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Genotypic and phenotypic uniformity among the population of *Pectobacterium atrosepticum* strains isolated during three growing seasons from potato fields in Poland

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Abstract *Pectobacterium atrosepticum* (Pba) are phytopathogenic, pectinolytic, non-sporulating, gram-negative rods causing blackleg and soft rot diseases in potato. They are ubiquitously present under temperate climate conditions and contribute to significant economic losses. Currently, there are no efficient control methods against soft rot *Pectobacteriaceae* (SRP). As limitation of the spread of SRP is solely prevention-based, we undertook broad characterization on both genotypic and phenotypic levels of the *P. atrosepticum* isolates acquired during monitoring of potato fields in Poland in 2013, 2014 and 2016. Of REP, ERIC and BOX-based genomic fingerprinting, the BOX-based approach turned out to be the most informative and allowed for classification of 118 Pba strains into six groups. The present study indicated that IV and I BOX profiles dominated among the investigated population. Finally, 23 Pba strains representing all BOX groups and originating from various growing seasons were selected for genotypic, phylogenetic and phenotypic studies. *rpoS*-based phylogeny revealed intraspecies variation (16 SNP sites) among the studied Pba strains, in contrast to the analyses relying on *gyrA* (1 SNP) and *recA* (0 SNP) sequences. Pba strains showed higher potency to macerate potato tissue at 20 °C than 28 °C. These isolates exhibited rather uniform production of plant cell wall degrading enzymes (pectinases, cellulases and proteases), lipases, siderophores and biofilm, however in the majority of cases inferior in comparison to *Dickeya solani* and *Pectobacterium carotovorum* strains. In conclusion, this study revealed genotypic and phenotypic uniformity in addition to high virulence potential of Pba strains isolated from potato fields during several growing seasons in Poland.

Keywords Soft rot · Blackleg · Pectinolytic bacteria · *Pectobacteriaceae*

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Introduction

Pectobacterium atrosepticum is a Gram(-), non-sporulating necrotrophic plant pathogenic bacterium responsible for blackleg and soft rot on potato (Perombelon & Kelman, 1980). It was isolated for the first time nearly 120 years ago by C. J. J. van Hall and assigned to *Bacillus atrosepticus* species (van Gijsegem et al., 2021a, b). Subsequently, due to improvement of characterization and differentiation methods, the taxonomic position of this phytopathogen was changed to *Erwinia carotovora* subsp. *atroseptica*, then *Pectobacterium carotovorum* subsp. *atrosepticum* to be finally classified as *P. atrosepticum* within the family *Pectobacteriaceae*, order *Enterobacterales* (Adeolu et al., 2016; Gardan et al., 2003; Hauben et al., 1998; Winslow et al., 1920).

Until the 1970s, *P. atrosepticum* was stated as a unique cause of potato blackleg and main pathogen responsible for potato soft rot disease in Europe (van Gijsegem et al., 2021a, b), putatively due to strict association with temperate climate conditions (Toth et al., 2003). Later on, other soft rot *Pectobacteriaceae* (SRP) species such as *Dickeya solani*, *Pectobacterium parmentieri* or *Pectobacterium brasiliense* started being detected on this continent, though *P. atrosepticum* was still frequently isolated in potato-growing countries and significantly contributed to the local SRP populations (Dees et al., 2017; Motyka-Pomagruk et al., 2021). To underline the importance of *P. atrosepticum,* this species was placed by Mansfield et al. (2012) on the top ten list of plant pathogenic bacteria. Moreover, the firstly published full genomic sequence of any SRP was that of *P. atrosepticum* SCRI1043 (Bell et al., 2004).

Importantly, *P. atrosepticum* in contrast to some other SRP, like *e.g. Pectobacterium carotovorum*, *P. brasiliense* or *Dickeya dadantii*, is regarded to have a narrow host range (Ma et al., 2007). *P. atrosepticum* causes blackleg and soft rot on potato, though strains from this species were also previously isolated from *Brassica rapa*, *Helianthus annuus*, *Solanum melongena*, *Zantedeschia aethiopica* or asymptomatic unspecified weeds originating from potato fields (Motyka-Pomagruk et al., 2021; Toth et al., 2021). The disease symptoms on potato include maceration of the inner tissue of the tubers known as soft rot, in addition to blackening of the stem base leading to wilting of the whole plant referred to as blackleg (Perombelon & Kelman, 1980). Blackleg symptoms are restricted to the field conditions, while soft rot is recognized in the fields, transportation, storage and during marketing of the tubers. However, *P. atrosepticum* has been isolated more frequently to the present day from affected potato stems than tubers (Motyka-Pomagruk et al., 2021; Śledź et al., 2000).

To observe the typical disease symptoms, a virulent pathogen needs to be present, as well as a susceptible host and environmental conditions favoring bacterial multiplication (Barrett et al., 2009). At first, *P. atrosepticum* cells infiltrate susceptible plant host *via* wounds or natural openings such as lenticels (Perombelon & Kelman, 1980). Afterwards, bacterial cells remain in the vascular tissue and plant intercellular spaces until the infection-favoring conditions occur (Toth et al., 2003). In the meantime, the pathogen produces extracellular polysaccharide (EPS), a component of the 3D biofilm structure, which is essential for facing water flow and plant defense mechanisms during colonization of the plant xylem (Gorshkov et al., 2016). In SRP, including the cells of *P. atrosepticum*, the quorum sensing mechanism strictly controls the transcription factors-dependent regulatory network, which is activated after reaching a required cell density threshold (Liu et al., 2008). As a result, efficient production of major and minor virulence factors begins (Liu et al., 2008). *P. atrosepticum* secretes plant cell wall degrading enzymes (PCWDEs) extracellularly, *via* Type I or Type II secretion systems. Pectinases, cellulases and proteases depolymerize main components of the plant cell wall, triggering leakage of the protoplast content, which is rich in nutrients highly valuable for the pathogen (Perombelon, 2002). Of the PCWDEs listed, pectinases, including pectate lyases, pectin lyases, pectin methyl esterases and polygalacturonases (Barras et al., 1994), contribute to the highest extent to the virulence of this pest. Moving to the additional PCWDEs, two cellulases (*CelV*/*CelB* in addition to *CelS*) and up to six putative proteases have been identified in *P. atrosepticum* (van Gijsegem et al., 2021a, b). Even though in a closely related SRP species inactivation of a single or several genes coding for the above-listed enzymes did not end up in a complete loss of bacterial pathogenicity, reduction in the resultant disease symptoms was noted (van Gijsegem et al., 2021a, b). Access to the nutrients is furthermore provided by the action of lipases and

nucleases, releasing readily available fatty acids and oligonucleotides, respectively (van Gijsegem et al., 2021a, b). Recently, a notable impact on the overall virulence of *P. atrosepticum* was attributed to the production of an iron-scavenging siderophore, namely enterobactin (Gorshkov et al., 2021).

Interestingly, previous genome-oriented studies based on polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) or amplified fragments length polymorphism (AFLP) suggested high genomic homogeneity within the *P. atrosepticum* species in contrast to, for instance, *P. carotovorum* (Darrasse et al., 1994; Ngadze et al., 2012; Waleron et al., 2002). The uniformity of the genomes of distinct *P. atrosepticum* strains is especially puzzling taking into consideration that these strains have been isolated for many years from all continents where potato plants are grown (van der Wolf et al., 2021a, b). Ubiquitous presence of *P. atrosepticum* strains results from efficient spread by various artificial and environmental routes. These disease-causing agents may be dispersed by shipping of latently infected plant material or transfer of plant debris containing SRP cells (Motyka et al., 2017). *P. atrosepticum* cells may also originate from alternative plant hosts or be spread by various vectors such as insects, maggots or nematodes (Rossmann et al., 2018; Toth et al., 2021). As other sources of these pathogens, there are listed waterways (McCarter-Zorner et al., 1984), soil (Peltzer & Sivasithamparam, 1988), air, aerosols and surfaces of field machinery or storage containers (Toth et al., 2021). Despite the high economic importance and common occurrence of *P. atrosepticum*, the currently applied methods to combat SRP, are solely prevention-based (Czajkowski et al., 2011). Monitoring of the presence of *P. atrosepticum* on potato fields followed by a detailed characterization of the acquired isolates seems crucial in finding a way to manage this pathogen.

In order to broaden the current knowledge on this phytopathogen, we extensively characterized the *P. atrosepticum* isolates acquired in years 2013-2016 during monitoring of potato fields in Poland for the presence of SRP (Motyka-Pomagruk et al., 2021). We applied Repetitive Extragenic Palindromic (REP), Enterobacterial Repetitive Intergenic Consensus (ERIC) and BOX-based repetitive sequence-based PCRs (rep-PCR) to reveal genomic variability among 118 *P. atrosepticum* strains. Subsequently, 23 representative *P. atrosepticum* strains exhibiting the 6 assigned BOX profiles and preferably originating from various growing seasons were selected for further genotypic and phenotypic analyses. *rpoS*, *gyrA* and *recA*-based phylogenies showed extremely high homogeneity among all 23 studied *P. atrosepticum* strains. In terms of phenotypic traits, the abilities of *P. atrosepticum* strains to macerate potato tissue, produce plant cell wall degrading enzymes (pectinases, cellulases and proteases), lipases, siderophores and biofilm were investigated in comparison to 5 reference strains belonging to the other SRP species.

Materials and methods

Bacterial isolation and identification

P. atrosepticum strains were isolated in 2013, 2014 or 2016 during monitoring of potato fields in Poland for the presence of blackleg- and soft rot-causing bacteria as described by (Motyka-Pomagruk et al., 2021; Potrykus et al., 2016; Zoledowska et al., 2018a).

Briefly, inspectors from the Regional Inspectorates of Plant Health and Seed Inspection Service in Poland (PH & SIS) and the employees of the collaborating plant breeding institutions collected samples of blackleg- or soft rot-affected potato plants or tubers (for more information on the studied samples see Motyka-Pomagruk et al., 2021). Shortly after arrival, 1 g of each plant or tuber tissue was manually homogenized in 10 mL phosphate buffer (pH = 7.2) in an extraction bag (Bioreba, Reinach, Switzerland). The obtained suspension was serially diluted to 10^{-6} in 0.85% NaCl solution. 100 µl of 10^{-5} and 10^{-6} dilutions were plated on Crystal Violet Pectate (CVP) medium (Hélias et al., 2012). After 48 h incubation at 28 °C, the observed cavity-forming colonies of pectinolytic bacteria, were picked and replated several times either on CVP or Trypticase Soy Agar (TSA) media to acquire axenic cultures.

Preliminary identification of pectinolytic isolates was performed with multiplex PCR (Potrykus et al., 2014a). Assignment to the *P. atrosepticum* species was based on acquisition of a specific 420 bp amplicon in a single PCR reaction conduced with Y45 and Y46 starters according to Frechon et al. (1998). The identified *P. atrosepticum* strains were frozen in 40%

(v/v) glycerol and kept at -80 °C in the collection of plant pathogenic bacteria of IFB UG & MUG.

Genotypic and phylogenetic analyses

Genomic DNA of the 23 selected *P. atrosepticum* isolates and 6 reference strains (Table 1) was isolated from single overnight cultures in Trypticase Soy Broth (TSB) (BTL, Łodz, Poland) with Genomic Mini AX Bacterial Kit (A&A Biotechnology, Gdansk, Poland) according to the manufacturer's instructions. The quality and concentration of the isolated DNA was assessed spectrophotometrically with a NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA) and stored at 4 °C for further use.

Genomic profiling of *P. atrosepticum* strains was based on three independent rep-PCRs targeting ERIC, REP and BOX elements according to Versalovic et al., (1991, 1994).

The isolated DNA was diluted to reach a concentration of 10 ng μL^{-1}. The rep-PCR reactions were performed in 25 µL PCR mixtures containing 5 µL of 10 ng μL^{-1} DNA, 2.5 µl of 10×concentrated Taq buffer with $(NH_4)_2SO_4$, 3.5 µl of 25 mM $MgCl_2$, 2.5 µl of 2 mM dNTPs, 0.1 µl of 100 µM ERIC1R and ERIC2 primers or REP1R and REP2 primers or BOX A1R primer, 0.4 µl of 5 U μl^{-1} *Taq* recombinant DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and the nuclease-free water (Invitrogen, Waltham, MA, USA). The PCR reactions consisted of the following steps: initial denaturation (95 °C, 7 min), 30 cycles of denaturation (94 °C, 1 min), annealing (52 °C, 1 min – ERIC; 40 °C, 1 min – REP or 53 °C, 1 min – BOX), and elongation (65 °C, 8 min) with a final single extension step (65 °C, 16 min). After mixing with 6×Loading Dye solution (Thermo Fisher Scientific, Waltham, MA, USA) the resultant amplicons were separated by electrophoresis (50 V, 4 h) in 1% agarose gel suspended in 0.5×Tris Borate EDTA (TBE) with the use of the Mupid-One (Takara, Kusatsu, Japan) system. GeneRuler™ 1 kb DNA ladder (Thermo Fisher Scientific, Waltham, USA) was utilized as a band size reference. Post separation, the gel was stained in 0.5 mg L^{-1} ethidium bromide solution (15 min), washed in distilled water (10 min) and visualized under UV light with Molecular Imager® ChemiDoc™ XRS transluminator (Bio-Rad, Hercules, CA, USA).

Phylogenetic analysis of the *P. atrosepticum* strains and the reference strains (Table 1) was based on the comparison of the *rpoS* (Waleron et al., 2008), *recA* (Waleron et al., 2002) and *gyrA* (Waleron et al., 2008) gene sequences. Amplification of the *rpoS*, *recA* and *gyrA* gene fragments was carried out as described previously (Waleron et al., 2002, 2008). Briefly, the PCR reaction was performed in a 50 µL mixture containing 1 µL of 10 ng μL^{-1} DNA, 5 µl of 10×concentrated Taq buffer with KCl, 4 µl of 25 mM $MgCl_2$, 5 µl of 1 mM dNTPs, 10 pmol of rpoS1 and rpoS2 primers in the case of *rpoS* gene, recA1 and recA2 primers regarding *recA* gene, gyrA1 and gyrA4 primers concerning *gyrA* gene and 1 U *Taq* recombinant DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA). The following steps were included: initial denaturation (95 °C, 3 min), 32 cycles of denaturation (94 °C, 1 min), annealing (55 °C, 1 min for *rpoS*; 47 °C, 1 min for *recA*; 56 °C, 1 min for *gyrA*), and elongation (72 °C, 2 min) with a final single extension step (72 °C, 5 min).

The resultant PCR products of 920 bp for *rpoS*, 737 bp for *recA* and 1018 bp for *gyrA*, respectively, were sequenced from both ends by a commercial company (Genomed, Warsaw, Poland), manually edited and subjected to multiple sequence alignment by the ClustalW algorithm in the MEGA X (Pennsylvania State University; www.megasoftware.net; (Kumar et al., 2018)) software. The *rpoS*, *recA* and *gyrA*-based phylogenetic trees were also constructed in MEGA X (Kumar et al., 2018). A maximum likelihood algorithm with the Tamura-Nei model was used. Bootstrap was set at the level of 1000 replicates. The lengths of the tree branches were associated with the number of substitutions per site. The corresponding gene sequences of *D. solani* IFB0099 were applied as the outgroups. The sequences of *rpoS*, *recA* and *gyrA* genes of *P. atrosepticum* isolates were submitted to the GenBank database and are freely available under the accession numbers listed in Table 2.

Phenotypic analyses

Overnight cultures in TSB of the 23 selected *P. atrosepticum* strains and the reference strains (Table 1) were centrifuged (10 min, 4779 g) and the cell pellet was washed twice in sterile 0.85% NaCl. Subsequently, either 0.5 McF or OD_{600}=0.1 bacterial suspensions in 0.85% NaCl were prepared with the use of densitometer DEN-1B (Biosan,

Table 1 Characteristics of the *Pectobacteriaceae* strains included in this study

L.p./Species	Strain no	Year of Isolation	Collection Site	Host	Reference
P. atrosepticum strains isolated in Poland					
1	IFB5650	2013	Kukły, Warmia-Masuria Province	Potato stem, cv. Iris	This study and Motyka-Pomagruk et al. (2021)
2	IFB5651	2013	Kukły, Warmia-Masuria Province	Potato stem, cv. Iris	This study and Motyka-Pomagruk et al. (2021)
3	IFB5652	2013	Kukły, Warmia-Masuria Province	Potato stem, cv. Iris	This study and Motyka-Pomagruk et al. (2021)
4	IFB5653	2013	Szkody, Warmia-Masuria Province	Potato stem, cv. Irga	This study and Motyka-Pomagruk et al. (2021)
5	IFB5654	2013	Kukły, Warmia-Masuria Province	Potato tuber, cv. Irga	This study and Motyka-Pomagruk et al. (2021)
6	IFB5655	2013	Berżniki, Podlasie Province	Potato stem, cv. Irga	This study and Motyka-Pomagruk et al. (2021)
7	IFB5656	2013	Słupia, Pomerania Province	Potato stem, cv. Tajfun	This study and Motyka-Pomagruk et al. (2021)
8	IFB5657	2013	Rokitnica, Lower Silesia Province	Potato tuber, cv. Lord	This study and Motyka-Pomagruk et al. (2021)
9	IFB5658	2013	Rokitnica, Lower Silesia Province	Potato tuber, cv. Lord	This study and Motyka-Pomagruk et al. (2021)
10	IFB5659	2014	Dunowo, West Pomerania Province	Potato tuber, cv. Tajfun	This study and Motyka-Pomagruk et al. (2021)
11	IFB5660	2014	Biskupiec, Warmia-Masuria Province	Potato stem, cv. Irga	This study and Motyka-Pomagruk et al. (2021)
12	IFB5661	2014	Lichtajny, Warmia-Masuria Province	Potato stem, cv. Cyprian	This study and Motyka-Pomagruk et al. (2021)
13	IFB5662	2014	Złotopole, Kujawy-Pomerania Province	Potato stem, cv. Kuba	This study and Motyka-Pomagruk et al. (2021)
14	IFB5663	2014	Wiercień Mały, Podlasie Province	Potato stem, cv. Eafana	This study and Motyka-Pomagruk et al. (2021)
15	IFB5664	2014	Bukowiec, Kujawy-Pomerania Province	Potato tuber, cv. Lord	This study and Motyka-Pomagruk et al. (2021)
16	IFB5665	2016	Tuszów Narodowy, Subcarpathia Province	Potato stem, cv. Red Sonia	This study
17	IFB5666	2016	Iłownica, Pomerania Province	Potato stem, cv. Vineta	This study
18	IFB5667	2016	Liniewo, Pomerania Province	Potato stem, cv. Vineta	This study

Table 1 (continued)

L.p./Species	Strain no	Year of Isolation	Collection Site	Host	Reference
19	IFB5668	2016	Tuszów Narodowy, Subcarpathia Province	Potato tuber, cv. Red Sonia	This study
20	IFB5669	2016	Małki, Kujawy-Pomerania Province	Potato tuber, cv. Red Anna	This study
21	IFB5670	2016	Małki, Kujawy-Pomerania Province	Accompanying weed	This study
22	IFB5671	2016	Małki, Kujawy-Pomerania Province	Potato stem, cv. Red Anna	This study
23	IFB5672	2016	Mielęcin, West Pomerania Province	Potato stem, cv. Skawa	This study
Reference strains of soft rot *Pectobacteriaceae*					
Pba	LMG 2386^{TS}	1957	Scotland	Potato stem	Potrykus et al. (2014a)
Pc	IPO 200	1974	The Netherlands	Potato	Potrykus et al. (2014a)
Pbr	LMG 21371	1999	Brasil	Potato cv. Elvira	Duarte et al. (2004)
Pwa	CFBP 3304	1985	Japan	Horseradish	Goto and Matsumoto (1987)
Ppa	SCC 3193	1980s	Finland	Potato	Nykyri et al. (2012)
Dsol	IFB0099	2005	Poland	Potato, cv. Santa	Slawiak et al. (2009)PP

Pba, *P. atrosepticum*; Pc, *P. carotovorum*; Pbr, *P. brasiliense*; Pwa, *P. wasabiae*; Ppa, *P. parmentieri*; Dsol, *D. solani*

Riga, Latvia) or spectrophotometer Specord 200 Plus (Analytik Jena, Jena, Germany), respectively, depending on the conducted assay.

Potato maceration assay

Potato slice maceration assay was performed as described previously (Zoledowska, et al., 2018a) with some modifications. Briefly, potato tubers cv. Lord were acquired from a local market, washed under tap water and surface-sterilized by immersing for 10 min in 10% NaOCl. After washing-off disinfectant residuals, the tubers were dried in a laminar flow cabinet. Additionally, 15 min UV-exposure was applied to both sides of the tubers. Surface-sterilized potato tubers were cut in 1 cm slices. Then, up to 3 holes (of 5 mm in diameter) were drilled in each potato slice. Potato slices were placed on moistened Whatman filter paper in sterile plastic boxes. 50 µl of $OD_{600} = 0.1$ bacterial suspension of the tested *P. atrosepticum* strain (Table 1) or the reference SRP strain (Table 1) were introduced into one hole. The control samples, *i.e.* 50 µl of 0.85% NaCl solution-inoculated holes were included. After covering the boxes with the lids, potato slices were incubated under anaerobic, humid conditions for 48 h at either 28 °C or 20 °C depending on the experiment. Subsequently, the diameters (in millimeters) of the resultant rotten spots were measured. The collected results corresponded to the potato tissue maceration capacity of the studied strains. Each experiment was repeated twice with seven replicates per tested strain.

Growth at different temperature conditions

Bacterial ability to grow at diverse temperatures, *i.e.* at 20 °C, 28 °C and 37 °C (Golanowska et al., 2017) was evaluated as follows: 5 µl of 0.5 McF bacterial suspension of the tested *P. atrosepticum* strain (Table 1) or the reference SRP strain (Table 1) were placed on the surface of TSA medium. The abilities of bacterial strains to form colonies after 72 h incubation at 20 °C, 28 °C or 37 °C were recorded based on visual observations. The experiment was repeated three times with two replicates each. A plus sign was assigned to strains exhibiting the ability to grow at the examined temperature, a minus sign was attributed to strains incapable of multiplying. If discrepancies

Table 2 The selected genomic and phenotypic traits of the *Pectobacteriaceae* strains included in this study

L.p./Species	Strain no	BOX profile	Accession numbers of *rpoS*, *recA* and *gyrA* sequences[a]	Growth on medium with 5% NaCl	Growth at temperature 20 °C	28 °C	37 °C
P. atrosepticum strains isolated in Poland							
1	IFB5650	II	OM105620 OM105597 OM158465	-	+	+	-
2	IFB5651	II	OM105621 OM105598 OM158466	-	+	+	-
3	IFB5652	II	OM105622 OM105599 OM158467	-	+	+	-
4	IFB5653	IV	OM105623 OM105600 OM158468	-	+	+	-
5	IFB5654	II	OM105624 OM105601 OM158469	-	+	+	-
6	IFB5655	I	OM105625 OM105602 OM158470	-	+	+	-
7	IFB5656	IV	OM105626 OM105603 OM158471	-	+	+	-
8	IFB5657	III	OM105627 OM105604 OM158472	-	+	+	-
9	IFB5658	I	OM105628 OM105605 OM158473	-	+	+	-
10	IFB5659	I	OM105629 OM105606 OM158474	-	+	+	-
11	IFB5660	V	OM105630 OM105607 OM158475	-	+	+	-
12	IFB5661	V	OM105631 OM105608 OM158476	-	+	+	-
13	IFB5662	IV	OM105632 OM105609 OM158477	-	+	+	-
14	IFB5663	IV	OM105633 OM105610 OM158478	-	+	+	-
15	IFB5664	I	OM105634 OM105611 OM158479	-	+	+	-
16	IFB5665	I	OM105635 OM105612 OM158480	-	+	+	-

Table 2 (continued)

L.p./Species	Strain no	BOX profile	Accession numbers of *rpoS*, *recA* and *gyrA* sequences[a]	Growth on medium with 5% NaCl	Growth at temperature 20 °C	28 °C	37 °C
17	IFB5666	IV	OM105636 OM105613 OM158481	-	+	+	-
18	IFB5667	VI	OM105637 OM105614 OM158482	-	+	+	-
19	IFB5668	I	OM105638 OM105615 OM158483	-	+	+	-
20	IFB5669	III	OM105639 OM105616 OM158484	-	+	+	-
21	IFB5670	III	OM105640 OM105617 OM158485	-	+	+	-
22	IFB5671	III	OM105641 OM105618 OM158486	-	+	+	-
23	IFB5672	IV	OM105642 OM105619 OM158487	-	+	+	-
Reference strains of soft rot *Pectobacteriaceae*							
Pba	LMG 2386^{TS}	I		-	+	+	-
Pc	IPO 200	NA		+	+	+	+
Pbr	LMG 21371	NA		+	+	+	+
Pwa	CFBP 3304	NA		-	+	+	-
Ppa	SCC 3193	NA		-	+	+	-/+
Dsol	IFB0099	NA		-	+	+	+

NA, not available; Pba, *P. atrosepticum*; Pcc, *P. carotovorum*; Pcbr, *P. brasiliense*; Pwa, *P. wasabiae*; Ppa, *P. parmentieri*; Dsol, *D. solani*; +, growth; -, lack of growth; -/+, inconclusive result

[a]The ordering of the accession numbers is as follows: *rpoS*, *recA* and *gyrA* gene sequences

were observed between the replicates the outcome was marked as inconclusive.

Growth on medium with 5% NaCl

The bacterial ability to grow at an elevated salinity level (5% NaCl) (Sarfraz et al., 2020) was evaluated as follows. 5 µl of 0.5 McF bacterial suspension of the tested *P. atrosepticum* strain (Table 1) or the reference SRP strain (Table 1) was placed on the surface of TSA + 5% NaCl medium. Incubation of the plates at 28 °C for 72 h followed. Bacterial ability to form visible colonies was assessed. The experiment was repeated three times with two replicates each.

Pectinases activity

Pectinases activity of bacterial strains was investigated on M63 + PGA medium as described previously (Reverchon et al., 1986) with some modifications. 1.5 µl of $OD_{600} = 0.1$ bacterial suspension of the tested *P. atrosepticum* strain (Table 1) or the reference SRP strain (Table 1) was placed on the surface of M63 + PGA medium. Incubation at 28 °C for 48 h followed. Subsequently, the medium was flooded with 10% $Cu(CH_3COO)_2$ for 10 min. The plates then stained blue, while white halo zones around bacterial colonies resulted from PGA degradation. The diameters of the halo zones were

measured. The experiment was repeated three times with four replicates each.

Cellulases activity

Cellulases activities of bacterial strains were investigated on M63 + CMC medium as described previously (Teather & Wood, 1982; Zoledowska et al., 2018a) with some modifications. 1.5 µl of $OD_{600} = 0.1$ bacterial suspension of the tested *P. atrosepticum* strain (Table 1) or the reference SRP strain (Table 1) was placed on the surface of M63 + CMC medium. Incubation at 28 °C for 48 h followed. Then the medium was flooded with 1% Congo red solution for 5 min and subsequently washed three times with 4 M NaCl. The plates stained red, while clear halo zones around bacterial colonies resulted from CMC degradation. The diameters of the above-mentioned halo zones were measured and corresponded to the cellulases activities of the studied strains. The experiment was repeated three times with four replicates each.

Proteases activity

Protease activity of bacterial strains was investigated on skimed milk agar medium as described previously (Ji et al., 1987) with some modifications. 1.5 µl of $OD_{600} = 0.1$ bacterial suspension of the tested *P. atrosepticum* strain (Table 1) or the reference SRP strain (Table 1) was placed on the surface of skim milk agar medium. Incubation at 28 °C for 48 h followed. The diameters of the halo zones around bacterial colonies were measured. The experiment was repeated three times with four replicates each.

Lipases activity

Lipases activities of bacterial strains were investigated on rhodamine medium as described previously (Samad et al., 1989) with some modifications. 1.5 µl of $OD_{600} = 0.1$ bacterial suspension of the tested *P. atrosepticum* strain (Table 1) or the reference SRP strain (Table 1) was placed on the surface of a rhodamine medium. Incubation at 28 °C for 48 h followed. The diameters of the halo zones around bacterial colonies were measured. The experiment was repeated twice with four replicates each.

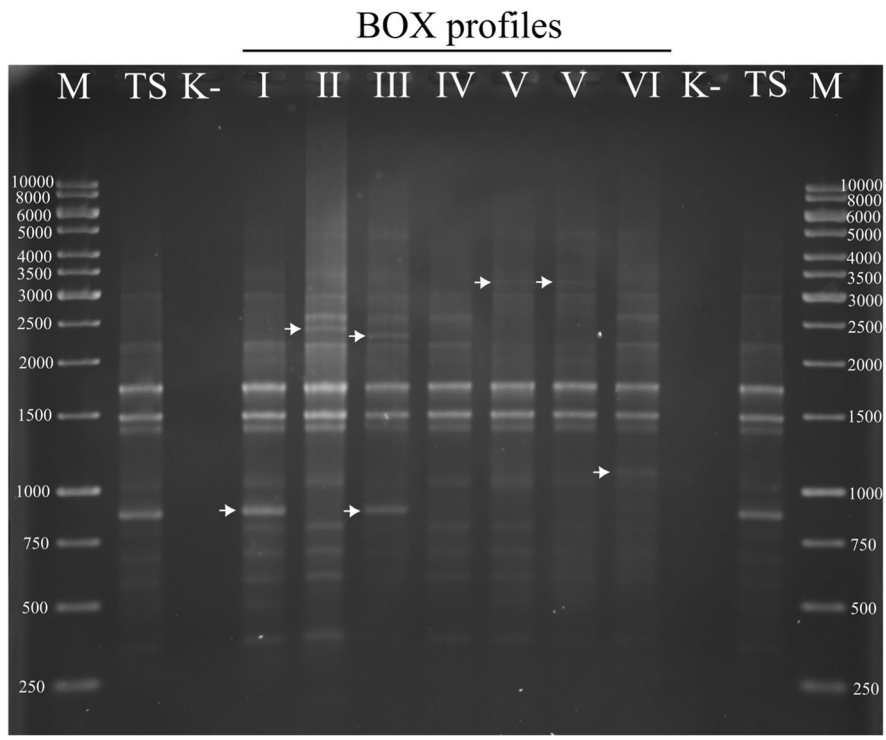

Fig. 1 The BOX genomic profiles assigned to *P. atrosepticum* strains isolated from potato fields in Poland. BOX profiles for the following representative *P. atrosepticum* strains were shown: BOX profile I of IFB5664 strain, BOX profile II of IFB5650 strain, BOX profile III of IFB5670 strain, BOX profile IV of IFB5662 strain, BOX profile V of IFB5660 and IFB5661 strains and BOX profile VI of IFB5667 strain. Arrows mark discriminatory bands of the differentiated BOX profiles. M: molecular marker GeneRuler™ 1 kb DNA ladder (Thermo Fisher Scientific, Waltham, USA). TS: BOX profile I attributed to *P. atrosepticum* type strain LMG 2386^{TS}, K-: PCR negative control

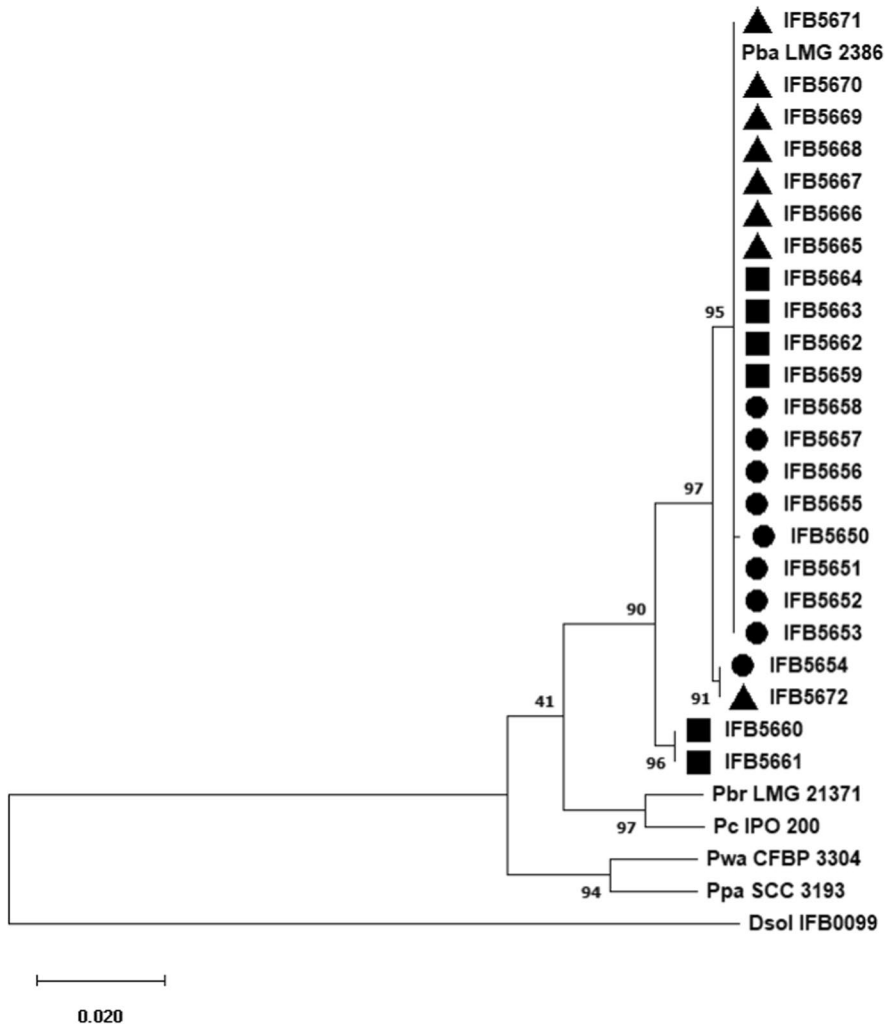

Fig. 2 Phylogenetic analysis of *P. atrosepticum* strains based on the sequences of *rpoS* gene. *P. atrosepticum* strains isolated in Poland were juxtaposed to the following *Pectobacteriaceae* reference strains: *P. atrosepticum* LMG 2386^{TS}, *P. brasiliense* LMG 21371, *P. carotovorum* IPO 200, *P. wasabiae* CFBP 3304 and *P. parmentieri* SCC 3193. *D. solani* IFB0099 was used as an outgroup. Multiple sequence alignment was performed with the ClustalW algorithm in the MEGA X software (Kumar et al., 2018). The tree was generated by application of a maximum likelihood algorithm with Tamura-Nei model also in MEGA X (Kumar et al., 2018). Bootstrap was set on the level of 1000 replicates. The lengths of the tree branches were associated with the number of substitutions per site. The year of isolation of the *P. atrosepticum* strains was marked with the following signs: a dot – 2013, a square – 2014 and a triangle – 2016

Siderophores production

Siderophores production of bacterial strains was investigated on a chrome azurol S (CAS) agar medium as described previously (Schwyn & Neilands, 1987) with some modifications. 1.5 µl of $OD_{600}=0.1$ bacterial suspension of the tested *P. atrosepticum* strain (Table 1) or the reference SRP strains (Table 1) was placed on the surface of CAS medium. Incubation at 28 °C for 48 h followed. The diameters of the orange halo zones around bacterial colonies were measured. The experiment was repeated three times with two replicates each.

Biofilm formation assay

Biofilm formation capacity of bacterial strains was investigated as described previously (Babinska et al., 2021) with some modifications. 10 µl of an overnight culture in TSB medium of the tested *P. atrosepticum* strain (Table 1) or the reference strain (Table 1; *Pseudomonas fluorescens* CCM2115; *Pseudomonas aeruginosa* PAO1) was inoculated into 1.5 ml volume microcentrifuge tube (Eppendorf, Germany) with 400 µl of M9 minimal medium. Static incubation at 18 °C for 16 h followed. The ring-shape biofilm was stained with 70 µl of 1% crystal violet for 20 min. The biofilm was then three times washed with distilled

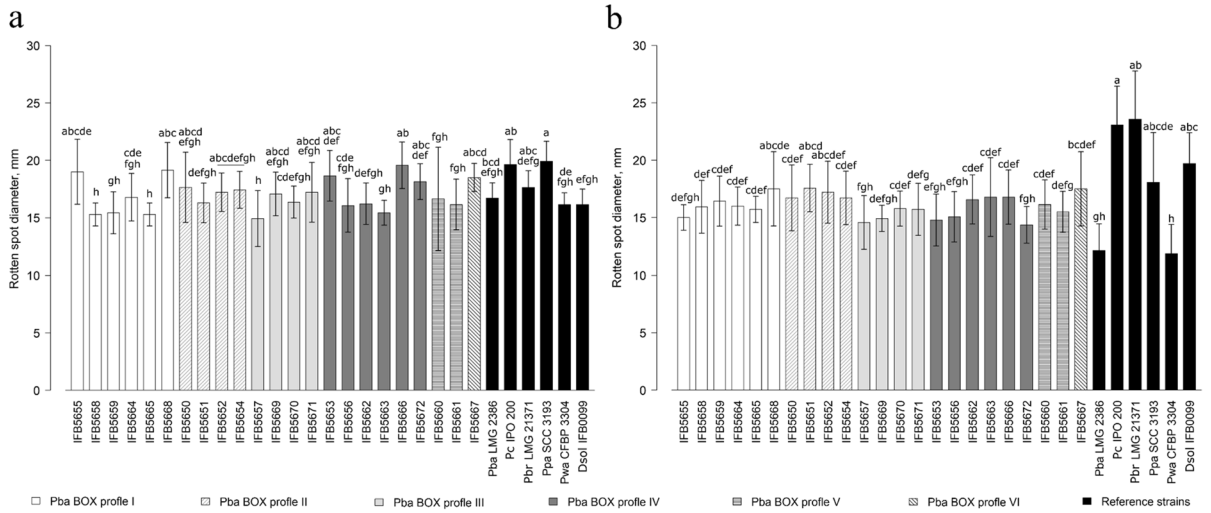

Fig. 3 Potato tissue maceration capacity of *P. atrosepticum* strains isolated from potato fields in Poland. Standard potato slice assay was performed, potato slices inoculated with bacterial suspension were incubated at 20 °C (**a**) or 28 °C (**b**) for 48 h and the observed rotten spot diameters were measured. The lengths of the columns (colored accordingly to the BOX profiles assigned to the *P. atrosepticum* strains) of the bar chart correspond to means, while standard deviations are shown on the error bars. Different letters mark maceration capacities that diverge in a statistically significant manner (according to Kruskal-Wallis test prior to a *post hoc* analysis applying Fisher's least significant difference criterion with Bonferroni correction) at $p < 0.05$. Pba – *Pectobacterium atrospeticum*; Pc – *Pectobacterium carotovorum*; Pbr – *Pectobacterium brasiliense*; Ppa – *Pectobacterium parmentieri*; Pwa – *Pectobacterium wasabiae*; Dsol – *Dickeya solani*

water and air-dried. For quantitative evaluation 600 µl of 96% ethanol was added to each biofilm-containing microcentrifuge tube. The absorbance of the extracted crystal violet, which corresponded to the biofilm-formation capacity was assessed spectrophotometrically at 565 nm with Victor2 I420 Multilabel Counter (Wallac, Finland) in a 96-well microplate (Sarstedt, Germany). *P. fluorescens* CCM2115 and *P. aeruginosa* PAO1 were included in this analysis as positive controls of potent biofilm producers. The experiment was repeated twice with four replicates each.

Statistical analysis and data visualization

The gathered data were analysed in R (R Core Team, 2021) version 3.1.3 with the use of the *agricolae* package. The equality of variances and the data normality were determined by Levene's and Shapiro-Wilk's tests, respectively. If the requirements for parametric analysis were fulfilled, the analysis of variance (ANOVA) followed by Tukey's honest significant difference test was performed (Fig. 4a). Otherwise, the Kruskal-Wallis test prior to the *post hoc* applying Fisher's least significant difference criterion with Bonferroni correction was utilized (Figs. 3, 4b, c, and 5). Details on the statistical tests applied for each dataset are presented in the Figure legends. $p < 0.05$ was applied. Inkscape version 0.92.3 (https://inkscape.org/) was used for assembling the Figures.

Results

Genotypic analysis of *P. atrosepticum* strains originating from potato fields in Poland

In years 2013–2014, 531 samples of potato plants or tubers were collected from potato fields or storage facilities in Poland and have been tested for the presence of bacterial phytopathogens from the *Pectobacteriaceae* family (Motyka-Pomagruk et al., 2021). For the current study we also analyzed 124 samples of potato stems, 88 potato tubers, and 47 accompanying weeds picked from potato fields or storage facilities in year 2016. Altogether, 118 strains (35, 41 and 42 from 2013, 2014 and 2016, respectively), that formed cavities on CVP medium were identified (Frechon et al.,

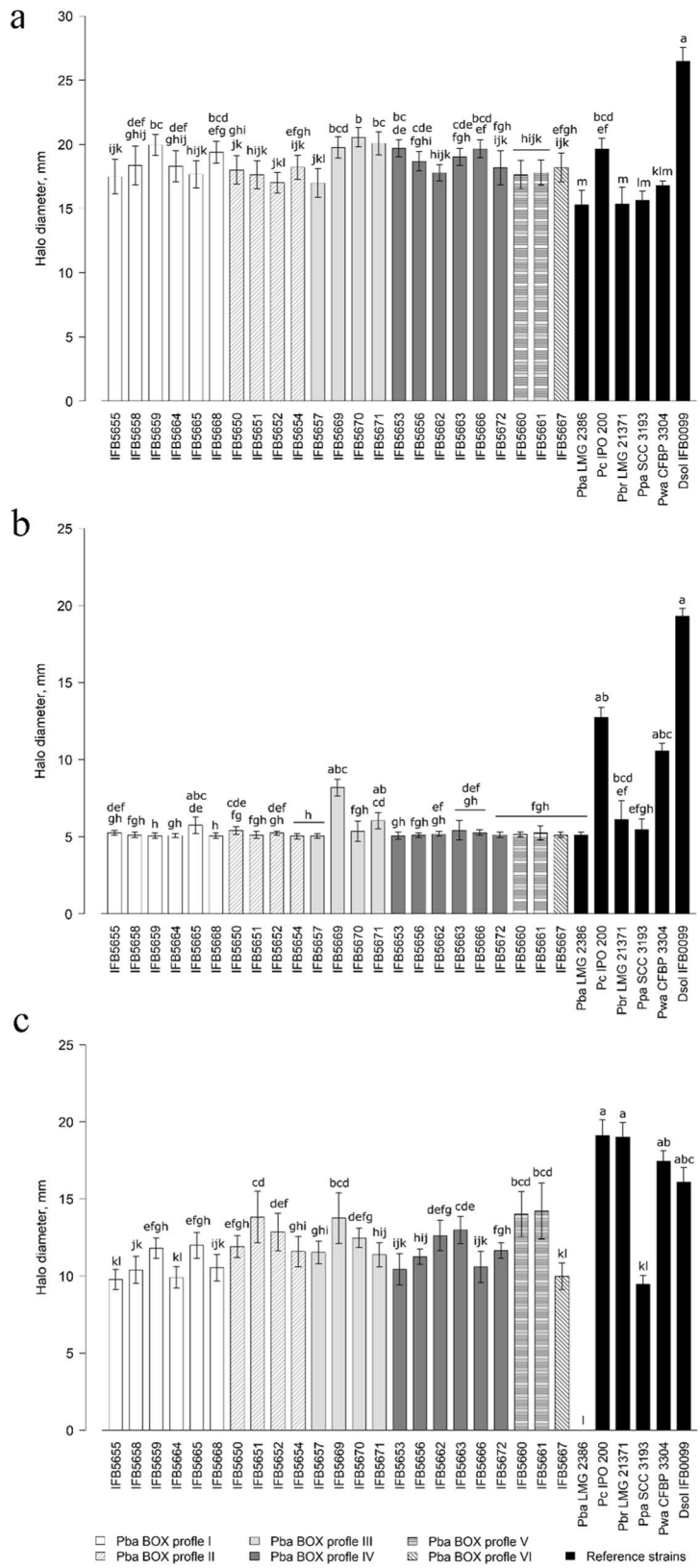

Fig. 4 The plant cell wall degrading enzyme activities of pectinases (**a**), cellulases (**b**) and proteases (**c**) among *P. atrosepticum* strains isolated from potato fields in Poland. M63 + PGA (**a**), M63 + CMC (**b**) and Skim milk (**c**) -based plate assays were performed. The bacteria-inoculated plates were incubated at 28 °C for 48 h and the resultant halo diameters around bacterial colonies were measured. The lengths of the columns (colored accordingly to the BOX profiles assigned to the *P. atrosepticum* strains) of the bar chart correspond to means, while standard deviations are shown on the error bars. Different letters mark enzyme activities that diverge in a statistically significant manner at $p < 0.05$ according either to ANOVA followed by Tukey's honest significant difference test (**a**) or Kruskal-Wallis test followed by a *post hoc* applying Fisher's least significant difference criterion with Bonferroni correction (**b**, **c**). Pba—*Pectobacterium atrospeticum*; Pc—*Pectobacterium carotovorum*; Pbr—*Pectobacterium brasiliense*; Ppa—*Pectobacterium parmentieri*; Pwa—*Pectobacterium wasabiae*; Dsol—*Dickeya solani*

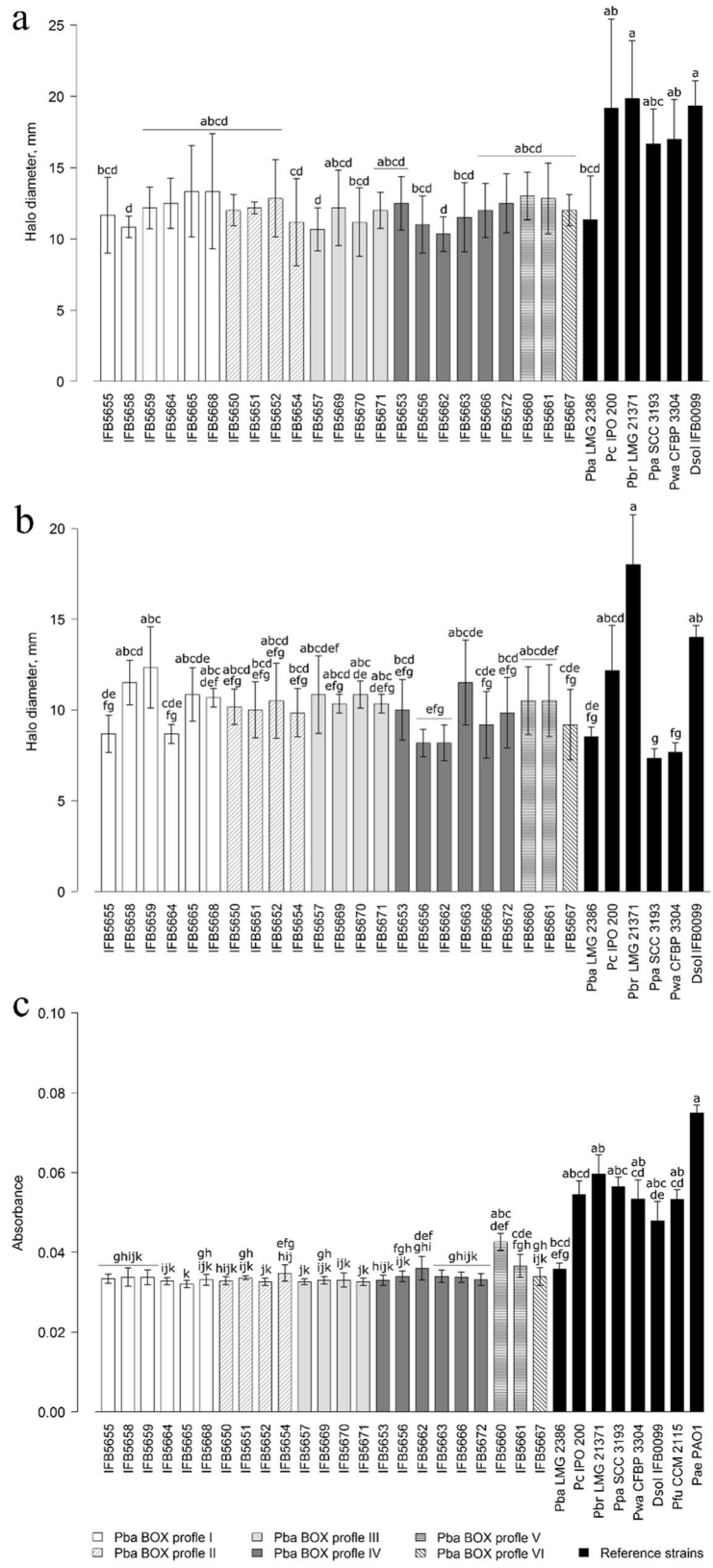

Fig. 5 Other virulence factors, *i.e.* lipases production (**a**), siderophores activity (**b**) and biofilm formation capacity (**c**) of *P. atrosepticum* strains isolated from potato fields in Poland. Rhodamine (**a**) and CAS (**b**) -based plate assays were performed. The bacteria-inoculated plates were incubated at 28 °C for 48 h and the resultant halo diameters around bacterial colonies were measured (**a**, **b**). Biofilm formation capacity (**c**) was examined as described previously (Babinska et al., 2021). Absorbance at 565 nm corresponding to the biofilm-formation ability (**c**) was assessed spectrophotometrically with Victor2 I420 Multilabel Counter (Wallac, Finland). The lengths of the columns (colored accordingly to the BOX profiles assigned to the *P. atrosepticum* strains) of the bar charts correspond to means, while standard deviations are shown on the error bars. Different letters mark abilities that diverge in a statistically significant manner (Kruskal-Wallis test followed by a *post hoc* applying Fisher's least significant difference criterion with Bonferroni correction) at $p < 0.05$. Pba—*Pectobacterium atrospeticum*; Pc—*Pectobacterium carotovorum*; Pbr—*Pectobacterium brasiliense*; Ppa—*Pectobacterium parmentieri*; Pwa—*Pectobacterium wasabiae*; Dsol—*Dickeya solani*; Pfu—*Pseudomonas fluorescens*; Pae—*Pseudomonas aeruginosa*

1998) as *P. atrosepticum*. This pool of strains was subjected to rep-PCR-based genomic profiling with the BOX (Fig. 1), ERIC (Supplementary Fig. 1) and REP primers (Supplementary Fig. 2). Of the above-listed tests, BOX-PCR turned out to be the most effective for differentiation of *P. atrosepticum* isolates (Fig. 1). It allowed for classification of 118 collected *P. atrosepticum* strains to 6 diverse BOX profiles I-VI (Table 3).

BOX-PCR-based genomic profile IV (Fig. 1) was the one most frequently observed (Table 3). The second most commonly detected (Table 3) genomic pattern I (Fig. 1) was characteristic for about 30% of the Polish strains and was also attributed to *P. atrosepticum* type strain LMG 2386^{TS}. The other genomic profiles (II, III, V and VI) have been assigned solely to several strains (Tables 2, 3). On the basis of the outcomes of the BOX-based genomic profiling, 23 *P. atrospeticum* strains (Tables 1, 2) exhibiting diverse genomic patterns and isolated in different years were selected as a representative pool for further phylogeny-oriented and phenotypic analyses.

Phylogenetic analysis of *P. atrosepticum* strains originating from potato fields in Poland

Of the performed *recA* (Supplementary Fig. 3), *gyrA* (Supplementary Fig. 4) and *rpoS*-based phylogenetic analyses, the one relying on *rpoS* (Fig. 2) revealed intraspecies variability within the Polish population of *P. atrosepticum*. The sequence of *recA* was identical among all the tested *P. atrosepticum* strains (Supplementary Fig. 3), while in terms of *gyrA,* there was only one SNP identified that differentiated IFB5665 (of BOX profile I) from the other *P. atrosepticum* strains (Supplementary Fig. 4). The *rpoS*-based analysis yielded 16 SNPs identified in the alignment generated for the studied *P. atrosepticum* strains. Regarding the phylogeny computed on *rpoS* sequences, the vast majority *i.e.* 19 of the studied *P. atrosepticum* strains in addition to *P. atrosepticum* type strain LMG 2386^{TS} grouped into a single clade (Fig. 2). This taxonomic group was most closely related to *P. atrosepticum* IFB5654 (of BOX profile II) and IFB5672 (of BOX profile IV) strains, which clustered together. Interestingly, the next clade consisted solely of *P. atrosepticum* IFB5660 and IFB5661 (Fig. 2), being the only strains in this study attributed with BOX profile V (Table 1). The observed tree branching in the part of Fig. 2 enclosing the *Pectobacteriaceae* reference strains reflected the established evolutionary relations between the included species, *i.e. P. brasiliense* LMG 21371 was located in the proximity to *P. carotovorum* IPO 200, while *P. wasabiae* CFBP 3304 was placed next to *P. parmentieri* SCC 3193. The tree was rooted on *D. solani* IFB0099.

Table 3 The abundance of the assigned BOX-based genomic profiles to *P. atrosepticum* strains isolated from potato fields in Poland in years 2013, 2014 and 2016

BOX-based genomic profile	No. of *P. atrosepticum* strains			
	2013	2014	2016	All years (%)
I	10	12	14	36 (30.5%)
II	7	0	0	7 (5.9%)
III	1	0	5	6 (5.1%)
IV	17	27	22	66 (55.9%)
V	0	2	0	2 (1.7%)
VI	0	0	1	1 (0.9%)

Phenotypic analysis of *P. atrosepticum* strains originating from potato fields in Poland

At first, we estimated the capacity of the representative *P. atrosepticum* strains to macerate potato tissue at different temperatures, *i.e.* at 20 °C or 28 °C (Fig. 3) in relation to the included *Pectobacteriaceae* reference strains (Table 1). It is worth to notice that *P. atrosepticum* strains of Polish origin showed higher capacity to macerate potato slices at the lower (Fig. 3A) than the higher temperature (Fig. 3B), as demonstrated by differences in the average rotten spot diameter (17.0 vs. 16.1 mm). Despite the fact that *P. atrosepticum* strains exhibited diverse BOX profiles, their ability to decay potato tissue was rather homogenous. Interesting observations concern the *Pectobacteriaceae* reference strains as *P. atrosepticum* type strain LMG 2386^{TS} and *P. wasabiae* CFBP 3304 showed also higher ability to macerate potato tissue at 20 °C than at 28 °C (Fig. 3). *P. brasiliense* LMG 21371 and *P. carotovorum* IPO 200 degraded potato more efficiently at 28 °C (Fig. 3).

Moving to the bacterial capacity to grow at different temperatures, all strains of *P. atrosepticum* and the other *Pectobacteriaceae* reference strains

multiplied at 20 °C and 28 °C (Table 2). Though, each of the included *P. atrosepticum* strains in addition to *P. wasabiae* CFBP 3304 were incapable of growing at 37 °C (Table 1). Of the reference strains analyzed *P. brasiliense* LMG 21371, *P. carotovorum* IPO 200 and *D. solani* IFB0099 were able to multiply at 37 °C (Table 2). In terms of *P. parmentieri* SCC 3193, the results of the conducted experiments were inconclusive (Table 2).

The bacterial ability to multiply in spite of higher salinity in the medium was examined. All the studied *P. atrosepticum* strains, similarly to *P. wasabiae* CFBP 3304, *P. parmentieri* SCC 3193 and *D. solani* IFB0099, were unable to grow in the medium supplemented with 5% NaCl (Table 2). Only *P. brasiliense* LMG 21371 and *P. carotovorum* IPO 200 reference strains were capable of multiplying in the medium of elevated salinity (Table 2).

Subsequently, the activities of PCWDEs being major virulence factors of soft rot *Pectobacteriaceae* were investigated. Concerning pectinase activity, the representative *P. atrosepticum* strains, independently of the assigned BOX profiles, revealed slight deviations in this feature (Fig. 4a). Notably, the mean pectinase activity of the Polish *P. atrosepticum* strains, which corresponded with the mean halo diameter of 18.52 mm, was higher than that of *P. atrosepticum* type strain LMG 2386^{TS}. There were just two reference strains, *D. solani* IFB0099 and *P. carotovorum* IPO 200, whose pectinase activity was either higher or comparable to that of the Polish *P. atrosepticum* strains (Fig. 4a).

Regarding the cellulase activity, the vast majority of the isolates of *P. atrosepticum* in addition to *P. atrosepticum* type strain LMG 2386^{TS} exhibited rather low, but homogeneous cellulase activity with no relation to the assigned BOX profiles (Fig. 4b). The mean halo diameter measured for *P. atrosepticum* strains from Poland was 5.37 mm. There were just three strains from the pool of *P. atrosepticum* of a slightly higher cellulase production, namely IFB5669, IFB5671 and IFB5665. However, the reference strains of *D. solani* IFB0099, *P. carotovorum* IPO 200 and *P. wasabiae* CFBP 3304 showed a notably elevated cellulase activity in contrast to the *P. atrosepticum* strains (Fig. 4b).

In terms of protease production, there was some variability among the investigated *P. atrosepticum* strains (Fig. 4c). Primarily, one-third of the *P. atrosepticum* strains, *i.e.* IFB5655, IFB5658, IFB5664, IFB5668, IFB5653, IFB5666 and IFB5667, showed low capacity to produce proteases and the intensity of this trait was similar to that of *P. parmentieri* SCC 3193 reference strain. We observed also *P. atrosepticum* strains, *i.e.* IFB5669, IFB5660 and IFB5661, of high proteases activity, which was comparable to that of *D. solani* IFB0099 and *P. wasabiae* CFBP 3304. Notably, the above-listed *P. atrosepticum* strains of either elevated or diminished protease activities had been assigned to diverse BOX genomic profiles. Interestingly, *P. atrosepticum* type strain LMG 2386^{TS} turned out not to possess any proteases activity under the applied experimental conditions, contrary to all the other strains belonging to this species yielding the mean halo diameter of 11.8 mm. Undoubtedly, *P. carotovorum* IPO 200 and *P. brasiliense* LMG 21371 indicated the highest protease production in this analysis (Fig. 4c).

The ability of *P. atrosepticum* strains to produce lipases, which are involved in distortion of the plant cellular membranes, was studied next. It turned out that the representative *P. atrosepticum* strains isolated in Poland together with *P. atrosepticum* type strain LMG 2386^{TS} revealed similar activities of this group of enzymes (Fig. 5a) with a mean halo diameter of 12.0 mm. Moreover, no correlation between the BOX-based genomic patterns attributed to *P. atrosepticum* strains and their capacity to secrete lipases was observed. Interestingly, reference strains belonging to the other species of the *Pectobacteriaceae* family, *i.e. P. brasiliense* LMG 21371, *P. carotovorum* IPO 200, *D. solani* IFB0099, *P. wasabiae* CFBP 3304 and *P. parmentieri* SCC 3193, showed higher lipase activity than the tested *P. atrosepticum* strains (Fig. 5a).

In terms of the siderophore production efficacy, that is essential for iron scavenging under the limiting environmental conditions, there were several strains of *P. atrosepticum*, *e.g.* IFB5656, IFB5662, and IFB5655 and *P. atrosepticum* type strain LMG 2386^{TS}, of diminished production of these molecules (Fig. 5b). However, the observed differences were not statistically significant in relation to majority of the other *P. atrosepticum* strains. Curiously, the above-mentioned *P. atrosepticum* strains of lower siderophore production belong to the groups attributed with either BOX profile I or IV. In general, the mean halo diameter, corresponding to the siderophore

production of the representative *P. atrosepticum* strains isolated in Poland, equaled 10.1 mm (Fig. 5b).

Regarding the bacterial ability to form biofilm, also high homogeneity among the studied *P. atrosepticum* strains was revealed (Fig. 5c). There was one strain, IFB5660 of BOX profile V, which showed elevated capacity (in a statistically significant manner) to generate biofilm. Overall, the mean absorbance value measured for the representative *P. atrosepticum* strains isolated in Poland was 0.034. Notably, the included reference strains classified to the other species of soft rot *Pectobacteriaceae* formed biofilm more efficiently than the investigated *P. atrosepticum* strains. In more detail, *P. brasiliense* LMG 21371, *P. parmentieri* SCC 3193, *P. carotovorum* IPO 200 and *P. wasabiae* CFBP 3304 generated this 3D structure more potently than even one of the included positive controls of efficient biofilm producers *i.e. P. fluorescens* CCM2115 (Fig. 5c).

Discussion

High dominance of *Pectobacterium* spp. over *Dickeya* spp. on the territory of Poland has been demonstrated in our previous study (Motyka-Pomagruk et al., 2021). Among the detected species of soft rot *Pectobacteriaceae*, the contribution of *P. atrosepticum* strains exceeded 26% in the period from 1996 until 2014 (Motyka-Pomagruk et al., 2021). It is worth to note that the majority of the SRP-oriented studies focused on emerging or newly-established *Pectobacteriaceae* species (Sarfraz et al., 2018; van der Wolf et al., 2014; Waleron et al., 2018, 2019; Zoledowska et al. 2018a, b). We examined the biodiversity and virulence of an important constituent of the SRP population in Poland being *P. atrosepticum*. Several studies on this species were also conducted in other countries due to the major contribution of *P. atrosepticum* to development of blackleg symptoms (van der Wolf et al., 2021a, b) and its status of a well-established pathogen of significant economic impact. So far, USA (Ma et al., 2018), Scotland (Toth et al., 2011), Canada (de Boer et al., 2012), Norway (Dees et al., 2017), Finland (Degefu, 2021), Northern Ireland (Zaczek-Moczydłowska et al., 2019), Pakistan (Sarfraz et al., 2020), Serbia (Arsenijević et al., 1994), Turkey (Ozturk et al., 2018), New Zealand (Pitman et al., 2008) or Nigeria (Monilola & Abiola, 2011) reported the occurrence and importance of this pathogen.

The diversity among *P. atrosepticum* strains isolated in different countries has been evaluated by using serological analyses, phage typing, PCR-RFLP and Random Amplification of Polymorphic DNA (RAPD) fingerprinting (Darrasse et al., 1994; Gross et al., 1991; Maki-Valkama & Karjalainen, 1994; Toth et al., 1999; Waleron et al., 2002). Application of the above-listed approaches, which differed in the number and types of markers used, resulted in the detection of 9 serogroups, 4 phage-typing groups, 2 PCR-RFLP profiles and nine RAPD profiles (Darrasse et al., 1994; Gross et al., 1991; Maki-Valkama & Karjalainen, 1994; Toth et al., 1999; Waleron et al., 2002). The first report on the phenotypic diversity of *P. atrosepticum* strains isolated in Poland was provided by Śledź et al. (2000). In that research, 8 *P. atrosepticum* biovars have been differentiated among approx. 750 isolates collected from potato fields in 1996 and 1997. Waleron et al. (2002) performed single gene (*recA*) PCR-RFLP-based genotyping on pectinolytic *Erwinia* (among them 13 strains of *P. atrosepticum* isolated in different countries were analysed) by digesting 730 bp fragments of the *recA* gene with each of the following 4 endonucleases: *Alu*I, *Hinf*I, *Tas*I and *Tru*1I (Waleron et al., 2002). This study indicated 2 diverse PCR-RFLP profiles among *P. atrosepticum* strains, emphasizing significantly lower intraspecies variation in this taxon than in *e.g. P. carotovorum* (18 distinctive profiles) or *Dickeya chrysanthemi* (15 diverse profiles) (Waleron et al., 2002). In the current study variability among the studied 118 *P. atrosepticum* strains collected in 2013, 2014 or 2016 in Poland has been shown by applying rep-PCR and distinction of 6 BOX profiles. In addition, the analyses of the sequences of 3 single housekeeping genes, *i.e. rpoS*, *gyrA* and *recA*, indicated the presence of 16 SNPs in the *rpoS* gene, 1 in the *gyrA* gene and no SNPs in the *recA* gene fragment. Besides, we have shown that the *rpoS*-based phylogeny was discriminative enough to reveal biodiversity within the *P. atrosepticum* species. From the agronomical point of view, low genetic variability in *P. atrosepticum* species could be beneficial in terms of studies focused on development of eradication or detection methods for this pathogen. The association between a restricted host range of this bacterium and the observed minor

genetic diversity can be hypothesized. In addition, our research revealed that the analysis of the repeated BOX sequences had higher discriminatory power for genotyping *P. atrosepticum* than the ERIC- and REP-based fingerprinting approaches.

Interestingly, we have demonstrated that the Polish *P. atrosepticum* strains multiply and macerate potato tissue under laboratory conditions more efficiently at 20 °C than 28 °C, which correlates with the research conducted earlier (Hasegawa et al., 2005). The herein investigated *P. atrosepticum* strains grew very well at 20 °C and 28 °C, while they were unable to multiply at 37 °C, which agrees with the published findings (Gardan et al., 2003; van der Wolf et al., 2021a, b). These observations are in accordance with the previously stated strict association between *P. atrosepticum* and the temperate climate conditions (Perombelon & Kelman, 1980; Toth et al., 2003). Even at subarctic and boreal regions of Greenland, the causal agents of soft rot and blackleg are identified as *P. atrosepticum* (de Neergaard et al., 2020). Further support for high virulence of *P. atrosepticum* at relatively low air temperatures was given by the fact that this species is able to infect earlier in summer (*i.e.* June or July) in comparison to other SRP (Motyka-Pomagruk et al., 2021; Tsuyama & Sakamoto, 1952).

In terms of bacterial resistance to elevated salinity, the Polish *P. atrosepticum* strains were unable to grow at 5% NaCl, which contrasts with the previously published findings (Gardan et al., 2003; van der Wolf et al., 2021a, b). Nonetheless, one of the reference strains belonging to the *P. brasiliense* species did show capacity to multiply when subjected to an elevated salinity agreeing with what was reported by Li et al. (2019) for another *P. brasiliense* strain. Likewise, the reference *P. carotovorum* strain did grow at 5% NaCl, agreeing with de Haan et al. (2008). Bacterial tolerance to salinity has been associated with potent production of exopolysaccharides and biofilm formation capacity that limit the uptake of Na^+ ions (Shultana et al., 2020). The occurrence of salt-intolerant *P. atrosepticum* seems agronomically relevant in a view of the saline-related abiotic stress, which affects plant cultivation on 6% of the total and approx. 20% of the irrigated land (Samant & Jawali, 2016).

In general, the ability of SRP to macerate plant tissue results from efficient production of numerous PCWDEs. Also proper synchronization of gene expression and release of the resultant virulence factors is crucial for the pathogenesis of *P. atrosepticum*. Pagel and Heitefuss (1990) revealed that polygalacturonase, pectate lyase, cellulase, protease and xylanase were detected in *P. atrosepticum*-inoculated potato tubers sequentially. It was shown that development of soft rot and blackleg symptoms relies on pectinase activity. In *P. atrosepticum*, these enzymes are encoded by the following genes: *pelB/C/Z* (*pel1/2/3*), *pelI*, *pelL/N*, *rhiE*, *pnl*, *pel*, *pehK*, *pehN* (*pehX*), *pehA*, *pehA2*, *paeY*, *pemA* and *pemB* (van Gijsegem et al., 2021a, b). In our study, the *P. atrosepticum* strains isolated in Poland showed high, rather uniform pectinase production. Similarly, the cellulase activity of these strains was homogeneous, with the exception of *P. atrosepticum* IFB5669. These outcomes agree with the work of Almomani et al. (2013), who described low variation in cellulase production between Jordanian *P. atrosepticum* isolates in relation to *P. carotovorum* strains. Here, the included reference strains of *P. carotovorum* and *D. solani* had higher cellulase activity than the *P. atrosepticum* strains, which is in agreement with the study of Ozturk et al. (2018). Moving to protease production, all Polish *P. atrosepticum* strains showed notable activity of this group of enzymes, which contradicts with the reference *P. atrosepticum* strain LMG 2386^{TS}. This observation matches the results of Potrykus et al. (2014b) for a closely related SRP species, *D. solani*. In that research *D. solani* IFB0223 strain showed no protease activity. Possibly the proteases of SRP might either degrade proteins associated with plant defense mechanisms or enable acquisition of amino acids for building up bacterial proteins (Heilbronn & Lyon, 1990). However, it should be taken into consideration that according to the current knowledge the contribution of cellulases and proteases to the overall virulence of SRP seems minor in comparison to pectinases (Marits et al., 1999).

Likewise, the studied *P. atrosepticum* strains turned out to secrete lipases less efficiently than the other SRPs included. Similar observations on the lowest lipolytic activity of *P. atrosepticum* among the members of the *Pectobacteriaceae* family were previously reported by Babinska et al. (2021). This phenotypic feature was also studied by Zoledowska, et al. (2018a), who found small variation in lipase production between diverse *P. parmentieri* strains of Polish origin. Here, we also included one reference strain of *P. parmentieri* that excreted more fatty acids than all the investigated *P. atrosepticum* strains. In

spite of obvious benefits for SRP resulting from digestion of host lipids for nutrient acquisition, the free fatty acids stimulate bacterial adhesion and therefore contribute to colonization and persistence of the pathogen (Stehr et al., 2003).

Additional molecules broadly engaged in plant-microbe interactions are siderophores. Gorshkov et al. (2018) noted significant upregulation of the enterobactin- and TonB transporter-related genes during plant colonization by *P. atrosepticum*. Subsequent studies on Δ*entA P. atrosepticum* knockout mutants, deprived of the enterobactin biosynthesis gene *entA*, revealed impaired virulence of this strain on salicylic acid-primed plants (Gorshkov et al., 2021). In spite of solely carrying an essential trace element required as a cofactor for multiple enzymes, siderophores were shown to be involved in the transfer of other metals than iron (also sequestration of toxic metals). Thus, these molecules also play a signaling role and assure protection from oxidative stress (Gorshkov et al., 2021; Kramer et al., 2020). All the *P. atrosepticum* strains studied exhibited siderophore production, though some variation in the intensity of this trait was noted. Interestingly, in the current research *D. solani* being a chrysobactin and achromobactin producer showed higher siderophore activity than the enterobactin-synthesizing *P. atrosepticum* strains.

Furthermore, we provided insights into the biofilm formation of *P. atrosepticum* strains from Poland. All these strains generated biofilms similarly, though less efficiently than the other reference SRP strains. The herein reported outcomes are in agreement with Pérez-Mendoza et al. (2011) who disclosed low biofilm-formation ability of *P. atrosepticum* SCRI1043 under standard laboratory conditions. In that study the ability to form biofilms by *P. atrosepticum* was correlated with significant levels of c-di-GMP, a signaling molecule whose concentration is dependent on an interplay between the pathogen and the plant host or changes due to environmental or physiological stimuli (Pérez-Mendoza et al., 2011). Moreover, *P. atrosepticum* SCRI1043 was reported to be capable of forming multicellular biofilm-like arrangements named bacterial emboli in the plant xylem vessels (Gorshkov et al., 2014). It seems intriguing that the chemical structure of major component of *P. atrosepticum* SCRI1043 EPS, which are polymers providing resistance to external factors and involved in incorporation of bacterial cells within the biofilm 3D matrix, was similar to the structure of *P. atrosepticum* SCRI1043 O-specific polysaccharide (OPS), a constituent of the lipopolysaccharide of this bacterium (Gorshkov et al., 2017).

In conclusion, characterization of *P. atrosepticum* strains isolated in Poland during three growing seasons provided not only scientific proofs for the significance of this phytopathogen for potato cultivation under temperate climate conditions, but also pointed to notable uniformity among the bacteria from this taxonomic group. As the current approaches to control SRP are solely prevention-based, proper identification of the disease-causing agent in addition to gathering knowledge on its biodiversity, virulence-associated traits and background mechanisms of interactions with the plant host, is crucial to build up a future perspective for development of procedures to combat the pest.

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Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files. The sequenced fragments of *rpoS*, *recA* and *gyrA* housekeeping genes of *P. atrosepticum* isolates are available in the GenBank database under the following accession numbers: OM105620, OM105597, OM158465, OM105621, OM105598, OM158466, OM105622, OM105599, OM158467, OM105623, OM105600, OM158468, OM105624, OM105601, OM158469, OM105625, OM105602, OM158470, OM105626, OM105603, OM158471, OM105627, OM105604, OM158472, OM105628, OM105605, OM158473, OM105629, OM105606, OM158474, OM105630, OM105607, OM158475, OM105631, OM105608, OM158476, OM105632, OM105609, OM158477, OM105633, OM105610, OM158478, OM105634, OM105611, OM158479, OM105635, OM105612, OM158480, OM105636, OM105613, OM158481, OM105637, OM105614, OM158482, OM105638, OM105615, OM158483, OM105639, OM105616, OM158484, OM105640, OM105617, OM158485, OM105641, OM105618, OM158486, OM105642, OM105619, OM158487.

Declarations

Human and/or animals rights Not applied.

Informed consent Not applied.

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