46). Published by the Norwegian Veterinary Institute. Retrieved from https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2016/fish-health-report-2015

Colquhoun, D. J. (2017). Other bacterial infections of fish. In Hjeltnes, B., Bornø, G., Jansen, M. D., Haukaas, A., & Walde, C. S. (Eds.), *The health situation in Norwegian aquaculture 2016* (pp. 74-75). Published by the Norwegian Veterinary Institute. Retrieved from https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2017/fish-health-report-2016

Cornelis, G. R. (1992). *Yersiniae*, finely tuned pathogens. In Hormaeche, C. E., Penn, C. W., & Smyth, C. J. (Eds.). Molecular biology of bacterial infection, in press. SGM symposium series vol. 49. Cambridge University Press, Cambridge. https://doi.org/10.1007/978-3-642-78624-2_11

Cornelis, G. R., Boland, A., Boyd, A. P., Geuijen, C., Iriarte, M., Neyt, C., Sory, M. P., & Stainier, I. (1998). The virulence plasmid of *Yersinia*, an antihost genome. *Microbiology and molecular biology reviews : MMBR*, *62*(4), 1315–1352. https://doi.org/10.1128/MMBR.62.4.1315-1352.1998

Costa, A. A., Leef, M. J., Bridle, A. R., Carson, J., & Nowak, B. F. (2011). Effect of vaccination against yersiniosis on the relative percent survival, bactericidal and lysozyme response of Atlantic salmon, *Salmo salar*. *Aquaculture*, 315, 201–206. https://doi.org/10.1016/j.aquaculture.2011.02.031

Dahiya, I., & Stevenson, R. M. (2010a). The ZnuABC operon is important for *Yersinia ruckeri* infections of rainbow trout, Oncorhynchus mykiss (Walbaum). *Journal of fish diseases*, *33*(4), 331–340. https://doi.org/10.1111/j.1365-2761.2009.01125.x

Dahiya, I., & Stevenson, R. M. (2010b). The UvrY response regulator of the BarA-UvrY two-component system contributes to *Yersinia ruckeri* infection of rainbow trout (Oncorhynchus mykiss). *Archives of microbiology*, *192*(7), 541–547. https://doi.org/10.1007/s00203-010-0582-8

Dalsgaard, I. , From, J., & Harlyck, V. (1984). First observation of *Yerslnia ruckeri* in Denmark. *Bulletin of the European Association of Fish Pathologists, 4*, 10.

Danley, M. L., Goodwin, A. E., & Killian, H. S. (1999). Epizootics in farm-raised channel catfish, *Ictalurus punctatus (*Rafinesque), caused by the enteric redmouth bacterium *Yersinia ruckeri*. *Journal of Fish Diseases 22*(6), 451-456.

Davies, R. L., & Frerichs, G. N. (1989). Morphological and biochemical differences among isolates of *Yersinia ruckeri* obtained from wide geographical areas. *Journal of Fish Diseases, 12*, 357-365. https://doi.org/10.1111/j.1365-2761.1989.tb00324.x

Davies R. L. (1990). O-serotyping of *Yersinia ruckeri* with special emphasis on European isolates. *Veterinary microbiology*, *22*(4), 299–307. https://doi.org/10.1016/0378-1135(90)90016-o

Davies, R. L. (1991a). Outer membrane protein profiles of *Yersinia ruckeri*. *Veterinary Microbiology, 26*(1–2), 125-140 https://doi.org/10.1016/0378-1135(91)90049-L

Davies, R. L. (1991b). Clonal analysis of *Yersinia ruckeri* based on biotypes, serotypes and outer membrane protein types. *Journal of Fish diseases, 14*(2), 221-228. https://doi.org/10.1111/j.1365-2761.1991.tb00591.x

Davies, R. L. (1991c). Virulence and serum-resistance in different clonal groups and serotypes of *Yersinia ruckeri. Veterinary Microbiology, 29*(3–4), 289-297. https://doi.org/10.1016/0378-1135(91)90136-4

Deshmukh, S., Raida, M. K., Dalsgaard, I., Chettri, J. K., Kania, P. W., & Buchmann, K. (2012). Comparative protection of two different commercial vaccines against *Yersinia ruckeri* serotype O1 and biotype 2 in rainbow trout (*Oncorhynchus mykiss*). *Veterinary immunology and immunopathology*, *145*(1-2), 379–385. https://doi.org/10.1016/j.vetimm.2011.12.014

Dinh Thanh, M., Agustí, G., Mader, A., Appel, B., & Codony, F. (2017). Improved sample treatment protocol for accurate detection of live *Salmonella* spp. in food samples by viability PCR. *PloS one, 12*(12), e0189302. https://doi.org/10.1371/journal.pone.0189302

Dulin, M. P., Huddleston, T., Larson, R. E. & Klontz, G. W. (1976). Enteric Redmouth Disease. Forest, Wildlife and Range Experiment Station, Bulletin number 8. Available from: Idaho Forest, Wildlife, and Range Experiment Station Collection, Digital Initiatives, University of Idaho Library. https://www.lib.uidaho.edu/digital/fwres/items/fwres89.html

Dwilow, A. G., Souter, B. W., & Knight, K. (1987). Isolation of *Yersinia ruckeri* from burbot, *Lota lota* (L.), from the Mackenzie River, Canada. *Journal of Fish Diseases 10*(4), 315-317.

Eissa, A.E., Moustafa, M., Abdelaziz, M., & Ezzeldeen, N.A. (2010). *Yersinia ruckeri* infection in cultured Nile tilapia, *Oreochromis niloticus*, at a semi-intensive fish farm in lower Egypt. *African Journal of Aquatic Science, 33*(3), 283-286 https://doi.org/10.2989/AJAS.2008.33.3.13.625

Ewing, W. H., Ross, A. J., Brenner, D. J., & Fanning, G. R. (1978). *Yersinia ruckeri* sp. nov., the redmouth (RM) bacterium. *International Journal of Systematic Bacteriology, 28*: 37-44.

Evenhuis, J. P., Lapatra, S. E., Verner-Jeffreys, D. W., Dalsgaard, I., & Welch, T. J. (2009). Identification of flagellar motility genes in *Yersinia ruckeri* by transposon mutagenesis. *Applied and environmental microbiology, 75*(20), 6630–6633. https://doi.org/10.1128/AEM.01415-09

Faguy, D. M., & Jarrell, K. F. (1999). A twisted tale: the origin and evolution of motility and chemotaxis in prokaryotes. *Microbiology (Reading, England)*, *145 (Pt 2)*, 279–281. https://doi.org/10.1099/13500872-145-2-279

Falcão, J. P., Brocchi, M., Proença-Módena, J. L., Acrani, G. O., Corrêa, E. F., & Falcão, D. P. (2004). Virulence characteristics and epidemiology of *Yersinia enterocolitica* and *Yersiniae* other than *Y. pseudotuberculosis* and *Y. pestis* isolated from water and sewage. *Journal of applied microbiology*, *96*(6), 1230–1236. https://doi.org/10.1111/j.1365-2672.2004.02268.x

Falush D. (2016). Bacterial genomics: Microbial GWAS coming of age. *Nature microbiology, 1,* 16059. https://doi.org/10.1038/nmicrobiol.2016.59

Fan, J., Zhao, L., Hu, Q., Li, S., Li, H., Zhang, Q., Zou, G., Zhang, L., Li, L., Huang, Q., & Zhou, R. (2022). Screening for Virulence-Related Genes via a Transposon Mutant Library of *Streptococcus suis* Serotype 2 Using a *Galleria mellonella* Larvae Infection Model. *Microorganisms*, *10*(5), 868. https://doi.org/10.3390/microorganisms10050868

FAO. 2022. The State of World Fisheries and Aquaculture 2022. Towards Blue Transformation. Rome, FAO. Available from https://doi.org/10.4060/cc0461en

Farmer, J. J., 3rd, Davis, B. R., Hickman-Brenner, F. W., McWhorter, A., Huntley-Carter, G. P., Asbury, M. A., Riddle, C., Wathen-Grady, H. G., Elias, C., & Fanning, G. R. (1985). Biochemical identification of new species and biogroups of *Enterobacteriaceae* isolated from clinical specimens. *Journal of clinical microbiology*, *21*(1), 46–76. https://doi.org/10.1128/jcm.21.1.46-76.1985

Feldman, M., Bryan, R., Rajan, S., Scheffler, L., Brunnert, S., Tang, H., & Prince, A. (1998). Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. Infection and *immunity, 66*(1), 43–51. https://doi.org/10.1128/IAI.66.1.43-51.1998

Fernández, L., Lopez, J. R., Secades, P., Menendez, A., Marquez, I., & Guijarro, J. A. (2003). *In vitro* and *in vivo* studies of the Yrp1 protease from *Yersinia ruckeri* and its role in protective immunity against enteric red mouth disease of salmonids. *Applied and environmental microbiology*, *69*(12), 7328–7335. https://doi.org/10.1128/AEM.69.12.7328-7335.2003

Fernández, L., Márquez, I., & Guijarro, J. A. (2004). Identification of specific *in vivo*-induced (ivi) genes in *Yersinia ruckeri* and analysis of ruckerbactin, a catecholate siderophore iron acquisition system. *Applied and environmental microbiology*, *70*(9), 5199–5207. https://doi.org/10.1128/AEM.70.9.5199-5207.2004

Fernández, L., Méndez, J., & Guijarro, J. A. (2007). Molecular virulence mechanisms of the fish pathogen *Yersinia ruckeri*. *Veterinary microbiology, 125*(1-2), 1–10. https://doi.org/10.1016/j.vetmic.2007.06.013

Fernández, L., Prieto, M., & Guijarro, J. A. (2007). The iron- and temperature-regulated haemolysin YhlA is a virulence factor of *Yersinia ruckeri*. *Microbiology (Reading, England)*, *153*(Pt 2), 483–489. https://doi.org/10.1099/mic.0.29284-0

Fernández, L., Secades, P., Lopez, J. R., Márquez, I., & Guijarro, J. A. (2002). Isolation and analysis of a protease gene with an ABC transport system in the fish pathogen Yersinia ruckeri: insertional mutagenesis and involvement in virulence. *Microbiology (Reading, England)*, *148*(Pt 7), 2233–2243. https://doi.org/10.1099/00221287-148-7-2233

Folkedal, O., Utskot, S. O., & Nilsson, J. (2021). Thermal delousing in anaesthetised small Atlantic salmon (*Salmo salar*) post-smolts: A case study showing the viability of anaesthesia prior to delousing for improved welfare during treatment for salmon lice. *Animal Welfare*, 30(2), 117-120. https://doi.org/10.7120/09627286.30.2.117

Fouz, B., Zarza, C., & Amaro, C. (2006). First description of non-motile *Yersinia ruckeri* serovar I strains causing disease in rainbow trout, *Oncorhynchus mykiss* (Walbaum), cultured in Spain. *Journal of fish diseases*, 29(6), 339–346. https://doi.org/10.1111/j.1365-2761.2006.00723.x

Frerichs, G. N., & Collins, R. O. (1984). Enteric redmouth disease in Scotland. *The Veterinary record*, *115*(2), 45–46. https://doi.org/10.1136/vr.115.2.45-a

Frerichs, G. N., Stewart, J. A., & Collins, R. O. (1985). Atypical infection of rainbow trout, *Salmo gairdneri* Richardson, with *Yersinia ruckeri*. *Journal of Fish diseases, 8*(4), 383-387. https://doi.org/10.1111/j.1365-2761.1985.tb00960.x

Fuhrmann, H., Bohm, K. H., & Schlotfeldt, H. J. (1983). An outbreak of enteric redmouth disease in West Germany. *Journal of Fish diseases, 6*(3), 309-311. https://doi.org/10.1111/j.1365-2761.1983.tb00080.x

Fuhrmann, H., Bohm, K. H., & Schlotfeldt, H. J. (1984). On the importance of enteric bacteria in the bacteriology of freshwater fish. *Bulletin of the European Association of Fish* Pathologists 4(3), 42-46.

Furones, M. D., Gilpin, M. J., Alderman, D. J., & Munn, C. B. (1990). Virulence of *Yersinia ruckeri* serotype I strains is associated with a heat sensitive factor (HSF) in cell extracts. *FEMS microbiology letters*, *54*(1-3), 339–343. https://doi.org/10.1016/0378-1097(90)90309-e

Furones, M. D., Gilpin, M. L., & Munn, C. B. (1993). Culture media for the differentiation of isolates of *Yersinia ruckeri*, based on detection of a virulence factor. *The Journal of applied bacteriology*, *74*(4), 360–366. https://doi.org/10.1111/j.1365-2672.1993.tb05139.x

Furones, M. D., Rodgers, C. J., & Munn, C. B. (1993). *Yersinia ruckeri*, the causal agent of enteric redmouth disease (ERM) in fish. *Annual Review of Fish Diseases, 3*, 0–125. https://doi.org/10.1016/0959-8030(93)90031-6

Garcia, J. A., Dominguez, L., Larsen, J. L., & Pedersen, K. (1998). Ribotyping and plasmid profiling of *Yersinia ruckeri*. *Journal of Applied Microbiology, 85*(6), 949–955. https://doi.org/10.1111/j.1365-2672.1998.tb05258.x

Gelev, I., Gelev, E., Steigerwalt, A., Carter, G., & Brenner, D. (1990). Identification of the bacterium associated with haemorrhagic septicaemia in rainbow trout as *Hafnia alvei*. *Research in Microbiology, 141*(5), 573–576. https://doi.org/10.1016/0923-2508(90)90021-h

Ghosh, B., Crosbie, P., Nowak, B. F., & Bridle, A. R. (2018). A highly sensitive, non-invasive qPCR-based strategy for direct quantification of *Yersinia ruckeri* in fish faeces. *Journal of fish diseases*, *41*(9), 1421–1428. https://doi.org/10.1111/jfd.12839

Gibello, A., Porrero, M. C., Blanco, M. M., Vela, A. I., Liébana, P., Moreno, M. A., Fernández-Garayzábal, J. F., & Domínguez, L. (2004). Analysis of the gyrA gene of clinical *Yersinia ruckeri* isolates with reduced susceptibility to quinolones. *Applied and environmental microbiology*, *70*(1), 599–602. https://doi.org/10.1128/AEM.70.1.599-602.2004

Gibello, A., Blanco, M. M., Moreno, M. A., Cutuli, M. T., Domenech, A., Domínguez, L., & Fernández-Garayzábal, J. F. (1999). Development of a PCR assay for detection of *Yersinia ruckeri* in tissues of inoculated and naturally infected trout. *Applied and environmental microbiology*, *65*(1), 346–350. https://doi.org/10.1128/AEM.65.1.346-350.1999

Giorgetti, G., Geschia, G., & Sarti, M. (1985). Evolution of bacterial pathology in italian fresh water trout farms. International conference of the *European Association of Fish Pathologists, 1985,* paper No. 13.

Gismervik, K, Gåsnes, S. K., Nielsen, K. V., & Mejdell C. M. (2019). Fish welfare. In Hjeltnes, B., Bang Jensen, B., Bornø, G., Haukaas, A., & Walde, C. S. (Eds.), *The health situation in Norwegian aquaculture 2018* (pp. 20-31). Published by the Norwegian Veterinary Institute. Retrieved from https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2019/fish-health-report-2018

Gismervik, K., Gåsnes, S. K., Nielsen, K. V., & Mejdell, C. M. (2020). Fiskevelferd [Fish welfare]. In Sommerset, I., Walde, C. S., Bang Jensen, B., Bornø, B., Haukaas, A., & Brun, E. (Eds.). *Fiskehelserapporten 2019* [Fish health report 2019] (pp. 24-41). In Norwegian. Published by the Norwegian Veterinary Institute. Retrieved from https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2020/fiskehelserapporten-2019

Glenn, R:A., Taylor, P.W., Pelton, E.H., Gutenberger, S.K., Ahrens, M.A., Marchant, L.M., & Hanson, K.C. (2015). Genetic Evidence of Vertical Transmission and Cycling of *Yersinia ruckeri* in Hatchery-Origin Fall Chinook Salmon *Oncorhynchus tshawytscha*. *Journal of Fish and Wildlife Management 6*(1), 44–54. https://doi.org/10.3996/012014-JFWM-010

Gudding, R., & Van Muiswinkel, W. B. (2013). A history of fish vaccination: science-based disease prevention in aquaculture. *Fish & shellfish immunology*, *35*(6), 1683–1688. https://doi.org/10.1016/j.fsi.2013.09.031

De Grandis, S.A., Krell, P.J., Flett, D.E., & Stevenson, R.M.W. (1988). Deoxyribonucleic Acid Relatedness of Serovars of *Yersinia ruckeri*, the Enteric Redmouth Bacterium. *International Journal of Systematic Bacteriology 38*(1), 49-55. https://doi.org/10.1099/00207713-38-1-49

De Grandis, S. A., & Stevenson, R. M. (1985). Antimicrobial susceptibility patterns and R plasmid-mediated resistance of the fish pathogen *Yersinia ruckeri*. *Antimicrobial agents and chemotherapy*, *27*(6), 938–942. https://doi.org/10.1128/AAC.27.6.938

Grave, K., Lillehaug, A., Lunestad, B. T., & Horsberg, T. E. (1999). Prudent use of antibacterial drugs in Norwegian aquaculture? Surveillance by the use of prescription data. *Acta veterinaria Scandinavica*, *40*(3), 185–195. https://doi.org/10.1186/BF03547016

Green, M., & Austin, B. (1983). The identification of *Yersinia ruckeri* and its relationship to other representatives of the *Enterobacteriaceae*. *Aquaculture, 34*(3-4), 185–192. https://doi.org/10.1016/0044-8486(83)90201-6

Grøtvedt, R., & Jansen, P. A. (2016). Salmon lice. In Hjeltnes, B., Walde, C., Bang jensen, B., & Haukaas, A. (Eds.). *The Fish Health Report 2015* (pp. 49-55). Published by the Norwegian Veterinary Institute. Retrieved from https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2016/fish-health-report-2015

Gudmundsdottir, B. K., Gudmundsdottir, S., Gudmundsdottir, S., & Magnadottir, B. (2014). Yersiniosis in Atlantic cod, *Gadus morhua* (L.), characterization of the infective strain and host reactions. *Journal of fish diseases*, *37*(6), 511–519. https://doi.org/10.1111/jfd.12139

Guilvout, I., Quilici, M. L., Rabot, S., Lesel, R., & Mazigh, D. (1988). BamHI restriction endonuclease analysis of *Yersinia ruckeri* plasmids and their relatedness to the genus *Yersinia* 42- to 47-megadalton plasmid. *Applied and environmental microbiology*, *54*(10), 2594–2597. https://doi.org/10.1128/aem.54.10.2594-2597.1988

Gulla, S. (2017). Yersiniosis. In Hjeltnes, B., Bornø, G., Jansen, M. D., Haukaas, A., & Walde, C. S. (Eds.), *The health situation in Norwegian aquaculture 2016* (pp. 78-82). Published by the Norwegian Veterinary Institute. Retrieved from https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2017/fish-health-report-2016

Gulla, S., Gu, J., & Olsen, A.B. (2019). Yersiniosis. In Hjeltnes, B., Bang Jensen, B., Bornø, G., Haukaas, A., & Walde, C. S. (Eds.), *The health situation in Norwegian aquaculture 2018* (pp. 70-73). Published by the Norwegian Veterinary Institute. Retrieved from https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2019/fish-health-report-2018

Gulla, S., & Olsen, A.B. (2020). Yersiniosis. In Sommerset, I., Walde, C. S., Bang Jensen, B., Bornø, B., Haukaas, A., & Brun, E. (Eds.). *Fiskehelserapporten 2019* [Fish health report 2019] (pp. 80-82). In Norwegian. Published by the Norwegian Veterinary Institute. Retrieved from https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2020/fiskehelserapporten-2019

Gulla, S., & Olsen, A.B. (2022). Yersiniosis. In Sommerset, I., Walde, C. S., Bang Jensen, B., Wiik-Nielsen, J., Bornø, G., Oliveira, V.H.S., Haukaas, A., & Brun, E. Norwegian Fish Health Report 2021 (pp. 109-110). Published by the Norwegian Veterinary Institute. Retrieved from https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2022/fish-health-report-2021

Gulla, S., Barnes, A. C., Welch, T. J., Romalde, J. L., Ryder, D., Ormsby, M. J., Carson, J., Lagesen, K., Verner-Jeffreys, D. W., Davies, R. L., & Colquhoun, D. J. (2018). Multilocus Variable-Number Tandem-Repeat Analysis of *Yersinia ruckeri* Confirms the Existence of Host Specificity, Geographic Endemism, and Anthropogenic Dissemination of Virulent Clones. *Applied and environmental microbiology*, 84(16), e00730-18. https://doi.org/10.1128/AEM.00730-18

Gunasena, D. K., Komrower, J. R., & Macintyre, S. (2003). The Fish Pathogen *Yersinia ruckeri* Possesses a TTS System. In Skurnik, M., Bengoechea, J. A., &, Granfors, K. (Eds.). *The Genus Yersinia* (pp. 105–107). Published by Kluwer Academic/Plenum Publishers, New York, 2003. https://doi.org/10.1007/0-306-48416-1_19

Haig, S. J., Davies, R. L., Welch, T. J., Reese, R. A., & Verner-Jeffreys, D. W. (2011). Comparative susceptibility of Atlantic salmon and rainbow trout to *Yersinia ruckeri*: relationship to O antigen serotype and resistance to serum killing. *Veterinary microbiology*, *147*(1-2), 155–161. https://doi.org/10.1016/j.vetmic.2010.06.022

Hall, M., Chattaway, M. A., Reuter, S., Savin, C., Strauch, E., Carniel, E., Connor, T., Van Damme, I., Rajakaruna, L., Rajendram, D., Jenkins, C., Thomson, N. R., & McNally, A. (2015). Use of whole-genus genome sequence data to develop a multilocus sequence typing tool that accurately identifies *Yersinia* isolates to the species and subspecies levels. *Journal of clinical microbiology*, *53*(1), 35–42. https://doi.org/10.1128/JCM.02395-14

Haller, J. C., Carlson, S., Pederson, K. J., & Pierson, D. E. (2000). A chromosomally encoded type III secretion pathway in *Yersinia enterocolitica* is important in virulence. *Molecular microbiology*, *36*(6), 1436–1446. https://doi.org/10.1046/j.1365-2958.2000.01964.x

Hares, M. C., Hinchliffe, S. J., Strong, P., Eleftherianos, I., Dowling, A. J., Ffrench-Constant, R. H., & Waterfield, N. (2008). The *Yersinia pseudotuberculosis* and *Yersinia pestis* toxin complex is active against cultured mammalian cells. *Microbiology (Reading, England)*, *154*(Pt 11), 3503–3517. https://doi.org/10.1099/mic.0.2008/018440-0

Heimbrook, M. E., Wang, W. L., & Campbell, G. (1989). Staining bacterial flagella easily. *Journal of clinical microbiology*, 27(11), 2612–2615. https://doi.org/10.1128/jcm.27.11.2612-2615.1989

Hernández, S. P. (2005). Responsible use of antibiotics in aquaculture. Food and Agriculture Organization of the United Nations, Rome, Italy. Fisheries technical paper. Available from: https://www.fao.org/3/a0282e/a0282e.pdf

Heymann, J. B., Bartho, J. D., Rybakova, D., Venugopal, H. P., Winkler, D. C., Sen, A., Hurst, M., & Mitra, A. K. (2013). Three-dimensional structure of the toxin-delivery particle antifeeding prophage of *Serratia entomophila*. *The Journal of biological chemistry*, *288*(35), 25276–25284. https://doi.org/10.1074/jbc.M113.456145

Hjeltnes, B. (Ed.) (2014). *Fish Health Report 2013*. Published by the Norwegian Veterinary Institute. Retrieved from https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2014/fish-healt-report-2013

Hjeltnes, B., Bæverfjord, G., Erikson, U., Mortensen, S., Rosten, T., & Østergård, P. (2012). Risk Assessment of Recirculation Systems in Salmonid Hatcheries. Published by the Norwegian Scientific Committee for Food Safety (VKM). Report series 2012, 1. Retrieved from https://vkm.no/english

Holland, P. M., Abramson, R. D., Watson, R., & Gelfand, D. H. (1991). Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. *Proceedings of the National Academy of Sciences of the United States of America*, *88*(16), 7276–7280. https://doi.org/10.1073/pnas.88.16.7276

Horne, S. M., & Prüss, B. M. (2006). Global gene regulation in *Yersinia enterocolitica*: effect of FliA on the expression levels of flagellar and plasmid-encoded virulence genes. *Archives of microbiology*, *185*(2), 115–126. https://doi.org/10.1007/s00203-005-0077-1

Huang, Y., Jung, A., Schäfer, W. J., Mock, D., Geovana Brenner Michae, G., Runge, M., Schwarz, S., & Steinhagen, D. (2015) Analysis of *Yersinia ruckeri* strains isolated from trout farms in northwest Germany. *Diseases of Aquatic Organisms 116*:243-249. https://doi.org/10.3354/dao02920

Huang, Y., Michael, G. B., Becker, R., Kaspar, H., Mankertz, J., Schwarz, S., Runge, M., & Steinhagen, D. (2014). Pheno- and genotypic analysis of antimicrobial resistance properties of *Yersinia ruckeri* from fish. *Veterinary microbiology*, *171*(3-4), 406–412. https://doi.org/10.1016/j.vetmic.2013.10.026

Hurst, M., Becher, S. A., Young, S. D., Nelson, T. L., & Glare, T. R. (2011). *Yersinia entomophaga* sp. nov., isolated from the New Zealand grass grub *Costelytra zealandica*. *International journal of systematic and evolutionary microbiology*, *61*(Pt 4), 844–849. https://doi.org/10.1099/ijs.0.024406-0

Hurst, M., Beattie, A., Jones, S. A., Laugraud, A., van Koten, C., & Harper, L. (2018). Serratia proteamaculans Strain AGR96X Encodes an Antifeeding Prophage (Tailocin) with Activity against Grass Grub (*Costelytra giveni*) and Manuka Beetle (*Pyronota Species*) Larvae. *Applied and environmental microbiology*, *84*(10), e02739-17. https://doi.org/10.1128/AEM.02739-17

Hyatt, D., Chen, G. L., Locascio, P. F., Land, M. L., Larimer, F. W., & Hauser, L. J. (2010). Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC bioinformatics*, *11*, 119. https://doi.org/10.1186/1471-2105-11-119

Iwobi, A., Heesemann, J., Garcia, E., Igwe, E., Noelting, C., & Rakin, A. (2003). Novel virulence-associated type II secretion system unique to high-pathogenicity *Yersinia enterocolitica*. *Infection and immunity*, *71*(4), 1872–1879. https://doi.org/10.1128/IAI.71.4.1872-1879.2003

Janda, J. M., & Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *Journal of clinical microbiology*, *45*(9), 2761–2764. https://doi.org/10.1128/JCM.01228-07

Jank, T., Eckerle, S., Steinemann, M., Trillhaase, C., Schimpl, M., Wiese, S., van Aalten, D. M., Driever, W., & Aktories, K. (2015). Tyrosine glycosylation of Rho by Yersinia toxin impairs blastomere cell behaviour in zebrafish embryos. *Nature communications*, *6*, 7807. https://doi.org/10.1038/ncomms8807

Joh, S. J., Kweon, C. H., Kim, M. J., Kang, M. S., Jang, H., & Kwon, J. H. (2010). Characterization of *Yersinia ruckeri* isolated from the farm-cultured eel *Anguilla japonica* in Korea. *Korean Journal of Veterinary Research 50*(1), 29-35.

Johnsen, S. I., Strand, D. A., Rusch J. C., & Vrålstad T. (2020). Environmental DNA (eDNA) Monitoring of Noble Crayfish *Astacus astacus* in Lentic Environments Offers Reliable Presence-Absence Surveillance – But Fails to Predict Population Density. *Frontiers in Environmental Science, 8*. https://doi.org/10.3389/fenvs.2020.612253

Jozwick, A. K. S., Graf, J., & Welch, T. J. (2017). The flagellar master operon *flhDC* is a pleiotropic regulator involved in motility and virulence of the fish pathogen *Yersinia ruckeri*. *Journal of applied microbiology*, 122(3), 578–588. https://doi.org/10.1111/jam.13374

Jozwick, A. K. S., LaPatra, S. E., Graf, J., & Welch, T. J. (2019). Flagellar regulation mediated by the Rcs pathway is required for virulence in the fish pathogen *Yersinia ruckeri*. *Fish & shellfish immunology*, 91, 306–314. https://doi.org/10.1016/j.fsi.2019.05.036

Keeling, S.E., Johnston, C., Wallis, R., Brosnahan, C.L., Gudkovs, N., & McDonald, W.L. (2012). Development and validation of real-time PCR for the detection of *Yersinia ruckeri*. Journal of Fish Diseases, 35: 119-125. https://doi.org/10.1111/j.1365-2761.2011.01327.x

Kennedy, D. A., & Read, A. F. (2017). Why does drug resistance readily evolve but vaccine resistance does not?. *Proceedings. Biological sciences*, *284*(1851), 20162562. https://doi.org/10.1098/rspb.2016.2562

De Keukeleire, S., De Bel, A., Jansen, Y., Janssens, M., Wauters, G., & Pierard, D. (2014). *Yersinia ruckeri*, an unusual microorganism isolated from a human wound infection. *New Microbes and New Infections, 2*(4), 134-135.

Kim, D. H., & Austin, B. (2006). Innate immune responses in rainbow trout (*Oncorhynchus mykiss*, Walbaum) induced by probiotics. *Fish & shellfish immunology*, *21*(5), 513–524. https://doi.org/10.1016/j.fsi.2006.02.007

Kolmogorov, M., Yuan, J., Lin, Y., & Pevzner, P. A. (2019). Assembly of long, error-prone reads using repeat graphs. *Nature Biotechnology.* https://doi.org/10.1038/s41587-019-0072-8

Kralik, P., & Ricchi, M. (2017). A Basic Guide to Real Time PCR in Microbial Diagnostics: Definitions, Parameters, and Everything. *Frontiers in Microbiology, 8*. https://doi.org/10.3389/fmicb.2017.00108

Kumar, G., Menanteau-Ledouble, S., Saleh, M., & El-Matbouli, M. (2015). *Yersinia ruckeri*, the causative agent of enteric redmouth disease in fish. *Veterinary research*, *46*(1), 103. https://doi.org/10.1186/s13567-015-0238-4

Kutyavin, I. V., Afonina, I. A., Mills, A., Gorn, V. V., Lukhtanov, E. A., Belousov, E. S., Singer, M. J., Walburger, D. K., Lokhov, S. G., Gall, A. A., Dempcy, R., Reed, M. W., Meyer, R. B., & Hedgpeth, J. (2000). 3'-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic acids research*, *28*(2), 655–661. https://doi.org/10.1093/nar/28.2.655

Latasa, C., Solano, C., Penadés, J. R., & Lasa, I. (2006). Biofilm-associated proteins. *Comptes rendus biologies*, *329*(11), 849–857. https://doi.org/10.1016/j.crvi.2006.07.008

Leatham, M. P., Stevenson, S. J., Gauger, E. J., Krogfelt, K. A., Lins, J. J., Haddock, T. L., Autieri, S. M., Conway, T., & Cohen, P. S. (2005). Mouse intestine selects nonmotile *flhDC* mutants of *Escherichia coli* MG1655 with increased colonizing ability and better utilization of carbon sources. *Infection and immunity*, *73*(12), 8039–8049. https://doi.org/10.1128/IAI.73.12.8039-8049.2005

LeJeune, J. T., & Rurangirwa, F. R. (2000). Polymerase chain reaction for definitive identification of *Yersinia ruckeri*. *Journal of veterinary diagnostic investigation* 12(6), 558–561. https://doi.org/10.1177/104063870001200611

Leo, J. C., & Skurnik, M. (2011). Adhesins of human pathogens from the genus *Yersinia*. *Advances in experimental medicine and biology*, *715*, 1–15. https://doi.org/10.1007/978-94-007-0940-9_1

Leo, J. C., Oberhettinger, P., Schütz, M., & Linke, D. (2015). The inverse autotransporter family: intimin, invasin and related proteins. *International journal of medical microbiology : IJMM*, *305*(2), 276–282. https://doi.org/10.1016/j.ijmm.2014.12.011

Lees, J., Bentley, S. (2016) Bacterial GWAS: not just gilding the lily. *Nature Reviews Microbiology 14,* 406. https://doi.org/10.1038/nrmicro.2016.82

Lesel, R., Lesel, M., Gavini, F., & Vulllaume, A. (1983). Outbreak of enteric redmouth disease in rainbow trout, *Salmo gairdneri* Richardson, in France. *Journal of fish diseases*, 6(4), 385-387. https://doi.org/10.1111/j.1365-2761.1983.tb00091.x

Lewin, A. S., Haugen, T., Netzer, R., Tøndervik, A., Dahle, S. W., & Hageskal, G. (2020). Multiplex droplet digital PCR assay for detection of *Flavobacterium psychrophilum* and *Yersinia ruckeri* in Norwegian aquaculture. *Journal of microbiological methods*, *177*, 106044. https://doi.org/10.1016/j.mimet.2020.106044

Lillehaug, A., Jensen, B. B., Toft, N., Qviller, L., Jansen, M. D., Brun, E., Nilsen, A., Hansen, H., & Hjeltnes, B. (2019). Changes in risk of infection. In Hjeltnes, B., Bang Jensen, B., Bornø, G., Haukaas, A., & Walde, C. S. (Eds.), *The health situation in Norwegian aquaculture 2018* (pp. 10-19). Published by the Norwegian Veterinary Institute. Retrieved from https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2019/fish-health-report-2018

Lindland, K. M., Gjerstad, B., Krøvel, A. V., & Ravagnan, E. (2019). Governing for sustainability in the Norwegian aquaculture industry. *Ocean & Coastal Management, 179*, 104827. https://doi.org/10.1016/j.ocecoaman.2019.104827

Liu, T., Wang, K. Y., Wang, J., Chen, D. F., Huang, X. L., Ouyang, P., Geng, Y., He, Y., Zhou, Y., & Min, J. (2016). Genome Sequence of the Fish Pathogen *Yersinia ruckeri* SC09 Provides Insights into Niche Adaptation and Pathogenic Mechanism. *International journal of molecular sciences*, *17*(4), 557. https://doi.org/10.3390/ijms17040557

Liu, T., Wang, E., Wei, W., Wang, K., Yang, Q., & Ai, X. (2019). TcpA, a novel *Yersinia ruckeri* TIR-containing virulent protein mediates immune evasion by targeting MyD88 adaptors. *Fish & shellfish immunology*, *94*, 58–65. https://doi.org/10.1016/j.fsi.2019.08.069

Liu, T., Li, L., Wei, W., Wang, K., Yang, Q., & Wang, E. (2020). *Yersinia ruckeri* strain SC09 disrupts proinflammatory activation via Toll/IL-1 receptor-containing protein STIR-3. *Fish & shellfish immunology*, *99*, 424–434. https://doi.org/10.1016/j.fsi.2020.02.035

Llewellyn, L. C. (1980). A bacterium with similarities to the redmouth bacterium and *Serratia liquefaciens* (Grimes and Hennerty) causing mortalities in hatchery reared salmonids in Australia. *Journal of Fish Diseases, 3*(1), 29–39. https://doi.org/10.1111/j.1365-2761.1980.tb00181.x

Nilsson, J., Moltumyr, L., Madaro, A., Kristiansen, T. S., Gåsnes, S. K., Mejdell, C. M., Gismervik, K., & Stien, L. H. (2019). Sudden exposure to warm water causes instant behavioural responses indicative of nociception or pain in Atlantic salmon. *Veterinary and animal science*, *8*, 100076. https://doi.org/10.1016/j.vas.2019.100076

Nowakiewicz, A., Ziółkowska, G., Zięba, P., Dziedzic, B. M., Gnat, S., Wójcik, M., Dziedzic, R., & Kostruba, A. (2015). Aerobic bacterial microbiota isolated from the cloaca of the European pond turtle (*Emys orbicularis*) in Poland. *Journal of wildlife diseases*, *51*(1), 255–259. https://doi.org/10.7589/2013-07-157

Manna, S.K., Samanta, S., Das, M.K., & Mishra, S.S. (2003). An outbreak of *Yersinia ruckeri* septicaemia in Indian Major Carps. *Journal of the Inland Fisheries Society of India, 35* (2) (2003), pp. 28-31

Maury, M. M., Tsai, Y. H., Charlier, C., Touchon, M., Chenal-Francisque, V., Leclercq, A., Criscuolo, A., Gaultier, C., Roussel, S., Brisabois, A., Disson, O., Rocha, E., Brisse, S., & Lecuit, M. (2016). Uncovering *Listeria monocytogenes* hypervirulence by harnessing its biodiversity. *Nature genetics, 48*(3), 308–313. https://doi.org/10.1038/ng.3501

McArdle, J. F., & Dooley-Martyn, C. (1985). Isolation of *Yersinia ruckeri* type I (Hagerman strain) from goldfish *Carassius auratus* (L.). *Bulletin of the European Association of Fish Pathologists 5*(1), 10-11

McClure, J. M., Lakhundi, S., Kashif, A., Conly, J. M., & Zhang, K. (2018). Genomic Comparison of Highly Virulent, Moderately Virulent, and Avirulent Strains From a Genetically Closely-Related MRSA ST239 Sub-lineage Provides Insights Into Pathogenesis. *Frontiers in microbiology*, *9*, 1531. https://doi.org/10.3389/fmicb.2018.01531

McDaniel, D. (1971). Hagerman redmouth: a new look at an old fish problem. *American Fishes and U.S. Trout News, 15*(5), 14-28.

Meier, W. (1986). Enteric redmouth disease: outbreak in rainbow trout in Switzerland. *Diseases of Aquatic Organisms, 2*, 81-82.

Menanteau-Ledouble, S., Lawrence, M. L., & El-Matbouli, M. (2018). Invasion and replication of *Yersinia ruckeri* in fish cell cultures. *BMC veterinary research, 14*(1), 81. https://doi.org/10.1186/s12917-018-1408-1

Menanteau-Ledouble, S., Nöbauer, K., Razzazi-Fazeli, E., & El-Matbouli, M. (2020). Effects of *Yersinia ruckeri* invasion on the proteome of the Chinook salmon cell line CHSE-214. *Scientific reports*, *10*(1), 11840. https://doi.org/10.1038/s41598-020-68903-5

Méndez, J., Fernández, L., Menéndez, A., Reimundo, P., Pérez-Pascual, D., Navais, R., & Guijarro, J. A. (2009). A chromosomally located *traHIJKCLMN* operon encoding a putative type IV secretion system is involved in the virulence of *Yersinia ruckeri*. *Applied and environmental microbiology*, *75*(4), 937–945. https://doi.org/10.1128/AEM.01377-08

Michel, C., Faivre, B., & De Kinkelin, P. (1986). A clinical case of enteric redmouth in minnows (*Pimephales promelas*) imported in Europe as bait-fish. *Bulletin of the European Association of Fish Pathologists 6*(4), 97- 99.

Midtlyng, P. J., Grave, K., & Horsberg, T. E. (2011). What has been done to minimize the use of antibacterial and antiparasitic drugs in Norwegian aquaculture? *Aquaculture Research, 42*, 28-34 https://doi.org/10.1111/j.1365-2109.2010.02726.x

Mitchum, D. L. (1981). Concurrent infections: ERM and furunculosis found in emerald shiners. *American Fisheries Society Fish Health Newsletter 9*(5).

Moreau, E., Thomas, T., Brevet, M., Thorin, C., Fournel, C., & Calvez, S. (2019). Mutations involved in the emergence of *Yersinia ruckeri* biotype 2 in France. *Transboundary and emerging diseases*, *66*(3), 1387–1394. https://doi.org/10.1111/tbed.13175

Mühlenkamp, M., Oberhettinger, P., Leo, J. C., Linke, D., & Schütz, M. S. (2015). *Yersinia* adhesin A (YadA)--beauty & beast. *International journal of medical microbiology : IJMM*, *305*(2), 252–258. https://doi.org/10.1016/j.ijmm.2014.12.008

Murros-Kontiainen, A., Fredriksson-Ahomaa, M., Korkeala, H., Johansson, P., Rahkila, R., & Björkroth, J. (2011). *Yersinia nurmii* sp. nov. *International journal of systematic and evolutionary microbiology*, *61*(Pt 10), 2368–2372. https://doi.org/10.1099/ijs.0.024836-0

Myhr, E., & Lillehaug, A. (1987). Yersiniose – 'ERM, Enteric redmouth disease, Rødmunnsjuke' – hos oppdrettsfisk: en oversikt med spesiell vekt på sjukdommen under norske forhold [Yersiniosis – 'ERM, Enteric redmouth disease, Redmouth disease' – in aquaculture: an overview with special emphasis on the disease in Norwegian circumstances]. In Norwegian. Veterinary leaflet. VD-NYTT, 1987, 3. pp 2-8. Published by Veterinæravdelingen, Oslo, Norway.

Navais, R., Méndez, J., Cascales, D., Reimundo, P., & Guijarro, J. A. (2014a). The heat sensitive factor (HSF) of *Yersinia ruckeri* is produced by an alkyl sulphatase involved in sodium dodecyl sulphate (SDS) degradation but not in virulence. *BMC microbiology*, *14*, 221. https://doi.org/10.1186/s12866-014-0221-7

Navais, R., Méndez, J., Pérez-Pascual, D., Cascales, D., & Guijarro, J. A. (2014b). The *yrpAB* operon of *Yersinia ruckeri* encoding two putative U32 peptidases is involved in virulence and induced under microaerobic conditions. *Virulence*, *5*(5), 619–624. https://doi.org/10.4161/viru.29363

Nelson, M. C., LaPatra, S. E., Welch, T. J., & Graf, J. (2015). Complete Genome Sequence of *Yersinia ruckeri* Strain CSF007-82, Etiologic Agent of Red Mouth Disease in Salmonid Fish. *Genome announcements, 3*(1), e01491-14. https://doi.org/10.1128/genomeA.01491-14

Nordmo, R., Sevatdal, S., & Ramstad, A., (1997). Experimental infection with *Vibrio salmonicida* in Atlantic salmon (*Salmo salar* L.): an evaluation of three different challenge methods. *Aquaculture 158,* 23-32.

Ohtani, M., Villumsen, K. R., Koppang, E. O., & Raida, M. K. (2015). Global 3D imaging of *Yersinia ruckeri* bacterin uptake in rainbow trout fry. *PloS one*, *10*(2), e0117263. https://doi.org/10.1371/journal.pone.0117263

O'Leary, P. J. (1977). Enteric Redmouth Bacterium of salmonids : a biochemical and serological comparison of selected isolates. Master of Science (M.S.) thesis, Microbiology. Oregon State University. Retrieved from https://ir.library.oregonstate.edu/concern/graduate_thesis_or_dissertations/5x21tj391

O'leary P. J., Rohovec J. S., & Fryer J. L. (1979). A further characterization of *Yersinia ruckeri* (Enteric Redmouth Bacterium). *Fish Pathology 14*(2) 71-78.

O'Leary, P. J., Rohovec, J. S., Sanders, J. E., & Fryer, J. L. (1982). Serotypes of *Yersinia ruckeri* and their immunogenic properties. Oregon State University Agricultural Experiment Station Technical paper no. 6235. Retrieved from https://repository.library.noaa.gov/view/noaa/10921

Ormsby, M. J., Caws, T., Burchmore, R., Wallis, T., Verner-Jeffreys, D. W., & Davies, R. L. (2016). *Yersinia ruckeri* Isolates Recovered from Diseased Atlantic Salmon (*Salmo salar)* in Scotland Are More Diverse than Those from Rainbow Trout (*Oncorhynchus mykiss*) and Represent Distinct Subpopulations. *Applied and environmental microbiology*, *82*(19), 5785–5794. https://doi.org/10.1128/AEM.01173-16

Ormsby, M. J., & Davies, R. L. (2021). Diversification of OmpA and OmpF of *Yersinia ruckeri* is independent of the underlying species phylogeny and evidence of virulence-related selection. *Scientific reports*, *11*(1), 3493. https://doi.org/10.1038/s41598-021-82925-7

Ormsby, M. J., Grahame, E., Burchmore, R., & Davies, R. L. (2019). Comparative bioinformatic and proteomic approaches to evaluate the outer membrane proteome of the fish pathogen *Yersinia ruckeri*. *Journal of proteomics*, *199*, 135–147. https://doi.org/10.1016/j.jprot.2019.02.014

O'Toole, R., Lundberg, S., Fredriksson, S. A., Jansson, A., Nilsson, B., & Wolf-Watz, H. (1999). The chemotactic response of *Vibrio anguillarum* to fish intestinal mucus is mediated by a combination of multiple mucus components. *Journal of bacteriology*, *181*(14), 4308–4317. https://doi.org/10.1128/JB.181.14.4308-4317.1999

Overton, K., Dempster, T., Oppedal, F., Kristiansen, T. S., Gismervik, K., & Stien, L. H. (2019). Salmon lice treatments and salmon mortality in Norwegian aquaculture: a review. *Reviews in Aquaculture*, *11*(4), 1398-1417. https://doi.org/10.1111/raq.12299

Pallen, Mark J.; Wren, Brendan W. (2007). Bacterial pathogenomics. *Nature 449* (7164), 835–842. https://doi.org/10.1038/nature06248

Papaparaskevas, J., Houhoula, D. P., Papadimitriou, M., Saroglou, G., Legakis, N. J., & Zerva, L. (2004). Ruling out *Bacillus anthracis*. *Emerging infectious diseases, 10*(4), 732–735. https://doi.org/10.3201/eid1004.030544

Percival, S. L., & Williams, D. W. (2014). Chapter Thirteen – *Yersinia*. In Percival, S. L., Yates, M. V., Williams, D. W., Chalmers, R. M., & Gray, N. F. (Eds.), *Microbiology of Waterborne Diseases, 2nd edition* (pp. 249-259). Published by Academic Press, Cambridge, USA. https://doi.org/10.1016/B978-0-12-415846-7.00013-5

Popovic, N. T., Hacmanjek, M. & Teskeredzic, E. (2001). Health status of rudd (*Scardinius erythrophthalamus hesperidicus* H.) in Lake Vrana on the Island of Cres, Croatia. *Journal of Applied Ichthyology 17*(1), 43-45.

Pujol, C., & Bliska, J. B. (2005). Turning Yersinia pathogenesis outside in: subversion of macrophage function by intracellular *yersiniae*. *Clinical immunology (Orlando, Fla.)*, *114*(3), 216–226. https://doi.org/10.1016/j.clim.2004.07.013

Raju, D., Rizzuti, D., & Jones, N. L. (2012). Cell culture assays to evaluate bacterial toxicity and virulence. *Methods in molecular biology (Clifton, N.J.)*, *921*, 77–88. https://doi.org/10.1007/978-1-62703-005-2_11

Ramos, H. C., Rumbo, M., & Sirard, J. C. (2004). Bacterial flagellins: mediators of pathogenicity and host immune responses in mucosa. *Trends in microbiology*, *12*(11), 509–517. https://doi.org/10.1016/j.tim.2004.09.002

Ren, T., Zamboni, D. S., Roy, C. R., Dietrich, W. F., & Vance, R. E. (2006). Flagellin-deficient *Legionella* mutants evade caspase-1- and Naip5-mediated macrophage immunity. *PLoS pathogens*, *2*(3), e18. https://doi.org/10.1371/journal.ppat.0020018

Renaud, N., Lecci, L., Courcol, R. J., Simonet, M., & Gaillot, O. (2012). Comparison of CHROMagar *Yersinia* and CIN Agar media for isolation of potentially virulent *Yersinia enterocolitica* in stools. ASM2012, Poster 3896. Retrieved from https://www.chromagar.com/wp-content/uploads/2021/12/O.GAILLOT_ASM_2012.pdf

Reuter, S., Connor, T. R., Barquist, L., Walker, D., Feltwell, T., Harris, S. R., Fookes, M., Hall, M. E., Petty, N. K., Fuchs, T. M., Corander, J., Dufour, M., Ringwood, T., Savin, C., Bouchier, C., Martin, L., Miettinen, M., Shubin, M., Riehm, J. M., Laukkanen-Ninios, R., … Thomson, N. R. (2014). Parallel independent evolution of pathogenicity within the genus *Yersinia*. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(18), 6768–6773. https://doi.org/10.1073/pnas.1317161111

Rintamäki, P., Valtonen, E. T. & Frerichs, G. N. (1986). Occurence of *Yersinia ruckeri* infection in farmed whitefish, *Coregonus peled* Gmelin and *Coregonus muksun* Pallas, and Atlantic salmon, *Salmo salar* L., in Northern Finland. *Journal of Fish Diseases, 9*(2), 137-140. 10.1111/j.1365-2761.1986.tb00993.x

Roberts, M. S. (1983). A report of an epizootic in hatchery reared rainbow trout, *Salmo gairdneri* Richardson, at an English trout farm, caused by *Yersinia ruckeri*. *Journal of Fish Diseases, 6*, 551-552. https://doi.org/10.1111/j.1365-2761.1983.tb00111.x

Robertson, P. A. W., O'Dowd, C., Burrells, C., Williams, P., & Austin, B. (2000). Use of *Carnobacterium* sp. as a probiotic for Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*, Walbaum). *Aquaculture, 185*(3–4), 235-243. https://doi.org/10.1016/S0044-8486(99)00349-X.

Robins-Browne, R. M., Cianciosi, S., Bordun, A. M., & Wauters, G. (1991). Pathogenicity of *Yersinia kristensenii* for mice. *Infection and immunity*, *59*(1), 162–167. https://doi.org/10.1128/iai.59.1.162-167.1991

Rodgers, C. J. (1991a). The usage of vaccination and antimicrobial agents for control of *Yersinia ruckeri*. *Journal of Fish Diseases, 14*(3), 291–301. https://doi.org/10.1111/j.1365-2761.1991.tb00826.x

Rodgers, C. J. (1991b). Epidemiological studies of the bacterial fish pathogen *Yersinia ruckeri*. Thesis (PhD), Microbiology. University of Plymouth, Faculty of Science and Technology. Retrieved from http://hdl.handle.net/10026.1/1112

Rodgers, C. J. (1992). Development of a selective-differential medium for the isolation of *Yersinia ruckeri* and its application in epidemiological studies. *Journal of Fish Diseases, 15*(3), 243–254. https://doi.org/10.1111/j.1365-2761.1992.tb00660.x

Rodgers, C. J. (2001). Resistance of *Yersinia ruckeri* to antimicrobial agents in vitro. *Aquaculture 196*(3-4), 0–345. https://doi.org/10.1016/s0044-8486(01)00546-4

Rodgers, C. J., & Hudson, E. B. (1985). A comparison of two methods for isolation of *Yersinia ruckeri* from rainbow trout (*Salmo gairdneri*). *Bulletin of the European Association of Fish Pathologists, 5*(4), 92-93.

Rodríguez, A., Rodríguez, M., Córdoba, J. J., & Andrade, M. J. (2015). Design of primers and probes for quantitative real-time PCR methods. *Methods in molecular biology (Clifton, N.J.)*, *1275*, 31–56. https://doi.org/10.1007/978-1-4939-2365-6_3

Rohde, J. R., Luan, X. S., Rohde, H., Fox, J. M., & Minnich, S. A. (1999). The *Yersinia enterocolitica* pYV virulence plasmid contains multiple intrinsic DNA bends which melt at 37 degrees C. *Journal of bacteriology*, *181*(14), 4198–4204. https://doi.org/10.1128/JB.181.14.4198-4204.1999

Romalde, J. L., MagariÑos, B., Barja, J. L., & Toranzo, A. E. (1993). Antigenic and Molecular Characterization of *Yersinia ruckeri* Proposal for a New Intraspecies Classification. *Systematic and Applied Microbiology, 16*(3), 411-419. https://doi.org/10.1016/S0723-2020(11)80274-2

Romalde, J. L., MagariÑos, B., Fouz, B., Bandin, I., Nunez, S., & Toranzo, A. E. (1995). Evaluation of BIONOR Mono-kits for rapid detection of bacterial fish pathogens. *Diseases of aquatic organisms, 21*. 25-34. https://doi.org/10.3354/dao021025

Ross, A. J., Rucker, R. R., & Ewing, W. H. (1966). Description of a bacterium associated with redmouth disease of rainbow trout (*Salmo gairdneri*). *Canadian journal of microbiology*, *12*(4), 763–770. https://doi.org/10.1139/m66-103

Roth, B. (2016). Avlusing av laksefisk med Optilice: Effekt på avlusing og fiskevelferd [Delousing of salmonid fish with Optilice: Effect of delousing and fish welfair]. In Norwegian. Report series 2016, 59. Published by Nofima, Tromsø, Norway. Retrieved from https://nofima.no/publikasjon/1408716/

Rosinski-Chupin, I., Sauvage, E., Mairey, B., Mangenot, S., Ma, L., Da Cunha, V., Rusniok, C., Bouchier, C., Barbe, V., & Glaser, P. (2013). Reductive evolution in *Streptococcus agalactiae* and the emergence of a host adapted lineage. *BMC genomics, 14*, 252. https://doi.org/10.1186/1471-2164-14-252

Rossez, Y., Wolfson, E. B., Holmes, A., Gally, D. L., & Holden, N. J. (2015). Bacterial flagella: twist and stick, or dodge across the kingdoms. *PLoS pathogens*, *11*(1), e1004483. https://doi.org/10.1371/journal.ppat.1004483

Rucker, R. R. (1966). Redmouth disease of rainbow trout (*Salmo gairdneri*). *Bulletin - Office international des epizooties*, *65*(5), 825–830.

Ruan, Y., & Braun, V. (1990). Hemolysin as a marker for *Serratia*. *Archives of microbiology, 154*(3), 221–225. https://doi.org/10.1007/BF00248958

Rudi, K., Naterstad, K., Drømtorp, S. M., & Holo, H. (2005). Detection of viable and dead *Listeria monocytogenes* on gouda-like cheeses by real-time PCR. *Letters in applied microbiology*, *40*(4), 301–306. https://doi.org/10.1111/j.1472-765X.2005.01672.x

Ryckaert, J., Bossier, P., D'Herde, K., Diez-Fraile, A., Sorgeloos, P., Haesebrouck, F., & Pasmans, F. (2010). Persistence of *Yersinia ruckeri* in trout macrophages. *Fish & shellfish immunology*, *29*(4), 648–655. https://doi.org/10.1016/j.fsi.2010.06.009

Savin, C., Criscuolo, A., Guglielmini, J., Le Guern, A. S., Carniel, E., Pizarro-Cerdá, J., & Brisse, S. (2019). Genus-wide *Yersinia* core-genome multilocus sequence typing for species identification and strain characterization. *Microbial genomics*, *5*(10), e000301. https://doi.org/10.1099/mgen.0.000301

Schill, W. B., Phelps, S. R., & Pyle S. W. (1984). Multilocus Electrophoretic Assessment of the Genetic Structure and Diversity of *Yersinia ruckeri. Applied and Environmental Microbiology 48*, 975 – 979. https://doi.org/10.1128/aem.48.5.975-979.1984

Schmid, M., Frei, D., Patrignani, A., Schlapbach, R., Frey, J. E., Remus-Emsermann, M., & Ahrens, C. H. (2018). Pushing the limits of *de novo* genome assembly for complex prokaryotic genomes harboring very long, near identical repeats. *Nucleic acids research*, *46*(17), 8953–8965. https://doi.org/10.1093/nar/gky726

Schlotfeldt, H. J., Böhm, K. H., Pfortmüller, F., & Pfortmüller, K. (1985). »Rotmaulseuche«/ERM (Enteric Redmouth Disease) in Nordwestdeutschland - Vorkommen, Therapie und Vakzinierungsergebnisse [»Redmouthdisease«/ERM (Enteric Redmouth Disease) in north-west Germany - occurrence, therapy and vaccination results]. In German. Veterinary leaflet. *Tierarztliche Umschau Zeitschrift fur alle Gebiete der Veterinarmedizin, 40*(12), pp. 985-995. Printed by Terra-Verlag, 7750 Konstanz, Germany.

Scott, C. J. W. (2012). Molecular studies on the fish pathogen *Yersinia ruckeri*. Thesis (PhD), Microbiology. Heriot-Watt University, School of Life Sciences. Retrieved from http://hdl.handle.net/10399/2610

Scott, A., Minnich, S.A., & Rohde, H.N. (2007). A Rationale for Repression and/or Loss of Motility by Pathogenic *Yersinia* in the Mammalian Host. In Perry, R. D., & and Fetherston, J. D. (Eds.). *The Genus Yersinia : From Genomics to Function by Jacqueline D. Fetherston (Advances in Experimental Medicine and Biology Volume 603).* Published by Springer, New York, USA. e-ISBN 978-0-387-72124-8

Seemann T. (2014). Prokka: rapid prokaryotic genome annotation. *Bioinformatics (Oxford, England)*, *30*(14), 2068–2069. https://doi.org/10.1093/bioinformatics/btu153

Secades, P., & Guijarro, J. A. (1999). Purification and characterization of an extracellular protease from the fish pathogen *Yersinia ruckeri* and effect of culture conditions on production. *Applied and environmental microbiology, 65*(9), 3969–3975. https://doi.org/10.1128/AEM.65.9.3969-3975.1999

Shah, S. Q. A., Karatas, S., Nilsen, H., Steinum, T. M., Colquhoun, D. J., & Sørum, H. (2012). Characterization and expression of the *gyrA* gene from quinolone resistant *Yersinia ruckeri* strains isolated from Atlantic salmon (*Salmo salar* L.) in Norway. *Aquaculture, 350–353*, 37-41, https://doi.org/10.1016/j.aquaculture.2012.04.022.

Shaowu, L., Di, W., Hongbai, L., & Tongyan, L. (2013). Isolation of *Yersinia ruckeri* strain H01 from farm-raised Amur Sturgeon *Acipenser schrencki* in China. *Journal of aquatic animal health*, *25*(1), 9–14. https://doi.org/10.1080/08997659.2012.728169

Sharma, M, Katoch, R C, Nagal, K B, Sambyal, D S & Asrani, R K. (1995). Isolation of *Yersinia ruckeri* from rainbow trout suffering from Enteric Red Mouth disease in Himachal Pradesh (India). *Journal of Aquaculture in Tropics 10*: 73-77.

Shea, D., Bateman, A., Li, S., Tabata, A., Schulze, A., Mordecai, G., Ogston, L., Volpe, J. P., Neil Frazer, L., Connors, B., Miller, K. M., Short, S., & Krkošek, M. (2020). Environmental DNA from multiple pathogens is elevated near active Atlantic salmon farms. *Proceedings. Biological sciences*, *287*(1937). https://doi.org/10.1098/rspb.2020.2010

Sica, M. G., Brugnoni, L. I., Marucci, P. L., & Cubitto, M. A. (2012). Characterization of probiotic properties of lactic acid bacteria isolated from an estuarine environment for application in rainbow trout (*Oncorhynchus mykiss*, Walbaum) farming. *Antonie van Leeuwenhoek*, *101*(4), 869–879. https://doi.org/10.1007/s10482-012-9703-5

Sommerset, I., Krossøy, B., Biering, E., & Frost, P. (2005). Vaccines for fish in aquaculture. *Expert review of vaccines*, *4*(1), 89–101. https://doi.org/10.1586/14760584.4.1.89

Song, H., Hwang, J., Yi, H., Ulrich, R. L., Yu, Y., Nierman, W. C., & Kim, H. S. (2010). The early stage of bacterial genome-reductive evolution in the host. *PLoS pathogens*, *6*(5), e1000922. https://doi.org/10.1371/journal.ppat.1000922

Sparboe, O., Koren, C., Håstein, T., Poppe, T.T., & Stenwig, H. (1986). The first isolation of *Yersinia ruckeri* from farmed Norwegian salmon. *Bulletin of The European Association of Fish Pathologists*, 6, 41-42.

Steiner T. S. (2007). How flagellin and toll-like receptor 5 contribute to enteric infection. *Infection and immunity*, *75*(2), 545–552. https://doi.org/10.1128/IAI.01506-06

Stave, J.W., Cook, T.M., & Roberson, B.S. (1987). Chemiluminescent responses of striped bass, Morone saxatilis (Walbaum), phagocytes to strains of *Yersinia ruckeri***.** *Journal of Fish Diseases 10*, 1-10 https://doi.org/10.1111/j.1365-2761.1987.tb00712.x

Stepkowski, T., & Legocki, A. B. (2001). Reduction of bacterial genome size and expansion resulting from obligate intracellular lifestyle and adaptation to soil habitat. *Acta biochimica Polonica*, *48*(2), 367–381.

Stevenson R., Flett D. & Raymond B.T. (1993) Enteric Redmouth (ERM) and other enterobacterial infections of fish. In Inglis, V., Roberts, R. J., & Bromage, N. R. (Eds.). *Bacterial Diseases of Fish.* Published by Wiley-Blackwell, Hoboken, New Jersey, USA.

Stevenson, R. M. W., & Airdrie, D. W. (1984). Serological variation among *Yersinia ruckeri* strains. *Journal of Fish Diseases 7*(4), 247–254. https://doi.org/10.1111/j.1365-2761.1984.tb00930.x

Stevenson, R. M. W. & Daly, J. G. (1982). Biochemical and serological characteristics of Ontario isolates of *Yersinia ruckeri*. *Canadian Journal of Fisheries and Aquatic Sciences 39*(6), 870-876.

Stevenson, R. M. W., & De Grandis, S. A. (1982). Variations in plasmid profiles and growth characteristics of *Yersinia ruckeri* strains, *FEMS Microbiology Letters, 15*(3), 199–202, https://doi.org/10.1111/j.1574-6968.1982.tb00067.x

Stige, L. F., Qviller, L., & Helgesen, K. O. (2022). Salmon lice – *Lepeophtheirus salmonis*. In Sommerset, I., Walde, C. S., Bang Jensen, B., Wiik-Nielsen, J., Bornø, G., Oliveira, V.H.S., Haukaas, A., & Brun, E. Norwegian Fish Health Report 2021 (pp. 120-128). Published by the Norwegian Veterinary Institute. Retrieved from https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2022/fish-health-report-2021

SVA (2019) Yersinios-rödmunsjuka (ERM) hos fisk [Yersiniosis-redmouth disease (ERM) in fish]. In Swedish. Published online by the Swedish veterinary institute. Last updated 2019-12-02. https://www.sva.se/amnesomraden/djursjukdomar-a-o/yersinios-rodmunsjuka-erm-hos-fisk

Sviland Walde, C., Bang Jensen, B., Pettersen, J. M., & Stormoen, M. (2021). Estimating cage-level mortality distributions following different delousing treatments of Atlantic salmon (*Salmo salar*) in Norway. *Journal of fish diseases*, *44*(7), 899–912. https://doi.org/10.1111/jfd.13348

Taksdal, T., Håstein, T., & Onstad, E. 1993. Sikdød i Begnavassdraget høsten 1990 og 1991 [Mortalities in European whitefish in Begnavassdraget autum of 1990 and 1991]. In Norwegian. Veterinary leaflet. *Norsk Veterinærtidsskrift 105*, 4.

Temprano, A., Yugueros, J., Hernanz, C., Sánchez, M., Berzal, B., Luengo, J.M., & Naharro, G. (2001). Rapid identification of *Yersinia ruckeri* by PCR amplification of *yruI–yruR* quorum sensing. *Journal of Fish Diseases*, 24, 253-261. https://doi.org/10.1046/j.1365-2761.2001.00261.x

Tennant, S. M., Skinner, N. A., Joe, A., & Robins-Browne, R. M. (2005). Homologues of insecticidal toxin complex genes in *Yersinia enterocolitica* biotype 1A and their contribution to virulence. *Infection and immunity*, *73*(10), 6860–6867. https://doi.org/10.1128/IAI.73.10.6860-6867.2005

Tinsley, J. W., Austin, D. A., Lyndon, A. R., & Austin, B. (2011). Novel non-motile phenotypes of *Yersinia ruckeri* suggest expansion of the current clonal complex theory. *Journal of fish diseases*, *34*(4), 311–317. https://doi.org/10.1111/j.1365-2761.2011.01237.x

Tobback, E., Decostere, A., Hermans, K., Haesebrouck, F., & Chiers, K. (2007). *Yersinia ruckeri* infections in salmonid fish. *Journal of fish diseases*, *30*(5), 257–268. https://doi.org/10.1111/j.1365-2761.2007.00816.x

Tobback, E., Decostere, A., Hermans, K., Ryckaert, J., Duchateau, L., Haesebrouck, F., & Chiers, K. (2009). Route of entry and tissue distribution of *Yersinia ruckeri* in experimentally infected rainbow trout *Oncorhynchus mykiss*. *Diseases of aquatic organisms*, *84*(3), 219–228. https://doi.org/10.3354/dao02057

Tobback, E., Decostere, A., Hermans, K., Van den Broeck, W., Haesebrouck, F., & Chiers, K. (2010). In vitro markers for virulence in *Yersinia ruckeri. Journal of fish diseases, 33*(3), 197–209. https://doi.org/10.1111/j.1365-2761.2009.01106.x

Uffelmann, E., Huang, Q. Q., Munung, N. S., de Vries, J., Okada, Y., Martin, A. R., Martin, H. C., Lappalainen, T., & Posthuma, D. (2021) Genome-wide association studies. *Nature Reviews Methods Primers 1*(59). https://doi.org/10.1038/s43586-021-00056-9

Ummey, S., Khan, S., Vijayakumar, P.P.N., & Ramya, A. (2021). Enteric Red Mouth disease and its causative bacterium, *Yersinia ruckeri*, in Indian Major Carps from culture ponds in Andhra Pradesh, India. *Aquaculture and Fisheries 6*(3), 289-299 https://doi.org/10.1016/j.aaf.2020.05.009

Valtonen, E. T., Rintamaki, P., & Koskivaara, M. (1992). Occurence and pathogenicity of *Yersinia ruckeri* at fish farms in northern and central Finland. *Journal of fish diseases, 15*(2), 163-171. https://doi.org/10.1111/j.1365-2761.1992.tb00650.x

Venecia, K., & Young, G. M. (2005). Environmental regulation and virulence attributes of the Ysa type III secretion system of *Yersinia enterocolitica* biovar 1B. *Infection and immunity, 73*(9), 5961–5977. https://doi.org/10.1128/IAI.73.9.5961-5977.2005

Verner-Jeffreys, D.W., Haig, S.J., Welch, T.J., Pond, M.J., Stone, D., Davies, R.L., & Gardner, R. (2011) Characterisation of a serotype O1 *Yersinia ruckeri* isolate from the Isle of Man: further evidence that O antigen serotype is not a reliable indicator of virulence. *Bulletin of the European Association of Fish Pathologists, 31*, pp. 86-91.

Vrålstad, T., Knutsen, A. K., Tengs, T., & Holst-Jensen, A. (2009). A quantitative TaqMan (R) MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci*. *Veterinary Microbiology 137*, 146–155.

Vu, N. T., Sang, N. V., Trong, T. Q., Duy, N. H., Dang, N. T., & Nguyen, N. H. (2019). Breeding for improved resistance to *Edwardsiella ictaluri* in striped catfish (*Pangasianodon hypophthalmus*): Quantitative genetic parameters. *Journal of fish diseases*, *42*(10), 1409–1417. https://doi.org/10.1111/jfd.13067

Vuillaume, A., Brun, R., Chene, P., Sochon, E., & Lesel, R. (1987). First isolation of *Yersinia ruckeri* from sturgeon, *Acipenser baeri* Brandt, in South West of France. *Bulletin of the European Association of Fish Pathologists 7*(1), 18.

Wade, J. (2019). Characterization of *Yersinia ruckeri* and enteric redmouth disease (ERM) to inform pathogen transfer risk assessments in British Columbia. DFO Canadian Science Advisory Secretariat Research Document 2019/022. available from: https://waves-vagues.dfo-mpo.gc.ca/Library/40853032.pdf

Waltman, W. D., & Shotts Jr., E. B. (1984). A Medium for the Isolation and Differentiation of *Yersinia ruckeri*. *Canadian Journal of Fisheries and Aquatic Sciences, 41*(5), 804–806. https://doi.org/10.1139/f84-093

Wang, E., Qin, Z., Yu, Z., Ai, X., Wang, K., Yang, Q., Liu, T., Chen, D., Geng, Y., Huang, X., Ouyang, P., & Lai, W. (2018). Molecular Characterization, Phylogenetic, Expression, and Protective Immunity Analysis of OmpF, a Promising Candidate Immunogen Against *Yersinia ruckeri* Infection in Channel Catfish. *Frontiers in immunology*, *9*, 2003. https://doi.org/10.3389/fimmu.2018.02003

Wangkahart, E., Scott, C., Secombes, C. J., & Wang, T. (2016). Re-examination of the rainbow trout (*Oncorhynchus mykiss*) immune response to flagellin: *Yersinia ruckeri* flagellin is a potent activator of acute phase proteins, anti-microbial peptides and pro-inflammatory cytokines in vitro. *Developmental and comparative immunology*, *57*, 75–87. https://doi.org/10.1016/j.dci.2015.12.017

Welch T. J. (2020). Characterization of a novel *Yersinia ruckeri* serotype O1-specific bacteriophage with virulence-neutralizing activity. *Journal of fish diseases, 43*(2), 285–293. https://doi.org/10.1111/jfd.13124

Welch, T. J., & LaPatra, S. (2016). *Yersinia ruckeri* lipopolysaccharide is necessary and sufficient for eliciting a protective immune response in rainbow trout (*Oncorhynchus mykiss*, Walbaum). *Fish & shellfish immunology*, *49*, 420–426. https://doi.org/10.1016/j.fsi.2015.12.037

Welch, T. J., Fricke, W. F., McDermott, P. F., White, D. G., Rosso, M. L., Rasko, D. A., Mammel, M. K., Eppinger, M., Rosovitz, M. J., Wagner, D., Rahalison, L., Leclerc, J. E., Hinshaw, J. M., Lindler, L. E., Cebula, T. A., Carniel, E., & Ravel, J. (2007). Multiple antimicrobial resistance in plague: an emerging public health risk. *PloS one*, *2*(3), e309. https://doi.org/10.1371/journal.pone.0000309

Welch, T. J., Verner-Jeffreys, D. W., Dalsgaard, I., Wiklund, T., Evenhuis, J. P., Cabrera, J. A., Hinshaw, J. M., Drennan, J. D., & LaPatra, S. E. (2011). Independent emergence of *Yersinia ruckeri* biotype 2 in the United

States and Europe. *Applied and environmental microbiology*, 77(10), 3493–3499. https://doi.org/10.1128/AEM.02997-10

Wheeler, R. W., Davies, R. L., Dalsgaard, I., Garcia, J., Welch, T. J., Wagley, S., Bateman, K. S., & Verner-Jeffreys, D. W. (2009). *Yersinia ruckeri* biotype 2 isolates from mainland Europe and the UK likely represent different clonal groups. *Diseases of aquatic organisms*, 84(1), 25–33. https://doi.org/10.3354/dao02039

White, R., Pellefigues, C., Ronchese, F., Lamiable, O., & Eccles, D. (2017). Investigation of chimeric reads using the MinION. *F1000Research, 6*, 631. https://doi.org/10.12688/f1000research.11547.2

Wick, R. R., Judd, L. M., Gorrie, C. L., & Holt, K. E. (2017). Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLoS computational biology*, *13*(6), e1005595. https://doi.org/10.1371/journal.pcbi.1005595

Willumsen, B. (1989). Birds and wild fish as potential vectors of *Yersinia ruckeri*. *Journal of Fish Diseases, 12, 275-277.* https://doi.org/10.1111/j.1365-2761.1989.tb00313.x

Wobeser, G. (1973). An Outbreak of Redmouth Disease in Rainbow Trout (*Salmo gairdneri*) in Saskatchewan. *Journal of the Fisheries Research Board of Canada 30*: 571-575.

Wrobel, A., Ottoni, C., Leo, J. C., Gulla, S., & Linke, D. (2018a). The repeat structure of two paralogous genes, *Yersinia ruckeri* invasin (*yrInv*) and a "*Y. ruckeri* invasin-like molecule", (*yrllm*) sheds light on the evolution of adhesive capacities of a fish pathogen. *Journal of structural biology*, *201*(2), 171–183. https://doi.org/10.1016/j.jsb.2017.08.008

Wrobel, A., Ottoni, C., Leo, J. C., & Linke, D. (2018b). pYR4 From a Norwegian Isolate of *Yersinia ruckeri* Is a Putative Virulence Plasmid Encoding Both a Type IV Pilus and a Type IV Secretion System. *Frontiers in cellular and infection microbiology*, *8*, 373. https://doi.org/10.3389/fcimb.2018.00373

Wrobel, A., Saragliadis, A., Pérez-Ortega, J., Sittman, C., Göttig, S., Liskiewicz, K., Spence, M. H., Schneider, K., Leo, J. C., Arenas, J., & Linke, D. (2020). The inverse autotransporters of *Yersinia ruckeri*, *YrInv* and *Yrllm*, contribute to biofilm formation and virulence. *Environmental microbiology*, *22*(7), 2939–2955. https://doi.org/10.1111/1462-2920.15051

Young, G. M., Schmiel ,D. H., & Miller, V. L. (1999). A new pathway for the secretion of virulence factors by bacteria: the flagellar export apparatus functions as a protein-secretion system. *PNAS USA 96*, 6456-6461.

9 Enclosed articles

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RESEARCH ARTICLE

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Biotyping reveals loss of motility in two distinct *Yersinia ruckeri* lineages exclusive to Norwegian aquaculture

Andreas Riborg[1,2] | Duncan J. Colquhoun[1,3] | Snorre Gulla[1]

[1]Norwegian Veterinary Institute, Ås, Norway

[2]Vaxxinova Norway AS, Bergen, Norway

[3]University of Bergen, Bergen, Norway

Correspondence
Andreas Riborg, Norwegian Veterinary Institute, Ås, Norway.
Email: andreas.riborg@vetinst.no

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Abstract

Non-motile strains of *Yersinia ruckeri*, known as *Y. ruckeri* biotype 2, now dominate amongst clinical isolates retrieved from rainbow trout internationally. Due to an acute increase in the number of yersiniosis cases in Norway in recent years, followed by introduction of widespread intraperitoneal vaccination against the disease, an investigation on the prevalence of *Y. ruckeri* biotype 2 in Norwegian aquaculture was conducted. We biotyped 263 *Y. ruckeri* isolates recovered from diseased salmonids in Norway between 1985 and 2020. A total of seven biotype 2 isolates were identified, four of which were collected between 1985 and 1987, and three of which belong to the current epizootic clone, isolated from two different sea-farms in 2017. Whole-genome sequencing revealed single non-synonymous nucleotide polymorphisms in the flagellar genes *flhC* in isolates from the 1980s, and in *fliP* in isolates from 2017. In both variants, motility was restored both by complementation with wild-type alleles *in trans* and via spontaneous mutation-driven reversion following prolonged incubation on motility agar. While biotype 2 strains do not yet seem to have become broadly established in Norwegian aquaculture, the seven isolates described here serve to document a further two independent cases of *Y. ruckeri* biotype 2 emergence in salmonid aquaculture.

KEYWORDS
aquaculture, Atlantic salmon, biotype, flagella, *Yersinia ruckeri*, yersiniosis

1 | INTRODUCTION

The Gram-negative bacterium *Yersinia ruckeri* causes yersiniosis, also known as enteric redmouth disease, predominantly in farmed salmonids (Busch, 1978; Ewing et al., 1978; Ross et al., 1966). Found throughout the world wherever salmonid fish are farmed, yersiniosis is considered primarily a disease of rainbow trout, but also affects farmed Atlantic salmon in Norway, Australia, Chile and the UK (Bastardo et al., 2011; Costa et al., 2011; Gulla et al., 2018; Wheeler et al., 2009). Several studies have demonstrated an epidemic population structure for *Y. ruckeri*, with both host species specific- and geographically limited lineages forming discrete clonal complexes (CC) (Barnes et al., 2016; Bastardo et al., 2011; Calvez et al., 2014). Most recently, multi-locus variable number of tandem repeat analysis (MLVA) revealed a single clonal complex (CC2) as responsible for the majority of yersiniosis outbreaks in rainbow trout globally, while distinct, geographically restricted clones were linked to outbreaks in farmed Atlantic salmon in Norway (CC1), the UK (CC4) and Australia (CC5) (Gulla et al., 2018).

While wild-type *Y. ruckeri* are flagellated and motile, non-flagellated, non-motile strains do occur (Davies & Frerichs, 1989). As lipase secretion is dependent upon the flagellar export apparatus (Evenhuis et al., 2009; Young et al., 1999), motility and lipase phenotypes are genetically linked with non-motile strains also lacking lipase activity (Welch et al., 2011). Non-motile, lipase negative strains are often termed biotype 2 (BT2), while motile, lipase-positive strains are termed biotype 1 (BT1) (Davies & Frerichs, 1989).

In recent years, non-motile BT2 *Y. ruckeri* strains have emerged independently on different continents following detrimental mutations in flagellar genes (Welch et al., 2011; Wheeler et al., 2009), and have become the dominating form associated with yersiniosis in rainbow trout in the USA and Europe (Fouz et al., 2006; Gulla et al., 2018; Welch et al., 2011; Wheeler et al., 2009). Observations of vaccine failure coinciding with BT2 emergence in the UK, Spain, USA and Australia (Austin et al., 2003; Fouz et al., 2006; Arias et al., 2007; Costa et al., 2011) have resulted in speculations around vaccine-escape as the primary selective force responsible (Wheeler et al., 2009; Welch et al., 2011; Tinsley et al., 2011; Barnes et al., 2016). Conversely, reports of BT2 from salmon farming industries worldwide are scarce, with only a single non-motile isolate reported from Norway in 1985 (Sparboe et al., 1986), a few UK isolates from the 1980s and 1990s (Wheeler et al., 2009), and the more recent emergence within a local endemic clonal complex in Australia (Barnes et al., 2016; Gulla et al., 2018).

In Norway, a single *Y. ruckeri* clonal complex (CC1) has almost entirely dominated the yersiniosis situation in salmon farming in both freshwater and seawater since the turn of the millennium (Gulla et al., 2018). In response to an increasing number of outbreaks in recent years, and as a supplement to immersion vaccination, many Norwegian farms now utilize intraperitoneally administered vaccines against yersiniosis, which have thus far proven to be effective in preventing outbreaks in large fish post sea transfer (Gulla & Olsen, 2020). Given the putative association between vaccination and BT2 development, the present study was performed to investigate possible emergence and spread of BT2 *Y. ruckeri* in Norwegian aquaculture.

A non-motile isolate from diseased rainbow trout in Sweden, NVI-11000, was also included in the study as Swedish *Y. ruckeri* BT2 strains have not, to the best of our knowledge, been characterized previously.

2 | MATERIALS AND METHODS

2.1 | Strains and culture

Detailed information on the 263 Norwegian *Y. ruckeri* isolates assessed by biotyping is provided in Table S1, including 46 isolates previously biotyped in our laboratory (Gulla et al., 2018) as well as BT2 isolate NVI-344, which was first described by Sparboe et al. (1986) and also mentioned by Wheeler et al. (2009) and Ormsby (2015) under the designation RD154. Isolates used in complementation and biotype reversion experiments are listed in Table 1. Bacterial cultures were prepared from cryopreserved stocks held at −80°C in Tryptic Soy Broth (TSB) with 20% glycerol. *Y. ruckeri* was cultured on 5% bovine blood agar (BA) or in TSB at 22°C. *E. coli* was cultured at 37°C on Luria agar (LA) or in Luria broth (LB), supplemented with 0.3 mM 2, 6-diaminopimelic acid (DAP) (Alfa Aesar, Ward Hill, Massachusetts, USA) for culture of *E. coli* MFD-pir (Ferrières et al., 2010). The pMJH-46 plasmid was maintained in *E. coli* SM10(λpir), grown with 100 mg/L ampicillin (Merck, Darmstadt, Germany) and 30 mg/L chloramphenicol (Merck) at 30°C. Agar and liquid media for maintaining the pAR3 plasmid vector and derivatives in *Y. ruckeri* and *E. coli* were supplemented with 50 mg/L kanamycin (Merck).

2.2 | Biotyping

Methods for phenotypic biotype assessment were adapted from those described by Evenhuis et al. (2009). Motility was assayed on semi-solid Tryptic Soy Agar (TSA) with 0.3% agar incubated at 22°C. The assay was prepared by surface-inoculating overnight BA cultures of *Y. ruckeri* at the centre of the TSA plate. A visible expanding zone of motile cells within the semi-solid TSA after 24 or 48 h was considered positive for motility. Lipase activity on Tween 80 medium was assayed by incubation at 22°C. Visible calcium precipitation after 48 h incubation was considered positive. Due to inconsistent calcium precipitation reactions for many CC1 isolates that also displayed flagellar motility (Figure S1), biotype assessment of isolates belonging to this lineage did not include lipase activity.

2.3 | General molecular techniques

DNA extraction for MLVA and qualitative PCRs was performed by boiling a bacterial colony for 7 min in nuclease-free water as described previously (Gulla et al., 2018). Genomic DNA templates for whole-genome sequencing, Sanger sequencing and PCR amplification for cloning was extracted with the Gentra Puregene Yeast/Bact. Kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations for Gram-negative bacteria. Plasmid templates for PCR amplification for cloning were purified with the Qiagen Plasmid Mini Kit (Qiagen).

Oligonucleotides (Table 2) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). HotStarTaq Master Mix (Qiagen) was used for PCR amplification prior to Sanger sequencing and for qualitative PCR assays, with standard cycling conditions according to the manufacturer's recommendations including 30 s annealing at 60°C and 60 s extension time. All PCR products were verified by agarose gel electrophoresis. Sanger sequencing was performed on an Avant 3500xl Genetic Analyzer (Applied Biosystems Waltham, MA, USA) using BigDye version 3.1 reagents according to manufacturer's description.

The MLVA genotyping was performed according to Gulla et al. (2019). Briefly, DNA extracted from each isolate was used as

TABLE 1 Bacterial strains and plasmids used for biotype reversion and complementation experiments

Bacterial strain or plasmid	Description	Source or references
Yersinia ruckeri strains		
NVI−344 (RD154)	CC10, BT2	This study; Sparboe et al. (1986); Wheeler et al. (2009)
NVI−492	CC10, BT1, source of wild-type *flhDC*	This study
NVI−10990	CC1, BT2	This study
NVI−10705	CC1, BT1, source of wild-type *fliP*	This study
Escherichia coli strains		
MFD-pir	MG1655 RP4−2-Tc::[ΔMu1::*aac(3)IV*-Δ*aphA*-Δ*nic*35-ΔMu2::*zeo*] Δ*dapA*::(*erm-pir*) Δ*recA*	Ferrières et al. (2010)
Plasmids		
pMJH−46	Source of the *bla* promoter	Addgene plasmid #67,272; Hossain et al. (2015)
pAR3	pBBR1 *oriT KanR amilCP*	This study (Materials S1)
pAR3-fliP	pAR3 containing the *bla* promoter and wild-type *fliP*	This study
pAR3-flhDC	pAR3 containing wild-type *flhDC*	This study

Note: NVI-344 is identical to the isolate described by Sparboe et al. (1986) and synonymous to RD154 mentioned in Wheeler et al. (2009) and Ormsby (2015).

MFD-pir was acquired from Biological Resource Center of the Institut Pasteur (CRBIP).

pMJH-46 was acquired from Addgene, deposited by Mark Liles.

A complete description of pAR3 is provided in Materials S1.

template in two five-plex PCR assays featuring fluorescently labelled primers, with subsequent capillary electrophoresis for size calling of PCR products and in silico calculation of 10-loci MLVA profiles. A minimum spanning tree based on MLVA results was generated in BioNumerics v7.6.3 (Applied Maths NV, Sint-Martens-Latem, Belgium).

2.4 | Whole-genome analyses

Genome assemblies for a total of 26 *Y. ruckeri* isolates (Table 3) of diverse origin, covering various genetic lineages and both biotypes, were utilized in the present study. Of these, seven were downloaded from the National Center for Biotechnology Information (NCBI), while the remaining 19 were generated as described below.

Sequencing libraries were prepared by use of either NEBNext Ultra DNA Library Prep- (New England Biolabs, Ipswich, MA, USA), TruSeq DNA PCR-Free- (Illumina, San Diego, CA, USA) or NexteraFlex- (Illumina, San Diego, CA, USA) kits. Subsequent Illumina sequencing was performed on either a HiSeq or MiSeq platform, with paired end read lengths of 125, 150 or 300 base pairs. See Table S2 for per-strain sequencing details. Adapter sequences in raw reads were removed and low-quality nucleotides trimmed with Trimmomatic v0.38 (Bolger et al., 2014), prior to de novo assembly using SPAdes v3.13.0 (Bankevich et al., 2012) with the -careful option.

All 26 *Y. ruckeri* genome assemblies were annotated with Prokka v1.13 (Seemann, 2014) utilizing default settings, prior to generation of a core gene alignment using Roary v3.12.0 (Page et al., 2015) with the MAFFT aligner. The alignment was concatenated with snp-sites v2.4.1 (Page et al., 2016) and a maximum likelihood tree was generated using MEGA v10.2 (Kumar et al., 2018) with 1000 bootstrap replicates and visualized in R using ggtree (Yu et al., 2017).

The flagellar sequence region (position 2355800–2427400) was extracted from the genome of *Y. ruckeri* NHV_3758 (accession no. CP023184.1) and used as a reference for alignment of flagellar genes (*flh*, *fli* and *flg*) in MEGA.

2.5 | Complementation experiments

Complementation experiments for restoration of motility in BT2 strains were performed by supplying wild-type variants of flagellar genes *in trans* on the plasmid vector pAR3 (Materials S1). This plasmid contains the pBBR1 plasmid origin, kanamycin resistance, amilCP chromoprotein and an origin-of-transfer which allows for mobilization by the chromosomally encoded conjugation machinery in the *E. coli* MFD-pir donor strain.

Linearized vector (by restriction with XbaI, New England Biolabs, Catalog # R0145) and target inserts were amplified by PCR with sequence-overlapping primers (Table 2), using Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs) as specified by the manufacturer, with annealing temperatures calculated with the NEB Tm Calculator tool. PCRs included 22 cycles of amplification, with extension at 72°C for 30 s or 60 s, respectively for amplification of inserts or the vector backbone. PCR products were verified by agarose gel electrophoresis.

TABLE 2 Primer sequences used during complementation experiments

Oligo name	Sequence (5′–3′)	Usage
fliH_f	GAGAGTGGCGAAATCGTRATTGG	Sanger sequencing, *fliH*
fliH_r	CAATTTGTCCAATGTAGACAACCAACG	
flhC_f	GCCACTTACTGCATGAGTTATCGTTG	Sanger sequencing, *flhC*
flhC_r	GCCAGACAGATAAGACATCCATATCG	
fliP_f	ATGATGTCCCTGCACTGTGAATCCAAAG	Sanger sequencing, *fliP*
fliP_r	GGGAGATTAACTGTAGAAACTTTGCG	
pAR3_f	TAATGACTAGTCAAGTGGCTCCTCGCTC	pAR3 vector backbone amplification
pAR3_r	TCTAGAGCTTGCCCTCATCTGTTACG	
fliP_OL_f	**GAAAAAGGAAGAGTATCTAGACTAGT**ATGATGTCCCTGCACTGTGAATCC	Complementation, *fliP* expression
fliP_OL_r	CGAGGAGCCACTTGACTAGTCATTAGGGAGATTAACTGTAGAAACTTTGCG	
bla_P_OL_f	CAGATGAGGGCAAGCTCTAGATTTCAGGTGGCACTTTTCGGGGAAATGTG	Complementation, *fliP* expression
bla_P_OL_r	**CATACTAGTCTAGATACTCTTCCTTTTTC**AATATTATTGAAGC	
flhDC_OL_f	CGTAACAGATGAGGGCAAGCTCTAGACCACATTCAGTTATGTCTTCCTTGC	Complementation, *flhDC*
flhDC_OL_r	GCGAGGAGCCACTTGACTAGTCATTACCAGACAGATAAGACATCCATATCG	
oriT_f	GCTTGCCCTCATCTGTTACG	PCR verification of vector
oriT_r	GTTCGTGTAGACTTTCCTTGGTG	

Note: Underlined nucleotides indicate overlapping sequence to the pAR3 vector backbone.
Bold nucleotides indicate overlap sequence between the *bla* promoter and *fliP*.

Strain NVI-10990 was complemented with pAR3-fliP, containing *fliP* (corresponding to nucleotides 2,381,401–2,382,198 in acc.no. CP023184) amplified with primers fliP_OL_f and fliP_OL_f from the motile CC1 isolate NVI-10705, coupled with the *E. coli* wild-type *bla* promoter for weak constitutive expression, amplified from pMJH-46 (acc.no. JQ070344, nucleotides 9530–9662) with primers bla_P_OL_f and bla_P_OL_r.

Strain NVI-344 was complemented with pAR3-flhDC, containing the *flhDC* operon (corresponding to nucleotides 2,428,041–2,426,303 in acc.no. CP023184) from a motile CC10 isolate, NVI-492, amplified using primers flhDC_OL_f and flhDC_OL_r.

The PCR-amplified backbone and respective inserts were assembled with NEBuilder HiFi (New England Biolabs) and cloned into chemically competent *E. coli* MFD-pir by heat shock according to the manufacturer's instructions. Kanamycin-resistant colonies were isolated followed by PCR-verification of the plasmid backbone with primers oriT_f and oriT_r, and of the respective inserts with primers fliP_f and fliP_r, and primers flhC_f and flhC_r. Assembly of the *bla* promoter and *fliP* was confirmed by PCR amplification using the bla_P_OL_f and fliP_OL_r primers, with an expected amplicon size of 986 bp.

Complementing plasmids were transferred to *Y. ruckeri* by conjugation, performed by combining two 25 μl PBS suspensions containing approximately equal densities of *E. coli* MFD-pir donor and *Y. ruckeri* recipients respectively. The 50 μl suspension was plated and incubated overnight at 28°C on non-selective LB-agar supplemented with DAP. The resulting bacterial lawn was collected and washed twice in 1 ml PBS by centrifugation (5 min at 6000G), followed by suspension of the bacterial pellet in 0.1ml PBS and plating on 50 mg/L kanamycin LA without DAP. Kanamycin-resistant transconjugants were isolated with subsequent species confirmation by MALDI-TOF (Biotyper Microflex LT; Bruker Daltonics, Bremen, Germany) and PCR-verification of the expression vector backbone with primers oriT_f and oriT_r. Complemented strains were cured of the vector by repeated subculture on non-selective agar media until kanamycin-sensitive colonies were recovered and confirmed negative for the vector by PCR with the oriT primer-set.

2.6 | Biotype reversion experiments

To facilitate reversion to BT1, BT2 strains NVI-344 and NVI-10990 were initially prepared in 10 parallels each consisting of 5 ml TSB-culture grown to stationary phase by overnight shaking at room temperature. From each replicate culture, a 10 μl droplet was deposited at the centre of a semi-solid TSA plate, which was then sealed with parafilm and incubated for up to 6 weeks at 22°C in a sealed box with a water reservoir to maintain humidity. Motile cells from the leading edge of expanding growth zones were isolated and flagellar genes *fliP* and *flhC* amplified by PCR and Sanger sequenced.

2.7 | Microscopy

Staining of flagella was performed using a tannic acid and alum mordant with a crystal violet stain as described by Heimbrook et al. (1989). Cells were grown overnight in TSB at 22°C without agitation, before being stained and observed by phase-contrast microscopy at 1000× magnification.

TABLE 3 26 *Yersinia ruckeri* genome assemblies downloaded from NCBI or produced in the current study for phylogenetic evaluation and alignment of flagellar genes

Strain	Biological source	Country	Year	Sero-type	Bio-type	MLVA-CC[a]	Accession no.	Genome reference
NVI–10990	*S. salar*	Norway	2017	O1	2	1	JAJIBN000000000	This study
NVI–10974	*S. salar*	Norway	2017	O1	2	1	JAJIBM000000000	This study
NVI–10705	*S. salar*	Norway	2016	O1	1	1	JAJJIH000000000	This study
NVI–9967	*S. salar*	Norway	2015	O1	1	1	JAJIBK000000000	This study
NVI–3629	*S. salar*	Norway	1996	O1	1	1	JAJIBH000000000	This study
NHV_3758	*S. salar*	Norway	1987	O1	1	1	CP023184	Wrobel et al. (2018)
NVI–494	*S. salar*	Norway	1987	O1	2	10	JAJIBF000000000	This study
NVI–344	*S. salar*	Norway	1985	O1	2	10	JAJIBV000000000	This study
NVI–492	*S. salar*	Norway	1987	O1	1	10	JAJIBE000000000	This study
NVI–9681	*S. salar*	Norway	2014	O2	1	3	JAJIBJ000000000	This study
NVI–6225	*S. salar*	Norway	2008	O2	1	3	JAJIBI000000000	This study
NVI–1347	*S. salar*	Norway	1988	O2	1	3	JAJIBG000000000	This study
NCTC12268	*O. mykiss*	Canada	1985	O5	1	s	JAJIBS000000000	This study
QMA0431	*S. salar*	Australia	2007	O1	2	5	GCA_001882895	Barnes et al. (2016)
QMA0427	*S. salar*	Australia	2004	O1	2	5	GCA_001883575	Barnes et al. (2016)
QMA0435	*S. salar*	Australia	2009	O1	1	5	GCA_001882945	Barnes et al. (2016)
NVI–11076	Salmon farm (b.f.)	Norway	2017	O1	1	7	JAJIBQ000000000	This study
NVI–11050	*S. salar* (e.f.)	Norway	2017	O1	1	9	JAJIBP000000000	This study
NVI–11000	*O. mykiss*	Sweden	2017	O1	2	2	JAJIBO000000000	This study
NCTC12266	*O. mykiss*	USA	pre 1990	O1	1	2	JAJIBR000000000	This study
CSF007–82	*O. mykiss*	USA	1982	O1	1	2	LN681231	Nelson et al. (2015)
ATCC29473	*O. mykiss*	USA	1961	O1	1	2	KN150747 & KN150748	Daligault et al. (2014)
NCTC12269	*O. mykiss*	Canada	pre 1990	O6	1	s	JAJIBT000000000	This study
BigCreek74	*O. tshawytscha*	USA	1974	O2	1	s	CP011078	Unpublished
NVI–10587	*S. salar* (e.f.)	Norway	2015	O1	1	8	JAJIBL000000000	This study
NCTC12270	*A. anguilla*	Denmark	pre 1990	O7	1	s	JAJIBU000000000	This study

Note: Abbreviations used: *O.* (*Oncorhynchus*), *S.* (*Salar*), *A* (*anguilla*), e.f. (egg-fluid), b.f. (biofilm).

Public nucleotide sequences identified as plasmids were not included for the phylogenetic analysis.

The two contigs of ATCC29473 were combined into a single file prior to analysis.

[a]MLVA clonal complex according to Gulla et al. (2018) or present study, with 's' indicating singleton or minor/undefined clonal complex.

3 | RESULTS

3.1 | Genotypic background

Ongoing efforts to MLVA genotype the collection of more than 800 Norwegian *Y. ruckeri* isolates cryopreserved at the Norwegian Veterinary Institute continue to verify the long-standing dominance of the CC1 lineage amongst domestic yersiniosis outbreaks (Gulla et al., 2018). Conversely, Norwegian isolates recovered in other contexts, e.g. from disease in individual wild fish, ovarian fluid of clinically healthy broodstock, or environmental biofilms, are, as previously described, much more diverse. Such isolates represent a wide range of putatively non- or low-virulent lineages, minor clonal complexes and singletons. Inclusion of a large number of older isolates in the present study further allowed identification of a previously unrecognized Norwegian clonal complex proposed here as CC10. CC10 currently harbours six isolates recovered between 1985 and 1988 from two geographically distant Norwegian salmon farms, and includes the single BT2 isolate previously reported from Norway (Sparboe et al., 1986), NVI-344 (also referred to as RD154). An up-to-date minimum spanning tree visualizing results from MLVA genotyping of 601 Norwegian *Y. ruckeri* isolates, and associated biotyping results (see below), is shown in Figure 1. MLVA further revealed that NVI-11000, isolated from rainbow trout in Sweden, belongs to CC2 (not shown in Figure 1), the dominant clonal complex amongst international rainbow trout isolates. CC2 has not yet been identified from Norway.

3.2 | Phenotypical analyses

Of 263 Norwegian *Y. ruckeri* isolates biotyped in the present study (see Table S1 and Figure S1), seven isolates, originating from four different locations and recovered over a period of 33 years, were identified as BT2 by being non-motile and lacking lipase activity. Four of these isolates originate from two neighbouring farms in northern Norway in the 1980s and belong to CC10. The remaining two CC10 isolates studied, from a different location in mid-Norway, are both motile BT1. The remaining three BT2 isolates, which were recovered in 2017 from two Atlantic salmon sea farms located in neighbouring fjords in mid-Norway, belong to CC1 and display identical MLVA profiles.

While lipase activity and motility were linked in all non-CC1 isolates, some motile CC1 isolates displayed varying lipase activity ranging from weak to absent (Figure S1). Such isolates were recorded here as BT1. Non-motile CC1 isolates (BT2) displayed no lipase activity.

3.3 | Whole-genome analyses

A core genome of 2838 genes (protein identity ≥97%) was identified across the 26 *Y. ruckeri* genomes assayed, resulting in a 14,670 bp SNP-alignment as the basis for phylogenetic reconstruction. Clustering in the resulting tree demonstrates deep branching

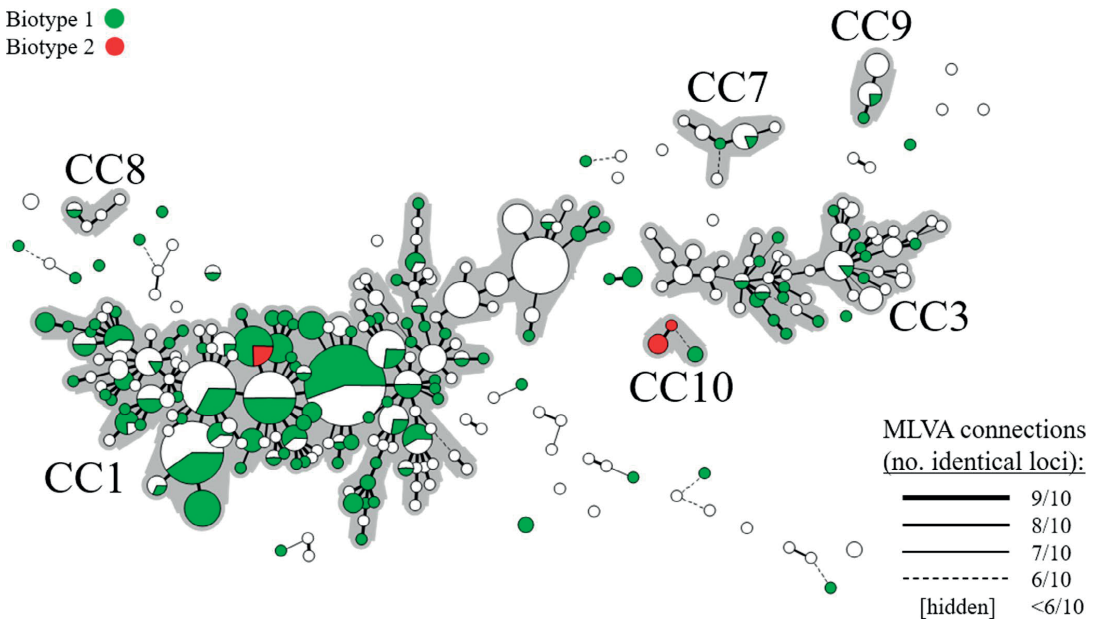

FIGURE 1 Minimum spanning tree based upon multi-locus variable number of tandem repeat analysis (MLVA) genotyping (Gulla et al., 2018) of 601 Norwegian *Y. ruckeri* isolates, collected from 1985 to 2020. Declining MLVA similarity correlates with the declining thickness of branch connections (see bottom right legend). Defined clonal complexes, interconnected throughout via ≥6/10 identical loci, are bound by a grey border. Biotyping results for assayed isolates are shown in green (biotype 1) and red (biotype 2)

between the MLVA-defined clonal complexes and highlights the independent origins of BT2 within CC's 1, 2, 5 and 10 (Figure 2).

Alignment of flagellar gene sequences extracted from the NVI-11000 genome assembly revealed the same 10 bp deletion in *fliR* as previously observed in BT2 isolates from rainbow trout in Denmark and Finland, *fliR*Δ2, which results in a frameshift from amino acid 147 onwards with the protein terminated early by a stop codon in position 169 (Welch et al., 2011).

Sequence alignment of flagellar genes from the NVI-344 genome with those of motile CC10 isolates revealed two non-synonymous SNPs not previously identified in *Y. ruckeri*, that is, *fliH (A205V)* and *flhC (G143V)*. Sanger sequencing of these two genes in all currently known CC10 isolates subsequently verified that while the *fliH*-variant is omnipresent within the CC10 lineage irrespective of biotype, the *flhC*-variant was found exclusively in BT2 isolates (Figure 3). The *flhC* mutation is situated in a sharp turn in the tertiary structure where FlhC binds a zinc ion and interacts with FlhD in the $FlhD_4C_2$ complex (Wang et al., 2006). This complex functions as a master transcriptional activator for flagellar and chemotaxis genes (Liu & Matsumura, 1994), and is also involved in the regulation of other genes (Bleves et al., 2002; Jozwick et al., 2017). Deletions or detrimental mutations in *flhDC* are known to silence expression of flagellar genes (Al Mamun et al., 1996; Chain et al., 2004; Jozwick et al., 2017; Monday et al., 2004).

Alignment of flagellar genes further revealed a single non-synonymous SNP, *fliP(A224D)* in NVI-10990, not present in any publicly available *Yersinia fliP* sequences. This SNP was also confirmed in the remaining non-motile CC1 isolates (NVI-10990 -10974 and -10975) by Sanger sequencing, while being absent in motile CC1 isolates. The flagellar export protein FliP functions with FliQ and FliR in a $P_5Q_4R_1$ complex, which constitutes the export gate of the flagellar secretion channel, a key component of the flagellar export apparatus (Kuhlen et al., 2018; Minamino & Macnab, 1999). Mutations in the export gate complex proteins have been shown to cause loss of motility in *Y. ruckeri* previously, specifically two variants of frameshift-inducing deletions in *fliR* (Welch et al., 2011). The *fliP(A224D)* mutation results in a shift in amino acid class from non-polar to polar close to the critical Asp197–Lys222 intramolecular bridge, which has been shown previously to result in motility defects when mutated (Kuhlen et al., 2018; Ward et al., 2018).

3.4 | Biotype reversion

From 10 replicate cultures each of isolates NVI-344 and NVI-10990, incubated on semi-solid agar for detection of spontaneous BT reversion, motile mutants were successfully recovered from a single culture of NVI-344, and from five cultures of NVI-10990.

Sanger sequencing of *flhC* in the reversal mutant of NVI-344 revealed a non-synonymous alanine-valine mutation at residue 143 of *flhC* (Figure 3). Although different from the wild-type glycine found in motile strains, alanine is more similar to glycine than to the branched amino acid valine found in the non-motile NVI-344.

Sanger sequencing of *fliP* in reverted mutants of NVI-10990 revealed non-synonymous SNPs in residue 224 in all five mutants, resulting in reversion to non-polar amino acid residues in this position (Figure 4). Reversion to the original wild-type allele of *fliP* was observed in one case.

All of the spontaneously reverted BT2 strains were phenotypically indistinguishable from their wild-type motile counterparts in motility and lipase assays, and by microscopic observation of flagella.

3.5 | Complementation experiments

Plasmid-mediated complementation of NVI-344 and NVI-10990 with wild-type variants of the *flhDC* operon and constitutively expressed *fliP*, respectively, restored motility and lipase activity for both strains (Table 4), with observable flagella in flagella-stained phase-contrast microscopy (Figure 5). Motility and lipase activity was lost when the strains were cured of the complementing plasmids.

4 | DISCUSSION

While non-motile BT2 *Y. ruckeri* mutants have emerged and established independently on multiple occasions in international farming of rainbow trout, often shortly after introduction of yersiniosis vaccines, these variants remain rarely reported from farmed Atlantic salmon. This study presents the first characterization of BT2 *Y. ruckeri* recovered from farmed salmon in Norway, where an increase in the use of yersiniosis vaccines has occurred in recent years. Two novel BT2 variants, belonging to distant phylogenetic lineages and isolated three decades apart, were detected amongst the 263 *Y. ruckeri* isolates biotyped. Neither of the two mutants seem to have established widespread dominance, however, and are now possibly extinct *in natura*, although at least one of the progenitor lineages still thrives. The mutations responsible for loss of motility were identified, and motility could in both cases be readily restored experimentally via culture-based reversion and plasmid-mediated complementation.

As for all previously characterized BT2 *Y. ruckeri*, the non-motile phenotypes of the two Norwegian variants described here are linked to mutations in essential flagellar genes. Both phenotype-altering mutations differed, however, from most previously documented cases of BT2 development in that they represented non-synonymous shifts rather than frameshifting deletions (Barnes et al., 2016; Welch et al., 2011). As detrimental non-synonymous SNPs are arguably less conspicuous than frameshift-inducing indels when exploring sequence data, they may be more difficult to identify as a likely cause for altered phenotype. This was certainly the case with BT2 isolate NVI-344, which displayed two unique non-synonymous SNPs in flagellar genes. The discovery of closely related motile isolates by MLVA genotyping was of key importance to enable identification of the responsible mutation by comparing the suspected genes between CC10 isolates of both biotypes. In

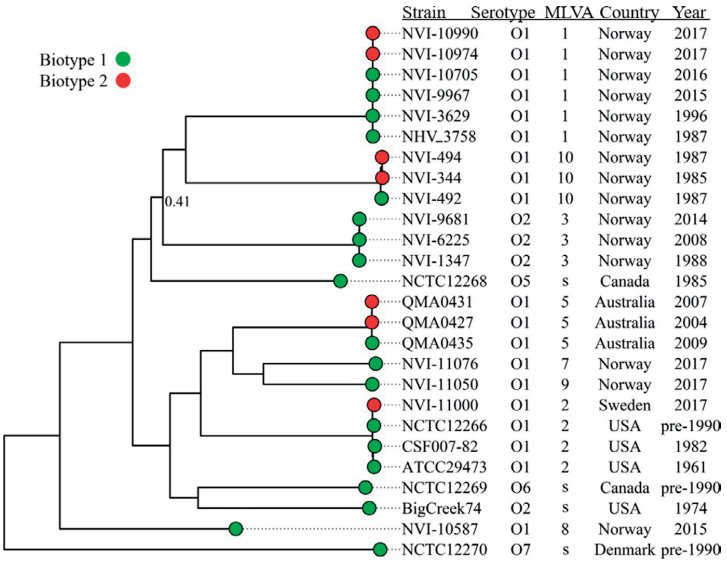

FIGURE 2 Maximum likelihood phylogenetic tree based on a core-gene alignment of 26 *Y. ruckeri* isolates produced with Roary using the MAFFT aligner. Assemblies were downloaded from NCBI or produced in current study (details in Table 3). Bootstrap values are from 1000 replicates with values >0.8 hidden. Multi-locus variable number of tandem repeat analysis (MLVA) 's' indicates the isolate does not belong to any of the MLVA clonal complexes defined here or by Gulla et al. (2018). Biotypes are shown in green (biotype 1) and red (biotype 2)

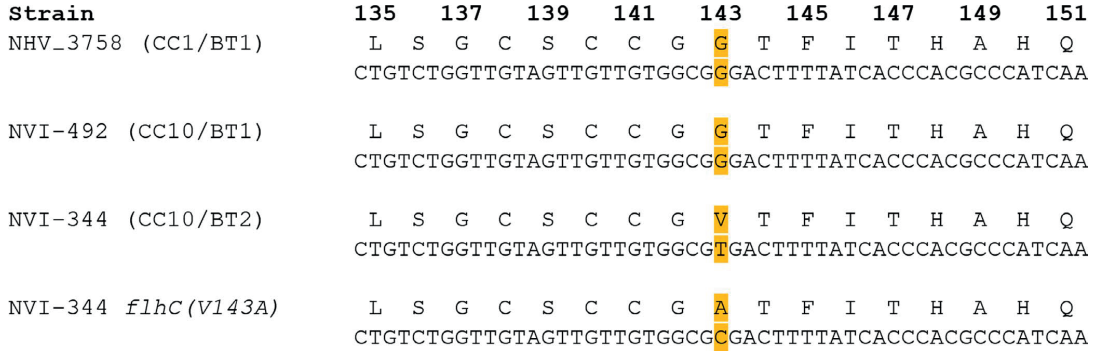

FIGURE 3 Protein- and DNA-sequence data for *flhC* amino acid positions 135–151 in the genomes of isolates NHV_3758 (CC1/BT1; accession no. CP023184), NVI-492 (CC10/BT1), NVI-344 (CC10/BT2), as well as the NVI-344 biotype reversal mutant

contrast, the causative SNP in BT2 isolate NVI-10990 was readily identifiable as several whole-genome sequences from very closely related motile isolates were already at hand. There is notably at least one previous record of BT2 where the causative mutation(s) could not be identified (Calvez et al., 2014; S. Calvez, pers. comm.), which together with the results presented here could suggest that, although more difficult to discover, BT2 development may arise as commonly by non-synonymous SNPs as by indels.

In addition to our findings in *Y. ruckeri* from Norwegian Atlantic salmon, we also investigated a single non-motile CC2 isolate (NVI-11000) recovered in 2017 from rainbow trout farmed in Sweden, revealing the same 10 bp *fliR* deletion as previously described in BT2 isolates from rainbow trout in Denmark and Finland (Welch et al., 2011). This finding contributes to further document the wide distribution of the various BT2 sub-lineages within the internationally dominant rainbow trout-associated CC2 lineage (Gulla et al., 2018). Notably, despite being a prominent producer of rainbow trout, CC2 has not yet been identified in Norway.

NVI-344, confirmed here as a member of the hitherto undescribed CC10 lineage, represents the first recorded instance of *Y. ruckeri* BT2, and indeed yersiniosis, in Norway (Sparboe et al., 1986). This relatively minor clonal complex is restricted to five clinical cases diagnosed in two Norwegian salmon farms between 1985 and 1988. Available information on the severity of these CC10 cases is limited, but the two affected farms being separated by more than 700 km indicates a wider historical distribution than that documented here. Moreover, while BT2 isolates were only detected in one of the farms, these isolations predate BT1 detections from the other farm, indicating that *Y. ruckeri* CC10 (with the BT1 phenotype) was likely also present in Norway at some point prior to 1985.

While identification of *Y. ruckeri* BT2 in CC10 is interesting in the context of independent BT2 emergences in salmonid aquaculture,

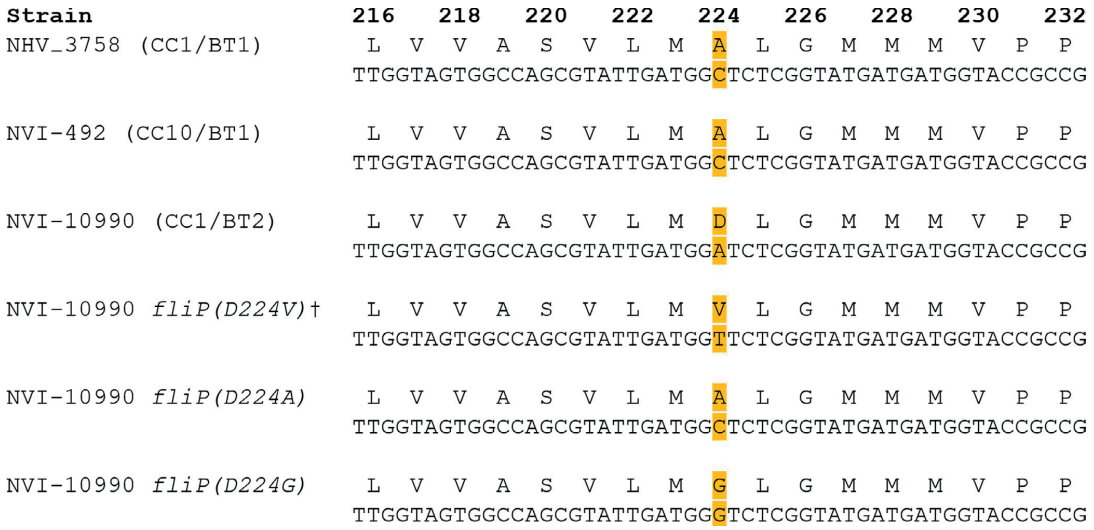

FIGURE 4 Protein- and DNA-sequence data for *fliP* amino acid positions 216–232 in the genomes of isolates NHV_3758 (CC1/BT1; accession no. CP023184), NVI-10990 (CC1/BT2), as well as the five NVI-10990 biotype reversal mutants. (†) NVI-10990 *fliP(D224V)* was observed in three cases

TABLE 4 Motility- (assessed on semi-solid TSA), lipase secretion- (assessed on Tween80 agar) and presence of flagella- (assessed by phase-contrast microscopy with flagella-stain) phenotypes of the two BT2 isolates NVI-344 (CC10) and NVI-10990 (CC1), respectively before and after reversion and complementation

Isolate	Motility	Lipase	Flagella
NVI-344	–	–	–
NVI-344 pAR3-flhDC	+	+	+
NVI-344 *flhC(V143A)*	+	+	+
NVI-10990	–	–	–
NVI-10990 pAR3-fliP	+	+	+
NVI-10990 *fliP(D224V)*[a]	+	+	+
NVI-10990 *fliP(D224A)*	+	+	+
NVI-10990 *fliP(D224G)*	+	+	+

[a]Three independent mutants of NVI-10990 displaying the same *fliP(D224V)* mutation and phenotype.

this lineage has not been registered since the late 1980s despite hundreds of later Norwegian isolates being genotyped, and is now possibly extinct. However, the three BT2 isolates belonging to the contemporary and only clinically relevant *Y. ruckeri* lineage in Norway in recent years, CC1, are of considerable current interest. While confirming that BT2 mutants have the potential to arise within this clonal lineage, the fact that of 204 biotyped CC1 isolates, collected over four consecutive decades, only three isolates from early 2017 displayed the BT2 phenotype, indicates a low historical and current prevalence in Norwegian aquaculture. The three isolates in question, NVI-10974, -10975 and -10990, share identical MLVA-profiles and the same causative BT-shifting SNP, indicating an unidentified common origin. Indeed, they were all recovered over a single month from two sea-farms in neighbouring fjords where a de-lousing vessel had visited both facilities shortly prior to the outbreaks, constituting perhaps the most likely route of transmission. Being that this phenotype within CC1 has not been detected since however, this might have represented a dead-end emergence.

While it has been hypothesized that BT2 development may be driven by lower levels of vaccine protection towards strains lacking the strongly immunogenic flagella, any causal correlation has yet to be verified. It is nevertheless interesting to note that the BT2 isolates detected within the exclusively Norwegian CC1 lineage were recovered shortly after introduction of widespread intraperitoneal vaccination within the Norwegian salmon farming industry, although it remains uncertain whether the affected fish had received such a vaccine.

The various *Y. ruckeri* lineages now known to harbour BT2 strains, that is, CC1, CC2, CC5 and CC10, all represent deep-branching phylogenetic lineages within the global *Y. ruckeri* population (Figure 2), demonstrating that BT2 mutations may likely arise within any lineage. As previously mentioned however, while BT2 *Y. ruckeri* have become remarkably successful in farming of rainbow trout, emerging on different continents following multiple independent mutation events within the clonal lineage CC2, this situation is not mirrored in international Atlantic salmon aquaculture. Early BT2 isolates from salmon in the UK in the 1980s and 1990s were then described as 'rainbow trout-like' (Wheeler et al., 2009), a supposition later confirmed by MLVA placing them within the globally dominating rainbow trout lineage CC2 (Gulla et al., 2018). A different *Y. ruckeri* lineage, CC4, has been associated with yersiniosis in farmed salmon in the UK in recent

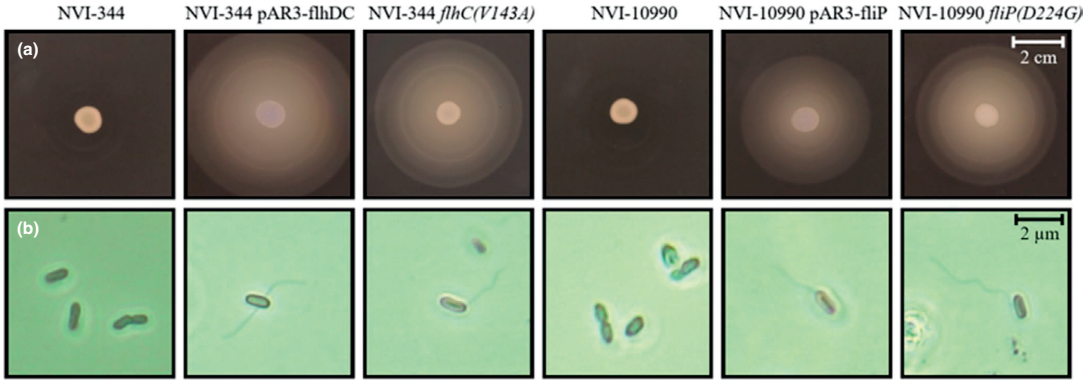

FIGURE 5 Motility assayed on semi-solid Tryptic Soy Agar (a) photographed after 24 h for motile strains and 48 h for non-motile strains, and phase-contrast microscopy with flagella stain (b). Microscopy images are representative except for NVI-10990 complemented with pAR3-fliP which displayed a lower proportion of flagellated cells (approximately 1 in 50). No flagellated cells were observed in the non-motile isolates NVI-344 and NVI-10990

years with no reports of BT2 development (Ormsby et al., 2016), while studies on isolates from Chilean salmon farming found exclusively BT1 (Bastardo et al., 2011). To our knowledge, the well-documented BT2 development within the salmon-associated CC5 from Australia (Barnes et al., 2016) represents the only known post-millennial BT2 development in 'salmon specific' lineages prior to the present study. One conceivable explanation why BT2 emergences have not established a similar dominance in salmon-associated lineages as seen within the rainbow trout-associated CC2, might relate to differing production forms for the two fish species. As such, both of the novel BT2 variants from Norwegian salmon characterized here were found over short time periods and from marine farms only. A mutant of this essentially freshwater pathogen arising in the marine environment, with harvested fish not returning to freshwater, will likely face a dead-end in terms of onwards transmission. As rainbow trout are generally farmed internationally in freshwater, and as *Y. ruckeri* appear well adapted for long-term survival in freshwater environments (Thorsen et al., 1992; Romalde et al., 1994; Coquet et al., 2002), these conditions will likely provide greater opportunities for persistence of eventual BT2 mutants and spread to subsequent fish stocks.

Although the success of *Y. ruckeri* CC1 in Norway over the past three decades cannot be attributed to biotype shift, CC1 does display a seemingly intrinsic slow-swimming phenotype in comparison to motile isolates within other examined lineages (Riborg, personal observation). Lipase activity appears linked to this low-motility trait as slow-swimming CC1 isolates display a weak or even negative lipase reaction while remaining motile (Figure S1). This situation therefore contradicts the conventional BT2 definition as described by Davies and Frerichs (1989), which regards lipase-activity and motility as positively correlated traits. This feature appears unique to CC1 as we find all other motile strains to be lipase positive. We further found that lipase activity may be increased to detectable levels in motile CC1 isolates by leaving a liquid culture static for several weeks prior to plating, while at the same time swimming-speed also increases to be on par with motile non-CC1 strains. Due to the phenotypic ambiguity thus observed, the lipase assay was discarded in the present study and biotyping of remaining CC1 isolates was based on motility assessment alone. Reduced motility has notably also been observed previously in the Australian salmon-pathogenic lineage CC5, where a successive reduction in motility was observed over time, in addition to some completely non-motile (BT2) strains also arising (Barnes et al., 2016).

Mechanisms for conditional expression of flagellar motility in *Y. ruckeri* are known, in particular from the temperature-dependant regulation of motility documented in other *Yersinia* species (Cornelis, 1992). Although *Y. ruckeri* is non-motile at lower temperatures, flagella are still expressed (O'Leary et al., 1979) and thus exposed to the immune system in a cold-blooded host. As such, Jozwick et al. (2019) recently demonstrated that flagellin expression was repressed in a motile CC2 strain during infection in rainbow trout. This was, however, a remarkably slow process requiring several days to achieve complete repression of *fliC* transcription, while transcription rates rapidly increased in the host *post mortem* (Jozwick et al., 2019). During infection, tighter regulation of motility could presumably provide some of the same advantages as potentially awarded by the BT2 phenotype, while at the same time retaining access to the flagella on demand. This might be a favoured strategy in some of the salmon-associated lineages, or perhaps a step along an evolutionary path towards ultimately dispensing of motility permanently.

During characterization of the two *Y. ruckeri* BT2 mutants NVI-344 and NVI-10990 it was further discovered that prolonged incubation on motility agar eventually produced spontaneous biotype reversions mutants, which could be easily observed and recovered (Figure 6). This highlights the risk of misidentifying BT2 isolates as slow-swimming BT1, if the agar plate is not frequently monitored throughout the incubation period. Interestingly, while all reverse mutants regained motility by single base conversion at the exact

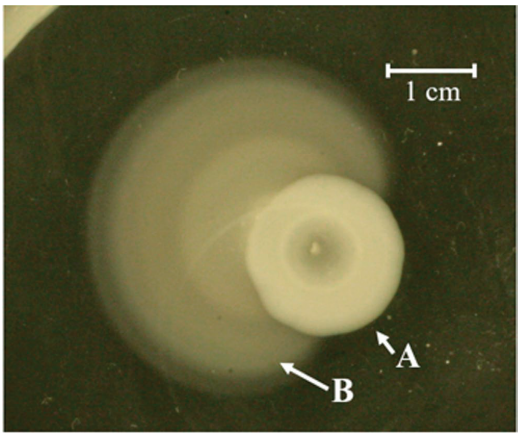

FIGURE 6 Spontaneous biotype reversion after prolonged incubation of the non-motile strain NVI-10990 (a) on semi-solid Tryptic Soy Agar. The motile mutant (b) appeared after 8 days of incubation

site suspected of causing a BT2-shift in the first place, most did not revert to the original amino acid residue found in the motile wild-type, but rather acquired alternative amino acids with similar characteristics. While motility on 0.3% agar appeared fully restored for these variants, it may be that protein function is affected negatively in other ways. Nonetheless, the possibility for spontaneous BT-reversion in *Y. ruckeri* documented here could imply that BT2 variants arising from point mutations in the wild are more flexible than those caused by frameshift-inducing deletions, where spontaneous reversion mutants are probably less likely to arise.

Aside from prolonged culture, motility in both NVI-10990 and NVI-344 could also be restored via plasmid complementation with wild-type functional copies of, respectively, *fliP* and *flhDC*. Complemented NVI-10990 displayed slower swimming than equivalent (CC1) motile isolates, correlating with the proportion of microscopically observable flagellated cells. Similar effects have been observed previously with plasmid-mediated complementation of *flhA(D256G)* and are likely due to competition from the chromosomally situated mutated gene (Welch et al., 2011), and in our specific case also affected by the expression levels of *fliP* from the *bla*-promoter. In contrast, motility in complemented NVI-344 was greatly enhanced, likely due to increased copy number of the plasmid-borne *flhDC* operon.

Identification and characterization of spontaneous biotype reversions, in combination with complementation experiments, verified that the identified mutations in *fliP* and *flhC* are responsible for the BT2 phenotype observed within *Y. ruckeri* lineages CC1 and CC10, respectively. Considering all previously characterized *Y. ruckeri* BT2 mutants have possessed mutations in genes encoding the flagellar export apparatus specifically (Barnes et al., 2016; Welch et al., 2011), the BT2-inducing mutation in *fliP* was unsurprising. The *flhC* mutation was, on the other hand, somewhat unexpected, as mutations in the *flhDC* operon have never before been linked to loss of motility in *Y. ruckeri*, and it has been proposed as a protected locus in this species (Jozwick et al., 2017). It may be the case that CC10 have other adaptations that allow for the loss of *flhDC* function in this lineage, or that some functionality is preserved with this missense mutation in *flhC* even though motility is lost. Mutations in the *flhDC* operon do, however, represent a common cause of motility-loss in other pathogens (Al Mamun et al., 1996; Chain et al., 2004; Monday et al., 2004) and it would represent a seemingly optimal mutation site in terms of preserving resources via silencing the transcription of all flagella-related operons. Interestingly, Jozwick et al. (2017) found their constructed *flhDC*-deletion mutant to be more virulent in challenge trials, in contrast to naturally occurring BT2 mutations known at the time. As such, it could be relevant to compare the virulence of the BT2 isolates in CC10 with their BT1 counterparts. However, given the current state of the yersiniosis situation in Norwegian aquaculture, with CC1 completely dominating and CC10 remaining absent for over 30 years, further examination of the effects of BT2 in *Y. ruckeri* CC1 should be prioritized.

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DATA AVAILABILITY STATEMENT

The genome assemblies have been deposited at DDBJ/ENA/GenBank under accession numbers JAJIBE000000000–JAJIBV000000000 and JAJJIH000000000, as described in Table 3.

ORCID

Andreas Riborg https://orcid.org/0000-0002-8741-9853
Snorre Gulla https://orcid.org/0000-0002-7135-9227

REFERENCES

Al Mamun, A. A., Tominaga, A., & Enomoto, M. (1996). Detection and characterization of the flagellar master operon in the four *Shigella* subgroups. *Journal of Bacteriology*, *178*(13), 3722–3726. https://doi.org/10.1128/jb.178.13.3722-3726.1996

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., Lesin, V. M., Nikolenko, S. I., Pham, S., Prjibelski, A. D., Pyshkin, A. V., Sirotkin, A. V., Vyahhi, N., Tesler, G., Alekseyev, M. A., & Pevzner, P. A. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of computational biology : a journal of computational molecular cell biology*, *19*(5), 455–477. https://doi.org/10.1089/cmb.2012.0021

Barnes, A. C., Delamare-Deboutteville, J., Gudkovs, N., Brosnahan, C., Morrison, R., & Carson, J. (2016). Whole genome analysis of *Yersinia ruckeri* isolated over 27 years in Australia and New Zealand reveals geographical endemism over multiple lineages and recent evolution under host selection. *Microbial Genomics*, *2*(11), e000095. https://doi.org/10.1099/mgen.0.000095

Bastardo, A., Bohle, H., Ravelo, C., Toranzo, A. E., & Romalde, J. L. (2011). Serological and molecular heterogeneity among *Yersinia ruckeri* strains isolated from farmed Atlantic salmon Salmo salar in

Chile. *Diseases of Aquatic Organisms*, *93*(3), 207–214. https://doi.org/10.3354/dao02296

Bleves, S., Marenne, M. N., Detry, G., & Cornelis, G. R. (2002). Up-regulation of the *Yersinia enterocolitica* yop regulon by deletion of the flagellum master operon *flhDC*. *Journal of Bacteriology*, *184*(12), 3214–3223. https://doi.org/10.1128/JB.184.12.3214-3223.2002

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics (Oxford, England)*, *30*(15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Busch, R. A. (1978). Enteric red mouth disease (Hagerman strain). *Marine Fisheries Review*, *40*, 467–472.

Calvez, S., Gantelet, H., Blanc, G., Douet, D. G., & Daniel, P. (2014). *Yersinia ruckeri* Biotypes 1 and 2 in France: Presence and antibiotic susceptibility. *Diseases of Aquatic Organisms*, *109*(2), 117–126. https://doi.org/10.3354/dao02725

Chain, P. S., Carniel, E., Larimer, F. W., Lamerdin, J., Stoutland, P. O., Regala, W. M., Georgescu, A. M., Vergez, L. M., Land, M. L., Motin, V. L., Brubaker, R. R., Fowler, J., Hinnebusch, J., Marceau, M., Medigue, C., Simonet, M., Chenal-Francisque, V., Souza, B., Dacheux, D., … Garcia, E. (2004). Insights into the evolution of *Yersinia pestis* through whole-genome comparison with *Yersinia pseudotuberculosis*. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(38), 13826–13831. https://doi.org/10.1073/pnas.0404012101

Coquet, L., Cosette, P., Quillet, L., Petit, F., Junter, G. A., & Jouenne, T. (2002). Occurrence and phenotypic characterization of *Yersinia ruckeri* strains with biofilm-forming capacity in a rainbow trout farm. *Applied and Environmental Microbiology*, *68*(2), 470–475. https://doi.org/10.1128/AEM.68.2.470-475.2002

Cornelis, G. (1992). *Yersiniae*, finely tuned pathogens. In C. E. Hormaeche, C. W. Penn, & C. J. Smyth (Eds.), *Molecular biology of bacterial infections: Current Status and Future Perspectives* (pp. 231-266). Society for General Microbiology symposium no. 49. Cambridge University Press.

Costa, A. A., Leef, M. J., Bridle, A. R., Carson, J., & Nowak, B. F. (2011). Effect of vaccination against yersiniosis on the relative percent survival, bactericidal and lysozyme response of Atlantic salmon, *Salmo salar*. *Aquaculture*, *315*, 201–206. https://doi.org/10.1016/j.aquaculture.2011.02.031

Daligault, H. E., Davenport, K. W., Minogue, T. D., Bishop-Lilly, K. A., Broomall, S. M., Bruce, D. C., Chain, P. S., Coyne, S. R., Frey, K. G., Gibbons, H. S., Jaissle, J., Koroleva, G. I., Ladner, J. T., Lo, C. C., Munk, C., Palacios, G. F., Redden, C. L., Rosenzweig, C. N., Scholz, M. B., & Johnson, S. L. (2014). Whole-Genome Yersinia sp. Assemblies from 10 Diverse Strains. *Genome Announcements*, *2*(5), e01055–e1114. https://doi.org/10.1128/genomeA.01055-14

Davies, R. L., & Frerichs, G. N. (1989). Morphological and biochemical differences among isolates of *Yersinia ruckeri* obtained from wide geographical areas. *Journal of Fish Diseases*, *12*, 357–365. https://doi.org/10.1111/j.1365-2761.1989.tb00324.x

Evenhuis, J. P., Lapatra, S. E., Verner-Jeffreys, D. W., Dalsgaard, I., & Welch, T. J. (2009). Identification of flagellar motility genes in *Yersinia ruckeri* by transposon mutagenesis. *Applied and Environmental Microbiology*, *75*(20), 6630–6633. https://doi.org/10.1128/AEM.01415-09

Ewing, W. H., Ross, A. J., Brenner, D. J., & Fanning, G. R. (1978). *Yersinia ruckeri* sp. nov., the redmouth (RM) bacterium. *International Journal of Systematic Bacteriology*, *28*, 37–44. https://doi.org/10.1099/00207713-28-1-37

Ferrières, L., Hémery, G., Nham, T., Guérout, A. M., Mazel, D., Beloin, C., & Ghigo, J. M. (2010). Silent mischief: Bacteriophage Mu insertions contaminate products of *Escherichia coli* random mutagenesis performed using suicidal transposon delivery plasmids mobilized by broad-host-range RP4 conjugative machinery. *Journal of Bacteriology*, *192*(24), 6418–6427. https://doi.org/10.1128/JB.00621-10

Fouz, B., Zarza, C., & Amaro, C. (2006). First description of non-motile *Yersinia ruckeri* serovar I strains causing disease in rainbow trout, *Oncorhynchus mykiss* (Walbaum), cultured in Spain. *Journal of Fish Diseases*, *29*(6), 339–346. https://doi.org/10.1111/j.1365-2761.2006.00723.x

Gulla, S., Barnes, A. C., Welch, T. J., Romalde, J. L., Ryder, D., Ormsby, M. J., Carson, J., Lagesen, K., Verner-Jeffreys, D. W., Davies, R. L., & Colquhoun, D. J. (2018). Multilocus variable-number tandem-repeat analysis of *Yersinia ruckeri* confirms the existence of host specificity, geographic endemism, and anthropogenic dissemination of virulent clones. *Applied and Environmental Microbiology*, *84*(16), e00730–e818. https://doi.org/10.1128/AEM.00730-18

Gulla, S., Mohammad, S. N., & Colquhoun, D. J. (2019). Multi-locus variable-number tandem-repeat analysis of the fish-pathogenic bacterium *Yersinia ruckeri* by multiplex PCR and capillary electrophoresis. *Journal of Visualized Experiments*, *148*, e59455. https://doi.org/10.3791/59455

Gulla, S., & Olsen, A. B. (2020). Yersiniosis. In I. Sommerset, C. S. Walde, B. Bang Jensen, B. Bornø, A. Haukaas, & E. Brun (Eds.), *The health situation in Norwegian aquaculture 2019* (pp. 80-82). Published by the Norwegian Veterinary Institute. Retrieved from https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2020/fish-health-report-2019

Heimbrook, M. E., Wang, W. L., & Campbell, G. (1989). Staining bacterial flagella easily. *Journal of Clinical Microbiology*, *27*(11), 2612–2615. https://doi.org/10.1128/jcm.27.11.2612-2615.1989

Hossain, M. J., Thurlow, C. M., Sun, D., Nasrin, S., & Liles, M. R. (2015). Genome modifications and cloning using a conjugally transferable recombineering system. *Biotechnology Reports (Amsterdam, Netherlands)*, *8*, 24–35. https://doi.org/10.1016/j.btre.2015.08.005

Jozwick, A. K., Graf, J., & Welch, T. J. (2017). The flagellar master operon *flhDC* is a pleiotropic regulator involved in motility and virulence of the fish pathogen *Yersinia ruckeri*. *Journal of Applied Microbiology*, *122*(3), 578–588. https://doi.org/10.1111/jam.13374

Jozwick, A., LaPatra, S. E., Graf, J., & Welch, T. J. (2019). Flagellar regulation mediated by the Rcs pathway is required for virulence in the fish pathogen *Yersinia ruckeri*. *Fish & Shellfish Immunology*, *91*, 306–314. https://doi.org/10.1016/j.fsi.2019.05.036

Kuhlen, L., Abrusci, P., Johnson, S., Gault, J., Deme, J., Caesar, J., Dietsche, T., Mebrhatu, M. T., Ganief, T., Macek, B., Wagner, S., Robinson, C. V., & Lea, S. M. (2018). Structure of the core of the type III secretion system export apparatus. *Nature Structural & Molecular Biology*, *25*(7), 583–590. https://doi.org/10.1038/s41594-018-0086-9

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, *35*(6), 1547–1549. https://doi.org/10.1093/molbev/msy096

Liu, X., & Matsumura, P. (1994). The *FlhD*/*FlhC* complex, a transcriptional activator of the *Escherichia coli* flagellar class II operons. *Journal of Bacteriology*, *176*(23), 7345–7351. https://doi.org/10.1128/jb.176.23.7345-7351.1994

Minamino, T., & Macnab, R. M. (1999). Components of the *Salmonella* flagellar export apparatus and classification of export substrates. *Journal of Bacteriology*, *181*(5), 1388–1394. https://doi.org/10.1128/JB.181.5.1388-1394.1999

Monday, S. R., Minnich, S. A., & Feng, P. C. (2004). A 12-base-pair deletion in the flagellar master control gene *flhC* causes nonmotility of the pathogenic German sorbitol-fermenting *Escherichia coli* O157:H- strains. *Journal of Bacteriology*, *186*(8), 2319–2327. https://doi.org/10.1128/JB.186.8.2319-2327.2004

Nelson, M. C., LaPatra, S. E., Welch, T. J., & Graf, J. (2015). Complete genome sequence of *Yersinia ruckeri* Strain CSF007-82, etiologic agent of red mouth disease in salmonid fish. *Genome Announcements*, *3*(1), e01491–e1514. https://doi.org/10.1128/genomeA.01491-14

O'Leary, P. J., Rohovec, J. S., & Fryer, J. L. (1979). A further characterization of *Yersinia ruckeri* (Enteric Redmouth Bacterium). *Fish Pathology*, *14*(2), 71–78. https://doi.org/10.3147/jsfp.14.71

Ormsby, M. J. (2015). *Comparative phenotypic, proteomic and genomic approaches to assess lipopolysaccharide and outer membrane protein diversity among isolates of* Yersinia ruckeri *recovered from Atlantic salmon and rainbow trout*. [PhD thesis, University of Glasgow]. Retrieved from https://theses.gla.ac.uk/7109/

Ormsby, M. J., Caws, T., Burchmore, R., Wallis, T., Verner-Jeffreys, D. W., & Davies, R. L. (2016). *Yersinia ruckeri* isolates recovered from diseased Atlantic salmon (*Salmo salar*) in Scotland are more diverse than those from rainbow trout (*Oncorhynchus mykiss*) and represent distinct subpopulations. *Applied and Environmental Microbiology*, *82*(19), 5785–5794. https://doi.org/10.1128/AEM.01173-16

Page, A. J., Cummins, C. A., Hunt, M., Wong, V. K., Reuter, S., Holden, M. T., Fookes, M., Falush, D., Keane, J. A., & Parkhill, J. (2015). Roary: Rapid large-scale prokaryote pan genome analysis. *Bioinformatics (Oxford, England)*, *31*(22), 3691–3693. https://doi.org/10.1093/bioinformatics/btv421

Page, A. J., Taylor, B., Delaney, A. J., Soares, J., Seemann, T., Keane, J. A., & Harris, S. R. (2016). SNP-sites: Rapid efficient extraction of SNPs from multi-FASTA alignments. *Microbial Genomics*, *2*(4), e000056. https://doi.org/10.1099/mgen.0.000056

Romalde, J. L., Barja, J. L., Magariños, B., & Toranzo, A. E. (1994). Starvation-survival processes of the bacterial fish pathogen *Yersinia ruckeri*. *Systematic and Applied Microbiology*, *17*(2), 161–168. https://doi.org/10.1016/S0723-2020(11)80002-0

Ross, A. J., Rucker, R. R., & Ewing, W. H. (1966). Description of a bacterium associated with redmouth disease of rainbow trout (*Salmo gairdneri*). *Canadian Journal of Microbiology*, *12*(4), 763–770. https://doi.org/10.1139/m66-103

Seemann, T. (2014). Prokka: Rapid prokaryotic genome annotation. *Bioinformatics (Oxford, England)*, *30*(14), 2068–2069. https://doi.org/10.1093/bioinformatics/btu153

Sparboe, O., Koren, C., Håstein, T., Poppe, T. T., & Stenwig, H. (1986). The first isolation of *Yersinia ruckeri* from farmed Norwegian salmon. *Bulletin of the European Association of Fish Pathologists*, *6*, 41–42.

Thorsen, B. K., Enger, O., Norland, S., & Hoff, K. A. (1992). Long-term starvation survival of Yersinia ruckeri at different salinities studied by microscopical and flow cytometric methods. *Applied and environmental microbiology*, *58*(5), 1624–1628. https://doi.org/10.1128/aem.58.5.1624-1628.1992

Tinsley, J. W., Lyndon, A. R., & Austin, B. (2011). Antigenic and cross-protection studies of biotype 1 and biotype 2 isolates of Yersinia ruckeri in rainbow trout, Oncorhynchus mykiss (Walbaum). *Journal of applied microbiology*, *111*(1), 8–16. https://doi.org/10.1111/j.1365-2672.2011.05020.x

Wang, S., Fleming, R. T., Westbrook, E. M., Matsumura, P., & McKay, D. B. (2006). Structure of the *Escherichia coli FlhDC* complex, a prokaryotic heteromeric regulator of transcription. *Journal of Molecular Biology*, *355*(4), 798–808. https://doi.org/10.1016/j.jmb.2005.11.020

Ward, E., Renault, T. T., Kim, E. A., Erhardt, M., Hughes, K. T., & Blair, D. F. (2018). Type-III secretion pore formed by flagellar protein *FliP*. *Molecular Microbiology*, *107*(1), 94–103. https://doi.org/10.1111/mmi.13870

Welch, T. J., Verner-Jeffreys, D. W., Dalsgaard, I., Wiklund, T., Evenhuis, J. P., Cabrera, J. A., Hinshaw, J. M., Drennan, J. D., & LaPatra, S. E. (2011). Independent emergence of *Yersinia ruckeri* biotype 2 in the United States and Europe. *Applied and Environmental Microbiology*, *77*(10), 3493–3499. https://doi.org/10.1128/AEM.02997-10

Wheeler, R. W., Davies, R. L., Dalsgaard, I., Garcia, J., Welch, T. J., Wagley, S., Bateman, K. S., & Verner-Jeffreys, D. W. (2009). *Yersinia ruckeri* biotype 2 isolates from mainland Europe and the UK likely represent different clonal groups. *Diseases of Aquatic Organisms*, *84*(1), 25–33. https://doi.org/10.3354/dao02039

Wrobel, A., Ottoni, C., Leo, J. C., Gulla, S., & Linke, D. (2018). The repeat structure of two paralogous genes, Yersinia ruckeri invasin (yrInv) and a "Y. ruckeri invasin-like molecule", (yrIlm) sheds light on the evolution of adhesive capacities of a fish pathogen. *Journal of structural biology*, *201*(2), 171–183. https://doi.org/10.1016/j.jsb.2017.08.008

Young, G. M., Schmiel, D. H., & Miller, V. L. (1999). A new pathway for the secretion of virulence factors by bacteria: The flagellar export apparatus functions as a protein-secretion system. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(11), 6456–6461. https://doi.org/10.1073/pnas.96.11.6456

Yu, G., Smith, D. K., Zhu, H., Guan, Y., & Lam, T. T. Y. (2017). ggtree: an r package for visualization and annotation of phylogenetic trees with their covariates and other associated data. *Methods in Ecology and Evolution*, *8*(1), 28–36. https://doi.org/10.1111/2041-210X.12628

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Supplemental table S1, strains originating from Norway evaluated in present study

Isolate	County	Year	Source	Serotype	MLVA-CC	Biotype	Biotype published previously
NVI-344 (RD154)	T	1985	*Salmo salar*	O1	10	2	Sparboe et al. 1986; Wheeler et al. 2009
NVI-486	T	1986	*Salmo salar*		10	2	
NVI-492	ST	1987	*Salmo salar*	O1	10	1	
NVI-494	T	1987	*Salmo salar*	O1	10	2	
NVI-495	TM	1987	*Oncorhynchus mykiss*	O2	s	1	
NVI-1175 (JR-1533)	NL	1987	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-1277	T	1987	*Salmo salar*		10	2	
NVI-1347	HL	1988	*Salmo salar*	O2	3	1	Gulla et al. 2018
NVI-1364	ST	1988	*Salmo salar*		10	1	
NVI-1365	NT	1988	*Salmo salar*	O1	s	1	Gulla et al. 2018
NVI-1366	NT	1988	Unknown	O2	3	1	Gulla et al. 2018
NVI-1367	NT	1988	Unknown	O2	3	1	Gulla et al. 2018
NVI-1389	NL	1989	*Salmo salar*	O1	s	1	
NVI-1594	S&F	1990	*Salmo salar*	O2	3	1	Gulla et al. 2018
NVI-1660	M&R	1990	*Salmo salar*	O2	3	1	Gulla et al. 2018
NVI-2135	OL	1991	*Coregonus lavaretus*	O2	s	1	Gulla et al. 2018
NVI-2197	OL	1991	*Coregonus lavaretus*	O2	s	1	Gulla et al. 2018
NVI-2205	OL	1991	*Coregonus lavaretus*	O2	s	1	Gulla et al. 2018
NVI-2274	HL	1992	*Salmo salar*	O2	3	1	Gulla et al. 2018
NVI-2275	S&F	1992	*Salmo salar*	O1	s	1	Gulla et al. 2018
NVI-2329	HL	1992	*Salmo salar*	O2	3	1	Gulla et al. 2018
NVI-2365	M&R	1992	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-2775	OL	1994	*Oncorhynchus mykiss*	O2	s	1	Gulla et al. 2018
NVI-2954	S&F	1994	*Salmo salar*	O2	3	1	Gulla et al. 2018
NVI-2966	HL	1994	*Salmo salar*	O1	s	1	Gulla et al. 2018
NVI-2970	HL	1995	*Salmo salar*	O1	s	1	Gulla et al. 2018
NVI-3629	M&R	1996	*Salmo salar*	O1	1	1	
NVI-3633	BR	1996	*Coregonus lavaretus*		3	1	
NVI-3736	RL	1997	*Salmo salar*		s	1	
NVI-3753	M&R	1997	*Salmo salar*		1	1	
NVI-3775	AK	1997	*Salmo salar*		1	1	
NVI-3793	M&R	1997	*Salmo salar*	O1	1	1	
NVI-3854		1998	*Salmo salar*	O1	1	1	
NVI-3878	M&R	1998	*Salmo salar*		1	1	
NVI-4017	M&R	1999	*Salmo salar*		s	1	
NVI-4046	M&R	1999	*Salmo salar*		1	1	
NVI-4063	M&R	1999	*Salmo salar*	O1	1	1	
NVI-4092	M&R	1999	*Salmo salar*	O1	1	1	
NVI-4193	NL	2000	*Salmo salar*		1	1	
NVI-4211	NL	2000	*Salmo salar*	O1	1	1	
NVI-4255	NL	2001	*Salmo salar*	O1	1	1	
NVI-4333	NL	2001	*Salmo salar*	O1	1	1	
NVI-4334	NL	2001	*Salmo salar*	O1	1	1	
NVI-4429	M&R	2001	*Salmo salar*	O1	1	1	
NVI-4431	NL	2001	*Salmo salar*	O1	1	1	
NVI-4439	M&R	2002	*Salmo salar*		1	1	
NVI-4508	ST	2002	*Oncorhynchus mykiss*			1	
NVI-4523		2002	*Salmo salar*	O1		1	
NVI-4566	ST	2002	*Salmo salar*	O1	1	1	
NVI-4605		2002	*Salmo salar*			1	
NVI-4636	M&R	2002	*Salmo salar*	O1	1	1	
NVI-4805	M&R	2003	*Salmo salar*	O1	1	1	
NVI-5306	ST	2005	*Salmo salar*	O1	1	1	
NVI-5523	M&R	2006	*Salmo salar*		1	1	
NVI-5570	M&R	2006	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-5635	HL	2006	*Salmo salar*	N.R.	s	1	
NVI-5899	ST	2007	*Salmo salar*	O1	1	1	
NVI-5983	M&R	2007	*Salmo salar*	O1	s	1	
NVI-6092	M&R	2008	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-6130	M&R	2008	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-6225	M&R	2008	*Salmo salar*	O2	3	1	Gulla et al. 2018
NVI-6287	M&R	2008	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-6288	M&R	2008	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-6390	ST	2008	*Salmo salar*		s	1	
NVI-6400	ST	2008	*Salmo salar*	O1	1	1	
NVI-6523	ST	2008	*Salmo salar*	O1	1	1	
NVI-6524	NT	2008	*Salmo salar*	O1	1	1	
NVI-6688	M&R	2009	*Salmo salar*		1	1	
NVI-6853	M&R	2009	*Salmo salar*		3	1	
NVI-6920	ST	2009	*Salmo salar*		1	1	
NVI-6939	M&R	2009	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-6940	M&R	2009	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-6941	ST	2009	*Salmo salar*	O1	1	1	
NVI-7059	M&R	2009	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-7080	ST	2009	*Salmo salar*	O1	1	1	

NVI-7107	M&R	2009	*Salmo salar*	O1	1	1	
NVI-7231	M&R	2010	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-7299	ST	2010	*Salmo salar*	O1	1	1	
NVI-7348	M&R	2010	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-7485	M&R	2010	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-7531	M&R	2010	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-7597	ST	2010	*Salmo salar*	O1	1	1	
NVI-7985	M&R	2011	*Salmo salar*	O1	1	1	
NVI-7989	NT	2011	*Salmo salar*	O1	s	1	
NVI-8331	FM	2012	*Salmo salar*	O2	3	1	Gulla et al. 2018
NVI-8507	M&R	2012	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-8508	M&R	2012	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-8509	ST	2012	*Salmo salar*	O1	1	1	
NVI-8524	M&R	2012	*Salmo salar*	O1	1	1	
NVI-8525	M&R	2012	*Salmo salar*	O1	1	1	
NVI-8526	M&R	2012	*Salmo salar*	O1	1	1	
NVI-8527	M&R	2012	*Salmo salar*	O1	1	1	
NVI-8618	M&R	2012	*Salmo salar*	O1	1	1	
NVI-8638	ST	2012	*Salmo salar*	O1	1	1	
NVI-8668	M&R	2012	*Salmo salar*	O1	1	1	
NVI-8670	M&R	2012	*Salmo salar*	O1	1	1	
NVI-8723	M&R	2012	*Salmo salar*	O1	1	1	
NVI-8725	M&R	2012	*Salmo salar*	O1	1	1	
NVI-8749	M&R	2012	*Salmo salar*	O1	1	1	
NVI-8780	M&R	2012	*Salmo salar*	O1	1	1	
NVI-8828	FM	2013	*Salmo salar*	O1	1	1	
NVI-8931	M&R	2013	*Salmo salar*	O1	1	1	
NVI-9018	M&R	2013	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-9055	M&R	2013	*Salmo salar*	O1	1	1	
NVI-9162	M&R	2013	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-9163	M&R	2013	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-9336	M&R	2013	*Salmo salar*	O1	1	1	
NVI-9591	T	2013	*Salmo salar*	O1	1	1	
NVI-9597	FM	2014	*Salmo salar*	O1	1	1	
NVI-9598	FM	2014	*Salmo salar*	O1	1	1	
NVI-9654	M&R	2014	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-9656	NT	2014	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-9680	NT	2014	*Salmo salar*	O1	1	1	
NVI-9681	M&R	2014	*Salmo salar*	O2	3	1	Gulla et al. 2018
NVI-9682	NT	2014	*Salmo salar*	O1	1	1	
NVI-9698	M&R	2014	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-9701	M&R	2014	*Salmo salar*	O1	1	1	
NVI-9706	M&R	2014	*Salmo salar*	O1	1	1	Gulla et al. 2018
NVI-9733	M&R	2014	*Salmo salar*	O1	1	1	
NVI-9750	M&R	2014	*Salmo salar*	O1	1	1	
NVI-9806	FM	2014	*Salmo salar*	O1	1	1	
NVI-9807	FM	2014	*Salmo salar*	O1	1	1	
NVI-9816	T	2014	*Salmo salar*	O1	1	1	
NVI-9817	FM	2014	*Salmo salar*	O1	1	1	
NVI-9824	FM	2014	*Salmo salar*	O1	1	1	
NVI-9837	ST	2014	*Salmo salar*	O1	1	1	
NVI-9928	ST	2015	*Salmo salar*	O1	1	1	
NVI-9934	ST	2015	*Salmo salar*	O1	1	1	
NVI-9949	M&R	2015	*Salmo salar*	O1	1	1	
NVI-9966	ST	2015	*Salmo salar*	O1	1	1	
NVI-9967	ST	2015	*Salmo salar*	O1	1	1	
NVI-9987		2015	*Salmo salar*	O1		1	
NVI-9992	ST	2015	*Salmo salar*	O1	1	1	
NVI-9997		2015	*Salmo salar*	O1		1	
NVI-9999		2015	*Salmo salar*	O1		1	
NVI-10024	ST	2015	*Salmo salar*	O1	1	1	
NVI-10025	NT	2015	*Salmo salar*	O1	1	1	
NVI-10050	M&R	2015	*Salmo salar*	O1	1	1	
NVI-10115	M&R	2015	*Salmo salar*	O1	1	1	
NVI-10123	M&R	2015	*Salmo salar*	O1	1	1	
NVI-10204	NT	2015	*Salmo salar*	O1	1	1	
NVI-10206	NT	2015	*Salmo salar*	O1	1	1	
NVI-10253	NT	2016	*Salmo salar*	O1		1	
NVI-10266	M&R	2016	*Salmo salar*	O1	1	1	
NVI-10307	T	2016	*Salmo salar*	O1	1	1	
NVI-10333	NT	2016	*Salmo salar*	O1	1	1	
NVI-10359	T	2016	*Salmo salar*	O1	1	1	
NVI-10401	M&R	2016	*Salmo salar*	O1	1	1	
NVI-10418		2016	*Salmo salar*	O1		1	
NVI-10429	FM	2016	*Salmo salar*	O1	1	1	
NVI-10433	M&R	2016	*Salmo salar*	O1	1	1	
NVI-10454	FM	2016	*Salmo salar*	O1	1	1	
NVI-10512	M&R	2016	*Salmo salar*	O1	1	1	
NVI-10515	FM	2016	*Salmo salar*	O1	1	1	

NVI-10516	T	2016	*Salmo salar*	O1	1	1	
NVI-10545		2016	*Salmo salar*	O1		1	
NVI-10571	ST	2016	*Salmo salar*	O1	1	1	
NVI-10587	ST	2015	*Salmo salar,* egg-fluid	O1	8	1	Gulla et al. 2018
NVI-10622	NT	2016	*Salmo salar*	O1	1	1	
NVI-10705	M&R	2016	*Salmo salar*	O1	1	1	
NVI-10711	T	2016	*Salmo salar*	O1	1	1	
NVI-10724	M&R	2016	*Salmo salar*	O1	1	1	
NVI-10728	ST	2016	*Salmo salar*	O1	1	1	
NVI-10730	ST	2016	*Salmo salar*	O1	1	1	
NVI-10892	M&R	2016	*Salmo salar*	O1	1	1	
NVI-10935	T	2016	*Salmo salar*	O1	1	1	
NVI-10946	M&R	2017	*Salmo salar*	O1	1	1	
NVI-10951	M&R	2017	*Salmo salar*	O1	1	1	
NVI-10974	M&R	2017	*Salmo salar*	O1	1	2	
NVI-10975	M&R	2017	*Salmo salar*	O1	1	2	
NVI-10976	M&R	2017	*Salmo salar*	O1	1	1	
NVI-10986	M&R	2017	*Salmo salar*	O1	1	1	
NVI-10989	M&R	2017	*Salmo salar*	O1	1	1	
NVI-10990	M&R	2017	*Salmo salar*	O1	1	2	
NVI-11024	ST	2017	*Salmo salar*	O1	1	1	
NVI-11028	ST	2017	*Salmo salar*	O1	1	1	
NVI-11036	M&R	2017	*Salmo salar*	O1	1	1	
NVI-11049	ST	2017	*Salmo salar,* egg-fluid	O1	9	1	Gulla et al. 2018
NVI-11050	ST	2017	*Salmo salar,* egg-fluid	O1	9	1	
NVI-11065	ST	2017	*Salmo salar,* egg-fluid	O1	s	1	
NVI-11073	ST	2017	Biofilm, aquaculture	O1	s	1	
NVI-11074	HL	2017	Biofilm, aquaculture	O1	7	1	Gulla et al. 2018
NVI-11076	HL	2017	Biofilm, aquaculture	O1	7	1	
NVI-11087	ST	2017	*Salmo salar*	O1	1	1	
NVI-11116	M&R	2017	*Salmo salar*	O1	1	1	
NVI-11117	M&R	2017	*Salmo salar*	O1	1	1	
NVI-11118	T	2017	*Salmo salar*		1	1	
NVI-11119	M&R	2017	*Salmo salar*	O1	1	1	
NVI-11120	M&R	2017	*Salmo salar*	O1	1	1	
NVI-11121	M&R	2017	*Salmo salar*	O1	1	1	
NVI-11122	T	2017	*Salmo salar*	O1	1	1	
NVI-11123	M&R	2017	*Salmo salar*	O1	1	1	
NVI-11124	M&R	2017	*Salmo salar*	O1	1	1	
NVI-11125	M&R	2017	*Salmo salar*	O1	1	1	
NVI-11126	M&R	2017	*Salmo salar*	O1	1	1	
NVI-11127	T	2017	*Salmo salar*	O1	1	1	
NVI-11128	T	2017	*Salmo salar*	O1	1	1	
NVI-11129	ST	2017	*Salmo salar*	O1	1	1	
NVI-11130	ST	2017	*Salmo salar*	O1	1	1	
NVI-11132	NT	2017	*Salmo salar*	O1	1	1	
NVI-11133	NT	2017	*Salmo salar*	O1	1	1	
NVI-11134	M&R	2017	*Salmo salar*	O1	1	1	
NVI-11135	ST	2017	*Salmo salar*	O1	1	1	
NVI-11136	M&R	2017	*Salmo salar*	O1	1	1	
NVI-11137	ST	2017	*Salmo salar*	O1	1	1	
NVI-11138	ST	2017	*Salmo salar*	O1	1	1	
NVI-11139	NT	2017	*Salmo salar*	O2	3	1	
NVI-11140	ST	2017	*Salmo salar*	O1	1	1	
NVI-11141	M&R	2017	*Salmo salar*	O1	1	1	
NVI-11201	NL	2018	*Salvelinus alpinus*	O1	1	1	
NVI-11203	M&R	2018	*Salmo salar,* egg-fluid	N.R.	s	1	
NVI-11204	ST	2018	*Salmo salar,* egg-fluid	O2	3	1	
NVI-11205	ST	2018	*Salmo salar,* egg-fluid	O2		1	
NVI-11206	ST	2018	*Salmo salar,* egg-fluid	O2	3	1	
NVI-11212	RL	2018	*Salmo salar*	O1	1	1	
NVI-11227	ST	2018	*Salmo salar*	O1	1	1	
NVI-11228	ST	2018	*Salmo salar*	O2	3	1	
NVI-11229	ST	2018	*Salmo salar*	O2	3	1	
NVI-11250	ST	2018	*Salmo salar*		1	1	
NVI-11251	ST	2018	*Salmo salar*		1	1	
NVI-11252	ST	2018	*Salmo salar*		1	1	
NVI-11253	ST	2018	*Salmo salar*		1	1	
NVI-11266	ST	2018	*Salmo salar*		1	1	
NVI-11267	ST	2018	*Salmo salar*		1	1	
NVI-11268	ST	2018	*Salmo salar*	O1	1	1	
NVI-11269	ST	2018	*Salmo salar*		1	1	
NVI-11270	ST	2018	*Salmo salar*		1	1	
NVI-11271	ST	2018	*Salmo salar*	O1	1	1	
NVI-11272	ST	2018	*Salmo salar*		1	1	
NVI-11273	ST	2018	*Salmo salar*		1	1	
NVI-11274	ST	2018	*Salmo salar*		1	1	
NVI-11275	ST	2018	*Salmo salar*		1	1	
NVI-11276	ST	2018	*Salmo salar*		1	1	

NVI-11277	ST	2018	*Salmo salar*		1	1
NVI-11278	ST	2018	*Salmo salar*	O1	1	1
NVI-11279	ST	2018	*Salmo salar*		1	1
NVI-11280	ST	2018	*Salmo salar*		1	1
NVI-11281	ST	2018	*Salmo salar*		1	1
NVI-11287	ST	2018	*Salmo salar*		1	1
NVI-11288	ST	2018	*Salmo salar*		1	1
NVI-11289	ST	2018	*Salmo salar*		1	1
NVI-11290	ST	2018	*Salmo salar*		1	1
NVI-11291	ST	2018	*Salmo salar*		1	1
NVI-11294	RL	2018	*Salmo salar*		1	1
NVI-11295	RL	2018	*Salmo salar*		1	1
NVI-11296	RL	2018	*Salmo salar*		1	1
NVI-11297	RL	2018	*Salmo salar*		1	1
NVI-11304	HL	2018	*Cyclopterus lumpus*		1	1
NVI-11305	M&R	2018	*Salmo salar*		1	1
NVI-11306	M&R	2018	*Salmo salar*		1	1
NVI-11307	M&R	2018	*Salmo salar*		1	1
NVI-11308	M&R	2018	*Salmo salar*		1	1
NVI-11309	M&R	2018	*Salmo salar*		1	1
NVI-11310	M&R	2018	*Salmo salar*		1	1
NVI-11311	ST	2018	*Salmo salar*		1	1
NVI-11312	ST	2018	*Salmo salar*		1	1
NVI-11367	RL	2018	*Salmo salar*		1	1
NVI-11369	M&R	2018	*Salmo salar*		1	1
NVI-11390	M&R	2019	*Salmo salar*		1	1
NVI-11391	M&R	2019	*Salmo salar*		1	1
NVI-11395	FM	2019	*Salmo salar*		1	1
NVI-11400	ST	2019	*Salmo salar*		1	1
NVI-11401	RL	2019	*Salmo salar*	O1	s	1

s: Do not belong to any defined MLVA clonal complex

N.R.: No reaction with any available antisera

Norwegian county abreviations: Finnmark (FM), Troms (T), Nordland (NL), Nord-Trøndelag (NT), Sør-Trøndelag (ST), Møre og Romsdal (M&R), ıland (HL), Rogaland (RL), Buskerud (BR) and Oppland (OL).

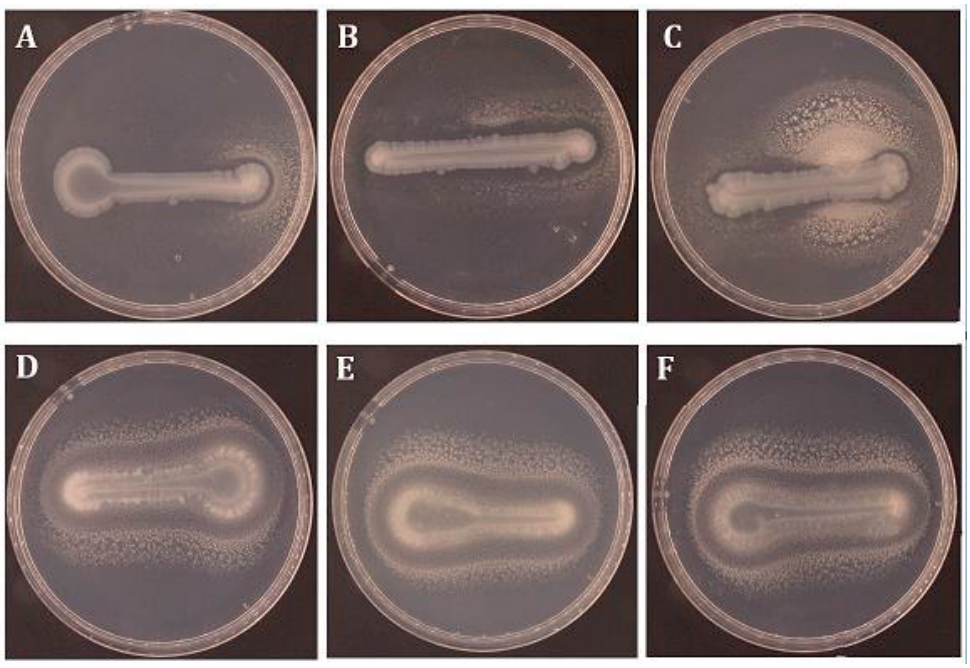

Figure S1

Lipase agar plates seeded with a 10µl droplet from a still overnight culture, streaked across the agar with an inoculation-loop. These particular plates were wrapped in parafilm to maintain humidity and incubated for two weeks to observe the slowly developing lipase reaction of the biotype 1 isolate NVI-10705, MLVA clonal complex CC1. While initially negative for lipase within 48h incubation, weak and/or variable lipase reactions were observed for three separate subcultures of NVI-10705 (A, B and C) after two weeks. The same subcultures of NVI-10705 display motility on 0.3% agar and visible flagella under phase-contrast microscopy (not shown). The NVI-10705 phenotype is typical for CC1 isolates. Lipase reactions typical for non-CC1 biotype 1 isolates, first visible after 24-48 hours incubation time, is observed in motile isolates from other genetic lineages. This is exemplified by NVI-492 CC10 (D), NVI-9681 CC3 (E) and NVI-11076 CC7 (F), where an excessive reaction is seen here due to the prolonged incubation time.

Table S2

Technical details on whole genome sequencing performed in this project.

Strain(s)	Library prep. kit	Sequencing technology	Read stats
NVI-10990	NEBNext Ultra DNA (New England Biolabs)	Illumina HiSeq	150 bp paired end
NVI-1347, NVI-6225, NVI-9681, NCTC12266, NCTC12268, NCTC12269, NCTC12270	TruSeq DNA PCR-Free (Illumina)	Illumina HiSeq	125 bp paired end
NVI-344, NVI-492, NVI-494, NVI-3629, NVI-9967, NVI-10587, NVI-10705, NVI-10974, NVI-11000, NVI-11050, NVI-11076	NexteraFlex (Illumina)	Illumina MiSeq	300 bp paired end

Description of the pAR1, pAR2 and pAR3 plasmids

Molecular techniques

Plasmids were maintained in *E. coli* cultured on Luria agar (LA) or in Luria broth (LB) with appropriate antibiotics (Table A). Plasmids were purified with the Qiagen Plasmid Mini Kit (Qiagen, Hilden, Germany) from overnight *E. coli* cultures incubated at 37°C, except for pMJH-46 and pORTMAGE-3 which were incubated at 30°C, with shaking. Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs, Ipswich, Massachusetts, USA, Catalog #M0494) was used for PCR amplification for cloning purposes.

Custom DNA oligonucleotides (Table B) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). PCR amplification for cloning purposes utilized 1 ng of purified plasmid DNA as template with 10 µl Q5 polymerase in 20 µl reactions containing 500 nM each of forward and reverse primers. Unless stated otherwise, the following PCR program was used: Initial denaturation at 98°C for 30 s, followed by 22 cycles of denaturation at 98°C for 5 s, primer annealing for 20 s and primer extension at 72°C for 60 s, followed by a final extension at 72°C for 2 minutes. Primer annealing temperatures were calculated with the NEB Tm Calculator tool (https://tmcalculator.neb.com) for each primerset. For shorter amplicons the primer extension step at 72°C was reduced to 30 s (*oriT* and R6K) or 10 s (T1, T2 and T3).

PCR for verification purposes was performed with HotStarTaq Master Mix (Qiagen) under standard cycling conditions according to the manufacturer's recommendations, using primer annealing at 60°C for 30 s and adjusting the extension times according to the expected product size.

PCR products were verified by gel electrophoresis in 1% agarose gels run with TBE buffer at 100V for 30-60 min depending on PCR product size.

Gel-purification was performed with QIAquick Gel Extraction Kit (Qiagen) from agarose gel bands excised with a sterile scalpel under UV light.

Chemically component *E. coli* cells were purchased or prepared according to Chan et al. (2013) using the $CaCl_2$ method with LB medium.

PCR amplified DNA was assembled with NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Catalog #E2621) and transformation of chemically component cells by heat-shock was performed as described by the NEBuilder manual.

pAR1

The pAR1 plasmid was generated as a conjugationally transferable plasmid with kanamycin resistance based on the low/medium copy number and broad host range plasmid origin pBBR1. Bidirectional terminators were included for isolation purposes. Blue chromoprotein amilCP was included as a visual indicator.

The bidirectional synthetic terminator (ST) BBa_B1006 (Huang 2007) was assembled by annealing oligonucleotides ST_f and ST_r generating dsDNA in a single primer extension step with 10 µl Q5 polymerase in a 20 µl reaction with 1000 nM of each primer. The reaction was subjected to 98°C denaturation for 30 s, followed by oligo annealing by stepwise reduction in temperature from 70°C to 60°C for 10 s per degree, and a single synthesis step at 72°C for 2 minutes. The product was then gel-purified from a 1.2% agarose gel to remove residual oligonucleotides. Purified ST was used as template for generating terminator 1 (T1) by amplification with primers T1_f and T1_r which introduces overlap sequence to the reverse primer region of the amilCP amplicon. Terminator 2 (T2) was synthesized by amplification of ST with primers T2_f and T2_r which introduces overlap sequence to the *KanR* promoter and *oriT*.

KanR and its native promoter was amplified from pJL1-eforRed with primers KanR_f and KanR_r.

oriT was amplified from pMJH46 with primers oriT_f and oriT_r.

amilCP and promoter was amplified from pGR-Blue with amilCP_f and amilCP_r primers, introducing overlapping sequence to the reverse primer region of the *KanR* amplicon.

pBBR1 was amplified from pORTMAGE-3 with pBBR1_f and pBBR1_r, introducing overlapping sequence to *oriT* and T1.

PCR products of *KanR*, *oriT*, amilCP, pBBR1, T1 and T2 were gel-purified and subsequently verified by agarose gel and quantified by comparison to DNA size/mass ladders (New England Biolabs) from gel electrophoresis images. The fragments were combined in equimolar amounts and assembled with NEBbuilder in a 60 min assembly reaction, following transformation of chemically component NEBturbo according to the manufacturer's recommendations. The pAR1 plasmid was recovered from *E.coli* isolates resistant to kanamycin and blue in color. All individual DNA fragments were verified by PCR. Functional tests of *oriT* and the broad-host range capabilities of pBBR1 were performed by conjugational transfer from *E. coli* MFD-pir to various gram-negative bacterial species (conjugation as described in the manuscript).

pAR2

The pAR2 plasmid was generated by modification of pAR1 by adding restriction sites and a R6K origin between *oriT* and pBBR1.

R6K was amplified from pUC18R6KT-mini-Tn7T-Km with primers R6K_f and R6K_r, generating overlapping sequence to *oriT*, with AciI and XbaI restriction sites, and pBBR1, with SacI and SpeI restriction sites.

pBBR1 was amplified from pORTMAGE-3 with pBBR1_2_f and pBBR1_2_r, resulting in a shorter variant of pBBR1 while introducing overlaps to T1, with Bam and SacI restriction sites, and to R6K.

The remaining backbone, from *oriT* to T1, was amplified from pAR1 with primers pAR2_BB_f and pAR2_BB_r.

All PCR-products were gel-purified and quantified as described for pAR1. Fragments were combined in equimolar amounts and assembled with NEBbuilder in a 20 min assembly reaction, transformation performed with chemically component NEBturbo, followed by PCR verification and functional tests as described for pAR1.

pAR3

The pAR3 plasmid was generated by modification of pAR2 by replacing R6K with a two-way terminator to fully isolate the region intended for inserts in the complementation experiments described in the manuscript.

pAR2 was linearized by restriction with XbaI (New England Biolabs, #R0145) followed by PCR amplification of the plasmid, except for the R6K region, with primers pAR3_BB_f and pAR3_BB_r.

T3 was amplified from ST with primers T3_f and T3_r.

The backbone and T3 amplicons were gel-purified, assembled, cloned and verified as described for pAR1.

Table A

Bacterial strain or plasmid	Description	Source or reference
***Escherichia coli* strains**		
MFD-pir	MG1655 RP4-2-Tc::[ΔMu1::*aac(3)IV-ΔaphA-Δnic*35-ΔMu2::*zeo*] Δ*dapA*::(*erm-pir*) Δ*recA*	Ferrières et al. 2010
NEB Turbo	F' *proA*$^{+}$*B*$^{+}$ *lacI*^{q} *ΔlacZM15 / fhuA2 Δ(lac-proAB) glnV galK16 galE15 R(zgb-210::Tn10)*Tet^{S} *endA1 thi-1 Δ(hsdS-mcrB)5*	New England Biolabs, Catalog#C2984H
Plasmids		
pGR-Blue	Source of amilCP 100 mg/l ampicillin	Addgene plasmid #68374 Deposited by Nathan Reyna Bradshaw et at., 2016

pJL1-eforRed	Source of *kanR* 50 mg/l kanamycin	Addgene plasmid #106320 Deposited by James Collins Huang et al., 2018
pMJH-46	Source of *oriT* 100 mg/l ampicillin & 30 mg/l chloramphenicol	Addgene plasmid #67272 Deposited by Mark Liles Hossain et al., 2015
pORTMAGE-3	Source of pBBR1 50 mg/l kanamycin	Addgene plasmid #72678 Deposited by Csaba Pál Nyerges et al., 2016
pUC18R6KT-mini-Tn7T-Km	Source of R6K 50 mg/l kanamycin	Addgene plasmid #64969 Deposited by Herbert Schweizer Choi et al 2005
pAR1	pBBR1, *oriT, KanR,* amilCP 50 mg/l kanamycin	This study
pAR2	pBBR1, R6K, *oriT, KanR,* amilCP 50 mg/l kanamycin	This study
pAR3	pBBR1, *oriT, KanR,* amilCP 50 mg/l kanamycin	This study

Plasmids with Addgene plasmid# were purchased from Addgene.
MFD-pir was acquired from Biological Resource Center of the Institut Pasteur (CRBIP).
Antibiotics were purchased from Merck (Darmstadt, Germany).
Media for growing *E. coli* MFD-pir was supplemented with 2, 6-diaminopimelic acid (Alfa Aesar, Ward Hill, Massachusetts, USA), and with erythromycin 30 mg/l while in pure culture and not carrying a plasmid.

Table B

Name	Sequence (5' to 3')	Template	Product
ST_f	CGCTCGTAAGAGGTCACTGACCTAACAAAAAAAAAACCCCGCCCCTGACAGGGCG	No template	ST
ST_r	CGATAACTGACTCTGCCACTCAAGACCAAAAAAAACCCCGCCCTGTCAGGGGCG		
T1_f	GCACGCAAACCTGTGGTCGCCTAACGCTCGTAAGAGGTCACTG	ST	T1
T1_r	CGATAACTGACTCTGCCAC		
KanR_f	CTCACGTTGTGTCTCAAAATCTCTG	pJL1-eforRed	*KanR*
KanR_r	CAATTCTGATTAGAAAAACTCATCGAGCATC		
oriT_f	GCTTGCCCTCATCTGTTACG	pMJH46	*oriT*
oriT_r	GTTCGTGTAGACTTTCCTTGGTG		
amilCP_f	GATGCTCGATGAGTTTTTCTAATCAGAATTGACATCGCATCTTTTTGTAC	pGR-Blue	*amilCP*
amilCP_r	TTAGGCGACCACAGGTTTGCGTGC		
pBBR1_f	GGTCTTGAGTGGCAGAGTCAGTTATCGAGGCGGTACAGCCGATAGTCTGG	pORTMAGE-3	pBBR1
pBBR1_r	GCCGGCGTAACAGATGAGGGCAAGCTGTATAAGAGACAGCTGGCCTGCC		
T2_f	GGGTACACCAAGGAAAGTCTACACGAACGCTCGTAAGAGGTCACTGACC	ST	T2
T2_r	CATCAGAGATTTTGAGACACAACGTGAGCGATAACTGACTCTGCCACTC		
pBBR1_2_r	GATTTAAATCCGGACTAGTGAGCTCTCATGCCAGCCCGTGGATATGTGGACG	pORTMAGE-3	pBBR1
pBBR1_2_f	GCATGCCTGCAGGATCCGAGCTCGAGGCGGTACAGCCGATAGTCTGGAAC		
R6K_f	GCTCTAGATTTAAATAACGTTGCTAGCTAACGCAGGAAAGAACATGGGCAGTTC	mini-Tn7T	R6K
R6K_r	GAGCTCACTAGTCCGGATTTAAATCTAAGGGCTTCTCAGTGCGTTACATCCC		
pAR2_BB_f	CTAGCAACGTTATTTAAATCTAGAGCTTGCCCTCATCTGTTACGCCG	pAR1	pAR2 backbone
pAR2_BB_r	GAGCTCGGATCCTGCAGGCATGCTGTACCGCCTCGATAACTGAC		
pAR3_BB_f	CTAGTGAGCTCTCATGCCAGCCC	pAR2	pAR3 backbone
pAR3_BB_r	AGCCACTTGACTAGTCATTATCTAGAGCTTGCCCTCATCTGTTACG		
T3_f	TAATGACTAGTCAAGTGGCTCCTCGCTCGTAAGAGGTCAC	ST	T3
T3_r	GCTGGCATGAGAGCTCACTAGCGATAACTGACTCTGCCAC		

References

Bradshaw, J. C., Gongola, A. B., & Reyna, N. S. (2016). Rapid Verification of Terminators Using the pGR-Blue Plasmid and Golden Gate Assembly. *Journal of visualized experiments : JoVE,* (110), 54064. https://doi.org/10.3791/54064

Chan, W. T., Verma, C. S., Lane, D. P., & Gan, S. K. (2013). A comparison and optimization of methods and factors affecting the transformation of Escherichia coli. *Bioscience reports*, 33(6), e00086. https://doi.org/10.1042/BSR20130098

Choi, K. H., Gaynor, J. B., White, K. G., Lopez, C., Bosio, C. M., Karkhoff-Schweizer, R. R., & Schweizer, H. P. (2005). A Tn7-based broad-range bacterial cloning and expression system. *Nature methods,* 2(6), 443–448. https://doi.org/10.1038/nmeth765

Ferrières, L., Hémery, G., Nham, T., Guérout, A. M., Mazel, D., Beloin, C., & Ghigo, J. M. (2010). Silent mischief: bacteriophage Mu insertions contaminate products of Escherichia coli random mutagenesis performed using suicidal transposon delivery plasmids mobilized by broad-host-range RP4 conjugative machinery. *Journal of bacteriology*, 192(24), 6418–6427. https://doi.org/10.1128/JB.00621-10

Hossain, M. J., Thurlow, C. M., Sun, D., Nasrin, S., & Liles, M. R. (2015). Genome modifications and cloning using a conjugally transferable recombineering system. *Biotechnology reports (Amsterdam, Netherlands)*, 8, 24–35. https://doi.org/10.1016/j.btre.2015.08.005

Huang, H. (2007). Design and characterization of artificial transcriptional terminators. [Master of Engineering Thesis, Massachusetts Institute of Technology] Retrieved from http://hdl.handle.net/1721.1/45981

Huang, A., Nguyen, P. Q., Stark, J. C., Takahashi, M. K., Donghia, N., Ferrante, T., Dy, A. J., Hsu, K. J., Dubner, R. S., Pardee, K., Jewett, M. C., & Collins, J. J. (2018). BioBits™ Explorer: A modular synthetic biology education kit. *Science advances*, 4(8), eaat5105. https://doi.org/10.1126/sciadv.aat5105

Nyerges, Á., Csörgő, B., Nagy, I., Bálint, B., Bihari, P., Lázár, V., Apjok, G., Umenhoffer, K., Bogos, B., Pósfai, G., & Pál, C. (2016). A highly precise and portable genome engineering method allows comparison of mutational effects across bacterial species. *Proceedings of the National Academy of Sciences of the United States of America*, 113(9), 2502–2507. https://doi.org/10.1073/pnas.1520040113

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RESEARCH ARTICLE

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qPCR screening for *Yersinia ruckeri* clonal complex 1 against a background of putatively avirulent strains in Norwegian aquaculture

Andreas Riborg[1,2] | Snorre Gulla[1] | David Strand[1] | Jannicke Wiik-Nielsen[1] | Anita Rønneseth[3] | Timothy J. Welch[4] | Bjørn Spilsberg[1] | Duncan J. Colquhoun[1,3]

[1]Norwegian Veterinary Institute, Ås, Norway

[2]Vaxxinova Norway AS, Bergen, Norway

[3]University of Bergen, Bergen, Norway

[4]National Centre for Cool and Coldwater Aquaculture, Leetown, West Virginia, USA

Correspondence
Duncan J. Colquhoun, Norwegian Veterinary Institute, Ås, Norway.
Email: duncan.colquhoun@vetinst.no

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Abstract

Although a number of genetically diverse *Yersinia ruckeri* strains are present in Norwegian aquaculture environments, most if not all outbreaks of yersiniosis in Atlantic salmon in Norway are associated with a single specific genetic lineage of serotype O1, termed clonal complex 1. To investigate the presence and spread of virulent and putatively avirulent strains in Norwegian salmon farms, PCR assays specific for *Y. ruckeri* (species level) and *Y. ruckeri* clonal complex 1 were developed. Following extensive screening of water and biofilm, the widespread prevalence of putatively avirulent *Y. ruckeri* strains was confirmed in freshwater salmon hatcheries, while *Y. ruckeri* clonal complex 1 was found in fewer farms. The formalin-killed bacterin yersiniosis vaccine was detected in environmental samples by both PCR assays for several weeks post-vaccination. It is thus important to interpret results from recently vaccinated fish with great care. Moreover, field studies and laboratory trials confirmed that stressful management procedures may result in increased shedding of *Y. ruckeri* by sub-clinically infected fish. Analysis of sea water sampled throughout thermal delousing procedures proved effective for detection of *Y. ruckeri* in sub-clinically infected populations.

KEYWORDS
aquaculture, Atlantic salmon, delousing, qPCR, *Yersinia ruckeri*

1 | INTRODUCTION

The gram-negative bacterium *Yersinia ruckeri* is the causative agent of yersiniosis, also known as enteric redmouth disease, a haemorrhagic septicaemia prevalent in farmed salmonids throughout the world (Bastardo et al., 2015; Davies & Frerichs, 1989; Ross et al., 1966). While yersiniosis is most commonly associated with farmed rainbow trout internationally, in Norway, the disease is restricted to farmed Atlantic salmon, *Salmo salar*, with occasional outbreaks in farmed Arctic char, *Salvelinus alpinus*. Although currently under relatively good control due to recent widespread adoption of intraperitoneal (ip) vaccination, the incidence of yersiniosis in Norwegian freshwater farms increased in the period 2006–2010, and in the sea-phase of culture c. 2013–2017 (Gulla & Olsen, 2020).

A number of different *Y. ruckeri* serotypes have been recognized (Davies, 1990; Romalde et al., 1993), but most disease-associated

variants belong to serotype O1 (McCarthy & Johnson, 1982; Davies, 1990; Barnes et al., 2016; Gulla, Barnes, et al., 2018). Recent studies have further revealed that this serotype is shared across a number of discrete genetic lineages, with some displaying a strong affinity towards specific host species (Barnes et al., 2016; Gulla, Barnes, et al., 2018). In Norwegian salmon farming, one such lineage, termed clonal complex 1 (CC1), was found responsible for all major yersiniosis outbreaks diagnosed since the late 1980s, while the serotype O2 lineage CC3 had caused sporadic and often less severe disease (Gulla, Barnes, et al., 2018). This study also identified a number of genetically diverse, putatively avirulent genotypes from freshwater environments and ovarian fluid of clinically healthy brood-fish. This verifies the presence in Norwegian salmon-farming environments of both established virulent and putatively avirulent *Y. ruckeri* strains, although their relative prevalence, within and across farms, remains uncharted.

Yersinia ruckeri is capable of establishing latent sub-clinical infections in salmonid fish (Ross et al., 1966). The infection may then be maintained within the population by intermittent shedding of the bacterium by asymptomatic carriers (Busch & Lingg, 1975; Hunter, Knittel & Fryer 1980). While the cause/s of the recurring outbreaks experienced during the freshwater phase of culture and the recent increase in the incidence of yersiniosis in large sea-farmed salmon in Norway remain unclear, these outbreaks could conceivably be associated with activation of such latent infections. Outbreaks in salmon at sea have been associated with transfer from freshwater or other stressful handling operations (Carson & Wilson, 2009; Gulla et al., 2019; Sparboe et al., 1986). In recent years, the introduction of non-medicinal delousing, where large numbers of fish are treated in a relatively small volume of water in closed systems (Roth, 2016), represents an additional stressful handling event for large fish at sea (Overton et al., 2019). Stress-induced outbreaks of disease, including yersiniosis, may follow such treatment (Gismervik et al., 2019; Gulla et al., 2019).

While subclinical infections are generally difficult to detect at low prevalence, screening for aquatic infectious agents utilizing a non-invasive, environmental DNA (eDNA)-based polymerase chain reaction (PCR) approach offers the possibility of screening the population as a whole (Bernhardt et al., 2021; Rusch et al., 2018; Shea et al., 2020; Strand et al., 2019). This methodology should be suitable to monitor levels of *Y. ruckeri* shedding from carrier fish. Several PCR assays have been previously developed for the detection of *Y. ruckeri* in both fish and environmental samples (Gibello et al., 1999; LeJeune & Rurangirwa, 2000; Temprano et al., 2001; Del Cerro et al., 2002; Bastardo et al., 2012; Keeling et al., 2012; Ghosh et al., 2018, Lewin et al., 2020). In recent years, PCR-based screening for *Y. ruckeri* has been commonly used as a biosecurity tool in Norwegian freshwater salmon farms, but reports from the industry of false-positive PCR test results have complicated their interpretation. Additionally, as both virulent and avirulent strains may be detected by *Y. ruckeri* PCRs specific at the species level, there is a need for assays, targeting relevant pathogenic strains, which in the current Norwegian context is *Y. ruckeri* CC1. We therefore sought to develop novel PCRs, specific to the *Y. ruckeri* species and *Y. ruckeri* CC1, respectively. These assays were utilized in tandem to assess eDNA-based PCR-screening for the general presence of *Y. ruckeri* and the virulent *Y. ruckeri* CC1 in Norwegian salmon aquaculture and to monitor shedding from sub-clinical and active infections.

2 | MATERIALS AND METHODS

2.1 | Strains and culture

Bacterial strains used for specificity testing (Table 2; Table 3) were cultured on suitable agar media and incubated at appropriate temperatures and durations. Species verification was performed with MALDI-TOF (Biotyper Microflex LT; Bruker Daltonics). Isolates of uncertain taxonomic status were classified and confirmed as non-*Y. ruckeri* by whole-genome-based analyses (Figure S1) as described previously (Riborg et al., 2022).For spiking experiments, *Y. ruckeri* CC1 strain NVI-10705 was cultured in Tryptic Soy Broth at 22°C with shaking until mid-log phase, from which a dilution series was prepared with sterile phosphate-buffered saline (PBS) chilled on ice and enumerated by plating on 5% bovine blood agar (BA) in triplicate. For the challenge trials, *Y. ruckeri* NVI-10705 was grown in Brain Heart Infusion Broth at 15°C with shaking for 20 hr, harvested by centrifugation and re-suspension in PBS, followed by enumeration on a cell counter (Casy Inovatis; Roche Diagnostics) and by plating of a 10-fold dilution series on BA. All BA plates were incubated at 22°C for 2 days prior to counting.

2.2 | DNA extraction

DNA templates for specificity testing were prepared from cultured colonies using the QIAamp DNA kit (Qiagen) according to the manufacturer's recommendations for Gram-positive or Gram-negative bacteria as appropriate, and assessed for purity and quantity with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

DNA for standard curves and determination of limits of detection were extracted from NVI-10705 with Gentra puregene (Qiagen) as recommended by the manufacturer for Gram-negative bacteria. Fluorometric quantification of DNA was done with a Qubit dsDNA HS Assay Kit on a Qubit 4 Fluorometer (Thermo Fisher Scientific).

Samples consisting of salmon tissue, biofilm swabs (eSwab with 1 ml Liquid Amies transport medium; Copan Diagnostics) and water filters (47 mm nitrocellulose 0.45 μm pore-size filters; Whatman; Cytiva) were processed by individual protocols prior to lysis and purification (using a common protocol) with the DNeasy Blood & Tissue kit (Qiagen). As such, environmental swabs were processed by vortexing for 5 s followed by transfer of 1 ml transport medium to a 1.5 ml Eppendorf tube. The sample material was then pelleted by centrifugation at 8000*g* for 10 min, and the supernatant was discarded. Tissue samples were transferred to 1.5 ml Eppendorf tubes and macerated with a sterile scalpel. Filters were gently folded and

transferred to 1.5 ml Eppendorf tubes. Macerated tissue and swab pellets were subsequently suspended in a lysis buffer consisting of 180 µl buffer ATL and 20 µl proteinase-K. Twice the volume of lysis buffer (400 µl) was added to tubes with filters to keep them submerged during lysis. Tissue, swab and filter samples were then lysed overnight at 56°C with agitation on a Thermomixer (Eppendorf AG). Post-lysis, filters were carefully removed with sterile tweezers while compressing the filter with a pipette to recover all of the lysate, prior to the addition of twice the volume of buffer AL and ethanol as described in the DNeasy manual. The resulting 1200 µl mixture was loaded onto a single spin column in two separate aliquots of 600 µl. All lysates were otherwise further processed according to the manufacturers description for purification of total DNA from animal tissues with the DNeasy Blood & Tissue kit. Extracted DNA was eluated in 100 µl buffer AE.

2.3 | PCR development

The CC1-specific locus was identified by alignment of genomes in Mauve (development version 20,150,226) (Darling et al., 2010), comparing *Y. ruckeri* CC1 and non-CC1 MLVA genotypes identified previously (Riborg et al., 2022), followed by BLAST searches (Altschul et al., 1997) on local and public databases with candidate sequences to identify CC1-specific targets suitable for PCR analysis. An intergenic region between the class C beta-lactamase (acc. no. WP_004721718) and a predicted AAA family ATPase (acc. no. WP_096823432) was identified as specific for, and ubiquitous within, *Y. ruckeri* CC1, and formed the basis for the CC1-specific PCR assay. By the same approach, a LuxR family transcriptional regulator (acc. no. WP_038241605) was found to be conserved across all of the investigated *Y. ruckeri* genomes and used as target for the *Y. ruckeri* species-specific assay. Primer and probe sequences (Table 1) were determined with Primer Express Software v3.0.1 (Applied Biosystems) and purchased from Thermo Fisher Scientific.

PCR reactions comprised 10 µl TaqMan Fast Advanced Master Mix (Applied Biosystems), primers and probes at 600 nM and 200 nM, respectively, and 5 µl of template DNA in 20 µl total reaction volume. The reactions were cycled in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories) with the following PCR program: UNG incubation at 50°C for 2 min, polymerase activation at 95°C for 20 s, followed by 50 cycles of 95°C for 3 s and 62°C for 20 s. Samples yielding exponential amplification curves with cycle threshold values of 42 or less were considered positive.

TABLE 1 Primer and probe sequences used in qPCR assays

Assay specificity	Name	Sequence (5′–3′)
Y. ruckeri	YrF	CTAATGTGCAGAGCGCAGATG
	YrR	GCGGACTGAATAACGATGATTG
	YrP	FAM-CCTGTACCGTCGTCAGG-MGB
Y. ruckeri CC1	YrCC1F	GAATTAGGCGCAACTCAATTTGAC
	YrCC1R	GCTGGTAAGGGATGTTATGTTTCA
	YrCC1P	VIC-TATGACGACTGAGTGTTTAC-MGB

Note: Minor groove binding (MGB) probes were labelled with FAM (6-carboxyfluorescein) or VIC (proprietary, Life Technologies).
Abbreviations: F, forward; P, Probe; R, Reverse.

2.4 | PCR validation

A dilution series of *Y. ruckeri* CC1 DNA was analysed in triplicate to investigate the amplification efficiency and linearity of both assays, and serve as standard curve for quantification. Limits of detection (LOD) were established by analysing 20 parallels of DNA samples in relevant dilutions. LOD was defined as the lowest amount producing at least 95% (≥19/20) positive results. The specificity of both PCR assays was tested using 1 ng purified DNA from a diverse panel of bacterial reference strains, clinical isolates primarily from diseased fish and isolates from aquaculture environments (Table 2). Specificity was further investigated by assaying a broad panel of *Y. ruckeri* isolated from various geographical and biological origins, serotypes and biotypes, including representatives from all known MLVA clonal complexes and some singletons as described by Gulla, Barnes, et al. (2018) (Table 3). The assays were also challenged with 100 ng DNA extracted from heart tissue of healthy Atlantic salmon and from *Y. ruckeri*-free biofilm (collected from an in-house research aquarium). Possible interference from a background matrix of non-target DNA was investigated by adding purified *Y. ruckeri* CC1 DNA equivalent to LOD directly to PCR reactions together with the salmon heart tissue or biofilm DNA templates. The ability to detect a low number of *Y. ruckeri* cells in the various sample types was investigated by spiking triplicates of 200 ml sterile PBS, 25 mg macerated salmon heart tissue and 25 mg (wet-weight) biofilm, with 100 µl of a *Y. ruckeri* CC1 2-fold dilution series (starting at 9.6×10^3 CFU ml^{-1}).

2.5 | Screening of freshwater hatcheries

Screening of freshwater hatcheries consisted of two distinct sample sets. Biofilm samples for both sample sets were collected by swabbing effluent pipes and the inner walls of tanks at the air/water interface. Samples were transported chilled, overnight to the laboratory, and stored at 4°C for up to 48 hr pending further processing.

2.5.1 | Sample-set one

Twenty-four Norwegian salmon hatcheries that had not experienced yersiniosis problems in recent years were sampled in 2017 as part of a previous study (Gulla, Wiik-Nielsen, & Colquhoun, 2018). Samples from hatcheries which had been identified in 2017 as PCR positive for *Y. ruckeri* (data not shown) were then re-analysed in the current work with the two novel PCR assays. This sample set also included salmon tissues (kidney and intestine), sampled on-site, suspended in

TABLE 2 Bacterial strains of various taxa used for specificity tests with the *Y. ruckeri* (Yr) and *Y. ruckeri* CC1 (YrCC1) qPCR assays

Bacterial species	Strain designation	Biological source	PCR Yr	PCR YrCC1
Aeromonas hydrophila	ATCC 14715	Silver salmon	–	–
Aeromonas salmonicida ssp. *salmonicida*	ATCC 14174	Brook trout	–	–
Arthrobacter globiformis	NCIMB 8907^T	Soil	–	–
Bacillus cereus	NVI-3588	Unknown	–	–
Brochothrix thermosphacta	NCFB 1676^T	Pork sausage	–	–
Carnobacterium piscicola	NCFB 2762^T	Cutthroat trout	–	–
Edwardsiella piscicida	NCIMB 14824^T	European eel	–	–
Escherichia coli	ATCC 25922	Clinical isolate	–	–
Francisella noatunensis ssp. *noatunensis*	NCIMB 14265^T	Atlantic cod	–	–
Moritella viscosa	NCIMB 13584^T	Atlantic salmon	–	–
Nocardia asterioides	NVI-6532	Unknown	–	–
Pasteurella sp.	NVI-9100	Lumpsucker	–	–
Pasturella skyensis	NCIMB 13593^T	Atlantic salmon	–	–
Photobacterium phosphoreum	NCIMB 1282^T	Unknown	–	–
Piscirickettsia salmonis	NVI-5692	Atlantic salmon	–	–
Proteus mirabilis	NCIMB 10823	Human, urine	–	–
Pseudomonas fluorescens	NCIMB 10067	Unknown	–	–
Renibacterium salmoninarum	ATCC 33209	Chinook salmon	–	–
Rhanella inusitata	NVI-11515	Biofilm, aquaculture	–	–
Rhanella sp.	NVI-11513	Biofilm, aquaculture	–	–
Undescribed *Yersiniaceae*	NVI-11511	Biofilm, aquaculture	–	–
Undescribed *Yersiniaceae*	NVI-11512	Biofilm, aquaculture	–	–
Undescribed *Yersiniaceae*	NVI-11514	Biofilm, aquaculture	–	–
Rhodococcus equi	NVI-6122	Unknown	–	–
Rhodococcus erythropolis	NCIMB 11148^T	Soil	–	–
Serratia marcescens	NCIMB 10351	Sheep	–	–
Serratia sp.	NVI-11516	Biofilm, aquaculture	–	–
Staphylococcus aureus	NCIMB 11787	Human, septic arthritis	–	–
Tenacibaculum finnmarkense	NVI-5134	Atlantic salmon	–	–
Vibrio salmonicida	NCMB 2262^T	Atlantic salmon	–	–
Vibrio splendidus	NVI-7628	Ballan wrasse	–	–
Vibrio tapetis	NVI-7627	Ballan wrasse	–	–
Yersinia entomophaga	NVI-2267	Aquaculture	–	–
Yersinia fredriksenii	NVI-1098	Unknown	–	–
Yersinia intermedia	NVI-11508	Biofilm, aquaculture	–	–
Yersinia intermedia	CCUG 26592	Domestic pig	–	–
Yersinia kristensenii	CCUG 26588	Domestic pig	–	–
Yersinia kristensenii	NVI-11509	Biofilm, aquaculture	–	–
Yersinia kristensenii	NVI-11510	Biofilm, aquaculture	–	–
Yersinia sp.	NVI-11061	Biofilm, aquaculture	–	–
Yersinia ruckeri (CC2)	ATCC 29473 ^{T}	Rainbow trout	+	–
Yersinia ruckeri (CC1)	NVI-10705	Atlantic salmon	+	+

TABLE 3 *Yersinia ruckeri* strains of various MLVA genotypes used for specificity tests with the *Y. ruckeri* (Yr) and *Y. ruckeri* CC1 (YrCC1) qPCR assays

Strain designation	Biological source	Country	Year	Serotype	Biotype	MLVA-CC[a]	PCR Yr	PCR YrCC1
NVI-1292	*Salmo salar*	Norway	1987	O1	1	1	+	+
NVI-8074	*Salmo salar*	Norway	2011	O1	1	1	+	+
NVI-9698	*Salmo salar*	Norway	2014	O1	1	1	+	+
NVI-9812	*Salvelinus alpinus*	Norway	2014	O1	1	1	+	+
NVI-10428	*Salmo salar*	Norway	2016	O1	1	1	+	+
NVI-10542	*Salmo salar*	Norway	2016	O1	1	1	+	+
NVI-10989	*Salmo salar*	Norway	2017	O1	1	1	+	+
NVI-10990	*Salmo salar*	Norway	2017	O1	2	1	+	+
DVJ-93010	*Oncorhynchus mykiss*	UK	1993	O1	1	2a	+	–
DVJ-93046	*Oncorhynchus mykiss*	UK	1993	O1	2	2a	+	–
CSF007-82	*Oncorhynchus mykiss*	USA	1982	O1	1	2b	+	–
TW-11.68	*Carassius auratus*	USA	1983	O1	1	2b	+	–
TW-F190	*Oncorhynchus mykiss*	USA	1995	O1	2	2b	+	–
NVI-1382	*Oncorhynchus mykiss*	Italy	1984	O1	1	2c	+	–
TW-F183	*Oncorhynchus mykiss*	USA	1995	O1	1	2c	+	–
NVI-9925	*Oncorhynchus mykiss*	Finland	2010	O1	2	2c	+	–
NVI-1347	*Salmo salar*	Norway	1988	O2	1	3	+	–
NVI-9681	*Salmo salar*	Norway	2014	O2	1	3	+	–
RD502	*Salmo salar*	UK	2010	O8/O1[b]	1	4	+	–
TW-11.43	*Oncorhynchus mykiss*	Australia	1959	O1	1	5	+	–
RD336	*Salmo salar*	UK	2001	O2	1	6	+	–
NVI-11077	Biofilm, aquaculture	Norway	2017	O1	1	7	+	–
NVI-11055	*Salmo salar*, egg-fluid	Norway	2017	O1	1	8	+	–
NVI-11054	*Salmo salar*, egg-fluid	Norway	2017	O1	1	9	+	–
RD154	*Salmo salar*	Norway	1985	O1	2	10	+	–
NVI-492	*Salmo salar*	Norway	1987	O1	1	10	+	–
NVI-11065	*Salmo salar*, egg-fluid	Norway	2017	O1	1	s	+	–
NVI-11073	Biofilm, aquaculture	Norway	2017	O1	1	s	+	–
TW-11.30	*Morone americana*	USA	1977	O1	N.D.	s	+	–
TW-11.57	*Salmo trutta*	USA	1980	O2	N.D.	s	+	–
NVI-1398	*Oncorhynchus mykiss*	Sweden	1986	O7	N.D.	s	+	–
YR122A	*Salmo salar*	Finland	1988	O6	N.D.	s	+	–
NVI-1389	*Salmo salar*	Norway	1989	O1	1	s	+	–
RD356	*Salmo salar*	UK	2005	O5/O1[b]	1	s	+	–
NVI-5635	*Salmo salar*	Norway	2006	N.R.	1	s	+	–
NVI-11065	*Salmo salar*, egg-fluid	Norway	2017	O1	1	s	+	–
NVI-11073	Biofilm, aquaculture	Norway	2017	O1	1	s	+	–
NCTC 12268	*Oncorhynchus mykiss*	Canada	1985	O5	1	s	+	–
NCTC 12269	*Oncorhynchus mykiss*	Canada	<1990	O6	1	s	+	–
NCTC 12270	*Anguilla anguilla*	Denmark	<1990	O7	1	s	+	–

Abbreviations: N.D., Not done; N.R., No reaction with any available *Y. ruckeri* antisera.

[a]MLVA clonal complex according to Gulla et al. (2018), with 's' indicating singleton or undefined clonal complex.

[b]Serotyping ambiguity dependent on strain-origin of antisera.

RNAlater (Qiagen) and kept chilled (during transport) for 24 h before being frozen at −20°C pending further processing.

2.5.2 | Sample-set two

The second sample set encompassed environmental samples alone, sampled as previously described from 16 Atlantic salmon hatcheries in Norway, collected between October 2019 and February 2020. These were analysed using the two novel quantitative PCR (qPCR) assays described in the present study. A single site was screened prior to vaccination, and then weekly for three consecutive weeks to investigate the possibility for detecting vaccine residues in the environment.

2.6 | Screening during active marine outbreak

To assess environmental *Y. ruckeri* levels during an active outbreak at sea, water samples were collected at a marine ongrowing site in Norway during a yersiniosis outbreak in large (4–5 kg), unvaccinated Atlantic salmon. Surface water samples (4 L) were collected in disposable plastic containers and filtered on-site through 0.45 μm pore-size nitrocellulose filters (Whatman) using a portable peristaltic pump (Masterflex portable sampler; Cole-Parmer) with a 47 mm inline filter-holder (Millipore). All equipment was thoroughly rinsed with ambient water between samplings. Filters were transported chilled to the laboratory for storage at −20°C pending further processing. Tissue samples (head kidney) from fish in the affected and neighbouring cages were collected with sterile scalpel and tweezers, suspended in RNAlater and transported chilled to the laboratory.

2.7 | Screening during thermal delousing

To investigate the presence of *Y. ruckeri* during delousing operations, water samples were collected from three different marine salmon farms in Norway during thermal delousing. The studied fish at site A were unvaccinated, clinically healthy but were exposed to *Y. ruckeri* at sea via an infected neighbouring cage. Fish treated at site B were vaccinated against yersiniosis (unknown method) but had experienced an outbreak of yersiniosis during the freshwater phase. Yersiniosis history and vaccination status for fish treated at site C are unknown. Clinical yersiniosis was not apparent in any of the populations at the time of sampling. Thermal delousing involves crowding of the fish which are then pumped into a treatment barge where they are exposed to heated sea water (28–34°C dependent on ambient sea water temperatures) for approximately 30 s, prior to being pumped back into the sea-cage (Roth, 2016).

Approximately 60,000–120,000 fish were treated in each of the treatments sampled. Water samples (0.5 L) were collected in disposable plastic containers from the sea cage prior to treatment, and from the treatment chamber prior to, during (twice) and post-treatment. Water samples were shipped to the lab chilled overnight and immediately filtered through an analytical test filter funnel (Nalgene Analytical Test Filter Funnel; Thermo Fisher Scientific) with 0.45 μm pore-size nitrocellulose filters (Whatman) using a peristaltic pump (Masterflex; Cole-Parmer). High water turbidity necessitated the use of multiple filters for some samples, in which case DNA was extracted from individual filters and independently analysed by qPCR with average values presented as results.

2.8 | Simulated thermal delousing in latently infected salmon

A trial was designed to emulate a field situation involving fish subclinically infected with *Y. ruckeri* from the freshwater phase being subjected to a stressful handling event in sea water, while monitoring shedding of *Y. ruckeri* by weekly sampling of eDNA. The trial made use of a cohabitant infection model where ip injected shedder fish were used to infect naïve cohabitants, as described below.

The challenge trials were conducted at the Industrial and Aquatic Laboratory (ILAB) with ~35 g Atlantic salmon of mixed sex. The fish were fed daily with a commercial diet (Nutra Olympic; Skretting AS). A water temperature of 14°C was maintained throughout the trial with water flow adjusted to maintain adequate dissolved oxygen levels. When necessary, fish were killed by tricaine methanesulfonate (Finquel MS-222) overdose. Shedders ($n = 60$) anaesthetized with MS-222 were marked subcutaneously using Visual Implant Elastomers (Northwest Marine Technology) and ip injected with 100 μl PBS suspension containing 3×10^6 CFU *Y. ruckeri* CC1 using a 0.5 mm gauge needle. Negative control shedders ($n = 60$) were injected with 100 μl sterile PBS. Naïve cohabitants ($n = 480$) were randomly distributed between eight 150 L tanks with freshwater, each tank receiving 60 fish. Fifteen infected shedders were then added to four of the tanks, while 15 negative control shedders were added to the four remaining tanks.

Following an initial period of mortality in shedders and cohabitants in the tanks holding infected shedders, mortality ceased and the fish were maintained as previously. After 7 weeks, the photoperiod was changed from 12 to 24 h light to induce smoltification, followed by a change from freshwater to natural sea water (32 ± 2 ppt) at week 10. During week 13, the number of fish in all tanks was adjusted to 42, with excess fish being killed and sampled (head kidney) for bacteriology (streak on BA) and qPCR to assess subclinical *Y. ruckeri* carrier status. One week later, all the fish were fasted for 24 h before two tanks containing presumptive sub-clinically infected fish and two control tanks were subjected to simulated thermal delousing. For each of these tanks, groups of three fish were sequentially held in a fine-meshed net and submerged for 30 s in a 100 L tank containing heated sea water (33.8°C). Additional oxygenation held O_2 values at or above saturation level during treatment. Treated fish were then immediately transferred to a recovery tank (50 L) containing 14°C oxygenated sea water. Following treatment of each tank, fish were returned to the 150 L holding tanks and water samples (1 L) were collected from the treatment and

recovery tanks for qPCR analysis. Fish from the remaining four tanks (two infected and two control tanks) not subjected to hot water were instead subjected to stress by handling and confinement by first reducing the water volume to 50 L and then further confining the fish by netting all of the fish for 1 min, repeated three times. After experimental stress (thermal or confinement), all fish were monitored until termination of the trial and were killed after a total of 20 weeks before being sampled for bacteriology and qPCR.

3 | RESULTS

3.1 | qPCR performance

Both PCR assays demonstrated linear performance over a 6 log range (Figure 1) with PCR efficiency of 99% and LODs equal to five *Y. ruckeri* genome equivalents. No amplification signals were observed on analysis of pure cultures of non-target bacterial species with either assay (Table 2). The *Y. ruckeri* species-specific assay was positive for all *Y. ruckeri* isolates tested, while amplification with the CC1 assay was observed exclusively for isolates confirmed as CC1 by MLVA genotyping (Table 3). No amplification was observed with DNA extracted from healthy salmon or presumed *Yersinia*-free biofilm, and no inhibition was observed when these templates were combined with *Y. ruckeri* CC1 genomic DNA. Spiking experiments verified the ability of both assays to detect low numbers of *Y. ruckeri* cells with 240 CFU per spiked sample detected in all triplicates of all sample types (water, salmon tissue and biofilm).

3.2 | Screening of freshwater hatcheries

Sample-set one consisted of DNA extracts from 11 freshwater hatcheries positive for *Y. ruckeri* in a screening study concluded in 2017 (Gulla, Wiik-Nielsen, & Colquhoun, 2018). Of 48 individual *Y. ruckeri* positive samples from these sites, CC1 was detected in only a single environmental sample from a single site, and in two kidney samples from dead fish at another site (Figure 2). Remaining kidney tissue samples from live fish were found negative by both assays. Positive environmental samples were thus heavily dominated by non-CC1 *Y. ruckeri*.

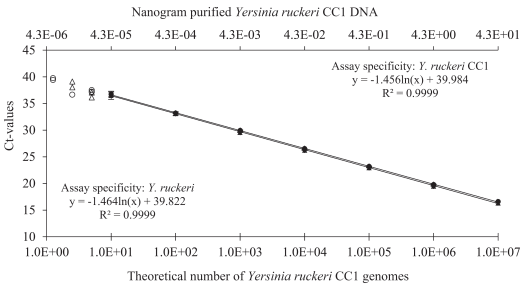

FIGURE 1 Standard curves based on qPCR analysis of serial dilutions of *Yersinia ruckeri* CC1 strain NVI-10705 purified DNA in triplicate. Upper x-axis show ng DNA, lower x-axis show estimated number of *Y. ruckeri* CC1 genomes. The assay specific to *Y. ruckeri* is indicated by triangles, the assay specific *Y. ruckeri* CC1 by circles. Triplicates with negative samples, represented by empty shapes without means, were excluded from standard curve calculations. qPCR, quantitative polymerase chain reaction

Sample-set two consisted of environmental swab samples collected from 16 Atlantic salmon hatcheries between October 2019 and February 2020. Due to a growing suspicion of detection of *Y. ruckeri* CC1 DNA from vaccine remnants in this sample set, the results were considered in relation to time since vaccination (Figure 3). While CC1 was detected at some sites that were not recently vaccinated (sites 5, 6 and 7), all produced high Ct-values (39–40) approaching the LOD. For recently vaccinated sites, the results for both assays correlated well, close to LOD. An exception was observed for site 15, which had a number of samples positive for *Y. ruckeri* with low Ct-values while being negative for CC1, indicating the presence of non-CC1 strains at this site. At sites 11 and 12, both of which were only partially vaccinated, *Y. ruckeri* CC1 was detected exclusively in samples from the recently vaccinated production units (Figure 4). Site 1 was screened prior to vaccination, and then weekly for three consecutive weeks using environmental swab samples. All samples were negative prior to vaccination, while all samples were positive 1 week post-vaccination. Most samples were still positive 2–3 weeks post-vaccination, albeit with increasingly high Ct-values (Figure 5).

3.3 | Screening during active marine outbreak

Yersinia ruckeri CC1 was detected in sea water (4 L) sampled adjacent to the affected sea cage and up to 100 m downstream from an active yersiniosis outbreak at a marine ongrowing site (Figure 6). Head kidney samples from salmon in the affected cage were all positive for *Y. ruckeri* CC1, while samples from neighbouring cages with ip vaccinated fish were negative with both assays (not shown).

3.4 | Screening during thermal delousing

Yersinia ruckeri and *Y. ruckeri* CC1 were detected in samples collected during thermal delousing treatment at two marine Atlantic salmon ongrowing sites (A and B), while at the third site (C) *Y. ruckeri* and *Y. ruckeri* CC1 were detected in the treatment chamber prior to, but not during treatment (Figure 7).

3.5 | Simulated thermal delousing

In the challenge trial designed to emulate a field situation involving stressful handling, mortality in ip infected shedder fish reached 100% in 10 days, while most of the cohabitant mortality occurred in weeks 2–4, eventually plateauing at 20–28% cumulative mortality

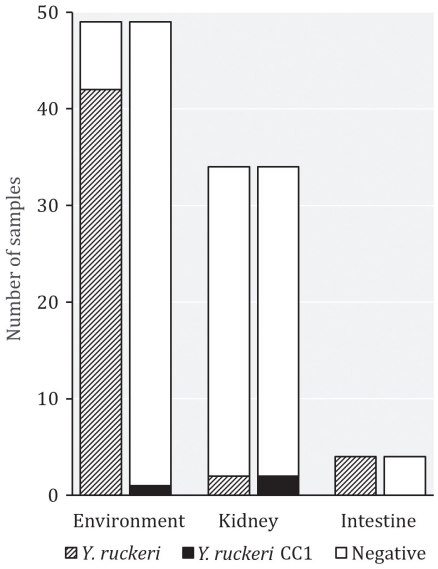

FIGURE 2 Stacked bar chart showing qualitative qPCR results from using the two novel assays on sample-set one. The sample material consisted of three different sample types, that is, environmental swabs, kidney and intestinal tissue, collected from 11 Atlantic salmon hatcheries. Samples were collected in 2017, prior to widespread use of ip vaccination against yersiniosis in Norway, from sites that had not experienced problems with the disease in recent years. qPCR, quantitative polymerase chain reaction

(Figure 8). *Y. ruckeri* CC1 was detected by qPCR in effluent water from all infected tanks during weeks 2–4, while mortality in cohabitants was ongoing. During the following weeks, *Y. ruckeri* CC1 was detected only sporadically from tank water, with Ct-values close to LOD and thus not readily visible in Figure 8. Change in photoperiod regimen or salinity did not seem to induce significant shedding. Thermal stress, however, resulted in significant shedding in both treatment and recovery tanks, with concentrations of *Y. ruckeri* CC1, as estimated by qPCR, similar to those observed during the active outbreak phase in the early weeks of the experiment (Figure 8). Unfortunately, water was not sampled for qPCR during the crowding treatment. *Y. ruckeri* was not detected in effluent water samples collected the week following stress exposures. Neither of the experimental stress events resulted in mortality during treatment, nor over the following weeks. From a total of 24 head kidney samples assayed for *Y. ruckeri* CC1 by qPCR in week 13, three were positive (12.5%), and at termination of the trial after 20 weeks, three out of 40 kidney samples were positive (7.5%). *Y. ruckeri* could not be isolated from kidney smears on BA at weeks 13 and 20. There were no mortalities nor *Y. ruckeri* detections in any of the negative control tanks throughout the experiment.

4 | DISCUSSION

The recent discovery in Norwegian salmon farming of a single virulent *Y. ruckeri* lineage (CC1), apparently co-existing alongside a

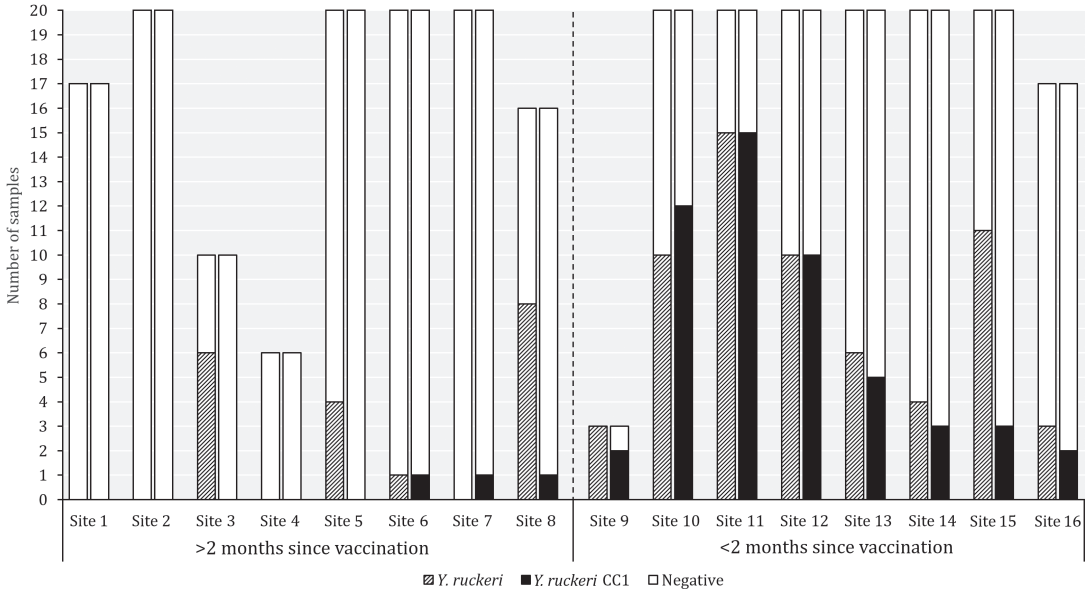

FIGURE 3 Stacked bar chart showing qualitative qPCR results from using the two novel assays on sample-set two. The sample material consisted of environmental swab samples from 16 Atlantic salmon hatcheries, collected in 2019 and 2020. Sites 9 through 16 had employed *Yersinia ruckeri* vaccines less than 2 months prior to sampling, while sites 1 through 8 did not. qPCR, quantitative polymerase chain reaction

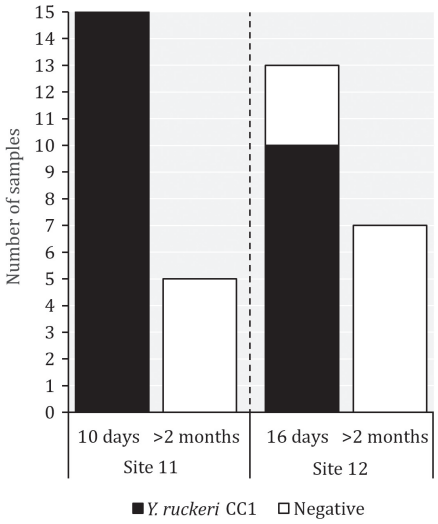

FIGURE 4 Stacked bar chart showing qualitative qPCR results for *Yersinia ruckeri* CC1 from environmental swab samples collected at two sites in sample-set two that were partially vaccinated, respectively 10 and 16 days prior to sampling (see also Figure 3). Results from production units that were not recently vaccinated ('>2 months') are also shown. The lowest Ct-values observed at each site were 28 (site 11) and 30 (site 12). qPCR, quantitative polymerase chain reaction

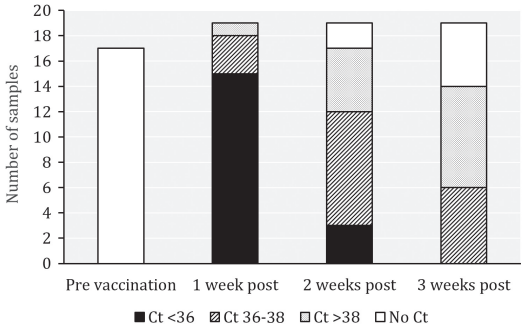

FIGURE 5 Stacked bar chart showing qPCR results for *Yersinia ruckeri* CC1 from environmental swab samples collected at site 1 (sample-set two, prevaccination data for this site are also depicted in Figure 3), a presumed *Y. ruckeri*-free freshwater aquaculture site, prior to vaccination against yersiniosis and weekly after vaccination. Samples were categorized by Ct-values to indicate relative amounts of *Y. ruckeri* DNA targets in them. The lowest Ct-values 1 week post-vaccination were 31 (one sample) and 32 (four samples). The lowest Ct-value 2 weeks post-vaccination was 35 (three samples). qPCR, quantitative polymerase chain reaction

diverse array of putatively avirulent strains unrelated to clinical disease (Gulla, Barnes, et al., 2018), has highlighted the need for more information relating to the distribution and prevalence of these

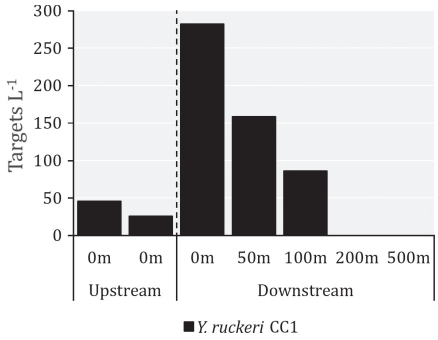

FIGURE 6 Quantification of *Yersinia ruckeri* CC1 (estimated DNA targets per litre) in filtered sea water samples (4 L) collected upstream and downstream of an Atlantic salmon sea cage during an active yersiniosis outbreak

bacteria in salmon production. Via development and use of two highly sensitive PCR assays, respectively, specific at the species- (*Y. ruckeri*) and genotype- (*Y. ruckeri* CC1) level, we were able to verify non-CC1 *Y. ruckeri* as prevalent and dominating across freshwater salmon farm environments in Norway. Moreover, during PCR surveys of sea water sampled during both field and experimental thermal delousing, we observed rapid shedding of *Y. ruckeri* CC1 from sub-clinically infected fish, emphasizing the potential biosecurity risks involved during such stressful procedures.

PCR is widely used as a biosecurity screening tool for fish-pathogenic agents in Norway, and such assays used for detection of *Y. ruckeri* have generally targeted the 16S rRNA gene. Specific 16S-based qPCR detection at the species level and beyond is, however, challenging due to the often highly conserved nature of this gene between closely related species (Cloud et al., 2000; Linton et al., 1997; Nishio et al., 1997; Ryu et al., 2013). In our experience, such assays directed at *Y. ruckeri* may generate false-positive signals when used on environmental samples, likely due to undescribed members of the *Yersiniaceae* carrying 16S rDNA sequence motifs near identical to *Y. ruckeri* (e.g., NVI-11511, -11,512 and -11,514, Table 2 and Figure S1). In light of these experiences, we chose to forgo the potential benefits of increased sensitivity offered by the multi-copy 16S rRNA gene, and instead focus on genetic loci present in *Y. ruckeri* but absent in other *Yersiniaceae* native to aquaculture environments. Development of PCRs for specific detection of pathogenic strains against a background of less-virulent or avirulent members of the same bacterial species offers an additional challenge. The availability of continuously growing public genome databases has, however, eased identification of genetic loci specific to particular taxa. Through scrutiny of publicly available *Y. ruckeri* genomes and genomes sequenced in our laboratory, we could thus establish a PCR specific for the single *Y. ruckeri* lineage (CC1) currently associated with serious disease in Norwegian salmon farming.

It is generally accepted that *Y. ruckeri* infections may persist sub-clinically at a low prevalence in affected fish stocks (Bruno &

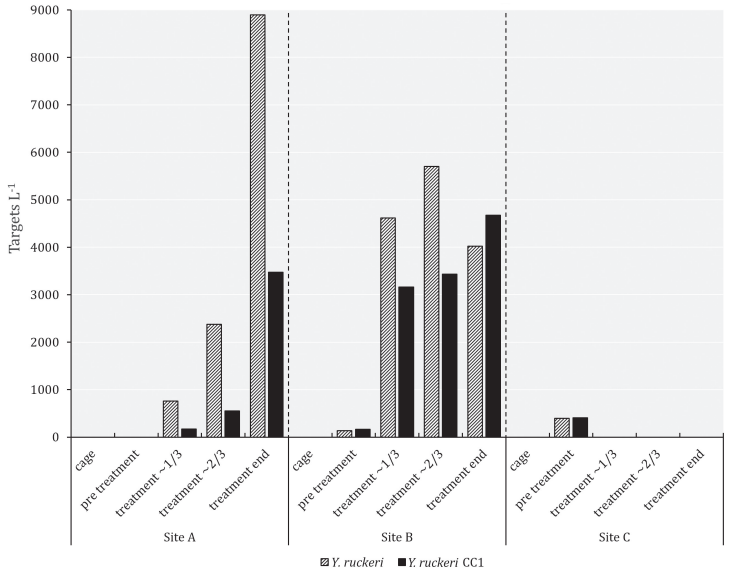

FIGURE 7 Quantification of *Yersinia ruckeri* and *Y. ruckeri* CC1 (estimated DNA targets per litre) in filtered sea water samples collected from sea cages ('cage') and treatment chambers ('pre treatment') prior to and during (at three intervals) thermal delousing. Two additional treatments were sampled at site C the following day, with the same equipment, where *Y. ruckeri* (not *Y. ruckeri* CC1) was only detected in the treatment chamber prior to one of the treatments (not shown)

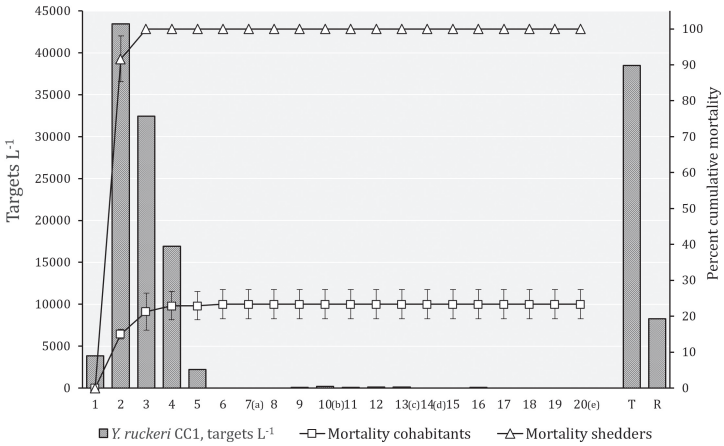

FIGURE 8 Cumulative mortalities (right vertical axis; percent) and quantification of *Yersinia ruckeri* CC1 (left vertical axis; estimated DNA targets per litre) in filtered water collected throughout the challenge trial. Sampling points as indicated on the horizontal axis are weekly samples (1–20), and sampling of tanks used for treatment (T) and recovery (R) of fish subjected to thermal stress (on far right). Sequential events indicated on the horizontal axis represent change in lightning regimen (a), change in salinity (b), fish number adjustments with kidney sampling (c), experimental stress (d) and trial termination with kidney sampling (e). *Y. ruckeri* CC1 quantification in weekly samplings are averages across four tanks, while quantification during thermal stress (T and R) are averages from two tanks. Cumulative mortality percentages are plotted as averages with bars indicating the observed range across four tanks

Munro, 1989; Willumsen, 1989) and that acute outbreaks of yersiniosis in Atlantic salmon may follow stressful management procedures (Gismervik et al., 2019). However, screening of fish-tissues for detection of such low-prevalence infections requires killing of many individuals and negative results will always be associated with a certain degree of uncertainty. On the other hand, screening of environmental DNA (eDNA) offers the possibility of surveying the host population as a whole, but is dependent on the particular pathogen of interest being shed from the infected fish. Although we found both biofilm and filtered water equally reliable for *Y. ruckeri* screening in salmon-farming environments, biofilm sampling was chosen, as this was quicker and less technically demanding, while

also producing easily transportable samples. As mature biofilms are not normally found within experimental aquarium facilities, however, we based our eDNA sampling during the laboratory challenge trial on filtered water samples.

Our screening studies suggest that Norwegian salmon farms are commonly colonized by *Y. ruckeri* genotypes other than CC1 (Figures 2 and 3). Recently vaccinated sites excluded, CC1 constituted a low proportion of *Y. ruckeri* detections in Atlantic salmon hatcheries. While this may suggest CC1 to be less prevalent than non-CC1 *Y. ruckeri* in general, it does not exclude the possibility that sub-clinically CC1 infected fish remain undetected. In our laboratory trials, consistent detection of water-borne *Y. ruckeri* CC1 was only possible during the active outbreak phase and during experimental stressing of sub-clinical carriers (Figure 8). Detection of water-borne *Y. ruckeri* was otherwise sporadic with Ct-values close to the LOD, indicating infrequent shedding from carrier fish.

While culture of *Y. ruckeri* from environmental sample material is generally challenging, MLVA genotyping directly on eDNA templates from two of the freshwater sites that were positive for *Y. ruckeri* while negative for *Y. ruckeri* CC1 corroborated the qPCR results by producing MLVA-profiles incompatible with *Y. ruckeri* CC1 (not shown). Low-virulent *Y. ruckeri* strains associated with salmonid aquaculture have been reported also from Australia (Barnes et al., 2016) and the UK (Verner-Jeffreys et al., 2011), possibly indicative of a natural, non-pathogenic presence in such freshwater environments. Previous studies have demonstrated the capability of *Y. ruckeri* to survive for extended periods of time in sterile freshwater (Thorsen et al., 1992), sediments and sand (Bomo et al., 2004; Romalde et al., 1994), and to form hardy biofilms on various materials (Coquet, Cosette, Junter, et al., 2002; Coquet, Cosette, Quillet, et al., 2002; Wrobel et al., 2020). It remains unclear, however, whether the putatively avirulent strains documented here depend upon proximity to, and/or interaction with, a salmonid hosts in order to thrive in these environments.

While suitable for environmental *Y. ruckeri* screening in general, the high sensitivity of both PCR assays developed here also rendered them capable of detecting eDNA presumably originating from *Y. ruckeri* vaccines for at least 3 weeks after administration by ip injection (Figures 3, 4 and 5). These vaccines consist of killed *Y. ruckeri* CC1 cells and thus also contain genomic DNA from the *Y. ruckeri* CC1 vaccine strain. Still, this apparent persistence of intact PCR targets from the vaccine was surprising as relatively rapid degradation of inactivated bacteria was expected in such systems, where microbial activity is high. Likely explanations include vaccine residues gradually leaking from the injection site and/or being deposited in biofilms in the production environment. The specific vaccine technology used or the common practice of co-injection of this water-based vaccine together with an oil-based multi-component vaccine yielding a depot effect, may influence both the degree of leakage and persistence of inactivated *Y. ruckeri* in these environments. Nevertheless, as some freshwater farms employ up to two rounds of yersiniosis vaccination by immersion, followed by subsequent ip administration, interpretation of *Y. ruckeri* PCR detections at such sites will inevitably carry some degree of uncertainty. Although PCR technologies have been described that do not amplify DNA from dead cells, for example, by use of viable/dead staining with Ethidium monoazide bromide or derivatives thereof (Soejima et al., 2007), these methods require significant optimization and are critically reliant on low turbidity (Fu et al., 2020; Santander et al., 2019), making them generally unsuitable for sample materials such as environmental swabs and filtered water from fish farms.

Historically, yersiniosis in sea-farmed Atlantic salmon in Norway has been considered a minor problem, primarily occurring in sub-clinically infected stocks shortly after sea transfer. In recent years, however, the disease has also become more common in larger sea-farmed fish, often manifesting within a couple of weeks following non-medicinal delousing. Such procedures, introduced in the face of increasing development of salmon-louse resistance to chemotherapeutants, may often cause acute mortality, skin damage and poor fish welfare (Folkedal et al., 2021; Nilsson et al., 2019; Overton et al., 2019; Sviland Walde et al., 2021), and are undoubtedly extremely stressful to the subjected fish. In this study, we found eDNA from sea water equally suitable for *Y. ruckeri* detection by PCR as compared to freshwater, with unambiguous detection of the bacterium by both developed assays as far as 100 m downstream of a salmon sea-cage experiencing an active *Y. ruckeri* CC1 infection (Figure 6). However, considering the dilution effect and the relatively high Ct-values observed, eDNA analyses are likely not sensitive enough for reliable detection of sub-clinical carrier status in sea-cage held populations of salmon.

In light of these findings, our investigation of *Y. ruckeri* in salmon at sea sites was instead focused on eDNA sampling during thermal delousing, a form of non-medicinal delousing where large numbers of fish are treated within a limited volume of heated water. Here, high amounts of *Y. ruckeri* CC1 were detected in treatment water on two farms during delousing of suspected sub-clinically infected salmon stocks (sites A and B in Figure 7). Interestingly, we did not detect the bacterium in water sampled from these sea-cages prior to treatment, strongly suggesting that *Y. ruckeri* shedding was provoked by handling and treatment. It should be noted that detections in the treatment chamber prior to delousing at sites B and C may represent inactivated *Y. ruckeri* post-disinfection procedures. Simulated thermal delousing on sub-clinically infected fish in the laboratory subsequently confirmed that the combination of handling and thermal stress did in fact result in significantly increased *Y. ruckeri* shedding (Figure 8). Despite the apparently low number of sub-clinically infected fish present at the time of experimental thermal treatment, as assessed by sampling from fish 1 week prior (12.5% positive), the amount of *Y. ruckeri* released into the treatment water corresponded to levels observed during the acute phase of yersiniosis earlier in the same experiment. However, no further clinical disease was recorded subsequently to stress exposure, possibly indicating a partly immunized population following the initial outbreak when sub-clinical infection was established, and/or that the stress involved was insufficient to induce another outbreak.

In conclusion, we developed two *Y. ruckeri* qPCR assays, specific at the species- and genotype- (CC1) level, respectively. While the CC1-specific assay is most relevant under the current Norwegian situation, the species-specific assay is suitable for international application. Using these two assays in combination we could readily detect *Y. ruckeri* in freshwater salmon-farm environments in Norway, where putatively avirulent strains of this bacterium were found to dominate. While this approach proved highly sensitive for *Y. ruckeri* screening in such environments, it is important to be aware that yersiniosis vaccination several weeks in advance of sampling may give rise to false-positive results. Screening of treatment water in marine salmon-farms undergoing thermal delousing further corroborated that yersiniosis outbreaks may follow due to stress-related shedding of *Y. ruckeri* from sub-clinical carrier fish, possibly exacerbated by physical damage experienced by the fish during treatment. Lab trials further verified that the thermal delouser treatment chamber, in which large numbers of fish are treated within a relatively small volume of water, represents an ideal eDNA sampling site for evaluating the *Y. ruckeri* carrier status of salmon stocks. Conceivably, sampling during such treatments or other stressful situations may also represent a relevant source of eDNA for detection of other infectious pathogens in marine aquaculture.

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DATA AVAILABILITY STATEMENT

This work was based on previously published genome sequences referenced in the text. Data regarding details on sites and samples areconfidential or deemed not relevant.

ORCID

Andreas Riborg https://orcid.org/0000-0002-8741-9853
Snorre Gulla https://orcid.org/0000-0002-7135-9227
Jannicke Wiik-Nielsen https://orcid.org/0000-0002-3450-2303
Anita Rønneseth https://orcid.org/0000-0002-7511-0234
Bjørn Spilsberg https://orcid.org/0000-0001-8579-4089
Duncan J. Colquhoun https://orcid.org/0000-0002-2737-6716

REFERENCES

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research*, *25*(17), 3389–3402. https://doi.org/10.1093/nar/25.17.3389

Barnes, A. C., Delamare-Deboutteville, J., Gudkovs, N., Brosnahan, C., Morrison, R., & Carson, J. (2016). Whole genome analysis of *Yersinia ruckeri* isolated over 27 years in Australia and New Zealand reveals geographical endemism over multiple lineages and recent evolution under host selection. *Microbial Genomics*, *2*(11), e000095. https://doi.org/10.1099/mgen.0.000095

Bastardo, A., Ravelo, C., & Romalde, J. L. (2012). Highly sensitive detection and quantification of the pathogen *Yersinia ruckeri* in fish tissues by using real-time PCR. *Applied Microbiology and Biotechnology*, *96*(2), 511–520. https://doi.org/10.1007/s00253-012-4328-1

Bastardo, A., Ravelo, C., & Romalde, J. L. (2015). Phylogeography of *Yersinia ruckeri* reveals effects of past evolutionary events on the current strain distribution and explains variations in the global transmission of enteric redmouth (ERM) disease. *Frontiers in Microbiology*, *6*, 1198. https://doi.org/10.3389/fmicb.2015.01198

Bernhardt, L. V., Lillehaug, A., Qviller, L., Weli, S. C., Grønneberg, E., Nilsen, H., & Myrmel, M. (2021). Early detection of salmonid alphavirus in seawater from marine farm sites of Atlantic salmon *Salmo salar*. *Diseases of Aquatic Organisms*, *146*, 41–52. https://doi.org/10.3354/dao03618

Bomo, A. M., Ekeberg, D., Stevik, T. K., Hanssen, J. F., & Frostegård, A. (2004). Retention and removal of the fish pathogenic bacterium *Yersinia ruckeri* in biological sand filters. *Journal of Applied Microbiology*, *97*(3), 598–608. https://doi.org/10.1111/j.1365-2672.2004.02342.x

Bruno, D. W., & Munro, A. L. S. (1989). Immunity in Atlantic salmon, *Salmo salar* L., fry following vaccination against *Yersinia ruckeri*, and the influence of body weight and infectious pancreatic necrosis virus (IPNV) on the detection of carriers. *Aquaculture*, *81*(3–4), 205–211. https://doi.org/10.1016/0044-8486(89)90146-4

Carson, J., & Wilson, T. (2009). Yersiniosis in fish. In *Australia and New Zealand standard diagnostic procedure* (pp. 1–19). Australia/New Zealand government. https://www.awe.gov.au/sites/default/files/sitecollectiondocuments/animal/ahl/ANZSDP-Yersiniosis.pdf

Cloud, J. L., Carroll, K. C., Pixton, P., Erali, M., & Hillyard, D. R. (2000). Detection of Legionella species in respiratory specimens using PCR with sequencing confirmation. *Journal of Clinical Microbiology*, *38*(5), 1709–1712. https://doi.org/10.1128/JCM.38.5.1709-1712.2000

Coquet, L., Cosette, P., Junter, G. A., Beucher, E., Saiter, J. M., & Jouenne, T. (2002). Adhesion of *Yersinia ruckeri* to fish farm materials: Influence of cell and material surface properties. *Colloids and Surfaces B: Biointerfaces*, *26*(4), 373–378. https://doi.org/10.1016/S0927-7765(02)00023-1

Coquet, L., Cosette, P., Quillet, L., Petit, F., Junter, G. A., & Jouenne, T. (2002). Occurrence and phenotypic characterization of *Yersinia ruckeri* strains with biofilm-forming capacity in a rainbow trout farm. *Applied and Environmental Microbiology*, *68*(2), 470–475. https://doi.org/10.1128/AEM.68.2.470-475.2002

Darling, A. E., Mau, B., & Perna, N. T. (2010). progressiveMauve: Multiple genome alignment with gene gain, loss and rearrangement. *PLoS One*, *5*(6), e11147. https://doi.org/10.1371/journal.pone.0011147

Davies, R. L. (1990). O-serotyping of *Yersinia ruckeri* with special emphasis on European isolates. *Veterinary Microbiology*, *22*(4), 299–307. https://doi.org/10.1016/0378-1135(90)90016-o

Davies, R., & Frerichs, G. (1989). Morphological and biochemical differences among isolates of *Yersinia ruckeri* obtained from wide geographical areas. *Journal of Fish Diseases*, *12*, 357–365. https://doi.org/10.1111/j.1365-2761.1989.tb00324.x

Del Cerro, A., Marquez, I., & Guijarro, J. A. (2002). Simultaneous detection of *Aeromonas salmonicida*, *Flavobacterium psychrophilum*, and *Yersinia ruckeri*, three major fish pathogens, by multiplex PCR. *Applied and Environmental Microbiology*, *68*(10), 5177–5180. https://doi.org/10.1128/AEM.68.10.5177-5180.2002

Folkedal, O., Utskot, S. O., & Nilsson, J. (2021). Thermal delousing in anaesthetised small Atlantic salmon (*Salmo salar*) post-smolts: A case study showing the viability of anaesthesia prior to delousing for improved welfare during treatment for salmon lice. *Animal Welfare*, *30*(2), 117–120. https://doi.org/10.7120/09627286.30.2.117

Fu, Y., Ye, Z., Jia, Y., Fan, J., Hashmi, M. Z., & Shen, C. (2020). An optimized method to assess viable *Escherichia coli* O157:H7 in agricultural soil using combined propidium monoazide staining and quantitative PCR. *Frontiers in Microbiology*, *11*, 1809. https://doi.org/10.3389/fmicb.2020.01809

Ghosh, B., Crosbie, P., Nowak, B. F., & Bridle, A. R. (2018). A highly sensitive, non-invasive qPCR-based strategy for direct quantification of *Yersinia ruckeri* in fish faeces. *Journal of Fish Diseases*, *41*(9), 1421–1428. https://doi.org/10.1111/jfd.12839

Gibello, A., Blanco, M. M., Moreno, M. A., Cutuli, M. T., Domenech, A., Domínguez, L., & Fernández-Garayzábal, J. F. (1999). Development of a PCR assay for detection of *Yersinia ruckeri* in tissues of inoculated and naturally infected trout. *Applied and Environmental Microbiology*, *65*(1), 346–350. https://doi.org/10.1128/AEM.65.1.346-350.1999

Gismervik, K., Gåsnes, S. K., Nielsen, K. V., & Mejdell, C. M. (2019). Fish welfare. In B. Hjeltnes, B. Bang Jensen, G. Bornø, A. Haukaas, & C. S. Walde (Eds.), *The health situation in Norwegian aquaculture 2018* (pp. 20–31). Published by the Norwegian Veterinary Institute. Retrieved from. https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2019/fish-health-report-2018

Gulla, S., Barnes, A. C., Welch, T. J., Romalde, J. L., Ryder, D., Ormsby, M. J., Carson, J., Lagesen, K., Verner-Jeffreys, D. W., Davies, R. L., & Colquhoun, D. J. (2018). Multilocus variable-number tandem-repeat analysis of *Yersinia ruckeri* confirms the existence of host specificity, geographic endemism, and anthropogenic dissemination of virulent clones. *Applied and Environmental Microbiology*, *84*(16), e00730–e00718. https://doi.org/10.1128/AEM.00730-18

Gulla, S., Gu, J., & Olsen, A. B. (2019). Yersiniosis. In B. Hjeltnes, B. Bang Jensen, G. Bornø, A. Haukaas, & C. S. Walde (Eds.), *The health situation in Norwegian aquaculture 2018* (pp. 20–31). Published by the Norwegian Veterinary Institute. Retrieved from. https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2019/fish-health-report-2018

Gulla, S., & Olsen, A. B. (2020). Yersiniosis. In I. Sommerset, C. S. Walde, B. Bang Jensen, B. Bornø, A. Haukaas, & E. Brun (Eds.), *The health situation in Norwegian aquaculture 2019* (pp. 80–82). Published by the Norwegian Veterinary Institute. Retrieved from. https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2020/fish-health-report-2019

Gulla, S., Wiik-Nielsen, J., & Colquhoun, D. J. (2018). Yersiniose i norsk lakseoppdrett: Kunnskapsstatus [Yersiniosis in Norwegian salmon aquaculture: knowledge status]. *Norsk Fiskeoppdrett*, *3*(2018), 50–53.

Keeling, S. E., Johnston, C., Wallis, R., Brosnahan, C. L., Gudkovs, N., & McDonald, W. L. (2012). Development and validation of real-time PCR for the detection of *Yersinia ruckeri*. *Journal of Fish Diseases*, *35*, 119–125. https://doi.org/10.1111/j.1365-2761.2011.01327.x

LeJeune, J. T., & Rurangirwa, F. R. (2000). Polymerase chain reaction for definitive identification of *Yersinia ruckeri*. *Journal of Veterinary Diagnostic Investigation*, *12*(6), 558–561. https://doi.org/10.1177/104063870001200611

Lewin, A. S., Haugen, T., Netzer, R., Tøndervik, A., Dahle, S. W., & Hageskal, G. (2020). Multiplex droplet digital PCR assay for detection of *Flavobacterium psychrophilum* and *Yersinia ruckeri* in Norwegian aquaculture. *Journal of Microbiological Methods*, *177*, 106044. https://doi.org/10.1016/j.mimet.2020.106044

Linton, D., Lawson, A. J., Owen, R. J., & Stanley, J. (1997). PCR detection, identification to species level, and fingerprinting of Campylobacter jejuni and Campylobacter coli direct from diarrheic samples. *Journal of Clinical Microbiology*, *35*(10), 2568–2572. https://doi.org/10.1128/jcm.35.10.2568-2572.1997

McCarthy, D. H., & Johnson, K. A. (1982). A serotypic survey and cross-protection test of North American field isolates of *Yersinia ruckeri*. *Journal of Fish Diseases*, *5*(4), 323–328.

Nilsson, J., Moltumyr, L., Madaro, A., Kristiansen, T. S., Gåsnes, S. K., Mejdell, C. M., Gismervik, K., & Stien, L. H. (2019). Sudden exposure to warm water causes instant behavioural responses indicative of nociception or pain in Atlantic salmon. *Veterinary and animal science*, *8*, 100076. https://doi.org/10.1016/j.vas.2019.100076

Nishio, T., Yoshikura, T., & Itoh, H. (1997). Detection of Methylobacterium species by 16S rRNA gene-targeted PCR. *Applied and Environmental Microbiology*, *63*(4), 1594–1597. https://doi.org/10.1128/aem.63.4.1594-1597.1997

Overton, K., Dempster, T., Oppedal, F., Kristiansen, T. S., Gismervik, K., & Stien, L. H. (2019). Salmon lice treatments and salmon mortality in Norwegian aquaculture: A review. *Reviews in Aquaculture*, *11*(4), 1398–1417. https://doi.org/10.1111/raq.12299

Riborg, A., Colquhoun, D. J., & Gulla, S. (2022). Biotyping reveals loss of motility in two distinct Yersinia ruckeri lineages exclusive to Norwegian aquaculture. *Journal of Fish Diseases*, *45*, 641–653. https://doi.org/10.1111/jfd.13590

Romalde, J. L., Barja, J. L., Magariños, B., & Toranzo, A. E. (1994). Starvation-survival processes of the bacterial fish pathogen *Yersinia ruckeri*. *Systematic and Applied Microbiology*, *17*(2), 161–168. https://doi.org/10.1016/S0723-2020(11)80002-0

Romalde, J. L., MagariÑos, B., Barja, J. L., & Toranzo, A. E. (1993). Antigenic and molecular characterization of *Yersinia ruckeri* proposal for a new intraspecies classification. *Systematic and Applied Microbiology*, *16*(3), 411–419. https://doi.org/10.1016/S0723-2020(11)80274-2

Ross, A. J., Rucker, R. R., & Ewing, W. H. (1966). Description of a bacterium associated with redmouth disease of rainbow trout (*Salmo gairdneri*). *Canadian Journal of Microbiology*, *12*(4), 763–770. https://doi.org/10.1139/m66-103

Roth, B. (2016). *Avlusing av laksefisk med Optilice: Effekt på avlusing og fiskevelferd. Rapport 59/2016* (p. 41. p (in Norwegian). Retrieved from). Nofima. https://nofima.no/publikasjon/1408716/

Rusch, J. C., Hansen, H., Strand, D. A., Markussen, T., Hytterød, S., & Vrålstad, T. (2018). Catching the fish with the worm: a case study on eDNA detection of the monogenean parasite Gyrodactylus salaris and two of its hosts, Atlantic salmon (Salmo salar) and rainbow trout (Oncorhynchus mykiss). *Parasites & Vectors*, *11*(1), 333. https://doi.org/10.1186/s13071-018-2916-3

Ryu, H., Henson, M., Elk, M., Toledo-Hernandez, C., Griffith, J., Blackwood, D., Noble, R., Gourmelon, M., Glassmeyer, S., & Santo Domingo, J. W. (2013). Development of quantitative PCR assays targeting the 16S rRNA genes of Enterococcus spp. and their application to the identification of enterococcus species in environmental samples. *Applied and Environmental Microbiology*, *79*(1), 196–204. https://doi.org/10.1128/AEM.02802-12

Santander, R. D., Meredith, C. L., & Aćimović, S. G. (2019). Development of a viability digital PCR protocol for the selective detection and quantification of live *Erwinia amylovora* cells in cankers. *Scientific Reports*, *9*(1), 11530. https://doi.org/10.1038/s41598-019-47976-x

Shea, D., Bateman, A., Li, S., Tabata, A., Schulze, A., Mordecai, G., Ogston, L., Volpe, J. P., Neil Frazer, L., Connors, B., Miller, K. M., Short, S., & Krkošek, M. (2020). Environmental DNA from multiple pathogens is elevated near active Atlantic salmon farms. *Proceedings Biological sciences*, *287*(1937), 20202010. https://doi.org/10.1098/rspb.2020.2010

Soejima, T., Iida, K., Qin, T., Taniai, H., Seki, M., Takade, A., & Yoshida, S. (2007). Photoactivated ethidium monoazide directly cleaves bacterial DNA and is applied to PCR for discrimination of live and dead bacteria. *Microbiology and Immunology*, *51*(8), 763–775. https://doi.org/10.1111/j.1348-0421.2007.tb03966.x

Sparboe, O., Koren, C., Håstein, T., Poppe, T. T., & Stenwig, H. (1986). The first isolation of *Yersinia ruckeri* from farmed Norwegian salmon. *Bulletin of the European Association of Fish Pathologists*, *6*, 41–42.

Strand, D. A., Johnsen, S. I., Rusch, J. C., Agersnap, S., Larsen, W., Knudsen, S., Møller, P., & Vrålstad, T. (2019). Monitoring a Norwegian freshwater crayfish tragedy - eDNA snapshots of invasion, infection and extinction. *Journal of Applied Ecology.*, *56*, 1661–1673. https://doi.org/10.1111/1365-2664.13404

Sviland Walde, C., Bang Jensen, B., Pettersen, J. M., & Stormoen, M. (2021). Estimating cage-level mortality distributions following different delousing treatments of Atlantic salmon (*Salmo salar*) in Norway. *Journal of Fish Diseases*, *44*(7), 899–912. https://doi.org/10.1111/jfd.13348

Temprano, A., Yugueros, J., Hernanz, C., Sánchez, M., Berzal, B., Luengo, J. M., & Naharro, G. (2001). Rapid identification of *Yersinia ruckeri* by PCR amplification of *yrul–yruR* quorum sensing. *Journal of Fish Diseases*, *24*, 253–261. https://doi.org/10.1046/j.1365-2761.2001.00261.x

Thorsen, B. K., Enger, O., Norland, S., & Hoff, K. A. (1992). Long-term starvation survival of *Yersinia ruckeri* at different salinities studied by microscopical and flow cytometric methods. *Applied and Environmental Microbiology*, *58*(5), 1624–1628. https://doi.org/10.1128/aem.58.5.1624-1628.1992

Verner-Jeffreys, D., Haig, S. J., Welch, T. J., Pond, M. J., Stone, D., Davies, R. L., & Gardiner, R. (2011). Characterisation of a serotype O1 *Yersinia ruckeri* isolate from the Isle of Man: Further evidence that O antigen serotype is not a reliable indicator of virulence. *Bulletin of the European Association of Fish Pathologists*, *31*, 86.

Willumsen, B. (1989). Birds and wild fish as potential vectors of *Yersinia ruckeri*. *Journal of Fish Diseases*, *12*, 275–277. https://doi.org/10.1111/j.1365-2761.1989.tb00313.x

Wrobel, A., Saragliadis, A., Pérez-Ortega, J., Sittman, C., Göttig, S., Liskiewicz, K., Spence, M. H., Schneider, K., Leo, J. C., Arenas, J., & Linke, D. (2020). The inverse autotransporters of *Yersinia ruckeri*, *YrInv* and *YrIlm*, contribute to biofilm formation and virulence. *Environmental Microbiology*, *22*(7), 2939–2955. https://doi.org/10.1111/1462-2920.15051

SUPPORTING INFORMATION

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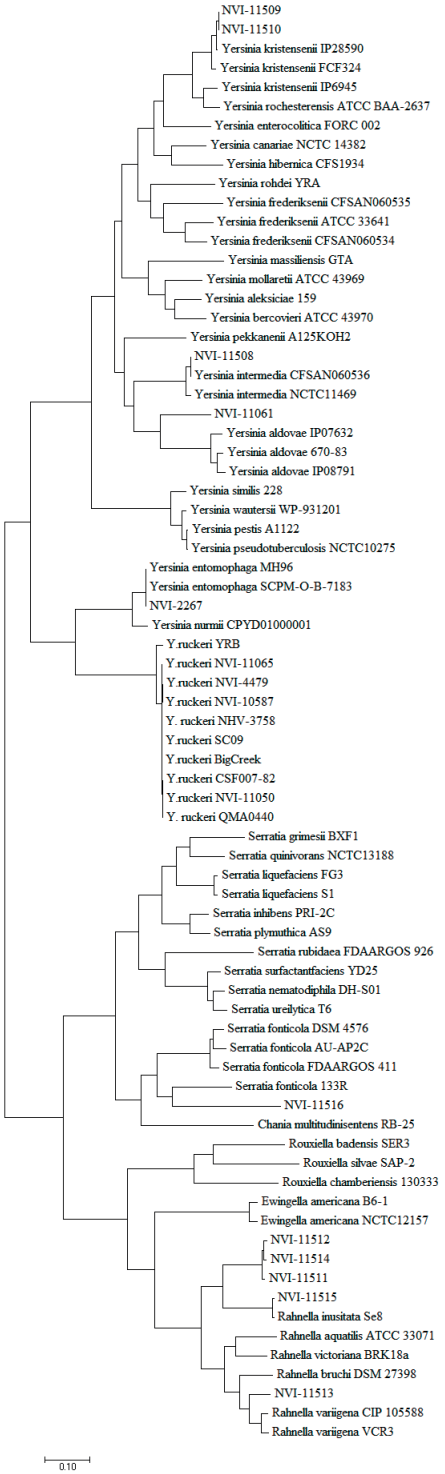
Core gene (n=250), Maximum likelihood tree, MEGA X

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roary -e --mafft -o output_folder -f gff_folder -n -g 160000 -i 90 -cd 100 -p 32 *.gff

Core genes      (100% <= strains <= 100%)       250
Soft core genes (95% <= strains < 100%)         138
Shell genes     (15% <= strains < 95%)          5753
Cloud genes     (0% <= strains < 15%)           83950
Total genes     (0% <= strains <= 100%)         90091
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III

Full title

Pan-genome survey of the fish pathogen *Yersinia ruckeri* links accessory- and amplified genes to virulence

Short title

The *Yersinia ruckeri* pan-genome reveals virulence-related accessory features

Author list

Andreas Riborg[1, 2 *], Snorre Gulla[1], , Eve Zeyl Fiskebeck[1], David Ryder[3], David W. Verner-Jeffreys[3], Duncan J. Colquhoun[1, 4], Timothy J. Welch[5].

[1] Norwegian Veterinary Institute, Ås, Norway
[2] Vaxxinova Norway AS, Bergen, Norway
[3] Centre for Environment, Fisheries and Aquaculture Science (CEFAS), Weymouth, Dorset DT4 8UB, United Kingdom
[4] University of Bergen, Bergen, Norway
[5] National Centre for Cool and Coldwater Aquaculture, USDA-ARS, Leetown, WV, USA

*Corresponding author
E-mail: andreasriborg@gmail.com

Abstract

While both virulent and putatively avirulent *Yersinia ruckeri* strains exist in aquaculture environments, the relationship between the distribution of virulence-associated factors and *de facto* pathogenicity in fish remains poorly understood. Pan-genome analysis of 18 complete genomes, representing established virulent and putatively avirulent lineages of *Y. ruckeri*, revealed the presence of a number of accessory genetic determinants. Further investigation of 68 draft genome assemblies revealed that the distribution of certain putative virulence factors correlated well with virulence and host-specificity. The inverse-autotransporter invasin *yrIlm* was, however, the only gene present in all virulent strains, while absent in lineages regarded as avirulent. Strains known to be associated with significant mortalities in salmonid aquaculture display a combination of serotype O1-LPS and *yrIlm*, with the well-documented highly virulent lineages, represented by MLVA clonal complexes 1 and 2, displaying duplication of the *yrIlm* locus. Duplication of the *yrIlm* locus was further found to have evolved over time in clonal complex 1, where some modern, highly virulent isolates display up to three copies.

Author summary

Yersinia ruckeri is the causative agent of enteric redmouth disease in aquaculture, primarily affecting salmonid fish. Acute outbreaks in aquaculture are generally caused by a limited number of highly virulent strains, while strains of significantly lower virulence are known to exist in the environment. While many *Y. ruckeri* virulence factors have been identified and studied, the relationship between their presence and a virulent phenotype remains poorly understood. In the present work, a pan-genome study across *Y. ruckeri* strains of varying virulence revealed a number of previously described virulence factors as present in all genome sequences. Others were also present in many avirulent strains. Although a number of genetic determinants were overrepresented in virulent lineages, only the presence of the inverse-autotransporter invasin-like gene *yrIlm* correlated fully and exclusively with a virulent phenotype. Highly virulent lineages display a combination of serotype O1-LPS and *yrIlm*, with *yrIlm* being amplified to up to three copies in highly virulent lineages affecting Atlantic salmon in Norway and rainbow trout globally.

Introduction

Yersinia ruckeri is the causative agent of enteric redmouth disease (ERM), also known as yersiniosis in salmonids and other cold-water species of fish. While the disease has been primarily associated with farmed rainbow trout internationally, it also affects farmed Atlantic salmon in Norway, Finland, Australia, Chile and in the UK (Sparboe et al. 1986; Rintamäki et al. 1986; Costa et al. 2011; Bastardo et al. 2011; Wheeler et al. 2009). It has further been isolated from a wide range of other fish species, both farmed (Gudmundsdottir et al. 2014; Barnes et al. 2016; Liu et al. 2016) and wild (Willumsen 1989; Barnes 2011; Wade 2019). In Norway, *Y. ruckeri* strains appear to fall into at least three categories regarding their ability to cause disease in farmed salmon. All serious yersiniosis outbreaks since the late 1980s have been caused by a single genetic lineage of highly virulent serotype O1, known as MLVA clonal complex 1 (CC1) (Gulla et al. 2018). Another MLVA clonal complex (CC3) belonging to serotype O2 is considered only mildly pathogenic, being associated primarily with sporadic detections or low-mortality outbreaks (Gulla et al. 2018). A genetically diverse third category of isolates, predominantly sharing the same serotype (O1) as virulent CC1, are also found in Norwegian aquaculture environments and healthy fish. Despite commonly being present, these heterogeneous non-CC1, non-CC3 isolates are presumed avirulent as they have never been associated with disease (Riborg et al. 2022b).

Differences in *Y. ruckeri* virulence are also recognised internationally, with serotype O1 generally associated with high virulence and serotype O2 isolates considered to be of low or moderate virulence (Bullock et al. 1978; McCarthy & Johnson 1982; Barnes 2011). Serotype O1 (CC5) isolates dominate amongst disease cases in Atlantic salmon in Australia, while outbreaks of yersinosis in rainbow trout in the USA, UK and mainland Europe are almost exclusively caused by the serotype O1 CC2 lineage (Barnes et al. 2016; Gulla et al. 2018). Reports of avirulent isolates from Australia (Barnes et al. 2016) and the UK (Verner-Jeffreys et al. 2011) may indicate an international situation similar to that in Norway, with disease outbreaks dominated by highly virulent clones against a background of essentially avirulent, environmental strains.

Differences in virulence between *Y. ruckeri* lineages must be reflected in their genomes. Identification of key genetic differences should thus broaden our understanding of this widespread and economically important aquaculture pathogen. A considerable degree of host-specificity is apparent amongst various *Y. ruckeri* lineages (Gulla et al. 2018), but cross-species virulence has been demonstrated, albeit at reduced efficiency (Haig et al. 2011), suggesting the presence of common virulence mechanisms. To gain a better understanding of the genetic elements necessary

for the virulent phenotype observed in certain strains, a diverse collection of *Y. ruckeri* strains was whole-genome sequenced and analysed together with publicly available genomes. As mobile elements and putative virulence factors may contain highly repetitive sequences, a hybrid Illumina and MinION nanopore long-read sequencing approach was utilized for selected representative strains to obtain complete circular and highly accurate genome assemblies.

Results & Discussion

Core-gene phylogenetic reconstruction

The maximum-likelihood phylogeny (Fig 1) inferred from 2388 core genes shared across the 86 *Y. ruckeri* genomes investigated verifies the existence of multiple discrete lineages separated according to host species and/or geography (Barnes et al. 2016), and accurately reflects the MLVA-based population structure described previously (Gulla et al. 2018). Disregarding the distantly related YRB lineage, the tree bifurcates into two lineages (A and B), one of which (lineage A) contains all of the well-documented virulent isolates. This includes CC2 found globally in rainbow trout (Gulla et al. 2018), CC5 from Australian salmon (Barnes et al. 2016) as well as those of non-O1 serotypes such as strains BigCreek74 and SC09. Lineage A also contains all disease-associated Norwegian isolates, belonging respectively to the currently dominating CC1 and to CC10 from the late 1980s (Sparboe et al. 1986; Riborg et al. 2022a), as well as the mildly virulent CC3 (serotype O2). Two notable exceptions to the association between virulence and affiliation to lineage A are CC7 and CC9, which in Norway are associated with biofilms in salmon rearing facilities and egg-fluid of apparently healthy salmon. Challenge trials with NVI-11076 (CC7) have demonstrated its avirulent nature in Atlantic salmon (manuscript in preparation). Isolates belonging to CC9 from Australia and New Zealand have been described as of low virulence, with New Zealand isolates being associated with screening of healthy fish or sporadic minor losses in Chinook salmon for which vaccination was not considered necessary (Barnes et al. 2016). The relatively close phylogenetic relationship between the well-documented virulent sub-lineages CC2 and CC5, and the putatively avirulent CC7 and CC9 (Fig 1), could facilitate identification of virulence-discriminatory genetic determinants in this part of the tree.

Juxtaposed to lineage A lies lineage B (further subdivided into B1 and B2), which contains isolates mostly originating from biofilm, health screening of clinically healthy farmed fish (CC8) and wild fish. In Norway, isolates from these sub-lineages are considered avirulent, as they have never been

106 associated with disease outbreaks despite being widely present in aquaculture environments.
107 While various serotypes are found within lineage B, most belong to serotype O1.

108 The strain YRB forms a distinct distal lineage together with a single isolate (NVI-3736) from wild
109 salmon in Norway. While relatively distantly related to the remaining isolates studied (Fig S1), both
110 genomes display the same distinguishing genetic characteristics of the species in terms of genome
111 size and gene content. The pangenome is considerably affected by including these two genomes,
112 increasing by 507 total genes while 129 genes are lost from the core (Fig S2), but status as core or
113 accessory for the virulence-related genetic determinants discussed later was not affected.

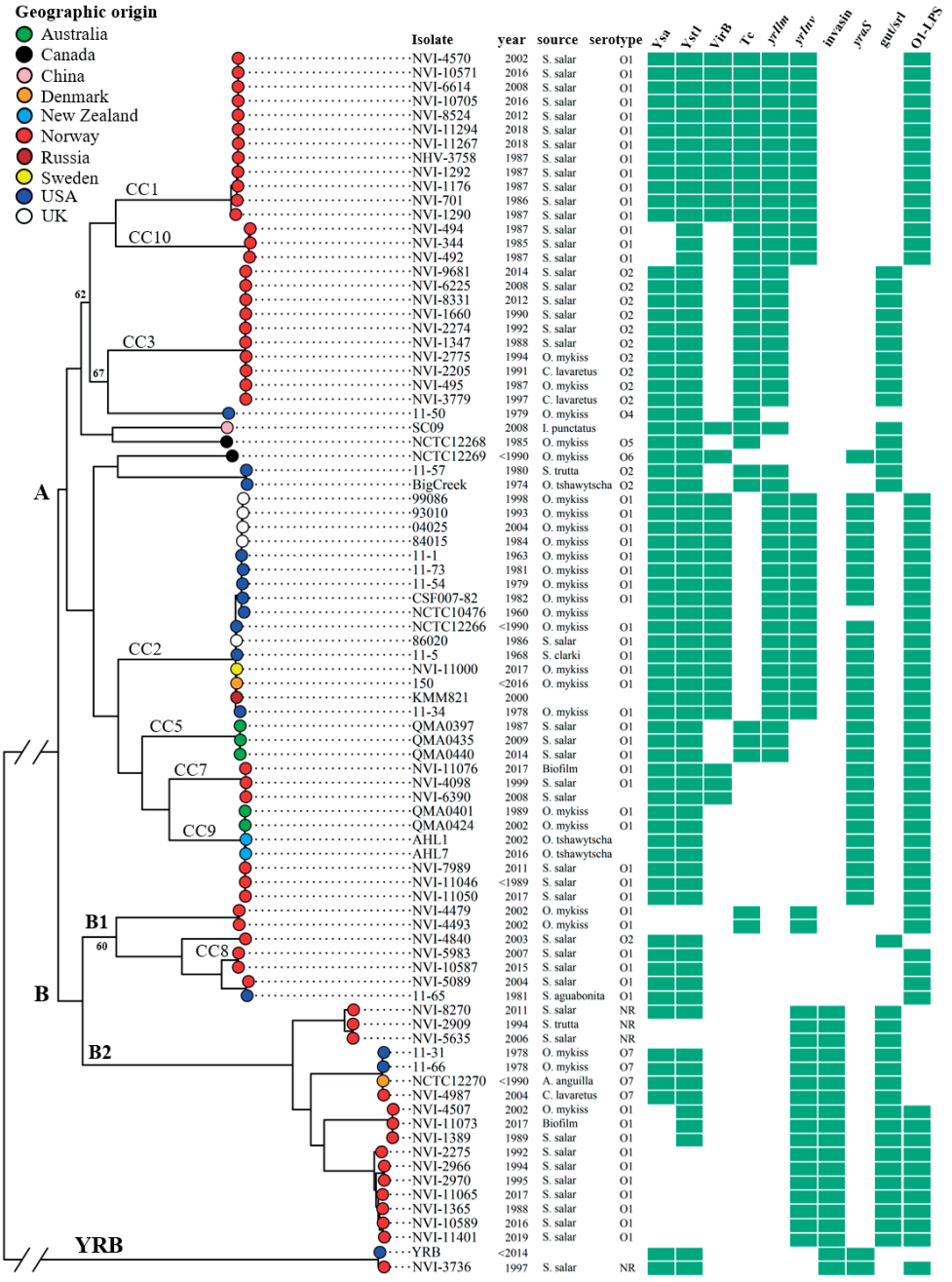
Geographic origin
Australia
Canada
China
Denmark
New Zealand
Norway
Russia
Sweden
USA
UK
Isolate
year
source
serotype
Ysa
Yst1
YirB
Tc
yrfIm
yrfInv
invasin
yreS
gutAsr1
O1-LPS
CC1
CC10
CC3
CC2
CC5
CC7
CC9
CC8
A
B
B1
B2
YRB
62
67
60
NVI-4570 2002 S. salar O1
NVI-10571 2016 S. salar O1
NVI-6614 2008 S. salar O1
NVI-10705 2016 S. salar O1
NVI-8524 2012 S. salar O1
NVI-11294 2018 S. salar O1
NVI-11267 2018 S. salar O1
NHV-3758 1987 S. salar O1
NVI-1292 1987 S. salar O1
NVI-1176 1987 S. salar O1
NVI-701 1986 S. salar O1
NVI-1290 1987 S. salar O1
NVI-494 1987 S. salar O1
NVI-344 1985 S. salar O1
NVI-492 1987 S. salar O1
NVI-9681 2014 S. salar O2
NVI-6225 2008 S. salar O2
NVI-8331 2012 S. salar O2
NVI-1660 1990 S. salar O2
NVI-2274 1992 S. salar O2
NVI-1347 1988 S. salar O2
NVI-2775 1994 O. mykiss O2
NVI-2205 1991 C. lavaretus O2
NVI-495 1987 O. mykiss O2
NVI-3779 1997 C. lavaretus O2
11-50 1979 O. mykiss O4
SC09 2008 I. punctatus
NCTC12268 1985 O. mykiss O5
NCTC12269 <1990 O. mykiss O6
11-57 1980 S. trutta O2
BigCreek 1974 O. tshawytscha O2
99086 1998 O. mykiss O1
93010 1993 O. mykiss O1
04025 2004 O. mykiss O1
84015 1984 O. mykiss O1
11-1 1963 O. mykiss O1
11-73 1981 O. mykiss O1
11-54 1979 O. mykiss O1
CSF007-82 1982 O. mykiss O1
NCTC10476 1960 O. mykiss
NCTC12266 <1990 O. mykiss O1
86020 1986 S. salar O1
11-5 1968 S. clarki O1
NVI-11000 2017 O. mykiss O1
150 <2016 O. mykiss O1
KMM821 2000
11-34 1978 O. mykiss O1
QMA0397 1987 S. salar O1
QMA0435 2009 S. salar O1
QMA0440 2014 S. salar O1
NVI-11076 2017 Biofilm O1
NVI-4098 1999 S. salar O1
NVI-6390 2008 S. salar
QMA0401 1989 O. mykiss O1
QMA0424 2002 O. mykiss O1
AHL1 2002 O. tshawytscha
AHL7 2016 O. tshawytscha
NVI-7989 2011 S. salar O1
NVI-11046 <1989 S. salar O1
NVI-11050 2017 S. salar O1
NVI-4479 2002 O. mykiss O1
NVI-4493 2002 O. mykiss O1
NVI-4840 2003 S. salar O2
NVI-5983 2007 S. salar O1
NVI-10587 2015 S. salar O1
NVI-5089 2004 S. salar O1
11-65 1981 S. aguabonita O1
NVI-8270 2011 S. salar NR
NVI-2909 1994 S. trutta NR
NVI-5635 2006 S. salar NR
11-31 1978 O. mykiss O7
11-66 1978 O. mykiss O7
NCTC12270 <1990 A. anguilla O7
NVI-4987 2004 C. lavaretus O7
NVI-4507 2002 O. mykiss O1
NVI-11073 2017 Biofilm O1
NVI-1389 1989 S. salar O1
NVI-2275 1992 S. salar O1
NVI-2966 1994 S. salar O1
NVI-2970 1995 S. salar O1
NVI-11065 2017 S. salar O1
NVI-1365 1988 S. salar O1
NVI-10589 2016 S. salar O1
NVI-11401 2019 S. salar O1
YRB <2014
NVI-3736 1997 S. salar NR

Fig 1: Maximum likelihood tree based on 2,387 genes, 49,389 core gene SNPs (16,762 SNPs were identified with the YRB lineage excluded; Fig S2), with presence/absence data for specific systems or genes. MLVA clonal complexes (CC) and lineages are indicated on branches. Bootstrap values as percentages of 200 replicates are indicated if < 75 percent. Branch lengths between the YRB lineage and remaining genomes are truncated (see Fig S1). Presence of genetic determinants indicated by green shading for Ysa Type 3 secretion system, Yst1 Type 2 secretion system, virB/virD4 Type 4 secretion system (VirB), Toxin complex (Tc), the putative invasins *yrllm*, *yrlnv* and undescribed invasin WP_042527435, sorbitol utilization genes (*gut*/*srl*), the alkyl sulphatase associated with SDS degradation (*yarS*), and the serotype O1-LPS synthesis cluster. Partial deletions of Ysa, Yst1 and the O1-LPS cluster are shown as absent if genes regarded as critical for function are not present. Serotype NR indicate no reaction with available antisera (O1, O2, O5).

Pan-genome analysis

Based on the phylogenetic reconstruction described above, representative isolates of putatively virulent and avirulent sub-lineages/CCs were selected for Nanopore sequencing to produce complete hybrid (Nanopore/Illumina) genome assemblies. Publicly available complete genome sequences (QMA0440, BigCreek74, SC09, CFS007, KMM821 and YRB) were also included (Table 1). As a large number of diverse plasmids were identified among these assemblies, most of which were exclusive to a single genome, plasmids were excluded from the pan-genome analysis and investigated separately. With plasmids excluded, most of the genetic diversity amongst the complete assemblies could be related to a limited number of major deletions and a large number of diverse mobile elements. Accessory mobile elements ranging from single insertion sequence (IS) elements and small transposons (Tn), to large integrative and conjugative elements (ICE), as well as prophages and various cryptic phage-like elements, were identified. A large portion of the accessory genome resides within a few specific regions of the genome (Fig 2).

Table 1: Complete genome assemblies and plasmids used for pan-genome analyses.

Strain	Lineage		Chromosome		Plasmids			Reference
	Main	Sub- / CC	accession	Size	Name	accession	Size	
NVI-10705	A	1	CP099805	3,809,437	pYR4 pYR5	CP099806 CP099807	115,589 5,191	Current
NVI-492	A	10	CP099813	3,654,750	pYR5	CP099814	5,286	Current
NVI-9681	A	3	CP099811	3,764,054	pYR7	CP099812	121,362	Current
SC09	A	s	CP025800	3,799,871	pLT pWKY	CP025802 CP025801	57,905 73,051	Liu et al. 2015
BigCreek74	A	s	CP011078	3,699,725	-			Unpublished
KMM821	A	2	CP071802	3,773,395	pYR2 pYR3	CP071803 CP071804	16,925 103,906	Unpublished
CFS007-82	A	2	LN681231	3,799,036	pYR2 pYR3	LN681229 LN681230	16,923 103,917	Nelson et al. 2015
QMA0440	A	5	CP017236	3,856,634	-			Barnes et al. 2016
NVI-11076	A	7	CP099808	3,763,098	-			Current
NVI-11050	A	9	CP099815	3,853,871	-			Current
NVI-4479	B1	s	CP098710	3,660,364	-			Current
NVI-4840	B1	s	CP098703	3,657,071	pYR8	CP098705	6,876	Current

					pYR9	CP098704	78,774	
NVI-5089	B1	s	CP098701	3,724,773	pYR10	CP098702	89,273	Current
NVI-10587	B1	8	CP099809	3,700,651	pYR11	CP099810	4,985	Current
NVI-8270	B2	s	CP098694	3,655,667	-			Current
NVI-11073	B2	s	CP098722	3,585,796	-			Current
NVI-11065	B2	s	CP098723	3,703,894	-			Current
YRB	YRB	s	CP009539	3,605,216	-			Johnson et al. 2015
NVI-11294†	A	1	CP098716	3,808,409	pYR4 pYR5	CP098717 CP098718	115,590 5,191	Current
NVI-11267†	A	1	CP098719	3,808,455	pYR4 pYR5	CP098720 CP098721	115,590 5,191	Current
NVI-10571†	A	1	CP098724	3,783,355	pYR4 pYR5	CP098725 CP098726	76,483 5,191	Current
NVI-8524†	A	1	CP098691	3,808,529	pYR4 pYR5	CP098692 CP098693	115,590 5,191	Current
NVI-6614†	A	1	CP098697	3,783,289	pYR4 pYR5 pYR6	CP098699 CP098700 CP098698	80,438 5,191 83,000	Current
NVI-4570†	A	1	CP098706	3,773,829	pYR4 pYR5 pYR6	CP098707 CP098709 CP098708	80,847 5,191 71,441	Current
NVI-1292†	A	1	CP098711	3,765,691	pYR4 pYR5	CP098712 CP098713	80,847 5,191	Current
NVH_3758†	A	1	CP023184	3,766,700	pYR4	CP032236	80,843	Wrobel et al. 2018a Wrobel et al. 2018b
NVI-1176†	A	1	CP098714	3,829,170	pYR4	CP098715	80,847	Current
NVI-701†	A	1	CP098695	3,758,144	pYR4	CP098696	80,847	Current

s: no MLVA CC defined

†: Not included in Fig 2. These CC1 assemblies vary only in complete- or partial prophage sequences (all of which are represented in Fig 2), plasmids, and *yrIlm*- copy number (Fig 5).

The significantly larger pYR4 variant (115,590bp) is due to a Mu-like prophage. The reduced size of pYR4 in NVI-10571 is due to loss of Tn-related sequences. KMM821 plasmids were identified as pYR2 and pYR3 by BLAST. pYR11 in NVI-10587 is nearly identical to *Aeromonas veronii* plasmid pWP3-W19-ESBL-03_3 (NZ_AP022041).

A deletion encompassing 20 genes within the O-antigen biosynthesis cluster in *Y. ruckeri* has been previously shown responsible for the O2 serotype (Barnes et al. 2016). Our dataset indicates that this deletion is a common feature of all non-O1 serotypes, including isolates that are unreactive to antisera from defined serotypes, implying that all serotypes derive from a common serotype O1 ancestor. BLAST searches based on ORFs from the LPS O1-antigen cluster revealed this phenomenon in all 86 genome assemblies studied here, with the exception of NVI-3736 in the YRB lineage. The O1-antigen cluster in this isolate appears to be intact but does not result in a visible reaction with O1-specific antisera. With the sole exception of NVI-3736, these finding are in agreement with a previous study that reported PCR targeting this region as being positive exclusively for serotype O1 strains (Welch & LaPatra 2015). The serotype O1 LPS-antigen cluster contains genes essential for synthesis of nonulosonic acid, a sialic acid component of the O-polysaccharide repeat of O1 *Y. ruckeri* (Welch & LaPatra 2015). Sialic acid-containing LPS is associated with host mimicry and evasion of the innate immune response in vertebrate hosts

(Severi, Hood & Thomas 2007), and its presence may thus explain the dominance of serotype O1 amongst highly virulent strains. Additionally, serotype O1 LPS has been shown to be critically important for resistance to serum killing in rainbow trout (Welch 2020) and this specific O1 antigen structure may possibly confer serum resistance.

The Yst1 type II (T2SS) and Ysa type III (T3SS) secretion systems are situated between core genes *mutS* and *fumA* (Liu et al. 2016). This locus may contain accessory transposons, and is partially or completely lost in several sub-lineages (Fig 3). With the exception of CC10, all serotype O1 sequences have a putative biofilm adhesin polymer cluster associated with this locus, similar to *pgaABCD* in *E. coli* which synthesizes and exports biofilm-enhancing linear homopolymers (Wang et al. 2014). Several non-O1 sequences alternatively display a putative O-antigen modification cluster at this site. BLAST searches against the complete dataset of all 86 assemblies link this O-antigen cluster with serotype O2, suggesting that gain of this O-antigen cluster along with loss of the O1-antigen cluster may be required for the O2 serotype. Another putative O-antigen cluster of similar size and genetic composition is located some 300 Kbp downstream (between *ushA* and *copA*) and also linked to the O2 serotype, but occurs also in serotypes O4 and O6. The pattern of distribution of these accessory features between serotype O2 strains and the distantly related strain YRB, is a strong indication of horizontal gene transfer involving fairly large genomic segments. Other putative O-antigen modification clusters are notably also present at various locations within the accessory genome and may be involved in producing other serotypes and/or subtypes of serotype O1, as described in some serotyping schemes (Romalde et al. 1993).

Another highly variable region may contain toxin complex (Tc) genes and a fimbria cluster, and in most strains various cryptic and prophage-related elements. These cryptic genes are similar in putative function but highly variable in sequence between strains and comprise ~10% of the accessory genome. Yet another highly variable region is found upstream of a putative large exoprotein (WP_045844312), which resembles a contact-dependent growth inhibition operon, and contains accessory O-antigen modification genes and oxidase enzymes in CC1 and some other serotype O1 strains. This region may also contain transposons carrying a putative peptidase or hypothetical genes.

DEAD-box helicases and restriction systems are common Tn payloads in *Y. ruckeri* (Barnes et al. 2016). Most strains have acquired several accessory restriction systems, and at least one DEAD-box helicase in addition to that present in the core genome. Restriction systems are also common within prophages and cryptic phage-like elements. While prophages make up a substantial portion of the

accessory genome, no known virulence-related prophages were identified, nor does the presence of any prophage seem to align with the virulence trait. The T6SS-like anti-feeding prophage (AFP), which has been previously related to virulence (Jank et al. 2015), is present in all assemblies.

The presence/absence of genes involved in sorbitol-utilization (Sorbitol-specific membrane transport and utilization, *gut*/*srl*; Barnes et al. 2016) and sodium dodecyl sulfate (SDS) degradation (*yraS* alkyl sulphatase, WP_080717331) varies between lineages and correlates with the phenotypes of CC1 (negative for both traits), CC2 (degrades SDS) and CC3 (utilizes sorbitol). Sorbitol utilization has previously been considered a trait specific to serotype O2 (O'Leary et al. 1982; Cipriano & Pyle 1985), although rare instances of sorbitol-fermenting serotype O1 strains have been reported (Stevenson & Airdrie I984; Rintamäki et al. 1986). In our dataset, the sorbitol utilization genes are present in most isolates that display deletion of the LPS O1-antigen cluster, i.e. non-O1 serotypes, but they are also present throughout lineage B2 which include several serotype O1 isolates (Fig 1). The ability to degrade SDS has been previously associated with virulence, as a 'heat-sensitive factor' (Furones et al 1990; Furones, Gilpin & Munn 1992), but this has since been refuted (Navais et al. 2014). Here, we find the presence of the alkyl sulphatase involved in primary degradation of SDS to correlate well with core-gene based phylogeny, but not with virulence or serotype. Disruption of *lacZ* was identified in isolates NVI-4840 (lineage B1) and NVI-8270 (lineage B2) due to insertion of, respectively, a transposase and transposase-related sequence. These isolates were found to be *ortho*-nitrophenyl-β-galactoside (ONPG) negative (API20E, not shown). ONPG negative *Y. ruckeri* strains have, to our knowledge, only been reported once previously (Stevenson & Daly 1982). The scarcity of reports of ONPG-negativity, and the sorbitol-fermenting serotype O1 phenotype, suggests that strains belonging to lineage B have been poorly represented in previous studies. Genes absent in the YRB lineage include the lac repressor, the formate hydrogenlyase cluster, and genes involved in L-ascorbate metabolism, although impact on phenotype is not known.

Of the wide variety of pilin/fimbriae gene clusters found both chromosomally- and in plasmids, many of which probably overlap in function, four were considered to be of particular interest. One (A) is present in 8 of the 16 complete genome sequences, but its presence does not correlate well with phylogeny or virulence. Another (B), located within the core-genome flagellar region, was reported by Barnes et al. (2016) to be exclusively missing in 'serotype O1 strains from the USA' i.e. CC2. Here, we found these genes also absent in CC7, and lineages B and YRB. Some adjacent hypothetical proteins, including a predicted outer membrane LPS/capsule polymerase, have nearly identical distributions, but are also present in CC2. The third (C) fimbria cluster is associated with

the Tc cluster, while the fourth (D) is present in all complete sequences, albeit with a duplicated pilin subunit gene in CC1.

A large putative ICE containing a conjugative plasmid-like (Tra) type IV secretion system (T4SS) is present in SC09 and in the putatively avirulent NVI-11076 (CC7). Another ICE, although with a virB/virD4-type T4SS, was found to be present in CC1 and CC2, as well as in strains SC09 and NVI-11076. This virB/virD4 ICE has been implicated in virulence previously (Liu et al. 2020).

Four accessory inverse-autotransporter invasin-like genes (IAT-invasins) were identified, some of which have been previously linked to *Y. ruckeri* virulence (Wrobel et al. 2018a; Wrobel et al. 2020). Of these four, individual strains carry up to two different IAT-invasins, situated in transposons or (apparently) seamlessly integrated into the genome at various genomic locations.

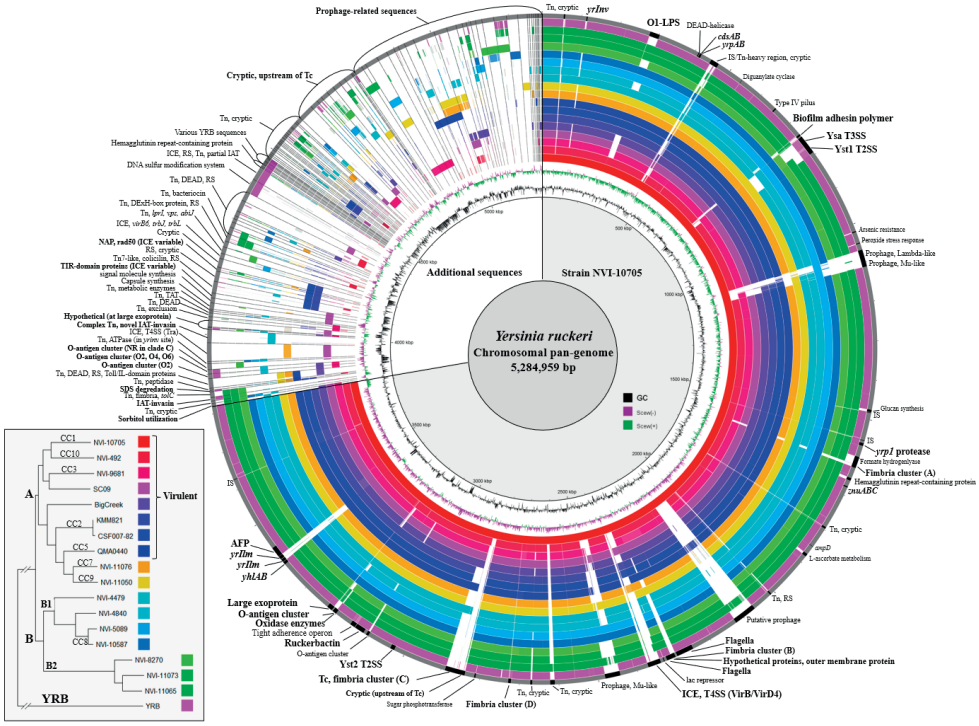

Fig 2: Maximum likelihood core-gene phylogenetic tree (bottom left) and circular representation of genomic comparisons generated with the BLAST Ring Image Generator (BRIG) from complete *Y. ruckeri* genome assemblies. Plasmid sequences were removed from the data prior to analysis. Lineages and MLVA clonal complexes (CC) are indicated on the phylogenetic tree tree branches, with strains regarded as virulent

labelled as such. The branch length of YRB is truncated (see Fig S1). Coloured rings represent genomic sequences used as BLAST queries, corresponding to the colour labelling on tree nodes. Ordering top-bottom on the tree and inner-outter on the circular representation is identical (virulent strains in innermost rings). Solid colour in each respective ring represents ≥95% nucleotide identity to the pan-genome reference. Cut-off identity for BLAST was 85%. The innermost ring (black line with nucleotide distances indicated) represents the pan-genome reference sequence, consisting of the chromosome of strain NVI-10705 for the first 3,818,566 bp, followed by additional *Y. ruckeri* chromosomal sequences with each segment separated by a 800 bp artificial gap with black fill, and ordered by commonality or general association. Prominent features within the NVI-10705 chromosome are labelled by black fill on the outermost ring (grey). Annular graphs towards the centre represent GC percent (black) and GC skew (purple and green). All chromosomal sequences from additional complete genomes belonging to CC1 (see Table 1) are represented, and vary from NVI-10705 only in prophage-related sequences (not shown) and plasmids (Table 1). Hemagglutinin repeat-containing protein is present in all sequences (n = 86) but divergent in YRB (82% nucleotide identity). Abbreviations used: Restriction system (RS), toxin-antitoxin (TAT), insertion sequence (IS), transposon (Tn), integrative and conjugative elements (ICE), Toxin Complex (Tc), inverse autotransporter (IAT), no reaction (NR), DEAD-box helicase (DEAD), Anti-feeding prophage (AFP).

Accessory virulence-associated determinants

In addition to accessory features identified during pan-genome analyses involving complete assemblies, the full dataset of 86 genomes was screened for the presence of several specific virulence factors described previously (Table 2). Many of these were found to be ubiquitous, also within the YRB outgroup, and are thus unlikely responsible for the observed variation in virulence between strains.

Table 2: Distribution of verified and putative virulence factors previously described or discovered here, across the 86 genome assemblies (BLASTn).

Name	Classification	Reference	Distribution
yrp1	Protease	Secades & Guijarro, 1999	Core
yrpAB	Peptidase	Navais et al. 2014	Core
yhlBA	Hemolysin	Fernández et al. 2007	Core
Yst2	Type II secretion system	Liu et al. 2016	Core
Anti-feeding prophage	Type VI-like secretion system	Jank et al. 2015	Core
Ruckerbactin	Siderophore iron acquisition	Fernández et al. 2004	Core
cdsAB	L-cysteine acquisition	Méndez et al. 2010	Core
znuABC	Zinc acquisition	Dahiya & Stevenson 2010a	Core
barA-uvrY	Response regulator	Dahiya & Stevenson 2010b	Core
ompF	Outer membrane protein	Wang et al. 2018	Core
filamentous hemagglutinin	Outer membrane protein	Ormsby et al. 2019	Core
Yst1	Type II secretion system	Liu et al. 2016	Shell

Ysa	Type III secretion system	Gunasena, Komrower & Macintyre 2004	Shell
yrIlm	IAT-invasin	Wrobel et al. 2018a	Shell
yrInv	IAT-invasin	Wrobel et al. 2018a	Shell
Unnamed invasin	IAT-invasin	this paper	Shell
yraS	HSF/SDS	Furones et al. 1990	Shell
Biofilm polymer cluster	Putative secreted polymer	this paper	Shell
Tc	Toxin complex	Barnes et al. 2016	Shell
VirB/VirD4	Type IV secretion system	Liu et al. 2020	Shell
Novel invasin	IAT-invasin	this paper	Cloud
STIR, *tcpA*	Putative secreted proteins	Liu et al. 2020	Cloud

Core indicates presence in 100%, shell in 15-95%, and cloud in less than 15% of genome assemblies (n=86). Some features relevant for virulence for which no particular gene or system have been attributed, e.g. phospholipase activity and various pilin/fimbriae clusters, were not screened. Plasmids are not shown. The biotype 2 trait which has a casual association with virulence is caused by non-synonymous SNPs or small indels (Welch et al. 2011) and thus outside the scope of this study. Rare deletions in Yst2, the tight adherence operon and iron acquisition have been described previously (Barnes et al. 2016) but were not observed amongst the sequences studied here.

Plasmids

Human-pathogenic *Yersinia* spp. are well known for plasmid-borne virulence (Cornelis et al. 1998), and the possibility of plasmid-mediated virulence in *Y. ruckeri* has been frequently discussed (De Grandis & Stevenson 1982; Stave, Cook & Roberson 1987; Guilvout et al. 1988; Garcia et al. 1998; Méndez et al. 2009; Wrobel et al. 2018b). A wide variety of plasmids were identified amongst the complete genome sequences studied here, ranging from small ~5 Kbp cryptic mobilizable plasmids, to large conjugative plasmids of up to ~120 Kbp carrying complete conjugative T4SS (Tra). While plasmids were identified in most sub-lineages/CCs considered virulent, they seem less common in the putatively avirulent ones, with several isolates displaying complete absence (Table 1). Additional plasmids not shown here notably exist amongst the non-complete genome assemblies. BLAST searches in all 86 genomes for plasmids identified within complete assemblies (BLASTn, replication protein) revealed them to be strictly sub-lineage/CC-specific with two exceptions, i.e. the small mobilizable cryptic plasmid pYR5 present in both CC1 and CC10, and the large conjugative plasmids pYR3 and pYR4, present in CC2 and CC1 respectively. Plasmids pYR3 and pYR4 may be considered as variants of the same plasmid as they share the same replicative and conjugative plasmid backbone (Wrobel et al. 2018b). A large pilin cluster and the conjugative T4SS present on pYR3 and pYR4 have been previously implicated in virulence (Méndez et al. 2009; Wrobel et al. 2018b), which is further supported here by their unique presence in the highly virulent CC1 and CC2. The variety of plasmids identified amongst virulent strains, and the complete absence of

plasmids in CC5 (also virulent), strongly indicates that no specific plasmid or the presence of plasmids in general are essential for virulence in salmonid fish. The omnipresence of pYR3/pYR4 across the highly virulent and successful CC1 and CC2 does however indicate some competitive advantage for this plasmid relating to virulence or survival in aquaculture environments.

The toxin complex locus

Insecticidal Toxin Complexes, or simply Toxin Complexes (Tc), are high-molecular weight secreted protein complexes known to provide insecticidal properties (Pinheiro & Ellar 2007), with activity against mammalian cells demonstrated for the Tc gene products of *Y. enterocolitica* and *Y. pestis* (Tennant et al. 2005; Hares et al. 2008). Although the presence of Tc genes in *Y. ruckeri* has been mentioned previously (Barnes et al. 2016; Cascales et al. 2017), no study has investigated them specifically. We found the presence of the Tc gene cluster, consisting of A, B and C toxin components TcA (WP_162486770), TcdB (WP_234049470) and RhsA (WP_096823580), to align well with pathogenicity towards Atlantic salmon (CC1, CC3 and CC10 in Norway, and CC5 in Australia). These genes are, however, absent in the rainbow trout-associated CC2, which notably constitutes the most studied *Y. ruckeri* sub-lineage to date, and this may be the reason for *Y. ruckeri* not being included in studies of *Yersinia* Tc genes (Fuchs et al. 2008; Springer et al. 2018). The Tc cluster has an identical distribution as fimbria cluster C located immediately upstream. A role in virulence or host-specificity for the Tc genes and/or this associated fimbria cluster seems likely given the distribution of these genes. They cannot be crucial components for *Y. ruckeri* virulence in general, however, given their absence in the highly virulent and globally prominent CC2.

The Yts1-Ysa locus

The Yts1 T2SS and Ysa T3SS of *Y. ruckeri* correspond to the Yts1 and Ysa of *Y. enterocolitica* (BLAST), located within the 199 kb 'plasticity zone' of enteropathogenic biotype 1B strains (Thomson et al. 2006). While some evidence suggests a role in survival within host macrophages (Bent et al. 2015), the exact role during infection of these secretion systems is not well understood. Both systems are nonetheless required for full virulence of *Y. enterocolitica* in mammalian hosts following per-oral administration (Haller et al. 2000; Iwobi et al. 2003; Venecia & Young 2005). The Yst1-Ysa1 locus is present throughout the deepest branching *Y. ruckeri* lineages, indicating its presence in the founding lineage, although it appears to be lost in several sub-lineages within lineage B which exclusively contain putatively avirulent strains. The phylogenetic placement of strains that have suffered deletions in the Yst1-Ysa1 locus suggests several independent deletion events, as does the variation in genes deleted (Fig 3). While the Ysa T3SS is largely conserved in

virulent strains, all investigated isolates of the virulent CC10 display a large deletion that includes the phosphorelay system, operator region, and a range of essential injectosome proteins, indicating that Ysa is not essential for virulence in Atlantic salmon. The loss of the Yts1 T2SS is, on the other hand, seemingly exclusive to certain putatively avirulent sub-lineages/CCs and may thus be required for virulence. However, the broad presence of both intact Ysa and Yts1 in several other putatively avirulent strains does not implicate these systems in playing a major role in enabling virulence.

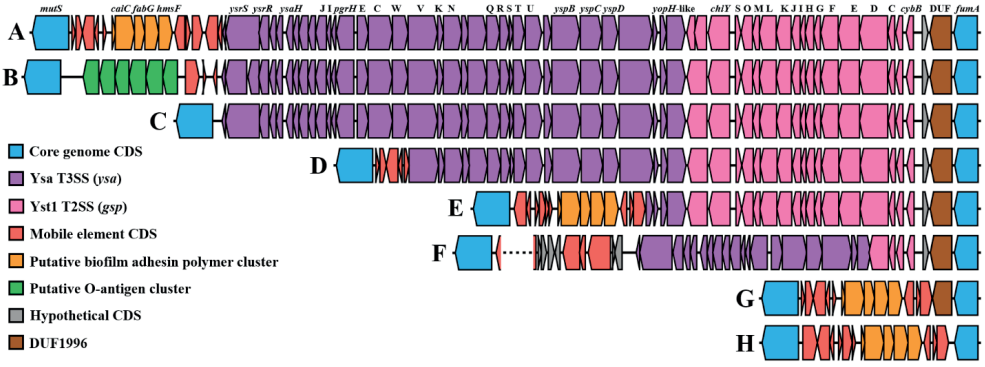

Fig 3: Genetic organization of the Ysa-Yts1 locus, between *mutS* and *fumA* in the *Y. ruckeri* core genome. Each gene is colored according to association with a system or other specific role as indicated by the panel on the left. Nomenclature for Ysa (*ysa*) and Yst1 (*gsp*) is according to Venecia and Young (2005) and Iwobi et al. (2003). The putative O-antigen cluster consists of protein sequences (green) WP_045844464-67 and WP_042527525. Genotypes, labelled A to H, represent the following sequences: (A) NVI-10705 and typical configuration of serotype O1 sequences that possess intact Ysa and Yst1; (B) NVI-9681 and typical configuration of serotype O2 with a O-antigen cluster in green; (C) SC09; (D) NVI-492 with partial Ysa deletion characteristic of CC10; (E) NVI-11073 with Ysa deletion; (F) NVI-5635 with partial Ysa and Yst1 deletion and a putative ICE inserted between *mutS* and the partial Ysa; (G) NVI-4479 with complete deletion of Ysa and Yst1; (H) NVI-11065 with complete deletion of Ysa and Yst1 including DUF 1996. The dotted line indicates a contig split (incomplete genome sequence).

Chromosomal type IV secretion system

T4SSs are capable of transmitting DNA and proteins to neighboring cells through a conjugative pilus and are essential components of conjugative plasmids and ICE, although specialized variants adapted for natural competence or virulence exist, and T4SS are key virulence factors in a number of bacterial species (Zhu et al., 2000; Cascales & Christie 2003; Voth, Broederdorf, & Graham 2012).

The virB/virD4-type T4SS ICE present in CC1, CC2 and strains SC09 and NVI-11076, have been linked to virulence in *Y. ruckeri* strain SC09 by a recent series of publications. These studies focused on a set of effector genes located immediately downstream of the core ICE components, the products of which are presumably secreted into host cells by the ICE T4SS or a different secretion system during infection (Liu et al. 2019; Liu et al. 2020). The downstream genes mobilized by the ICE are however quite variable between strains (Liu et al. 2020), and in other *Y. ruckeri* strains/lineages generally have functions associated with DNA-repair and recombination (Fig 4). While the effector genes are present exclusively in strain SC09 (Table 2), the presence of this virB/virD4-type T4SS ICE in the highly virulent CC1 and CC2, while uncommon in *Y. ruckeri* otherwise, may indicate some role in virulence. Besides secretion of effectors, some specialized T4SS contribute to virulence by the T4SS pilus conferring attachment to host cells (Vayssier-Taussat et al. 2010), and even non-pilus T4SS surface structures may grant cell-to-cell adhesive capabilities (Gonzalez-Rivera et al. 2019). As such, while this locus cannot be essential for virulence in salmonids, a potential role in *Y. ruckeri* virulence should not be ruled out.

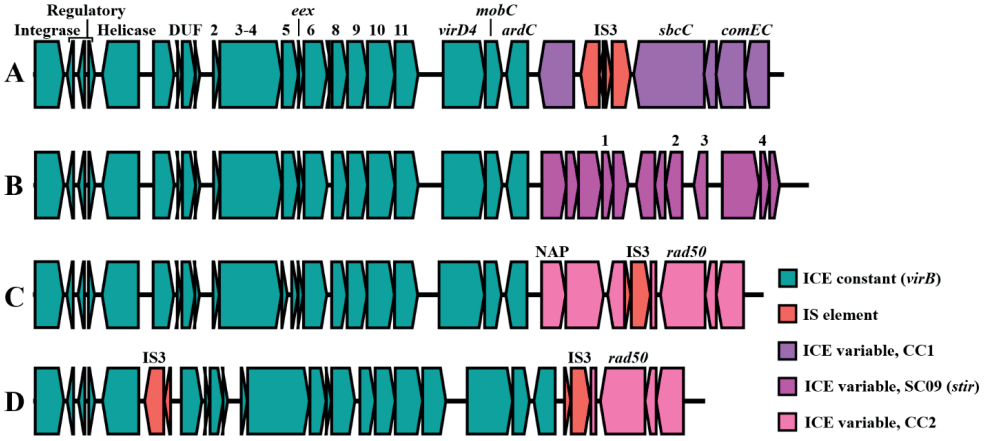

Fig 4: Genetic organization of the accessory VirB/VirD4 ICE and associated genes. Genes are coloured according to function or association with each variant. Genes indicated by number represent *virB2* - *virB11*. *virB3* and *virB4* are fused. Genotypes A to D represent the following sequences: (A) NVI-10705 and typical configuration in CC1 isolates with downstream genes exclusive to CC1; (B) SC09 with downstream genes exclusive to this strain; (C) CFS007-82 with typical configuration of CC2 isolates; (D) NVI-11076 (CC7) with some of the downstream genes present in CC2.

Inverse autotransporter invasins

The inverse autotransporter (IAT) protein family, also known as type Ve-secreted proteins, are characterized by an N-terminal β-barrel domain embedded in the outer membrane of Gram-negative bacteria. This structure functions both as an anchor and facilitator for secretion of the C-terminal domain of the same protein, which becomes exposed on the surface of the cell (Tsai et al. 2010). These proteins are often referred to as adhesins or invasins as they have been shown to facilitate adhesion to and invasion of host cells or tissues (Tsai et al. 2010; Leo et al. 2015; Sadana et al. 2017). Two IAT-invasins in *Y. ruckeri* have been studied in detail recently in a series of papers that describe *yrIlm* and *yrInv* and their contribution to biofilm formation and virulence (Wrobel et al. 2018a; Wrobel et al. 2020). In our dataset, we identified four IAT-invasins, all consisting of an N-terminal inverse-autotransporter domain, an array of bacterial immunoglobulin-like domains (BIG), and a C-terminal lectin-like domain.

yrIlm contains up to 22 nearly identical BIG-domains, is situated between the virulence factors *yhlA* hemolysin and AFP (Wrobel et al. 2018a), within a composite transposon flanked by IS256 transposase direct repeats (Fig 5a). Strikingly, the distribution of *yrIlm* seems to correlate fully with virulence, being present in all sub-lineages well documented as causing yersiniosis in fish, while being absent from all those considered avirulent (Fig 1). Its presence in the virulent Australian CC5 (Atlantic salmon), and absence in CC7 and CC9, may explain the observed difference in virulence between these relatively closely related sub-lineages.

The paralogous and notably shorter *yrInv* is also present in many virulent strains, although occurring throughout lineage B2 while absent in the virulent CC5, and can therefore not be considered essential for, or an enabler of, virulence. Furthermore, an IAT-invasin (WP_042527435) gene is widespread amongst *Yersinia* species at >60% AA identity, identical protein domain-organization and chromosomal location (between *aroP* and *ampE*), to our knowledge previously undescribed, is present in *Y. ruckeri* lineages B2 and YRB and thus appears to be negatively correlated with pathogenicity in salmonids. Finally, a novel IAT-invasin [accession pending] represents a paralogue of *yrInv* and *yrIlm,* with size and protein domain-organization similar to *yrInv*, although found exclusively in two nearly identical isolates within lineage B (NVI-4479, NVI-4493) and is thus not of significance for *Y. ruckeri* virulence.

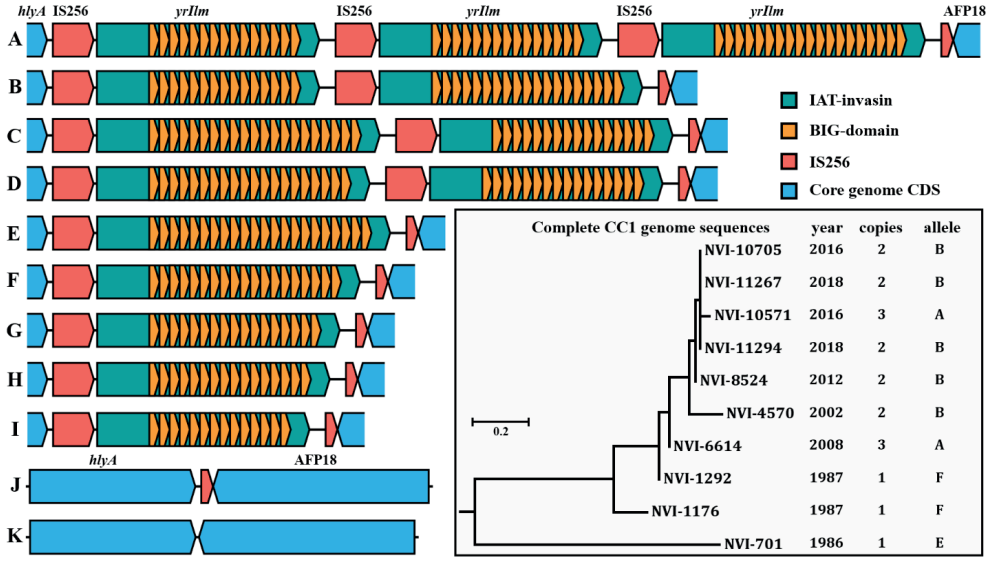

Fig 5: Genetic organization of the *yrIlm* locus, between *yhlA* and AFP18 in the *Y. ruckeri* core genome and maximum likelihood core gene phylogeny of complete CC1 genomes indicating year of isolation, *yrIlm* copy number and variant (bottom right). The complete sequence of strain NVH-3758 (CP023184; not included in this tree), isolated in Norway in 1987, contains a single copy of *yrIlm* (Wrobel et al. 2018a). Locus variants, labelled A to K, represent the following complete genomes: (A) NVI-6614 and NVI-10571 (CC1); (B) most modern CC1; (C) CFS007-82 (CC2); (D) KMM821 (CC2); (E) NVI-701 (CC1) and NVI-9681 (CC3); (F) NVI-1176 and NVI-1292 (CC1), and QMA0440 (CC5); (G) BigCreek74; (H) NVI-492 (CC10); (I) SC09; (J) empty site with partial IS256 in lineage B2; (K) empty site with no IS256 in lineages B1 and YRB, and MLVA clonal complexes CC7 and CC9.

yrIlm gene amplification

Gene amplification involving complex transposons is a mechanism utilized by bacteria to rapidly increase expression levels of a beneficial protein under extreme selection pressure, and it is often associated with increased antibiotic resistance following amplification of resistance cassettes (Sandegren & Andersson 2009). Amplification of *yrIlm* is present in both of the complete genome sequences for CC2 (Fig 5). The temporal (1982/2000) and spatial (USA/Russia) separation of these strains indicates that *yrIlm* duplication may be a characteristic feature of CC2. In contrast, early isolates (late 1980s) of the exclusively Norwegian CC1, contain only a single *yrIlm* copy, whereas all later isolates are found to harbour two or three copies, suggesting gradual gene amplification over the last few decades. (Fig 5b).

Not only does amplification of *yrIlm* likely increase expression levels, the additional allele displays differing numbers of BIG-repeats, likely affecting the length of the protein extending from the cell surface which may in turn affect adherence to host cells or other surfaces. However, as most available *Y. ruckeri* genome sequences are based on short read sequencing technologies that are inherently unsuitable for accurately sequencing such complex repeat structures, amplification of *yrIlm* may be more widespread than revealed here.

While the presence of flanking direct repeats may facilitate amplification, these also make the element prone to loss by homologous recombination in the absence of a positive selection pressure (Sandegren & Andersson 2009). Loss of *yrIlm* by homologous recombination seems to have occurred in some lineages in which a sequence corresponding to a single partial IS256 remains at this locus (Fig 5). The complete absence of IS256 sequence in lineages B1 and YRB, as well as in CC7 and CC9, indicates loss by a different mechanism, or that the element was never present in these lineages.

Conclusions

A relatively small number of highly virulent strains of *Y. ruckeri* are capable of causing serious disease outbreaks in salmonid aquaculture. Our findings point to the presence of a single gene, the IAT-invasin *yrIlm*, as the likely main contributor to virulence in Atlantic salmon and rainbow trout pathogenic strains, as this gene is present in all virulent sub-lineages and is amplified in the highly virulent CC1 and CC2. Amplification of *yrIlm* may contribute towards explaining the increasing impact of yersiniosis in Norwegian aquaculture from the mid-2000s and onwards (Gulla et al. 2018; Gulla & Olsen 2020). In virulent strains of non-O1 serotypes, where *yrIlm* is present, the lack of serotype O1 LPS may be responsible for the oft-cited lower virulence of such strains. While a multitude of other virulence-associated accessory features are also present in the pan-genome, some are more common in virulent lineages and likely provide some general or host-specific benefits. However, they seem to be less critical as they are generally not omnipresent amongst virulent strains. Further analysis is needed to verify the role of YrIlm and the importance of *yrIlm* copy number in virulence. In addition, further work is needed to define the role of the other accessory genetic determinants that may or may not contribute to variance in virulence and host specialization.

Materials and Methods

Strains & culture

Detailed information on the *Y. ruckeri* isolates sequenced is provided in Table S1. Isolates were cultured at 22°C on 5% bovine blood agar (BA) or in tryptic soy broth (TSB). Sorbitol and ONPG phenotypes were assessed with API20e (BioMérieux, Marcy-l'Étoile, France) according to the manufacturers recommendations, although incubated at 22°C for 48 hours. SDS phenotype was assessed on TSA agar plates with 1% SDS prepared according to Furones, Gilpin & Munn (1992), with colonies surrounded by crystalline deposits after 48h incubation at 22°C interpreted as a positive result. MLVA typing was performed according to Gulla, Mohammad & Colquhoun (2019) or data derived from previous work (Gulla et al. 2018; Riborg et al. 2022a). Serotypes were derived from previously published data or typed using in-house polyclonal rabbit antisera for *Yersinia ruckeri* O1, O2 and O5 with the slide agglutination technique.

Preperation of sequencing templates

For Illumina sequencing, DNA templates were extracted from pure cultures on BA with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations for Gram-negative bacteria. For nanopore sequencing, DNA templates were extracted from overnight culture in TSB with the Gentra Puregene Yeast/Bact. Kit (Qiagen) following the manufacturer's descriptions for Gram-negative bacteria. DNA templates were assessed for purity with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), quantified by Qubit dsDNA HS Assay Kit on a Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and checked for integrity by agarose gel electrophoresis.

Whole genome sequencing

Genome assemblies were downloaded from the National Center for Biotechnology Information (NCBI), derived from previous work (Riborg et al. 2022a), or generated from 300bp paired end sequencing libraries prepared with NexteraFlex (Illumina, San Diego, CA, USA) and sequenced with the MiSeq platform (Illumina). Trimmomatic v0.38 (Bolger, Lohse & Usadel, 2014) was used for trimming adapter sequences, cropping low-quality nucleotides from raw reads and filtering out raw reads prior to *de novo* assembly with SPAdes v3.13.0 or v3.9.0 (Bankevich et al. 2012) using the '--careful' option and otherwise default settings, or with Unicycler v0.4.8 (Wick et al. 2017) with default settings ('normal' mode) (see S1 Table).

Phylogenetic analysis

Annotation generated and downloaded genome assemblies was performed with Prokka v1.13 (Seemann, 2014) with default settings. Core-gene alignments were generated using Roary v3.12.0 (Page et al. 2015) with the MAFFT aligner, using a 95% identity cut-off, with a core definition of presence in 100% of isolates (option '-e --mafft -i 95 -cd 100'), and concatenated using snp-sites v2.4.1 (Page et al. 2016). Maximum likelihood trees were generated with MEGA v10.2 (Kumar et al. 2018), bootstrap re-sampling (200 replicates) was used to assess branch support . Trees were visualized with MEGA (Figs 2, 5b and S1), or in R (R Core Team, 2012) with the ggtree package (Yu et al. 2017) (Fig 1).

Generation of complete genome assemblies

For generation of complete genome assemblies, to complement the already/publicly available complete assemblies, strains were selected based on core-gene phylogeny to cover the majority of relevant *Y. ruckeri* strains/lineages. Sequencing libraries for each strain selected for nanopore sequencing were prepared from 500 ng of genomic DNA with the Oxford Nanopore ligation sequencing kit (SQK-LSK109, Oxford Nanopore Technologies (ONT), Oxford, UK), following the protocol for Flongle as described by the manufacturer, with no fragmentation performed. External reagents used were the NEBNext Ultra II end repair/dA-tailing module (New England Biolabs, catalog #E7546) and AMPure XP paramagnetic beads (Beckman Coulter Life Sciences, CA, USA, catalog #A63880). Sequencing libraries were loaded onto Flongle flow cells (FLO-MIN106, ONT) and sequenced for 24 h with a MinION sequencer (ONT). Base calling was performed with Guppy v4.0.15 (ONT). Base called reads were filtered NanoFilt v2.7.1 (De Coster et al 2018) by quality ('-q 8') and length (8Kbp, '-l 8000'). To ease hybrid assembly, base called reads were filtered by increasing length until an uncompressed file size of approximately 500MB was obtained. Hybrid assemblies were produced with Unicycler with default settings by supplying trimmed Illumina reads and base called, filtered nanopore reads for each strain.

To assess assembly quality of the highly repetitive *yrIlm*-region (between *yhlA* and AFP18 in the core genome), the region was, for the hybrid assemblies, aligned with nanopore-only assemblies produced with Flye version 2.9 (Kolmogorov et al. 2019; genome size set to 4Mbp and otherwise default options), as well as with five individual base called filtered nanopore reads spanning the entire *yrIlm*-region. In cases where Unicycler was in agreement with all individual nanopore reads, the Unicycler assembly was accepted. Otherwise, if the Flye assembly was in agreement with all individual nanopore reads, the Flye assembly was polished by mapping Illumina reads with

Bowtie2 v2.3.4 (Langmead & Salzberg 2012) and the consensus of mapped reads was extracted with sam2consensus v2 (developed by Edgardo M. Ortiz & Deise J. P. Gonçalves). The consensus was then used as a template to manually edit the Unicycler-produced hybrid assembly to account for missing repeats (mapped consensus sequence added) or excessive repeats (sequence deleted). Individual nanopore reads successfully aligning to both *yhlA* and AFP18 were always in agreement with each other in terms of the presence and number of duplicated *yrllm* and internal 300bp BIG-domain repeats.

Secondary circular contigs were identified as plasmids by BLAST and/or identification of a plasmid replication protein. Plasmids were removed from assemblies prior to complete pan-genome analyses.

Pan-genome analysis

The complete genome of strain NVI-10705 (CC1) was selected as the base for building the pan-genome. Each additional genome was utilized as a reference sequence with the current pan-genome aligned as a query with BLAST Ring Image Generator (BRIG) v0.95 (Alikhan et al. 2011), using the option 'perc_identity 85' and otherwise default BLAST settings. Genetic features not already present in the pan-genome were characterized according to annotation (Prokka annotations and annotations of the assemblies downloaded from NCBI) or BLASTp, or by genetic context (i.e. new hypothetical genes that were intermixed with prophage genes were characterized as 'prophage-related') and added to the pan-genome.

In silico screening analysis

All genomes were assayed for novel and previously described putative virulence factors (including serotype- and metabolism-related) by BLASTn. Genes found to be split between different contigs in the Illumina-only sequences (e.g. *yrllm*) were assayed using a truncated version as a query. For gene clusters (e.g. Tc genes and putative O-antigen clusters) and larger systems (e.g. secretion systems and O1-LPS), nucleotide sequences of three genes evenly spaced throughout the cluster were used as queries. In cases where these three searches did not agree (e.g. for Ysa and Yst1 in some instances), sequences were assessed individually by manual inspection to obtain presence/absence data for Fig 1.

Genetic maps (Figs 3, 4, 5) were visualized with DNAplotlib v1.0 (Der et al. 2017) based on annotated sequence data. BIG-domains in IAT-invasins were identified by BLASTp.

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Supplementary figures

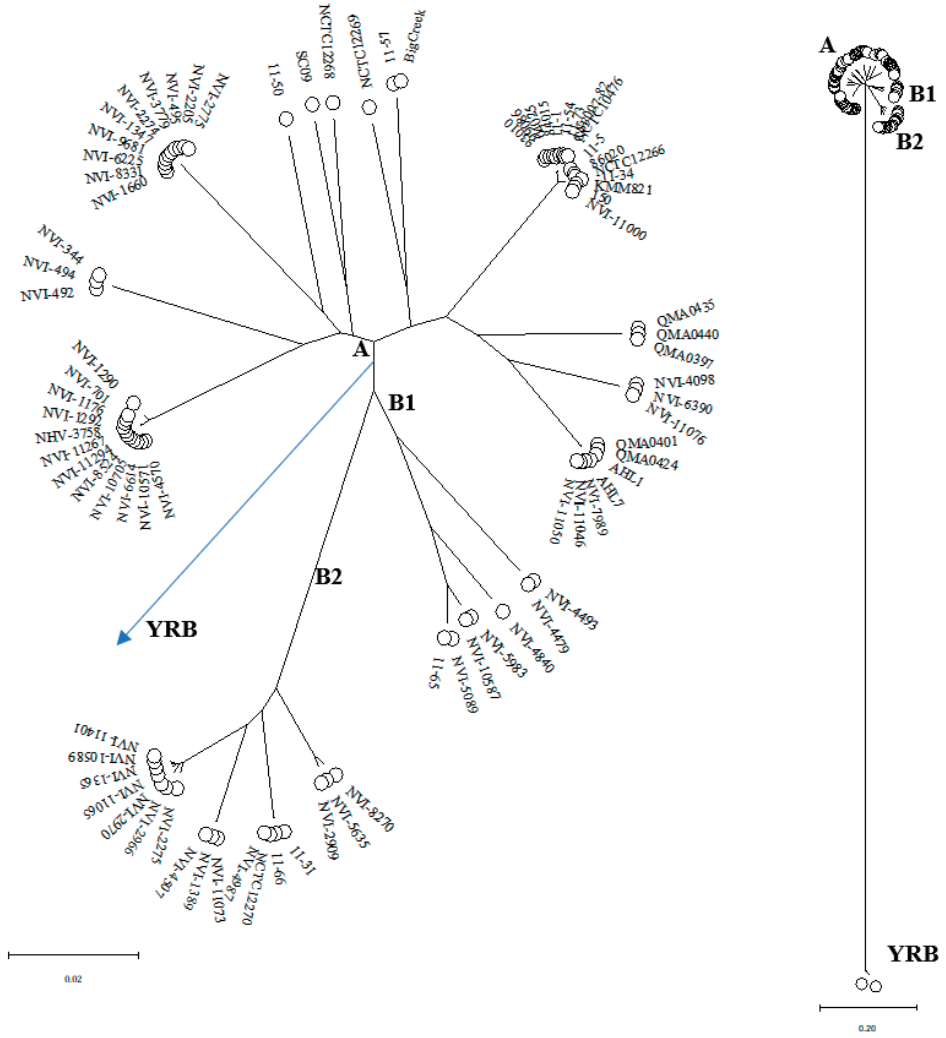
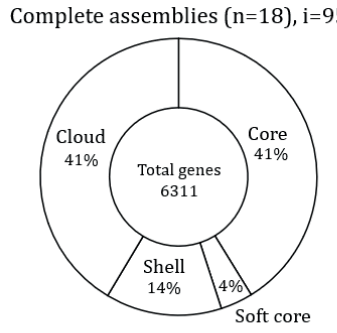
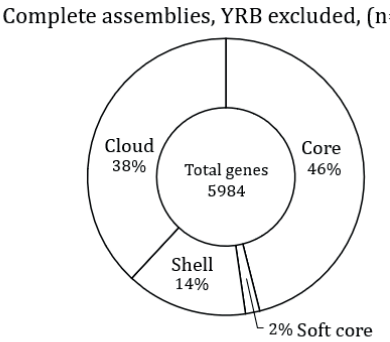


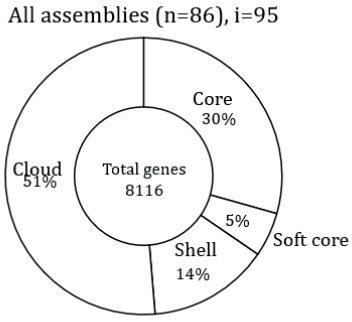
Fig S1: Core gene phylogeny, radial-style ML trees. The left tree is a subtree with lineage YRB excluded, with the attachment point of the YRB branch indicated by blue arrow. The right tree is the complete tree. See Fig 1 for bootstrap details and relevant metadata for each sequence.



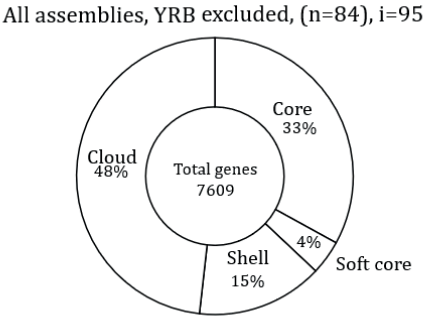
Core genes	(99% <= strains <= 100%)	2601
Soft core genes	(95% <= strains < 99%)	239
Shell genes	(15% <= strains < 95%)	857
Cloud genes	(0% <= strains < 15%)	2614
Total genes	(0% <= strains <= 100%)	6311



Core genes	(99% <= strains <= 100%)	2756
Soft core genes	(95% <= strains < 99%)	100
Shell genes	(15% <= strains < 95%)	849
Cloud genes	(0% <= strains < 15%)	2279
Total genes	(0% <= strains <= 100%)	5984



Core genes	(100% <= strains <= 100%)	2387
Soft core genes	(95% <= strains < 100%)	422
Shell genes	(15% <= strains < 95%)	1148
Cloud genes	(0% <= strains < 15%)	4172
Total genes	(0% <= strains <= 100%)	8129



Core genes	(100% <= strains <= 100%)	2516
Soft core genes	(95% <= strains < 100%)	306
Shell genes	(15% <= strains < 95%)	1126
Cloud genes	(0% <= strains < 15%)	3676
Total genes	(0% <= strains <= 100%)	7624

552
553 **Fig S2:** Summary statistics from Roary output respectively for complete genome assemblies only
554 (top) and all assemblies (bottom), with (left) and without (right) YRB lineage genomes. The core
555 gene alignment from 'All assemblies (n=86)' was used to generate the trees in Fig 1 and Fig S1.

References:

Alikhan, N. F., Petty, N. K., Ben Zakour, N. L., & Beatson, S. A. (2011). BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. BMC genomics, 12, 402. https://doi.org/10.1186/1471-2164-12-402

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., Lesin, V. M., Nikolenko, S. I., Pham, S., Prjibelski, A. D., Pyshkin, A. V., Sirotkin, A. V., Vyahhi, N., Tesler, G., Alekseyev, M. A., & Pevzner, P. A. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of computational biology : a journal of computational molecular cell biology, 19*(5), 455–477. https://doi.org/10.1089/cmb.2012.0021

Barnes, A. C. (2011). Enteric redmouth disease (ERM) (*Yersinia ruckeri*). In Woo, P. T. K., & Bruno, D. W. (Eds.), Fish diseases and disorders. Volume 3: viral, bacterial and fungal infections (pp. 484-511). Published by CABI. http://dx.doi.org/10.1079/9781845935542.0000

Bent, Z. W., Poorey, K., Brazel, D. M., LaBauve, A. E., Sinha, A., Curtis, D. J., House, S. E., Tew, K. E., Hamblin, R. Y., Williams, K. P., Branda, S. S., Young, G. M., & Meagher, R. J. (2015). Transcriptomic Analysis of Yersinia enterocolitica Biovar 1B Infecting Murine Macrophages Reveals New Mechanisms of Extracellular and Intracellular Survival. Infection and immunity, 83(7), 2672–2685. https://doi.org/10.1128/IAI.02922-14

Bastardo, A., Bohle, H., Ravelo, C., Toranzo, A. E., & Romalde, J. L. (2011). Serological and molecular heterogeneity among *Yersinia ruckeri* strains isolated from farmed Atlantic salmon *Salmo salar* in Chile. *Diseases of aquatic organisms*, *93*(3), 207–214. https://doi.org/10.3354/dao02296

Bullock, G. L., Stuckeye, H.M., & Shotts, J.R. (1978). Enteric redmouth bacterium: comparison of isolates from different geographic areas. *Journal of Fish Diseases, 1*(4), 351–356. doi:10.1111/j.1365-2761.1978.tb00039.x

Cascales, E., & Christie, P. J. (2003). The versatile bacterial type IV secretion systems. *Nature reviews. Microbiology*, *1*(2), 137–149. https://doi.org/10.1038/nrmicro753

Cascales, D., Guijarro, J. A., García-Torrico, A. I., & Méndez, J. (2017). Comparative genome analysis reveals important genetic differences among serotype O1 and serotype O2 strains of Y. ruckeri and provides insights into host adaptation and virulence. *MicrobiologyOpen*, *6*(4), e00460. https://doi.org/10.1002/mbo3.460

Cornelis, G. R., Boland, A., Boyd, A. P., Geuijen, C., Iriarte, M., Neyt, C., Sory, M. P., & Stainier, I. (1998). The virulence plasmid of *Yersinia*, an antihost genome. *Microbiology and molecular biology reviews : MMBR*, *62*(4), 1315–1352. https://doi.org/10.1128/MMBR.62.4.1315-1352.1998

De Coster, W., D'Hert, S., Schultz, D. T., Cruts, M., & Van Broeckhoven, C. (2018). NanoPack: visualizing and processing long-read sequencing data. *Bioinformatics (Oxford, England)*, *34*(15), 2666–2669. https://doi.org/10.1093/bioinformatics/bty149

Dahiya, I., & Stevenson, R. M. (2010a). The ZnuABC operon is important for Yersinia ruckeri infections of rainbow trout, Oncorhynchus mykiss (Walbaum). *Journal of fish diseases*, *33*(4), 331–340. https://doi.org/10.1111/j.1365-2761.2009.01125.x

Dahiya, I., & Stevenson, R. M. (2010b). The UvrY response regulator of the BarA-UvrY two-component system contributes to Yersinia ruckeri infection of rainbow trout (Oncorhynchus mykiss). *Archives of microbiology*, *192*(7), 541–547. https://doi.org/10.1007/s00203-010-0582-8

Der, B. S., Glassey, E., Bartley, B. A., Enghuus, C., Goodman, D. B., Gordon, D. B., Voigt, C. A., & Gorochowski, T. E. (2017). DNAplotlib: Programmable Visualization of Genetic Designs and Associated Data. *ACS synthetic biology*, *6*(7), 1115–1119. https://doi.org/10.1021/acssynbio.6b00252

Dillard, J. P., & Seifert, H. S. (2001). A variable genetic island specific for *Neisseria gonorrhoeae* is involved in providing DNA for natural transformation and is found more often in disseminated infection isolates. *Molecular microbiology*, *41*(1), 263–277. https://doi.org/10.1046/j.1365-2958.2001.02520.x

Fernández, L., Márquez, I., & Guijarro, J. A. (2004). Identification of specific in vivo-induced (ivi) genes in Yersinia ruckeri and analysis of ruckerbactin, a catecholate siderophore iron acquisition system. *Applied and environmental microbiology*, *70*(9), 5199–5207. https://doi.org/10.1128/AEM.70.9.5199-5207.2004

Fuchs, T. M., Bresolin, G., Marcinowski, L., Schachtner, J., & Scherer, S. (2008). Insecticidal genes of *Yersinia* spp.: taxonomical distribution, contribution to toxicity towards *Manduca sexta* and *Galleria mellonella*, and evolution. *BMC microbiology*, *8*, 214. https://doi.org/10.1186/1471-2180-8-214

Furones, M. D., Gilpin, M. J., Alderman, D. J., & Munn, C. B. (1990). Virulence of Yersinia ruckeri serotype I strains is associated with a heat sensitive factor (HSF) in cell extracts. *FEMS microbiology letters*, *54*(1-3), 339–343. https://doi.org/10.1016/0378-1097(90)90309-e

Furones, M. D., Gilpin, M. L., & Munn, C. B. (1993). Culture media for the differentiation of isolates of Yersinia ruckeri, based on detection of a virulence factor. *The Journal of applied bacteriology*, *74*(4), 360–366. https://doi.org/10.1111/j.1365-2672.1993.tb05139.x

Garcia, J. A., Dominguez, L., Larsen, J. L., & Pedersen, K. (1998). Ribotyping and plasmid profiling of *Yersinia ruckeri*. *Journal of Applied Microbiology*, 85(6), 949–955. doi:10.1111/j.1365-2672.1998.tb05258.x

De Grandis, S.A. & Stevenson, R.M.W. (1982). Variations in plasmid profiles and growth characteristics of *Yersinia ruckeri* strains. *FEMS Microbiology Letters,* Volume 15, Issue 3, Pages 199-202. https://doi.org/10.1111/j.1574-6968.1982.tb00067.x

Gudmundsdottir, B. K., Gudmundsdottir, S., Gudmundsdottir, S., & Magnadottir, B. (2014). Yersiniosis in Atlantic cod, *Gadus morhua* (L.), characterization of the infective strain and host reactions. *Journal of fish diseases*, *37*(6), 511–519. https://doi.org/10.1111/jfd.12139

Guilvout, I., Quilici, M. L., Rabot, S., Lesel, R., & Mazigh, D. (1988). BamHI restriction endonuclease analysis of *Yersinia ruckeri* plasmids and their relatedness to the genus *Yersinia* 42- to 47-megadalton plasmid. *Applied and environmental microbiology*, 54(10), 2594–2597. https://doi.org/10.1128/aem.54.10.2594-2597.1988

Gulla, S., & Olsen, A. B. (2020). Yersiniosis. In Sommerset I., Walde, C. S., Bang Jensen, B., Bornø, B., Haukaas, A., & Brun, E. (Eds.), *The health situation in Norwegian aquaculture 2019* (pp. 80-82). Published by the Norwegian Veterinary Institute. Retrieved from https://www.vetinst.no/rapporter-og-publikasjoner/rapporter/2020/fish-health-report-2019

Gulla, S., Barnes, A. C., Welch, T. J., Romalde, J. L., Ryder, D., Ormsby, M. J., Carson, J., Lagesen, K., Verner-Jeffreys, D. W., Davies, R. L., & Colquhoun, D. J. (2018). Multilocus Variable-Number Tandem-Repeat Analysis of Yersinia ruckeri Confirms the Existence of Host Specificity, Geographic Endemism, and Anthropogenic Dissemination of Virulent Clones. *Applied and environmental microbiology*, *84*(16), e00730-18. https://doi.org/10.1128/AEM.00730-18

Gunasena, D.K., Komrower, J.R., & Macintyre, S. (2004). The Fish Pathogen *Yersinia ruckeri* Possesses a TTS System. In: Skurnik, M., Bengoechea, J.A., & Granfors, K. (eds) The Genus Yersinia. Advances in Experimental Medicine and Biology, vol 529. Springer, Boston, MA. https://doi.org/10.1007/0-306-48416-1_19

Haig, S. J., Davies, R. L., Welch, T. J., Reese, R. A., & Verner-Jeffreys, D. W. (2011). Comparative susceptibility of Atlantic salmon and rainbow trout to *Yersinia ruckeri*: relationship to O antigen serotype and resistance to serum killing. *Veterinary microbiology*, *147*(1-2), 155–161. https://doi.org/10.1016/j.vetmic.2010.06.022

Haller, J. C., Carlson, S., Pederson, K. J., & Pierson, D. E. (2000). A chromosomally encoded type III secretion pathway in *Yersinia enterocolitica* is important in virulence. *Molecular microbiology*, *36*(6), 1436–1446. https://doi.org/10.1046/j.1365-2958.2000.01964.x

Hares, M. C., Hinchliffe, S. J., Strong, P., Eleftherianos, I., Dowling, A. J., Ffrench-Constant, R. H., & Waterfield, N. (2008). The Yersinia pseudotuberculosis and Yersinia pestis toxin complex is active against cultured mammalian cells. *Microbiology (Reading, England)*, *154*(Pt 11), 3503–3517. https://doi.org/10.1099/mic.0.2008/018440-0

Hofreuter, D., Odenbreit, S., & Haas, R. (2001). Natural transformation competence in *Helicobacter pylori* is mediated by the basic components of a type IV secretion system. *Molecular microbiology*, *41*(2), 379–391. https://doi.org/10.1046/j.1365-2958.2001.02502.x

Iwobi, A., Heesemann, J., Garcia, E., Igwe, E., Noelting, C., & Rakin, A. (2003). Novel virulence-associated type II secretion system unique to high-pathogenicity *Yersinia enterocolitica*. *Infection and immunity*, *71*(4), 1872–1879. https://doi.org/10.1128/IAI.71.4.1872-1879.2003

Jank, T., Eckerle, S., Steinemann, M., Trillhaase, C., Schimpl, M., Wiese, S., van Aalten, D. M., Driever, W., & Aktories, K. (2015). Tyrosine glycosylation of Rho by *Yersinia* toxin impairs blastomere cell behaviour in zebrafish embryos. *Nature communications*, *6*, 7807. https://doi.org/10.1038/ncomms8807

Johnson, S. L., Daligault, H. E., Davenport, K. W., Jaissle, J., Frey, K. G., Ladner, J. T., Broomall, S. M., Bishop-Lilly, K. A., Bruce, D. C., Coyne, S. R., Gibbons, H. S., Lo, C. C., Munk, A. C., Rosenzweig, C. N., Koroleva, G. I., Palacios, G. F., Redden, C. L., Xu, Y., Minogue, T. D., & Chain, P. S. (2015). Thirty-Two Complete Genome Assemblies of Nine *Yersinia* Species, Including *Y. pestis, Y. pseudotuberculosis*, and Y. *enterocolitica*. *Genome announcements*, *3*(2), e00148-15. https://doi.org/10.1128/genomeA.00148-15

Kolmogorov, M., Yuan, J., Lin, Y., & Pevzner, P. A. (2019). Assembly of long, error-prone reads using repeat graphs. *Nature biotechnology*, *37*(5), 540–546. https://doi.org/10.1038/s41587-019-0072-8

Langmead, B., & Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nature methods*, *9*(4), 357–359. https://doi.org/10.1038/nmeth.1923

Leo, J. C., Oberhettinger, P., Schütz, M., & Linke, D. (2015). The inverse autotransporter family: intimin, invasin and related proteins. *International journal of medical microbiology : IJMM*, *305*(2), 276–282. https://doi.org/10.1016/j.ijmm.2014.12.011

Liu, T., Li, L., Wei, W., Wang, K., Yang, Q., & Wang, E. (2020). *Yersinia ruckeri* strain SC09 disrupts proinflammatory activation via Toll/IL-1 receptor-containing protein STIR-3. *Fish & shellfish immunology*, *99*, 424–434. https://doi.org/10.1016/j.fsi.2020.02.035

Liu, T., Wang, K. Y., Wang, J., Chen, D. F., Huang, X. L., Ouyang, P., Geng, Y., He, Y., Zhou, Y., & Min, J. (2016). Genome Sequence of the Fish Pathogen *Yersinia ruckeri* SC09 Provides Insights into Niche Adaptation and Pathogenic Mechanism. *International journal of molecular sciences*, *17*(4), 557. https://doi.org/10.3390/ijms17040557

Liu, T., Wang, E., Wei, W., Wang, K., Yang, Q., & Ai, X. (2019). TcpA, a novel *Yersinia ruckeri* TIR-containing virulent protein mediates immune evasion by targeting MyD88 adaptors. *Fish & shellfish immunology*, *94*, 58–65. https://doi.org/10.1016/j.fsi.2019.08.069

McCarthy, D. H., Johnson, K. A. (1982). A serotypic survey and cross-protection test of North American field isolates of *Yersinia ruckeri*. *Journal of fish diseases, 5*: 323-328 https://doi.org/10.1111/j.1365-2761.1982.tb00487.x

Méndez, J., Reimundo, P., Pérez-Pascual, D., Navais, R., Gómez, E., & Guijarro, J. A. (2011). A novel cdsAB operon is involved in the uptake of L-cysteine and participates in the pathogenesis of *Yersinia ruckeri*. *Journal of bacteriology*, *193*(4), 944–951. https://doi.org/10.1128/JB.01058-10

Méndez, J., Fernández, L., Menéndez, A., Reimundo, P., Pérez-Pascual, D., Navais, R., & Guijarro, J. A. (2009). A chromosomally located traHIJKCLMN operon encoding a putative type IV secretion system is involved in the virulence of Yersinia ruckeri. *Applied and environmental microbiology*, *75*(4), 937–945. https://doi.org/10.1128/AEM.01377-08

Navais, R., Méndez, J., Cascales, D., Reimundo, P., & Guijarro, J. A. (2014). The heat sensitive factor (HSF) of *Yersinia ruckeri* is produced by an alkyl sulphatase involved in sodium dodecyl sulphate (SDS) degradation but not in virulence. *BMC microbiology*, *14*, 221. https://doi.org/10.1186/s12866-014-0221-7

Nelson, M. C., LaPatra, S. E., Welch, T. J., & Graf, J. (2015). Complete Genome Sequence of *Yersinia ruckeri* Strain CSF007-82, Etiologic Agent of Red Mouth Disease in Salmonid Fish. *Genome announcements, 3*(1), e01491-14. https://doi.org/10.1128/genomeA.01491-14

Ormsby, M. J., Grahame, E., Burchmore, R., & Davies, R. L. (2019). Comparative bioinformatic and proteomic approaches to evaluate the outer membrane proteome of the fish pathogen *Yersinia ruckeri*. *Journal of proteomics*, *199*, 135–147. https://doi.org/10.1016/j.jprot.2019.02.014

Pinheiro, V. B., & Ellar, D. J. (2007). Expression and insecticidal activity of *Yersinia pseudotuberculosis* and *Photorhabdus luminescens* toxin complex proteins. *Cellular microbiology*, *9*(10), 2372–2380. https://doi.org/10.1111/j.1462-5822.2007.00966.x

Puri, A. W., Owen, S., Chu, F., Chavkin, T., Beck, D. A., Kalyuzhnaya, M. G., & Lidstrom, M. E. (2015). Genetic tools for the industrially promising methanotroph *Methylomicrobium buryatense*. *Applied and environmental microbiology*, *81*(5), 1775–1781. https://doi.org/10.1128/AEM.03795-14

R Core Team (2012). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, http://www.R-project.org/

Riborg, A., Colquhoun, D. J., & Gulla, S. (2022a). Biotyping reveals loss of motility in two distinct Yersinia ruckeri lineages exclusive to Norwegian aquaculture. *Journal of fish diseases*, 10.1111/jfd.13590. Advance online publication. https://doi.org/10.1111/jfd.13590

Riborg, A., Gulla, S., Strand, D., Wiik-Nielsen, J., Rønneseth, A., Welch, T. J., Spilsberg, B., & Colquhoun, D. J. (2022b). qPCR screening for *Yersinia ruckeri* clonal complex 1 against a background of putatively avirulent strains in Norwegian aquaculture. *Journal of fish diseases*, *45*(8), 1211–1224. https://doi.org/10.1111/jfd.13656

Rintamäki, P., Valtonen, E.T., & Frerichs, G.N. (1986). Occurrence of *Yersinia ruckeri* infection in farmed whitefish, *Coregonus peled* Gmelin and *Coregonus muksun* Pallas, and Atlantic salmon, *Salmo salar* L., in northern Finland. Journal of Fish Diseases, 9: 137-140. https://doi.org/10.1111/j.1365-2761.1986.tb00993.x

Romalde, J. L., MagariÑos, B., Barja, J. L., & Toranzo, A. E. (1993). Antigenic and Molecular Characterization of *Yersinia ruckeri* Proposal for a New Intraspecies Classification. *Systematic and Applied Microbiology, 16*(3), 411-419. https://doi.org/10.1016/S0723-2020(11)80274-2.

Sadana, P., Mönnich, M., Unverzagt, C. and Scrima, A. (2017), Structure of the *Y. pseudotuberculosis* adhesin InvasinE. *Protein Science, 26*: 1182-1195. https://doi.org/10.1002/pro.3171

Sandegren, L., & Andersson, D. I. (2009). Bacterial gene amplification: implications for the evolution of antibiotic resistance. *Nature reviews. Microbiology*, *7*(8), 578–588. https://doi.org/10.1038/nrmicro2174

Secades, P., & Guijarro, J. A. (1999). Purification and characterization of an extracellular protease from the fish pathogen Yersinia ruckeri and effect of culture conditions on production. *Applied and environmental microbiology*, *65*(9), 3969–3975. https://doi.org/10.1128/AEM.65.9.3969-3975.1999

Severi, E., Hood, D. W., & Thomas, G. H. (2007). Sialic acid utilization by bacterial pathogens. *Microbiology (Reading, England)*, *153*(Pt 9), 2817–2822. https://doi.org/10.1099/mic.0.2007/009480-0

Sparboe, O., Koren, C., Håstein, T., Poppe, T.T., & Stenwig, H. (1986). The first isolation of *Yersinia ruckeri* from farmed Norwegian salmon. *Bulletin of The European Association of Fish Pathologists*, 6, 41-42.

Springer, K., Sänger, P. A., Moritz, C., Felsl, A., Rattei, T., & Fuchs, T. M. (2018). Insecticidal Toxicity of *Yersinia frederiksenii* Involves the Novel Enterotoxin YacT. *Frontiers in cellular and infection microbiology*, *8*, 392. https://doi.org/10.3389/fcimb.2018.00392

Stave, J., Cook, T., & Roberson, B. (1987). Chemiluminescent responses of striped bass, *Morone saxatilis* (Walbaum), phagocytes to strains of *Yersinia ruckeri*. *Journal of Fish Diseases,10*, 1–10. https://doi.org/10.1111/j.1365-2761.1987.tb00712.x

Stevenson, R.M.W., & Daly, J.G. (1982). Biochemical and Serological Characteristics of Ontario Isolates of *Yersinia ruckeri*. *Canadian Journal of Fisheries and Aquatic Sciences, 39*(6), 870–876. doi:10.1139/f82-118

Tennant, S. M., Skinner, N. A., Joe, A., & Robins-Browne, R. M. (2005). Homologues of insecticidal toxin complex genes in *Yersinia enterocolitica* biotype 1A and their contribution to virulence. *Infection and immunity*, *73*(10), 6860–6867. https://doi.org/10.1128/IAI.73.10.6860-6867.2005

Thomson, N. R., Howard, S., Wren, B. W., Holden, M. T., Crossman, L., Challis, G. L., Churcher, C., Mungall, K., Brooks, K., Chillingworth, T., Feltwell, T., Abdellah, Z., Hauser, H., Jagels, K., Maddison, M., Moule, S., Sanders, M., Whitehead, S., Quail, M. A., Dougan, G., Parkhill, J., & Prentice, M. B. (2006). The complete genome sequence and comparative genome analysis of the high pathogenicity *Yersinia enterocolitica* strain 8081. *PLoS genetics*, *2*(12), e206. https://doi.org/10.1371/journal.pgen.0020206

Tsai, J. C., Yen, M. R., Castillo, R., Leyton, D. L., Henderson, I. R., & Saier, M. H., Jr (2010). The bacterial intimins and invasins: a large and novel family of secreted proteins. *PloS one*, *5*(12), e14403. https://doi.org/10.1371/journal.pone.0014403

Vayssier-Taussat, M., Le Rhun, D., Deng, H. K., Biville, F., Cescau, S., Danchin, A., Marignac, G., Lenaour, E., Boulouis, H. J., Mavris, M., Arnaud, L., Yang, H., Wang, J., Quebatte, M., Engel, P., Saenz, H., & Dehio, C. (2010). The Trw type IV secretion system of Bartonella mediates host-specific adhesion to erythrocytes. *PLoS pathogens*, *6*(6), e1000946. https://doi.org/10.1371/journal.ppat.1000946

Venecia, K., & Young, G. M. (2005). Environmental regulation and virulence attributes of the Ysa type III secretion system of *Yersinia enterocolitica* biovar 1B. *Infection and immunity, 73*(9), 5961–5977. https://doi.org/10.1128/IAI.73.9.5961-5977.2005

Verner-Jeffreys, D., Haig, S. J, Welch, T. J, Pond, M. J, Stone, D., Davies, R. L, & Gardiner, R. (2011). Characterisation of a serotype O1 *Yersinia ruckeri* isolate from the Isle of Man: Further evidence that O antigen serotype is not a reliable indicator of virulence. *Bulletin of the European Association of Fish Pathologists, 31*, 86.

Voth, D. E., Broederdorf, L. J., & Graham, J. G. (2012). Bacterial Type IV secretion systems: versatile virulence machines. *Future microbiology*, *7*(2), 241–257. https://doi.org/10.2217/fmb.11.150

Wade, J. 2019. Characterization of Yersinia ruckeri and enteric redmouth disease (ERM) to inform pathogen transfer risk assessments in British Columbia. DFO Canadian Science Advisory Secretariat Research Document 2019/022 available from: https://waves-vagues.dfo-mpo.gc.ca/Library/40853032.pdf

Wang, X., Preston, J. F., 3rd, & Romeo, T. (2004). The pgaABCD locus of *Escherichia coli* promotes the synthesis of a polysaccharide adhesin required for biofilm formation. *Journal of bacteriology*, *186*(9), 2724–2734. https://doi.org/10.1128/JB.186.9.2724-2734.2004

Welch T. J. (2020). Characterization of a novel *Yersinia ruckeri* serotype O1-specific bacteriophage with virulence-neutralizing activity. *Journal of fish diseases*, *43*(2), 285–293. https://doi.org/10.1111/jfd.13124

Welch, T. J., Verner-Jeffreys, D. W., Dalsgaard, I., Wiklund, T., Evenhuis, J. P., Cabrera, J. A., Hinshaw, J. M., Drennan, J. D., & LaPatra, S. E. (2011). Independent emergence of *Yersinia ruckeri* biotype 2 in the United States and Europe. *Applied and environmental microbiology, 77*(10), 3493–3499. https://doi.org/10.1128/AEM.02997-10

Wick, R. R., Judd, L. M., Gorrie, C. L., & Holt, K. E. (2017). Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. *PLoS computational biology*, *13*(6), e1005595. https://doi.org/10.1371/journal.pcbi.1005595

Willumsen, B. (1989). Birds and wild fish as potential vectors of *Yersinia ruckeri*. *Journal of Fish Diseases*, *12*, 275-277. https://doi.org/10.1111/j.1365-2761.1989.tb00313.x

Wrobel, A., Ottoni, C., Leo, J. C., Gulla, S., & Linke, D. (2018a). The repeat structure of two paralogous genes, *Yersinia ruckeri* invasin (*yrInv*) and a "*Y. ruckeri* invasin-like molecule", (*yrIlm*) sheds light on the evolution of adhesive capacities of a fish pathogen. *Journal of structural biology*, *201*(2), 171–183. https://doi.org/10.1016/j.jsb.2017.08.008

Wrobel, A., Ottoni, C., Leo, J. C., & Linke, D. (2018b). pYR4 From a Norwegian Isolate of *Yersinia ruckeri* Is a Putative Virulence Plasmid Encoding Both a Type IV Pilus and a Type IV Secretion System. *Frontiers in cellular and infection microbiology*, *8*, 373. https://doi.org/10.3389/fcimb.2018.00373

Wrobel, A., Saragliadis, A., Pérez-Ortega, J., Sittman, C., Göttig, S., Liskiewicz, K., Spence, M. H., Schneider, K., Leo, J. C., Arenas, J., & Linke, D. (2020). The inverse autotransporters of *Yersinia ruckeri*, *YrInv* and *YrIlm*, contribute to biofilm formation and virulence. *Environmental microbiology*, *22*(7), 2939–2955. https://doi.org/10.1111/1462-2920.15051

Zhu, J., Oger, P. M., Schrammeijer, B., Hooykaas, P. J., Farrand, S. K., & Winans, S. C. (2000). The bases of crown gall tumorigenesis. *Journal of bacteriology*, *182*(14), 3885–3895. https://doi.org/10.1128/JB.182.14.3885-3895.2000

strain designation	method	version	technology	accession	strain available from
NVI-492	Unicycler	0.4.8	Illumina MiSeq + Oxford Nanopore	see Table 1	Norwegian Veterinary Institute (NVI)
NVI-9681	Unicycler	0.4.8	Illumina HiSeq + Oxford Nanopore	see Table 1	Norwegian Veterinary Institute (NVI)
NVI-10587	Unicycler	0.4.8	Illumina MiSeq + Oxford Nanopore	see Table 1	Norwegian Veterinary Institute (NVI)
NVI-11050	Unicycler	0.4.8	Illumina MiSeq + Oxford Nanopore	see Table 1	Norwegian Veterinary Institute (NVI)
NVI-11076	Unicycler	0.4.8	Illumina MiSeq + Oxford Nanopore	see Table 1	Norwegian Veterinary Institute (NVI)
NVI-10705	Unicycler	0.4.8	Illumina MiSeq + Oxford Nanopore	see Table 1	Norwegian Veterinary Institute (NVI)
NVI-10571	Unicycler	0.4.8	Illumina MiSeq + Oxford Nanopore	see Table 1	Norwegian Veterinary Institute (NVI)
NVI-11065	Unicycler	0.4.8	Illumina MiSeq + Oxford Nanopore	see Table 1	Norwegian Veterinary Institute (NVI)
NVI-11073	Unicycler	0.4.8	Illumina MiSeq + Oxford Nanopore	see Table 1	Norwegian Veterinary Institute (NVI)
NVI-11267	Unicycler	0.4.8	Illumina MiSeq + Oxford Nanopore	see Table 1	Norwegian Veterinary Institute (NVI)
NVI-11294	Unicycler	0.4.8	Illumina MiSeq + Oxford Nanopore	see Table 1	Norwegian Veterinary Institute (NVI)
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NVI-4570	Unicycler	0.4.8	Illumina MiSeq + Oxford Nanopore	see Table 1	Norwegian Veterinary Institute (NVI)
NVI-4840	Unicycler	0.4.8	Illumina MiSeq + Oxford Nanopore	see Table 1	Norwegian Veterinary Institute (NVI)
NVI-5089	Unicycler	0.4.8	Illumina MiSeq + Oxford Nanopore	see Table 1	Norwegian Veterinary Institute (NVI)
NVI-6614	Unicycler	0.4.8	Illumina MiSeq + Oxford Nanopore	see Table 1	Norwegian Veterinary Institute (NVI)
NVI-701	Unicycler	0.4.8	Illumina MiSeq + Oxford Nanopore	see Table 1	Norwegian Veterinary Institute (NVI)
NVI-8270	Unicycler	0.4.8	Illumina MiSeq + Oxford Nanopore	see Table 1	Norwegian Veterinary Institute (NVI)
NVI-8524	Unicycler	0.4.8	Illumina MiSeq + Oxford Nanopore	see Table 1	Norwegian Veterinary Institute (NVI)
11-1	SPAdes	3.9.0	Illumina MiSeq	JANAUW000000000	United States Department of Agriculture - Agricultural Research Service - National Center for Cool and Cold Water Aquaculture (USDA-ARS-NCCCWA)
11-31	SPAdes	3.9.0	Illumina MiSeq	JANAUV000000000	United States Department of Agriculture - Agricultural Research Service - National Center for Cool and Cold Water Aquaculture (USDA-ARS-NCCCWA)
11-34	SPAdes	3.9.0	Illumina MiSeq	JANAUU000000000	United States Department of Agriculture - Agricultural Research Service - National Center for Cool and Cold Water Aquaculture (USDA-ARS-NCCCWA)
11-5	SPAdes	3.9.0	Illumina MiSeq	JANAUT000000000	United States Department of Agriculture - Agricultural Research Service - National Center for Cool and Cold Water Aquaculture (USDA-ARS-NCCCWA)
11-54	SPAdes	3.9.0	Illumina MiSeq	JANAUS000000000	United States Department of Agriculture - Agricultural Research Service - National Center for Cool and Cold Water Aquaculture (USDA-ARS-NCCCWA)
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NVI-8331	SPAdes	3.13.0	Illumina HiSeq	JANATI000000000	Norwegian Veterinary Institute (NVI)

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Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no