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Isolation and Characterisation of *Yersinia ruckeri* Bacteriophages from Selected Fish Sites in Norway

Isolering og karakterisering av *Yersinia ruckeri* bakteriofager fra ulike fiskeoppdrettslokaliteter i Norge

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Summary

Title: Isolation and Characterisation of *Yersinia ruckeri* Bacteriophages from Selected Fish Sites in Norway

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There has been an increase of yersiniosis in Norwegian salmonid production in both freshwater and seawater stages in recent years. The disease is characterised by septicaemia and may cause high mortalities in fry. Antibiotics and vaccines are used for prevention and control of outbreaks. In this thesis we investigate the presence of *Y. ruckeri* bacteriophages in fish sites with a known previous outbreak of yersiniosis. Five water samples were collected from two different freshwater fish farming sites in Norway and used as sources for bacteriophages. A *Y. ruckeri* serotype O1 isolate, representing the serotype isolated from salmon with yersiniosis in Norway, was used to screen and propagate the phages. A total of 66 plaques were harvested following the initial screening. All of the water samples were represented with positive results. Out of the 66 isolates, 36 phages were verified with a culture lysis and a plaque formation test. 6 of the most potent isolates in terms of bacteriolysis were selected for further characterisation. Unfortunately, the NMBU got closed due to the Coronavirus outbreak and so the characterisation part of this work could not be completed.

The results of this study show that bacteriophages against *Y. ruckeri* are present and can easily be isolated and utilized as supplements in the treatment or control of yersiniosis. Follow-up studies to characterise them and establish their host range should be done as a next step.

Definitions and abbreviations

Bacteriophage (phage) = a virus that lives within a bacterium, replicating itself and eventually destroying the bacterial cell

BKD = Bacterial kidney disease

CFU = Colony forming units

ERM = Enteric red mouth disease

FTS = Flow-through systems

LB = Luria Bertani

OD = Optical density

Plaque = a clearing in the bacterial lawn where the bacteriophage has killed the cells

RAS = Recirculating aquaculture system

Introduction

Norwegian salmonid production

The Atlantic salmon (*Salmo salar* L.*)* is an anadromous species of fish, characterised by a lifespan taking place in freshwater for spawning and early life stages, and migration to the sea for the remaining parts of its life. After 1-4 years at sea the Atlantic salmon migrates back to the river where it was born to spawn. This phenomenon is called homing and takes place late in the fall. The fertilised eggs hatch in the spring, and for the first weeks of their life the alevins get nutrition from the attached yolk sack. After 5-6 weeks they start eating and are now called fry. They live as fry in the river for at least a year before they migrate to the sea. In cold rivers the fry can stay for up to 5 years. During the migration they transform into smolt, ready for a life in the seawater (1).

The production of farmed Atlantic salmon in Norway, which mimics the natural lifespan as briefly described above, started in the 1960s. The first Norwegian farmed salmon was harvested in 1971. Since then, the size of the industry and the technology has had a massive development, and today Norway stands at half of the worlds production of farmed salmon.

At present, the farming of salmon consists of intensive production and small margins, making the fish farms vulnerable to infective pathogens and disease (2).

During the 1980s, bacterial infections represented the largest challenge for the salmon industry in Norway, resulting in widespread use of antibiotics. However, following the development of several effective vaccines and introduction of routine vaccination, bacterial infections and mortalities fell dramatically (Fig. 1), and so did the use of antibiotics (3). Today, all farmed salmonids are vaccinated before they are put to sea.

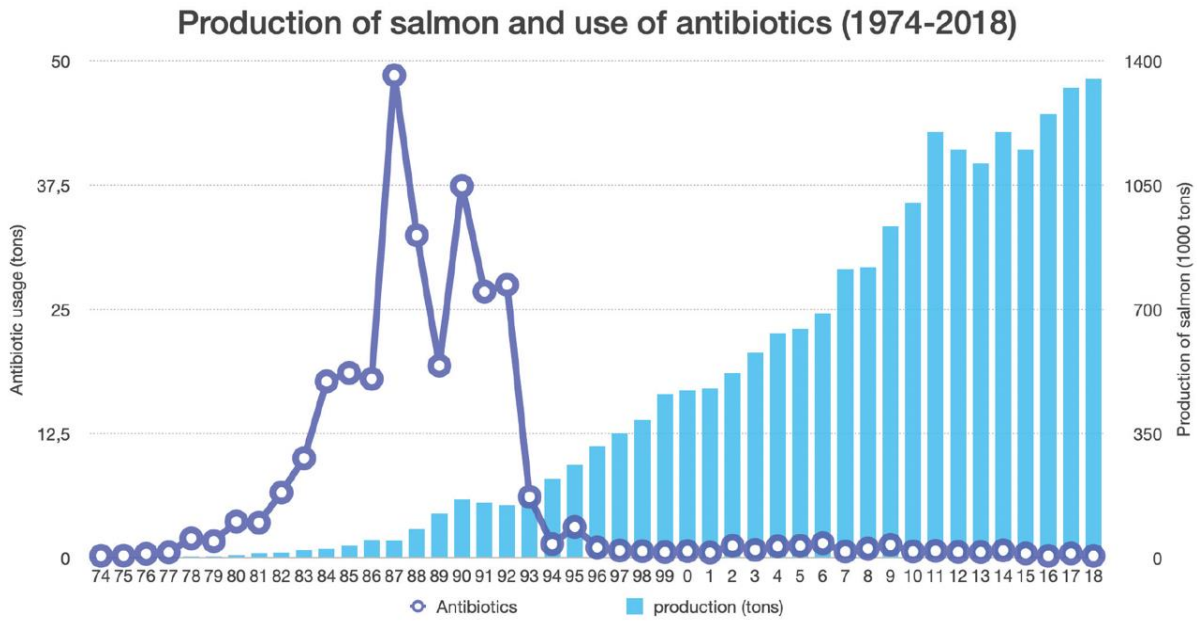

Figure 1: The effect of vaccination on the use of antibiotics and the growth of the production in Norwegian salmon farming from 1974 to 2018. Vibriosis vaccination started in 1977, cold water vibrosis vaccination started in 1987 and furunculosis vaccination started in 1990. Adapted from Håstein, Gudding and Evensen (3).

The production of farmed salmon can be separated according to water environments in which the fish are kept. The first life stages are in fresh water. The eggs hatch and develop to alevins, then fry and parr to smolt. In the development from parr to smolt the fish undergo physiological transformation to adapt from fresh to saline water, making them ready to be moved into net-pens in seawater. In total, the freshwater stage in salmon farming takes from 10 to 18 months. The seawater stage usually lasts from 14-22 months. In this time the post-smolts grow to market size (4-6 kg) before harvest.

Freshwater salmon facilities

Traditionally, the land-based freshwater salmon production sites have been based on flow through systems (FTS) connected to a nearby river, as the system requires access to a large amount of water. However, this approach makes it difficult to control water intake where pathogens and water quality are concerned. The temperature of the intake water will vary by seasons, and for many locations, will be below the optimal temperature of growth for a great part of the year. Extra costs are required for facilities to heat the water in order to achieve the desired growth during early life stages (4).

Due to the growth of the Norwegian aquaculture industry, not many facilities have access to sufficient water supply to support a flow through system. Furthermore, the development in technology has allowed more complicated water treatment systems, called recirculating aquaculture systems (RAS), with the advantages of better control of water quality parameters, biosecurity and a greatly reduced need of water. The number of production sites with RAS technology has increased both in Norway and in other countries (5). RAS, as the name indicates, functions by recirculating the water internally in the facility, and up to 99 % of the water can be recirculated (6). The water is treated through several steps to remove sludge particles and

microbes to ensure good water quality and living environment for the fish. This includes a biological filter consisting of nitrifying bacteria that convert the toxic ammonia (NH_3) and nitrite (NO_2^-) to the non-toxic nitrate (NO_3).

The benefits of RAS compared to flow-through systems are, in addition to limited use of water, improved biosecurity and less environmental impact due to full control of water quality parameters and water treatment (7). However, the systems are complicated and require good management to prevent poor water quality and growth of opportunistic pathogens (8). A study performed by Badiola et. al. (7) concluded that operation of a biofilter, control of solid particles in the water and management due to lack of training and knowledge, are the hardest tasks that may lead to system failure in RAS (7).

The RAS technology allows for land-based production of salmon from roe to market sized fish as it can be used with both freshwater and seawater. Experience with land-based post-smolt production is minimal, currently only one full-scale RAS salt-water facility in Norway is producing salmon with this technology, the facility is owned by Fredrikstad Seafoods AS. According to a risk assessment done by SINTEF, transfer of infection from the intake water or from fish brought from other production sites are defined as two of the most important risks factors regarding disease control (9). In addition, there is a probability of new or unpredicted diseases and welfare problems as a result of a new way of producing salmon.

Bacterial infections in the Norwegian salmonid industry

Bacteria are everywhere in the environment. Some are obligate pathogens specialised in infecting and causing disease in cells and tissues, while others are opportunistic pathogens

found in the normal bacterial flora or in the environment of the fish only causing disease during certain circumstances. Such circumstances could be poor water quality, stress, malnutrition and other factors connected to the bacteria, the host or the environment. Animals living in aquatic environments are at high risk of exposure to pathogenic bacteria from other animals, as bacteria spread easily in water and may enter a host through feeding or drinking (10). This effect will be reinforced in environments such as fish farming where the density of fish is high. Bacteria that cause disease in Norwegian salmon farming are usually gram negative, yet a few are gram positive (11).

In the early 1990s, bacterial infections caused severe problems in the salmon industry, furunculosis caused by *Aeromonas salmonicida* subsp. s*almonicida,* was the most important one (11). After the development of efficient vaccines against furunculosis and cold-water vibriosis, the incidence of these diseases decreased dramatically, and bacterial infections are no longer a large problem in today's salmon production in Norway.

The Fish health report from 2019 (12) describes the situation of bacterial infections in Norwegian fish farming. Very few outbreaks of notifiable diseases e.g. furunculosis and bacterial kidney disease (BKD) caused by *Aeromonas salmonicida* subspecies *salmonicida* and *Renibacterium salmoninarum,* respectively, have been detected in recent years. Another bacterial disease of significance is winter ulcers caused by *Moritella viscosa*, often in combination with *Tenacibaculum* ssp. and *Aliivibrio wodanis.* (12) This was seen along the entire coast of Norway in 2019 with highest prevalence in the northern parts, although the exact prevalence is difficult to estimate as this infection is not notifiable; and is quite easily diagnosed in the field therefore not well recorded. Nevertheless, the situation regarding these bacterial agents seems to be stable.

Yet, there was an increase in cases of pasteurellosis in 2019. The disease is caused by bacteria in the *Pasteurella* family, but the species that causes disease in salmon is not known. Yersiniosis was first detected in 1985 and has been one of the most important bacterial diseases in Norwegian salmonid industry since then(12) . Pasteurellosis was seen in 14 facilities in the western parts of Norway and is referred to as an emerging disease. In contrast, after a rise in yersiniosis caused by *Yersinia ruckeri* from 2012-2015, the number of cases decreased after 2016. Only 18 cases distributed over 12 localities were reported in 2019, and the decrease is probably due to higher vaccination rates. However, some facilities are still having problems with recurring outbreaks in the freshwater stage (12). With the ongoing problems in RAS facilities, which will be discussed later, it is a highly relevant bacterium to focus on and study further.

Yersinia ruckeri

Yersinia ruckeri is a facultative anaerobic gram-negative rod, belonging to the Enterobacteriaceae family. As most of the bacteria in this family, *Y. ruckeri* are motile with peritrichous flagella, although, strains missing the flagella also exist (13). The bacteria in the Enterobacteriaceae family are found in the environment both as harmless environmental bacteria and opportunistic pathogens, but they may also live as pathogenic bacteria for humans, animals or fish, mainly associated with disease in the intestinal system.

Y. ruckeri is known to cause enteric red mouth disease (ERM) in rainbow trout and yersiniosis in Atlantic salmon. Yersiniosis is a septicaemic disease affecting several organ systems. Internal pathological changes of yersiniosis include congestion, intestinal haemorrhage, petechial bleedings on serosa membranes, ascites, swollen kidney and enlarged spleen (13). Externally, petechia of the skin and exophthalmos are signs of the disease. Mandibular haemorrhage is a characteristic symptom in young trout affected by enteric red mouth disease

and is what has given its name. However, this trait is not present in the clinical manifestation in Atlantic salmon. Outbreaks among fry, or fingerlings, in freshwater may cause high mortality, potentially occurring without previous external signs of disease (14). The transfer of *Y. ruckeri* occurs horizontally between fish, either through contact with sick fish or through asymptomatic carriers. Carriers are important in the spread of the infection, and shedding of the bacteria and transfer from carriers to healthy fish (steelhead trout) has been demonstrated when the fish is exposed to stressful conditions (15).

Y. ruckeri can be categorized based on its serotype, biotype or outer membrane proteins (13). Serotype refers to the antigens on the cell surface and biotype 1 and 2 are based on the ability to ferment sorbitol (13). Serotype O1 biotype 1 and 2 are the most important in enteric red mouth disease in rainbow trout, and serotype O1 is considered to be most virulent (16). A study performed by Gulla et. al (16) analysing bacteria from clinical outbreaks of yersiniosis in Atlantic salmon between 1995 and 2017, identified serotype O1 in most of the cases (77%) and O2 in a few (12%). Interestingly, all the virulent bacteria belonged to the same clonal complex (CC1), while *Y. ruckeri* isolated from fish farms without yersiniosis outbreaks did not belong to this complex (16). Other clones of serotype O1 and other serotypes have been detected in Norwegian salmon farming, but these have not been linked to clinical infections. In 2018, detection of O1 biotype 2 was reported (17). In contrast to the dominating biotype 1, the biotype 2 is non-motile and non-phospholipase secreting. Findings indicate that this strain may be associated with less protection from the vaccine. The vaccine is based solely on *Y. ruckeri* serotype O1, biotype 1, and flagellar proteins act as important antigens for salmon immunization. As the biotype 2 bacteria lack the flagellum, the vaccine is less effective providing less protection against this bacterial strain (12). Notably, the biotype 2 has developed independently in several continents, which has led to the hypothesis that its occurrence is driven

by the vaccination of rainbow trout. The strain has been associated with disease outbreak in already vaccinated rainbow trout in other countries (18, 19).

Yersiniosis in Norwegian farmed salmon

The Norwegian Veterinary Institute annually publishes reports on fish health in Norwegian aquaculture. The information is gathered from official data of the Food Safety Authorities, data from the diagnostic lab at the Veterinary institute, data from private laboratories and a survey conducted towards employees in the fish health service and inspectors of the Norwegian Food Safety Authorities. In Norwegian aquaculture, yersiniosis has mainly been associated with farmed Atlantic salmon in freshwater facilities and in smolt recently transferred to sea (12). In recent years, an increase in number of cases has been observed, mainly due to more outbreaks at sea. Several of these outbreaks have occurred in larger salmon (>1 kg) months after sea transfer (17, 20). Asymptomatic carriers of *Y. ruckeri* in fish are well described (13), and it is likely that yersiniosis outbreaks in sea-transferred fish are caused by carriers initially infected in the freshwater facilities. Stress has been shown to be a trigger of release of bacteria from carriers to healthy fish (15). Notably, many of the outbreaks in larger salmon were reported by the farmers to begin shortly after treatment of sea lice (12), suggesting that stress triggered the outbreaks.

However, the number of outbreaks in freshwater facilities has remained stable in recent years (12, 17, 20). According to the fish health report from 2017, yersiniosis has been identified as a particular problem in RAS facilities with high mortality and recurring outbreaks in some facilities (20). Furthermore, it is recognized that *Y. ruckeri* is able to form biofilms (13, 21),

and this has been demonstrated to be the source of recurrent infections in freshwater facility fish farms (22).

Prophylaxis and treatment of Yersiniosis in Norwegian fish farming

Several vaccines targeting bacterial infections are available for use in Norwegian salmon farming. Close to all farmed salmon transferred to sea, are vaccinated against furunculosis (*Aeromonas salmonicida subsp. Salmonicida),* vibriosis *(Vibrio anguillarum)* and cold water vibriosis *(Vibrio salmonicida)* by the use of an multicomponent oil-adjuvanted vaccine. In addition, there are several commercially available vaccines against other bacterial and viral infections, as part of the multicomponent vaccine or as single component vaccines. Some vaccines are forbidden by law to use in Norway or in certain regions of the country, because of control programmes of certain diseases. Oil-adjuvanted vaccines are the main reason that the use of antibiotics in Norwegian fish farming has been reduced spectacularly since the early 1990s (23). *Yersinia ruckeri* vaccine was until recently only available as an inactivated immersion vaccine intended for salmon fry. This water-based vaccine, has in recent years been used as an injection vaccine in combination with the oil-adjuvant multicomponent vaccine, to prevent yersiniosis outbreaks after sea transfer. Recently, an injection vaccine has also become available on the marked (24). Reports on reduced numbers of outbreaks of yersiniosis in salmon at sea in 2018 and 2019, are likely due to the increased use of this vaccine as an injection vaccine. The effect of vaccination is promising, and farmers report that the vaccine is necessary to continue the production of farmed salmon (12).

Antibiotic resistance is a pressing problem not only in aquaculture, but also for animal and human health worldwide. Notably, previous research has documented that use of antibiotics in

animal and fish farming can promote resistance in human pathogens, as most of the antibiotics used for animals are also used in human medicine (10). As such, this underlines the importance of keeping the use of antibiotics in aquaculture to a minimum.

As already stated, following the introduction of several efficient vaccines, the use of antibiotics in Norwegian fish farming decreased dramatically since the early 1990s (23). Compared to other salmon producing countries, such as Chile, the current use of antibiotics in Norwegian aquaculture is low (25). Between 2013-2017 approximately 120-810 kg antibiotics were used annually for the 1,3 million tonnes of salmonids produced each year (20, 26). There was an increase in the use of antibiotics in 2017 and 2018, however, this was not due an increase in the total number of treatments, but as a result of a few incidents of yersiniosis treatment of larger fish that require high amounts (26). In 2019, the consumption of antibiotics decreased to the 2015-2016 levels (12). To prevent development of resistance in fish pathogenic bacteria, and the spread of resistance genes to pathogens and environmental bacteria, restricting the use of antimicrobials in Norwegian aquaculture is advised (20).

Yersiniosis in fish farming is most commonly treated using the quinolone oxolinic acid. Other antibiotics holding marked authorisation in Norway is florfenicol and sulphadiazin/trimethoprim (27) . Quinolones are a group of synthetic antimicrobials that inhibit nucleic acid synthesis (28). Most quinolones used today have a fluoride group added to the molecule and are termed fluoroquinolones (29). (Fluoro)quinolones inhibit type II topoisomerase enzymes, DNA gyrase and topoisomerase IV, which have roles in bacterial replication. DNA gyrase is involved in the unwinding of supercoiled DNA during DNA synthesis making the DNA strands available for separation and replication, and topoisomerase IV is involved in separating DNA strands (29). Inhibition of these topoisomerases spoil

replication of the bacterial genome and the effect is consequently bactericidal. Resistance towards fluoroquinolones are usually due to mutations in the genes of the bacterium encoding DNA gyrase and topoisomerase IV (30). Both enzymes consist of four subunits encoded by the genes *GyrA* and *GyrB* for DNA gyrase and *parC* and *parE* for topoisomerase IV. Different single base pair mutations in the same position in *GyrA*, leading to substitution of one amino acid have shown to enhance resistance to quinolones (30, 31). These chromosomal mutations are the most common cause of resistance to quinolones, this was also the case for the *Y. ruckeri* reported with less susceptibility to oxolinic acid in Norwegian fish farming in 2017 (20). The risk of horizontal spread of this type of chromosomal mutation is considered low, resistance genes on a mobile element would make up a bigger threat regarding spread of resistance. Plasmid encoded resistance to quinolones has also been found (29), although not yet in *Y. ruckeri*, even though, plasmid bound resistance to other groups of antimicrobials have been detected in *Y. ruckeri* (31). The emergence of quinolone resistance genes on mobile genetic elements represent a potential risk of spread of quinolone resistance genes in *Y. ruckeri*.

Bacteriophages

Bacteriophages (commonly referred to as phages) are a numerous group of small viruses that infect bacteria. They are the most abundant organism on the planet and are found in high numbers in aquatic environments (32). Bacteriophages are often highly specialised, only infecting a strain of a bacterial species, although phages of a wider host range also have been shown (33-35). Bacteriophages are grouped into either lytic or lysogenic (temperate) bacteriophages, based on their life cycle (36). Their morphology differs from simple to more complex structures. Bacteriophages are important tools used in biotechnology and research and they have potential as antimicrobial agents.

Structure and morphology

Most bacteriophages have a genome consisting of double-stranded (ds) DNA (28). The morphology of bacteriophages differs in its complexity, but all virions consist of a capsid protecting the viral genome. The capsid is made of capsid proteins called protomers and is classified in three groups according to structure: Helical, icosahedral (20 sided) and complex (28). Complex bacteriophages may consist of both icosahedral and helical components as well as other structures. Most bacteriophages are naked viruses, but bacteriophages with an outer membrane called envelope have been discovered. In marine environments, bacteriophages of the families *Myoviridae, Siphoviridae* and *Podoviridae* are the most common (37). These families make up the order *Caudovirales* – the tailed phages (38). Many of the characterised *Yersinia ruckeri* bacteriophages fall under the family *Podoviridae* in the genus of T7 viruses and have ds DNA (39, 40). Tailed phages use their tail receptor-binding proteins to attach to receptors on the bacteria which makes it possible for the phage to enter the bacteria. Also, tailed phages have evolved mechanisms which make the virion offspring able to more easily spread from infected cells after cell lysis (41).

Lifecycle

In general, the lifecycle of viruses, including bacteriophages, consists of the following steps. Attachment to the host cell, penetration of host cell membrane and entry of the phage nucleic acid into the cell. Replication of genome and multiplication and assembly of virion particles. And finally release of virus particles (28).

Two different life strategies are found among bacteriophages: Lytic and lysogenic. Lytic bacteriophages are referred to as virulent or infective bacteriophages, and use host cells to reproduce themselves, ending in lysis of the host cells. The phage attaches to the receptors of the bacterial cell membrane, transferring its DNA into the bacterium. As soon as the nucleic acid is inside the cell, it starts producing early proteins to break the cell's own DNA. This makes the phage control the replicating machinery of the host cell, the phage then uses the host cells replicating machinery to replicate its own genome and making new phage particles. The host cell bursts and releases 100-200 new phages out in the environment, ready to infect new hosts.

The lysogenic stage is a dormant condition in which the bacteriophage integrates its DNA into the bacterial host genome, becoming what is called a prophage. The genome of the phage is too small to disturb the bacteria, but it will be replicated passively along with the bacterial genome as the bacteria divide. If the bacteria are exposed to stressors such as chemicals, UV-light or low access to nutrients, the prophage may be activated and extracts itself from the host genome, starting the process of a lytic lifecycle. Phages that hold this ability and can shift between lysogenic and lytic life cycle are called temperate bacteriophages.

Phage therapy

Bacteriophages were discovered independently in 1915 by two researchers, Twort and d'Herelle. D'Herelle started experimenting with phages as therapeutic agents against infections, but when penicillin were discovered only a decade later, research on phage therapy was left in favour of the much more efficient and easily used antibiotics, at least in the western world (34, 42). In the former Soviet Union however, phage therapy was used during the Winter War to treat wounds and dysentery in soldiers, and further research and use of phage therapy continued after the war ended (42, 43). Today, the Eliava Institute in Tbilisi Georgia stands out as the

oldest institution of phage research, founded in 1923 by George Eliava and Felix d'Herelle himself. According to themselves, they hold the world's largest and most diverse bacteriophage collection, and offer phage treatment to patients from all over the world, including infections caused by antimicrobial resistant bacteria (44). As antimicrobial resistance has emerged as a problem in both human- and veterinary medicine, phage therapy has again gained interest as a potential alternative method of controlling disease, also in western medicine. Lytic phages have the potential of becoming efficient treatment of bacterial infections in aquaculture. One of the biggest advantages using phages instead of antibiotics is their specific pathogenicity, only killing the unwanted bacteria without affecting the host or the hosts normal bacterial flora (34, 35). Also, bacteriophages are considered natural and not harmful for human consumption and food safety, they are easy to administer, and they can be used as a preventive treatment, or as bio-sanitization in cases of struggling with recurring infections (34).

According to Hyman (45), bacteriophages intended for phage therapy should be screened for several characteristics. They should give positive results in culture lysis test, as this indicates that the phage is able to kill bacteria faster than the bacteria replicates, and the rate of mutant bacteria resistant to the phage must be low. The phages should be obligate lytic bacteriophages, and they best not have the potential of transduction as this potentially can transfer virulence genes between bacteria. Bacteriophages holding toxic genes should be excluded because of the potential of creating more virulent bacteria, and the host range of the bacteriophage should be determined to evaluate the suitability of the isolated phage for phage therapy (45).

The bacteriophages host specificity is a challenge in phage therapy, choosing the right host when isolating bacteriophages is key to finding phages active against the clinical isolate (45). As with antibiotics, bacteria have several mechanisms to develop resistance against phages. Blocking phage attaching to the bacterial membrane by altering receptors, preventing phage

DNA entering the cell and destroying phage DNA are some of the mechanisms bacteria use as a defence against phage attack (32, 34). This is a challenge in phage therapy, but studies indicate that the problem can be delayed by using cocktails of phages rather than a single phage in therapy (46). The evolution between host and phage is still a field in need of more research, but it is reasonable to assume that the phage has mechanisms to respond to bacterial resistance (32).

Temperate phages can go from a lysogenic to a lytic stage and may in this process transfer genes from one bacterium to another. This process is called transduction and may cause spread of antibiotic resistance genes or virulence genes among bacteria. Worst case, the phages can make a non-virulent bacterium become virulent, a so called phage conversion (34). This is important to avoid, so when using phages for phage therapy obligate lytic phages are preferred in this kind of treatment.

Due to the wish to keep antibiotic treatments to a minimum and the possibility of disease outbreaks in already vaccinated fish, bacteriophages make an interesting alternative to controlling yersiniosis in Norwegian salmon farming. Currently, one product offering biocontrol using bacteriophages is available on the Norwegian marked. $\text{CUSTUS}_{\text{YRS}}$ by ACD Pharma contains bacteriophages active against *Yersinia ruckeri* and has been available for a few years. The product is neither advertised as a treatment, nor is it approved as a pharmaceutical, but it aims to prevent outbreaks of yersiniosis by applying it in the water before stressful situations. Although there is an existing commercial product on *Y. ruckeri* bacteriophages, literature on *Y. ruckeri* bacteriophages in general and in the Norwegian salmonid industry in particular, is very limited and is required to explore phage therapy as a preventive measure against yersiniosis in farmed Atlantic salmon. x

Objective

The objective of this study was to investigate the presence of *Y. ruckeri* bacteriophages in selected salmonid farming facilities in Norway.

In order to fulfil this objective, the work was divided into the following work packages:

1. Isolation of *Y. ruckeri* bacteriophages from environmental samples.
2. Characterisation of bacteriophages isolated in the work package 1.

Materials and methods

Bacterial strain used for propagation of *Y. ruckeri* bacteriophages

The *Y. ruckeri* strain used in the laboratory work for this thesis was kindly provided by Drs. Duncan Colquhoun and Snorre Gulla of the Norwegian Veterinary Institute. The specific strain of *Yersinia ruckeri* is a serotype O1 within clonal complex 1 (CC1) isolated from Atlantic salmon in Trøndelag in 2018. The strain is the commonest clinical isolate of *Y. ruckeri* infections in Norway (16). Bacteria was stored in 40 % glycerol stock at -80 ºC and grown on LB plates at 20 ºC until single colonies were visible (1-2 days). To propagate phages, bacteria propagated in LB broth or agar supplemented with 10 mM $CaCl_2$ was always used.

Quantification of bacteria

To estimate the amount of bacteria for use in isolating phages, a correlation between bacterial turbidity by spectrophotometer based on the Optic Density (OD) and quantification by dilution were done. *Yersinia ruckeri* with a known OD value (0,25 and 0,49) was diluted 10-fold in LB broth six times. One ml of each dilutions was then plated in triplicate on LB agar plates and incubated at 20 °C overnight. The bacterial colony forming units (cfu) on each plate were then counted. The number of cfu/ml in the original sample was calculated as (cfu/ml = number of

colonies per plate/ dilution factor). This method was then used to estimate the bacterial growth curve. For this, 100 µl of bacteria initially archived at -80 °C was thawed and added to 5 ml LB broth. The OD values were measured from the start and every hour for 7 hours using a spectrophotometer (GENios Tecan Microplate Reader, software XFluor v. 4.40) at a wavelength of 595 nm. As control, LB broth without *Y. ruckeri* was used. In that way we knew when to expect the bacteria to be in exponential growth and how long it would take to get an OD of 0,25-0,30 after adding 100 µl of bacteria in 5 ml LB broth.

An additional bacterial growth curve was made with the aim to reach an OD of 0,25 within fewer hours. *Y. ruckeri* that was added to 5 ml LB broth and incubated on a shaker overnight had an OD value of 0,65 the following day. Two hundred µl of the bacteria from the day before was added in 5 ml LB broth and the OD measured every hour using a spectrophotometer (GENios Tecan Microplate Reader, software XFluor v. 4.40) for 5 hours. The spectrophotometer measured the absorbance, with a wavelength of 595 nm, of 350 µl of bacterial culture in a 96 well microplate. A control was included as described above.

Water samples (Sources of *Y. ruckeri* phages)

The water samples used in the project were collected from Norwegian salmon farming facilities in June 2019, details are listed in Table 1. Both facilities had outbreak of yersiniosis prior to sampling, but there were no signs of active infection during sampling.

Table 1 Overview of locations and type of facility from which water samples used in this study originated.

Location - county	**Production facility**	**Sampling site**
Finnmark	Smolt facility RAS and flow through system	Greywater
		Greywater winter
		Drain - initial feeding unit
		Drain
Møre og Romsdal	Smolt facility Flow through system	Drain

After collection and transportation to the lab, all water samples were filtered using a 0,22 µm filter to remove particles and bacteria. Filtered water samples were stored in 50 ml plastic tubes at 4 ºC. From this point, filtered water sample was called phage source.

Enrichment of phage sources

The concentration of bacteriophages in water or environmental samples may be inherently low, or this could be due to other reasons such as long transportation or extended storage. In order to increase the starting concentration therefore, the phage sources were enriched by mixing one part of the phage source with 4 parts Luria-Bertani (LB) broth, followed by inoculation with one colony of *Y. ruckeri* from an LB agar plate. This was then incubated overnight at room temperature on a shaker. Next day, the mixture was filtered using a 0,22 µm syringe filter.

Initial screening of phage source (Soft agar overlay method)

Screening of samples for bacteriophages was done using a modified protocol from "Molecular Cloning, a laboratory manual (47). 200 µl (enriched and filtered) phage source was mixed with 100 µl bacterial culture (OD_{595} 0,25-0,3) in a 15 ml tube. The mixture was put on ice for 10-15 min to allow phages to attach to the bacteria. The mixture was added to 2,2 ml of pre-heated

(45 °C) 0,5% soft agar, mixed carefully, and poured over LB agar plates. The soft agar on plates was allowed about 5 -10 min to solidify and then inspected for bubbles and uneven agar. These areas were marked with a pen to avoid confusion with plaques after incubation. The plates were put in a moist (wet paper towels) plastic bag and incubated at 20 ºC overnight. Duplicates were made for all the phage sources from the Finnmark locality. A negative control was made following the same protocol, but the phage source was replaced by filtered LB broth. Plates were inspected for plaque formation the following day.

Isolation of bacteriophage (plaque purification - Soft agar overlay method)

Protocol modified from "Molecular cloning, a laboratory manual" by Sambrook and Russel (47). To isolate the phages, plaques were picked using the following procedure: 100 µl pipette tip was used to pick a presumed plaque by stabbing at the centre and then dipping it in 100 µl LB broth. Plaques from the same plate at this stage were treated as different phages, marked accordingly and stored in 1,5 ml Eppendorf tubes at 4 ºC until use. Next, the isolated plaques were mixed with 100 µl of a fresh bacterial culture (4hrs – overnight; OD 0,25-0,3), incubated on ice for 10-15 minutes before transferring to fresh 2,2 ml 0,5% heated (45 °C) soft agar and poured over a LB agar plate, swirled once or twice and set to solidify. Bubbles or uneven spots of agar were marked before incubation. The plates were inverted, put in a plastic bag with wet paper towels, and incubated at a 20 ºC until next day. Visible plaques were picked by sticking a sterile pipette tip at the centre of a plaque and transferring this to 100 µl of phage medium (LB broth supplemented with 10 mM $CaCl_2$). Isolated plaques were stored in 1,5 ml Eppendorf tubes at 4 ºC until required.

Purification/verification of phage (Optical density- based method)

This purification method was used as a rapid verification step of presumed phages isolated in steps above. 100 µl of the isolated phage was mixed with bacterial culture (100 µl *Y. ruckeri* in

5 ml LB broth supplemented with 10 mM $CaCl_2$) in exponential phase (OD of 0,25-0,3) and incubated on a shaker at room temperature for 4 hours. The OD values of samples were measured after 2 and 4 hours. A reduction in OD value relative to the control was suggestive of a lytic phage. As a precaution, the soft agar overlay method was also used in addition to this method. Following measuring the OD after 4 hours, 200 µl of the phage/bacteria mixture was added to pre-heated 3 ml of 0,5 % soft agar, mixed carefully, poured and gently swirled on to a LB agar plate. The agar plate was then incubated at 20 °C overnight as described above. Visible plaques on the agar plate with a corresponding reduction in OD value was suggestive of a lytic phage. The phage/bacteria cultures were then centrifuged, and the supernatant collected, filtered and kept at 4 °C. Six of the samples that produced the least OD increase, compared to the controls, were picked for quantification and further characterisation.

Quantification of phages

To estimate the concentration of phages in a sample, serial dilutions of the verified and purified phages were done. The phage samples were diluted into 10-fold dilutions in LB broth six times. 100 µl of each dilution was added to 200 µl of bacteria in log phase (2,5 OD), mixed and incubated on ice for 20 min. The phage/bacteria mix was added to 3 ml of 0,5 % soft agar, mixed carefully and poured and swirled over onto a LB agar plate. Controls without *Y. ruckeri* phages were also used. Following solidification of the soft agar, the plates were inverted and put in a plastic bag with wet paper towels and incubated overnight at 20 °C. The following day, the plaques were counted and the concentration calculated according to the formula: Plaques / ml = number of plaques per plate / dilution factor.

Characterisation of bacteriophages by electron microscopy

Five ml each of two samples (9 and 10) and a negative control were prepared as described above, i.e. the phages were propagated in fresh LB broth with *Y. ruckeri* in log phase. After

harvesting, the phages were separated from the bacteria first by centrifugation (10000 x g at 4 °C for 10 minutes) in a desktop centrifuge. The supernatant containing phages were harvested and the pellet discarded. The supernatant was then filtered through a 0,22 µm filter to exclude the bacteria.

To concentrate the phages, the samples were centrifuged at 150000 x g in a desktop ultracentrifuge (Berkman) at 4 °C for 90 minutes. The supernatant was removed and the pellet resuspended in a residual volume of approximately 10 µl. It was estimated that in this way, the samples had been concentrated 100 times.

For electron microscopy, the samples were examined at the Imaging centre at NMBU. A droplet of each sample was placed on parafilm. A 400 mesh carbon-coated Copper-grid with formvar was placed on a droplet of the sample for 30 min. The grid was then carefully blotted on filter paper and then placed on a droplet of 4 % uranylacetat for 1 min to give contrast to the particles. Excess solution was blotted off carefully and samples were examined by a transmission electron microscopy at 80 kV.

Results

Bacterial growth curve

Figure 2 shows growth patterns of 3 replicates of *Y. ruckeri* over 7 hours with 100 µl bacteria stored at – 80 °C as starting volume in a total volume of 5 ml. The replicates were all growing at the same rate, reaching an OD of 0,25 after approximately 6 hours. When a volume of 200 µl of bacteria, instead of 100 µl was used, only a slight difference in terms of time to 0,25-0,3

was observed, that being a little more than 5 hours. However, when 200 µl of bacteria (OD 0,65) was used the replicates reached OD 0,25 after only 4 hours (Fig. 3).

Also, when a total volume of 3 ml instead of 5 ml was used, the clones of bacteria reached OD 0,25 after only 4 hours (results not shown).

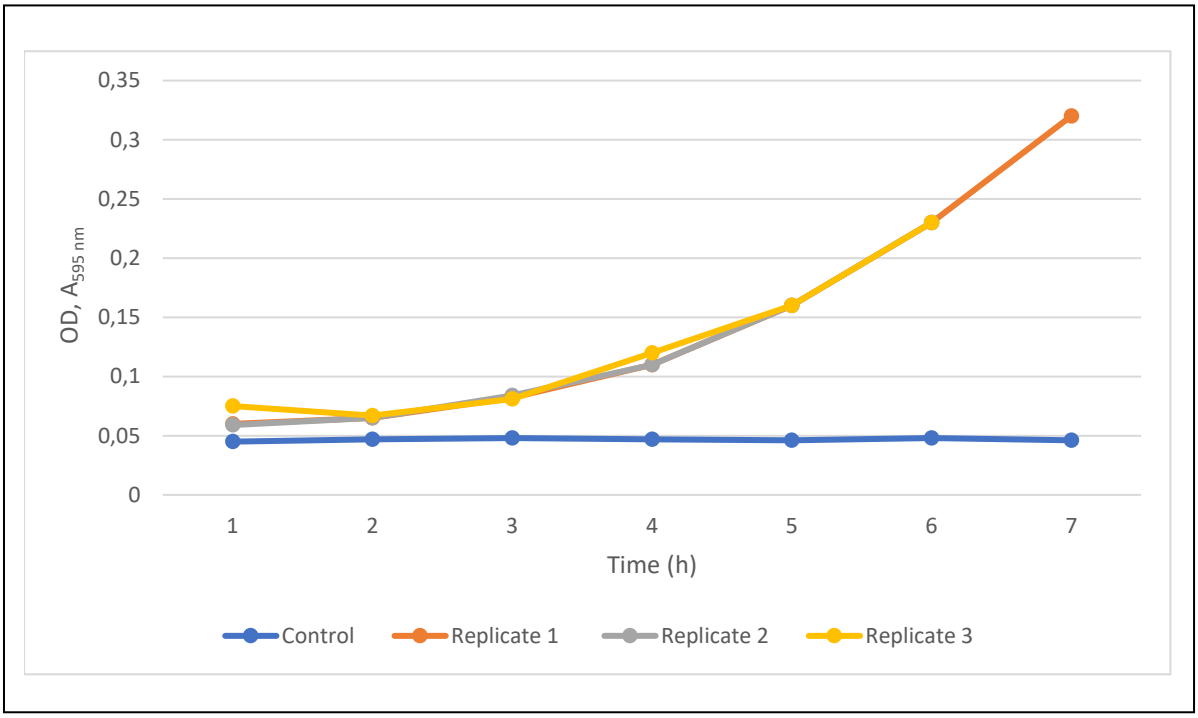

Figure 2: Growth of *Yersinia ruckeri* replicates in LB broth supplemented with 10 mM $CaCl_2$ incubated on shaker at room temperature. *Yersinia ruckeri* replicates 1-3 had a starting volume of 100 µl thawed *Y. ruckeri* in 5ml LB broth with 10 mM $CaCl_2$. The control was LB broth with 10 mM $CaCl_2$ only.

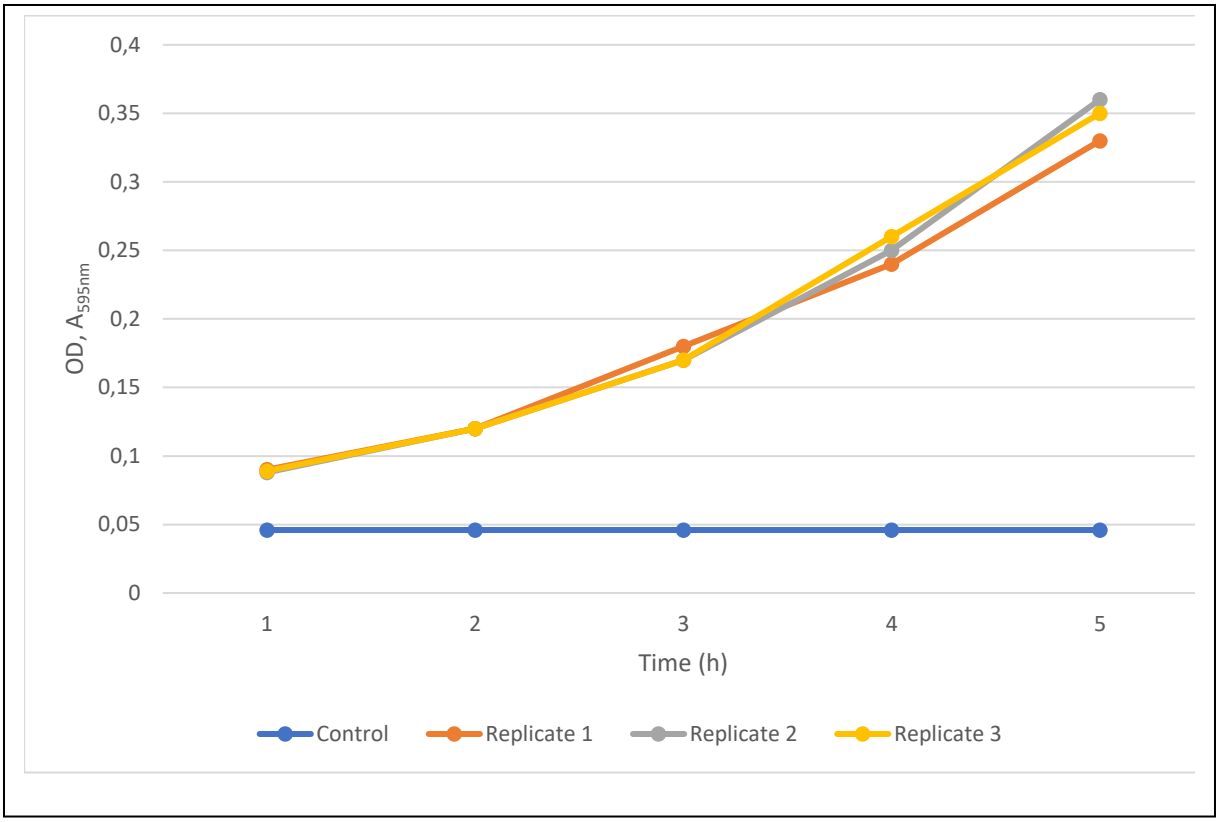

Figure 3: Growth of *Yersinia ruckeri* replicates in LB broth supplemented with 10 mM $CaCl_2$ incubated on shaker at room temperature. *Yersinia ruckeri* replicates 1-3 with starting volume of 200 µl bacteria (OD 0,65) in 5ml LB broth with 10 mM $CaCl_2$. The control consists of only LB broth with 10 mM $CaCl_2$

Quantification of bacteria

To quantify bacteria, first the bacteria was grown either to an OD of 0.25 or 0.49. The serial dilutions of the bacteria were made using LB broth to a concentration of 10^{-6}. Each of these dilutions were then plated in triplicate on LB agar plates and incubated overnight. All concentrations from undiluted to 10^{-4} had too numerous bacteria to count. Only the two lowest dilutions had a countable number of colonies on agar plates.

Table 2: An OD of 0,49 resulted in countable colonies in the highest dilution. An OD of 0,25 resulted in countable colonies in two of the dilutions.

Dilution factor	**OD = 0,49**	**OD = 0,25**
1:10	numerous	numerous
1:100	numerous	numerous
1:1 000	numerous	numerous
1:10 000	numerous	numerous
1:100 000	numerous	Plate 1: 323 cfu Plate 2: 339 cfu Plate 3: 307 cfu
1:1 000 000	Plate 1: 152 cfu Plate 2: 129 cfu Plate 3: 103 cfu	Plate 1: 29 cfu Plate 2: 21 cfu Plate 3: 32 cfu

The correlation between the OD and the concentration of bacteria in the suspension (cfu/ml) was not linear. The control (0 cfu/ml) had an OD value of 0,046. An OD of 0,49 was calculated to give $1,28*10^8$ cfu/ml. The concentration of bacteria at which phage samples were added, i.e. OD 0,25 was equivalent to $1,51*10^7$ cfu/ml.

Screening and isolation of bacteriophages – plaque assays

Water samples were assigned identification numbers on the basis of their sources (Table 3); subsequent letters or numbers in the sample id represent different presumed phages or replicates. A total of 20 plaques were sampled from the initial screening and these formed the

basis for purification. Plaques differed in size, but most of them were pinpoint sized clearings best visible if held against the light (Fig. 4).

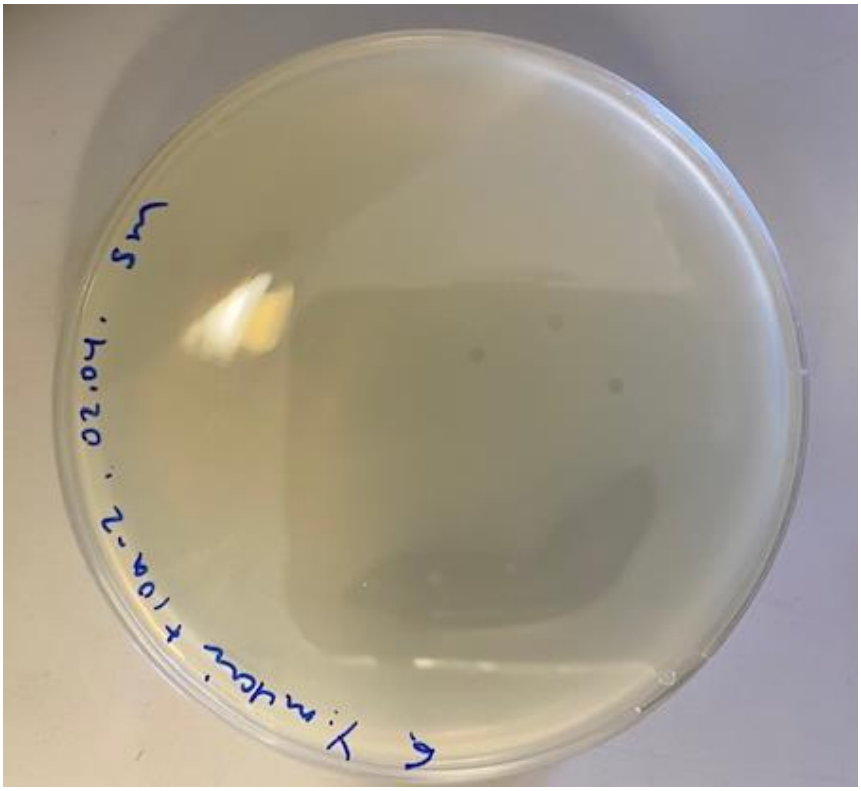

Figure 4: Sample 10A: Example of visible plaques on agar plate.

All environmental samples yielded positive results in the initial screening albeit not from all duplicates (Table 3).

Table 3: Overview of results from initial screening of phage source and isolation of bacteriophages (plaque purification protocol). Under sampling site column, letter F indicates sample originates from the Finnmark lokality, letter M indicates the sample originates from the Mid-Norway locality. (1) and (2) in front of sample name indicates duplicates of the sample.

		Initial screening of phage source			Isolation of bacteriophages (plaque purification)			
Sampling site	**Sample ID**	**Result**	**No. of plaques**	**Plaque ID**	**Plaque ID**	**Result**	**No. of plaques**	**Plaque ID**
F greywater (1)	1	Pos	3	1a, 1b, 1c	1a	Pos	8	1a1-8
					1b	Neg		
					1c	Neg		
F greywater (2)	4	Failed						
F greywater winter (1)	7	Pos	3	7a, 7b, 7c	7a	Neg		
					7b	Neg		
					7c	Neg		
F greywater winter (2)	9	Pos	4	9a, 9b, 9c, 9d	9a	Pos	2	9a1, 9a2
					9b	Pos	25	9b1-25
					9c	Pos	3	9c1-3
					9d	Neg		
F drain- initial feeding (1)	8	Pos	2	8a, 8b	8a	Pos	2	8a1, 8a2
					8b	Neg		
F drain - initial feeding (2)	10	Pos	3	10a, 10b, 10c	10a	Pos	4	10a1-4
					10b	Pos	8	10b1-8
					10c	Pos	2	10c1, 10c2
F drain (1)	2	Neg						
F drain (2)	3	Pos	3	3a, 3b, 3c	3a	Pos	2	3a1, 3a2
					3b	Pos	3	3b1-3
					3c	Neg		
M Drain	6	Pos	2	6a, 6b	6a	Neg		
					6b	Pos	7	6b1-7
		Total	20			Total	66	

Each plaque was assumed to be a different phage and so from the 20 harvested during the initial screening, several phages (3-25) were obtained (Fig. 5). As a result, 66 phages were collected after the second round.

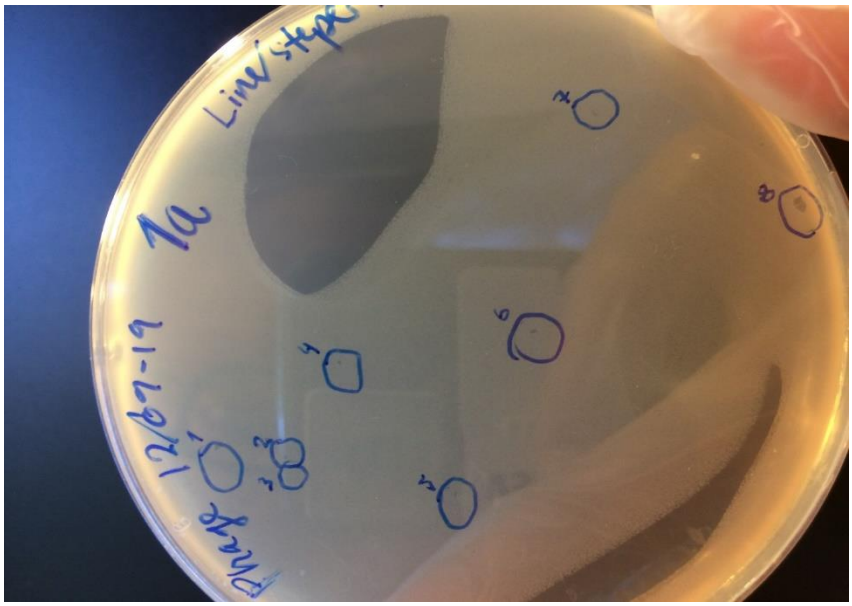

Figure 5: Example of results from the isolation of bacteriophage (plaque purification). Showing isolation of phage from plaque 1a from initial screening of water sample, resulting in 8 phages (1a1-8) after the purification protocol.

Final purification/verification of phage

To verify the plaques more conclusively, a double approach combining the optical density-based and soft agar overlay methods were done. This resulted into 36 tentative phages from the 66. The reduction in OD value compared to the control ranged from 0,008-0,0753 (Table 4) and plaque formation (Fig. 6). Out of the 36 isolates that had reduced OD value, 6 samples were picked, being the samples with the most pronounced reduction in OD values.

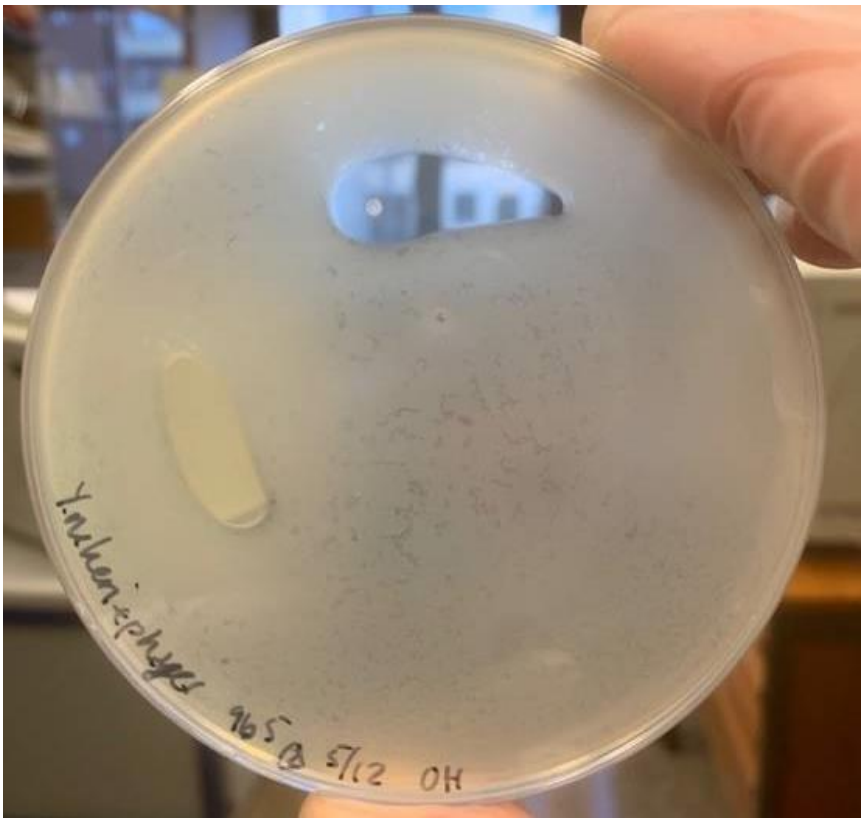

Figure 6: Isolate 9b5(2): Plaque formation in phage/bacteria mix in 3ml of 0,5 % soft agar on a LB plate with 10 mM $CaCl_2$

Table 4: OD values of *Yersinia ruckeri* after 4hrs following infection with different presumed phages and incubation with shaking at room temperature. The samples marked yellow were selected for further analysis.

Tube label	OD_f	Delta OD
LB broth (control)	0,041	0
Y.ruckeri (control)	0,606	0
9b-5 (2)	0,5307	0,0753
9b-5 (1)	0,5356	0,0704
3b-1	0,5374	0,0686
9b-11	0,5451	0,0609
1a-1	0,5486	0,0574
3b-3	0,5527	0,0533
1a-3	0,5538	0,0522
10a-2	0,5595	0,0465
3b-2	0,5628	0,0432
9c-3	0,5631	0,0429
6b-2	0,5636	0,0424
10b-7	0,5669	0,0391
6b-3	0,5670	0,0390
6b-1	0,5725	0,0335
9b-16	0,5743	0,0317
6b-7	0,5750	0,0310
9b-21	0,5785	0,0275
1a-7	0,5785	0,0275
9b-1	0,5790	0,0270
9b-13	0,5811	0,0249
8a-1	0,5816	0,0244
9b-11	0,5817	0,0243
1a-4	0,5845	0,0215
8a-2	0,5875	0,0185
9b-7	0,5879	0,0181
1a-4	0,5882	0,0178
10b-3	0,5891	0,0169
1a-6	0,5895	0,0165
10b-3	0,5897	0,0163
10a-3	0,5899	0,0161
9b-16	0,5908	0,0152
6b-4	0,5923	0,0137
9b-11	0,5934	0,0126
9b-10	0,5943	0,0117
9b-3	0,541	0,006
10a-4	0,6052	0,0008
9b-4	0,0549	-0,002
9b-17	0,6142	-0,0082
10b-5	0,491	-0,01
10b-6	0,492	-0,011
10c-2	0,495	-0,014
10a-1 (2b)	0,567	-0,017
9b-6	0,564	-0,014
6b-6 (1)	0,568	-0,018
3d-2 (2)	0,573	-0,023
6b-6 (2)	0,573	-0,023
10a-1 (3)	0,576	-0,0305
9c-2	0,6365	-0,0305
10c-1	0,513	-0,032
10b-4 (2)	0,583	-0,033
1d-5 (1)	0,586	-0,036
9b-25 (16)	0,591	-0,041
9b-20	0,593	-0,043
9b-25 (1a)	0,594	-0,044
10b-4 (1)	0,598	-0,048
1d5-2	0,601	-0,051
3a-1	0,601	-0,051
9a-2	0,601	-0,051
9a-1	0,601	-0,051
10b-1	0,6593	-0,0533
10a-1 (2a)	0,605	-0,055
6b-5 (2)	0,607	-0,057
9b-19	0,612	-0,062
6b-5 (1)	0,613	-0,063
1a-2	0,616	-0,066
3d-2 (1)	0,678	-0,128

Transmission electron microscopy

To confirm the presence and characterise the type of phages, three samples and a control were processed by transmission electron microscopy. Preliminary results showed the presence of capsids-like structures consistent with that of bacteriophages (Fig. 7) in one of the samples (10A). The sizes of the structures (approximately 200 nm) fall within the expected size of bacteriophages as shown elsewhere (48). Further studies to confirm our findings for example by preparing fresh, more concentrated phage samples for analysis could not be done due to Covid 19 restrictions at NMBU. This meant that no further work on characterization of phages could be done.

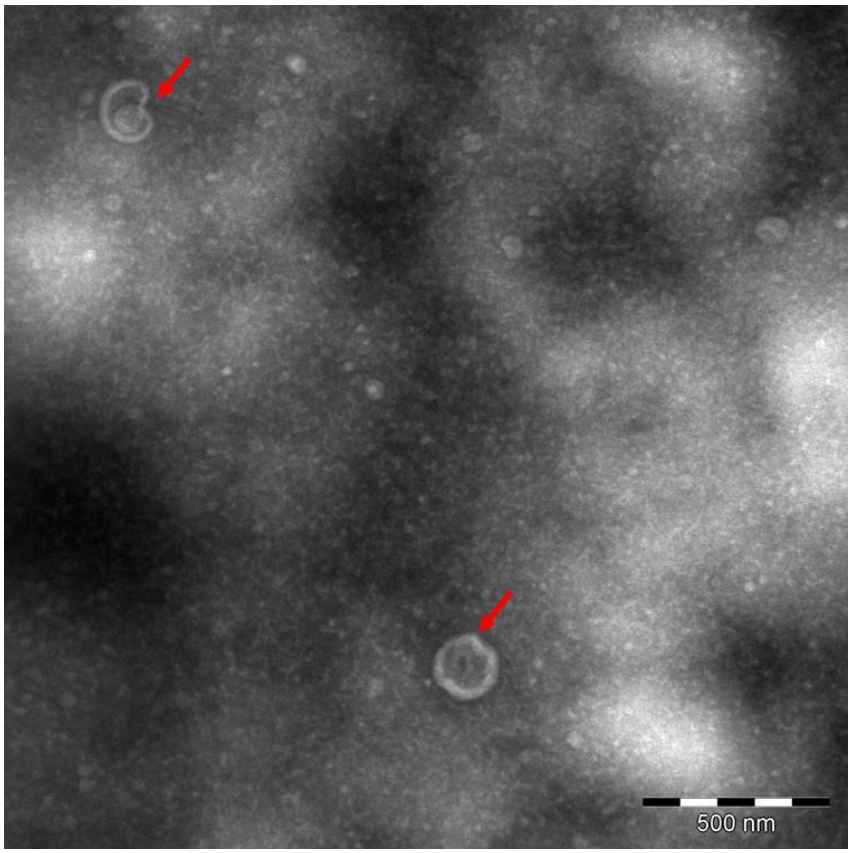

Figure 7: Transmission electron micrographs of suspected bacteriophages (arrows) against *Yersinia ruckeri*.

Discussion

Very little is known about *Y. ruckeri* bacteriophages. On PubMed for example, only two publications (33, 40) are available while there is only one non-peer reviewed thesis (49) in Google search and this is the only one that focuses on *Y. ruckeri* phages in Norwegian salmonids. This gap in knowledge makes *Y. ruckeri* bacteriophages an interesting topic to focus on and the findings of the present study suggest the availability of *Y. ruckeri* phages in the Norwegian salmonid farming industry, consistent with previous reports (40, 49, 50). This provides potential for alternative approaches to control *Y. ruckeri* infections in the industry.

Isolation of bacteriophages against *Y. ruckeri* from environmental samples

The water samples were collected from two different facilities in different parts of the country. From the location in Finnmark county we had four different sampling sites within the facility. Both facilities had outbreaks of yersiniosis prior to sampling. Although bacteriophages active against *Y. ruckeri* have been isolated from sewage (33), the best chances of isolating phages is to look where the host is present (45). Selecting water samples from production sites with confirmed problems with *Y. ruckeri*, was important when searching for bacteriophages as it gave a better likelihood to find phages for target bacteria (45). Having confirmed infection with the *Y. ruckeri* O1 CC1 strain in the facilities where the water samples were collected, increases the chances of isolating bacteriophages active against this strain. Most bacteriophages are thought to have a narrow host range, only able to infect one single strain of bacteria. However, Madhusudana Rao and Lalitha (34) state that the true host range for most phages are unknown, and both Nakai and Park (35) and Stevenson (33) isolated phages with a broader host range, the latter having worked with *Y. ruckeri* phages.

For the Finnmark locality, infection with the serovar O1 clonal complex 1 was confirmed approximately one month before sampling. Unfortunately, we were not able to obtain information about the bacterial strain causing the previous outbreak in the Mid-Norway location. Isolation of *Y. ruckeri* from the water sample and typing of the bacteria comparing it to the chosen strain used in the experiment, would have given us more information about host specificity of the phages isolated from this water sample. Supposedly, if the *Y. ruckeri* strain causing disease was not similar to the serovar we used in our thesis, we may have obtained less phages, due to the narrow host range of most phages.

Despite lack of information about the bacterial strain in the Mid-Norway location, we managed to isolate 7 plaques from this phage source. For the Finnmark locality we isolated in total 59 plaques, but this included 4 different sampling sites, and duplicates of all the samples, giving us an average of 7,4 plaques per sample. Hence, the Mid-Norway location was corresponding with the results from the other sampling sites. Five of the isolated plaques from the Mid-Norway sample came out positive on the culture lysis test, while two plaques came out negative. These results indicate that the phages are indeed active against the host strain used in the experiment, meaning that the previous outbreak in this locality is caused by the same strain or that the isolated bacteriophages have a host range involving more than one bacterial strain of *Y. ruckeri.*

As for the different sampling sites from the Finnmark locality, only one of the four sets of duplicates came out positive on both replicates. This was the water sample from the drain of the initial feeding unit, and the results may indicate that a higher density of phages were present in this water sample compared to the others. Yersiniosis is known as a disease that is more acute in young fish (fry and fingerling), while it manifests more often as a chronic infection in larger fish (21). Acute infections will spread more rapidly among the fish, resulting in a higher number

of bacterial shedding and thus more hosts for replication for the bacteriophages. As a result, the bacteriophages will increase in number, which may be a possible reason for a higher density of phages in this sample. However, further investigation would be needed for confirmation of this hypothesis.

The isolation protocol was performed with phages from the 20 plaques in the initial screening of water samples. Negative results were obtained from 9 out of 20 (45%) of the plaques. 11 of the initial 20 plaques (55%) gave positive results, yielding 66 phages. The number of phages had more than tripled. This was to be expected as the concentration of phages were expected to increase with each isolation. Possible reasons for the high number of negative results may be due to artefacts in the soft agar being mistaken as plaques in the screening of the water samples. Hockett and Baltrus (51) considered right temperature of the soft agar as a critical parameter for good results when using this method. Making sure the agar is thoroughly melted will avoid a grainy appearance on the surface of the plate, which makes the result hard to interpret. They claim that it will be possible to interpret the results despite the grainy overlay, but that this depends on the strength of the inhibition. In our case, a negative result probably represents a true negative. If not, the phage has a weak ability to lyse the bacteria, and in either case, it would not be of interest for the purpose of phage therapy. Other reasons for lack of plaque formation may be because of too high temperature of the soft agar when mixed with the bacteria/phage mixture, as this could result in killing of the bacteria. The tubes with soft agar were melted in the microwave and then placed in heated water (45 °C) right up until use. A better way of handling the soft agar, as proposed by Hockett and Baltrus (51), would have been to keep the tubes at 55-60 °C to prevent grainy overlay, but give it more time too cool down before adding the bacteria, to prevent killing of the bacteria.

During the lab work, we were three different people running different protocols sometimes at different times. Variations between the way we performed the tasks likely influenced the results that we obtained although it is not easy to pin point what contribution this had to our study. Anderson et. al. (52) states that despite this method being the most used method for detection of phages, poorly reproduceable results because of the risk of several parameters influencing the results, is one of the weaknesses. Also, Hyman (45) states that when testing for plaque formation, one can get false negatives in the sense that the phages reproduce and kill the bacteria, but still do not form visible plaques because of limited diffusion in agar or low productivity. However, these phages would probably not be very useful in phage therapy due to their low productivity (45).

Using a spectrophotometer as a tool to verify whether there were phages present in the bacterial culture depends on having isolates that are productive, resulting in lysis of the bacteria. If there were phages in the mixture, resulting in non-productive lysis, there would be no reduction in OD and therefore a false negative result (45). Yet, the culture lysis tests tend toward having false positives rather than false negatives as broth components may lead to bacterial killing (45).

Working with the bacteria we noticed that the growth varied a lot from the bacterial curve we established in the beginning of our lab work. There was, not surprisingly, a difference in the growth if we used bacteria from the -80 °C freezer, -18 °C freezer, 4 °C refrigerator or incubated in room temperature overnight, but there was also a big difference in growth when we used bacteria stored at the same temperature. These differences in bacterial growth made it hard to plan and predict the lab work. It is well known that differences in storage temperatures have an

influence on the viability of the bacteria. Others have used cryopreserved bacteria as a means for avoiding this problem (49).

In the final purification step, when filtering the supernatant of the isolates, we mistakenly used a filter that had a diameter of 1,2 μm instead of 0,2 μm on some of the isolates. *Y. ruckeri* bacillus are approximately 0.75 μm in diameter and 1–3 μm in length (21), so even though uninfected and resistant bacteria are removed by centrifugation, it is possible that some bacteria may have crossed through the filter along with the phages. In that way, bacteria that might have developed resistance against the phage could have been transferred to the phage/bacteria mix in the quantification step upholding the turbidity and growing on the plates. As this was confined to only a few of the first samples we purified we do not believe it affected our overall result.

Out of the 66 phages collected from the second round, 36 of them were recovered in the third round. Some plaques could have been false positives, but it was expected that more of would be true phages given the number of re-isolation. In the beginning, none of the isolates we tried to verify had a drop in OD value, yet some seemed to have plaque formation. Since we made one tube of bacteria for each phage it resulted in some unevenness in growth and potentially made the comparison with the control less significant. However, for the last 41 isolates one single batch of bacteria was made, in that way all the bacteria hit the exponential phase simultaneously. Most of our verified phages came from the remaining 41 isolates, so we suspect that if we had made one batch of bacteria and separated them into tubes after reaching an OD of 0,25 from the beginning we might have verified more plaques.

In this study, we did not re-use bacteria that had been exposed to the phages, since bacterial cells that rapidly develop resistance against the phage could have potentially given us false

negatives as well as they grow and uphold turbidity in the mix. Also, cell debris from already lysed bacteria can bind to and inactivate free phages (45). To be able to trust our results more, we plated out the first 25 isolates to see if there were plaque formation in correlation to the OD drop. Only one of the isolates had a drop in OD (and there was clearly plaque formation on the plate), but some plates had plaque formation and not OD drop. This strengthened our suspicion that we had some false negatives.

After the final purification step, the isolated phages were kept on 4 °C for about a month before the quantification, and when proceeding with the protocol it seemed like the phages were inactivated somehow. Isolates that previously had shown activity suddenly gave neither an OD drop nor plaque formation. Proceeding to the quantification of the phages we tried out different ratios of bacteria and phages for the dilutions, but we did not get any plaque formation on the plates, not even on those with the highest dilution. We tried to enrich the isolates with *Yersinia ruckeri* in exponential phase for four hours, but with no success; the phages still showed no activity. In a study from 1984, Stevenson et al. saw that two out of 18 *Y. ruckeri* phages lost all plaque formation activity after storage at 4 °C for 20 days, and while the rest of the phages remained active they showed a loss in infectivity titer after 80 days (33). This may be the case with our phages.

Characterisation of bacteriophages by transmission electron microscopy

The findings of this study revealed only two capsid-like structures following examination by transmission electron microscopy. These structures were in line with the expected sizes of bacteriophages (48) but these results are only preliminary because of too few phages observed, and also no tails were observed as reported elsewhere (40). The concentration of bacteriophages used in this study was too low to confidently detect bacteriophages follow-up experiment could not be done due to Covid 19 restrictions at NMBU.

In by Welch and colleagues (40), *Yersinia ruckeri* bacteriophages specific to serotype O1 *Y. ruckeri* were examined by Transmission electron microscopy. They revealed virion particles with 70 nM polyhedral heads and short tails which correlate with the *Podoviridae* phage family (40). Stevenson and Airdrie (33) too described *Y. ruckeri* bacteriophages in the electron microscope, and his phages were also tailed with icosahedral or octahedral heads.

Further characterisation of the isolated phages using PCR could also not be done due to the same restrictions of related Covid 19 at NMBU.

Conclusions

The work from this thesis has resulted in isolation of bacteriophages from water samples collected at two different freshwater facilities for farmed Atlantic salmon in Norway. All the protocols have been performed using *Y. ruckeri* serotype O1 clonal complex 1, described as the most common pathogenic isolate of *Y. ruckeri* found in yersiniosis outbreaks in Norwegian aquaculture. The results suggest that the isolated bacteriophages are active against the *Y. ruckeri* strain used, and that the bacteriophages are present in freshwater facilities, both with flow-through systems and RAS, of farmed Atlantic salmon that have experienced yersiniosis outbreak. Isolation of the phages can be done by relatively simple, but labor intensive laboratory methods. Further investigation of other localities with and without previous infections would give more evidence regarding the presence of such phages in Norwegian fish farms. Similar screening of seawater locations could also be an objective for further research.

Bacteriophages against *Y. ruckeri* may represent a potential as biocontrol as a supplement to vaccines combating yersiniosis infection in freshwater facilities for farmed Atlantic salmon.

Further characterisation, evaluation and testing of the isolated bacteriophages is necessary before such use of these bacteriophages is explored.

Acknowledgements

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Sammendrag

Tittel: Isolering og karakterisering av *Yersinia ruckeri* bakteriofager fra ulike fiskeoppdrettslokaliteter i Norge

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En økende forekomst av yersiniose har de senere år rammet norsk oppdrettslaks både i settefiskfasen og sjøfasen. Sykdommen gir septikemi og kan gi stor dødelighet blant yngel. Antibiotika og vaksiner brukes for å forebygge og kontrollere sykdommen, men interessen for sykdomskontroll ved hjelp av bakteriofager er økende. I denne oppgaven undersøker vi forekomst av bakteriofager fra anlegg som har hatt utbrudd av yersiniose. Det ble hentet inn 5 vannprøver fra to ulike settefiskanlegg. Vannprøvene ble brukt som kilde til bakteriofagene og et kjent *Y. ruckeri* serotype O1 isolat, et av det mest forekommende i oppdrettsanlegg i Norge,

ble brukt for å isolere og oppformere fagene. Etter en runde isolering og en runde rensing hadde vi positive prøver fra alle vannprøvene, totalt 66 isolerte plakk. Av disse ble 36 bakteriofager verifisert ved hjelp av en bakteriolyse- og en plakk-dannende test. De 6 isolatene som var mest potente i form av bakteriolyse ble valgt ut for videre karakterisering. Uheldigvis ble NMBU stengt på grunn av Koronavirus-utbruddet, så vi hadde ikke mulighet til å fullføre karakteriseringen.

Resultatene av denne studien viser at bakteriofager aktive mot *Y. ruckeri* er til stede i anlegg som har hatt et yersiniose-utbrudd. Videre studier for å karakterisere og etablere vertsspesifisiteten skulle blitt gjort som neste steg i dette arbeidet.

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Attachments

Appendix I

Protocols

All protocols are modifications of protocols from Molecular Cloning, a laboratory manual by Sambrook and Russel (47).

Quantification of bacteria

- Make a bacteria suspension of known OD, preferably 0.25-0.3
- Make serial six 10 fold dilutions of the bacteria in LB broth
- Plate 1ml of the different dilutions in triplicates on agar plates
- Incubate overnight at 20 degrees
- The next day, count bacterial colonies formed on each plate
- Calculate the number of cfu/ml in the original sample

$$\text{cfu/ml} = \frac{\text{number of colonies per plate}}{\text{dilution factor}}$$

Enrichment of phage source

Due to long transportation and thus risk of old water samples, phage source was enriched.

- Mix 1 part phage source (water) with 4 parts Luria-Bertani (LB) broth with 10mM $CaCl_2$
- Inoculate mix with one colony of *Y. ruckeri*
- Incubated overnight in room temperature in shaker
- Next day: Filter using 0,22µm syringe filter

Initial screening of phage source

- Mix 200µl (enriched and filtered) phage source with 100µl bacterial culture (OD=0,25-0,3) in a 15 ml tube
- Put the mix on ice for 10-15 min for the phage to attach to the bacteria.
- Add the mix to 2,2ml 0,5% soft agar, mix carefully
- Poured the mix on room temperature LB with 10mM $CaCl_2$ plates, make the agar cover the whole plate
- Wait maximum 10 minutes for the plate to solidify
- Put the plates in a plastic bag and incubated at 20 ºC overnight.
- Make a control following the same protocol, but replace phage source by filtered LB + $CaCl_2$ broth.
- Next day: Should be visible plaques on the plates

Isolation of bacteriophage (plaque purification)

Isolation:

- Stab the surface of the agar plate right above the center of the plaque using a 100µl pipette (set to 20µl).
- Put the content into 100µl LB broth with 10mM $CaCl_2$.
- Store phage in fridge at 4 degrees until use.

Purification:

- Mix 100µl isolated phage (from last step) with 100µl bacterial culture (OD 0,25-0,3) in a 15 ml tube
- Keep on ice for 10-15 minutes
- Add the mix to 2,2 ml 0,5% soft agar and mix carefully to prevent bubbles, pour it over plate and swirl once or twice, set to dry

    - PS: Only swirl once or twice, the agar is not supposed to cover the entire plate. The uncovered parts of the plate will function as a control.
- Invert plate and put in a plastic bag with wet paper towels, incubate at 20 degrees overnight
- Next day: Individual plaques should be visible on the lawn. Pick plaques following the same procedure as described above. If there are no plaques and the lawn has bacterial growth = no phages. If there are no plaques nor bacterial growth too much phage may have been added → Swirl fewer times.
- Store isolated plaques in 1,5 ml Eppendorf tubes at 4 ºC

Final purification/verification of phage

- To 5ml LB broth with 10mM $CaCl_2$, add 100µl bacteria from the freezer. Incubate at room temperature with shaking for about 5hrs (until OD=0.25-0.3)
- Add 100ul of phage extract and incubate for 4 hrs. Measure 350ul of this (OD) at 2hrs and then 4hrs.
- After 4hrs, get 200ul of the phage/bacteria mix and add to 300 ml of 0,5% soft agar and mix carefully to prevent bubbles, pour it over plate and swirl once or twice, set to dry (Do not allow over-drying)
    - Invert plate and put in a plastic bag with wet paper towels, incubate at 20°C overnight
- Centrifuge the remaining bacteria/phage mixture from the step above at 10.000rpm, 4°C for 10 min. Carefully harvest the supernatant and filter it through 0.22ul or 0.45ul using a syringe into a clean Eppendorf tube.
- Store purified phage in Eppendorf tubes at 4°C.

Quantification of phages

- To 5ml LB broth with 10mM $CaCl_2$, add 100µl bacteria from the freezer. Incubate at room temperature with shaking for about 5hrs (until OD=0.25-0.3)
- Make 10 fold dilutions of sample of purified phages up to 10^6
- Add 100ul of phage sample (each dilution) to 200ul of bacteria (OD 2.5), mix and incubate on ice for 20 min.
- Add to 300 ml of 0,5% soft agar and mix carefully to prevent bubbles, pour it over LB plate and swirl once or twice, set to dry (Do not allow over-drying)
    - Invert plate and put in a plastic bag with wet paper towels, incubate at 20°C overnight
- Next day: count the plaques on the plates where it is possible and calculate backwards the concentration in the original.

$$\text{plaques/ml} = \frac{\text{number of plaques per plate}}{\text{dilution factor}}$$

Characterisation of bacteriophages by electron microscopy

- Propagate the phage sample of choice (and a negative control) in fresh LB broth with *Y. ruckei* in log phase.
- Harvest and separate the phages from the bacteria first by centrifugation at 10000 x g at 4 °C for 10 minutes in a desktop centrifuge.
- Harvest the supernatant and filter through a 0,22 filter to exclude the bacteria, and discard the pellet

- To concentrate the phages, centrifuge the sample at 150000 x g in a desktop ultracentrifuge (Berkman) at 4 °C for 90 minutes. Remove the supernatant and resuspend the pellet in a residual volume of approximately 10 µl.
- Place one droplet of each sample on parafilm. Place a 400 mesh carboncoated coppergrid with formvar on the droplet of the sample for 30 min. Carefully blot the grid on filter paper and place on a droplet of 4 % uranylacetat for 1 min. Blot off excess solution and examine in transmission electron microscopy at 80 kV.

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