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Characterization of *Dickeya* and *Pectobacterium* strains obtained from diseased potato plants in different climatic conditions of Norway and Poland

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Abstract Soft rot and blackleg of potato caused by pectinolytic bacteria lead to severe economic losses in potato production worldwide. To investigate the species composition of bacteria causing soft rot and black leg of potato in Norway and Poland, bacteria were isolated from potato tubers and stems. Forty-one Norwegian strains and 42 Polish strains that formed cavities on pectate medium were selected for potato tuber maceration assays and sequencing of three housekeeping genes (*dnaX*, *icdA* and *mdh*) for species identification and phylogenetic analysis. The distribution of the species causing soft rot and blackleg in Norway and Poland differed: we have demonstrated that mainly *P. atrosepticum* and *P. c.* subsp. *carotovorum* are the causal agents of soft rot and blackleg of potatoes in Norway, while *P. wasabiae* was identified as one of the most important soft rot pathogens in Poland. In contrast to the other European countries, *D. solani* seem not to be a major pathogen of potato in Norway and Poland. The Norwegian and Polish *P. c.* subsp. *carotovorum* and *P. wasabiae* strains did not cluster with type strains of the respective species in the phylogenetic analysis, which underlines the taxonomic complexity of the genus *Pectobacterium.* No correlation between the country of origin and clustering of the strains was observed. All strains tested in this study were able to macerate potato tissue. The ability to macerate potato tissue was significantly greater for the *P. c.* subsp. *carotovorum* and *Dickeya* spp., compared to *P. atrosepticum* and *P. wasabiae*.

Keywords Black leg · Pectinolytic bacteria · Phylogeny · Soft rot · *Solanum tuberosum*

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Introduction

The *Enterobacteriaceae* family contains several important plant pathogens belonging to the genera *Pectobacterium* and *Dickeya* (previously named pectinolytic *Erwinia* spp.) which cause diseases in a wide range of plants, including potato (*Solanum tuberosum*) where they cause soft rot of tubers and blackleg of field-grown plants (McCarter-Zorner et al. 1984; McCarter-Zorner et al. 1985; Czajkowski et al. 2011). Diseases caused by pectinolytic bacteria lead to severe economic losses in potato production worldwide and members of the genera *Pectobacterium* and *Dickeya* are considered to be bacterial plant pathogens of high

importance in Europe and elsewhere (van der Wolf and De Boer 2007; Toth et al. 2011). Thus far, *Pectobacterium* species are considered to be the main cause of soft rot and blackleg of potato, but *Dickeya solani* has spread throughout Europe in relatively short time and is predicted to increase in importance of those diseases the coming years (Toth et al. 2011; Charkowski 2015; Potrykus et al. 2016).

P. carotovorum is a heterogeneous species within the pectinolytic bacteria that includes two subspecies with validly published names; *P. carotovorum* subsp. *carotovorum* and *P. carotovorum* subsp. *odoriferum* (Gallois et al. 1992; Gardan et al. 2003). *P. carotovorum* subsp. *brasiliense,* first described as the causal agent of blackleg disease of potatoes in Brazil, still lacks valid acceptance as a third subspecies of *P. carotovorum* (Duarte et al. 2004; Zhang et al. 2016). Members of *P. carotovorum* subspecies and *Dickeya* species are widely distributed in the world compared to *Pectobacterium atrosepticum*, which is mainly associated with potato in temperate regions (Czajkowski et al. 2011; Toth et al. 2011). *Pectobacterium wasabiae* was first identified as a pathogen on Japanese horseradish (Gardan et al. 2003). A number of strains originally identified as *P. c.* subsp. *carotovorum* have recently been reclassified as *P. wasabiae* (Pitman et al. 2010; Nabhan et al. 2012; Nykyri et al. 2012; Waleron et al. 2013). In 2005, *Pectobacterium chrysanthemi* was elevated into the novel genus named *Dickeya,* which includes the soft rot and blackleg pathogens *D. dadantii, D. dianthicola,* and *D. solani,* besides other species (Samson et al. 2005; Brady et al. 2012; van der Wolf et al. 2014).

The symptoms on potato plants and tubers caused by *Pectobacterium* spp. and *Dickeya* spp. are indistinguishable. Soft rot of tubers is initiated at the stolon end, in wounds or through lenticels, under wet conditions 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 (Kim et al. 2009). Blackleg develops at the stem base of the growing plants after rotting of the mother tuber and is characterized by a slimy, wet, black rot spreading from the mother tuber up the stem (Czajkowski et al. 2011). Soft rot and black leg can develop in plants and tubers from contaminated seed tubers or on tubers in storage. The major source of dissemination of the bacteria is contaminated seed tubers (Pérombelon 2002).

The pathogenesis of the pectinolytic bacteria is influenced and modulated by temperature, humidity and oxygen availability (Pérombelon 2002). Most likely, as a result of increasing temperatures, introduction of new bacterial species as well as shifts in bacterial populations currently present in Europe have been observed (Degefu et al. 2013; Potrykus et al. 2016). New species and subspecies of pectinolytic bacteria (e.g. *D. solani, P. c.* subsp. *brasiliense* and *P. wasabiae*) have been disseminated in Europe, including Poland and Norway. Rising temperatures and increased precipitation in particular, are recognized as important factors that may affect spreading and survival of pectinolytic bacteria, as well as disease development in plants (Toth et al. 2011).

The objectives of the study were to investigate the species composition of pectinolytic bacteria (*Dickeya*/*Pectobacterium* species) recently isolated from diseased potato tubers and stems in Poland and Norway; to measure the strains ability to macerate potato tissue; and to investigate the phylogenetic relatedness between the strains.

Materials and methods

Bacterial strains

The strains used in this study were obtained from symptomatic potato tubers and stems or latently infected tubers in Norway and Poland during the growing season in 2013. The majority of the Norwegian samples originated from south-eastern Norway which is the most important potato production area in the country, whereas Polish samples were collected from all of the potato-growing areas in Poland. In Norway, small pieces of potato tissue were excised from the border between healthy and diseased tissue and then homogenized in 0.9 ml sterile phosphate buffer saline (SPBS) and incubated for 30 minutes at room temperature. A 50 μl aliquot of the homogenate were plated out on modified Bulmer crystal violet pectate (MBCVP) plates (Woodward and Robinson 1990). Nine of the Norwegian strains were isolated from latently infected tubers by shaking tuber stolon ends or pieces of potato peel in 25 ml SPBS over night at 4°C, prior to transfer to MBCVP plates. Plates were incubated at two temperatures (room temperature and 37°C) for 48 h and bacterial colonies that formed cavities on MBCVP plates were 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 28°C. All Norwegian pectinolytic strains were initially identified by fatty acid methyl ester (FAME) analysis (Sasser 1990).

In Poland, pectinolytic bacteria were isolated from 185 potato samples, exhibiting symptoms of soft rot or black leg, obtained from The Polish Inspectorate of Plant Health and Seed Inspection Service and Plant Breeding and Acclimatization Institute - National Research Institute in 2013. One gram of symptomatic potato tissue (either tuber or stem) was placed in an extraction bag (Bioreba, Basel, Switzerland) in 9 ml of SPBS, homogenized with a hand homogenizer (Bioreba, Basel, Switzerland) and serially diluted in sterile 0.85% NaCl. The 10^{-6} and 10^{-5} dilutions of the homogenate were subsequently plated on crystal violet pectate (CVP) plates (Hélias et al. 2012) and incubated for 24 h at 28°C. Bacterial colonies forming cavities on CVP plates were transferred to tryptic soy agar (TSA) plates (Oxoid, USA). An initial identification of the Polish strains was done based on multiplex PCR described by Potrykus *et al.* (2014). For identification of *P. wasabiae* and *D. solani* additional PCR protocols were used (De Boer et al. 2012; Pritchard et al. 2013).

A representative panel of 41 Norwegian and 42 Polish pectinolytic strains was selected for further analysis. All strains were kept in 40% glycerol (v/v) at -80°C for storage. The reference strains and strains isolated for this study are presented in Table 1.

DNA sequencing and phylogenetic analyses

For DNA isolation, pure cultures of pectinolytic bacteria were grown on NGA plates over night at 28°C. Bacteria were harvested from the plates and added directly to the lysis buffer of the DNeasy Blood and tissue kit (Qiagen, GmbH, Hilden, Germany). DNA was isolated according to the manufacturer's instructions.

Partial sequences of three conserved housekeeping genes, *dnaX* (DNA polymerase III subunit tau), *icdA* (isocitrate dehydrogenase) and *mdh* (malate dehydrogenase), were amplified as described in Slawiak et al. (2009a) (*dnaX*) and Ma *et al.* (2007) (*icdA* and *mdh*). The primer sequences are shown in Table 2. The PCR mixture (25 μl) contained 0.4 mM of each primer, 0.2 mM deoxynucleoside triphosphates, 1.5 U AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA), 2.5 μl GeneAmp® 10X PCR Buffer (Applied Biosystems, Foster City, CA) and 2 μl template. The PCR cycle for amplification of *dnaX* consisted of denaturation at 94°C for 3 min; 35 cycles of 94 °C for 1 min, 59°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. The PCR protocol for amplification of *icdA* and *mdh* was the same except for the annealing temperature, which was 52°C. Purified PCR amplicons were sequenced in both directions at GATC Biotech, Germany, using the same primer set as for the PCR amplification. Sequences were assembled, manually edited and aligned using the CLC Main Workbench 7. Sequences from reference strains of *D. dianthicola* (CFBP 1200^T (AOOB01000001)), *P. atrosepticum* (CFBP 1526^T (ALIV01000029)), *P. c.* subsp. *brasiliense* (CFBP 6617^T (NZ_JQOE00000000)), *P. c.* subsp. *carotovorum* (CFBP 2046^T (AODT01000000)); NCPPB 3395 (JQHN01000001); WPP14 (ABVY01000000)) and *P. wasabiae* (CFBP 3304^T (AKVS01000000); NCPPB 3702 (JQOH01000001); RNS08.42.1A (JMDL01000000); WPP163 (NC_013421)) obtained from NCBI were included for comparison. Maximum parsimony analyses of the alignments of *dnaX*, *icdA* and *mdh* and a concatenated alignment of the three genes were performed using the neighbour-joining method implemented in CLC Main Workbench 7 followed by construction of phylogenetic trees. Jukes Cantor model was used for analysis and a consensus tree was built on the basis on 1000 bootstraps. The obtained gene sequences were deposited in GenBank under accession numbers: KX819318- KX819566.

Potato tuber maceration assay

Tuber maceration test was conducted according to Laurila *et al.* (2008) with modifications. Bacterial strains were streaked onto Luria Bertani agar plates and incubated for 48 h at 25°C. Bacteria were scraped from the plates, suspended in sterile distilled water, and the density was adjusted to A_{600} = 1.0 (±0.2) using a spectrophotometer, (Hitachi U-1900, Tokyo, Japan). The reference strains *D. dianthicola* (NCPPB 3345), *D. solani* (NCPPB 4479 = IPO 2222), *P. atrosepticum* (CFBP 1526), *P. c.* subsp. *carotovorum* (CFBP 2046) and *P. wasabiae* (CFBP 3304), were included in the assay for comparison. Tubers of cultivar Irys, stored at 4°C for 2 months, were surface-disinfected by submerging them into a 1% sodium hypochlorite solution for 15

Table 1 Strains of *Pectobacterium* spp. and *Dickeya* spp. included in this study

Strain	Species[1]	Isolation source	Origin	County/ Voivodeship	Maceration ability* (rotten tissue, gram)	Motility** 0.3% agar	Cellulase activity**
IFB0485	Ddth	Potato (stem)	Poland	Masovian	2.4±0.2	22.3±5.9	16.8±0.3
Dsol 748-2-2-12	Ds	Potato (stem)	Norway	Hedmark	11.0±5.7	29.8±0.5	24.5±0.3
IFB0458	Ds	Potato (stem)	Poland	Greater Poland	15.6±1.0	10.5±0.6	26±0.4
IFB5444	Pba	Potato (stem)	Poland	Warmian-Masurian	9.8±0.5	16.3±1.3	7.0±0.4
IFB5445	Pba	Potato (stem)	Poland	Warmian-Masurian	13.2±0.6	19.8±1.7	6.3±0.3
IFB5446	Pba	Potato (stem)	Poland	Warmian-Masurian	0.9±0.0	43.8±1.7	21.5±0.6
IFB5447	Pba	Potato (stem)	Poland	Warmian-Masurian	0.3±0.0	16.8±0.5	6.3±0.6
IFB5448	Pba	Potato (tuber)	Poland	Warmian-Masurian	7.2±0.6	8.5±1.0	7.5±0.3
IFB5449	Pba	Potato (stem)	Poland	Podlaskie	0.3±0.2	6.8±1.4	7.3±0.5
IFB5450	Pba	Potato (stem)	Pol-and	Pomeranian	0.5±0.1	12.0±0.7	5.5±0.3
IFB5451	Pba	Potato (stem)	Poland	Pomeranian	0.2±0.1	20.8±3.1	11.3±1.0
IFB5452	Pba	Potato (stem)	Poland	Pomeranian	0.1±0.1	9.0±0.4	14.5±0.3
IFB5453	Pba	Potato (stem)	Poland	Pomeranian	7.1±0.1	48.3±1.7	11.8±0.9
IFB5454	Pba	Potato (stem)	Poland	Lower Silesian	0.1±0.1	24.3±0.9	6.3±0.3
IFB5455	Pba	Potato (tuber)	Poland	Lower Silesian	8.1±1.6	5.3±0.5	13.8±0.5
IFB5456	Pba	Potato (tuber)	Poland	Podlaskie	0.3±0.2	15.0±0.4	9.0±0.4
IFB5457	Pba	Potato (stem)	Poland	Podkarpackie	1.1±0.4	11.8±0.5	3.5±0.6
OKDS 42-1-12	Pba	Potato (stem)	Norway	Hedmark	9.7±0.0	4.8±0.6	7.3±1.3
OKDS 44-1-12	Pba	Potato (stem)	Norway	Hedmark	5.6±0.4	21.8±0.6	9.3±0.6
OKDS 47-1-12	Pba	Potato (stem)	Norway	Hedmark	8.3±0.5	32.5±1.2	15.0±0.4
OKDS 48-1-12	Pba	Potato (stem)	Norway	Hedmark	8.8±0.1	12.8±0.8	7.0±0.4
OKDS 49-1-12	Pba	Potato (stem)	Norway	Hedmark	6.7±0.3	12.5±0.6	8.3±0.5
OKDS 51-1-12	Pba	Potato (stem)	Norway	Hedmark	9.1±0.8	21.5±0.6	9.5±0.9
OKDS 53-1-12	Pba	Potato (stem)	Norway	Hedmark	0.9±0.1	19.5±0.6	4.5±0.3
OKDS 56-1-12	Pba	Potato (stem)	Norway	Hedmark	1.1±0.1	27.0±0.9	11.8±1.2
Øst 1-1-13	Pba	Potato (tuber)	Norway	Hedmark	7.0±0.1	13.3±0.9	8.8±0.3
Peik kirkej. 1-13	Pba	Potato (stem)	Norway	Akershus	7.0±0.6	12.3±0.6	6.8±0.3
Peik kirkej. 2-13	Pba	Potato (stem)	Norway	Akershus	6.6±0.5	10.5±0.6	6.8±0.3
Peik kirkej. 3-13	Pba	Potato (stem)	Norway	Akershus	8.0±0.8	35.8±0.3	8.3±0.5
PK 1085-1-3-13	Pba	Potato (tuber)	Norway	Troms	7.3±0.7	25.5±1.4	6.5±0.6
PK 782-1-1-13	Pba	Potato (stem)	Norway	Hedmark	10.4±0.6	11.0±0.4	7.3±0.3

Table 1 (continued)

Strain	Species[1]	Isolation source	Origin	County/ Voivodeship	Maceration ability* (rotten tissue, gram)	Motility** 0.3% agar	Cellulase activity**
PK 782-1-2-13	Pba	Potato (stem)	Norway	Hedmark	11.3±0.6	22.3±1.1	9.0±0.4
Sol 1-1-13	Pba	Potato (tuber)	Norway	Hedmark	6.9±0.1	12.8±0.9	7.3±0.3
Sol 1-3-13	Pba	Potato (tuber)	Norway	Hedmark	8.1±0.6	19.3±0.5	10.8±0.3
Sol 3-3-13	Pba	Potato (tuber)	Norway	Hedmark	9.3±0.4	10.0±0.4	7.8±0.3
Ewelina 52	Pcc	Potato (stem)	Poland	West Pomeranian	13.0±0.8	35.8±0.9	19.5±0.6
IFB5502	Pcc	weed	Poland	Warmian-Masurian	0.1±0.1	39.0±1.1	20.5±0.3
IFB5503	Pcc	Potato (stem)	Poland	Lower Silesian	0.6±0.2	27.5±1.6	16.0±0.4
IFB5504	Pcc	Potato (tuber)	Poland	Podkarpackie	0.2±0.2	26.3±1.0	20.0±0.4
IFB5505	Pcc	Potato (tuber)	Poland	Warmian-Masurian	4.4±1.7	22.3±1.3	14.0±0.4
IFB5506	Pcb	Potato (tuber)	Poland	Podlaskie	16.5±2.2	24.3±0.9	9.8±0.5
IFB5508	Pcb	Potato (stem)	Poland	Greater Poland	21.3±0.5	36.5±0.9	13.0±0.4
IFB5509	Pcc	Potato (stem)	Poland	Pomeranian	10.0±2.6	34.3±0.5	17.5±0.3
IFB5512	Pcc	Potato (tuber)	Poland	Podkarpackie	9.4±0.8	31.5±1.3	17.8±0.5
na 992-11-13	Pcc	Potato (tuber)	Norway	Hedmark	12.4±0.5	42.3±1.0	24.5±0.3
na 992-20-13	Pcc	Potato (tuber)	Norway	Hedmark	12.7±2.0	20.5±0.6	15.8±0.3
na 992-6-1-13	Pcc	Potato (tuber)	Norway	Hedmark	13.1±0.3	16.0±0.4	10.0±0.4
na 992-9-1-13	Pcc	Potato (tuber)	Norway	Hedmark	1.9±0.6	16.0±0.4	11.0±0.4
Øst 3-3-13	Pcc	Potato (tuber)	Norway	Hedmark	12.4±0.5	35.0±0.4	14.8±0.3
Øst 3-4-13	Pcc	Potato (tuber)	Norway	Hedmark	12.5±0.5	31.5±0.9	14.5±0.3
PK 1045-1-2-13	Pcc	Potato (tuber)	Norway	Møre og Romsdal	7.2±1.5	38.8±1.3	14.5±0.3
PK 1045-1-3-13	Pcc	Potato (tuber)	Norway	Møre og Romsdal	11.1±1.0	32.5±0.6	12.8±0.3
PK 1045-1-4-13	Pcc	Potato (tuber)	Norway	Møre og Romsdal	10.6±0.5	19.8±0.9	10.0±0.6
PK 57-1-1-13	Pcc	Potato (tuber)	Norway	Hedmark	9.4±1.5	16.5±0.6	17.5±0.5
PK 57-1-2-13	Pcc	Potato (tuber)	Norway	Hedmark	13.0±0.6	35.0±5.5	14.5±0.3
PK 60-1-13	Pcc	Potato (tuber)	Norway	Hedmark	10.8±0.2	31.5±0.6	12.8±0.3
PK 60-2-13	Pcc	Potato (tuber)	Norway	Hedmark	10.9±0.1	24.8±0.5	13.8±0.3
sk 992-12-13	Pcc	Potato (tuber)	Norway	Hedmark	19.5±0.0	25.5±0.6	15.5±0.5
sk 992-16-13	Pcc	Potato (tuber)	Norway	Hedmark	12.6±1.0	32.0±8.7	15.8±0.3
sk 992-19-13	Pcc	Potato (tuber)	Norway	Hedmark	18.9±2.0	10.8±0.5	19.5±0.3
sk 992-20-13	Pcc	Potato (tuber)	Norway	Hedmark	16.8±1.0	25.8±1.1	16.3±0.3
sk 992-9-1-13	Pcc	Potato (tuber)	Norway	Hedmark	14.6±0.1	39.8±0.9	10.8±0.3

Table 1 (continued)

Strain	Species[1]	Isolation source	Origin	County/ Voivodeship	Maceration ability* (rotten tissue, gram)	Motility** 0.3% agar	Cellulase activity**
Sol 2-2-13	Pcc	Potato (tuber)	Norway	Hedmark	13.9±0.2	33.8±0.9	18.5±0.3
Sol 4-4-13	Pcc	Potato (tuber)	Norway	Hedmark	16.9±1.0	31.5±3.5	23.8±0.8
Sol 5-2-13	Pcc	Potato (tuber)	Norway	Hedmark	12.1±0.1	18.3±3.7	12.3±0.6
Denar 51	Pwa	Potato (stem)	Poland	Kuyavian-Pomeranian	11.6±1.6	25.3±0.5	5.5±0.3
IFB5400	Pwa	Potato (tuber)	Poland	West Pomeranian	1.7±1.1	12.3±1.3	7.3±0.9
IFB5407	Pwa	Potato (stem)	Poland	Pomeranian	0.1±0.1	22.0±1.1	10.5±0.3
IFB5408	Pwa	Potato (root)	Poland	Pomeranian	0.3±0.1	22.3±1.3	11.3±0.5
IFB5412	Pwa	Potato (stem)	Poland	West Pomeranian	0.2±0.2	25.8±3.0	10.8±0.3
IFB5415	Pwa	Potato (stem)	Poland	Pomeranian	1.5±0.1	27.5±1.2	18.0±3.6
IFB5420	Pwa	Potato (stem)	Poland	Lower Silesian	0.4±0.2	25.0±2.6	10.5±0.3
IFB5425	Pwa	Potato (stem)	Poland	Greater Poland	0.4±0.3	25.5±0.6	13.3±0.3
IFB5427	Pwa	weed	Poland	Podkarpackie	2.5±0.1	24.5±1.6	11.5±0.3
IFB5430	Pwa	Potato (tuber)	Poland	Kuyavian-Pomeranian	11.2±1.3	41.5±0.6	11.5±0.3
IFB5497	Pwa	Potato (stem)	Poland	Pomeranian	2.3±1.2	22.5±1.2	10.8±0.5
IFB5498	Pwa	Potato (root)	Poland	Pomeranian	0.3±0.1	24.3±0.9	9.8±0.3
IFB5500	Pwa	Potato (stem)	Poland	Kuyavian-Pomeranian	1.1±0.1	29.0±6.8	12.3±0.6
IFB5501	Pwa	Potato (stem)	Poland	Lower Silesian	0.1±0.0	26.0±0.4	12.5±0.3
IFB5507	Pwa	Potato (stem)	Poland	Greater Poland	11.9±0.6	19.5±0.9	10.8±0.3
IFB5511	Pwa	Potato (stem)	Poland	Masovian	2.5±1.2	4.5±0.3	0.0±0.0
IFB5513	Pwa	Potato (tuber)	Poland	Masovian	1.3±0.6	12.3±0.9	11.8±0.3
Pwas Pol al.	Pwa	Potato (stem)	Norway	Aust-Agder	8.0±1.6	28.5±5.2	10.8±0.5
CFBP 1526	Pba[2]	Potato	Scotland, 1957	NA	2.3±0.46	NA	NA
CFBP 2046	Pcc[2]	Potato	Denmark, 1952	NA	6.4±1.42	NA	NA
CFBP 3304	Pwa[2]	Wasabi	Japan, 1985	NA	3.8±1.91	NA	NA
NCPPB 3345	Ddth[3]	Potato	France, 1984	NA	2.1±0.31	NA	NA
NCPPB 4479 = PRI2222	Ds[3]	Potato	The Netherlands, 2010	NA	7.3±0.98	25.5±3.2	22.3±0.3

*= Ten tubers were inoculated per strain and the average amount of rotten tissue (gram) measured after 3 days of incubation (±SE). **=mean diameter (±SE). [1] Ds = *Dickeya solani*; Ddth = *Dickeya dianthicola*; Pba = *Pectobacterium atrosepticum*; Pcb = *Pectobacterium carotovorum* subsp. *brasiliense*; Pcc = *Pectobacterium carotovorum* subsp. *carotovorum; Pectobacterium wasabiae.* [2] IRM-CFBP, International Center for Microbial Resources – French Collection for Plant-associated Bacteria, INRA-ACO-UA, France. [3] NCPPB, The Food and Environment Research Agency, United Kingdom; NA = not available

Table 2 Primers used in this study for PCR amplification and sequencing

Target gene	Primer	Sequence (5'→ 3')	Sequence length of amplicon	Reference
dnaX	dnaXf dnaXr	TATCAGGTYCTTGCCCGTAAGTGG TCGACATCCARCGCYTTGAGATG	535	Slawiak *et al.* 2009a
icdA	icdA400F icdA977R	GGTGGTATCCGTTCTCTGAACG TAGTCGCCGTTCAGGTTCATACA	520	Ma *et al.* 2007
mdh	mdh86F mdh628R	CCCAGCTTCCTTCAGGTTCAGA CTGCATTCTGAATACGTTTGGTCA	460	Ma *et al.* 2007

min, then rinsed in distilled water and air dried at room temperature one day before inoculation. The tubers were wounded by piercing them with a steel rod (diameter: 2 mm; length: 10 mm). The wounds were inoculated with 10 μl of bacterial suspension and covered with white vaseline and parafilm. Ten tubers were inoculated per strain and additionally 10 tubers were inoculated with water as negative control. The tubers were placed in plastic boxes and sprayed with water. The boxes were covered with lids and incubated for 3 days at 26°C. After incubation, the tubers were cut vertically through the inoculation points and weight of rotten tissue was measured. The experiment was carried out twice.

Plate assays for biochemical and physiological characterization

Bacterial strains were grown overnight on TSA, harvested and suspended in sterile 0.85% sodium chloride (NaCl), then adjusted to a density of A_{600} = 0.1. For evaluation of the swimming and swarming capacities, four aliquots of 2.5 μl of the bacterial suspensions were spotted onto nutrient broth (NB, Oxoid, USA) supplemented with 0.3% and 0.8% agar (Kearns 2010). The diameters of the bacterial colonies were measured after 24 hours at 28°C. The activity of secreted cellulases was evaluated in a plate assay with carboxymethyl cellulose (CMC). Four aliquots of 2.5 μl of the bacterial suspension were spotted onto the minimal media M63Y + 1% CMC (Hankin and Anagnostakis 1977) and plates were incubated for 72 h at 28°C. Cellulase activity was visualized by staining with 0.5% Congo red for 5 min and de-staining in 2M NaCl solution for 10 min, after which the diameter of the halo around the colonies was measured. All plate assays were carried out in four replicates.

Statistical analysis

Analysis of variance (ANOVA), general linear model, was performed using Minitab 16 to compare the statistical significance of the observed differences in the ability to macerate potato tubers by strains belonging to *Pectobacterium* and *Dickeya* species.

Results

In 2013, pectinolytic bacteria were isolated from symptomatic or latently infected potato tubers and stems in Norway and Poland. Forty-one Norwegian strains and 42 Polish strains that formed cavities on CVP media were selected for potato tuber maceration assays and sequencing of three housekeeping genes (*dnaX*, *icdA* and *mdh*) for species identification and phylogenetic analysis. The Norwegian strains were initially identified by FAME analysis, as either *P. carotovorum* or *P. atrosepticum,* in addition to one *D. solani* strain and the Polish strains were initially classified as *P. atrosepticum, P. carotovorum, P. wasabiae*, *D. dianthicola* or *D. solani* by PCR.

Phylogenetic analysis

Partial sequences of the genes *dnaX* (444 nucleotides), *icdA* (452 nucleotides) and *mdh* (449 nucleotides) obtained from the Norwegian and Polish strains were subjected to Basic Local Alignment Search Tool (BLAST) to determine the species identity of the strains. Twenty-one Norwegian strains and nine Polish strains were identified as *P. carotovorum*; 18 Norwegian strains and 14 Polish strains belonged to *P. atrosepticum*; one Norwegian and 17 Polish strains were identified as

P. wasabiae; and only three strains were assigned to the *Dickeya* genus (*D. solani*: one Norwegian and one Polish strain; *D. dianthicola*: one Polish strain) (Table 1). Further, the partial sequences of *dnaX*, *icdA* and *mdh* were aligned with reference sequences obtained from GenBank. The three alignments were concatenated and a maximum parsimony phylogram was constructed using the neighbor-joining method (Fig. 1). The pectinolytic bacteria identified by BLAST analysis grouped separately into four main clusters corresponding to three *Pectobacterium* species: *P. atrosepticum*, *P. carotovorum*, *P. wasabiae;* and *Dickeya* spp.. Phylogenetic analysis revealed greatest variation among *P. carotovorum* strains regardless of country of origin. *P. carotovorum* grouped into three main clusters: one group with the type strain of *P. c.* subsp. *carotovorum* (CFBP 2046) and *P. c.* subsp. *carotovorum* strain (WPP 14) obtained from the GenBank; a second cluster contained the majority of the Norwegian and the Polish strains and the *P. c.* subsp. *carotovorum* strain NCPPB 3395. Moreover, two of the Polish strains (IFB5506 and IFB5508), initially identified as *P. carotovorum*, clustered in a third subgroup together with the type strain of *P. c*. subsp. *brasiliense* (CFBP 6617). The type strain of *P. wasabiae* (CFBP 3304), isolated from Japanese horseradish, clustered separately from the Norwegian and Polish strains of *P. wasabiae,* but together with the *P. wasabiae* strain NCPPB 3702, isolated from Japanese horseradish. The Norwegian and Polish strains of *P. wasabiae* clustered with *P. wasabiae* strains isolated from potato in Finland (SCC3193), France (RNS 08.42.1A) and Wisconsin, USA (WPP 163).

Less variation was observed among the *P. atrosepticum* strains. However, five of the *P. atrosepticum* strains (IFB5457, OKDS44-1-12, OKDS47-1-12, OKDS51-1-12 and OKDS53-1-12) constituted a separate sub-cluster on the basis of 99.3 % identity to the rest of the strains within this species. One Norwegian and one Polish strain clustered together with the type strain of *D. solani* (IPO 2222), while one of the Polish strains grouped with the type strain of *D. dianthicola* (CFBP 1200). The phylogenetic trees computed separately for each of the genes *dnaX*, *icdA* and *mdh* showed the same resolution of the taxonomic relationships as for the concatenated tree (data not shown). No single strain appeared in the cluster of a different species in any of the trees.

Potato tuber maceration assay

All strains tested in this study were able to macerate potato tissue. Ten tubers were inoculated per strain and the average amount of rotten tissue measured after 3 days of incubation was in the range of 0.1 g to 21.3 g (Table 1). ANOVA test (general linear model) was performed to assess the statistical significance of the observed differences in the ability of the strains to macerate potato tubers. In general, maceration was significantly greater for the Norwegian strains compared to the Polish ones ($P = 0.005$). The ability to macerate potato tissue was significantly greater for the *P. c.* subsp. *carotovorum* and *Dickeya* spp. ($P = 0.001$), compared to *P. atrosepticum* and *P. wasabiae*. The ability of the reference strains to macerate potato tissue decreased in the following order: *D. solani* (NCPPB 4479), followed by *P. c.* subsp. *carotovorum* (CFBP 2046), *P. wasabiae* (CFBP 3304), *P. atrosepticum* (CFBP 1526) and *D. dianthicola* (NCPPB 3345). The plant part origin of the pectinolytic bacteria from where it was isolated did not influence the virulence of the strains ($P = 0.369$). No maceration was observed on the control tubers inoculated with water.

Biochemical and physiological characterization of the strains

Swimming and swarming motility assays were performed in order to assess the flagellar mediated motility. All 83 strains tested were motile on 0.3% agar, however, none of them showed motility on 0.8% agar. Furthermore, all but one of the strains (IFB5511) showed activity of secreted cellulases in a plate assay with carboxymethyl (1% CMC medium).

Discussion

The objective of this study was to characterize and compare the pectinolytic bacteria populations in two countries with different climatic conditions (temperature and humidity). For this, 41 strains of pectinolytic bacteria from Norway and 42 strains from Poland were collected and characterized.

The initial species identification of the pectinolytic strains was done either by FAME or by multiplex PCR (Potrykus et al. 2014). A comparison of the results from

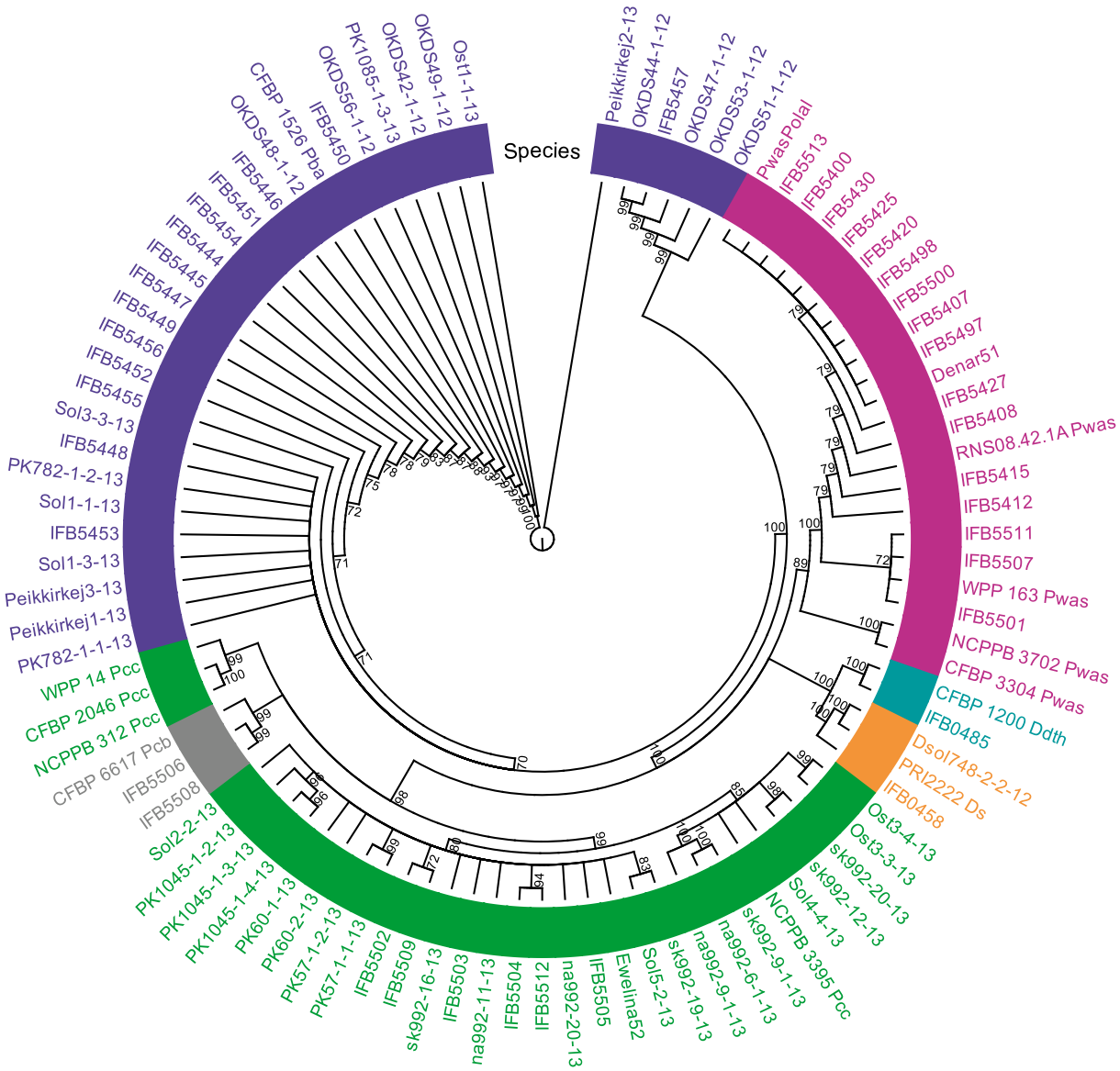

Fig. 1 Maximum parsimony phylogeny of 83 Norwegian and Polish *Pectobacterium* sp. and *Dickeya* sp. strains, produced by neighbor joining method, using concatenated gene portions of *dnaX*, *icdA* and *mdh*. Jukes Cantor model was used for analysis and the consensus tree was built on the basis on 1000 bootstraps. Ddth (petrol blue): *Dickeya dianthicola;* Ds (orange): *Dickeya solani*; Pba (blue): *Pectobacterium atrosepticum*; Pcb (gray): *Pectobacterium carotovorum* subsp. *brasiliense;* Pcc (green): *Pectobacterium carotovorum* subsp. *carotovorum;* Pwa (pink): *Pectobacterium wasabiae*. The sequences of the following strains were obtained from the GenBank: Ddth CFBP 1200^{T}; Pba CFBP 1526^{T}; Pcb CFBP 6617^{T}; Pcc CFBP 2046^{T}, NCPPB 3395, WPP 14; Pwa CFBP 3304^{T}, NCPPB 3702, RNS08.42.1A, WPP 163

species identification by FAME and multiplex PCR with the sequencing results from this study, revealed that many of the strains initially identified as *P. carotovorum* were in fact *P. wasabiae*. This confirms the limitations of FAME analysis, multiplex PCR (Potrykus et al. 2014) and *P. wasabiae* specific PCR based on "PhR/PhF"-primers (De Boer et al. 2012) as classification methods for current populations of pectinolytic bacteria.

Identification by sequencing of the three housekeeping genes revealed 32 strains of *P. atrosepticum*, 28 strains of *P. c.* subsp. *carotovorum,* 18 strains of *P. wasabiae* and three *Dickeya* sp. strains. Two of the Polish strains (IFB5506, IFB5508) initially identified as

P. carotovorum clustered with the type strain of *P. c.* subsp. *brasiliense* (CFBP 6617). *P. c.* subsp. *brasiliense* is previously identified in Poland (Waleron et al. 2015) and more investigations need to be done to confirm the sub-species identity of the two Polish strains included in this study.

The distribution of the species in Norway and Poland differed: *P. atrosepticum* and *P. c.* subsp. *carotovorum* were the most common species causing soft rot and blackleg in Norway, while *P. c.* subsp. *carotovorum* and *P. wasabiae* were found most frequently in potato samples in Poland. The abundance of *P. wasabiae* in Poland confirmed findings of a previous study by Waleron et al. (2013). The limitations of the most common identification methods have likely led to the underestimation of *P. wasabiae* worldwide. The re-identification of many *P. c.* subsp. *carotovorum* strains as *P. wasabiae,* including the Finish *P. wasabiae* strain SCC3193, shows that the latter species has already been present in potato in Europe for many years (De Boer et al. 2012; Nabhan et al. 2012; Nykyri et al. 2012; Waleron et al. 2013). In Norway, only a few strains of *P. wasabiae* have been obtained so far, including one presented in this study. This could be due to the relatively strict plant health regulations in Norway that prohibit import of seed potatoes and hence limits the introduction of new potato pathogens. On the contrary, seed potatoes are imported to Poland from other European countries. Besides that, about 2% of the total potato production area in Poland is used for seed potato production; however, Poland is not subjected to strict plant health regulations (Dzwonkowski 2016; Stypa and Urbanowicz 2016).

D. solani was isolated in Norway in 2013 from a diseased potato plant sampled in an experimental field where imported seed potatoes were planted. Similarly, only a few strains of *D. solani* has been identified in Poland in 2013. However, a higher number of *D. solani* strains was isolated in Poland in 2009, 2010 and 2011 (Slawiak et al. 2009b; Potrykus et al. 2016). The decrease in *D. solani* incidences in Poland may be due to rejection of diseased seed lots after testing for the bacteria. Moreover, the prevalence of *Dickeya* species is increasing in Europe (Toth et al. 2011; Degefu et al. 2013; Gill et al. 2014; van der Wolf et al. 2014; Potrykus et al. 2016), but this does not seem to be true for Norway thus far.

Phylogenetic analysis of the strains showed grouping of the species *P. atrosepticum*, *P. carotovorum, P. wasabiae* and *Dickeya* spp. into distinct clusters (Fig. 1). A very recent re-evaluation of the taxonomy of *Dickeya* spp. and *Pectobacterium* spp. reveal that a number of genomes deposited in GenBank are misnamed and that some strains might represent new species (Zhang et al. 2016).

The greatest intraspecific variation was observed among the *P. carotovorum* strains, which is consistent with previous studies (Nabhan et al. 2012; Pasanen et al. 2013). Interestingly, five of the Norwegian strains designated as *P. carotovorum* clustered together with *P. c.* subsp. *carotovorum* NCPPB 3395 which has been suggested to represent a novel species in a recently published re-evaluation of the taxonomy of *Dickeya* spp. and *Pectobacterium* spp. (Zhang et al. 2016). The re-evaluation study is based on phylogenomics and systematic analysis of eighty-three genomes from the two genera. Despite the limitation of our study with the inclusion of only three housekeeping genes, the same clustering pattern can be observed for the ten strains included from the study by Zhang *et al*. (2016). Interestingly, none of the Norwegian or Polish *P. wasabiae* strains grouped with the type strain of *P. wasabiae* (CFBP 3304), but with the two *P. wasabiae* strains RNS08.42.1A and WPP 163, isolated from potato plants in France and USA, respectively, which were suggested to be a subspecies of *P. wasabiae* (Zhang et al. 2016) or a separate species (Waleron et al. 2013). A high level of homogeneity was observed among the *P. atrosepticum* strains, which also has been demonstrated in earlier studies (Darrasse et al. 1994; Nabhan et al. 2012).

Strains isolated from either potato tubers or stems did not form any sub-clusters based on their plant part origin. However, results from another phylogenetic study based on the intergenic spacer region (IGS), indicated a clear separation of the strains associated with stems or tubers (Pasanen et al. 2013). No correlation between the country of origin and clustering of the strains was observed in the phylogenetic analysis. The lack of correlation between geographical origin and classification of the strains was also observed by Nabhan et al. (2012). Despite the geographic separation of at least 700 km and the relatively strict Norwegian import regime since 1914, it might be suggested that the Norwegian and the Polish strains share the same origin. Considering the speed at which bacteria evolve, a more likely explanation is that the selected housekeeping genes are conserved and did not provide resolution

sufficient for studying intraspecific variation. Still, some migration of the bacteria cannot be excluded, i.e. on illegally transported potatoes and other plants, or in soil attached to the wheels of vehicles, etc.

All strains tested in this study were able to macerate potato tissue, which was in agreement with our expectations due to the initial selection of colonies that formed cavities on CVP plates. In general, the average maceration rate was significantly greater for the Norwegian strains than the Polish ones. This may indicate differences in expression of virulence genes among the bacterial strains. However, when looking at the maceration rate of the individual strains, the Polish *P. carotovorum* strain IFB5508 showed the highest maceration rate among all strains. Furthermore, the most aggressive *P. atrosepticum* and *P. c.* subsp. *carotovorum* strains from Norway and Poland did not differ significantly in maceration rate. The ability to macerate potato tissue was significantly greater for *P. carotovorum* and *Dickeya* spp. ($P = 0.001$), compared to *P. atrosepticum* and *P. wasabiae*. The observed intraspecies variation in maceration ability is in agreement with previous studies (Laurila et al. 2008; Toth et al. 2011). There were only two *D. solani* strains identified in this study, and their maceration ability was relatively high. This is in agreement with former studies indicating high maceration ability of *D. solani* strains (Slawiak et al. 2009a; Slawiak et al. 2009b; van der Wolf et al. 2014; Potrykus et al. 2016). The optimal temperature for efficient production of lytic enzymes is in the range between 12 and 24°C (Latour et al. 2007), which could have influenced the maceration rate of the different strains. In general, the greater maceration ability for *P. carotovorum*, compared to *P. atrosepticum* and *P. wasabiae*, could be partly explained by the temperature used in this study (26°C), which was slightly more favorable for *P. c.* subsp. *carotovorum*. The reference strains used in this study showed maceration ability in agreement with the optimal temperature requirements for the species. The higher the temperature, the greater maceration, with the exception of the strain *D. dianthicola* (NCPPB 3345), which was not significantly different from *P. atrosepticum* (CFBP 1526) (Table 1).

Our results showed that multilocus phylogenetic analysis using housekeeping genes present in nearly all enterobacteria, provides information for a robust phylogeny. We have demonstrated that mainly *P. atrosepticum* and *P. c.* subsp. *carotovorum* are the causal agents of soft rot and blackleg disease of potatoes in Norway, while *P. c.* subsp. *carotovorum* and *P. wasabiae* have been identified as the most important soft rot pathogens in Poland. The recent re-evaluation of the taxonomy of *Dickeya* and *Pectobacterium* (Zhang et al. 2016) underlines the complexity of the genera and some of the Norwegian and Polish strains did cluster with representatives for newly suggested species or subspecies of *P. c.* subsp. *carotovorum* and *P. wasabiae*, respectively. In contrast to the other European countries, *D. solani* seems not to be a major pathogen of blackleg and soft rot of potato, neither in Poland or Norway.

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