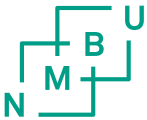

Norwegian University of Life Sciences
Faculty of Biosciences
Department of Plant Sciences

Philosophiae Doctor (PhD)
Thesis 2018:92

Potato soft rot *Pectobacteriaceae* in Norway – seed potato health, association with insects and molecular mechanisms of disease development

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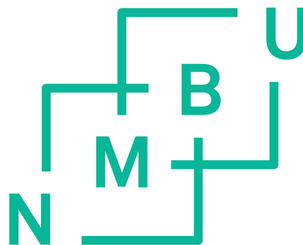

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"Dr. Leach stated in the introduction that when insects are involved in plant disease, three organisms must be considered and understood: the insect, the pathogen, and the host plant. He stressed the need to blend the sciences of entomology, microbiology, and botany, and attempted to do this throughout his book. He noted that successful control measures, with few exceptions, are dependent upon a knowledge of the methods by which pathogens are transmitted and the relative significance of each method. At the time of writing the book Dr. Leach noted that transmission in general was treated casually, and the role of insects in the spread of pathogens and development of plant diseases had not received the attention it deserved. His book remained for almost 20 years as the only text in the field. When confronted with the suggestion that insects do not transmit diseases, only the pathogens that incite them, Dr. Leach would respond with 'Do not quibble. To get all of this in the title of a book is too cumbersome.'"

- Gallegly and Barnett (1989)

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Abstract

Soft rot *Pectobacteriaceae* are among the most problematic pathogens of a wide variety of crop plants and ornamentals. Among their hosts is potato, the most important food crop in Norway, where the bacteria cause soft rot of the tubers and blackleg of the stem. The soft rot *Pectobacteriaceae* currently comprise 18 recognized bacterial species of the genera *Dickeya* and *Pectobacterium*, which are somewhat diverse in aspects like geographical distribution, preferred temperature for growth and virulence, typical host range, as well as the disease symptoms they cause on different hosts. Nevertheless, they all feed on rotten host tissue, which they macerate by secreting a variety of plant cell wall degrading enzymes into the intercellular space.

In this work, several aspects of soft rot *Pectobacteriaceae* biology and virulence were investigated with the ultimate goal of contributing to the improvement of potato seed health. To gain more insight into the taxonomy and phylogeny of these pathogens, the genome sequencing of two isolates, which appeared atypical in previous phylogenetic analysis, was performed. This lead to the description of the new species *Pectobacterium polaris*, which subsequently was found in old isolate collections in Poland and the Netherlands. The identification of this new species may lead to improved detection tools, which facilitate sensible management strategies.

The health of Norwegian potato seed of varied quality and different cultivars was assessed in a three-year field study. This was done to investigate the influence of environmental and host factors, as well as testing the implementation of large scale molecular testing of the pathogens' infection prevalence. The bacteria were found to be near-ubiquitous, regardless of potato cultivar or seed quality, except for seed of the highest quality. These tubers quite reliably tested low in infection prevalence and disease was rarely observed compared to lower quality seed. Climate, particularly precipitation seemed to play the most important role in infection prevalence. Disease incidence for blackleg was partially determined by the host, as demonstrated by one cultivar (Innovator), which seemed less susceptible to this disease. It also performed best in a test, where tubers from 15 different cultivars were infected with *P. polaris* and *P. atrosepticum*. In this test, it was also shown that temperature was the factor that had the overall largest influence on rot outcome. This study gave insight into the

status of Norwegian seed potatoes in regard to soft rot and important environmental factors in disease.

In addition, a two-year field study was performed to examine the presence of the pathogens in insects that were caught in potato fields. The insect were tested by qPCR, which allowed the selection of specimens which contained a high amount of bacteria. These insect were identified by DNA sequencing, and were found to belong to over 90 different species. In the two years, 15 and 20 % of tested insects contained a high amount of soft rot bacteria. A high percentage of insects in a field where clean seed potatoes were grown, was carrying the bacteria, which is highly relevant, because it is not clear how clean seed potatoes are initially infected. Remarkably, the seed corn maggot (*Delia platura*) was the species that most often carried a high amount of the bacteria. The same species and closely related flies were previously shown to transmit the bacteria to potato and other plants. By showing the high number of insects that carried the soft rot bacteria, and identifying the insects as known vector species, it was demonstrated that insects may play a much more important role in the infection of clean seed than previously assumed.

Furthermore, the transcriptomes of the type strain of *P. polaris*, as well as a strain of *P. carotovorum* subsp. *carotovorum* were analysed during early and progressing tuber infection by RNA sequencing. This was done to examine the regulation of virulence factors in the bacteria over the development of disease. In both examined strains, important virulence genes, encoding e.g. the enzymes that degrade host cell walls, as well as several bacterial secretion systems, decreased in expression over time. This, indicated a shift in prioritized function in the investigated bacteria. A better understanding of the molecular processes and their trajectory in progressing infection may help in devising and optimizing counterstrategies.

Sammendrag

Bløtråtebakterier er blant de mest problematiske skadegjørere i mange forskjellige kulturplanter, inkludert potet der de forårsaker sykdommene bløtråte og stengelråte. Bløtråtebakterier består per i dag av 18 kjente bakteriearter fra slekten *Dickeya* og *Pectobacterium.* Disse er noe ulike med hensyn på geografisk distribusjon, foretrukket temperatur for vekst og sykdomsspredning (virulens), typisk vertsspekter, i tillegg til hvilke sykdomssymptomer de gir. De likevel næres alle av vertens råtne vev, som de bløter opp ved å skille ut en rekke plantecelleveggdegraderende enzymer i det intercellulære rommet.

Dette arbeidet har undersøkt flere aspekt ved bløtråtebakteriers biologi og virulens. Målet har vært å bidra til forbedring av settepotetenes helse. For å få bedre innsikt i taksonomi og fylogeni av disse bakteriene, har genomene blitt sekvensert av to isolat som virket atypiske i tidligere fylogenetiske analyser. Dette førte til beskrivelse av den nye arten *Pectobacterium polaris,* som etterpå ble funnet igjen i eldre samlinger av isolat i Polen og Nederland. Identifiseringen av denne nye arten kan føre til forbedrede deteksjonsmetoder, som igjen kan gjøre det lettere å tilrettelegge for tilpassede bekjempelsesstrategier.

En treårig feltstudie ble også gjennomført, der helsen til norske settepoteter av ulike sorter og varierende kvalitet ble vurdert. Dette ble gjort for å undersøke faktorer knyttet til potetsorter og miljø, i tillegg til å teste implementeringen av storskala molekylær testing av utbredelsen av bløtråtebakterier. Undersøkelsen viste at bløtråtebakterier var nesten allestedsnærværende, uavhengig av potetsort og kvalitet. Et unntak var knoller av høyeste kvalitet. Disse knollene testet nokså pålitelig lavt i infeksjonsutbredelse, og sammenlignet med i knoller av lavere kvalitet, var sykdom sjeldent observert. Klima, og særlig nedbør, spilte den viktigste rolle for infeksjonsutbredelse. Forekomst av stengelråte var delvis bestemt av potetsorten. Dette kom særlig fram i en sort (Innovator), som var minst mottakelig mot denne sykdommen. Den kom også best ut i en test, der 15 forskjellige sorter ble infisert med *P. polaris* og *P. atrosepticum.* I denne testen kom det også fram at temperatur var den faktoren som hadde størst effekt på utvikling av råten. Denne studien ga innsikt i helsestatusen til norske settepoteter med hensyn på bløtråte og viktige miljøfaktorer som påvirker sykdommen.

I tillegg ble en toårig feltstudie gjennomført for å undersøke tilstedeværelse av bløtråtebakterier i insekter som ble fanget i potetåkrer. Insektene ble testet med qPCR, som gjorde det mulig å velge ut eksemplarer som inneholdt store mengder bakterier. Disse insektene ble identifisert med DNA-sekvensering, og viste seg å tilhøre over 90 ulike arter. I løpet av de to årene, inneholdt 15 og 20 % av de testede insektene store mengder av bløtråtebakterie, inkludert et felt der rene settepoteter ble dyrket. Dette er interessant fordi man ikke sikkert vet hvordan settepoteter blir først infisert. Bønneflue (*Delia platura*) var den arten som oftest bar med seg store mengder av bakterien. Den samme arten, og nært beslektede fluer, har i tidligere studier vist seg å overføre bakterien til poteter og andre planter. Det høye antallet insekter som var bærere av bløtråtebakterier og identifisering av kjente vektorarter, viste at insekter potensielt spiller en mye viktigere rolle i smitte av rene poteter enn først antatt.

Transkriptomet av typestammen til *P. polaris*, i tillegg til en stamme av *P. carotovorum* subsp. *carotovorum*, ble analysert under infeksjon av potetknoller ved hjelp av RNA-sekvensering. Dette ble gjort for å undersøke regulering av virulensfaktorer i bakterien i ulike utviklingsstadier av sykdommen. I begge de undersøkte stammene ble viktige virulensgener, som f.eks. koder for enzymer som bryter ned vertens cellevegg, i tillegg til flere bakterielle sekresjonssystemer, redusert i uttrykk over tid. Dette indikerte et skifte i de prioriterte funksjonene i den undersøkte bakterien. Bedre forståelse av de molekylære prosessene og framdrift av infeksjonen, kan hjelpe med å utvikle og optimalisere bekjempelsesstrategier.

List of publications

I. Dees, M. W., Lysøe, E., Rossmann, S., Perminow, J., & Brurberg, M. B. (**2017**). *Pectobacterium polaris* sp. nov., isolated from potato (*Solanum tuberosum*). International Journal of Systematic and Evolutionary Microbiology, 67(12), 5222-5229.

II. Rossmann, S., Dees, M. W., Torp, T., Le, V. H., Skogen, M., Glorvigen, B., van der Wolf, J., & Brurberg, M. B. (**manuscript**). Investigation of factors determining potato diseases caused by soft rot Pectobacteriaceae using quantitative PCR assays.

III. Rossmann, S., Dees, M. W., Perminow, J., Meadow, R., & Brurberg, M. B. (**2018**). Soft rot *Enterobacteriaceae* are carried by a large range of insect species in potato fields. Applied and Environmental Microbiology, 84(12), e00281-00218.

IV. Rossmann, S., Lysøe, E., Dees M. W., & Brurberg, M. B. (**manuscript**). Transcriptome analysis of two *Pectobacterium* strains shows initial spike and successive downregulation of secretion systems and virulence factors during soft rot progression in potato.

1 Introduction

This work comprises research on various aspects of the ecology, taxonomy, and molecular mechanisms of bacterial plant pathogens in the genera *Pectobacterium* and *Dickeya*. The variety of research disciplines spanned by this thesis is a testament to the amount of unanswered questions that remain in the understanding of these bacteria. Simultaneously, findings from almost a century of experimental work were discussed in the context of the obtained results, demonstrating the perpetual relevance of these questions.

1.1 Soft rot *Pectobacteriaceae*

Bacteria of the genera *Pectobacterium* and *Dickeya* cause soft rots and were recently reclassified from *Enterobacteriaceae* into the new family *Pectobacteriaceae* (Adeolu et al., 2016). Although a taxonomically diverse group, soft rot *Pectobacteriaceae* (SRP) are plant pathogens, and cause similar symptoms on a large variety of hosts. Their host-range includes a diverse set of crops such as cabbage, carrot, corn, cucumber, horse radish, rutabaga, pepper, potato, and tomato, as well as a large number of ornamental plants (Charkowski, 2018; Ma et al., 2007). The economic impact of diseases caused by SRP, as well as the long history of research conducted on them, has led to the inclusion of both *Dickeya* and *Pectobacterium*, in the 'Top 10 plant pathogenic bacteria in molecular plant pathology' (Mansfield et al., 2012).

During infection, SRP secrete a wide array of plant cell wall degrading enzymes (PCWDEs), including pectinases, cellulases, and protease, which vary between different SRP strains and species in efficiency and numbers (Dees et al., 2017a; Glasner et al., 2008). The phylogenetic complexity of *Dickeya* and *Pectobacterium* is reflected in the large and continuously growing list of described species. At its original conception in 2005, the genus *Dickeya* contained the six species *D. chrysanthemi*, *D. dadantii*, *D. dianthicola*, *D. dieffenbachiae*, *D. paradisiaca*, and *D. zeae* (Samson et al., 2005). Since then, three new species, *D. aquatica, D. fangzhongdai*, and *D. solani* have been identified, and *D. dieffenbachiae* was reclassified as *D. dadantii* subsp. *dieffenbachiae* (Brady et al., 2012; Parkinson et al., 2014; Tian et al., 2016; van der Wolf et al., 2014).

For *Pectobacterium,* five species out of a total of ten have been newly described or reclassified over the last five years. The species described currently are *P. aroidearum*, *P. atrosepticum*, *P. betavasculorum*, *P. cacticidum*, *P. carotovorum*, *P. parmentieri*, *P. peruviense*, *P. polaris*, *P. punjabense*, *P. wasabiae*, and the proposed genomospecies "*Candidatus* Pectobacterium maceratum" (Dees et al., 2017b; Gardan et al., 2003; Khayi et al., 2016; Nabhan et al., 2013; Sarfraz et al., 2018; Shirshikov et al., 2018; Waleron et al., 2018b). Historically *P. carotovorum* was used as a catchall species for many SRP, due to their similar symptomatology and morphology. Currently, it contains four subspecies, *P. c. actinidae*, *P. c.* subsp. *brasiliense*, *P.c.* subsp. *carotovorum*, and *P. c.* subsp. *odoriferum* (Nabhan et al., 2012a; Zhang et al., 2016). However, these have been suggested to be elevated to the species level after *in silico* analysis (Zhang et al., 2016). *P. c.* subsp. *carotovorum* still contains distinct phylogenetic clusters, which complicates detection of this most common SRP subspecies and may lead to further reclassification (Dees et al., 2017b; Nabhan et al., 2012b; Zhang et al., 2016).

1.2 Potato farming and important diseases

Potato (*Solanum tuberosum* L.), is among the most important crops worldwide and a cornerstone of Norwegian agriculture, and identity. The starchy tubers commonly referred to as 'potatoes' are the harvested product and the typical seed material in potato farming. Seed potatoes can be stored for many months in cold, dark conditions and retain their ability to sprout and give rise to new plants, which produce multiple offspring. In some regions, such as North America and India, it is common to cut the tubers into seed pieces that retain their ability to sprout instead of using whole tubers in order to save costs (Kumar et al., 2015; Webster et al., 2018).

Several pathogenic bacteria, fungi, and oomycetes are responsible for devastating potato diseases that are transmitted via seed tubers (Wustman, 2007). The oomycete *Phytophthora infestans* causes late blight, and is infamous as the causal agent of the Irish potato famine (Nowicki et al., 2012). Dry rot, caused by *Fusarium* spp. is a fungal disease that causes tissue degradation in stored and planted tubers (Wustman, 2007). Gangrene, a fungal disease caused by *Boeremia* spp. is characterized by dark lesions on the surface of tubers that may appear similar to dry rot (Wustman, 2007). Several

pathogens cause skin blemish diseases that are primarily reducing the quality and value of the yield due to their unappealing effect on tuber appearance, although they may also affect seed performance in some cases. In Norway, the skin blemish diseases (scab and scurf) common scab (*Streptomyces* spp.), black dot (*Colletotrichium coccodes*), black scurf (*Rhizoctonia solani*), powdery scab (*Spongospora subterranea*), silver scurf (*Helminthosporium solani*), and skin spot (*Polyscytalum pustulans*) are very common (Nærstad et al., 2012). The most important bacterial diseases besides the ones caused by SRP are bacterial wilt (*Ralstonia solanacearum*), bacterial ring rot (*Clavibacter michiganensis* ssp. *sepedonicus*), common scab (*Streptomyces* spp.), and the zebra chip disease caused by "*Candidatus* Liberibacter solanacearum" (Munyaneza, 2012; van der Wolf & de Boer, 2007). In addition to these fungal and bacterial diseases, several viruses, such as e.g. *Potato virus X* (PVX), *Potato virus Y* (PVY), and *Potato leafroll virus* (PLRV), cause devastating diseases in potato (Loebenstein et al., 2013; Wang et al., 2011).

The carry-over and an accumulation of tuber-borne pathogens from one field generation to the next is an inherent problem of using tubers as seed material. Seed potato regulations and certification schemes are therefore employed in most nations in order to limit and control pathogen spread and consequently minimize yield losses. Certification schemes mostly rely on grades according to disease symptoms, pathogen testing, adherence to certain planting practices, and the number of generations a given seed has been used (UNECE, 2004). A maximum number of generations before the seed is disqualified is included in most of these seed potato regulations, regardless of other factors. In Norway, seed potato production is governed by the regulation FOR-1996-07-02-1447, which defines, among other things, certification standards, and a maximum of seven generations in the field before loss of certification (Norway, 1996).

In order to satisfy these demands for new, healthy planting material, it is necessary to generate tubers independently from previous seeds. Initially, stem cuttings were taken and grown in a controlled greenhouse environment, primarily to combat virus infections (Cole & Wright, 1967). Currently, aseptic tissue cultures are propagated *in vitro* into 'microtubers', and then multiplied using hydroponic or aeroponic systems to obtain so called 'minitubers' (Ahloowalia, 1994; Ritter et al., 2001). In Norway,

minitubers are generated using meristem cultures and hydroponics, and multiplied in dedicated field sites for the first generation by a single producer, Overhalla Klonavlssenter AS (Aspeslåen et al., 2016).

1.3 SRP as potato pathogens

1.3.1 Pathogenic species in potato

Not all of the *Dickeya* and *Pectobacterium* species listed in section 1.1 are pathogens of potato. Traditionally, *P. atrosepticum*, *P. c.* subsp. *carotovorum*, and *Dickeya* sp. have been viewed as common potato pathogens (Pérombelon, 2002). While the genetic homogeneity of *D. solani* isolates suggests that it is a newly emerged species, other SRP, such as *P. polaris* and *P. parmentieri* were found in older isolate collections, demonstrating that they have been potato pathogens long before their description (Khayi et al., 2016; van der Wolf et al., 2014; Waleron et al., 2018a).

While *P. atrosepticum* and *P. c.* subsp. *carotovorum*, are still regarded important potato pathogens, outbreaks of other species have been of major concern. *D. solani*, *P. c.* subsp. *brasiliense*, and *P. parmentieri* were responsible for considerable yield losses in large parts of Europe. *D. dianthicola* is commonly isolated from symptomatic plants in the U.S., and has also been found on potatoes in Europe and Australia (Ma et al., 2018; Toth et al., 2011; van der Wolf et al., 2014; van der Wolf et al., 2017; Wright et al., 2018). *D. dadantii* was found to be the dominant SRP species on potato in South Africa and Zimbabwe (Ngadze et al., 2012).

In Norway, *Dickeya* species are considered not present. However, *D. solani* was detected twice, which led to the destruction of the affected crop and the implementation of counter measures by the Norwegian food health authorities (Dees et al., 2017a; Rossmann et al., 2018a). The most common SRP species in Norway seem to be *P. atrosepticum*, P. *c.* subsp. *carotovorum,* and *P. parmentieri*, based on a large collection of SRP from the last years (Dees et al., 2017a). Although definitely present, the distribution and frequency of *P. polaris* in Norway is not yet known, since routine assays are not yet available (Dees et al., 2017b).

1.3.2 Symptoms caused by SRP in potato

All diseases caused by SRP follow the same basic pattern. Upon successful and sustained infection of the host, the bacteria produce and secrete a wide array of plant cell wall degrading enzymes (PCWDEs) by which the host's tissue is macerated (Pérombelon, 2002). The degraded components then serve as nutrition for the SRP, which rapidly multiply. Utilizing this basic mechanisms, SRP can cause a variety of distinct rotting symptoms on different potato organs. The two diseases that are most characteristic for SRP in potato are soft rot and blackleg (Figure 1).

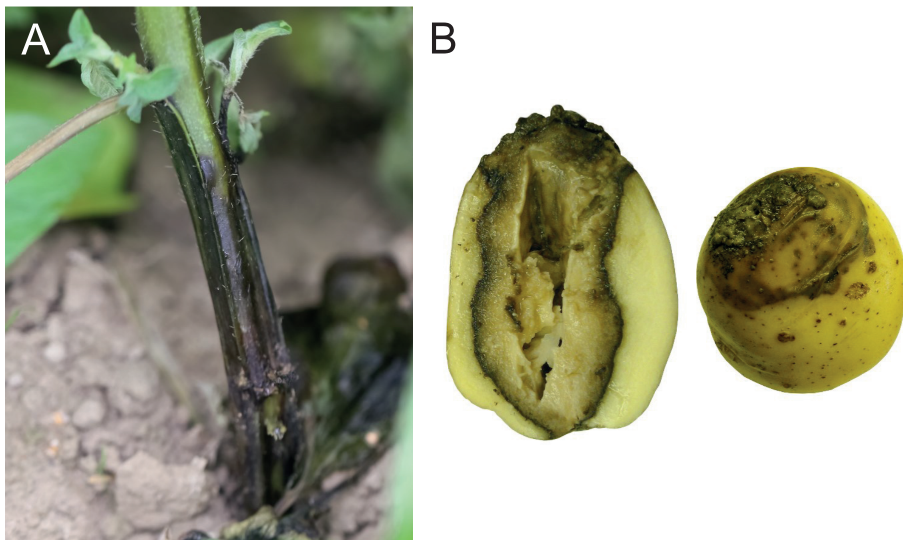

Figure 1. Symptoms of soft rot *Pectobacteriaceae* disease on potato stem and tubers. (**A**) Blackleg on the stem of a young potato plant (photo: Merete Wiken Dees). (**B**) Severe soft rot on a halved (left), and whole (right) potato tuber (photo: May Bente Brurberg)

Soft rot is a disease of the tuber that is characterized by a wet, slimy mass of macerated tissue, often with a clear border zone, if the tuber is not completely broken down, and often a strong odour (Charkowski, 2018). The outside view of the whole tuber shown in Figure 1 is typical, as the thin skin of the tubers often remains intact, while the tissue directly underneath it is degraded, making symptomatic regions soft to the touch. Blackleg is a disease of the potato stem that owes its name to the distinctive black colour that develops, and it typically affects the lower part of the stem (Charkowski, 2018). Blackleg occurs after systemic infection of the plant following soft rotting of the mother tuber and upward movement of the bacteria through the xylem (Pérombelon & Kelman, 1987; Pérombelon et al., 1989). While a

number of SRP can cause blackleg, *P. c.* subsp. *carotovorum* is unable to (Charkowski, 2018; Pérombelon, 2002). SRP can also cause stem rots with lighter colouring and a different progression. While blackleg is historically defined as a consequence of infection in the mother tuber, aerial stem rot occurs after infection of above-ground plant wounds and translocation of the bacteria through the xylem (Czajkowski et al., 2010a; Pérombelon & Kelman, 1987; Pérombelon, 2002). Furthermore, *Dickeya* spp. causes stem symptoms similar to blackleg, so called stem wet rot (slow wilt), which is characterized by more pronounced decay of the vascularity and less apparent symptoms on the stem surface (de Boer et al., 2012; van der Wolf & de Boer, 2007).

1.3.3 Environmental and host factors

Several environmental and host factors influence the course of infection, of the different SRP species. Temperature can greatly influence the occurrence of different SRP species and determine their success in causing disease. Different species were shown to have different growth range and optimum temperatures, as well as different optimum temperatures for activity of their PCWDEs (du Raan et al., 2016; Smadja et al., 2004). Interestingly, the optimum temperatures for growth and enzyme activity were different, suggesting a trade-off between fast growth and maximum maceration (Smadja et al., 2004). Of the tested species, *P. atrosepticum* had the lowest temperature range and was able to grow between 18 and 31°C (optimum 26-27°C), which is consistent with the relative predominance of *P. atrosepticum* in cooler climate zones (du Raan et al., 2016). For comparison, *D. solani* had the highest optimum temperature with 35°C, and was able to grow between 24 and 42°C (du Raan et al., 2016).

In addition to temperature, humidity and oxygen availability play a big role in SRP infection. Under wet soil conditions, SRP are able to make full use of their motility and can infect nearby plants through roots and lenticels (Czajkowski et al., 2010b; Pérombelon, 2002). Rain drops, but also agricultural practices (haulm killing) may contribute to aerosol formation and spread SRP over large distances (Perombelon et al., 1979; Quinn et al., 1980). Most importantly, however, strong rainfall and poor field draining will lead to waterlogging around tubers and roots. This creates low-oxygen conditions, leading to stressed plants with reduced ability to fend off pathogens, while

the SRP are facultative anaerobes that tolerate such situations well (Burton & Wigginton, 1970; de Boer & Kelman, 1978; Pérombelon, 1992). Good control of temperature, humidity, and oxygen levels during storage is therefore central in controlling soft rot disease. Under good storage conditions, SRP decrease in numbers over time (van Vuurde & de Vries, 1994). Thorough drying of the tubers before storage, evenly distributed cold temperatures and good ventilation are necessary to prevent outbreak and spreading of SRP (Czajkowski et al., 2011).

Some plant nutrients have been shown to decrease host susceptibility against SRP diseases. High calcium levels contributed to host resistance and were able to reduce disease incidence in the greenhouse and field (Bain et al., 1996; McGuire & Kelman, 1984). High nitrogen levels were shown to decrease the susceptibility of *Philodendron selloum* to *Dickeya* sp., but lead to stunted plant growth (Haygood et al., 1982). Likewise, high nitrogen fertilization lead to decreased blackleg incidence compared to low nitrogen fertilization (Graham & Harper, 1966).

As for the potato host, tested commercial cultivars varied significantly in their ability to withstand tuber and stem rot in several studies (Chung et al., 2013; Lyon, 1989; Pasco et al., 2006; Rossmann et al., 2018b; Rouffiange et al., 2014; Tzeng et al., 1990). However, breeding for resistance against SRP has historically been given low priority and is not trivial due to the lack of identified resistance sources (Czajkowski et al., 2011). Nevertheless, susceptibility to tuber soft rot was decreased by crossing with the wild potato species *Solanum brevidens*, suggesting that breeding may be employed to control SRP diseases to some extent (Tek et al., 2004).

1.3.4 SRP transmission and control

In the farming of potatoes, tubers are used as seed material, which favours the transmission of tuber-borne pathogens like SRP from one field generation to the next. Once rot breaks out in storage, SRP infect adjacent tubers and either rot a large amount of stored material or get carried into the next field generation. In addition to storage conditions and environmental aspects that favour transmission in the field, farming routines are assumed to contribute substantially to transmission. SRP may

spread by smearing during mechanical haulm cutting, harvest and tuber handling (e.g. grading), and especially seed cutting, where practiced (Czajkowski et al., 2011).

No chemical control agents are employed to combat SRP diseases in the field or storage, since no sustainable and ecologically viable options are available (Czajkowski et al., 2011). Some biological control agents have been evaluated, but none have proven particularly effective in the field (Czajkowski et al., 2011). However, certain approaches like target specific bacteriophages or rhizome bacteria that block quorum sensing signals, a key component of SRP disease onset, show promise and may lead to future control applications in the field or storage (Cirou et al., 2010; Lim et al., 2013). Effective available strategies target the avoidance of infection, transmission, and clean seed material. To avoid infection and disease outbreak, good hygiene and farming practices are central, e. g. regular and thorough cleaning of harvesting and sorting machines, use of whole tubers instead of cut seed, draining to avoiding waterlogging in the field, and allowing for proper wound healing and drying of tubers before storage (Czajkowski et al., 2011). By far the most efficient measure in minimizing SRP levels and disease incidence is the use of clean seed (Rossmann et al., 2018b). However, the generation of clean seed from tissue culture is expensive and currently can not supply sufficient material for general use without prior multiplication by dedicated seed growers.

1.3.5 Association of SRP and insects

While the spread of SRP via seed tubers, machines, aerosols, and motile bacteria in the soil is well documented and discussed, these pathways largely rely on the ubiquitous presence of SRP in the vicinity of the tubers or plants. Initially seed tubers grown from tissue culture and multiplied in the greenhouse are free of the pathogens, as shown for Norwegian P2 seed (Rossmann et al., 2018b). During multiplication in the field however, the clean seed material becomes infected with SRP and infection rates can rapidly increase within few field generations (Boomsma et al., 2013; Czajkowski et al., 2011; Rossmann et al., 2018b). While the rapid increase may be explained by the transmission ways outlined above, initial infection is not. Insect transmission has therefore been suspected to be involved in the infection of clean seed with SRP.

The first observation of SRP being associated with insects was made in Minnesota in 1925, when Dr. Julian Gilbert Leach suspected the seed maggot (*Delia platura*) to transmit SRP to healthy potato plants (Leach, 1925). He observed *D. platura* laying eggs near potato tubers shortly after planting and hypothesized that the larvae transmitted SRP to tubers by boring into them (Fig. 2; Leach, 1926). Leach tested those observations extensively and produced ample evidence supporting a close, potentially symbiotic, relationship between *D. platura* and SRP (Leach, 1926, 1931, 1934). His observations included the presence of SRP on the egg surface but not inside the eggs of *D. platura*, high mortality of sterile larvae which could be amended by supplying SRP, overwintering of the bacteria inside the pupae, and SRP growing in the path of freshly hatched, wandering *D. platura* larvae on sterile medium. When discussing the conclusions made in the work of J. G. Leach, it is important to bear in mind the limited availability of diagnostics and understanding of SRP diseases at the time of the experiments.

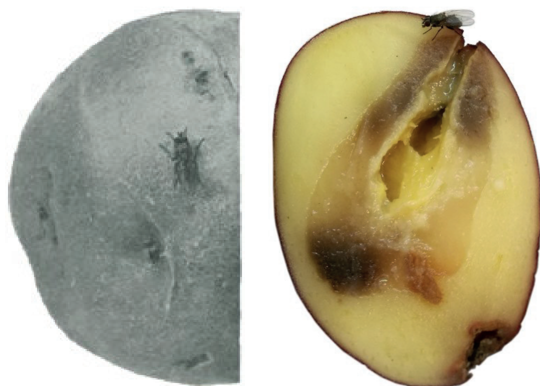

Figure 2. Flies on potato tubers. Left: *Delia platura* (female adult) on potato seed piece, photo: Leach (1926). Right: Unidentified adult fly on rotting tuber (cultivar Lunarossa), three days after inoculation with *P. polaris* strain NIBIO1006

Shortly after Leach's findings on *D. platura*, *D. radicum* (cabbage maggot) and and *D. antiqua* (onion maggot) were described to transmit SRP to their respective hosts, brassica crops and onions (Bonde, 1930; Doane, 1953; Doane, 1959; Doane & Chapman, 1964; Johnson, 1930). Later, it was shown that artificially inoculated *Delia platura* adults can transmit *Pectobacterium carotovorum* subsp. *carotovorum* to healthy potato plants in a cage experiment (Phillips & Kelman, 1982). There, it was also shown that a specific, artificially introduced SRP strain can persist in a cull pile

and was found in various insects around the pile. The introduced SRP strain was detected with specific antibodies in harvested tubers from plants that grew up to 26 m away from the cull pile.

Another insect vector for SRE that has received some attention is *Drosophila*. The first account of *D. melanogaster* transmitting SRP between infected and healthy potato plants was made by Molina et al. (1974). Later experiments showed that at least some SRP strains have developed to survive in and on *D. busckii* and *D. melanogaster* for up to 72 h (Brewer et al., 1981). Subsequently *P.c. subsp. carotovorum* strain Ecc15 was shown to evoke and immune response in *D. melanogaster*, and the interaction was shown to be promoted by a single gene, the *evf* (*Erwinia* virulence factor) gene (Basset et al., 2000; Basset et al., 2003). Furthermore, the *evf* gene increased bacterial survival in the gut of *D. melanogaster* (Muniz et al., 2007; Quevillon-Cheruel et al., 2009). Unrelated to potato, *Dickeya dadantii* was shown to be pathogenic to the pea aphid *Acyrthosiphon pisum* by expressing the *cytABCD* cluster of genes, which have homology to target-specific insect cytotoxins encoded by *Bacillus thuringiensis* (Grenier et al., 2006).

Attempts to identify insects that may transmit SRP to clean potatoes were made in a field for the multiplication of stem cutting material in Scotland in the 1970s (Graham et al., 1976). Almost 2500 insects were collected over four consecutive years using different collection methods. *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) was isolated from 15 samples (11 individual and 4 bulked) belonging to the genera *Leptocera* (5 samples), *Fannia* (2 samples), *Drosophila* (1 sample) and *Vespa* (1 sample). The rest of the samples were only determined to order level with 4 unidentified Diptera samples and 2 unidentified Hymenoptera samples. SRP contaminated dipterous insects were observed before the emergence of symptoms on the previously clean plants, indicating a possible transmission by some of these taxonomically diverse insects and some SRP source other than the clean potato plants. Simultaneously, nearby waste potato piles were sampled for insects (Harrison et al., 1977). There, SRP were isolated from between 3 and 6% of the tested insects, which were identified as belonging to 12 genera within the order Diptera.

The association of SRP and Diptera, particularly *Delia* species, has also been shown in current work. In recent microbiome studies of blowflies and houseflies, as well as

cactophilic *Drosophila* species, SRP were commonly found associated with those flies (Junqueira et al., 2017; Martinson et al., 2017). Furthermore, a *Pectobacterium* sp. strain present in the *D. radicum* gut microbiome was shown to be able to break down phenolic plant components that are toxic to the insect (Welte et al., 2016).

Despite all of the efforts summarized above, insect transmission and interaction remain some of the least understood components in SRP ecology, and are only mentioned briefly, if at all, in reviews on potato soft rot and blackleg diseases (Charkowski, 2018; Czajkowski et al., 2011; Pérombelon, 2002).

1.3.6 Molecular mechanisms

SRP can remain latent in tubers for several months, and latent infection is widespread (Pérombelon, 2002; Rossmann et al., 2018b). Latent infection is maintained until environmental and/or physiological conditions favour disease outbreak. On a molecular level, growth to a sufficient number of SRP after latent infection is accompanied by the so-called 'stealth mode', the tight regulation of virulence factor expression and manipulation of host defence (Charkowski et al., 2012; Toth & Birch, 2005). This 'stealth mode' was proposed as an antonym to the perceived 'brute-force' nature of SRP infection, which presupposed rapid breakdown of host cell walls by a variety of PCWDEs to be the main aspect of SRP virulence (Toth & Birch, 2005). The network regulating virulence is fairly complex, and the discovery of disease factors beyond PCWDEs has contributed to a diversified understanding of SRP infection.

The central role of the various cellulases, pectinases, proteases and other enzymes that are collectively referred to as PCWDEs remains undisputed. However, the diversity, amount and efficiency of these enzymes vary between SRP species and strains (Dees et al., 2017a; Glasner et al., 2008). Most PCWDEs are exported through the type II secretion system (T2SS), also referred to as the 'out system' (Charkowski et al., 2012; Corbett et al., 2005; Coulthurst et al., 2008; Pineau et al., 2014). In addition, the type III secretion system (T3SS), which consists of similar components as the bacterial pilus, transports at least three different effector proteins into the host cell (Charkowski et al., 2012). However, the T3SS is not present in all SRP (Kim et al., 2009). The function of the three effectors, HrpN, DspE/F, is not understood, but they were shown to elicit a hypersensitive response (localized cell death as plant defence) in leaves of multiple hosts and may therefore be useful in causing cell death in early

infection, and expand the range of tissue that SRP can infect (Hogan et al., 2013; Holeva et al., 2004; Kim et al., 2011). The type VI secretion system (T6SS) is composed of components that are homologous to the baseplate and tail of bacteriophages and is commonly implied in antagonism or other interactions between bacteria (Russell et al., 2014). However, T6SS upregulation was observed during SRP infection repeatedly, implying either a function in the interaction between SRP and host, or SRP and other bacteria, including SRP (Liu et al., 2008; Mattinen et al., 2008; Rossmann et al., 2018c). While its function during virulence is not understood, knockout of T6SS genes in *P. atrosepticum* resulted in hypervirulence, suggesting some role in disease modulation (Mattinen et al., 2008).

An extensive amount of research is dedicated to understanding the function and hierarchy of regulators that govern virulence onset. In *Pectobacterium*, the key repressor of virulence onset appears to be the RNA binding protein RsmA, which interacts with the mRNA of PCWDEs, motility regulators, and the T3SS master-regulator HrpL (Charkowski et al., 2012; Chatterjee et al., 1995; Kõiv et al., 2013; Liu et al., 1998; Mukherjee et al., 1998). To release the repression by RsmA, transcription of the small RNA *rsmB* is upregulated, which inactivates RsmA by competing for binding (Liu et al., 1998). The quorum sensing (QS) molecule *N*-acyl-homoserine lactone (AHL), which is synthesized by ExpI and sensed by the receptor ExpR has been shown to influence *rsmA* transcription directly. In the absence of AHL, ExpR drives *rsmA* transcription, and stops doing so when AHL is bound (Chatterjee et al., 1995; Cui et al., 2005). This leads to a QS-driven switch from stealth to brute force, implying that virulence onset only occurs when cell density is sufficient to ensure successful disease outbreak (Liu et al., 2008). Additionally, the transcriptional regulator HexA was shown to interact with the RsmAB system by repressing *rsmB* transcription, thereby effectively repressing virulence onset (Charkowski et al., 2012; Mukherjee et al., 2000). The transcription of *hexA* itself is modified by the motility regulator FlhDC, which in turn is inhibited post-transcriptionally by RsmA (Cui et al., 2008). While these mechanisms are connected to motility via FlhDC and cell density via QS, they also incorporate host signals from the micro-environment, e.g. via the two-component system ExpS/ExpA, which activates *rsmB* and is modified by FlhDC (Cui et al., 2001; Cui et al., 2008). The kinase ExpS activates the response regulator ExpA by phosphorylation upon presence of plant phenolic compounds in the cell's

environment in *Pseuodomonas* and is assumed to have an analogous function in *Pectobacterium* (Yamazaki et al., 2012). The ExpS/A system is required for virulence and may act partially independently from RsmAB (Broberg et al., 2014; Charkowski et al., 2012). Furthermore, KdgR supresses virulence onset via activation of RsmA transcription, and is inactivated by binding 2-Keto-3-deoxygluconate (KDG), a pectin breakdown product (Liu et al., 1999; Nasser et al., 1992). The products of the *vgu* operon, consisting of *vguABCD*, sense gluconate (Mole et al., 2010). A deletion-inserstion mutant in the operon showed hypervirulence in tubers, as well as lowered *flhD*, *rsmB* and *hexA* expression, while in leaves, the knockout showed increased *kdgR* expression and lowered virulence at 3 and 2.5 h post inoculation (hpi) respectively (Mole et al., 2010). While this clearly suggests involvement of gluconate sensing in virulence regulation, the exact mechanism is not clear, particularly in the context of the other discussed regulators.

Most of the discussed work was conducted through gene knockouts or microarray profiling and focused on the switches from latent infection to virulence onset and from stealth mode to brute force. RNA-sequencing (RNA-seq) has been used to study the transcriptome of potato during SRP infection and the transcriptome of *P. atrosepticum* during infection of *Nicotiana benthamiana* stems, as well as *P. atrosepticum* short RNAs during stress (Gorshkov et al., 2018; Kwenda et al., 2016a; Kwenda et al., 2016b). The first transcriptome profiling of *Pectobacterium spp*. during infection of potato tubers has shown that virulence genes in *P. c.* subsp. *carotovorum* and *P. polaris* are upregulated strongest at 24 hpi, with a consecutive decrease at 48 and 72 hpi (Rossmann et al., 2018c). Among the virulence associated genes, T3SS expression decreased first, and T2SS and T6SS expression later. Furthermore, expression of some PCWDEs decreased during the later phases of infection while it increased for others. During these phases, the expression of the known virulence regulators was not consistent with expectations based on their role in virulence onset, suggesting the involvement of yet unknown regulation mechanisms (Rossmann et al., 2018c).

2 Objectives

This thesis aimed to examine different aspects of potato soft rot bacteria in Norway, and thereby expand on the knowledge base that informs control measures to improve potato seed health. It was conducted as part of the project POTTIFRISK (Matfondavtale grant 244207), which includes several fungal diseases in addition to soft rot *Pectobacteriaceae* (SRP) to support Norwegian seed tuber production by providing comprehensive research data. At start-up, the outline for this thesis (Rossmann, 2016) stated the following research questions to be investigated in the course of the work:

1. Which SRP species are currently present in Norwegian potato seed material and how are they distributed?
2. How do environmental factors contribute to disease outbreak and severity in SRP infected potato plants?
3. Can the currently used detection and identification methods for SRP be improved and standardized for routine testing of potato seed material?
4. Are there insect vectors involved in SRP spreading in potato fields and initial infection of healthy potato seed material?
5. Which previously undescribed molecular mechanisms are involved in early disease development of SRP in potato?

The collective effort over the last decades to shed light on the taxonomy of the SRP belonging to the genera *Pectobacterium* and *Dickeya*, is outlined in section 1.1. Accurate detection and prediction tools that may be incorporated in control schemes rely on the ability to distinguish and correctly address distinct SRP groups. Establishing a common nomenclature and categorization for similar organisms enables scientific progress and facilitates more efficient collaboration (Cohan, 2002). To understand the differences between species, extensive genome sequence data is a helpful tool. Accordingly, the genomes of two Norwegian *Pectobacterium* isolates that stood out in routine testing due to their high aggressiveness and distinct phylogeny in previous work were sequenced, and their taxonomic position was determined (Dees et al., 2017; **Paper I**).

As elaborated in section 1.3.3, environmental and host factors greatly affect SRP prevalence and disease occurrence. Understanding these factors in an agricultural context is a key aspect of effective control of SRP diseases and meaningful seed certification. Furthermore, the implementation of such measures relies on the ability to test for SRP prevalence on an industrial scale. Central factors such as potato cultivars, seed production and certification schemes, climate, and the length of the growing season greatly vary between the potato producing regions around the world. The three-year field experiment therefore served three goals: assessing the health of Norwegian seed potatoes, investigating the influence of environmental factors on disease incidence and SRP prevalence, and establishing feasible and accurate testing routines by using molecular tools (**Paper II**).

In spite of the long history of research on insect-SRP interactions and the role of insects in the infection of potato, the current knowledge summarized in section 1.3.5 is sparse compared to that on other aspects of SRP ecology and aetiology. Although ambitious and carefully executed, the majority of the previous experiments on SRP transmission from insects to potato suffered from severe methodological limitations, and a partial lack of knowledge on the identity and mode-of-action of the SRP. Furthermore, these studies, as well as the later work on the (molecular) interaction of SRP with insects focused mainly on *Delia* and *Drosophila* species, and few select SRP strains. This work aimed at broadening the perspective by investigating randomly collected insects found in potato fields, including the only field in Norway where minitubers are multiplied. Those insects were tested for the presence of SRP using quantitative real-time PCR (qPCR) assays, and simultaneously identified using DNA barcoding (**Paper III**).

The sequenced type strain of *P. polaris*, together with a Norwegian isolate of *P. c.* subsp. *carotovorum,* were investigated by transcriptome analysis during infection of potato tubers and growth on minimal medium (**Paper IV**). This was done to expand on the previous work outlined in section 1.3.6, which largely utilized gene knockouts or model organisms to examine molecular processes involved in virulence onset. While some transcriptome studies contributed to the understanding of key virulence components using microarrays, they also focused on early virulence and partially utilized artificial media or model hosts (Broberg et al., 2014; Liu et al., 2008; Mattinen

et al., 2008). Consequently, the molecular mechanisms that facilitate SRP disease are largely understood from a limited perspective, which is centred on the switch from latent to active infection. In the presented work, RNA-seq was used to investigate the transcriptome of *Pectobacterium* during infection of potato tubers over multiple days (**Paper IV**).

To address the five questions proposed above, the work presented here offers scientific contributions on several major aspects of SRP biology and control. The first question was addressed with a taxonomic focus in **paper I**, with a focus on distribution frequency in seed material in **paper II**, and the geographical distribution and identity of SRP species was partially investigated for SRP found in insects in **paper III**. The second question was addressed in a three-year field study, which is discussed in **paper II**. This paper also deals with the third question by establishing a testing routine suited for large-scale investigation of SRP prevalence in potato seed. The fourth question was investigated in a study of over 2000 individual insects, which were caught in ten potato fields across Norway's major potato farming regions, including the location where minitubers are multiplied for clean seed supply (**Paper III**). The fifth question was addressed in a transcriptome study of *P. c.* subsp. *carotovorum* and *P. polaris* described in **paper IV**.

3 Results and discussion of the main experiments

In the course of this work, several important observations on the biology of SRP and the status of Norwegian seed potatoes were made. These observations range from novel concepts in basic SRP research, such as the description of a new bacterial species, to highly applicable findings with potentially large implications on farming practices, e.g. the ubiquitous presence of SRP association with a heterogeneous insect population in potato fields. In this section, the most important findings are highlighted and discussed.

3.1 *Pectobacterium polaris* - a newly identified SRP species

As outlined in section 1.1, the taxonomy of SRP is complex and is currently still being resolved. The increased availability of genome sequences and the establishment of reliable bioinformatics tools for phylogenetic analysis have greatly contributed to recent efforts in resolving SRP taxonomy. In the course of this work, the genomes of two *Pectobacterium* strains (NIBIO1006 and NIBIO1392) were sequenced using a PacBio RS II platform at the Norwegian sequencing centre in Oslo, and the reads were assembled into a single contiguous sequence (contig) for each strain (**Paper I**). The contigs were circularized into chromosomes, genes and coding sequences (CDS) were predicted and annotated by homology to public sequences. The chromosome of NIBIO1006 had a length of 4.8 Mbp and 4252 genes were annotated, while NIBIO1392 had a length of 5.0 Mbp and contained 4375 genes, among other features (Table 1).

Table 1. Overview of the features identified in the genome assemblies of *P. polaris* strain NIBIO 1006 and NIBIO1392 (GenBank CP017481 and CP017482) detailed in **paper IV**.

	NIBIO1006	**NIBIO1392**
Assembly length (bp)	4 826 824	5 008 416
Total genes	4252	4375
Functional CDS	4056	4221
5S/16S/23S rRNAs	8/7/7	8/7/7
tRNAs	77	77
CRISPR arrays		4
Incomplete prophage regions	3	2

The two strains were identified to represent a different species from *P. c.* subsp. *carotovorum*, which they were originally identified as, and other known *Pectobacterium* species by average nucleotide identity (ANI). The new species was named *Pectobacterium polaris* after the potato cultivar Polaris that the type strain NIBIO1006 was isolated from. The new species was confirmed to be more aggressive than the *P. c.* subsp. *carotovorum* type strain NCPPB312 in a tuber maceration assay on the potato cultivar Romera. Further maceration tests with 15 other potato cultivars showed NIBIO1006 to be significantly more aggressive than a *P. atrosepticum* strain at 24 °C, but not significantly different at 20 °C (**Paper II**). This, and the fact that both sequenced *P. polaris* strains were able to grow at 39 °C, which *P. atrosepticum* is typically not, suggests *P. polaris* to have a higher temperature optimum, likely in the range of *P. c.* subsp. *carotovorum* and *P. c.* subsp. *brasiliense*, its two closest relatives (**Paper I;** du Raan et al., 2016). Phenotypical differences from *P. c.* subsp. *carotovorum* in the standard biochemical characterization using API 50Ch and ID 32E strips were not found. Since publication, strains previously identified as *P. c.* subsp. *carotovorum* were reclassified as *P. polaris* in a collection of Polish SRP isolates (Waleron et al., 2018a). Furthermore, preliminary results from a data mining study in a proprietary collection of SRP genome sequences suggested *P. polaris* to be present in the Netherlands, based on 16S and multilocus sequence analysis (MLSA) clustering of strains isolated from potato (Rossmann et al., 2017)

3.2 Environmental disease factors and Norwegian seed health

Disease outcomes in the field and in storage are largely unpredictable, due to the combination of environmental and host factors that influence it, as well as the variety among SRP, which are described in sections 1.1, 1.3.2, and 1.3.3. To investigate the prevalence of SRP in Norwegian seed tubers and correlate environmental and host factors with disease incidence, a three-year field study was conducted (**Paper II**). Seed potatoes of varying qualities according to the Norwegian certification scheme of the cultivars Asterix, Innovator, and Lady Claire were grown in 2015, 2016, and 2017. Blackleg incidence was registered by visual inspection shortly before haulm killing. At planting and harvest, as well as after 3 months of storage, 8 samples of each seed

lot were taken in 2015 and 6 samples in 20016 and 2017. Each sample contained 25 randomly selected tubers, which were incubated at 16 °C for two weeks (dark) before analysis. Then, each sample was inspected for soft rot symptoms, and the tubers were cut in half and sealed in vacuum bags. After five days in vacuum at 20 °C, the liquid forming in the bags by tuber degradation was sampled, and the quantity of SRP DNA was determined using qPCR.

In this way, it was established that SRP were present in all seed lots, regardless of quality, year and cultivar, but P2 material contained significantly lower amounts of SRP and were practically free in most cases. In 2015, SRP prevalence increased on average from planting to harvest and further during storage. In 2016, it was higher at planting than in 2015 and slightly increased at harvest, but a strong decline was observed during storage. In 2017, prevalence was overall very low but increased during storage. In 2017, the offspring of five seed lots grown in 2016 were replanted. These seed lots, although by definition of lower quality due to the consequential downgrading from generation to generation, all had lower SRP prevalence at harvest in 2017 than they did at harvest in 2016, regardless of quality and cultivar. Furthermore, seed quality as indicated by certification grade was not reliably correlated with blackleg incidence in the field or soft rot symptoms of the tubers. Taken together, this indicates that the climate during the growing season, rather than the quality according to seed certification dictate SRP prevalence, and to some extent disease incidence. Particularly heavy precipitation late in the growing season seems to favour the SRP accumulation from harvest to storage with a potentially lasting effect into the following growing season. The predominant role of the climate was confirmed in analysis of variance (ANOVA) models, using the year of the experiment, cultivar and quality of the tubers as categorical factors and results from the qPCR assay, and disease incidence as continuous responses.

For disease incidence, particularly blackleg, cultivar seemed to have a great influence, as Innovator over all years showed almost no blackleg symptoms. Tuber rot was almost never observed at planting, but was found at an average frequency of approximately 17 % at harvest and 15 % after storage. In regard to disease incidence, P2 seeds also performed substantially better than lower qualities. Overall, seed quality did not reliably inform field outcome or SRP prevalence, supporting the

demand for large scale testing for pathogens as formulated by Pérombelon (2000). Most importantly, the SRP quantification and disease incidence assessment clearly show the value of clean seed, as represented by P2 material, and the potentially rapid spread of SRP and the diseases they cause after one or few field generations.

The role of cultivar, infecting SRP species and temperature was investigated *in vitro* and added some insight into the hierarchy of these factors in determining disease severity. Out of 15 tested cultivars, only the least susceptible (Innovator) and the most susceptible (Berber) had a rot outcome that was significantly different from the other cultivars after three days, regardless of the influence of the tested temperatures and SRP strains (Fig. 3). In an ANOVA model it was determined that of all tested factors, temperature had by far the greatest influence on rot outcome. Nevertheless, all tested factors and factor interactions significantly contributed to the observed differences in rot outcome. On average, tubers were more macerated at 24 °C than at 20 °C, and at the higher temperature, *P. polaris* macerated significantly more tissue than *P. atrosepticum*.

The low amount of tuber maceration of Innovator, combined with the low blackleg incidence in the field strongly suggests it to be less susceptible to SRP, although others have observed it to be somewhat susceptible to blackleg caused by *Dickeya* spp. (Rouffiange et al., 2014). Large scale assessment of commonly used potato cultivars upon challenge with different SRP in different conditions may eventually lead to the development of reliable predictive tools.

Figure 3. Tuber maceration of 15 potato cultivars three days after inoculation with *P. atrosepticum* PK-782-1-2-13 (Pba) and *P. polaris* NIBIO1006 (Ppol).

3.3 Insect-SRP association in the potato field

The relevance of clean seed tubers as a main factor in avoiding yield losses due to SRP disease has been stressed throughout section 1.2, 1.3.2 and 1.3.4 and is supported by the results obtained in **paper II**. As outlined in section 1.3.3, insects have been suspected to transmit disease for a long time and could explain the initial infection of previously clean seed material in spite of good farming and hygiene practices. Since no contemporary data exists on this topic, and it has received little focus, despite its potentially substantial impact on potato farming, this work included a two-year field survey of flying insects present in potato fields across Norway and their association with SRP (**Paper III**). Insects were collected in ten fields using yellow sticky traps, which were either posted next to blackleg symptomatic plants, or in a minimum distance of 10 m from any symptomatic plant. Sampling included the certified clean seed production site where minitubers are generated and multiplied into P2 seeds for the Norwegian market. From over 2000 individual insects, total DNA was isolated and tested for the presence and quantity of SRP, using a generic qPCR assay. To focus on specimens which could credibly infect plants, further investigation focused on insects which carried more than approximately 10.000 bacteria as inferred by qPCR.

Insects that satisfied this condition were found in all tested fields and both years, with 15 % in 2015 and 23 % in 2016. Distance of the traps from a symptomatic plant did not have an impact on the frequency of SRP being found in insect samples. In fact, the lowest percentages were observed on traps that were next to symptomatic plants. A large percentage of the insects found in the field for clean seed production tested positive, even though no symptomatic plants were conceivably present in those fields. This suggests that insects are associated with SRP regardless of the presence of obvious nearby infection sources. Conversely, the direct uptake of SRP from symptomatic plants in the field was suggested by the presence of 3 insects with high amounts of SRP, which tested positive for *Dickeya solani* in a field where the symptomatic plant also tested positive for *D. solani*. This was a clear indication of direct uptake, since *Dickeya solani* is a quarantine pathogen in Norway and was only found in that field in 2015.

The insects that tested positive were identified by DNA barcoding and BOLD database searches. The most commonly associated genus was *Delia*, with the most common

species being *Delia platura*. This strongly supports earlier work on the association of SRP and *Delia* and suggests vector association in the field to be much more common than previously assumed. Furthermore, more than 90 different species were found to be associated with a high amount of SRP, indicating a large potential for undiscovered vector species. For most of the identified species, only one individual was found to carry SRP, suggesting that potentially short and random associations of SRP with insects are common. Whether such association can lead to the infection of healthy plants is unknown, although it is certainly conceivable that stochastic transmission may occur e.g. when randomly associated insects visit plant wounds.

The longevity of SRP-insect associations was investigated by testing cultured turnip root flies (*Delia floralis*), cabbage moths (*Plutella xylostella*), and green lacewing larvae (*Chrysoperla carnea*) which had been reared under clean conditions for several generations or were commercially obtained in the case of the lacewing larvae. Most of the *D. floralis* adult flies tested positive for SRP (66 %), while of the cabbage moths only 13 % did. One out of 40 lacewing larvae tested positive. This strongly suggests that several *Delia* species indeed retain SRP even in capture. Furthermore, the near absence of SRP in the carnivorous lacewing larvae compared to the presence in the herbivorous *P. xylostella* and *D. florilega* supports a possibly more general function, or more likely presence of SRP in insects that live off plant material. While this is somewhat expected due to the fact that SRP reside on plant material, the retention in culture may indicate a closer association with a biological function beyond transmission.

3.4 Gene regulation during infection of potato tubers

Previous findings on the molecular processes during virulence onset (see section 1.3.6) showed SRP infection to involve multiple layers of control and a variety of disease mechanisms. However, the overall gene expression in SRP during tuber infection, especially as disease progresses, has largely been inferred from data on early infection. To better understand gene expression over the whole infection process, as well as further study *P. polaris,* RNA sequencing (RNA-seq) was performed on a *P. c.* subsp. *carotovorum* strain and the *P. polaris* type strain NIBIO1006 during infection of Asterix minitubers (**Paper IV**). As a non-infection control, the bacteria

were also grown on M9 minimal medium agar plates. Solid medium was chosen over liquid medium to better reflect a comparable growth situation in regard to humidity and oxygen levels, as well as surface adhesion and motility incentives for the bacteria. Of all samples, three biological replicates were sequenced, resulting in a total of 24 samples ([3*3 time points*2 strains on potato] + 3*2 strains on M9). An independent sample set was generated to test the results of the RNA-seq by quantitative reverse transcription PCR (RT-qPCR).

The overall amount of up- and downregulated genes remained similar when comparing infection over the three time points to *in vitro* growth. When comparing later infection (48 and 72 hpi) to early infection (24 hpi), an increasing amount of genes was differentially expressed in both strains. Although approximately the same number of genes were upregulated and downregulated in each time point and strain, virulence associated genes were overwhelmingly downregulated at 48 and 72 hpi compared to 24 hpi (Figure 4).

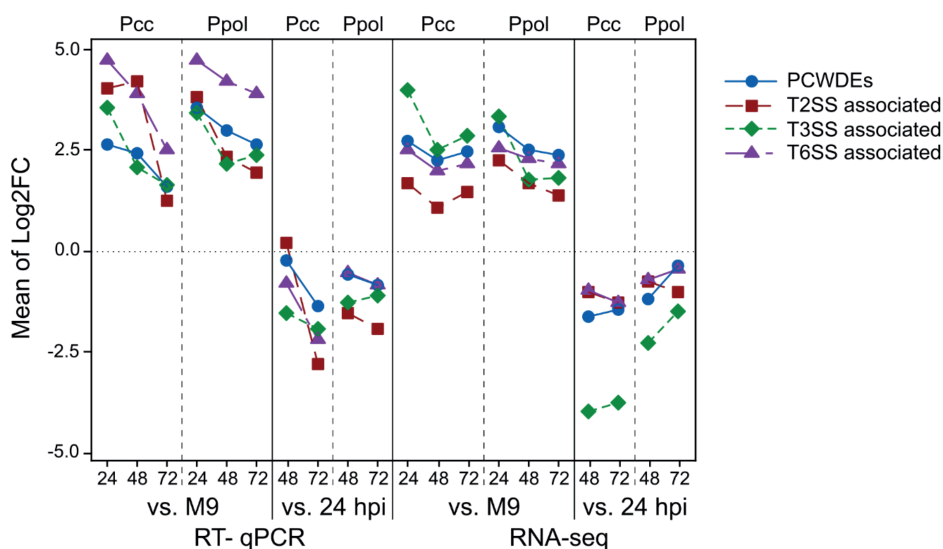

Figure 4. Differential expression of virulence associated genes in *P. c.* subsp. *carotovorum* (Pcc) and *P. polaris* (Ppol) during progressing infection. Mean Log2FC of genes encoding PCWDEs (blue dots), T2SS associated genes (red squares), T3SS associated genes (green diamonds), and T6SS associated genes (purple triangles) when comparing different time points of tuber infection to bacteria grown on M9 (vs. M9), or early infection at 24 hpi (vs. 24 hpi). Results are shown for RNA-seq (right), and a subset of the same genes tested by RT-qPCR in an independent experiment.

The analysis of virulence factors focused on four major groups of genes: Plant cell wall degrading enzymes (PCWDEs), type II secretion system (T2SS), type III secretion system (T3SS), and type VI secretion system (T6SS) associated genes. As summarized in section 1.3.6, the cell wall breakdown and partially understood secretion system activity are likely the central components of SRP disease. Although one could argue that PCWDEs are T2SS associated, they were analysed separately, because export via the T2SS is suggested for most, but not all investigated enzymes (Corbett et al., 2005; Coulthurst et al., 2008; Pineau et al., 2014). All of the investigated groups of virulence associated genes were on average upregulated compared to M9 over all time points, but downregulated at 48 and 72 hpi compared to infection at 24 hpi (Figure 4). However, some genes encoding PCWDEs did not follow that trend and kept increasing in expression over time. Furthermore, T3SS associated genes seemed to be downregulated stronger than the other gene groups at 48 hpi compared to early infection. At 72 hpi the difference was less pronounced. This suggested a general decrease in the expression of virulence associated genes as infection progressed with potentially different regulation mechanisms for the various components of disease. The analysis of differential expression in the known regulators did not yield results that were consistent with regulation in virulence onset (**Paper IV**).

Some components, such as the *vgu*-operon, together with *kdgR* may play a role in the downregulation of virulence functions by processing signals related to host-derived metabolites. A downregulation the quorum sensing (QS) elicitor synthesis and response gene observed in *P. polaris* may also play a role. An explanation for the function of this observed downregulation could be a shift in the community structure, as the infection progresses (Figure 5). Since RNA was isolated from a homogenized sample of rotten tissue, a larger fraction of the community may be involved in metabolism of host components instead of active infection at later time points. A QS-gradient may play a role in the regulation of such community-wide signalling.

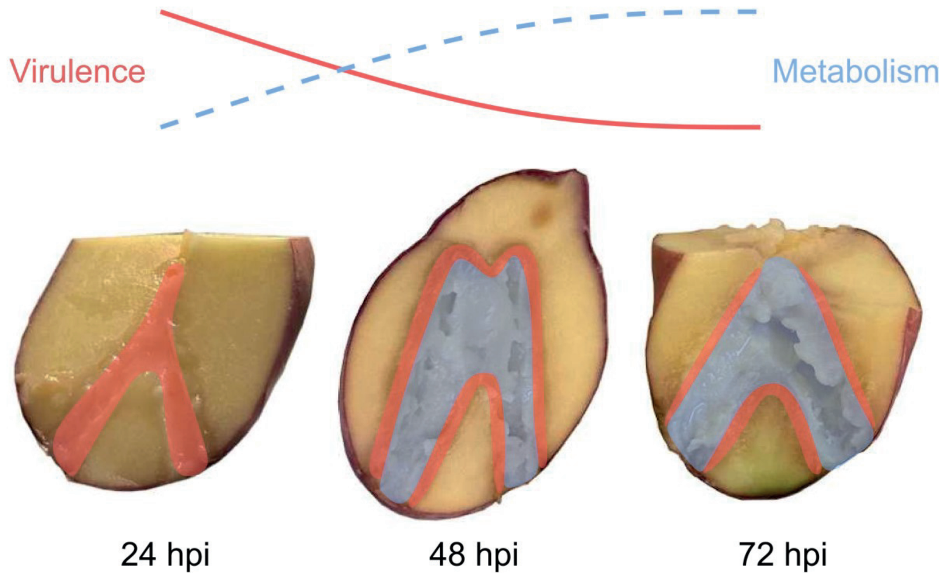

Figure 5. Illustration of suggested shifting SRP community function during infection over three days in potato tubers. Compared to the infection at 24 hpi (left), the amount of bacteria directly in contact with uninfected potato tissue (red) becomes smaller at later time points relative to the total amount of bacteria. As infection progresses, an increasing number of bacteria is found in macerated host tissue (blue). These two fractions could have different biological priorities, leading to an average decrease in virulence function and an average increase in metabolic functions measured over the whole community.

4 Conclusions and future perspective

As demonstrated in **paper I**, systematic genome sequencing of atypical strains greatly contributes to the understanding of SRP taxonomy. The discovery and description of the new species *Pectobacterium polaris* represented a further step in resolving the correct grouping of isolates classified as *P. c.* subsp. *carotovorum*. Eventually, sufficient resolution of SRP taxonomy through nucleotide sequences will allow for a complete set of inexpensive molecular diagnostic tools, which can be used to reliably detect, quantify and discriminate SRP (e.g. specific qPCR assays). Such tools will be immensely helpful in the design of comprehensive standardized testing schemes to inform predictive models and effective pest control programs.

The investigation of Norwegian seed and the factors that influence disease described in **paper II** suggests several important conclusion for potato farming. Most fundamentally, the near-universal presence of SRP in certified Norwegian seed was shown by molecular testing. Conversely, the relative health and desired quality was demonstrated for early generation (P2) seed, which performed well in regard to disease incidence and SRP prevalence. However, even this clean seed material was in some cases accumulating SRP over the growing season and offspring did not reliably perform well. In regard to environmental factors, precipitation was identified as potentially playing a major role in resilience of the harvested tubers in storage. In the tuber inoculation tests, temperature was the most impactful factor in determining rot severity over host cultivar, and infecting SRP species. Over the course of the work, the cultivar Innovator was suggested to be least susceptible to disease caused by SRP, particularly *P. atrosepticum* and *P. polaris*.

Additionally, the sampling and testing strategy employed in **paper II** demonstrated the feasibility of large-scale testing by qPCR from enrichment extract collected after vacuum incubation. Similar testing methods, together with better information on cultivar susceptibility and SRP identity, could be employed to improve seed certification and inform predictive models as a part of comprehensive control schemes.

The work presented in **paper III** showed the broad presence of SRP in insects, regardless of pre-existing infection in the seed tubers. Most commonly the pathogens

were carried by known vectors of the genus *Delia*, giving new weight to hypotheses first formulated in the early 20th century. The results strongly suggest that SRP are more commonly transmitted by insects than commonly assumed and that more focus should be put on this aspect, particularly in the production of clean seed material.

Future research should focus on the nature of the association between SRP and insects as well as identifying transmission frequency, with the goal of assessing the threat and devising sensible control strategies. To examine the nature of the association, it is of central importance to identify potential molecular mechanisms that facilitate the association of SRP with e.g. *Delia* species. Preliminary results of data mining in public and proprietary databases show the *evf* gene, which increases survival in the gut of *Drosophila melanogaster*, to only be present in a small subset of *P. c. carotovorum* isolates (Rossmann et al., 2017). The same is true for the *cyt*-cluster that facilitates virulence in the pea aphid, but was only found in *Dickeya* isolates. Future research should therefore attempt to identify more general mechanisms, which are suggested to exist by the results in this work. Determining transmission frequency may not be trivial due to the high variety of insect species that could potentially be vectors under the right conditions. The demonstrated scale of the issue, will hopefully raise awareness and motivate future research on this topic.

The analysis of molecular disease factors during soft rot in potato tubers, summarized in **paper IV**, showed the downregulation of most investigated virulence genes after initial strong expression at 24 hpi. This was likely an indicator of functional diversification of the bacterial community, leading to an overall lowered expression of virulence genes, although still substantially higher than in a non-virulence situation for most genes. How this switch, indicated by a progressively changing transcriptome over time, is governed on a molecular level did not become clear from the analysis of known regulators. Regulators involved in the sensing of host-derived metabolites, as well as quorum sensing mechanisms may conceivably play a role. Further research on the function of the T3SS and T6SS in tuber infection could clarify the observed transcriptome shifts and explain the difference in time points and intensity of expression shifts for genes associated with those two secretion systems. Furthermore, in-depth analysis of the separate fractions of SRP in several zones of the

progressing rot may reveal the modes of regulation involved in modulating disease after onset.

Overall, the work collected in this thesis provided several important and novel findings on diverse aspects of SRP disease and biology. Although some parts of this work were conducted with a focus on Norwegian agriculture, the major conclusions are highly relevant to the international research community and efforts to improve plant health.

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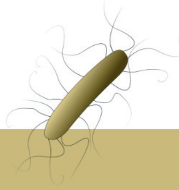
Papers I-IV



Paper I

INTERNATIONAL JOURNAL OF **SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY**

TAXONOMIC DESCRIPTION
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MICROBIOLOGY SOCIETY

Pectobacterium polaris sp. nov., isolated from potato (*Solanum tuberosum*)

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Abstract

The genus *Pectobacterium*, which belongs to the bacterial family *Enterobacteriaceae*, contains numerous species that cause soft rot diseases in a wide range of plants. The species *Pectobacterium carotovorum* is highly heterogeneous, indicating a need for re-evaluation and a better classification of the species. PacBio was used for sequencing of two soft-rot-causing bacterial strains (NIBIO1006^T and NIBIO1392), initially identified as *P. carotovorum* strains by fatty acid analysis and sequencing of three housekeeping genes (*dnaX*, *icdA* and *mdh*). Their taxonomic relationship to other *Pectobacterium* species was determined and the distance from any described species within the genus *Pectobacterium* was less than 94 % average nucleotide identity (ANI). Based on ANI, phylogenetic data and genome-to-genome distance, strains NIBIO1006^T, NIBIO1392 and NCPPB3395 are suggested to represent a novel species of the genus *Pectobacterium*, for which the name *Pectobacterium polaris* sp. nov. is proposed. The type strain is NIBIO1006^T (=DSM 105255^T=NCPPB 4611^T).

The bacterial family *Enterobacteriaceae* includes several significant plant pathogens that cause diseases in different agricultural crops worldwide. The soft-rotting *Enterobacteriaceae*, which include the genera *Pectobacterium* and *Dickeya*, cause soft rot diseases in a wide range of plants, including potato (*Solanum tuberosum*) [1–3]. Species of these genera (*Pectobacterium carotovorum*, *Pectobacterium atrosepticum*, *Dickeya dadantii* and *Dickeya solani*) are ranked among the most significant plant pathogenic bacteria in terms of economic and scientific importance [4]. Since potato is the world's third most important food crop after rice and wheat, and is produced on all continents except Antarctica [5], these pathogens lead to reduced crop yields and severe economic losses for potato producers worldwide [6].

Soft rot of tubers is typically initiated at the stolon end and results in maceration of the plant tissue by plant cell-wall-degrading enzymes, such as pectinases, cellulases and proteases that are the main virulence factors of these bacteria [7]. Blackleg disease is characterized by a slimy, wet, black rot and develops at the stem base of the growing plants [1].

The genus *Pectobacterium* was first proposed in 1945 to include the pectinolytic *Enterobacteriaceae* [8], but the classification has been subject to extensive revision over the last decade and it is likely that several of the genome-sequenced *Pectobacterium* strains have been incorrectly assigned to *P. carotovorum* [9–12]. *P. carotovorum* is a heterogeneous species that includes the subspecies *P. carotovorum* subsp. *carotovorum*, *P. carotovorum* subsp. *odoriferum* and *P. carotovorum* subsp. *brasiliense* [9, 13, 14]. Members of the *P. carotovorum* subspecies are widely distributed [1] and have been isolated from several host plants, soil and water [2, 3, 15].

This study aimed to establish the taxonomic status of two strains (NIBIO1006^T and NIBIO1392), initially classified as members of *P. carotovorum* by fatty acid analysis and sequencing of three housekeeping genes (*dnaX*, *icdA* and *mdh*); and of strain NCPPB3395 from the Netherlands, previously assigned to *P. c.* subsp. *carotovorum*. NIBIO1006^T was obtained from potato tubers showing severe symptoms of soft rot in Norway in 2010, and NIBIO1392 was obtained from a latently infected tuber in 2013 as previously described [16]. NIBIO1006^T was selected for whole genome sequencing because of its aggressiveness on potato tubers. NIBIO1392 was selected for genome sequencing based on phylogenetic results in a previous study [16] where it clustered together with NCPPB3395, separately from *P. c.* subsp. *carotovorum* NCPPB312^T.

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Keywords: *Enterobacteriaceae*; genomics; potato pathogen; *Solanum tuberosum*; phylogenomics; taxonomy.
Abbreviations: ANI, average nucleotide identity; CDS, coding sequence; dDDH, digital DNA-DNA hybridization; GGD, genome-to-genome distance; GI, genomic island; MCP, methyl-accepting chemotaxis protein.
The GenBank/EMBL/DDBJ accession numbers for the whole genome sequences of the *Pectobacterium polaris* strains NIBIO1006^T and NIBIO1392 are CP017481 and CP017482, respectively.
One supplementary table and two supplementary figures are available with the online Supplementary Material.

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For genome sequencing, genomic DNA was extracted using Genomic-tips 500/G (Qiagen) according to the manufacturer's instructions. A library was prepared using the Pacific Biosciences 20 kb library preparation protocol, and size selection of the final library was performed using BluePippin (Sage Science) with a 9 kb cut-off. The libraries were sequenced, at the Norwegian Sequencing Centre, using a PacBio RS II sequencer (Pacific Biosciences), and P6-C4 chemistry with 360 min movie time on one single-molecule real-time (SMRT) cell. The reads were assembled using HGAP v3 (Pacific Biosciences, SMRT Analysis Software v2.3.0). The Minimus2 software of the AMOS package was used to circularize the contig, which was confirmed by a dot plot to contain the same sequence at the beginning and end of the contig. RS_Resequencing.1 software (SMRT Analysis version v2.3.0) was used to map reads back to the assembled and circularized sequence in order to correct the sequence after circularization. The genomes were annotated using the NCBI Prokaryotic Genome Annotation Pipeline [17], GeneMarkS+ v3.3 and the Rapid Annotation System Technology (RAST) server [18]. The complete genome sequence of NIBIO1392 consisted of one single circular chromosome of 5 008 416 bp and contained 4221 coding sequences (CDSs), while the genome sequence of NIBIO1006^T consisted of one single circular chromosome of 4 826 824 bp and contained 4056 CDSs. The genomes of both strains contained eight copies of 5S rRNA genes, seven copies of 16S rRNA genes, seven copies of 23S rRNA genes, 77 tRNA genes, as well as five and six non-coding RNA genes, respectively. The DNA G+C contents of both strains were 52 %. The whole genome sequences of strains NIBIO1006^T and NIBIO1392 were deposited in GenBank under accession numbers CP017481 and CP017482, respectively.

The multiple genome alignment tools Mauve [19] and Parsnp [20] were used to investigate the phylogenetic relationships among NIBIO1006^T, NIBIO1392 and NCPPB3395 and other published *Dickeya* and *Pectobacterium* genomes. An alignment of the 16S rRNA gene of *Dickeya* species and *Pectobacterium* species was performed using the neighbour-joining method implemented in CLC Main Workbench 7 followed by reconstruction of a phylogenetic tree. The Jukes–Cantor model was used for analysis and a consensus tree was built on the basis on 1000 bootstraps. NIBIO1006^T and NIBIO1392 did not cluster with *P. c.* subsp. *carotovorum* NCPPB312^T in phylogenetic analysis based on the 16S rRNA gene and whole genome alignment, but resided in the same cluster as NCPPB3395 (Fig. 1). The 16S rRNA sequences of NIBIO1006^T and NIBIO1392 were deposited in GenBank under accession numbers MG022083 and MG022082, respectively.

Pairwise comparison of the genomes of NIBIO1006^T and NIBIO1392 to other published *Pectobacterium* and *Dickeya* genomes was performed using the average nucleotide identity (ANI) calculator (Table 1) [21]. Strains NIBIO1006^T and NIBIO1392 shared an ANI of 96.9 %. They shared 92.9 and 93.0 % identity with *P. c.* subsp. *carotovorum* NCPPB312^T, which is below the suggested cut-off value of 95–96 % to delineate bacterial species [22]. Moreover, both strains were 96 % identical to *P. carotovorum* NCPPB3395, which has been suggested to represent a novel species in a recently published re-evaluation of the taxonomy of *Dickeya* species and *Pectobacterium* species [12].

The genome-to-genome-distance calculator GGDC 2 (http://ggdc.dsmz.de/) was used to predict digital DNA–DNA hybridization (dDDH) values [23]. The estimated dDDH value was 67.7 and 68.1 % when NCPPB3395 was used as query (Table 2). The estimated dDDH values when strain NCPPB312^T was used as query were as low as 52.4 and 52.5 %. These results are in agreement with the ANI values and clustering of the strains in the phylogenetic analysis, and hence support the proposition of a novel *Pectobacterium* species, *Pectobacterium polaris* sp. nov., which includes NIBIO1006^T, NIBIO1392 and NCPPB3395.

The genomes of NIBIO1006^T and NIBIO1392 were screened for horizontally acquired DNA using the IslandViewer search tool [24] (Fig. 2). For strain NIBIO1392, 53 putative genome islands (GIs) that range from 4.0 to 98.4 kb were detected, of which the largest consisted of 98 408 bp with 83 predicted protein coding regions. For strain NIBIO1006^T, 36 putative GIs that range from 4.2 to 16.4 kb were detected, and the largest consisted of 16 439 bp and was predicted to encode 14 proteins. Genes encoding tRNAs, transposases and integrases, as well as genes related to prevention of host death, flagella, transcriptional regulation and the type VI secretion system, could be identified in the GIs of both strains. Furthermore, genes related to antibiotic biosynthesis, phages, tellurite resistance, toxin–antitoxin systems and the type IV secretion system were identified in the GIs of strain NIBIO1392. Genome alignment with Mauve comparing NIBIO1006^T and NIBIO1392 clearly showed putative GIs and a large inverted region in NIBIO1006^T compared to NIBIO1392 (Fig. S1, available in the online Supplementary Material).

The PHAST server [25] was used to identify and annotate prophage sequences within the bacterial genomes. Two incomplete prophage regions of 19.2 kb (PHAGE_Burkho_BcepMu_NC_005882) and 21.7 kb (PHAGE_Entero_P4_NC_001609) were identified in *P. polaris* NIBIO1392. For *P. polaris* NIBIO1006^T, three incomplete prophage regions of 23.4 kb (PHAGE_Burkho_BcepMu_NC_005882), 8.5 kb (PHAGE_Escher_HK639_NC_016158) and 18.5 kb (PHAGE_Entero_P4_NC_001609) were identified.

TXSScan [26, 27] on the Mobyle-based webserver [28] was used to predict the presence of secretion systems in *P. polaris* NIBIO1006^T and NIBIO1392. Genes related to all six secretion systems (Types I–VI) known from *Pectobacterium* were present entirely or in parts in the two genomes. In addition, 26 and 27 genes encoding putative proteases were identified in *P. polaris* NIBIO1392 and NIBIO1006^T, respectively. Both strains encode two putative metalloproteases, which may play different roles in virulence: either by attacking plant cell-wall

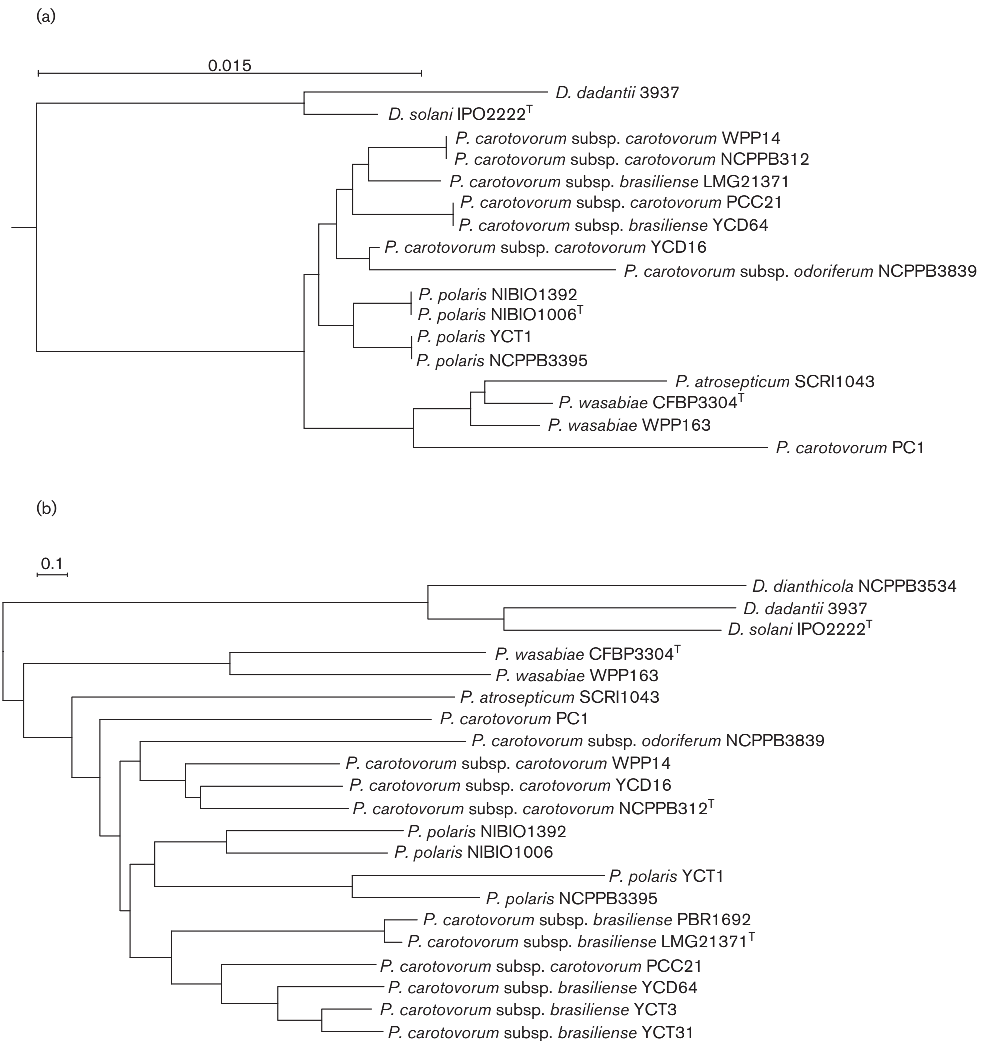

Fig. 1. Phylogenetic trees of *Pectobacterium* species and *Dickeya* species strains based on the 16S rRNA gene (a) and whole genome alignment using Parsnp (b). The 16S rRNA tree was made using the neighbour–joining method. The Jukes–Cantor model was used for analysis and the tree was built on the basis on 1000 bootstraps. Bar, 0.015 changes per nucleotide position.

proteins or by degrading enzymes secreted by the pathogen [29]. *Pectobacterium* species primarily cause disease by the coordinated and prolific production of a variety of plant cell-wall-degrading enzymes [30]. A total of 12 genes encoding putative pectate lyases and two genes encoding cellulases were identified in each of the sequenced strains. Genes related to nitrogen fixation (*nifA*, *B*, *E*, *H*, *N*, *Q*, *W* and *X*) were found in both strains, including the gene encoding the key N_2-fixing regulator NifA [31]. Nitrogen fixation is a potential advantage for survival in the soil or rhizosphere and a nitrogen (N_2) fixation gene cluster was reported from the first complete *Pectobacterium* genome, that of *P. atrosepticum* [32]. There are 34 genes encoding putative transmembrane chemoreceptors known as methyl-accepting chemotaxis proteins (MCPs) in each of the two genomes, which is about the same number as reported for *P. atrosepticum* strain 1043 (36 MCP genes), and considerably more than in other sequenced enterobacterial genomes [32]. MCPs allow bacteria to track gradients of

Table 1. Pairwise ANI between the novel strains and other *Pectobacterium* and *Dickeya* strains, with their respective genome sizes and numbers of CDSs

	Genome size (Mb)	Number of CDSs	Pairwise ANI (%)	
			NIBIO1006	NIBIO1392
NIBIO1006^{T}	4.8	4056	–	96.9
NIBIO1392	5.0	4221	96.9	–
P. carotovorum subsp. *carotovorum* NCPPB3395	4.6	3849	96.0	96.0
P. carotovorum subsp. *carotovorum* NCPPB312^{T}	4.8	4113	92.9	93.0
P. carotovorum subsp. *carotovorum* PCC21	4.8	4109	93.7	93.7
P. carotovorum subsp. *brasiliense* LMG 21371^{T}	4.8	4114	93.5	93.5
P. carotovorum subsp. *carotovorum* PC1	4.9	4228	89.5	89.5
P. carotovorum subsp. *odoriferum* BC S7	4.9	3855	92.2	92.3
P. atrosepticum SCRI1043	5.1	4300	88.9	88.8
P. parmentieri CFBP8475^{T}	5.0	4454	88.16	88.16
P. wasabiae CFBP3304^{T}	5.0	4371	88.2	88.2
D. solani IPO2222^{T}	4.9	4022	79.3	79.5
D. dadantii 3937	4.9	4215	79.6	79.6
D. dianthicola NCPPB3534	4.9	4151	79.2	79.3

Table 2. Genome-to-genome distance (GGD) and digital DNA–DNA hybridization (dDDH) estimations of *P. polaris* sp. nov. compared with related *Pectobacterium* and *Dickeya* strains

dDDH >70 % indicates that the compared strains belong to the same species

Reference genome	Query genome	dDDH (%) (formula 2)	Confidence interval (%)	GGD
P. polaris NIBIO1006^{T}	*P. polaris* NIBIO1392	74.4	71.4–77.2	0.03
	P. carotovorum subsp. *carotovorum* NCPPB3395	68.1	65.1–70.9	0.04
	P. carotovorum subsp. *carotovorum* NCPPB312^{T}	52.4	49.7–55	0.07
	P. carotovorum subsp. *carotovorum* PCC21	55.7	53–58.4	0.06
	P. carotovorum subsp. *brasiliense* LMG 21371^{T}	54.4	51.7–57.1	0.06
	P. carotovorum subsp. *carotovorum* PC1	40.4	38–43	0.10
	P. carotovorum subsp. *odoriferum* BCS7	49.2	46.6–51.9	0.07
	P. atrosepticum SCRI1043	38.5	36.1–41.1	0.10
	P. parmentieri CFBP8475^{T}	36.6	34.1–39	0.11
	P. wasabiae CFBP3304^{T}	36.8	34.4–39.4	0.11
	D. solani IPO2222^{T}	21.1	18.9–23.6	0.21
	D. dadantii 3937	21.4	19.2–23.9	0.20
	D. dianthicola NCPPB3534	20.8	18.6–23.3	0.21
P. polaris NIBIO1392	*P. polaris* NIBIO1006^{T}	74.4	71.4–77.2	0.03
	P. carotovorum subsp. *carotovorum* NCPPB3395	67.7	64.7–70.5	0.04
	P. carotovorum subsp. *carotovorum* NCPPB312^{T}	52.5	49.8–55.1	0.07
	P. carotovorum subsp. *carotovorum* PCC21	55.5	52.8–58.2	0.06
	P. carotovorum subsp. *brasiliense* LMG 21371^{T}	54.4	51.7–57.1	0.06
	P. carotovorum subsp. *carotovorum* PC1	40.5	38–43	0.10
	P. carotovorum subsp. *odoriferum* BCS7	49.5	46.9–52.1	0.07
	P. atrosepticum SCRI1043	38.5	36–41	0.10
	P. parmentieri CFBP8475^{T}	36.5	34.1–39.1	0.11
	P. wasabiae CFBP3304^{T}	36.8	34.4–39.4	0.11
	D. solani IPO2222^{T}	21.2	19–23.6	0.21
	D. dadantii 3937	21.5	19.3–23.9	0.20
	D. dianthicola NCPPB3534	20.9	18.6–23.3	0.21

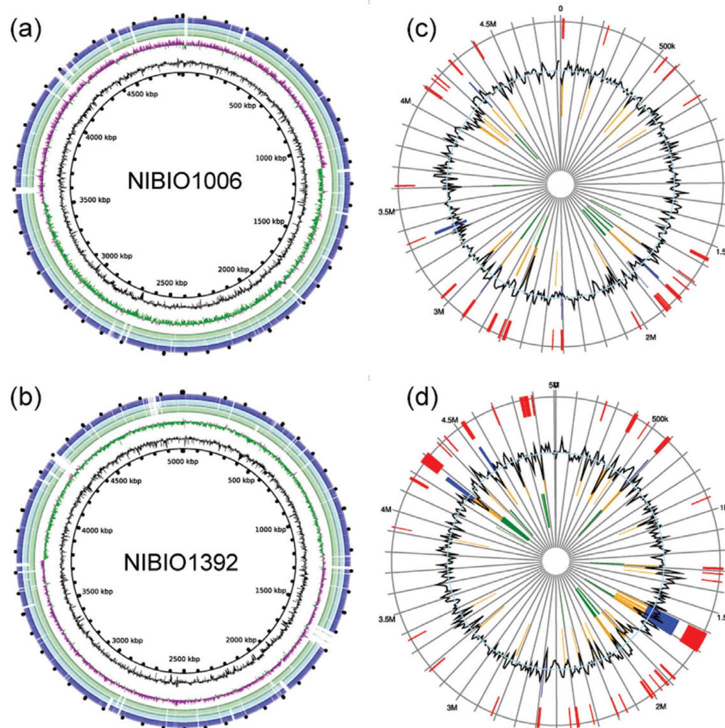

Fig. 2. Genome comparisons and predicted GIs. Genome alignment of *P. polaris* NIBIO1006^T (a) and *P. polaris* NIBIO1392 (b) to *P. carotovorum* strains PC1 and PCC21 using BLAST Ring Image Generator (BRIG) [37]. (a) The rings from inner to outer representing *P. polaris* NIBIO1006^T, G+C content, GC skew, *P. polaris* NIBIO1392, and *P. carotovorum* strains PC1 and PCC21. (b) The rings from inner to outer representing *P. polaris* NIBIO1392, G+C content, GC skew, *P. polaris* NIBIO1006^T, and *P. carotovorum* strains PC1 and PCC21. GIs of strains NIBIO1006^T (c) and NIBIO1392 (d) as predicted by the IslandViewer web server. Predicted GIs are coloured based on the tools used: IslandPick, based on comparative genomics (green); SIGI-HMM, which predicts GIs based on a hidden Markov model (orange); IslandPath-DIMOB, which predicts GIs based on features associated with GIs, such as sequence bias, tRNAs, and integrases and transposases (blue); and an integration of the three methods, IslandPath-DIMOB, SIGI-HMM and IslandPick, which predicts based on comparative genomics (red). The black line represents the percentage G+C content.

attractive and repellent chemicals in their environment and mediate chemotactic behaviours [33, 34]. The relatively high number of MCPs in plant pathogens, compared to enterobacterial animal pathogens, may indicate that *Pectobacterium* strains have adapted to a fluctuating environment, which is consistent with their widespread presence [29].

The aggressiveness of the two *P. polaris* strains NIBIO1006^T and NIBIO1392 was confirmed by a maceration assay on potato tubers essentially as described by Laurila *et al.* [35]. *In vitro* tubers of the cultivar Romera, obtained from Overhalla Klonavlsenter AS, were wounded by piercing them at two sites and the wounds were inoculated with 30 µl of bacterial suspension (A_{600}=0.8). Ten tubers were inoculated per strain and additionally 10 tubers were inoculated with PBS as a negative control. The tubers were placed in plastic boxes and incubated at room temperature for 72 h. After incubation, the tubers were cut vertically through the inoculation points, the macerated tissue was carefully scraped out and the weight of rotten tissue (grams) was recorded. The reference strain *P. c.* subsp. *carotovorum* NCPPB312^T was included in the assay for comparison. The ability to macerate potato tissue was significantly greater for *P. polaris* NIBIO1006^T (5.8 g, SE: 0.27) and NIBIO1392 (5.8 g, SE: 0.20) than for *P. c.* subsp. *carotovorum* NCPPB312^T (5.0 g, SE: 0.16) (Fig. S2).

Biochemical characterization of *P. polaris* NIBIO1006^T, NIBIO1392 and NCPPB3395 and *P. c.* subsp. *carotovorum* NCPPB312^T were done using API 50CH and ID 32E strips (bioMérieux), according to the manufacturer's instructions. The *P. polaris* strains NIBIO1006^T, NIBIO1392 and NCPPB3395 tested positive for amygdalin, arbutin, D-xylose,

Table 3. Phenotypic characterization of *P. polaris* sp. nov. (strains NIBIO1006^T, NIBIO1392 and NCPPB3395) and *P. c.* subsp. *carotovorum* NCPPB312^T

Characteristic	NIBIO1006^T	NIBIO1392	NCPPB3395	NCPPB312^T
Growth at 37 °C	+	+	+	+
Growth at 39 °C	+	+	+	+
Growth on lysogeny broth agar with 5 % (w/v) NaCl	+	+	+	+
Potassium 2-ketogluconate	–	–	–	–
Potassium 5-ketogluconate	–	–	–	–
L-Arginine	–	–	–	–
Adonitol	–	–	–	–
Starch	–	–	–	–
Amygdalin	+	+	+	+
Arbutin	+	+	+	+
L-Aspartic acid 4-nitroanilide	–	–	–	–
Cellobiose	+	+	+	+
D-Arabinose	–	–	–	–
D-Arabitol	–	–	–	–
D-Fucose	–	–	–	–
Dulcitol	–	–	–	–
D-Xylose	+	+	+	+
Erythritol	–	–	–	–
Aesculin ferric citrate	+	+	+	+
D-Fructose	+	+	+	+
D-Galactose	+	+	+	+
Galacturonic acid	–	–	–	–
Gentiobiose	+	+	+	+
D-Glucose	+	+	+	+
Glycerol	+	+	+	+
Glycogen	–	–	–	–
Potassium gluconate	–	–	–	–
Inositol	+	+	+	+
Inulin	–	–	–	–
Lactose	+	+	+	+
L-Arabinose	+	+	+	+
L-Arabitol	–	–	–	–
L-Lysine	–	–	–	–
L-Fucose	–	–	–	–
5-Bromo-3-indoxylnonanoate	–	–	–	–
L-Xylose	–	–	–	–
D-Lyxose	–	–	–	–
Maltose	–	–	–	–
D-Mannitol	+	+	+	+
Methyl α-D-glucopyranoside	–	–	–	–
Methyl α-D-mannopyranoside	–	–	–	–
Methyl β-D-xylopyranoside	–	–	–	–
Melibiose	+	+	+	+
Melezitose	–	–	–	–
D-Mannose	+	+	+	+
Sodium malonate	–	–	–	–
N-Acetyl glucosamine	+	+	+	+
L-Ornithine	–	–	–	–
Palatinose	–	–	–	–
Raffinose	+	+	+	+
L-Rhamnose	+	+	+	+
D-Ribose	+	+	+	+

Table 3. cont.

Characteristic	NIBIO1006^T	NIBIO1392	NCPPB3395	NCPPB312^T
Sodium pyruvate	–	–	–	–
Sucrose	+	+	+	+
Salicin	+	+	+	+
L-Sorbose	–	–	–	–
D-Sorbitol	–	–	–	–
4-Nitrophenyl-β-D-galactopyranoside	+	+	+	+
4-Nitrophenyl-β-D-glucopyranoside	+	+	+	+
4-Nitrophenyl-β-D-glucuronide	–	–	–	–
5-Bromo-4-chloro-3-indolyl-*N*-acetyl-β-D-glucosamine	–	–	–	–
D-Tagatose	–	–	–	–
Trehalose	+	+	+	+
Turanose	–	–	–	–
Urea	–	–	–	–
Xylitol	–	–	–	–
4-Nitrophenyl-α-D-galactopyranoside	+	+	+	+
4-Nitrophenyl-α-D-glucopyranoside	–	–	–	–
4-Nitrophenyl-α-D-maltopyranoside	–	–	–	–

aesculin ferric citrate, D-fructose, D-galactose, gentiobiose, D-glucose, glycerol, inositol, lactose, L-arabinose, D-mannitol, melibiose, D-mannose, *N*-acetyl glucosamine, raffinose, L-rhamnose, D-ribose, sucrose, salicin, 4-nitrophenyl-β-D-galactopyranoside, 4-nitrophenyl-β-D-glucopyranoside, trehalose and 4-nitrophenyl-α-D-galactopyranoside (Table 3). None of the tested compounds distinguished the four strains tested.

Fatty acid methyl ester contents were determined for *P. polaris* NIBIO1006^T, NIBIO1392 and NCPPB3395, *P. c.* subsp. *carotovorum* NCPPB312^T and *D. solani* IPO2222^T (Table S1) according to Sasser [36]. The whole-cell fatty acids of all strains were dominated by straight-chain components, while no clearly discriminative fatty acid component was detected among *Pectobacterium* strains. *D. solani* IPO2222^T, analysed for comparison, showed a clearly different pattern, lacking straight-chain acids $C_{10:0}$, $C_{13:0}$ and $C_{11:0}$.

In conclusion, DNA–DNA relatedness analysis, whole-genome sequences and phylogenetic data indicate that NIBIO1006^T, NIBIO1392 and NCPPB3395 represent a novel species, distinct from *P. c.* subsp. *carotovorum* (NCPPB312^T), for which the name *Pectobacterium polaris* sp. nov. is proposed.

DESCRIPTION OF *PECTOBACTERIUM POLARIS* SP. NOV.

Pectobacterium polaris (po.la′ris. N.L. gen. n. *polaris* pertaining to the potato cultivar Polaris, which was the source of the type strain).

Cells are Gram-negative and motile. Grows on lysogeny broth agar with 5 % (w/v) NaCl, and at 37 and 39 °C. Causes soft rot on potato tubers. Using API 50CH and ID 32E assays, positive for amygdalin, arbutin, D-xylose, aesculin ferric citrate, D-fructose, D-galactose, gentiobiose, D-glucose, glycerol, inositol, lactose, L-arabinose, D-mannitol, melibiose, D-mannose, *N*-acetyl glucosamine, raffinose, L-rhamnose, D-ribose, sucrose, salicin, 4-nitrophenyl-β-D-galactopyranoside, 4-nitrophenyl-β-D-glucopyranoside, trehalose and 4-nitrophenyl-α-D-galactopyranoside. Clusters separately in phylogenetic analyses based on the 16S rRNA gene and whole genome alignment of *Dickeya* species and *Pectobacterium* species strains.

The type strain is NIBIO1006^T (=DSM 105255^T=NCPPB 4611^T) and was obtained from potato tubers of cv. Polaris showing severe symptoms of soft rot in Norway in 2010. The DNA G+C content of the DNA of the type strain is 52 mol%. NIBIO1392 and NCPPB3395 are additional strains of the species.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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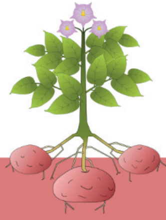

Paper II

Investigation of factors determining potato diseases caused by soft rot *Pectobacteriaceae* using quantitative PCR assays.

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Abstract

Potato soft rot *Pectobacteriaceae* (SRP) cause large yield losses and are persistent in seed lots once established. In Norway, different *Pectobacterium* species are the predominant cause of soft rot and blackleg disease. This work aimed to evaluate the potential of real-time PCR for quantification of SRP in seed tubers, as well as investigating the status of potato seed health with respect to SRP in Norway. A total of 34 seed potato lots, including some certified, were grown and monitored over three consecutive years. All seed lots contained a quantifiable amount of SRP, with very few samples being free of the pathogens. A high SRP abundance based on qPCR assay, as well as a high symptom incidence in certified seed were observed, suggesting that current criteria for seed certification are insufficient to determine tuber health and predict field outcomes. Consistently good performance of first generation seed lots with respect to blackleg and soft rot incidence, as well as low quantity of SRP in these seed lots demonstrated the importance of clean seed potatoes. Climatic conditions during the growing season seemed to govern disease incidence and SRP prevalence more than seed grade. The impact of temperature, potato cultivar and *Pectobacterium* species on tuber soft rot development were further examined in an *in vitro* experiment, which showed that temperature was the most important factor in nearly all cultivars. Large-scale quantification of latent infection and predictive models that include contributing factors like climate, and cultivar are needed to reduce soft rot and blackleg.

Keywords: Agriculture, Soft rot, *Pectobacterium*, Potato, Seed health, Climate

Introduction

Potatoes are the fourth most important food crop worldwide and the most important in Norway (FAOSTAT). Bacterial soft rot diseases, including blackleg and stem rot, caused by members of the genera *Pectobacterium* and *Dickeya* are a serious challenge in potato production worldwide. These genera are genetically diverse, composed of various species with partly different host preferences and geographical locations (Dees et al. 2017a; Ma et al. 2007). Globally, *Dickeya solani*, *D. dianthicola*, *D. dadantii*, *Pectobacterium atrosepticum*, *P*. *carotovorum* subsp. *brasiliense* and *P. parmentieri* are responsible for the most devastating outbreaks (van der Wolf et al. 2014; Ma et al. 2018; van der Wolf et al. 2017). In Norway, *Pectobacterium atrosepticum*, *Pectobacterium carotovorum* subsp. *carotovorum*, and to a lesser extent, *Pectobacterium parmentieri* and *Pectobacterium polaris* seem to be the dominant potato soft rot species (Dees et al. 2017a; Dees et al. 2017b).

Soft rot bacteria (SRP) cause disease by secreting a variety of plant cell wall degrading enzymes that macerate the plant tissue leading to a typically soft rot. The enzyme composition is species and strain dependent, and efficiency of the enzymes varies with temperature (Smadja et al. 2004; Glasner et al. 2008). In potato, tuber soft rot can occur both in the soil and in storage after uptake. Rotting of the seed tubers can prevent emergence of the plants, which can lead to yield losses in infected seed lots (Pérombelon et al. 1988).Tissue maceration can also occur in roots, stolons and the stem (Czajkowski et al. 2010b). Blackleg is a rotting lesion of the stem, signified by its black colour, typically occurring after soft rot of the mother tuber and spread of the pathogen through the vascular system of a sprouted plant (Pérombelon et al. 1989). Entry of SRP in aboveground wounds of the plant can lead to systemic infection and aerial stem rot, a lighter rot of the haulm (Pérombelon 2002; Czajkowski et al. 2010a). While all of the above-mentioned *Pectobacterium* and *Dickeya* species cause similar symptoms, *P.c.* subsp. *carotovorum* has rarely been isolated from blackleg lesions (de Haan et al. 2008; Pérombelon 2000). It is unclear whether *Pectobacterium polaris* causes blackleg in the field, since it was only recently described (Dees et al. 2017b).

Farming machinery and contact between tubers in storage are thought to facilitate transmission of SRP from diseased to healthy plants and tubers (Czajkowski et al. 2011). Transmission can also occur during field growth. The bacteria are motile, given sufficient soil moisture, and can infect roots and tubers of plants in their proximity by entering lenticels or wounds, as demonstrated for a GFP-labelled *Dickeya* strain (Czajkowski et al. 2010b). SRP can therefore rapidly accumulate within a few field generations (Czajkowski et al. 2011). The main source of infection of an initially unaffected seed lot is still unknown. Recently, large-scale presence of SRP in insects found in Norwegian potato fields was demonstrated, including in a field with first generation prebasic seed (Rossmann et al. 2018). This may offer an explanation for the initial infection of clean seed.

The development of blackleg or soft rot symptoms in a given plant or tuber is difficult to predict, and seems to depend on many factors, such as environmental conditions, the phytobiome, health status and genotype of the host, and the infecting soft rot strain (Pérombelon 2002; Czajkowski et al. 2011; Dees et al. 2017a; van der Wolf et al. 2017). Water saturated soil and consequently low oxygen levels around the roots and tuber, stress the plants and favour bacterial growth (Pérombelon 2002). Such conditions often

occur after heavy rainfall in fields with inadequate water draining capacity. Furthermore, *D. solani* and *P. c.* subsp. *brasiliense*, as well as some *P. c.* subsp. *carotovorum* strains seem to prefer warmer temperatures than *P. atrosepticum* (du Raan et al. 2016). Additionally, calcium amendments in soils with low calcium content have been shown to decrease susceptibility of tubers to soft rot (Ngadze et al. 2014). While most commercial potato cultivars are susceptible to soft rot, cultivars with less susceptibility towards blackleg and soft rot have been uncovered in various studies (Chung et al. 2013; Lyon 1989). However, reliable screening for cultivars that are less susceptible to blackleg and soft rot is not trivial due to the influence of environmental factors on disease development (Czajkowski et al. 2011).

SRP can remain latent for a long time, complicating efficient detection and control. Several diagnostic methods of SRP detection and identification are available, including immunological assays, fatty acid analysis, and other biochemical assays. Currently, molecular tools, mainly quantitative real-time PCR (qPCR), are most commonly used for detection and identification in research and plant health diagnostics, due to their accuracy and efficiency (Czajkowski et al. 2015). However, in seed certification schemes, symptom incidence of soft rot and blackleg in field inspections are routinely used for quality assessment.

During the last years, major blackleg occurrence, and incidents of non-emergence due to rotting seed tubers have been reported by many Norwegian seed growers. This work aimed to investigate the impact of environmental and host factors contributing to seed health, and evaluate qPCR as a diagnostic tool for the assessment of SRP infection on a large scale. For this, SRP were quantified in 34 seed lots over three years, and symptoms were assessed on the plants and tubers. The role of potato cultivar, SRP species and temperature during infection was examined further for 15 common Norwegian potato cultivars in a controlled environment.

Materials and methods

Potato material

Norwegian seed potatoes are certified according to health status, producer certification and number of generations after production of clones from tissue culture. An overview of the different stages in Norwegian seed potato production is given in Table 1. While detailed requirements vary, the Norwegian certification scheme is comparable to current potato seed certification requirements given by the European Union (EU), as described in the Council Directive 2002/56/EC. Seed lots with qualities varying from Pre-Basic 2 (P2) to Certified (C) were selected for this work, in addition to some disqualified lots and some of unknown quality (Table 2). The three cultivars Asterix (table potato), Innovator (fries), Lady Claire (crisps), were examined. The tubers used in this study were provided by various seed producers, and Pre-Basic 2 (P2) material was provided by Overhalla Klonavlssenter AS (Minituber and prebasic seed centre).

Table 1. Overview over the Norwegian certified seed potato quality classes.

Seed class	Generation[a]	Producer	Source	Main intended purpose
Minituber/ Pre-Basic 1 (P1)	1	Minituber and prebasic seed centre	Tissue culture	Generation of healthy seed material, further multiplication in Pre-Basic (P) generations
Pre-Basic 2 (P2)	2	Minituber and prebasic seed centre	P1	Supply for Basic (B) seed producers, further multiplication under Pre-Basic (P) standards
Pre-Basic 3 (P3)	3	Pre-Basic certified seed producer	P2	Supply for Basic (B) seed producers, further multiplication under Pre-Basic (P) standards
Pre-Basic 4 (P4)	3(4)	Pre-Basic certified seed producer	P3	Supply for Basic (B) seed producers
Basic 1 (B1)	4	Seed producer	Pre-Basic	Controlled generation of Basic 2 (B2) and certified (C) seed
Basic 2 (B2)	5	Seed producer	Pre-Basic or B1	Controlled generation of Basic 3 (B3) and certified (C) seed Production of potatoes for consumption and industry
Basic 3 (B3)	5 (6)	Seed producer	Pre-Basic or B1/B2	Controlled generation of certified (C) seed, Production of potatoes for consumption and industry
Certified (C)	5 (7)	Seed producer	Any of the above	Production of potatoes for consumption and industry

[a]Earliest generation usually receiving the classification with the latest possible given in parentheses.

Table 2. Cultivars, seed lots, and their quality according to the Norwegian certification scheme, used in the field study. Abbreviations used: D = disqualified seed, U = Unknown quality, otherwise as described in Table 1. Numbers in front of the letter were used as identifiers for the individual seed lot and are not indicative of quality

	Seed lot number	1	2	3	4	5	6
2015	**Asterix**	1D	2D	3D	4D	5D	6D
	Innovator	1U	2U	3U	4U	5U	
	Lady Claire	1U	2U	3U	4U		
2016	**Asterix**	P2	1C	2C	3C		
	Innovator	P2	1B	2B (B1)	3B		
	Lady Claire	P2	1B (B1)	2B (B3)	3B (B2)		
2017	**Asterix**	P2	“P3” (Replant of 2016 P2)	1C	2C		
	Innovator	P2	“P3” (Replant of 2016 P2)	“B2” (Replant of 2016 B1)	2B		
	Lady Claire	P2	“P3” (Replant of 2016 P2)	“B2” (Replant of 2016 B1)	U		

Field trial

The 34 examined seed lots were grown at a test field site located in Hedmark, a county and potato district in the southeast of Norway, for three consecutive years (Table 2). They were grown in multiple (non-random) rows per seed lot, sorted by cultivar with 25 cm planting distance. Some material was grown over two consecutive years after storage. Farming conditions on the field were typical concerning fertilization, as well as interval treatment for weed, fungus and insect control. Haulms were killed chemically (Diquat).

Assessment of soft rot and blackleg, and quantification of SRP by qPCR

Blackleg symptoms were registered by thorough field inspection toward the end of the growing season, 80 days (2015), 86 days (2016), and 97 days (2017) after planting. The tubers were analysed just before planting, immediately after harvest and after three months of storage. At each sampling time, eight (2015) or six (2016, 2017) samples of 25 tubers were analysed from each seed lot. Tubers were incubated for two weeks at 16°C. Samples were rinsed in tap water, air-dried, and visually assessed for symptoms of soft rot. Tubers were cut in halves and packed into separate vacuum bags per sample of 25 tubers. Bags were vacuum-sealed using common kitchen appliances (Food Sealer Pro, OBH Nordica; corresponding Food Sealer bags) and left for 5 days at 20°C (modified from Boomsma et al. 2013). The liquid that formed in the bags due to tuber degradation was taken out (5x1 mL if sufficient material) and used for DNA extraction using KingFisher Cell and Tissue DNA kit (Thermo Scientific) on the Kingfisher Duo Prime platform according to the manufacturer's instructions. SRP DNA was detected and quantified from six samples per seed lot using the generic PEC TaqMan primer/probe set from Pritchard et al. (2013) as described before (Rossmann et al. 2018). Quantities were calculated based on a standard curve of genomic DNA from *P. atrosepticum* strain OKDS 42-1-12 (Dees et al. 2017a).

Tuber infection test

First-generation clonal potato tubers (P2) of 15 different cultivars commonly used in Norwegian potato farming were inoculated with two SRP strains and incubated at two temperatures to measure the impact of these factors on rot progression. The cultivars used in this experiment were Asterix, Beate, Berber, Cerisa, Erika, Fakse, Folva, Hassel, Innovator, Lady Britta, Lady Claire, Lunarossa, Mandel, Nansen, and Rutt. The *Pectobacterium* strains used for inoculation were *Pectobacterium atrosepticum* strain PK-782-1-2-13 (Dees et al. 2017a) and *Pectobacterium polaris* strain NIBIO1006 (Dees et al. 2017b). The two strains were selected because they are more aggressive than other strains of their respective species. The tuber infection assay was performed as previously described with modifications (Dees et al. 2017b; Laurila et al. 2008). The bacteria were grown on LB plates at room temperature for 2-3 days, and suspended in phosphate-saline buffer (PBS) to an optical density (OD) of 0.8 ±0.05 at a wavelength of 600 nm. For each strain, 10 µL was inoculated into wounds of each of 15 tubers of each cultivar. The wounds were created using a pipette tip, which was left in the wound to reduce air contact. Five tubers of each cultivar were inoculated with PBS as a control. The inoculated tubers were placed in trays (50 x 29 x 6 cm), lined with a filter paper soaked with 50 mL Milli-Q H_2O, and each tray was wrapped in plastic to promote high humidity and low oxygen conditions. The trays were incubated at a room temperature of 24.2°C ±0.6 (SD) for 3 days. Separate trays were used for each cultivar. After incubation, the macerated tissue was removed with a scalpel and weighed. The same experimental setup was used in a culture

room with a temperature of 19.5°C ±0.2 (SD) for 3 days. The two temperatures are rounded to 20 and 24 °C throughout the manuscript.

Statistical analysis

Analysis of variance (ANOVA) was performed for the three response variables SRP infection prevalence, observed blackleg incidence, and soft rot incidence. The statistical models were fitted to data with year, quality, and cultivar as categorical factors and some of the other response variables as covariates, if appropriate. The response variables were SRP infection prevalence, as measured by qPCR, which was log10-transformed to achieve normality and homogeneous variance, blackleg incidence in percent per seed lot, and average soft rot incidence, measured in mean symptomatic tubers from the 25-tuber samples of a given seed lot.

The response variable tuber infection was modelled by an ANOVA model using cultivar, temperature, strain, and all their interactions as independent factors. Grouping according to statistical significance by cultivar, as well as all combinations of cultivars, temperatures, and strains, was done using Tukey's pairwise comparisons with 5 % significance level. The calculations were performed using the statistical software Minitab 17.2.1.

Climate data

Climate data was obtained from two measurement stations in the Kongsvinger municipality in Hedmark county. The average monthly temperature and precipitation data was obtained from the eKlima database of the Norwegian Meteorological Institute, reporting from a site approx. 8 km from the field site. The ground temperature at a distance of approx. 0.5 km from the field site was obtained through Agrometeorology Norway.

Results

A total of 34 seed potato lots were grown and monitored over three consecutive years (Table 2). In 2017, offspring of five of the seed lots from 2016 were grown in addition to new seed lots. In 2016 and 2017, P2 seeds were grown as an indicator of clean seeds with minimal SRP presence.

Quantification of SRP infection prevalence

After visual assessment, the samples were incubated in conditions that favour SRP multiplication, and the infection prevalence was quantified by measuring the amount of bacterial DNA in each sample in a qPCR assay. All seed lots contained a quantifiable amount of bacteria and the quantities varied between the 25-tuber samples measured from a given seed lot (Figure 1). The quantification showed differences in the SRP prevalence between samples taken at planting, harvest and after storage in all years (Figure 1). In 2015, the overall amount of SRP present in the samples seemed to increase steadily from planting, to the last sampling after three months of storage. In 2016, the overall amount between planting and harvest seemed similar to samples in 2015, but a decrease was measured after storage. In 2017, the average levels were similarly low between samples taken at planting and harvest but increased overall in samples after storage. While not all seed lots followed these trends in a given year, the trends were largely consistent between seed lots and cultivar. A notable exception are the Innovator seed lots in 2017, which contained low SRP quantities at all sampling points, compared to the other cultivars.

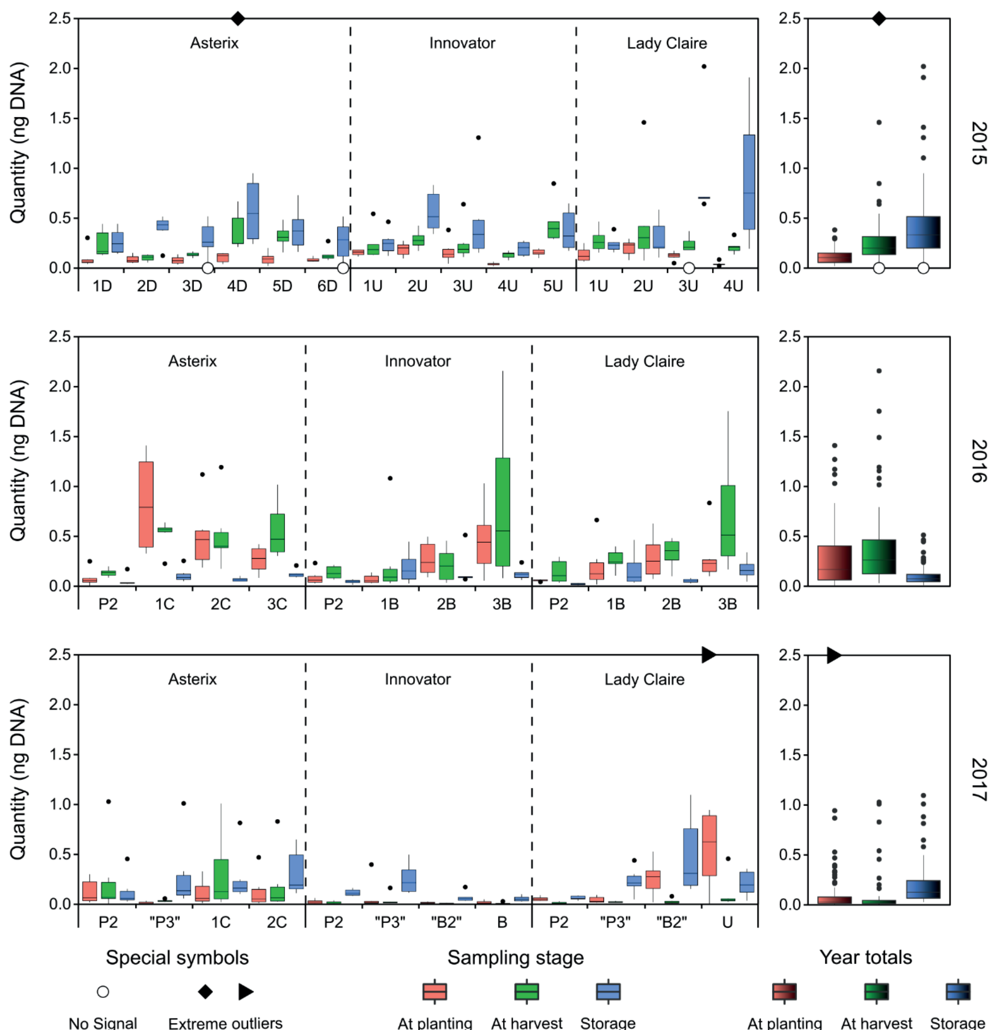

Figure 1. SRP infection prevalence in the tested potato seed lots of for each of the years 2015-2017. Seed lots quality and identity (x-axes) is detailed in Table 2. The quantity of SRP was calculated from qPCR results using the generic PEC assay after vacuum incubation enrichment. The quantity is given in ng DNA, based on a dilution series of pure genomic DNA. The colours of individual bars indicate the three sampling stages, planting (red), harvest (green), and after 3 months of storage (blue). The overall quantities at planting (red-black), harvest (green-black) and after 3 months of storage (blue-black) are shown on the right of the detailed plots for each year. The boxes indicate the median (black line) and the first and third quartile with the whiskers indicating 1.5 inter-quartile range. Dots indicate values outside this range (outliers). Two outliers outside the range of the diagram are indicated by a diamond in 2015 (12.9 ng) and an arrowhead in 2017 (5.6 ng). Replicates with no detectable signal (0 ng) are emphasized by white circles.

The climate during the growing seasons varied between the years in the area of the field site (Figure 2).

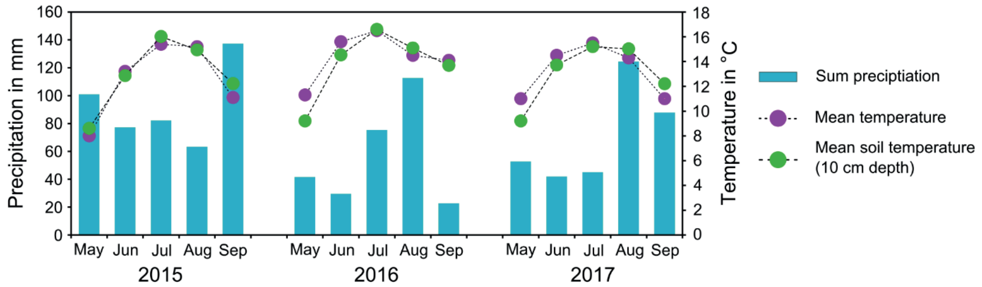

Figure 2. Climate at the field location during the growing seasons 2015-2017. Shown are the total precipitation in mm (blue bars), mean air temperature in °C (purple dots), and mean soil temperature at 10 cm depth in °C (green dots) for the months May – September (abbreviated).

Blackleg incidence

In all years, blackleg symptomatic plants were observed towards the end of the growing season (Figure 3). The total percentages of plants with clear blackleg symptoms were 0.6 % in 2015, 1.2 % in 2016, and 0.5 % in 2017. The cultivar and quality seemed to be the most important determinants of blackleg occurrence, as Asterix consistently showed the largest overall proportion of symptomatic plants in all three years (1.7 % on average), and Innovator showed no symptoms in 2015 and 2017, and a very low percentage of plants that developed blackleg in 2016 (0.1%). While plants grown from P2 seeds were generally free from symptoms or showed very low incidence of blackleg, the replanted tubers harvested from the Asterix P2 seed lot (Asterix, 2017, "P3") showed a 2 % incidence of blackleg symptoms. Otherwise, the seed classifications did not determine the blackleg incidence reliably. In the case of plants from B1 seed for Lady Claire, high incidence was observed in 2016, while the offspring tubers produced plants were without blackleg symptoms in 2017 ("B2"). Overall, blackleg occurrence varied with the seed lot and potato cultivar, and to a lesser extent between years and seed quality, apart from P2.

Figure 3. Blackleg incidence in the field for three potato cultivars in late summer over three years. The incidence is shown as the percentage of symptomatic plants out of the total number of plants for a given seed lot. The seed lots are split by year and cultivar. For each seed lot, the quality is given as indicated in Table 2.

Soft rot symptoms on tubers

The number of samples that contained rotten tubers and the number of rotten tubers in those samples was assessed by visual inspection (Figure 4).

Figure 4. Overview of soft rot symptoms in tuber samples from all examined seed lots at planting, harvest, and after three months of storage over three years. Each column represents one seed lot and each cell one sample of 25 tubers. The numbers and colours indicate the number of symptomatic tubers in a sample (0=green, 1 to 12= yellow to red). The total percentage of samples with symptomatic tubers (Samples) and symptomatic tubers (tubers) are given for each year (bottom) and each sampling time point (right). The seed lots are sorted by cultivar and shown in the same order as in Table 2. Missing samples due to insufficient material are indicated with x.

Tubers with soft rot symptoms were observed for almost all tested seed batches in all years, but only in two cases for all samples of one seed lot. Before planting, only 1.6 % of samples contained symptomatic tubers over all three years. However, at harvest and after three months of storage, the percentage of samples with symptomatic tubers rose to 17.8 and 15.5 % respectively. Over the three years, the total percentages of samples with symptomatic tubers increased from 9.2 % in 2015, 11.4 % in 2016, and to 16.2 % in 2017. Except for 2016, Innovator tubers showed the lowest number of samples with symptomatic tubers. P2 tubers and their offspring, showed no samples with symptomatic tubers in 2016, except for one Innovator replicate at harvest. In 2017, Asterix P2 had samples with soft rot symptoms at harvest and after storage, while the P2 seed lots of Innovator and Lady Claire were entirely free of symptoms. The highest number of symptomatic tubers was found after storage in the Innovator B1 seed lot in 2016 and the Lady Claire seed lot of unknown quality in 2017.

Main factors in SRP infection prevalence and disease incidence

To explore which factors are most informative in explaining the observed variability between samples, ANOVA models were fitted to the data measured at planting, at harvest and after storage, some results shown in figure 5. The models ranged in their ability to explain the variability of the three measured response variables with R^2 values ranging from 21 to 72 %. For SRP prevalence at all sampling times and soft rot incidence at harvest and after storage, the year of the experiment seems to have largest influence, indicated by the highest F-value, and smallest p-value, among the main effects of the investigated factors, year, cultivar, and quality.

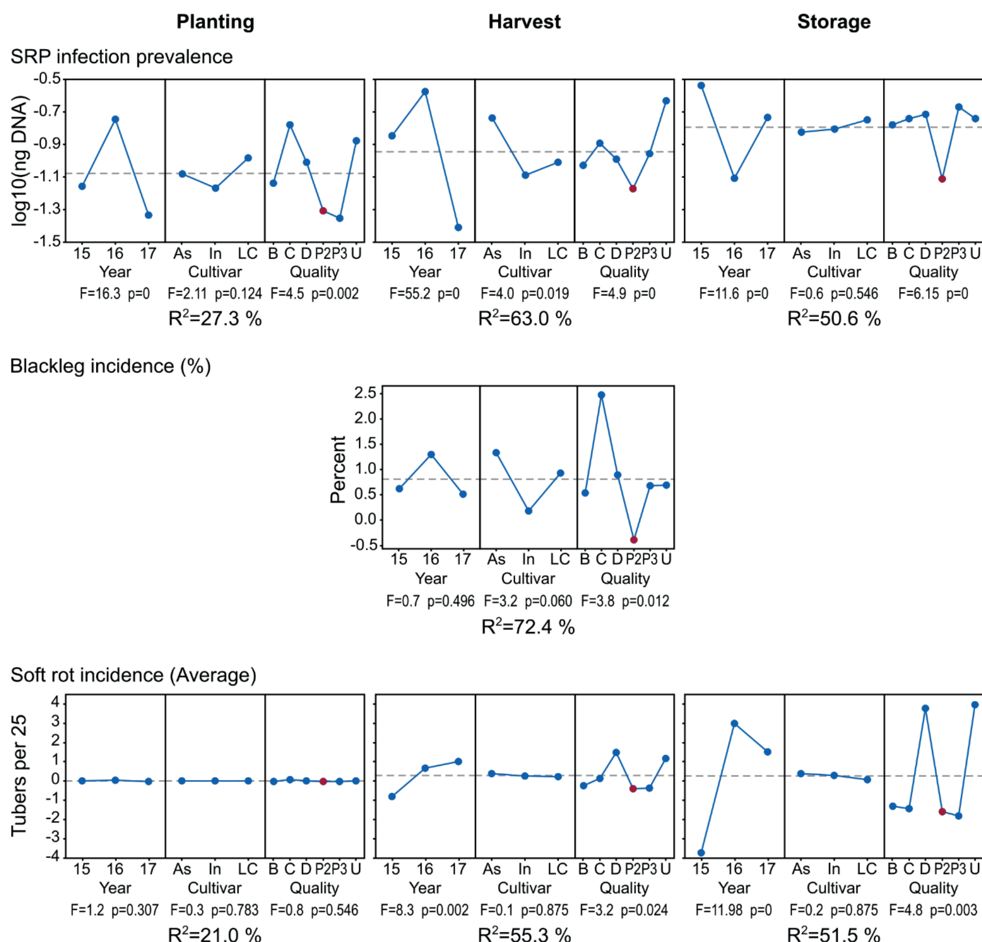

Figure 5. Estimated main effects in ANOVA models for factors for SRP infection prevalence, blackleg and soft rot incidence. Shown are the estimated means of these three response variables modelled based on the data shown in Figures 1, 3 and 4. The three examined main factors were the year of sampling, the three investigated cultivars Asterix (As), Innovator (In) and Lady Claire (LC), and the seed quality according to certification, as given in Table 2. Models were calculated separately for the three sampling points planting (left column), harvest (middle column) and storage (right column). The highest investigated seed quality (P2) is highlighted in red. Overall means are indicated by grey dotted lines. For each model, the F-value and *p*-value for the factors year, cultivar and quality as well as the R^2-value are shown. Model details are shown in Supplementary table 2.

For blackleg incidence, quality according to certification and cultivar seem to best explain the observed variability, $p = 0.012$ for quality and $p = 0.060$ for cultivar (Supplementary table 2). The estimated mean blackleg incidence for the quality grade B, declined seed tubers, and tubers of unknown quality, as well as the offspring of P2 seed ("P3") were between 0.5 and 1 %, while quality grade C was at 2.5% and P2 at less than 0 %. Seed tubers of the quality P2 had the lowest estimated means, or second lowest after "P3", for all models. Other qualities did not behave consistently in relation to each other according to their estimated means (Figure 5). The three tested cultivars were only a significant factor in explaining the observed variability of SRP infection prevalence at harvest ($p = 0.019$) but not in any other responses. Most of the used ANOVA models fit the data well. For some of the models there were some relatively small departures from the normality assumption, in particularly for soft rot incidence at planting and after storage.

Tuber infection of 15 potato cultivars at two different temperatures

To test different potato cultivars for tuber susceptibility to *Pectobacterium*, tubers were infected with two different strains of Pectobacterium and incubated in a controlled environment at two temperatures. Large overall differences in the amount of tissue rotten by *P. atrosepticum* and *P. polaris* were observed at 20 and 24 °C (Figure 6). At 24 °C, *P. polaris* macerated more tissue on average in 13 out of 15 cultivars, whereas *P. atrosepticum* macerated more tissue on average at 20 °C in 10 out of 15 cultivars, albeit with smaller margins. Table 3 shows the results of the fitted ANOVA model ($R^2 = 73.8$ %) according to which all factors and factor interactions significantly contributed to the observed variability in tissue maceration. While all factors were significant, temperature had by far the largest F-value, which indicates a larger impact in determining tissue maceration compared to the other factors and factor interactions. A visualization of the estimated mean maceration values for the main factors cultivar, temperature, and strain is provided in Supplementary figure 1. The differences between the cultivars over both temperatures and strains, as determined by Tukey's pairwise comparison method, were only statistically significant in some cases (Supplementary table 1). Due to the significant impact of temperature, infecting strain and factor interactions, most cultivars could not be reliably and unambiguously ranked in their overall susceptibility to *Pectobacterium* in this experiment. However, grouping by pairwise comparisons indicate that Berber and Innovator each were statistically distinct from the other tested varieties (Table 4). Innovator was least susceptible to infection and Berber most susceptible. Pairwise comparison of tuber infections split by all three factors showed that all samples except those with the highest and lowest estimated means, belonged to multiple groups, and did not show a clear split of groups according to a single factor (Supplementary table 2).

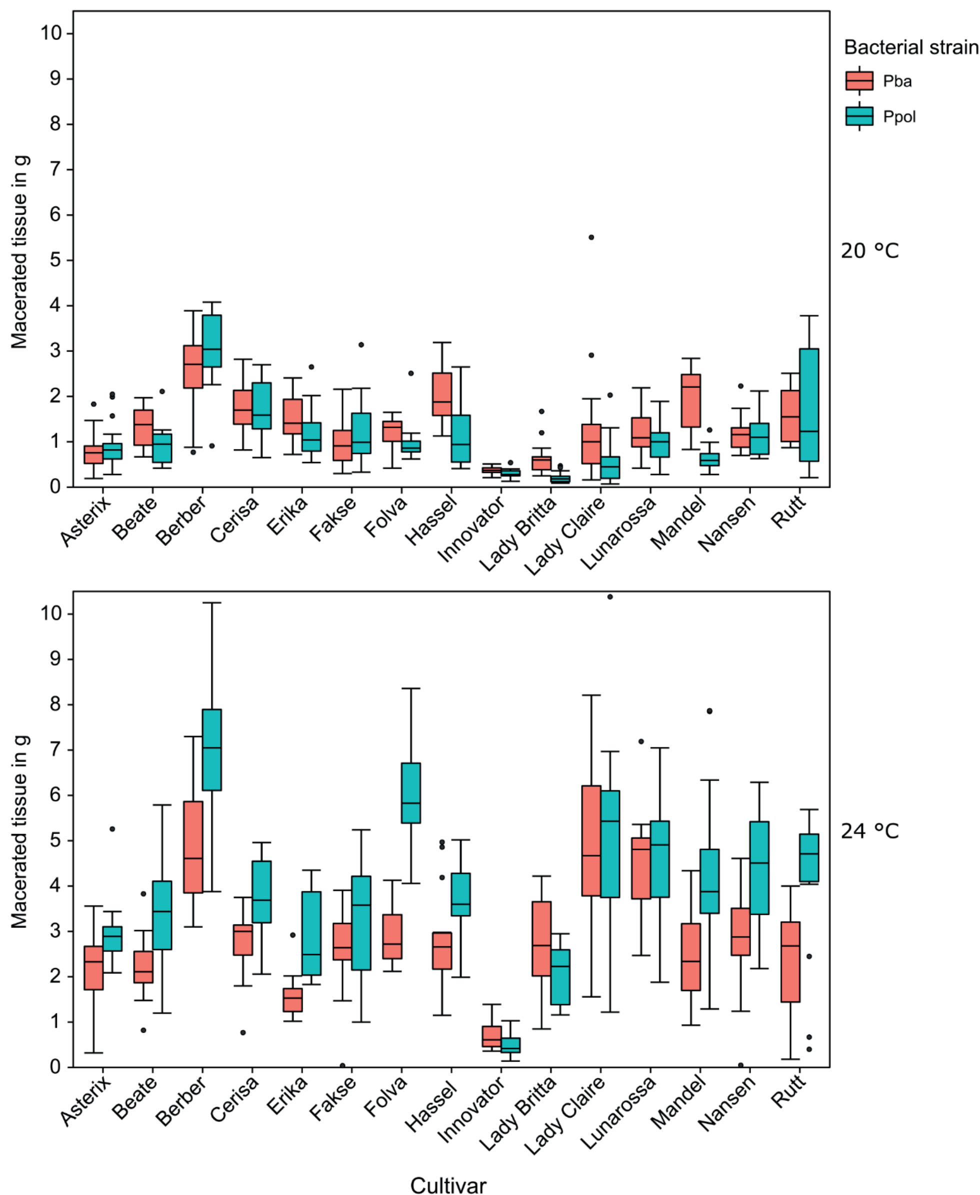

Figure 6. Amount of macerated tuber tissue from 15 potato cultivars at two different temperatures three days after inoculation with two different *Pectobacterium* strains. Shown is the macerated tissue in g of 15 tubers per sample. The temperatures during incubation were 20 °C (top) and 24 °C (bottom). Pba (red) and Ppol (blue) refer to the two strains *Pectobacterium atrosepticum* strain PK-782-1-2-13 and *Pectobacterium polaris* NIBIO1006. The boxes indicate the median (black line) and the first and third quartile with the whiskers indicating 1.5 upper and lower inter-quartile range. Dots indicate values outside this range (outliers).

Table 3. Results from fitting the ANOVA model to the tuber infection test data using cultivar, temperature, strain, and all their interactions as independent factors. Given are the degrees of freedom (DF), adjusted sum of squares (Adj. SS), adjusted mean squares (Adj. MS), F-value, and *p*-value associated with each factor.

Source	DF	Adj. SS	Adj. MS	F-value	*p*-value
Cultivar	14	606.5	43.3	49.6	0.000
Temperature	1	1011.6	1011.6	1158.4	0.000
Strain	1	34.7	34.7	39.8	0.000
Cultivar*Temperature	14	215.7	15.4	17.6	0.000
Cultivar*Strain	14	64.7	4.6	5.3	0.000
Temperature*Strain	1	92.4	92.4	105.9	0.000
Cultivar*Temperature*Strain	14	56.1	4.0	4.6	0.000
Error	867	757.1	0.9		
Total	926	2888.8			

Table 4. Grouping results from Tukey's pairwise comparisons, using 5 % significance level, of the main effects of the cultivars based on the ANOVA model fitted to the tuber infection test data. Given are the number of observations (N) and the estimated mean macerated tissue in g (Mean in g). Cultivars that do not share a letter indicate that they are significant different, based on the estimated means.

Cultivar	N	Mean in g	Grouping							
Berber	58	4.3	A							
Lady Claire	60	3.0		B						
Lunarossa	60	2.8		B	C					
Folva	60	2.8		B	C					
Cerisa	60	2.5		B	C	D				
Rutt	60	2.5		B	C	D				
Hassel	60	2.4		B	C	D				
Nansen	60	2.4		B	C	D				
Mandel	60	2.3			C	D	E			
Fakse	60	2.0				D	E	F		
Beate	60	2.0				D	E	F	G	
Erika	60	1.8					E	F	G	
Asterix	89	1.7						F	G	
Lady Britta	60	1.4							G	
Innovator	60	0.5								H

Discussion

Certified seed lots were assessed for SRP infection by large scale molecular testing after enrichment in vacuum and symptom monitoring of seed potatoes, plants and harvested tubers before and after storage. Enrichment of SRP in vacuum resulted in quantities that were reliably measurable in nearly all samples. The near-ubiquitous presence of SRP in all seed lots demonstrates the widespread potential for disease development. Even the P2 lots that were included in 2016 and 2017 to offer a baseline and contrast to seed lots in 2015, contained some SRP, albeit in lower quantities compared to other certified material.

Overall trends of SRP infection prevalence in tubers before planting, at harvest, and after storage varied heavily between the years (Figure 1). In all years, haulm destruction and harvest were conducted in mid and late September respectively, but the climate during the growing seasons varied over the three years (Figure 2). This suggests substantial impact of the environmental conditions during the experiment. The most pronounced difference between the three years was the overall higher precipitation in 2015 and 2017, compared to 2016. In 2015, the overall and September precipitation was highest. High precipitation might weaken the tubers' defences by creating low oxygen conditions through waterlogging (Burton and Wigginton 1970; Pérombelon and Lowe 1975). This could be particularly impactful after haulm destruction, when bacteria can multiply on the dying plant organs and may wash into the soil or form aerosols under heavy rainfall (Quinn et al. 1980; Burgess et al. 1994). In addition, wet soil may promote the spread of SRP through active motility of the bacteria or washing of the bacteria from diseased to healthy material. At harvest, moisture may promote contamination of healthy tubers by increasing smearing of bacteria via machines, tools and direct contact.

The increase in SRP infection prevalence from harvest to storage in 2015 indicates that after high September precipitation, the SRP quantity persists, and that tuber health may be compromised throughout storage. In 2016, tubers showed high SRP quantities across cultivars at planting, which decreased to harvest and decreased further in storage. This is an indication that the dryer and warmer 2016 climate, especially in September, might have remedied the rather poor condition of the seed lots. The fact that this effect was more pronounced after storage, suggests an impact of climate during the growing season on long-term seed health. By replanting some of the offspring of the 2016 seed lots in 2017, it was observed that the decreased SRP quantity after storage persisted to planting, and in some cases harvest. However, most 2017 seed lots showed increased SRP quantities at harvest, likely because of high precipitation late in the growing season (September) and lower temperatures at harvest.

Normally, it is expected that under good storage conditions, SRP infection in tubers decreases over time (van Vuurde and de Vries 1994), as was suggested by lowered SRP infection prevalence after storage in 2016 (Figure 1). The synopsis of climate and SRP infection prevalence overall suggests that high precipitation during the growing season, particularly close to harvest, compromises the ability of the tubers to fend off SRP and/or may increase the risk of transmission between tubers. Conversely, a dryer growing season and harvest window might promote tuber defence throughout storage and provide seed with relatively lower SRP quantity in the next season.

The proposed influence of the climate on long-term seed tuber health may explain some of the observations made in the assessment of blackleg incidence (Figure 3). The high

overall blackleg incidence in 2016 may reflect the compromised seed tuber health of the mother tubers grown in 2015. Similarly, the total absence of blackleg incidence in Lady Claire plants in 2017 may be explained by the susceptibility of mother tubers grown in a drier climate in 2016. Blackleg incidence in 2015 may have been driven by the high consistent precipitation early on in the growing period. Moreover, the blackleg incidence seems to have been influenced significantly by potato cultivar. Plants from all Asterix seed lots, except for P2 quality seed, developed blackleg in all years, while Innovator rarely developed any, despite the presence of SRP in the tubers. Lady Claire plants performed overall worse than Innovator but better than Asterix in blackleg incidence, and had no symptomatic plants in 2017. This suggests that Innovator and Lady Claire might have a lower susceptibility to blackleg, than Asterix. However, they seem to be similarly susceptible to SRP pressure under specific climatic conditions.

Plants from P2 seed tubers displayed reliably low blackleg incidence, few soft rot symptomatic tubers (Figure 4), and harbored low levels of SRP according to the enrichment assay in all three tested cultivars (Figure 1). With the exception of P2 seeds, the certification grades displayed inconsistent behavior between the years and tested cultivars. In 2016, the high incidence the 1B seed lot of Lady Claire compared to 2B and 3B, as well as the Asterix seed lots compared to the disqualified 2015 lots are the most dramatic example of this. Moreover, the same Lady Claire 1B seed lot, replanted in the next year (“B2”, Lady Claire, 2017), was completely free of symptomatic plants.

The observations in the replanted seed lots in 2016 and 2017 point to a major shortcoming in the current seed certification method; It does not sufficiently account for the potential of rapid accumulation of SRP and rise in disease incidence, such as observed in the case of the 2017 “P3” progeny of the 2016 Asterix P2 lot. Furthermore, it assumes a continuous and irreversible rise in SRP levels and disease risk. The replanted progeny of the Innovator P2 and Lady Claire B1 lots, however, showed lower blackleg incidence (Figure 3) and overall SRP prevalence (Figure 1) in 2017 compared to 2016. Similarly, the progeny of Lady Claire P2 slightly improved in both of these properties. The findings in this study support previous concerns about the efficiency of field inspection-based seed certifications to prevent blackleg. As such, it strengthens the demand for quality control that offers higher predictive value by integrating models that include quantification of SRP, and other more reliable determinants (Pérombelon 2000). The use of halved tubers and enrichment using inexpensive instruments (vacuum sealer, suitable plastic bags) is cost-efficient, quick, and yields samples that are easier to quantify, compared to industrial scale skin peeling and SRP extraction, which was previously suggested (Pérombelon 2000). Furthermore, testing just the skin and stolon heel was shown to miss a significant part of the tuber-associated inoculum (Boomsma et al. 2013). The implementation of a qPCR assay after enrichment, as performed here, shows the feasibility of this method in large-scale potato seed testing.

Tubers with soft rot symptoms were counted for the samples used for the SRP quantification (Figure 4). The seed lots with a high number of samples containing symptomatic tubers did not correspond to the seed lots with high blackleg incidence (Figure 3 and Figure 4). Since the bacteria can rapidly spread and elicit symptoms in previously healthy tubers, the number of rotten tubers in one sample may be less informative. However, the trends seen in the SRP quantification (Figure 1) are somewhat reflected in the soft rot occurrence (Figure 4). In 2015, the samples with symptomatic tubers increased from harvest to storage, while they decreased or remained equal in most

seed lots in 2016. This further supports the notion that the precipitation late in the growing season has an overall influence on the resilience of tubers in storage. In 2017, the number of samples with symptomatic tubers remained relatively consistent between harvest and storage.

Asterix and Lady Claire tubers displayed a high amount of soft rot symptoms compared to Innovator in 2015 and 2017. In 2016, Asterix had an overall lower amount of symptomatic samples compared to the other years, while Lady Claire had none. Innovator had symptomatic samples in all seed lots after harvest but only in one after storage. In conclusion, Innovator seems to be least susceptible among the three cultivars tested in the field, based on the low blackleg incidence and mostly low amount of soft rot symptomatic samples. Earlier work in Switzerland showed Innovator to be susceptible to blackleg in the field after inoculation with *Dickeya dianthicola,* with large variations between years that were similar to other cultivars in that study (Rouffiange et al. 2014). The low susceptibility of Innovator to blackleg observed here may be specific to Norwegian conditions, where the climate during the growing season is comparatively cool and *Pectobacterium* species are predominant (Dees et al. 2017a).

The ANOVA models for SRP infection prevalence, blackleg incidence, and soft rot incidence confirm the large role that the climate played overall (Figure 5). It was assumed that the differences between the years were largely caused by climatic differences. While the differences in blackleg incidence were not explained well by differences between the years, this was the most influential factor in determining SRP prevalence and soft rot incidence according to the models. The data analysis indicates that the infection prevalence at planting and harvest and the blackleg incidence followed similar patterns over the years and for the three tested cultivars. However, the cultivar in particular did not significantly contribute to explaining the observed variability in all cases.

While quality often was a significant factor in determining the outcome of the observations, the behaviour of the particular seed grades was not stable over the different observations. The P2 grade seed tubers were ubiquitously estimated to perform well, but B and C grade seed tubers, as well as the offspring of the P2 seed ("P3") varied widely relative to each other. For example, C-grade seed tubers are estimated to have a much higher blackleg incidence than all other grades, whereas B and disqualified seed are similar. In infection prevalence, C-grade tubers were estimated to be infected with the highest amount of bacteria, whereas after storage, all seed grades except for P3 were estimated to carry a similar inoculum. Some of the unexpected results, particularly for C-grade seed tubers could be explained by some overlap in the effect of the cultivar, since almost all C-grade seed tubers were Asterix (Table 2). However, the raw data discussed above as well as the strong variation of estimated means of B, C, and "P3" tubers relative to P2 across sampling points and examined responses does not fit expectations in regard to the relative performance of the certified seed. The results of the infection prevalence as measured by qPCR were not correlated with blackleg or soft rot disease incidence in the general linear models (Supplementary table 2). Furthermore, the attempt to fit regression models to the data for disease incidence with infection prevalence as a regressor were unsuccessful (not shown). As a result, the measured prevalence in the presented form could not be used to predict disease outcomes. An improved workflow with direct enrichment, omitting the two-week incubation at 16 °C before analysis, could yield results, which can better predict field outcomes and disease incidence. The

incubation employed here could favour the multiplication of other pathogens which may be in competition with SRP (Kõiv et al. 2015). Similarly, the multiplication rate of SRP in the used vacuum enrichment method, and how it is influenced by other present microbes, is not known. While it gives a solid overview of infection prevalence, the qPCR assay after enrichment is not necessarily suited to quantify the initial amount of SRP.

The presented ANOVA models were a useful tool in exploring the central factors in the measured aspects of SRP pathology and confirmed climate to play a central role in infection prevalence. However, overall the models were not sufficiently powerful to serve a predictive function. Nevertheless, diversified data, more sophisticated modelling approaches, such as machine learning, and improved standardized molecular testing of SRP infection prevalence may eventually yield reliable prediction tools for field outcomes and prevention strategies. A balanced approach that combines the feasibility on a large scale presented here with a more direct quantification of bacterial numbers may yield additional insight into e.g. threshold infection levels that lead to disease.

The evaluation of tuber infection of 15 different cultivars under controlled conditions offered a simplified, yet important perspective on the results of the field trial (Figure 6). The high F-value of temperature compared to the other factors in the ANOVA model (Table 3) indicates that temperature was the most impactful factor in the progression of soft rot in this experiment. While the impact of the infecting strain was significant (Table 3), the difference between the two was much larger at 24°C than at 20°C (interactions between temperature and strain), due to the overall higher maceration by *P. polaris* at (Figure 6). This suggests that symptom progression, while overall tied to temperature, can become more severe in the presence of soft rot strains and species that are able to thrive at a higher temperature. Norway, unlike countries with a warmer climate, has so far not experienced outbreaks of species like *D. solani* or *P. c.* subsp. *brasiliense*, which thrive at warmer temperatures (du Raan et al. 2016). The data presented here suggests that such outbreaks are to be expected in Norway, as warmer growing seasons with heavy rainfalls and humid weather are predicted to occur (Hanssen-Bauer et al. 2009). Although the cultivar of the infected tubers was a significant factor in the ANOVA model, Berber and Innovator were the only cultivars with a distinct impact on soft rot outcome averaged over the two temperatures and infecting strains, as shown by the results in table 4 based on Tukey's pairwise comparisons. While Innovator was least susceptible to the tested strains, Berber was macerated significantly more than other cultivars.

The results of the tuber infection reflect the observations made for Innovator in the field experiment. There, Innovator had less overall SRP prevalence, blackleg incidence and rotten samples than Asterix and Lady Claire, suggesting a generally lower susceptibility. However, Lady Claire was significantly more susceptible than Asterix and Innovator in the tuber infection, but Asterix and Lady Claire did not seem to perform as clearly different in the field experiment. This may be explained by the impact of the infecting strain, as the SRP causing blackleg and tuber rot in the field experiment are likely to be different from the ones used in the tuber infection. SRP species and strains are known to vary heavily in their ability to cause disease (du Raan et al. 2016; Smadja et al. 2004; Lebecka et al. 2018). Furthermore, the ability to cause blackleg differs between *Pectobacterium* species. *P.c.* subsp. *carotovorum*, for instance, is commonly assumed not to cause blackleg. While a subgroup of *P. c.* subsp. *carotovorum* was isolated from blackleg lesions (de Haan et al. 2008), this subgroup was later determined to belong to *P. parmentieri* (van der Wolf et al.

2017). Based on this, the tuber infection conducted here is not a sufficient indicator of the tested cultivars' abilities to withstand systemic infection and blackleg occurrence under field conditions, especially due to the low number of tested strains and limited investigated temperature range. It nevertheless offers insight into the hierarchy of factors in tuber soft rot and suggests temperature to be the most important factor, followed by potato cultivar and infecting strain. This is supported by similar work on other cultivars and SRP strains (Lebecka et al. 2018; Dees et al. 2017a).

In conclusion, enrichment of SRP from halved tubers in vacuum and subsequent quantification by qPCR were implemented as a cost-efficient option for large-scale tuber testing and assessment of seed tuber health. Although, the obtained results did not reliably correlate with disease incidence, improvements in the workflow may yield data, which can be used in better diagnostic and predictive tools. The data presented here suggests that the climate during the growing season has the greatest impact on blackleg incidence and the health status of the tubers. The potato cultivar also plays a role in the overall susceptibility SRP but seems to be less important than the health condition of the seed lot and climate during the growing season. The ubiquity of SRP and seemingly inevitable acquisition in the field need to be addressed through higher volumes of clean early generation seed, stricter hygiene regimes and certification methods with better predictive value.

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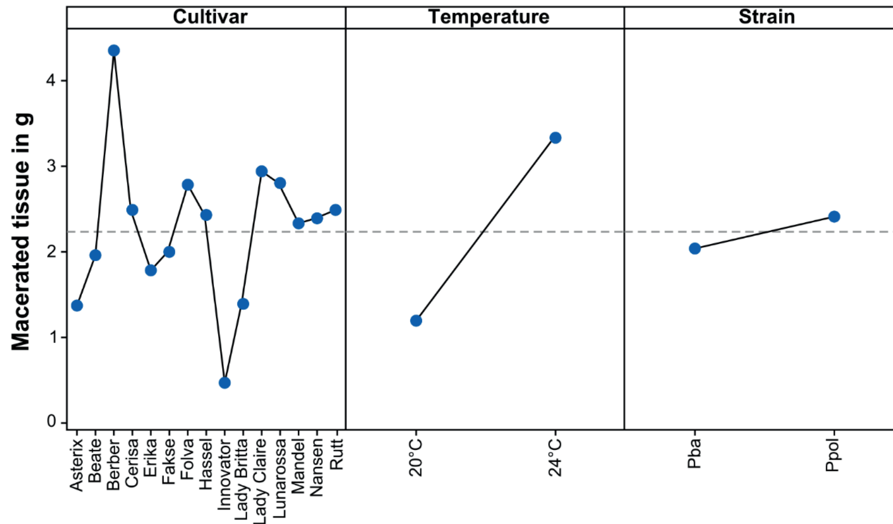
Supplementary figures and tables



Supplementary figure 1. Estimated main effects in tuber infection. Shown are the estimated main effect means for macerated tissue in g according to the ANOVA model for the 15 tested potato cultivars, the two tested temperatures 20 ℃ and 24 ℃, and the two tested strains of Pectobacterium atrosepticum (Pba) and P. Polaris (Ppol). Overall means are indicated by grey dotted lines.

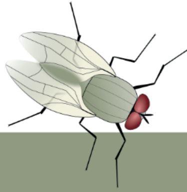
Supplementary table 1. Complete grouping results from Tukey's pairwise comparisons, using 5 % significance level, for all combinations of the 15 cultivars, 2 temperatures (incubated at 20 ℃ or 24 ℃), and 2 strains (infection with P. atrosepticum, Pba, or P. Polaris, Ppol), based on the ANOVA model fitted to the tuber infection data. Shown are the 60 combinations of cultivars, temperatures, and strains (Sample), the number of observations for each sample (N), and the estimated mean macerated tissue in g (Mean). Samples that do not share a letter indicate that they are significant different, based on the estimated means.

Sample	N	Mean	Grouping																						
Berber 24°C Ppol	15	6.8	A																						
Folva 24°C Ppol	15	6.1	A	B																					
Lady Claire 24°C Ppol	15	5.1		B	C																				
Berber 24°C Pba	15	4.9		B	C																				
Lady Claire 24°C Pba	15	4.9		B	C																				
Lunarossa 24°C Ppol	15	4.5			C	D																			
Lunarossa 24°C Pba	15	4.5			C	D																			
Nansen 24°C Ppol	15	4.5			C	D	E																		
Mandel 24°C Ppol	15	4.3			C	D	E	F																	
Rutt 24°C Ppol	15	4.1			C	D	E	F	G																
Cerisa 24°C Ppol	15	3.7			C	D	E	F	G	H															
Hassel 24°C Ppol	15	3.7			C	D	E	F	G	H															
Beate 24°C Ppol	15	3.4				D	E	F	G	H	I														
Fakse 24°C Ppol	15	3.2				D	E	F	G	H	I	J													
Berber 20°C Ppol	13	3.0					E	F	G	H	I	J	K												
Asterix 24°C Ppol	14	2.9						F	G	H	I	J	K	L											
Folva 24°C Pba	15	2.9						F	G	H	I	J	K												
Erika 24°C Ppol	15	2.9							G	H	I	J	K	L											
Hassel 24°C Pba	15	2.9							G	H	I	J	K	L											
Nansen 24°C Pba	15	2.8							G	H	I	J	K	L											
Cerisa 24°C Pba	15	2.8							G	H	I	J	K	L											
Lady Britta 24°C Pba	15	2.7								H	I	J	K	L	M										
Fakse 24°C Pba	15	2.6								H	I	J	K	L	M	N									
Berber 20°C Pba	15	2.5								H	I	J	K	L	M	N									
Mandel 24°C Pba	15	2.5								H	I	J	K	L	M	N	O								
Rutt 24°C Pba	15	2.5								H	I	J	K	L	M	N	O								
Beate 24°C Pba	15	2.2									I	J	K	L	M	N	O	P							
Asterix 24°C Pba	15	2.1									I	J	K	L	M	N	O	P							
Lady Britta 24°C Ppol	15	2.1									I	J	K	L	M	N	O	P	Q						
Hassel 20°C Pba	15	2.0									I	J	K	L	M	N	O	P	Q	R					
Mandel 20°C Pba	15	1.9										J	K	L	M	N	O	P	Q	R	S				
Rutt 20°C Ppol	15	1.8											K	L	M	N	O	P	Q	R	S	T			
Cerisa 20°C Pba	15	1.8											K	L	M	N	O	P	Q	R	S	T			
Cerisa 20°C Ppol	15	1.7											K	L	M	N	O	P	Q	R	S	T	U		
Rutt 20°C Pba	15	1.6											K	L	M	N	O	P	Q	R	S	T	U	V	
Erika 24°C Pba	15	1.6											K	L	M	N	O	P	Q	R	S	T	U	V	W
Erika 20°C Pba	15	1.5												L	M	N	O	P	Q	R	S	T	U	V	W
Lady Claire 20°C Pba	15	1.3													M	N	O	P	Q	R	S	T	U	V	W
Beate 20°C Pba	15	1.3													M	N	O	P	Q	R	S	T	U	V	W
Fakse 20°C Ppol	15	1.3														N	O	P	Q	R	S	T	U	V	W
Lunarossa 20°C Pba	15	1.2														N	O	P	Q	R	S	T	U	V	W
Folva 20°C Pba	15	1.2														N	O	P	Q	R	S	T	U	V	W
Nansen 20°C Pba	15	1.2														N	O	P	Q	R	S	T	U	V	W
Erika 20°C Ppol	15	1.2														N	O	P	Q	R	S	T	U	V	W
Hassel 20°C Ppol	15	1.2														N	O	P	Q	R	S	T	U	V	W
Nansen 20°C Ppol	15	1.1															O	P	Q	R	S	T	U	V	W
Folva 20°C Ppol	15	1.0																P	Q	R	S	T	U	V	W
Fakse 20°C Pba	15	1.0																P	Q	R	S	T	U	V	W
Lunarossa 20°C Ppol	15	1.0																P	Q	R	S	T	U	V	W
Beate 20°C Ppol	15	0.9																P	Q	R	S	T	U	V	W
Asterix 20°C Ppol	30	0.9																		R	S	T	U	V	W
Asterix 20°C Pba	30	0.8																			S	T	U	V	W
Innovator 24°C Pba	15	0.7																	Q	R	S	T	U	V	W
Lady Britta 20°C Pba	15	0.6																			S	T	U	V	W
Mandel 20°C Ppol	15	0.6																			S	T	U	V	W
Lady Claire 20°C Ppol	15	0.6																			S	T	U	V	W
Innovator 24°C Ppol	15	0.5																				T	U	V	W
Innovator 20°C Pba	15	0.4																					U	V	W
Innovator 20°C Ppol	15	0.3																						V	W
Lady Britta 20°C Ppol	15	0.2																							W

Supplementary table 2. Results for the main effects shown in figure 5 from fitting the corresponding ANOVA models including the described covariates. For each model and source of variation, the degrees of freedom (DF), adjusted sum of squares (Adj. SS), adjusted mean squares (Adj. MS), F-value, and p-value are given. The ANOVA models are from SRP infection prevalence (DP) and soft rot incidence (SR) at planting (1), harvest (2), and after storage (3), as well as from blackleg incidence (B).

Model	Source	DF	Adj. SS	Adj. MS	F-value	*p*-value
DP1	Year	2	9.2680	4.6340	16.32	0.000
	Quality	5	5.5617	1.1123	3.92	0.002
	Cultivar	2	1.1965	0.5982	2.11	0.124
	Soft rot incidence (planting)	1	0.2312	0.2312	0.81	0.368
	Error	214	60.7708	0.2840		
	Total	224	83.5867			
DP2	Year	2	17.0994	8.54971	55.21	0.000
	Quality	5	3.7751	0.75502	4.88	0.000
	Cultivar	2	1.2460	0.62301	4.02	0.019
	Soft rot incidence (planting)	1	0.0088	0.00877	0.06	0.812
	Soft rot incidence (harvest)	1	0.0046	0.00463	0.03	0.863
	SRP infection prevalence (planting)	1	3.7478	3.74778	24.20	0.000
	Error	211	32.6742	0.15485		
	Total	223	88.3698			
DP3	Year	2	2.3247	1.16233	11.57	0.000
	Quality	5	3.0896	0.61792	6.15	0.000
	Cultivar	2	0.1221	0.06104	0.61	0.546
	Soft rot incidence (planting)	1	0.2816	0.28163	2.80	0.096
	Soft rot incidence (harvest)	1	0.0568	0.05682	0.57	0.453
	Soft rot incidence (storage)	1	0.0074	0.00742	0.07	0.786
	SRP infection prevalence (planting)	1	0.0667	0.06673	0.66	0.416
	SRP infection prevalence (harvest)	1	0.3506	0.35063	3.49	0.063
	Error	207	20.8038	0.10050		
	Total	221	42.0761			
SR1	Year	2	0.009288	0.004644	1.23	0.307
	Cultivar	2	0.001857	0.000929	0.25	0.783
	Quality	5	0.015462	0.003092	0.82	0.546
	Avg. SRP infection prevalence (planting)	1	0.005844	0.005844	1.55	0.223
	Error	28	0.105564	0.003770		
	Total	38	0.133636			
SR2	Year	2	2.29529	1.14765	8.28	0.002
	Cultivar	2	0.03714	0.01857	0.13	0.875
	Quality	5	2.20480	0.44096	3.18	0.024
	Blackleg incidence	1	0.09582	0.09582	0.69	0.414
	Avg. soft rot incidence (planting)	1	0.05555	0.05555	0.40	0.533
	Avg. SRP infection prevalence (planting)	1	0.00004	0.00004	0.00	0.986
	Avg. SRP infection prevalence (harvest)	1	0.07367	0.07367	0.53	0.473
	Error	24	3.32775	0.13866		
	Total	37	7.44499			
SR3	Year	2	24.7137	12.3568	11.98	0.000
	Cultivar	2	0.3197	0.1598	0.15	0.857
	Quality	5	24.5135	4.9027	4.75	0.003
	Avg. SRP infection prevalence (planting)	1	1.2674	1.2674	1.23	0.278
	Avg. SRP infection prevalence (harvest)	1	0.2012	0.2012	0.20	0.662
	Avg. SRP infection prevalence (storage)	1	2.1825	2.1825	2.12	0.158
	Error	26	26.8200	1.0315		
	Total	38	55.3153			

B	Year	2	0.7932	0.39660	0.72	0.496
	Cultivar	2	3.4739	1.73697	3.16	0.060
	Quality	5	10.3825	2.07649	3.78	0.012
	Avg. SRP infection prevalence (planting)	1	0.3162	0.31621	0.58	0.455
	Avg. SRP infection prevalence (harvest)	1	0.0147	0.01467	0.03	0.872
	Avg. soft rot incidence (planting)	1	0.6966	0.69657	1.27	0.271
	Avg. soft rot incidence (harvest)	1	0.3797	0.37969	0.69	0.414
	Error	24	13.1867	0.54945		
	Total	37	47.8102			



Paper III

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INVERTEBRATE MICROBIOLOGY

Soft Rot *Enterobacteriaceae* Are Carried by a Large Range of Insect Species in Potato Fields

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ABSTRACT Pathogenic soft rot *Enterobacteriaceae* (SRE) belonging to the genera *Pectobacterium* and *Dickeya* cause diseases in potato and numerous other crops. Seed potatoes are the most important source of infection, but how pathogen-free tubers initially become infected remains an enigma. Since the 1920s, insects have been hypothesized to contribute to SRE transmission. To validate this hypothesis and to map the insect species potentially involved in SRE dispersal, we have analyzed the occurrence of SRE in insects recovered from potato fields over a period of 2 years. Twenty-eight yellow sticky traps were set up in 10 potato fields throughout Norway to attract and trap insects. Total DNA recovered from over 2,000 randomly chosen trapped insects was tested for SRE, using a specific quantitative PCR (qPCR) TaqMan assay, and insects that tested positive were identified by DNA barcoding. Although the occurrence of SRE-carrying insects varied, they were found in all the tested fields. While *Delia* species were dominant among the insects that carried the largest amount of SRE, more than 80 other SRE-carrying insect species were identified, and they had different levels of abundance. Additionally, the occurrence of SRE in three laboratory-reared insect species was analyzed, and this suggested that SRE are natural members of some insect microbiomes, with herbivorous *Delia floralis* carrying more SRE than the cabbage moth (*Plutella xylostella*) and carnivorous green lacewing larvae (*Chrysoperla carnea*). In summary, the high proportion, variety, and ubiquity of insects that carried SRE show the need to address this source of the pathogens to reduce the initial infection of seed material.

IMPORTANCE Soft rot *Enterobacteriaceae* are among the most important pathogens of a wide range of vegetables and fruits. The bacteria cause severe rots in the field and in storage, leading to considerable harvest losses. In potato, efforts to understand how soft rot bacteria infect and spread between healthy plants have been made for over a century. Early on, fly larvae were implicated in the transmission of these bacteria. This work aimed at investigating the occurrence of soft rot bacteria in insects present in potato fields and at identifying the species of these insects to better understand the potential of this suspected source of transmission. In all tested potato fields, a large proportion of insects were found to carry soft rot bacteria. This suggests a need to give more weight to the role of insects in soft rot ecology and epidemiology to design more effective pest management strategies that integrate this factor.

KEYWORDS plant pathology, soft rot, insect vectors, potato

Soft rot *Enterobacteriaceae* (SRE) are pathogenic organisms of the genera *Pectobacterium* and *Dickeya* that cause soft rots in plant species from 50% of angiosperm plant orders, including a wide variety of economically important crops, such as potatoes, tomatoes, onions, peppers, and cabbage (1). In potatoes, SRE cause soft rot in both tubers and stems. The bacteria enter potato tubers through lenticels and through

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fresh wounds on the tubers, roots, and above-ground parts of the plant (2). Blackleg symptoms follow soft rot in an infected seed tuber piece and a subsequent spreading of the pathogen through the vascular system (3).

The SRE species responsible for the most significant pre- and postharvest losses in potato are *Pectobacterium atrosepticum*, *Pectobacterium carotovorum* subsp. *brasiliensis*, *Pectobacterium carotovorum* subsp. *carotovorum*, *Pectobacterium parmentieri*, *Dickeya dianthicola*, and *Dickeya solani* (4–6). Some isolates of *Pectobacterium carotovorum* subsp. *carotovorum* were recently reclassified into the new species *Pectobacterium polaris* (7).

Infected seed tubers are considered to be the most important source of bacteria, and mechanical handling during planting and harvest contributes substantially to the spread among tubers (4, 8). The production of seed potatoes is initiated with minitubers originating from *in vitro* plant cultures that are free of SRE when planted in the field. These are multiplied in the field for economic reasons, and during multiplication, a steady increase in SRE levels can be observed in each field generation (4). The mechanisms of the initial infection of clean source material, such as tissue culture clones or stem cuttings, remain unexplained, although aerosols and insects have long been considered possible sources of SRE transmission (9, 10).

Insects function as alternative hosts and vectors of numerous phytopathogens, and various transmission systems have been identified (11). Such findings were used to develop and implement more efficient prevention strategies by targeting the insect vector instead of the pathogen, as exemplified in the control of various plant viruses through a decrease in their vectors (12).

Previous research on insects as vectors of SRE in potato has largely focused on *Delia platura* and *Drosophila melanogaster*. In the early 20th century, it was first observed that *D. platura* (seedcorn maggot) laid eggs near tubers shortly after planting. It was hypothesized that the larvae frequently transmitted SRE to the tubers by boring into them (13). Later, it was shown that artificially inoculated *D. platura* adults transmitted *P. carotovorum* subsp. *carotovorum* to healthy potato plants in a cage experiment (14). Similarly, *Delia radicum* (cabbage root fly) and *Delia antiqua* (onion fly) were shown to transmit SRE to their respective host plants (15–17). Furthermore, it was demonstrated that SRE could be transmitted from infected to healthy potato plants by *D. melanogaster* (18) and that some strains were able to survive in *Drosophila* spp. for at least 72 h (19).

In addition to the detailed work done on *Delia* and *Drosophila* species, studies of other insects potentially involved in SRE transmission were previously attempted around two potato waste dumps (9) and one field site for the propagation of clean seed material (20) in Scotland. However, since these studies were conducted in the 1970s, the detection of SRE required enrichment on artificial medium, and insect identification relied on morphological taxonomy. Despite strong indications of insects as a source of initial SRE infection in the field that was tested, the efforts were only moderately conclusive, since the isolation of bacteria was partially done from bulked insects and some that were not identified beyond their taxonomical order. The morphological identification of the few insect specimens found to carry SRE in those studies showed, among others, *Leptocera* spp., *Scaptomyza* spp., *Scatopse* spp., *Delia* spp., *Drosophila* spp., and unidentified Diptera (true fly) specimens (9, 20).

Phytopathogens that are transmitted by insect vectors can be described as having various degrees of vector specificity (21). Vector specificity is considered high if the phytopathogen is transmitted by one or a few insect species, as is the case for *Pantoea stewartii* and its vector, the corn flea beetle (11). Conversely, if a phytopathogen is transmitted by many different insect species, it has a low vector specificity; *Erwinia amylovora*, for example, is transmitted by a broad range of pollinator species (11) and has been detected in various insect pests (22). Research on insect vectors of SRE has not yielded sufficient data to address vector specificity.

The potential contribution of insects to initial SRE infection of potato and other crops, as well as to the dissemination within and between fields, remains unclear. The objective of this study, therefore, was to examine the presence of SRE in insects in

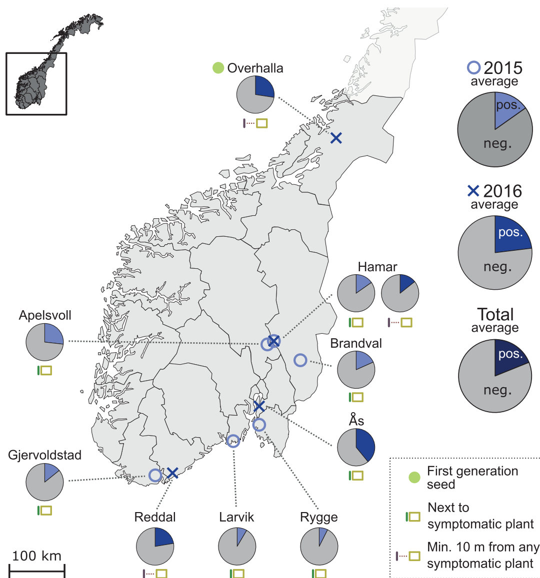

FIG 1 Map of Norway with indicators for all field locations in 2015 (O) and 2016 (×) and associated proportions of samples that tested positive (pos.; blue) and negative (neg.; gray) for SRE using the PEC assay. Names of the field locations are given above each pie chart. Overall proportions for 2015 (top), 2016 (center), and in total (bottom) are given on the right. Distances of the traps from any symptomatic plants are indicated under each pie chart. Fields with traps set up in a minimum (Min.) distance of 10 m from any symptomatic plant did not necessarily contain symptomatic plants. Further details are given in Table S1. (Map templates are from Geonorge.)

multiple potato fields by molecular methods suited for a sufficiently sensitive and efficient detection of pathogens from individual insects and more accurate identification of insect species. The test sites included fields where plants were symptomatic and one location where seed material was propagated from clean tubers generated from tissue culture. The field where clean seed tubers were grown was particularly informative in our examination of the potential of insect-borne SRE to contribute to initial infection. The chosen scope was intended to reveal new potential insect vectors that were not found in previous work and give an indication of the overall distribution of SRE over various insect species. Showing this distribution is a first step in the identification of possible vector candidates for SRE, thereby allowing for appropriate control measures to be developed.

RESULTS

A substantial proportion of insect samples contains SRE. To assess the potential of insects to present a viable inoculum for SRE transmission, individual insects sampled from potato fields in Norway using sticky traps (see Fig. S1 in the supplemental material) were examined for two consecutive years (Fig. 1).

The presence of SRE was tested by using a quantitative PCR (qPCR) assay targeting all *Dickeya* and *Pectobacterium* species (23). The threshold for a positive test (quantification cycle [C_q] < 28) was chosen conservatively to only include insects with a high load of SRE and corresponded to between 10,000 and 100,000 CFU, as determined by a dilution series experiment (Fig. S2). SRE were isolated from an insect that tested positive and caused soft rot symptoms when inoculated in SRE-free minitubers (Fig. S3).

Insects from all traps in all fields contained large amounts of SRE, with percentages ranging from 4% to 39% of insects from a given trap (Table S1). Out of 2,122 tested insects in total, 19% were positive for SRE with the chosen threshold. The overall

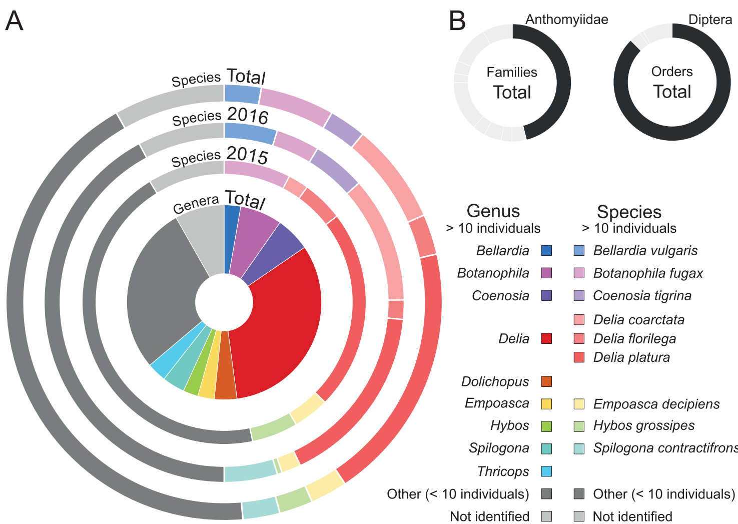

FIG 2 Identification, classification, and proportions of insect specimens that tested positive for SRE. (A) Genera of insect specimens that tested positive over both years (inner circle), as well as species that tested positive in 2015 (second circle), 2016 (third circle), and over both years (outer circle). Only taxa with more than 10 representatives over both years are shown, with the rest represented as "other." (B) Insect families and orders for specimens that tested positive over both years with the most prevalent family (Anthomyiidae) and order (Diptera) are highlighted in black, and others are in light gray.

percentage of insects that tested positive varied between 15% in 2015 and 23% in 2016 (Fig. 1).

A diverse group of insect species carries SRE. The insect specimens that tested positive for SRE in the qPCR assay were regarded as potential vectors due to the large amount of bacteria they contained. Species identification of the insects by DNA barcoding was successfully performed for 367 of the 401 SRE-positive insect samples (Fig. 2A). The identified specimens belonged to at least 91 different insect species, with 95% of the identified species belonging to the order Diptera (Table S2).

The families most commonly found to carry a large amount of SRE were *Anthomyiidae*, in 46% of the identified samples, and *Muscidae*, in 14% of the identified samples (Table S2). *Delia* was the dominant genus among the samples that tested positive, with 36% in 2015, 30% in 2016, and 32% in total (Fig. 2A). The most prominent species was *D. platura*, making up 19% of all positive samples. The positive specimens collected from traps in the northernmost field (Overhalla) were dominated by *Delia coarctata*, with only one individual being identified as *D. platura*, whereas *D. coarctata* only tested positive in the other locations sporadically (Table S2). In addition to Diptera, a number of Hemiptera (true bugs), mainly leafhoppers of the species *Empoasca decipiens*, tested positive in both years.

Although the proportions of the identified species were mostly stable across years, some species varied in abundance (Fig. 2A). This likely resulted from single species, with many individuals that tested positive occurring in one of the examined locations exclusively (Table S2).

Dissemination of SRE by insects within and between fields. The SRE species *D. solani* was identified in three individual insects from one field in 2015 and isolated from a symptomatic plant in the same field that year (Table 1). The finding of *D. solani* was unexpected, since it is an invasive species that was previously detected only once in Norway, in a quarantine field with imported seed material in 2012 (24). Since then, all certified seed potato lots are tested for *D. solani*, and there have been no detections in these.

TABLE 1 SRE species detected in symptomatic plants and insects from potato fields[a]

Field by yr	Source	No. of positive organisms or presence/absence of organisms detected					PEC C_q < 28[b]
		Dickeya solani	*Pectobacterium atrosepticum*	*Pectobacterium carotovorum* subsp. *brasiliensis*	*Pectobacterium carotovorum* subsp. *carotovorum*	*Pectobacterium parmentieri*	
2015							
Apelsvoll	Insects	3	6	0	NA	2	38
	Potato plants	+	+	−	−	−	
Brandval	Insects	0	6	0	NA	2	26
	Potato plants	−	+	−	−	−	
Gjervoldstad	Insects	0	24	0	NA	2	45
	Potato plants	−	+	−	−	−	
Hamar	Insects	0	11	0	NA	1	43
	Potato plants	−	+	−	+	−	
Larvik	Insects	0	0	0	NA	1	8
	Potato plants	−	+	−	−	−	
Rygge	Insects	0	1	0	NA	0	11
	Potato plants	−	+	−	+	−	
2016							
Hamar	Insects	0	0	0	NA	5	40
Overhalla	Insects	0	13	0	NA	34	103
Reddal	Insects	0	27	0	NA	10	62
Ås	Insects	0	0	0	NA	3	25

[a]Identification of SRE was done by using species-specific TaqMan assays on insects that tested positive in the PEC assay, or by FAME analysis of isolates from blackleg lesions of potato plants adjacent to traps. The numbers refer to insect specimens that tested positive for each of the species-specific TaqMan assays, for each field. The SRE species that were isolated from the symptomatic potato plants adjacent to the traps are indicated as "found" (+) and "not found" (−) based on the FAME identification. The insect data for *Pectobacterium carotovorum* subsp. *carotovorum* are not available (NA) since there was no specific TaqMan assay for it. In the 2016 fields, symptomatic potato plants were not tested.

[b]The number of insects that tested positive for SRE with the general PEC TaqMan assay in each field. Out of 401 positive insects, 10 were not tested with species-specific TaqMan assays due to a limited amount of DNA.

Insects trapped from a field dedicated to the propagation of germfree minitubers in Overhalla tested positive for SRE, with a relatively high percentage compared to the other fields where traps were tested (Fig. 1). A source of the bacteria within those fields was very unlikely due to the quality of the seed tubers.

Generally, increasing the distance of the traps from plants with blackleg did not lead to a lower percentage of insects that tested positive. Insects collected from a minimum distance of 10 m from a plant with blackleg tested positive in 22% of the samples. For insects trapped in immediate proximity of symptomatic plants, 16% samples tested positive (Table S1).

Abundance of insect species carrying SRE shows two extremes. While our data indicate a large variety of insect species to be capable of carrying large amounts of SRE, the number of individuals that tested positive differed widely between species. Two extremes were observed in the identified species, an abundance of species with few individuals that tested positive, versus few species with a large number of individuals that tested positive (Fig. 3). As the most extreme in the second group, *D. platura* alone represents one-fifth of all identified individuals. Together with nine other species, *D. platura* makes up more than 50% of the individuals shown to carry a high number of SRE. The remaining individuals belong to at least 79 different species, with eight or fewer individuals observed over both years. For 50 species, only one individual tested positive over both years (Fig. 3 and Table S2).

Laboratory-reared *Delia floralis* contains large amounts of SRE. To investigate further the relationship between SRE and *Delia* spp., we tested individuals from two generations of a long-term laboratory rearing of *D. floralis* (turnip root fly). Of these, 66% of 94 individuals tested positive for SRE using the rather conservative C_q level of 28 (Fig. 4). For comparison, we tested two other laboratory-reared insect species, *Plutella xylostella* (cabbage moth), because of its similar rearing conditions, and carnivorous *Chrysoperla carnea* (common green lacewing) larvae. The number of specimens positive for SRE was significantly higher in *D. floralis* samples than in the other tested

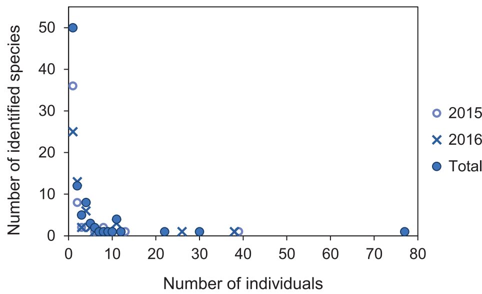

FIG 3 The relationship between the number of insect species and number of individuals for a given species. Number of individuals (*x* axis) refers to the number of instances of a species being identified, while number of identified species (*y* axis) refers to the number of instances where one species was identified with the corresponding amount of individuals (*x* axis). Samples for 2015 (O), 2016 (×), and in total (filled circles) are shown.

species, as well as in the samples trapped in the fields (Fig. 4). For *P. xylostella*, 13% of 94 of the specimens tested positive for SRE, and for the *C. carnea* larvae, only one out of 40 specimens tested positive for SRE. Furthermore, the average amount of SRE was significantly larger in *D. floralis* specimens than in *P. xylostella* and *C. carnea* specimens. Interestingly, adult individuals of both *C. carnea* and the closely related *Chrysoperla lucasina* (one each) that feed on pollen and nectar tested positive in the wild trapped samples (Table S2).

DISCUSSION

SRE have a broad host plant spectrum and can be found in rotting lesions of wild and cultivated plants (1), which might attract a variety of insects for egg deposition or feeding. It is therefore reasonable to assume that a number of different insect species encounter SRE in various amounts, depending on their behavior. The results shown in Fig. 2 support this assumption. The results suggest that Diptera are more likely to acquire or have SRE as members of their microbiome than other insects. However, the bias toward Diptera might be inherent to the sampling method in terms of the color of

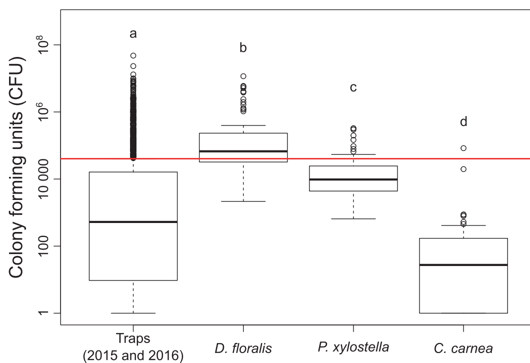

FIG 4 Number of SRE in insect samples from traps and from laboratory rearings, with median (black line) and distribution of all samples. The CFU were calculated using a linear approximation for the relationship between C_q and CFU values from the dilution series data (Fig. S2); samples from 1:10^3 to 1:10^6 were used to create the linear approximation. For *Delia floralis*, 94 samples of adult flies from two consecutive generations were tested (47 each); for *Plutella xylostella*, 94 samples of adult moths were tested; and for *Chrysoperla carnea*, 40 samples of larvae were tested. The red line indicates the calculated CFU corresponding to the C_q 28 threshold used in the field samples. Letters a to d indicate significantly different groups of samples according to a Mann-Whitney test ($P < 0.05$); all combinations were tested for both C_q and calculated CFU values.

the sticky traps (yellow) and that they were mounted above ground, excluding ground-dwelling insect species.

More than half of the identified insect species that tested positive for SRE were represented by only one individual in both years (Fig. 3). Since only individuals that tested positive were identified, it is not possible to infer the proportion of individuals of a given species that were carrying SRE. Contamination of some individuals on the traps by aerosols or cross-contamination from other insects that carried many SRE cannot be excluded. However, aerosol contamination would in principle be expected to be higher for samples taken close to symptomatic plants, but the proportions of positive samples were comparable (Table S1). Cross-contamination was assumed to be negligible, since the sampled insects rarely were in contact with each other on the traps. Likely explanations for species testing positive in few individuals could be that these species were either not abundantly present at the time and location of the experiment, not trapped, not tested, or simply are not commonly associated with SRE. If they were not commonly associated, this would suggest that at least some of the species that tested positive and were identified might not be dedicated vectors for SRE. However, some degree of stochastic transmission from these individuals is conceivable, given a sufficient presence of insects carrying large amounts of the pathogen. SRE have been shown to cause systemic infections upon inoculation in wounded tubers, stems, and leaves of potato under suitable conditions (25). This suggests a potential mechanism for stochastic transmission of SRE by various insects that visit and cause plant wounds. In such cases, SRE could be applied to and transferred between wounds by insects that retain the bacteria on their surface or mouthparts. Alternatively, SRE could be introduced during wounding by insects that carry SRE internally for a short period.

D. platura stood out as the Diptera species that carried SRE most frequently in both years of the study. In addition, six other *Delia* species were frequent carriers of SRE, which supports earlier work done on the relationship between SRE and various *Delia* species (13, 16, 17). The ecology of *Delia* species explains the acquisition of SRE at the larval stage, either from rotten plant tissue or by vertical transmission from the mother via the egg surface (13). SRE infection of plants through *Delia* spp. has been shown from the larvae to the seed material of their host (13, 16, 17), as well as from adult flies to wounded petioles and leaves of potato plants (14). In addition to the transmission, long-term survival of SRE in the pupae of *D. platura* that overwinter buried in the soil (26) offers a favorable means for the bacteria to survive the winter in spite of prolonged freezing periods in temperate climates. Normally, SRE survive poorly in the environment in a temperate climate (27).

In addition to Diptera, some specimens of the hemipteran leafhopper *E. decipiens* tested positive for SRE. *E. decipiens* has been shown to transmit "*Candidatus* Phytoplasma asteris" to daisies by feeding on leaves (28) and has previously been described as a potato pest (29). Plant pests, like *E. decipiens*, are likely vector candidates, since leafhoppers actively damage the plant tissue by their stylet-like mouthparts that they use for sucking plant sap (30), thereby creating suitable conditions for SRE infection (25). Dedicated efforts to show the transmission of SRE to potato or other plants by the different insect species that were identified here are needed to show how effectively they function as vectors for SRE.

A general function of SRE in herbivorous insect species might explain the presence of SRE in so many insects, as SRE are notorious producers of a variety of plant cell wall-degrading enzymes (PCWDEs) that are secreted to the extracellular environment (31). The notion of SRE as a functional component of the insect microbiome for the digestion of plant material is supported by the presence of SRE in the tested *D. floralis* laboratory rearing and a smaller, yet persistent, amount of SRE in most of the reared *P. xylostella* moths. However, the overall ratio of wild samples showing a low or no signal in the qPCR assay contradicts this assumption. In two general microbiome studies in Diptera from other ecological contexts, it was recently reported that some individuals carried large amounts of SRE, while other individuals of the same species with an otherwise comparable microbiome did not (32, 33).

The results from the field traps (Fig. 2), in combination with the findings from the laboratory-reared insects (Fig. 4), support the notion of a mutualistic relationship between multiple *Delia* species and SRE, as hypothesized in early work on *Delia* spp. (13, 16, 17). There, it was suggested by experiments with sterilized eggs that *D. platura* larvae needed SRE to survive and to develop normally under laboratory conditions. The relationship was therefore suggested to be specific or even symbiotic (13). In the case of the tested laboratory rearing of *D. floralis*, the last introduction of wild individuals to this was 5 years before testing. The results from the *D. floralis* samples therefore support the assumption that SRE are natural members of the microbiome of *Delia* species and are significantly more prevalent than in *P. xylostella* and *C. carnea*. Thus, it is likely that SRE and *Delia* species mutually add to their respective potentials to cause damage in their hosts. The relationships between SRE and specific insect groups might have various mutualistic facets. It was recently shown that a *Pectobacterium* sp. strain present in the *Delia radicum* gut microbiome was able to break down plant components that are toxic to the insect (34).

The detection of *D. solani* in insect samples from the Apelsvoll field (Table 1) suggests that some of the detected SRE originated from symptomatic plants in the tested fields. Sources of *D. solani* outside the field are highly unlikely, since this SRE species was not detected anywhere else in Norway that year, despite regular screening for it. Insects that take up large amounts of SRE from within the field might contribute to transmission between plants. However, being in vicinity to a symptomatic plant did not increase the proportion of positive samples in the traps in 2015 compared to 2016. More importantly, the traps set up in a field dedicated to the propagation of germfree minitubers contained a relatively high proportion of insects that tested positive compared to the other fields that year (Fig. 1, Overhalla). This suggests that there are inoculum sources outside the field or that SRE are part of the natural microbiome of some insects. The high proportion of individual specimens that tested positive at this field site shows the potential of insect-borne SRE to contribute to the initial infection of SRE-free plant material. Due to the high variety of identified insect species, this suggests a need to explore cultivation methods that minimize contact of the plants with insects to reduce initial SRE infection in seed production. Additionally, the detection of *Dickeya solani* in three insects suggests that insect trapping and bulk testing might be a tool that could be employed to monitor such quarantine pathogens that are commonly associated with insects.

Based on the results from this study, it is proposed that the SRE transmission in potato fields and other affected ecosystems is facilitated by a diverse range of potential vectors (Fig. 3). It seems that SRE have neither a low nor high vector specificity but rather represent a hybrid case, where the two mechanisms are acting simultaneously. While *D. platura* appeared to have the highest vector potential, other (*Delia*) species might be dominant depending on host plant prevalence, climatic conditions, and other factors impacting insect species composition. The background level of species carrying SRE with low or no vector potential, due to less likely acquisition and transmission scenarios, is suggested to be ubiquitous, as per the data shown in this work (Fig. 3). To test this, samples from different cultured and wild ecosystems under various climatic conditions need to be analyzed. Previous work suggests, for example, that *Drosophila* species act as a vector in other ecological contexts (18, 19), while they are nearly absent based on the data from this study (Table S2).

The work presented here suggests that the insect-borne SRE present in potato fields are more ubiquitous and heterogeneous than previously assumed. The results showed that at least 91 distinct insect species carried SRE in potato fields, including fields in which germfree tubers from tissue culture were propagated. This points to the potentially important role of a wide variety of insects in the ecology of SRE and may have implications for the initial infection of clean seed material and the currently employed control strategies for soft rot pathogens.

MATERIALS AND METHODS

Insect collection. Insects were collected using two to four yellow sticky traps in each of nine potato fields across the main potato-growing districts of Norway for two consecutive years (Fig. 1). In 2015, traps were set up next to potato plants with blackleg symptoms in six different fields for 6 to 10 days in summer (July to August). In 2016, three fields were sampled, including one field dedicated to the generation of prebasic 2 (P2; Norwegian seed certification) seeds from minitubers. That year, the traps were set up in a minimal distance of 10 m from any plant showing blackleg symptoms. Upon arrival at the Norwegian Institute of Bioeconomy Research, the traps were stored at −20°C.

One additional yellow sticky trap was set up adjacent to plants artificially inoculated with *Pectobacterium atrosepticum*, in Ås, Norway. Fully grown plants were inoculated by piercing the stem with a sterilized toothpick that was scraped over a bacterial lawn grown on LB agar plates (per liter, 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 15 g agar). After lesions had developed at 10 days postinoculation, the trap was set out for 7 days in the beginning of August. From this trap, 64 insects were cut in half immediately after collection from the trap. One-half was used for qPCR testing with the PEC primer/probe set, while the other half was stored in 25% glycerol at −20°C before plating the bacteria.

DNA isolation. Insects were picked from the traps individually using xylene substitute (Sigma-Aldrich) to dissolve the glue of the traps (2015) or by careful removal without dissolving the glue (2016). While it was attempted to pick insects randomly off the traps, individuals were always included if the species appeared to occur ≤3 times on a trap and was distinguished by a marked phenotype. The number of tested insects per trap varied with the number of insects present on a given trap. The total DNA from each picked insect was isolated using the protocol recommended by the Canadian Centre for DNA Barcoding (35). The isolated DNA (50 μl per sample) was stored at −20°C.

qPCR for SRE detection. All individual insect samples were tested for the presence and quantity of SRE DNA using the PEC TaqMan assay, which amplifies a 119-bp sequence from SRE strains with high specificity (23). The reactions were conducted using 2 μl of DNA in a 20-μl reaction volume of SsoAdvanced universal probes supermix (Bio-Rad), in a CFX96 Touch real-time PCR detection system (Bio-Rad), with 3 min of initial denaturation at 95°C, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Samples containing a large amount of SRE DNA (threshold set at $C_q < 28$) as determined by this qPCR analysis were used for species identification.

To find the relationship between the C_q signal and the number of CFU of SRE, a dilution series of *Pectobacterium polaris* strain NIBIO1006 (7) was tested. Three dilution series were produced from 3 × 1 ml of an overnight (o/n) culture of *P. polaris*, each grown from a single colony in LB broth at 28°C. Aliquots of undiluted culture and six 10-fold dilution steps until a 1:1,000,000 dilution for each of the three series were plated on LB medium. The colonies were counted after 48 h at room temperature for the two highest dilutions. The remaining dilutions were pelleted at 6,000 × *g* for 10 min and resuspended in vertebrate lysis buffer (35), and the DNA was isolated as described for the insect samples (see above). The three dilution series were tested with the PEC primer/probe set, each in three qPCR replicates. According to these tests, the threshold C_q of 28 in the PEC assay corresponds to approximately 80,000 CFU of *P. polaris* for the protocol used in the insect experiments (Fig. S2).

Species identification of SRE. Specific TaqMan assays for *Dickeya solani*, *Pectobacterium atrosepticum*, *Pectobacterium parmentieri*, and *Pectobacterium carotovorum* subsp. *brasiliensis* were used to determine the species of the SRE present (Table 1). Due to large heterogeneity within *Pectobacterium carotovorum* subsp. *carotovorum*, no species-specific test is available. For the potato samples, SRE were isolated from the blackleg lesions of plants next to the traps collected in 2015. Eighteen diseased potato stems from six different fields with blackleg or stem rot symptoms were washed thoroughly under running water. Small pieces of tissue were then excised close to the border between healthy and diseased tissue and soaked in 0.5 ml sterile phosphate-buffered saline (PBS) for 30 min. The resulting extracts were streaked on modified Bulmer crystal violent pectate (MBCVP) plates (36) and incubated at two temperatures (room temperature and 37°C). After 48 h, bacterial colonies were picked from cavities indicating pectolytic activity and transferred to nutrient glucose agar plates (NGA; 23 g nutrient agar [Difco, USA], 5 g yeast extract, 10 g glucose, 1,000 ml distilled water) for growth at 25°C. All pectolytic isolates were initially identified by fatty acid methyl ester (FAME) analysis (37), and most of them were identified as either *Pectobacterium carotovorum* subsp. *carotovorum* or *Pectobacterium atrosepticum*. One isolate, identified by FAME as *Dickeya chrysanthemi* biovar V, was further analyzed with qPCR and species-specific primer/probe sets and was determined to be *Dickeya solani*.

All insect samples that were tested with the PEC assay and identified by sequencing were also tested with species-specific primer/probe sets for *D. solani*, *P. atrosepticum*, *P. carotovorum* subsp. *brasiliensis*, and *P. parmentieri* (Table 2). Real-time PCR was performed as described for the PEC assay, except for the assay for *P. carotovorum* subsp. *brasiliensis*, where the primer concentrations were adjusted as described in the original publication (38).

Sequencing of selected insect samples. DNA barcoding was done by PCR amplification of the mitochondrial cytochrome *c* oxidase subunit I (COI) from selected insect samples using the LCO1490/HCO2198 primer set (39). The PCR amplification was done as follows: 94°C denaturation (3 min), followed by 5 cycles of 94°C (30 s), 45°C (30 s), and 72°C (1 min), followed by 35 cycles of 94°C (30 s), 54°C (30 s), and 72°C (1 min), and a final elongation at 72°C for 10 min. The protocol was modified from the 2-step protocol for insect DNA barcoding (40). In a total reaction volume of 25 μl, 3 μl of 1:100-diluted DNA isolated from the insect samples was added. The COI amplicon was Sanger sequenced in both directions (GATC Biotech, Germany). The obtained sequences were trimmed in the 3′ and 5′ regions and the forward and reverse sequences assembled into a consensus. The consensus sequences were used for

TABLE 2 Primers and TaqMan probes used throughout this work

Target	Name	Primer or probe sequence (5′–3′)			Reference
		Forward primer	Reverse primer	TaqMan probe	
All SRE	PEC	GTGCAAGCGTTAATCGGAATG	CTCTACAAGACTCTAGCCTGTCA GTTTT	CTGGGCGTAAAGCGCACGCA	23
Dickeya solani	SOL-C	GCCTACACCATCAGGGCTAT	ACACTACAGCGCGCATAAAC	CCAGGCCGTGCTCGAAATCC	23
Pectobacterium atrosepticum	ECA	CGGCATCATAAAAACACGCC	CCTGTGTAATATCCGAAAGGTGG	ACATTCAGGCTGATATTCCCC CTGCC	23
Pectobacterium parmentieri	Pw	TCTGTTCAATGTCAACGCAGGTA	AGGTAACCGCAATTTGCTCAA	TGTGCGCAACCTG	38
Pectobacterium carotovorum subsp. *brasiliensis*	Pcbr	TGCGGGTTCTGCGTTTC	TGGCGCGTTCGCAATAT	CAAGGCACGATACG	38
Insect COI barcode region	COI Folmer	GGTCAACAAATCATAAAGATA TTGG	TAAACTTCAGGGTGACCAAAA AATCA		39

species identification in the BOLD online interface for COI barcode identification with the Species Level Barcode Records database (41).

Accession number(s). All identified nucleotide sequences were deposited in the GenBank database under the accession numbers MG673557 to MG673923.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00281-18.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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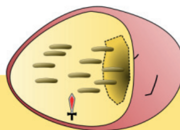

Paper IV

Transcriptome analysis of two *Pectobacterium* species shows initial spike and successive downregulation of secretion systems and virulence factors during soft rot progression in potato

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Abstract

Soft rot and blackleg caused by various *Pectobacterium* species are among the most important diseases of potato. The rot symptoms in potato and other host plants are primarily caused by a battery of plant cell wall degrading enzymes that the bacteria secrete during the infection process. In addition, several different secretion systems are mobilised during infection. While quite a lot is known about the virulence onset and its regulation by various molecular networks, information about the processes in the later stages of infection is scarce. Moreover, the *Pectobacterium* genus is heterogeneous and strains belonging to the various species can differ significantly from each other in their aggressiveness, and virulence properties. In this work, the transcriptomes of two aggressive strains of the two species *P. carotovorum* subsp. *carotovorum* and *P. polaris* were investigated during infection of potato tubers at 24, 48 and 72 hours after inoculation (hpi) by RNA sequencing. The transcriptomes were compared to that of bacteria grown *in vitro* on minimal medium, and transcriptomes from later infection time points (48 and 72 hpi) were compared to early infection (24 hpi). It was shown that the overall expression of plant cell wall degrading enzymes and secretion system associated genes were upregulated in infection compared to *in vitro* growth, but downregulated in the later phases of infection compared to the early infection phase. This downregulation could not be sufficiently explained by the expression of known virulence regulators such as the RsmA/B or the ExpA/S systems. The observed expression shift likely reflects a shift in the biological processes in the bacterial community isolated from infected tissue over time.

Keywords: *Pectobacterium*, transcriptome, RNA-seq, potato, virulence, secretion systems

Introduction

With a broad host range, large phylogenetic diversity and a barrage of weaponized enzymes, species of the genus *Pectobacterium* are among the most important plant pathogenic bacteria. Together with closely related species of the genus *Dickeya*, *Pectobacterium* spp. cause soft rot and blackleg in potato, inflicting great economical damage in fields and storage worldwide (Czajkowski et al., 2011). *Pectobacterium* species inflict rots on species from more than 30% of angiosperm orders, including major crops like sugar beet, potato, pepper, onion, carrot, and various *Brassica* species (Charkowski, 2018; Ma et al., 2007). They produce several different pectinases, cellulases, proteases, and other enzymes that facilitate the efficient breakdown of plant cell walls (Glasner et al., 2008). The export of pectinases and other plant cell wall degrading enzymes (PCWDEs) is facilitated via the type II secretion system (T2SS) (Charkowski et al., 2012; Coulthurst et al., 2008; Pineau et al., 2014). The often indistinguishable symptoms caused by different *Pectobacterium* species may obfuscate their large phylogenetic variety. *P. carotovorum* is the most phylogenetically diverse species and currently comprises four subspecies (Nabhan et al., 2012; Zhang et al., 2016). In addition to *P. carotovorum*, nine distinct species have been identified so far, five of which have only been recognized within the last five years (Dees et al., 2017b; Khayi et al., 2016; Nabhan et al., 2013; Sarfraz et al., 2018; Waleron et al., 2018).

Recent efforts determined the virulence potential of more than 80 *Pectobacterium* and *Dickeya* strains isolated from potato tubers in Norway and Poland, and found large differences in the tuber maceration ability, motility, and cellulase activity of those strains (Dees et al., 2017a). A majority of the isolated strains were identified as *Pectobacterium* sp., and a phylogenetically diverse collection of *P. carotovorum* subsp. *carotovorum* strains displayed the highest tissue maceration ability and cellulase activity. *P. polaris* was recently identified as a new species after genome sequencing, which was performed due to its high aggressiveness (Dees et al., 2017b). Before establishing their taxonomic distinctiveness, isolates of this species were classified as *P.c.* subsp. *carotovorum.*

Previous studies of single genes and transcriptomes have uncovered various molecular components of *Pectobacterium* virulence onset. The current model of the molecular network underlying *Pectobacterium* virulence is summarized in Charkowski et al. (2012). A key control mechanism is the RsmAB system, in which the small RNA binding protein RsmA is the central repressor of virulence and motility functions. Virulence onset is partly explained through deactivation of RsmA repression by binding of the noncoding *rsmB* transcript to the protein. By decreasing the amount of free RsmA, the translation of PCWDEs, motility regulators, and the type 3 Secretion System (T3SS) master regulator, alternative sigma factor HrpL, is increased. This release of RsmA repression marks the virulence onset and is, among other factors, governed by the detection of organic acids in the bacterial periphery (Charkowski et al., 2012). HexA, a transcriptional regulator, interacts with the RsmAB system by supressing *rsmB* expression via the two-component system GacS/A, and regulates PCWDE production, while it in turn is regulated by the motility regulator FlhDC (Cui et al., 2008; Harris et al., 1998). A knockout mutant of *rsmB* is non-virulent, presumably due to a lack of RsmA deactivation, however, virulence is restored by also knocking out a metabolic regulatory gene (*metJ*), which encodes a repressor of the methionine biosynthesis regulon (Cubitt et al., 2012). A third layer of regulation is the repressor KdgR, which represses the expression of PCWDEs and *rsmB* in

the absence of pectin degradation products (Y. Liu et al., 1999). While this links KdgR to the RsmAB system, it is notable that KdgR expression is modulated via the gluconate sensor operon *vguABCD* in potato leaf infection (Mole et al., 2010). The knockout of the *vgu*-operon also led to hypervirulence in potato tubers, suggesting a role of the glucose metabolite gluconate in moderating virulence.

Furthermore, a microarray transcriptome analysis of *P. atrosepticum* revealed a central role for quorum sensing (QS) in the switch from latency to virulence (H. Liu et al., 2008). This work also showed that a knockout strain, incapable of synthesizing the QS elicitor, had lowered expression of genes encoding T3SS and Type VI secretion system (T6SS) components. The T6SS was shown to be expressed during *Pectobacterium* virulence in another study, but its role is not understood (Mattinen et al., 2008). The T3SS and its secreted proteins DspE and HrpN were previously described to be necessary for *P. atrosepticum* to cause stem lesions in potato (Holeva et al., 2004). While no function has been described for the T3SS in tuber rot, DspE was shown to elicit cell death in potato leaves and was hypothesized to expand the tissue range for successful *Pectobacterium* infection (Hogan et al., 2013). Only some *Pectobacterium* spp. strains capable of macerating potato tuber tissue encode a functional T3SS, suggesting that it may not serve a general function in virulence (Kim et al., 2009).

In this work the transcriptome of two *Pectobacterium* species was analysed during tuber soft rot using RNA-seq. The goal of this work was to elucidate the molecular mechanisms involved in tuber soft rot onset and progression for two aggressive strains of *Pectobacterium*.

Material and Methods

Bacterial cultures

P. carotovorum subsp. *carotovorum* strain PK1045-1-2-13 (Pcc) and *Pectobacterium polaris* strain NIBIO1006 (Ppol), were used (Dees et al., 2017a; Dees et al., 2017b). Before the experiments, bacteria were grown on LB plates overnight at room temperature. Bacterial suspensions for potato infection and plating on M9 minimal medium were created by resuspending bacteria in phosphate-buffered saline (PBS), and adjusting the suspensions to an optical density of 0.8 ± 0.03 at 600 nm. As a non-infection control, 70 µL were plated on each of nine M9 minimal medium agar plates and incubated at room temperature next to the infected potatoes. M9 minimal medium and LB plates were prepared according to standard protocols (CSH protocols, 2009, 2010).

Infection of potatoes

First generation seed potato tubers (*Solanum tuberosum*), so called minitubers, of the cultivar Asterix were used for infection. Minitubers can be assumed to be free from pathogens, since they are multiplied from tissue culture generated microtubers using hydroponics. Per strain, nine tubers were inoculated by piercing them with a pipette tip and injecting 30 µL of bacterial suspension. The pipette tip remained in the wound to avoid healing of the tissue. Each tuber was injected at two sites to obtain sufficient amounts of infected material. Three tubers and M9 plates were inoculated with PBS as a negative control. The infected tubers were incubated in boxes lined with moist filter paper at room temperature (approx. 21 °C). At 24, 48 and 72 hours post inoculation (hpi), the infected, soft rotten tissue was removed from three tubers per strain using a sterile scalpel blade (Figure 1). Bacteria were collected from three plates per strain at the same time points. Each sample was frozen in liquid N_2 immediately after collection and stored at -80 °C until RNA isolation. The infection of potatoes and plating of control material was repeated in an independent experiment to generate material for quantitative reverse transcription PCR (RT-qPCR) analysis. Instead of first generation seed (minitubers), certified seed of the grade Basic 2 (B2) according to the Norwegian seed certification scheme, of the cultivar Asterix was used, because minitubers were not available. Due to the uncertain quality compared to minitubers, they were surface sterilised by submerging them in a 1% chlorine solution for 15 minutes.

Isolation and processing of RNA

All samples from macerated potato tissue and bacteria grown on plate were homogenized by crushing them in liquid N_2 using a pestle and mortar. RNA was isolated from the homogenized samples using the Spectrum Plant Total RNA kit (Sigma Aldrich) according to the manufacturer's instructions, with the inclusion of the optional on-column DNase digestion to avoid genomic DNA contamination (On-Column DNase I Digestion Set, Sigma-Aldrich). The isolated RNA was analysed in the 2100 Bioanalyzer (Agilent) using the RNA 6000 Nano Kit, and ribosomal RNA was depleted using the RiboMinus Transcriptome Isolation Kit for bacteria (ThermoFisher) according to the instructions. For each strain and time point, three macerated tissue and M9 samples were sequenced. The 36 samples where indexed and mixed into one pool, and then sequenced using six lanes on an Illumina HiSeq 2500 (2x125 bp) by the Norwegian Sequencing Centre.

Read mapping and sample quality

The resulting sequence files (six forward, six reverse) from each sample where concatenated to one forward and one reverse fastq file. Raw reads were trimmed for

adaptor sequences, ambiguous bases (limit = 2) and quality (limit = 0.05), and short reads with less than 15 bp after trimming were discarded. Trimmed reads were mapped to the annotated genes of the *P. polaris* strain NIBIO1006 genome (GenBank: CP017481.1) using the following parameters: inclusion of intergenic regions, default costs (mismatch = 2, insertion/deletion = 3), length and similarity fraction 0.8, and single count for paired reads. Trimming and mapping were performed using the CLC Genomics Workbench (version 9.5.2). The NIBIO1006 genome should offer optimal read mapping for the *P. polaris* reads, but not necessarily the *P.c.* subsp. *carotovorum* reads, read mapping was also performed using the *P. c.*subsp. *carotovorum* strain PC1 (NCBI assembly GCA_000023605.1) genome as a reference, to compare performance. The read mapping against the NIBIO1006 genome was superior in the number and percentage of annotated genes with reads mapped to them, for both *Pectobacterium* species (Supplementary table 1). The NIBIO1006 mapping was therefore deemed more informative for both species and used for all analyses throughout this work.

The samples of bacteria grown on M9 minimal medium at 48 and 72 hpi of both species showed widely inconsistent expression values, both among the biological replicates, and compared to all other samples (Supplementary figure 1). This strongly indicates bad sample quality, either due to genomic DNA contamination or partial transcript degradation, and these samples were therefore excluded from analysis. The samples from M9 minimal medium at 24 hpi were used as the reference for non-infection, and are referred to as “M9 control” throughout this work for readability.

Differential gene expression

The analysis of differentially regulated genes was performed using the Empirical analysis of Differential Gene Expression (EDGE) algorithm, as implemented in the CLC Genomics Workbench (version 7.5.1) on raw expression count values, as recommended for the EDGE algorithm (Robinson et al., 2010). EDGE was performed with a total count filter cut-off of 5.0, estimated tagwise dispersions, and with False Discovery Rate (FDR) correction of *p*-values. For downstream analysis, differentially expressed genes with $p < 0.05$ (FDR corrected *p*-values) were regarded as statistically significant. For heat map representation of gene expression in RNA-seq data and clustering of sample groups a constant of 0.01 was added to the raw expression values before log2 transformation.

Reverse transcription PCR for quantification of genes of interest

For testing candidate genes after RNA-seq analysis, RNA was isolated from an independent sample set, as described above. Complementary DNA (cDNA) was generated using the iScript Advanced cDNA Synthesis kit (Biorad), and a no-RT control was included for all samples. For cDNA generation, 300 ng of RNA (according to Bioanalyzer measurements) were used. The cDNA was tested for genomic DNA contamination using the *ffh* housekeeping gene primers. In samples where the quantity ratio of *ffh* in cDNA/no-RT control was less than 10 000/1 (difference in quantification cycle [Cq]< 9.5), new cDNA was generated after an additional DNAse digestion step of the RNA. The second DNAse digestion was performed using the DNase I Amplification Grade kit (Sigma Aldrich) according to the manufacturer’s instructions, and 15 µL of the digested RNA solution were used in cDNA generation directly following DNase digestion. After the additional DNase digestion, all samples were completely or sufficiently free of genomic DNA, as none of the samples showed an *ffh* quantity of more than 1/10 000 for no-RT control/cDNA in the RT-qPCR.

Primers for 27 genes (Table 1) were designed using the CLC Genomics Workbench (version 7.5.1) primer design tools on the basis of the closest homologous coding sequences in three *Pectobacterium* genome assemblies, *P. polaris* strain NIBIO1006 (GenBank: CP017481.1), *P. carotovorum* subsp. *carotovorum* strain PC1 (NCBI assembly GCA_000023605.1) and strain PCC21 (NCBI assembly GCA_000294535.1). Primer pairs were designed to anneal at a temperature of around 60 °C and amplify not more than 180 bp, ideally around 100 bp. Specificity of the Primers for the genes of interest and absence of target sequences in potato were determined by Primer BLAST on NCBI (Ye et al., 2012).

Table 1. Primers used in this work. "Locus tag" refers to the ID given in the annotation of the *P. polaris* strain NIBIO1006 genome (GenBank: CP017481.1). All primers amplified the target gene from *P. polaris* strain NIBIO1006. Primers that also amplified the target from *P. c.*subsp. *carotovorum* strain PK1045-1-2-13 (Pcc) are marked with 'X'.

Locus tag	Gene name	Forward sequence (5'-3')	Reverse sequence (5'-3')	Amplicon size (bp)	Source	Pcc
BJJ97_09455	*avrXca*	AGATGCGTGGAGATGCGGA	TCACTTCTGGCACGGCTT	129	a	X
BJJ97_05585	*cel*	AAATCACCACGTCCGAAG	AGCAGCCGATCAAACATCA	82	a	X
BJJ97_09110	*cel*	GGTTTGCCAATTTAACGCAG	CGTGCGTGGTGAGGAAAT	147	a	X
BJJ97_16200	*dspE*	ACAGTCTGGTGGTGGATAA	AGCTTTTGGTATTGCCGTT	138	a	X
BJJ97_04370	*expI*	CACAATCGTCAGCCATCC	AACCCTTTCCTGCTTTTCT	97	a	
BJJ97_04365	*expR*	CATGACGAGTGGAGAGAGA	CCCAAGGGAAAGGTGTGA	97	a	
BJJ97_21040	*ffh*	ATGGGCGATGTGCTTTCACT	TCAAACCCATCGCCTTTCTT	101	b	X
BJJ97_18225	*flhC*	GCTGCAAAGGGATGTTCATT	GGAAGGGGGTTGGCACAAA	77	a	X
BJJ97_18230	*flhD*	CACCATCTGCGGTAAAGTC	CTGGCGCAACGCTTAATT	108	a	X
BJJ97_16335	*hrcN*	TCAGCCGCACTGCATTCA	TTCGACAAACGCGCTCTC	100	a	X
BJJ97_16325	*hrcV*	CAGACGCAGAACGAAACC	ACCGGAACGTGGTGAAAT	176	a	
BJJ97_16265	*hrpF*	GTCCCTTAACCCCATTCAG	ACGTTCAGCGCAATATCA	81	a	
BJJ97_16240	*hrpN*	GGCGAAGCAGGATGACAA	AGGTTGGTATTGCCGGTA	144	a	
BJJ97_02945	*pelA*	TGGTTCGAAAATGCGCTG	ATTGATGTGAGGGGAGGAC	150	a	
BJJ97_10320	*pelE*	TAACACCACCAATGGCCC	TGAAGTTGCCCTGTACGA	89	a	
BJJ97_14120	*pelL*	CTGACTTCGAGAGCGTTT	ACATCCCCACCCTTGTTG	124	a	X
BJJ97_16205	*pel*	TGAAGATGCGCTGACGGT	GCGCTACTGTTGGTGATTTC	69	a	X
BJJ97_20435	*pae*	ACTTTCCGCCCCTTTCAC	GCTGGTCTCTGATAATGATGGT	179	a	X
BJJ97_14830	*pel*	CACCAGCAGGAAATCGCA	ACATCAAAACCGTCAGCCA	88	a	X
BJJ97_21105	*recA*	GGTGAGCTGGTTGATCTGGG	GCATTCGCTTTACCCTGACC	101	b	X
BJJ97_16270	*hrcU*	GAACAGGCAGAAGCGGAA	TGTCCGGCTTGCGTAAT	104	a	X
BJJ97_16290	*hrpA*	CGCTCAGGCACAGAAAAT	CTTTCTGCCCGGAGTTCA	95	a	X
BJJ97_16350	*hrcQ*	ATTCAGCAGAGCAACACG	GAAAACAGCGCCAGCCAGA	121	a	
BJJ97_16280	*hrcJ*	TCTTCTGCTGATCGGGCT	GGTTTTGTCCACCCGTTTCT	139	a	X
BJJ97_05545	*vgrG*	ACCCCAATCTGGCACTCAA	TGCTGTAGGTGGTCGGTTC	111	a	X
BJJ97_21250	*tssK*	AACGAAGATCGCCTCCAG	GGGGCTTTGTCTATCCAGTTG	145	a	X
BJJ97_21240	*tssH*	TCCGCCAGAAACCGTATT	GTTCACCTTTATCAAACGCCT	99	a	X
BJJ97_21205	*hcp*	GCCAACTCCATGCTATATCAG	TTTCGTCTTCGTGGCCTTC	113	a	X
BJJ97_21200	*vgrG*	TTACCGGCACATAAAACGC	ACACCATCCATATCTTTCTGAG	134	a	X

[a]This work

[b](Takle et al., 2007)

RT-qPCR was performed with the same conditions and reagent mixtures for all primers. Reactions were performed in 10 µL final reaction volume with 5 µL Sso Advanced Universal SYBR Green Supermix (Biorad), 0.5 µL of each primer (10 pmol/µL), 3 µL H_2O, and 1 µL cDNA (1:10 diluted). The RT-qPCR was conducted in a CFX96 Touch Real-Time PCR system (Biorad) with the following cycling conditions: 3 min at 95 °C followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C, followed by a melting curve analysis from 65 to

95 °C in 0.5 °C increments. All reactions were performed in technical duplicates. Primer efficiency was determined by testing a dilution series from $1:10^1$ to $1:10^6$ in 10x dilution steps for all primers. A mixture of equal volumes from all cDNA samples was used in these dilution curve experiments. Arbitrary quantities of GOIs were calculated from these dilution curves and normalized using the averaged quantities of the housekeeping genes *ffh* and *recA*. Normalized quantities were used to calculate log2-fold changes (log2FC) of GOI quantity in bacteria from macerated potato tissue compared to bacteria from M9 minimal medium.

Results

The tuber soft rot after inoculation with the two *Pectobacterium* species progressed typically (Figure 1).

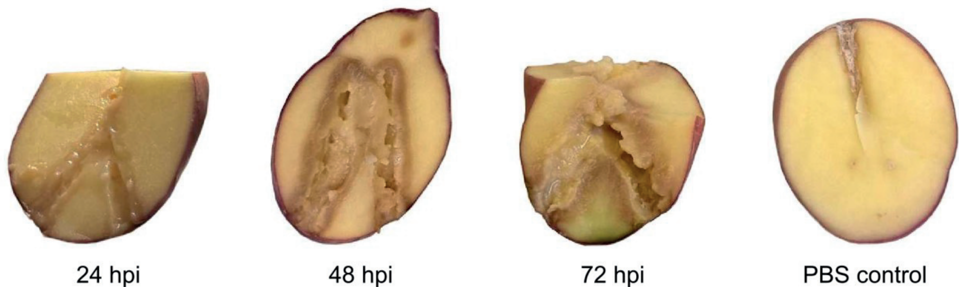


Figure 1. Samples used for RNA-seq experiment. Shown is the rot progression of *P. polaris* strain NIBIO1006 in potato tubers (*Solanum tuberosum* cv. Asterix) at 24, 48 and 72 hours post inoculation (hpi) and the control inoculation with phosphate-buffered saline (PBS) at 72 hpi. The depicted examples are representative of three biological replicates per time point, and similar to rot symptoms caused by *P. c.* subsp. *carotovorum* strain PK 1045-1-2-13.

In the RNA-seq, clustering of average transformed expression values of all genes during infection clearly separated the sample groups by species (Supplementary figure 2). Within the species, the infected samples were separated from the M9 control, with progressively larger distances between consecutive time points. This suggested overall sanity of the generated data in regard to expression differences between the respective sample groups.

Differentially expressed genes

A large number of genes were differentially expressed in *Pectobacterium carotovorum* subsp. *carotovorum* strain PK1045-1-2-13 (Pcc) and *Pectobacterium polaris* strain NIBIO1006 (Ppol) during infection of potato tubers at three time points, compared to bacteria grown on M9 plates (Figure 2A). In both *Pectobacterium* species, between 1077 and 1482 of the 4252 annotated genes were up- or downregulated over all three time points. When comparing bacteria at the later stages of infection (48 and 72 hpi) to the early phase of infection (24 hpi), fewer genes were differentially expressed than when comparing bacteria during infection to bacteria grown on M9 (Figure 2B), and there was an increase of differentially expressed genes (DEGs) with time in both strains.

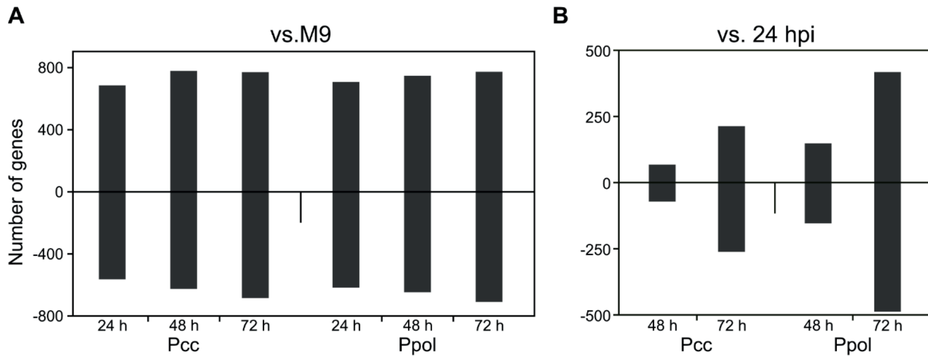


Figure 2. Differentially expressed genes (DEGs) for *P. c.* subsp. *carotovorum* (Pcc) and *P. polaris* (Ppol) during infection. The number of DEGs (FDR < 0.05) at three time points during infection compared to M9 control (**A**), and at 48 and 72 hpi compared to early infection (**B**) are shown. The number of DEGs with significantly higher expression in the sample compared to the respective reference (upregulated DEGs) is displayed as positive, the number of DEGs with a significantly lower expression (downregulated DEGs) is displayed as negative.

In the comparison of bacteria during infection to the M9 control, a majority of the DEGs were identical during all three time points for both species, with 820 for Pcc and 784 for Ppol, while the proportion of unique DEGs was largest at 72 hpi, with 343/1456 (Pcc), and 349/1482 (Ppol) (Figure 3).

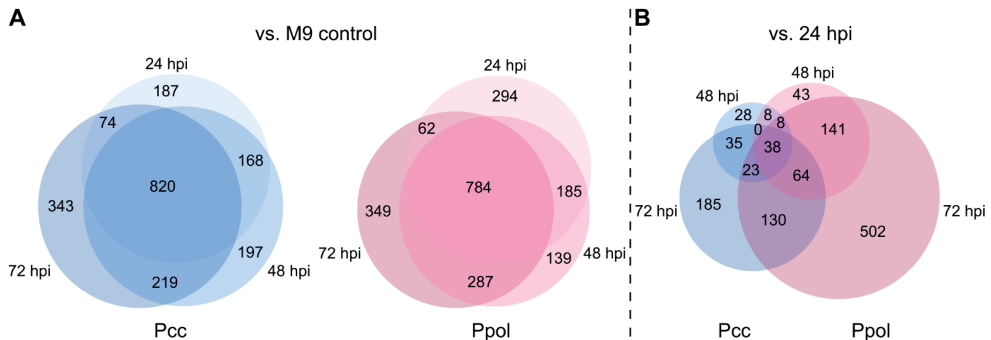
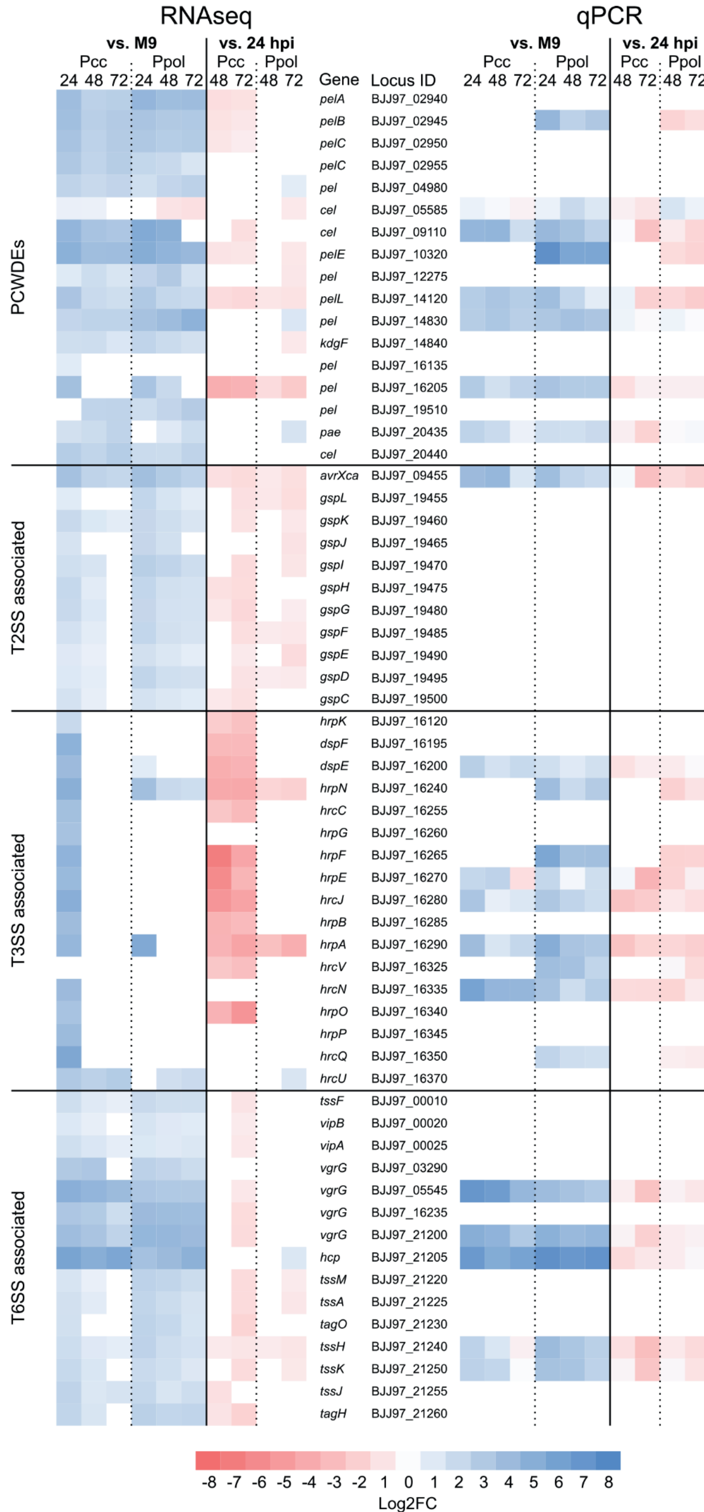


Figure 3. Overlapping and unique DEGs at 24, 48 and 72 hours post inoculation (hpi) for *P. c.* subsp. *carotovorum* (Pcc) and *P. polaris* (Ppol) during infection. The numbers of statistically significant DEGs (FDR < 0.05) at 24, 48 and 72 hpi during infection compared to M9 control (A), and progressing infection compared to early infection (B) are shown per species and time point. Circle and overlap size is proportional to the number of DEGs in each group.

When comparing expression in the later versus the early phase of infection, most of the DEGs at 48 hpi were also differentially expressed at 72 hpi, with 96/140 for Pcc and 251/302 for Ppol (Figure 3). A total of 271 DEGs were overlapping between Pcc and Ppol at 48 and 72 hpi.



RNAseq
qPCR
vs. M9
vs. 24 hpi
Pcc
Ppol
24 48 72
Gene
Locus ID
PCWDEs
T2SS associated
T3SS associated
T6SS associated
pelA BJJ97_02940
pelB BJJ97_02945
pelC BJJ97_02950
pelC BJJ97_02955
pel BJJ97_04980
cel BJJ97_05585
cel BJJ97_09110
pelE BJJ97_10320
pel BJJ97_12275
pelL BJJ97_14120
pel BJJ97_14830
kdgF BJJ97_14840
pel BJJ97_16135
pel BJJ97_16205
pel BJJ97_19510
pae BJJ97_20435
cel BJJ97_20440
avrXca BJJ97_09455
gspL BJJ97_19455
gspK BJJ97_19460
gspJ BJJ97_19465
gspI BJJ97_19470
gspH BJJ97_19475
gspG BJJ97_19480
gspF BJJ97_19485
gspE BJJ97_19490
gspD BJJ97_19495
gspC BJJ97_19500
hrpK BJJ97_16120
dspF BJJ97_16195
dspE BJJ97_16200
hrpN BJJ97_16240
hrcC BJJ97_16255
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hrpO BJJ97_16340
hrpP BJJ97_16345
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vipB BJJ97_00020
vipA BJJ97_00025
vgrG BJJ97_03290
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hcp BJJ97_21205
tssM BJJ97_21220
tssA BJJ97_21225
tagO BJJ97_21230
tssH BJJ97_21240
tssK BJJ97_21250
tssJ BJJ97_21255
tagH BJJ97_21260
-8 -7 -6 -5 -4 -3 -2 1 0 1 2 3 4 5 6 7 8
Log2FC

Figure 4. Differential expression of virulence factors in two *Pectobacterium* strains according to RNA-seq and qPCR quantification. The log2-fold changes (log2FC) are the average of three biological replicates of *P. c.* subsp. *carotovorum* (Pcc) and *P. polaris* (Ppol) sampled during infection at 24, 48 and 72 hours post inoculation (hpi) compared to bacteria grown on M9 plates, and 48 and 72 hpi compared to 24 hpi. For RNA-seq (left), log2FC is only shown for significant differential expression (FDR < 0.05). Gene names and locus tags, based on the annotated *P. polaris* genome (GenBank CP017481.1) and homology searches (Supplementary table 2) are given in the middle.

Virulence associated genes

The large number of DEGs in the comparison of bacteria during infection and the M9 control reflected a major shift in metabolism and cell physiology, as shown by overrepresented biological functions in the form of gene ontology (GO) terms (Supplementary figure 3). Furthermore, several groups of virulence associated genes were upregulated during infection. The analysis of molecular virulence determinants in this work focused on PCWDEs, T2SS, T3SS and T6SS associated genes, since these are commonly described to be key factors in disease, and were well represented by DEGs, allowing for detailed investigation. All members (60 genes) of these four groups identifiable by annotation or previous description with differential expression in at least one time point were investigated (Figure 4).

When comparing bacteria during infection to the M9 control, all of the investigated virulence associated genes were upregulated, or differential expression was not significant, except for one cellulase (BJJ97_05585), which was significantly downregulated in Ppol at 48 and 72 hpi. A majority of the genes were upregulated at several time points during infection for both species. However, T3SS associated genes showed a different expression pattern for the two species, with 16/17 upregulated genes for Pcc, and only 3/17 for Ppol, at 24 hpi (Figure 4).

In the comparison between the later phase of infection (48 and 72 hpi) and the early infection (24 hpi), the large majority of the virulence genes were downregulated, except five genes that were significantly upregulated in Ppol at 72 hpi (Figure 4). Of these, three were encoding PCWDEs, and two were associated with the T3SS and T6SS. For nine of the examined genes, no differential expression was observed during the progressing of the infection.To validate the RNA-seq results, the expression of 27 genes was measured by RT-qPCR in an independent sample set and normalized to the expression of two housekeeping genes (Supplementary table 3). The RT-qPCR results supported the observations made in the RNA-seq, with few exceptions (6.8 % of the genes analysed with RT-qPCR). On average, the four examined gene groups (PCWDEs, T2SS, T3SS, T6SS) were upregulated when comparing bacteria during infection to the M9 control, and downregulated when comparing the later phases of infection to early infection (Figure 5). This was observed for both *Pectobacterium* species in the RNA-seq analysis and largely in the RT-qPCR analysis.

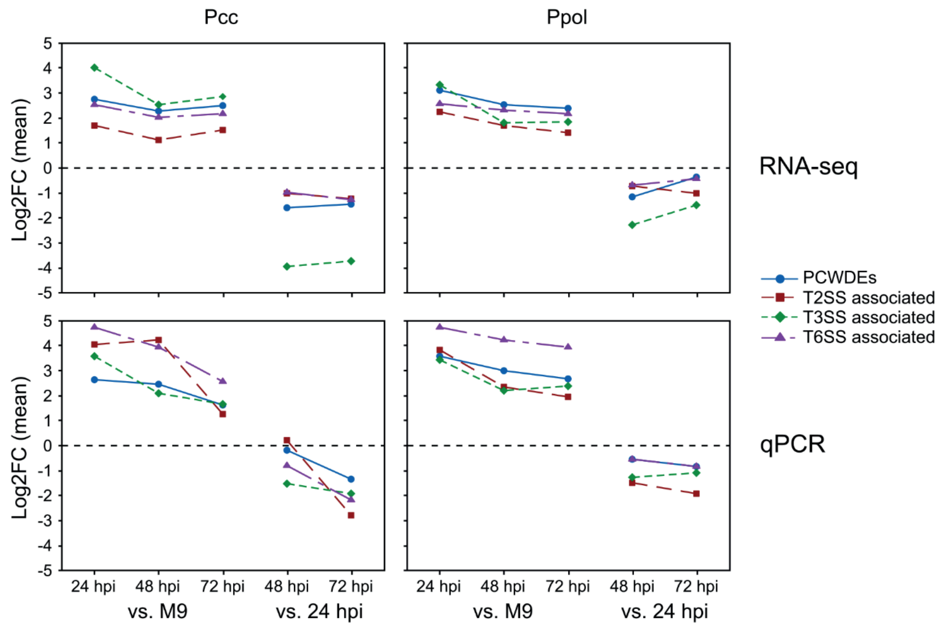

Figure 5. Differential expression of virulence associated gene groups in *P.c.* subsp. *carotovorum* (Pcc) and *P. polaris* (Ppol) during infection. The gene groups are encoding plant cell wall degrading enzymes (blue), T2SS (red), T3SS (green), and T6SS (purple). The log2FC values are shown for bacteria during infection of potato tubers at 24, 48, and 72 hours post infection (hpi) compared to bacteria grown at M9 plates, and for bacteria during later infection (48 and 72 hpi) compared to early infection (24 hpi). For RNA-seq (upper panels), the average log2FC for significantly differentially expressed genes in each group is shown, for qPCR (lower panels), the average log2FC of all measured genes in each group is shown.

In addition to these key disease factors, the differential expression of known virulence regulators was examined to allow speculation on the mechanisms driving the observed changes (Figure 6). Differential expression was not reliably observed in the 16 examined genes belonging to the following groups: master, motility and T3SS regulators, as well was quorum sensing genes and the gluconate sensing genes in the *vgu*-operon. The motility regulators was upregulated over time in both species compared to the M9 control as well as to early infection. The *vgu*-operon was mostly upregulated compared to the M9 control. The quorum sensing genes were downregulated at 48 and 72 hpi in both species compared to early infection but also the M9 control.

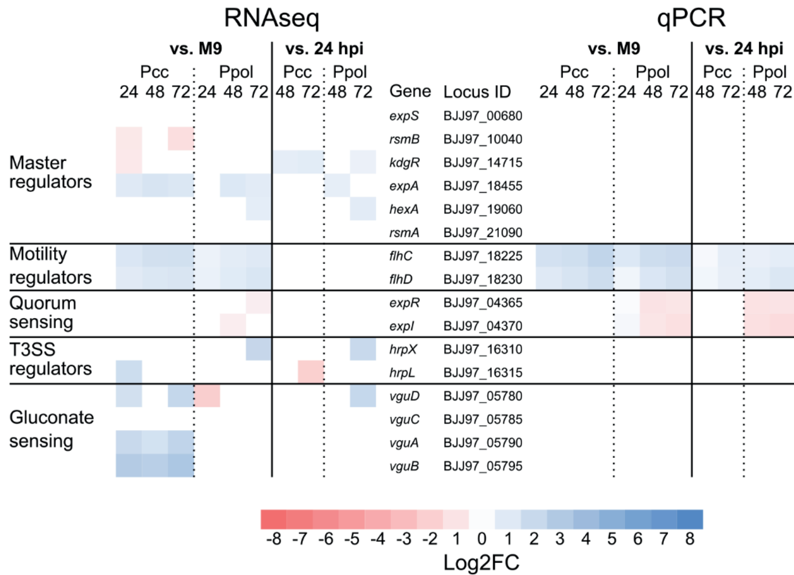

Figure 6. Differential expression of known virulence regulators in two *Pectobacterium* strains during infection. The log2-fold changes (log2FC) are shown for the average of three biological replicates of *P. c.* subsp. *carotovorum* (Pcc) and *P. polaris* (Ppol) sampled during infection at 24, 48 and 72 hpi compared to bacteria grown on M9 plates and the early phase of infection (24 hpi). For RNA-seq (left), log2FC is only shown for significant differential expression (FDR < 0.05). Gene names and locus tags, based on the annotated *P. polaris* genome (GenBank CP017481.1) and homology searches (Supplementary table 2) are given in the middle.

Clustering of samples by expression

To identify groups of genes with similar expression intensity, and visualize the expression according to RNA-seq analysis, the examined virulence and regulator genes were clustered by overall expression in individual samples from bacteria during infection at 24, 48 and 72 hpi, as well as bacteria grown on M9 plates (Figure 7). T3SS associated genes, except for the T3SS secreted effectors *dspE* and *hrpN*, had a weak overall expression compared to the other genes. They mostly clustered in two groups at the outermost branches of the tree (V and VI) together with the *vgu*-operon and QS genes. The highest overall expression relative to the other examined genes was observed for some PCWDEs, T6SS associated genes, and the motility regulators, as well as the T2SS secreted *avrXca*. These genes clustered in the third distant branch (IV). The three inner branches (I-III) were occupied by PCWDEs, and T6SS and T2SS associated genes, as well as the master regulator genes. The QS genes *expI* and *expR* clustered distinctly, within the T3SS associated genes and showed no or very low expression in Pcc samples.

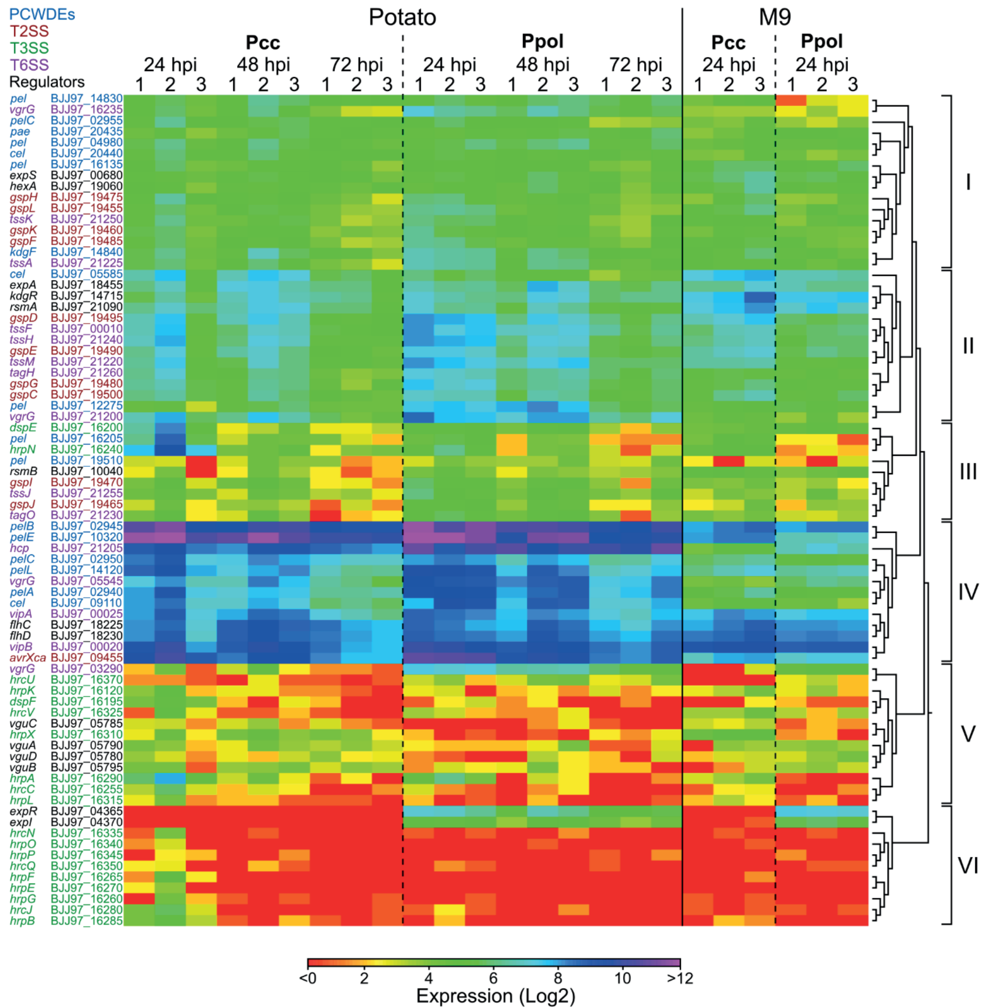

Figure 7. Heat map of expression values of selected virulence associated and regulator genes for all examined samples of *P. c. subsp. carotovorum* (Pcc) and *P. polaris* (Ppol), based on RNA seq analysis. Samples were taken during infection of potato tubers at 24, 48 and 72 hours post inoculation (hpi), or at 24 hpi from M9 minimal medium bacterial plates (M9) in three biological replicates each. Gene names and locus tags are given on the left, and are colour coded to indicate their association with the investigated gene groups given on the top left. A hierarchical clustering based on the transformed expression values is given by the clustering tree. The clustering is based on Euclidian distance measurement of the average linkage and major clusters are identified with roman numerals.

Discussion

General properties of differential gene expression in *Pectobacterium* when comparing infection to the M9 control and infection at the later time points (48 and 72 hpi) to early infection (24 hpi) were assessed. The comparison to the M9 control was conducted to gain insight into processes specific to infection in potato tubers compared to bacterial colonisation on a non-host solid surface. The later time points in infection compared to 24 hpi were compared to investigate virulence factors specific to the different phases of disease progression. Expression patterns were investigated in detail for 60 virulence associated genes and 16 known regulators, and partially validated by RT-qPCR. While RNA-seq provides insight on a transcriptome level, it only allows conclusions on differential expression when the observed difference is large enough to be regarded as statistically significant. On the other hand, RT-qPCR provides reliable information on more subtle gene expression differences but has to focus on few genes. In cases where the RNA-seq results are inconclusive due to statistical insignificance of differential expression, the RT-qPCR therefore can provide additional information.

The numbers of differentially expressed genes (DEGs) were similar across time points and strains when comparing bacteria during infection to bacteria grown on M9 plates (Figure 2A). When comparing the later phases of infection to the early phase of infection (24 hpi), the number of DEGs was overall lower than when comparing to the M9 control, showing a larger difference between infection and *in vitro* growth than the change that occurs over the course of infection. In both species, the number of DEGs in the advanced infection relative to the early phase was approximately three times higher at 72 hpi than at 48 hpi, showing a large shift in the transcriptome over time during soft rot progression. Ppol showed a much larger number of DEGs than Pcc, indicating a more pronounced difference of gene expression over time in Ppol.

The overlap of DEGs at the different time points showed that the genes that were differently expressed during infection compared to the M9 control are largely the same over time (Figure 3A). Nevertheless, the larger overlap between 24 and 48 hpi, as well as 48 and 72 hpi compared to 72 and 24 hpi, shows a progressive shift of expression over time in both strains. This becomes more apparent in the comparison of advanced and early infection, where most genes that are differentially expressed at 48 hpi continue to be differentially expressed at 72 hpi in both strains (Figure 3B). In the latter comparison, the overlap between species shows that a number of genes that are differentially regulated during disease development are shared between the two species. Furthermore, a large overlap of general DEG functions between Pcc and Ppol during infection was observed in an enrichment analysis of GO-terms (Supplementary figure 3).

PCWDEs, and their main secretion route, the T2SS, are key molecular components in *Pectobacterium* soft rot progression (Charkowski et al., 2012). The breakdown of the complex polysaccharides and other components in the host cell wall through cellulases and pectin degrading enzymes is responsible for the visible rot symptoms. The T3SS, although it only secretes few known effectors in *Pectobacterium*, has been shown to play a role in disease in numerous studies (e.g. Hogan et al., 2013; Holeva et al., 2004; H. Liu et al., 2008). The T6SS is probably the least understood virulence component in *Pectobacterium* spp., but was observed to be involved in infection in previous studies (H. Liu et al., 2008; Mattinen et al., 2008).

Genes associated with plant cell wall degradation (PCWD), T2SS, T3SS, and T6SS were heavily represented by DEGs during infection relative to growth on M9 plates, particularly in early infection. In both strains, genes associated with PCWD, the T2SS and T6SS were most reliably upregulated during infection relative to *in vitro* growth (Figure 4). This shows that these genes are specific to a virulence context in both species. The general upregulation of genes encoding PCWDEs, and T2SS associated components in a virulence context is expected, due to their key role in disease (Corbett et al., 2005; Coulthurst et al., 2008; Pineau et al., 2014). Similarly, the general upregulation of T6SS associated genes is congruent with previous observations (H. Liu et al., 2008; Mattinen et al., 2008). Genes associated with the T3SS were upregulated significantly mostly at 24 hpi in Pcc but not commonly differentially expressed at later time points, or in Ppol. However, the RT-qPCR, likely due to its higher sensitivity, showed that the T3SS genes were upregulated throughout the infection, in spite of not being significantly different according to the RNA-seq. While most virulence associated genes were upregulated during infection, a majority of the virulence associated genes showed the highest log2FC values at 24 hpi and their expression decreased gradually at 48 and 72 hpi in both species (Figure 4). This is reflected in widespread significant downregulation when comparing 48 and 72 hpi samples to 24 hpi samples. The T3SS associated genes in Pcc showed particularly strong downregulation at 48 hpi compared to the T2SS and T6SS associated genes, which exhibited overall less extreme, albeit still negative, log2FC values. PCWDE showed downregulation of about half of the examined genes in the late versus early phase of the infection. The trends were reflected well in the RT-qPCR analysis, although T3SS transcripts are less downregulated in Pcc at 48 hpi, suggesting either a difference in intensity or exact time point of the expression shift in the two independent sample sets, or a more accurate measurement by RT-qPCR (Figure 4). Overall, the change from 24 to 48 hpi seems to be more intense than between 48 and 72 hpi for the investigated virulence genes. On average, T2SS, T3SS, and T6SS associated genes as well as genes encoding PCWD functions were upregulated during infection relative to growth *in vitro*, but were downregulated during progression of the infection (Figure 5). This suggests a strong initial spike of virulence associated gene expression during infection, and thereafter a gradual downregulation of these genes. This is in contrast to the generally much higher number of DEGs at 72 hpi in both species, which indicated that general transcriptome changes were continuously amplifying from 24 to 72 hpi (Figure 2, Figure 3).

Some of the examined genes did not follow the postulated expression spike at 24 hpi and consecutive downregulation. A number of PCWDEs in particular were not significantly up- or down regulated, or were steadily more upregulated during infection (Figure 4). Furthermore, the downregulation of T6SS associated genes was mostly significant at 72 hpi in Pcc in the RNA-seq, with Ppol being less pronounced, and similar observations in the RT-qPCR. This suggests different modes of regulation among the different genes encoding PCWD functions, as well as between the T3SS and T2SS/T6SS genes, and different behaviour between the two investigated species, either in timing of the expression shift or the involved regulation mechanisms. This was supported by the clustering of genes according to expression data from the RNA-seq (Figure 7). While the T3SS associated genes overwhelmingly clustered together in two groups, the genes encoding PCWDEs varied widely in expression intensity and patterns, and clustered in multiple groups across the tree. The T2SS and T6SS associated genes similarly did not cluster together, due to a comparable variety in expression intensity. The clustering also

suggested that the important QS genes *expI* and *expR* were completely absent in Pcc. However, when mapping the transcriptome sequencing reads to a Pcc genome, moderate expression was detected for these genes in Pcc during infection and *in vitro* (Supplementary figure 4), in a similar intensity to Ppol. Since QS signals are highly specific to a given bacterial species or strain to discriminate between self and non-self signals, high sequence variability and therefore low mapping success of reads from these transcripts on genomes of a different strain may be expected (Sjöblom et al., 2006).

The potential differences in mapping success for the RNA-seq analysis, and potentially differing amplification efficiency for RT-qPCR complicate conclusions on differences between the two investigated species. Since the genome of the investigated Ppol strain was used for mapping, and primers were designed on the basis of this genome, it is likely that mapping and amplification efficiency was higher for Ppol than for Pcc. This, for instance, suggests that the observed stronger peak expression of T3SS genes of Pcc at 24 hpi, is likely a true difference between the two bacterial species or strains (Figure 7). This suggests either a truly lower expression, or an earlier downregulation of T3SS associated genes in Ppol during tuber infection. Overall, the expression patterns and intensities during infection appear similar between the two investigated species (Figure 7).

While the roles of T3SS and T6SS in *Pectobacterium* virulence are unclear, it seems from this transcriptome analysis that the T3SS is more important in the early virulence, while T6SS plays a role in the progressing infection, according to the more sustained expression of those genes, mainly in Ppol. It was previously shown that the T3SS and the effector DspE are necessary for virulence in potato stem infections, and play a role in early virulence on potato leaves by causing cell death (Hogan et al., 2013; Holeva et al., 2004). However, that study did not show a modification of host gene expression by DspE, and a phenotype for T3SS mutants in tuber infection is not sufficiently established for *Pectobacterium* sp. (Kim et al., 2011). For the T6SS, initial strong upregulation followed by steady downregulation of the T6SS genes *vasH* and *vasK* during tuber infection over 96 hours compared to *in vitro* growth was previously shown (Mattinen et al., 2008). For PCWDEs, the seeming redundancy seems to have functional significance in the progression of infection, since the genes appeared not to be expressed in the same manner over time, as some were downregulated, some were upregulated, and some were at the same level over the investigated course of the infection.

The transcriptome data is useful to examine possible regulatory mechanisms underlying the observed transcriptional changes during infection. While established models for virulence regulation focus on disease onset, the regulators involved may be overlapping. Motility, has commonly been described to be associated with virulence (Andresen et al., 2010; Charkowski et al., 2012; Cui et al., 2008). However, based on the data presented here, expression of the motility regulator genes *flhC* and *flhD* that control expression of virulence genes in addition to the flagellar operon, seem to be progressively increasing during infection, as opposed to other virulence associated genes (Figure 4, Figure 6). This contrasts earlier observations, where the knockout of the *flhDC* complex was shown to have decreased virulence, which was explained through the accumulation of the virulence repressor HexA in the mutant and consecutive lack of *rsmB* expression, leading to continuous repression of virulence transcripts by RsmA (Cui et al., 2008). The assumption that FlhDC enables the expression of virulence genes by ultimately blocking RsmA function was reinforced in later work that uncovered the repression of *flhDC* expression by RcsB

(Andresen et al., 2010). In the results presented here, however, a relative downregulation of most virulence associated genes in spite of a relative upregulation of *flhDC* was observed, suggesting no positive regulatory effect of FlhDC on virulence gene expression in prolonged infection.

Furthermore, differential expression of the master regulator gene of virulence onset, *rsmA*, was not significant at any time point during infection, neither compared to *in vitro* growth, nor compared to early infection (Figure 6). While *rsmB* expression was slightly lower at 24 hpi and 72 hpi in Pcc, when comparing infection to the M9 control, it was not significantly different for Ppol, or when comparing progressing to early infection. While both, *rsmA* and *rsmB* were continuously expressed (Figure 7), the lack of consistently significant differential expression does not warrant conclusions on their involvement in the observed downregulation of virulence genes, and may suggest these changes to have been largely independent of RsmAB and FlhDC.

The QS genes *expL* and *expR* encode for an enzyme the facilitates the synthesis of the QS signal and a transcriptional regulator that is inactivated upon binding the QS signal respectively. Both were continuously expressed but were not significantly different, or downregulated during infection compared to *in vitro* growth. According to the RT-qPCR, they were slightly upregulated at 24 hpi and then downregulated during infection compared to growth *in vitro* and to early infection. This suggests expression of the QS elicitor and receiver to be similar or slightly lower during infection than during *in vitro* growth. QS was previously shown to be a necessary, but not sufficient, component for the initial switch from a non-virulent state to the production of PCWDEs, and upregulation of other putative virulence factors, among them T3SS and T6SS associated genes (H. Liu et al., 2008; Põllumaa et al., 2012). The slight downregulation of the QS genes during rot progression may reflect an overall change in growth stage of the bacterial community, as this has been shown to impact QS expression previously (Crépin et al., 2012). The observed downregulation of the QS genes here may impact regulation of some of the examined virulence associated genes. However, QS does not seem to be as universally impactful in regulating gene expression of PCWDEs, T3SS and T6SS associated genes during later infection as it was shown to be during virulence onset (H. Liu et al., 2008).

Another previously described regulatory mechanism is the gluconate-dependent signalling via *vgu* genes, which has been shown to alter expression of PCDWEs and T3SS components (Mole et al., 2010). In that study, a *vgu*-operon knockout mutant exhibited increased expression of *kdgR* and *hrpN* during infection of potato leaves, and was hypervirulent on tubers with decreased *flhD*, *hexA* and *rsmB* expression. The resulting model proposed for the role of the *vgu*-operon in tuber infection assumed gluconate-dependent activation of *flhD* and *hexA* transcription via the *vgu* operon, leading to lower virulence through HexA dependent modulation of *rsmB* and direct repression of genes encoding PCWDEs (Harris et al., 1998; Mole et al., 2010). While *vguA*, *vguB* and *vguD* showed upregulation in Pcc infection relative to *in vitro* growth, they were not differentially expressed over the course of the infection (Figure 6). In Ppol, *vguD* was slightly upregulated during infection at 24 hpi relative to *in vitro* growth, and significantly upregulated at 72 hpi. The pectin-breakdown sensitive virulence repressor KdgR, involved in repression of pectate lyases, was upregulated during the later phases of infection compared to the early phase of infection in both species. The proposed reduced expression of some PCDWEs, as well as the KdgR mediated repression of HrpN in the

presence of gluconate and pectin breakdown products via the *vgu*-operon expression is therefore consistent with the observed transcriptome changes during rot progression, at least in Pcc (Mole et al., 2010). The proposed involvement of HexA and *rsmB*, however, was not reflected in the examined data, since both show only limited differential expression, and rather stable expression over time (Figure 6, Figure 7).

The ExpA/S two-component system was shown to modulate some virulence functions independently of RsmA in *P. wasabiae* (Broberg et al., 2014; Eriksson et al., 1998). However, while the response regulator gene *expA* was upregulated during infection compared to *in vitro* growth, it was not commonly differentially expressed over the course of infection. The histidine kinase gene *expS*, was stably expressed over all samples and not significantly different at any time point or growth condition (Figure 6, Figure 7). Overall, the previously described molecular regulation mechanisms in virulence onset do not appear to play an obvious role in the observed late downregulation of virulence related genes during the later phases of infection, with the possible exception of gluconate-dependent signalling via the *vgu*-operon and KdgR and some potential involvement of QS-dependent regulation.

In addition to the mechanisms that facilitated it, the reason for the observed downregulation of virulence associated genes during later phases of infection is not immediately apparent. On a physiological level, the most likely explanation for the observed initial spike and successive downregulation of virulence associated genes during rot progression, is a differentiation of functions within the bacterial community in different zones of infected and rotting tissue. At 24 hpi, a relatively large fraction of bacteria is in direct contact with intact potato tissue. During progression of the infection, the amount of macerated tissue grows faster compared to the zone where bacteria are in contact with intact tissue (Figure 1). Therefore, a smaller fraction of the analysed transcriptome may have been involved in active virulence over the examined time points. If community structure is the cause of the observed expression shift after initial onset, it could be governed by metabolites with host origin, such as pectin degradation products (e.g. gluconate), which would support the potential involvement of VguABCD and KdgR. A QS gradient with high concentration of QS signal around active maceration zones and lower concentration in tissue with progressed rot could function as a part of this regulation. This would give additional meaning to the observed net decrease in QS associated transcripts beyond the described dependence on growth phase (Crépin et al., 2012).

In conclusion, this work shows a previously undescribed downregulation of virulence associated genes during the progression of infection in tubers relative to a peak expression observed initially (24 hpi). This downregulation does not seem to have progressed evenly among or within different gene groups. T3SS associated transcripts were downregulated earliest, while T2SS and T6SS associated transcripts were downregulated later, and some PCWDE encoding genes remained evenly expressed or were progressively upregulated over time. This suggests various modes of regulation for different virulence associated genes during the progression of the infection. The observed expression changes are likely a result of the shifting structure of the bacterial community. Functional studies are needed to examine the molecular basis of this regulation. Future work on the function of the Type III and VI secretion systems in *Pectobacterium* tuber

infection may help in understanding the differences in regulation of these two gene groups during rot progression.

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Supplementary material

Supplementary methods – Read mapping against the PC1 genome

For the read mapping against the *P. c.*subsp. *carotovorum* strain PC1 (NCBI assembly GCA_000023605.1) genome, trimmed reads were mapped with mapping to gene regions only, default costs (mismatch = 2, insertion/deletion = 3), length and similarity fraction of 0.8, and single count for paired reads using the CLC Genomics Workbench (version 9.5.2).

Supplementary table 1. Mapping outcomes of read mapping from two *Pectobacterium* species to two different reference genomes. The two reference genomes used for mapping were *P. polaris* strain NIBIO1006 (GenBank: CP017481.1), and *P. c.* subsp. *carotovorum* strain PC1 (NCBI assembly GCA_000023605.1). Reads were generated by RNA-sequencing of *P. c.* subsp. *carotovorum* strain PK1045-1-2-13 (Pcc) and *P.polaris* strain NIBIO1006 (Ppol). Given for each mapping outcome are the number of genes and the percentage of annotated genes.

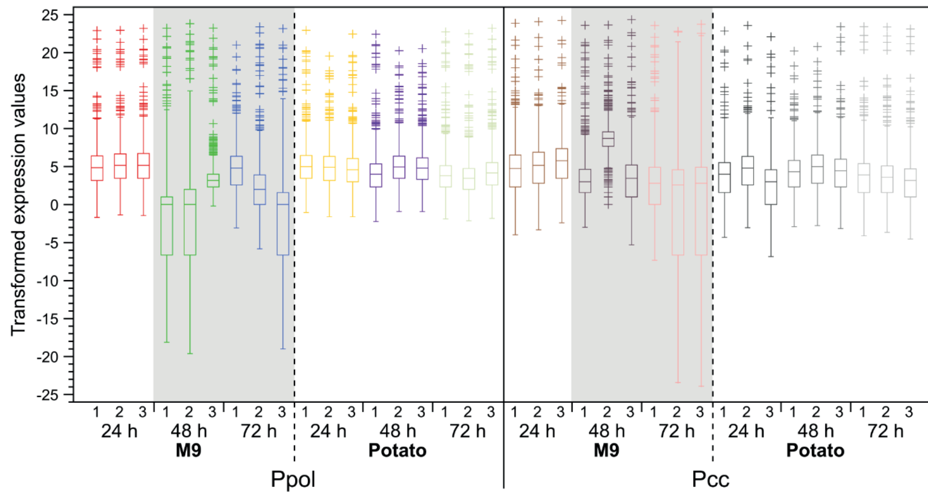
	NIBIO1006		**PC1**	
	Genes	%	Genes	%
Annotated genes	4252		4290	
with mapped reads from Pcc	3716	87.4	3673	85.6
no mapped reads from Pcc	536	12.6	617	14.4
with mapped reads from Ppol	4171	98.1	3610	84.1
no mapped reads from Ppol	81	1.9	680	15.9
without any mapped reads	74	1.7	520	12.1

Supplementary table 2. Identity of selected genes discussed in this work. The genes were identified by blastx search on NCBI's non-redundant protein sequence (nr) and UniProtKB (SwissProt) database with the following parameters: genetic code 6, word size 6, BLOSUM62 matrix, gap existence/ extension 11/1, and low complexity filtering. The protein identity is given by the protein's short name and, in some cases superfamily (SF).

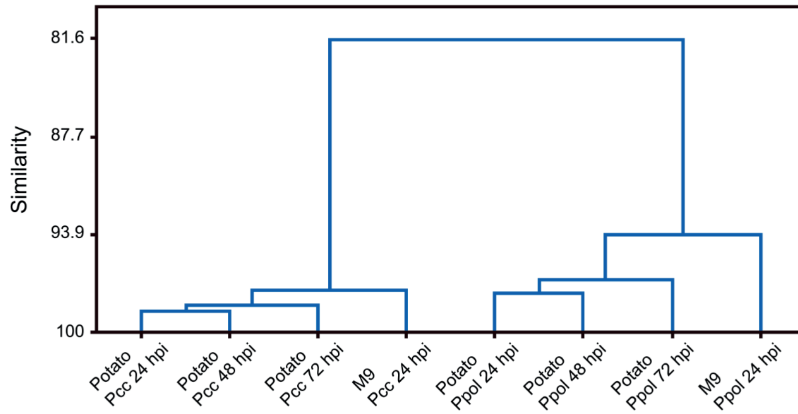
NIBIO1006 locus tag	Protein Name	Description
BJJ97_00010	TssF	Type VI secretion system baseplate subunit
BJJ97_00020	VipB	Type VI secretion system contractile sheath large subunit
BJJ97_00025	VipA	Type VI secretion system contractile sheath small subunit
BJJ97_02940	PL-6-SF	Pectate lyase A
BJJ97_02945	PL-6-SF	Pectate lyase B
BJJ97_02950	PL-6-SF	Pectate lyase C
BJJ97_02955	PL-6-SF	Pectate lyase C
BJJ97_03290	VgrGa/b	Type VI secretion system tip protein
BJJ97_04365	ExpR	QS-receptor/LuxR family transcriptional regulator
BJJ97_04370	ExpI/NAT-SF	QS-elicitor/Acyl-homoserine-lactone synthase
BJJ97_04980	ExoPL	Pectate disaccharide-lyase
BJJ97_05545	VgrGa/b	Type VI secretion system tip protein
BJJ97_05585	CMCase	Endoglucanase/Carboxymethylcellulase
BJJ97_09110	Glyco_hydro_1-SF	Exotoxin/Arabinogalactan endo-1,4-beta-galactosidase
BJJ97_09455	AvrXca	avirulence Protein
BJJ97_10320	PL	Pectate lyase E
BJJ97_12275	PL-6-SF	Pectate lyase
BJJ97_14120	PL	Pectate lyase L
BJJ97_14830	ExoPL	Pectate disaccharide-lyase
BJJ97_14840	KdgF/cupin-like SF	Pectin degradation/cupin-domain containing
BJJ97_15845	VgrG-SF	Type VI secretion system tip protein
BJJ97_15850	PAAR_2	Type VI secretion system protein
BJJ97_16120	HrpK	Type III effector HrpK
BJJ97_16135	PL 2	Periplasmic pectate lyase
BJJ97_16200	AvrE	Type III effector AvrE
BJJ97_16205	PL	pectate lyase/Type III effector
BJJ97_16235	VgrG	Type VI secretion system tip protein
BJJ97_16240	HrpN	Type III effector HrpN
BJJ97_16255	EscC/YscC/HrcC	Type III secretion system outer membrane ring protein
BJJ97_16260	HrpG/T3SS	Type III secretion system (response regulator HrpG)
BJJ97_16265	HrpF-SF	Type III secretion protein/Serine kinase
BJJ97_16270	HrpE/YscL	Type III secretion apparatus protein
BJJ97_16280	EscJ/YscJ/HrcJ	Type III secretion system inner membrane ring protein
BJJ97_16285	YscI/HrpB/ PscI	Type III secretion basal body protein
BJJ97_16290	HrpA	Type III secretion pilin HrpA
BJJ97_16310	HrpX	Type III secretion sensor kinase HrpX
BJJ97_16315	HrpL	Type III secretion system regulator/RNA polymerase sigma factor
BJJ97_16325	EscV/YscV/HrcV	Type III secretion system export apparatus protein
BJJ97_16335	EscN/YscN/HrcN	Type III secretion system ATPase
BJJ97_16340	HrpO	Type III secretion system protein
BJJ97_16345	HrpP	Type III secretion system protein
BJJ97_16350	YscQ/HrcQ	Type III secretion apparatus protein
BJJ97_16355	EscR/YscR/HrcR	Type III secretion apparatus protein
BJJ97_16370	EscU/YscU/HrcU	Type III secretion export apparatus switch
BJJ97_18225	FlhC	Flagellar transcriptional regulator
BJJ97_18230	FlhD	Flagellar transcriptional regulator
BJJ97_19510	PL-6-SF	Pectate lyase
BJJ97_20435	Pae	Pectin acetylesterase
BJJ97_20440	PeA	Pectinesterase A
BJJ97_21200	VgrGa/b	Type VI secretion system tip protein
BJJ97_21205	Hcp	Type VI secretion system effector Hcp family
BJJ97_21220	TssM	Type VI secretion system membrane subunit
BJJ97_21225	TssA/VasJ-SF	Type VI secretion system component protein
BJJ97_21230	TagO/VasI-SF	Type VI secretion system-associated protein
BJJ97_21240	TssH	Type VI secretion system secretion system ATPase
BJJ97_21250	TssK	Type VI secretion system baseplate subunit
BJJ97_21255	TssJ	Type VI secretion system lipoprotein
BJJ97_21260	TagH	Type VI secretion system-associated FHA domain protein

Supplementary table 3. Normalized gene expression of selected genes according to RT-qPCR for two Pectobacterium strains at three time points. Values are shown for P. c. subsp. carotovorum strain PK1045-1-2-13 (Pcc) and P. polaris strain NIBIO1006 (Ppol). For each strain and time point, gene expression for bacteria isolated from macerated potato tissue (potato) at 24, 48 and 72 hpi and grown on minimal M9 bacterial plates (M9) at 24 hpi is shown. Values represent the average expression in three biological replicates ± standard deviation. Expression for each replicate was normalized using the average expression of the housekeeping genes ffh and recA of that replicate. Genes are identified by their locus tag on the P. polaris strain NIBIO1006 (GenBank: CP017481.1) genome and the primers used in the RT-qPCR (Table 1). For primers that only amplified from Ppol, Pcc samples are marked with 'x'.

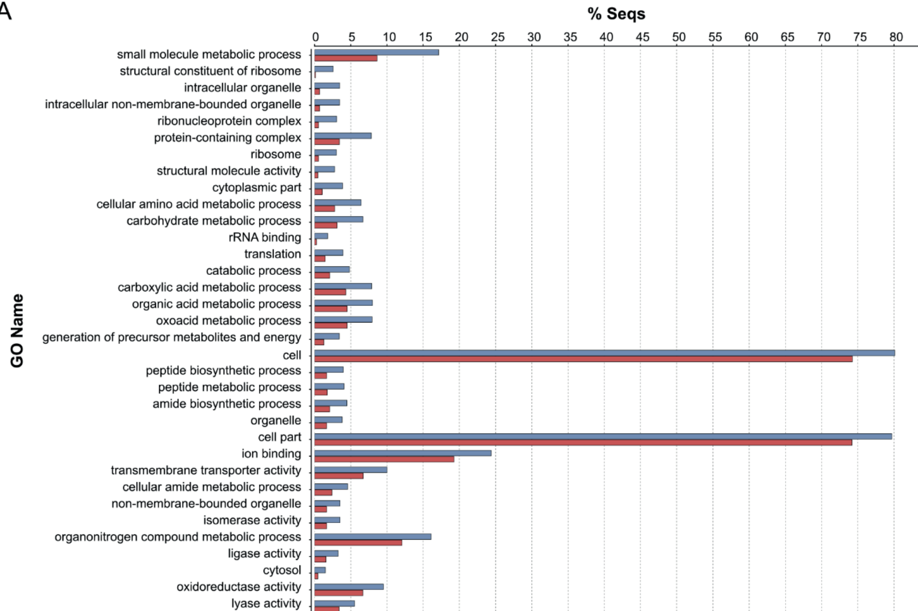
		Potato						M9	
		Pcc			Ppol			Pcc	Ppol
Locus tag	Primer	24 hpi	48 hpi	72 hpi	24 hpi	48 hpi	72 hpi	24 hpi	24 hpi
BJJ97_09455	*avrXca*	0.82±0.28	0.95±0.88	0.12±0.05	3.64±0.26	1.29±0.68	0.97±0.59	0.05±0.02	0.25±0
BJJ97_05585	*cel*	2.26±0.74	1.66±0.88	1.12±0.48	0.37±0.1	0.96±0.93	0.53±0.43	1.48±0.36	0.24±0.02
BJJ97_09110	*cel*	1.31±0.2	1.39±1.14	0.2±0.22	3.91±2.71	2.45±1.97	1.2±0.72	0.06±0	0.21±0.09
BJJ97_16200	*dspE*	1.93±0.38	0.87±0.21	1.24±0.44	1.55±1.11	0.97±0.14	1.46±0.71	0.3±0.07	0.49±0.03
BJJ97_04370	*expI*	x	x	x	2.05±0.28	0.91±0.68	0.8±0.58	x	1.76±0.22
BJJ97_04365	*expR*	x	x	x	1.53±0.23	0.7±0.5	0.76±0.54	x	1.46±0.13
BJJ97_18255	*ffh*	1.35±0.41	1.52±0.47	2.32±0.38	0.73±0.19	1.15±0.78	1.28±0.17	0.48±0.14	0.34±0.03
BJJ97_18230	*flhC*	0.94±0.26	1.2±0.41	1.57±0.2	0.5±0.24	0.89±0.42	1.12±0.06	0.46±0.08	0.38±0.02
BJJ97_16335	*flhD*	2.27±0.08	0.84±0.5	0.81±0.47	2.98±1.23	0.86±0.85	1.78±1.46	0.04±0.01	0.26±0.04
BJJ97_16325	*hrcN*	x	x	x	3.06±1.87	2.68±3.02	1.07±0.76	x	0.22±0.19
BJJ97_16265	*hrcV*	x	x	x	7.71±5.33	2.11±2.34	2.21±2.77	x	0.17±0.04
BJJ97_16240	*hrpF*	x	x	x	6.68±2.49	1.74±1.26	3.25±2.77	x	0.46±0.06
BJJ97_02945	*hrpN*	x	x	x	5.01±1.54	1.41±1.03	2.08±1.5	x	0.24±0.03
BJJ97_10320	*pelA*	x	x	x	4.39±0.68	1.59±1.19	1.23±0.88	x	0.03±0
BJJ97_14120	*pelE*	0.44±0.21	0.64±0.57	0.12±0.1	2.1±0.92	0.69±0.46	0.47±0.26	0.06±0.03	0.15±0.01
BJJ97_16205	*pelL*	2.77±0.83	1.11±0.44	2±0.36	1.24±0.82	0.86±0.44	0.86±0.11	0.36±0.06	0.11±0
BJJ97_20435	*pel*	1.49±0.32	1.04±0.07	0.41±0.26	1.26±0.17	1.17±0.2	1.47±0.25	0.27±0.08	0.37±0.03
BJJ97_14830	*pae*	1.35±0.49	1.85±0.5	1.25±0.7	1.12±0.17	1.58±0.39	1.2±0.24	0.2±0.05	0.13±0.02
BJJ97_16270	*pel*	0.32±0.08	0.4±0.38	0.03±0.04	1.67±0.43	0.47±0.34	1.22±1.09	0.07±0.03	0.37±0.05
BJJ97_16290	*recA*	6.19±2.97	0.99±0.53	1.79±1.51	1.32±0.87	0.42±0.26	0.33±0.15	0.4±0.11	0.04±0.01
BJJ97_16350	*hrcU*	x	x	x	5.41±1.69	3.62±3.27	3.32±3.36	x	1.07±0.26
BJJ97_16280	*hrpA*	6.43±3.34	1.03±0.27	1.39±1.72	1.18±0.44	0.66±0.49	0.46±0.19	0.63±0.25	0.13±0.03
BJJ97_01585	*hrcQ*	1.37±0.68	1.49±0.95	1.62±0.56	0.22±0.06	0.76±0.84	0.61±0.44	1.34±0.4	0.15±0.02
BJJ97_05545	*hrcJ*	1.72±0.84	1.16±0.32	0.27±0.13	1.53±0.64	1.14±0.47	0.82±0.29	0.01±0.01	0.1±0.01
BJJ97_21250	*vgrG*	2.01 ± 1.22	1.61 ± 0.9	0.35 ± 0.23	1.06 ± 0.28	0.95 ± 0.29	0.49 ± 0.06	0.34 ± 0.16	0.09 ± 0.01
BJJ97_21240	*tssK*	2.8 ± 1.12	1.19 ± 0.27	0.37 ± 0.21	1.48 ± 0.31	0.85 ± 0.41	0.53 ± 0.08	0.5 ± 0.18	0.09 ± 0.01
BJJ97_21205	*tssH*	2.76 ± 0.33	0.99 ± 0.21	1.5 ± 0.86	1.29 ± 0.42	0.85 ± 0.02	1.11 ± 0.13	0.03 ± 0.01	0.01 ± 0
BJJ97_21200	*hcp*	1.01 ± 0.34	0.78 ± 0.13	0.26 ± 0.09	1.16 ± 0.39	0.74 ± 0.13	0.88 ± 0.32	0.03 ± 0.01	0.04 ± 0.01

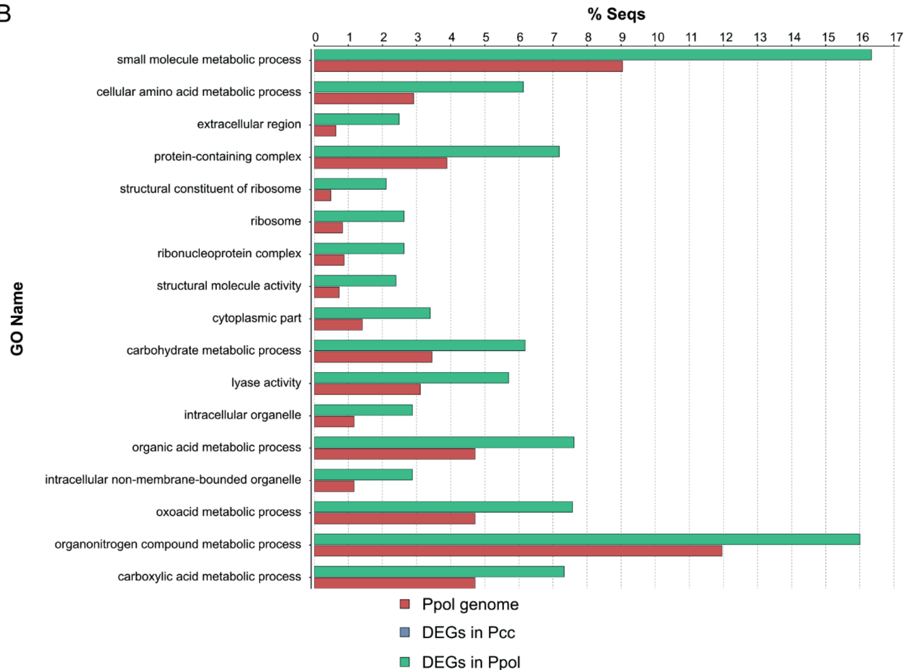


Supplementary figure 1. Distribution of transformed (log2) expression values for *P. polaris* strain NIBIO1006 (Ppol) and *P. c. subsp. carotovorum* strain PK1045-1-2-13 (Pcc) at 24, 48 and 72 hpi. The box plot shows expression values of all genes from each of three biological replicates of bacteria from macerated potato tissue (potato), and M9 minimal medium (M9). Values were transformed by adding a constant of 0.01 and log2 transformation. Box borders mark the 25- and 75-percentile, line in the box indicates the median, whiskers mark the 1.5 interquartile range, and outliers are marked by '+'. Grey background highlights samples that were not included in further analysis due to inconsistent expression ranges and large variation among replicates.

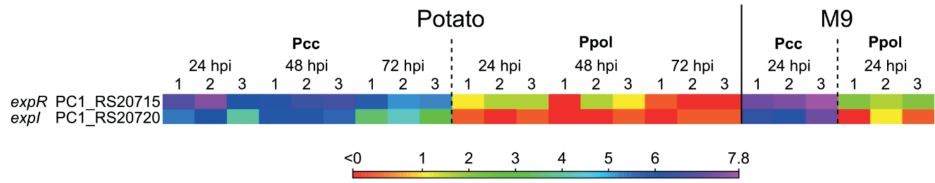


Supplementary figure 2. Similarity of overall gene expression in *P. c.* subsp. *carotovorum* (Pcc) and *P. polaris* (Ppol) at 24, 48 and 72 hpi on potato and 24 hpi on M9 medium. The clustering was performed on transformed expression values of all annotated genes averaged over three biological replicates per sample group. Groups were clustered by average linkage, and the similarity measured by Pearson correlation coefficient distance using Minitab (version 17.2.1).





Supplementary figure 3. Significantly overrepresented GO-terms in DEGs of P. c. subsp. carotovorum (Pcc) and P. polaris (Ppol) during infection. Significant overrepresentation in GO-term frequency (percentage of all genes) compared to the complete Ppol genome was analysed by Fisher's exact test (FDR < 0.05) in Blast2GO (version 5.2.0). Shown are overrepresented GO-terms in a list of all DEGs of Pcc (A) and Ppol (B) over all time points during infection compared to growth on M9 plates.



Supplementary figure 4. Expression of the quorum sensing genes expR and expI for read mapping to the *P. c.* subsp. *carotovorum* strain PC1 (NCBI assembly GCA_000023605.1) genome. Samples were taken during infection of potato tubers at 24, 48 and 72 hours post inoculation (hpi), or at 24 hpi from M9 minimal medium bacterial plates (M9) in three biological replicates each. Gene names and locus tags are given on the left. Expression values were log2-transformed and intensity is indicated on the bottom.

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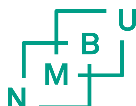

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