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Regulators Involved in *Dickeya solani* Virulence, Genetic Conservation, and Functional Variability

Marta Potrykus,[1] Małgorzata Golanowska,[1] Nicole Hugouvieux-Cotte-Pattat,[2] and Ewa Lojkowska[1]

[1]Department of Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdansk and Medical University of Gdansk, Kladki 24, 80-822 Gdansk, Poland; [2]CNRS UMR5240 Microbiologie Adaptation et Pathogénie, Université de Lyon 1, INSA de Lyon, F-69621Villeurbanne, France

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Bacteria from the genus *Dickeya* (formerly *Erwinia chrysanthemi*) are plant pathogens causing severe diseases in many economically important crops. A majority of the strains responsible for potato disease in Europe belong to a newly identified *Dickeya solani* species. Although some ecological and epidemiological studies have been carried out, little is known about the regulation of *D. solani* virulence. The characterization of four *D. solani* strains indicates significant differences in their virulence on potato, although they are genetically similar based on genomic fingerprinting profiles. A phenotypic examination included an analysis of virulence on potato; growth rate in culture; motility; Fe^{3+} chelation; and pectate lyase, cellulase, protease, biosurfactant, and blue pigment production. Mutants of four *D. solani* strains were constructed by inactivating the genes coding either for one of the main negative regulators of *D. dadantii* virulence (*kdgR, pecS*, and *pecT*) or for the synthesis and perception of signaling molecules (*expI* and *expR*). Analysis of these mutants indicated that PecS, PecT, and KdgR play a similar role in both species, repressing, to different degrees, the synthesis of virulence factors. The thermoregulator PecT seems to be a major regulator of *D. solani* virulence. This work also reveals the role of quorum sensing mediated by ExpI and ExpR in *D. solani* virulence on potato.

Bacteria from the genus *Dickeya* (formerly *Erwinia chrysanthemi*) are pathogens that cause severe diseases in many economically important crops. They have been listed in the top 10 most important bacterial plant pathogens based on their economic impact (Mansfield et al. 2012). The genus *Dickeya* consists of five species: *Dickeya chrysanthemi, D. dadantii, D. dianthicola*, *D. zeae*, and *D. paradisiaca* (Brady et al. 2012; Samson et al. 2005). Bacteria that belong to *D. dadantii* and *D. zeae* pose a threat to potato production in tropical climates (DeLindo and French 1981) while *D. dianthicola*, the pathogen of carnation, has become established in Europe over the last 40 years, causing black-leg and soft rot diseases in potato (Toth et al. 2011). The strains, isolated over the past 10 years from soft-rot- and black-leg-affected potato plants in numerous European countries, appeared to belong to a new *Dickeya* group (Slawiak et al. 2009b). Their characteristics, based on the disease symptoms, biochemical characterization, *dnaX* and *recA* sequencing, repetitive extragenic palindromic polymerase chain reaction (REP-PCR), and pulsed-field gel electrophoresis (PFGE) profiles, indicate a high level of homogeneity (Degefu et al. 2013; Slawiak at al. 2009b). Because these strains could not be classified into any previously described *Dickeya* spp., they were recently classified in a new species, *D. solani* (Van der Wolf et al. 2014). *D. solani* strains are highly virulent to potato plants and can initiate the disease from a low inoculum level (as low as 10 cells/tuber). They have a high optimal temperature range for disease development and growth (from 25 to 39°C) and they spread more easily through the plant vascular system than other *Dickeya* spp. (Czajkowski et al. 2010; Toth et al. 2011).

Most of the information concerning the molecular mechanisms driving the initiation and spread of the soft rot disease comes from studies on a few *Dickeya* strains: *D. dadantii* 3937 (Hugouvieux-Cotte-Pattat et al. 1996) and *Dickeya* sp. strain EC16 (Tamaki et al. 1988). Bacteria can initially live on the surface and inside the intercellular spaces of the plant, without causing any disease symptoms (Pérombelon 1992). When they encounter favorable conditions such as warm temperature, high humidity, and low oxygen availability, they multiply and begin to produce pectinases, resulting in the initiation of the disease (Hugouvieux-Cotte-Pattat et al. 1996; Pérombelon 2002). Bacteria from the genus *Dickeya* can invade and destroy the plant tissue via the coordinated expression of several genes encoding virulence factors, mainly plant cell-wall-degrading enzymes (PCWDE). *D. dadantii* strain 3937 secretes at least nine endopectate lyases and three accessory pectinases (Hassan et al. 2013; Hugouvieux-Cotte-Pattat et al. 1996; Kazemi-Pour et al. 2004), three proteases (Kazemi-Pour et al. 2004), and a cellulase (Py et al. 1991). Other factors can help bacteria to survive in harsh environments such as the surface of the plant, water, or soil. These factors include bacterial features such as iron capture, mobility, and the production of biosurfactants or pigments (Enard et al. 1988; Pérombelon 2002; Raaijmakers et al. 2009).

The bacteria from the genus *Dickeya* are able to sense their environment, and the induction of production of PCWDE and other virulence factors depends essentially on this ability. The appropriate production and secretion of these molecules requires an elaborate regulatory network that can act in a concerted manner in response to complex environmental signals (Charkowski et al. 2012; Hugouvieux-Cotte-Pattat et al. 1996; Kepseu et al. 2010; Reverchon and Nasser 2013). Studies on the regulation of *Dickeya* virulence led to the identification of 10 major regulators: namely, KdgR, PecS, PecT, CRP, H-NS, Fis, Fur,

Corresponding author: E. Lojkowska; Telephone: +48 58 5236426; Fax: +48 58 5236360; E-mail: ewa.lojkowska@biotech.ug.edu.pl

GacA, SlyA, and MfbR (Charkowski et al. 2012). Moreover, these studies suggest the existence of quorum-sensing (QS) mechanisms: an acyl-homoserine lactone (AHL) regulation system (ExpR and ExpI) (Reverchon et al. 1998) and the recently discovered Vfm system, responsible for the secretion and sensing of an as-yet-unidentified molecule (Nasser et al. 2013). These regulators not only trigger the synthesis and secretion of PCWDE but also that of other virulence factors such as exopolysaccharide production and motility (Charkowski et al. 2012).

In the *D. dadantii* 3937 virulence regulatory network, there are three principal negative transcriptional regulators: KdgR, PecT, and PecS (Condemine and Robert-Baudouy 1987; Praillet et al. 1996; Surgey et al. 1996). These three elements act independently on PCWDE synthesis and secretion. The KdgR transcriptional repressor is released from its target DNA in the presence of pectin degradation products such as 2-keto-3-deoxygluconate (Hugouvieux-Cotte-Pattat et al. 1996). The KdgR regulon includes the genes encoding proteins involved in pectin degradation, the catabolism of rhamnogalacturonides, the Out secretion system, some glycolytic steps, and sugar efflux transport (Rodionov et al. 2004). The PecT transcriptional regulator is involved in the regulation of PCWDE and exopolysaccharide synthesis. It was recently proposed that PecT functions as a thermoregulator of the target genes (Hérault et al. 2014). Variations in PecT intracellular concentrations have significant effects on these genes and PecT production is repressed by the nucleoid-associated protein H-NS and its own autoregulation but is activated by KdgR (Nasser and Reverchon 2002; Rodionov et al. 2004). PecS is a global regulator of the symptomatic phase of the disease. It directs the production and secretion of PCWDE and also the production of indigoidine, biosurfactant, and flagella (Hommais et al. 2008; Mhedbi-Hajri et al. 2011). The signal responsible for PecS derepression is still unknown. The QS system mediated by ExpR and ExpI can activate *pecS* expression. Reciprocally, PecS is able to repress the expression of *expR* and of the Vfm cluster (Nasser et al. 2013). *D. dadantii* 3937 and *D. solani* are able to produce three different AHL: N-(3-oxohexanoyl)-homoserine lactone (3-oxo-C6-HSL), N-(hexanoyl)-homoserine lactone (C6-HSL) and N-(3-oxooctanoyl)-homoserine lactone (3-oxo-C8-HSL) (Crepin et al. 2012). The QS mechanism involving ExpR and ExpI has been shown to be of little importance in *D. dadantii* 3937 (Mhedbi-Hajri et al. 2011) but it plays a significant role in the mobility and virulence of *D. zeae* EC1 (Hussain et al. 2008).

Little is known about the regulation of *D. solani* virulence. Here, we report the characterization of four *D. solani* strains that differ significantly in their level of virulence on potato although they appear to be genetically similar based on REP-PCR and PFGE profiles. The regulation of *D. dadantii* 3937 virulence is well documented but it remains unclear whether this regulation is similar in other *Dickeya* spp. To investigate the effect in *D. solani* of certain virulence regulatory factors, mutants of four *D. solani* strains were constructed by inactivating the genes coding either for one of the main virulence negative regulators (*kdgR, pecS*, and *pecT*) or for the synthesis and perception of signaling molecules (*expI* and *expR*). To our knowledge, this is the first report detailing the regulatory network controlling the virulence of *D. solani* strains.

RESULTS

Genetic characterization of four *D. solani* strains with different virulence levels.

The four *D. solani* wild-type strains (IFB0099, IFB0123, IFB0158, and IFB0223) were selected on the basis of their differential abilities to cause disease symptoms on potato tubers and chicory leaves (Fig. 1). As regards their genetic characterization, these strains appeared to be homogeneous based on their similar banding patterns in REP-PCR, with a REP primer pair, and in PFGE (Fig. 2). The *dnaX* sequence was 100% identical in the four tested strains and, furthermore, it was identical to other *D. solani dnaX* sequences available in the GenBank database. Three of the examined strains (IFB0099, IFB0123, and IFB0158) were isolated from symptomatic potato plants in Poland or The Netherlands, while the strain IFB0223 was derived from the rhizosphere of asymptomatic potato plant in Germany. Statistical analysis of the diseases symptoms showed that the most virulent *D. solani* strains on both chicory leaves and potato tubers were IFB0099 and IFB0158 isolated in Poland. The strains IFB0123 and IFB0223 were less virulent. On the potato tubers, strain IFB0123, isolated in The Netherlands, caused intermediate disease symptoms while IFB0223, isolated in Germany, showed the lowest virulence (Fig. 1).

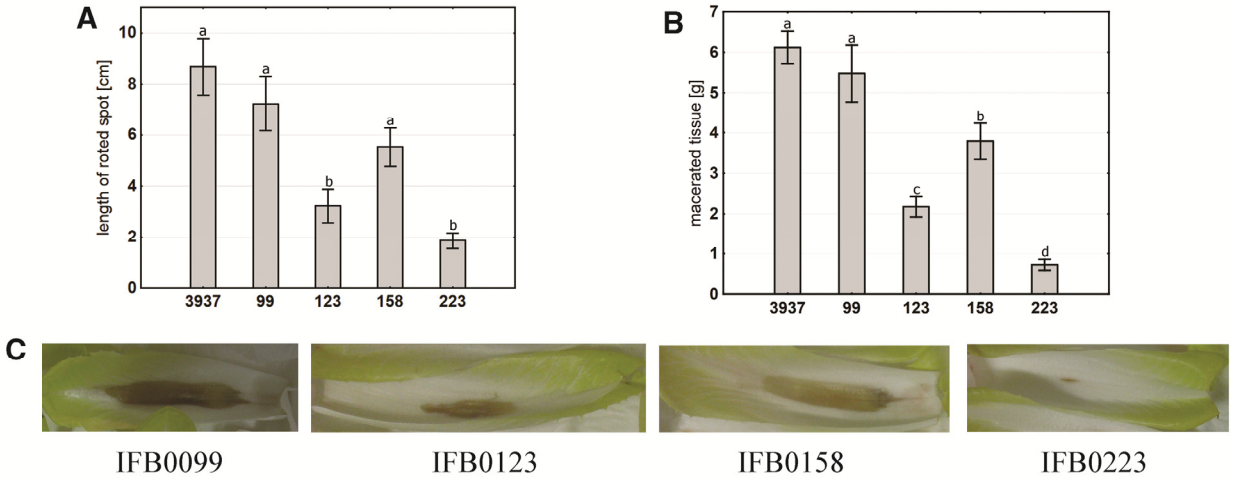

Fig. 1. Virulence of the selected *Dickeya solani* strains and of *D. dadantii* 3937 on chicory leaves and potato tubers. **A,** Virulence on chicory leaves 24 h postinoculation, measured by the length of the macerated area (cm) ($n = 3$; $F = 11.6$); **B,** virulence on potato tubers in grams of macerated tissue, 48 h postinoculation; ($n = 6$; $F = 34.2$); **C,** examples of symptoms observed on chicory leaves. Strains used are *D. dadantii* 3937 (3937) and the *D. solani* strains IFB0099 (99), IFB0123 (123), IFB0158 (158), and IFB0223 (223). For both plant species, experiments were performed twice. Within each figure, columns with different letters are significantly different based on an analysis of variance followed by Fisher test at $P < 0.05$, with the F values indicated above for each test.

We performed further characterization of the four selected *D. solani* strains together with *D. dadantii* 3937 used for comparison. *D. dadantii* 3937 is the *Dickeya* strain that has been the most extensively analyzed (Hugouvieux-Cotte-Pattat et al. 1996). Its genome has been sequenced (Glasner et al. 2011), regulation of the virulence program has been described (Reverchon and Nasser 2013), and a range of well-characterized mutants is available.

Phenotypic characterization of the *D. solani* strains.

First of all, the growth curve of each strain in Luria-Bertani (LB) medium was determined (Fig. 3A). For statistical analysis, we compared the cell densities obtained after 16 h (early stationary phase). The two *D. solani* strains that appeared to be the most virulent (IFB0099 and IFB0158) grew to higher densities than the other strains. Strains IFB0123 and *D. dadantii* 3937 grew to intermediate densities. The least virulent strain (IFB0223) grew to the least density.

Statistical analysis of the growth rates of these strains (Fig. 3B) divided them into two groups. The two most virulent strains (IFB0099 and IFB0158) and *D. dadantii* 3937 grouped together, with growth rates of 0.33, 0.32, and 0.39 per hour, respectively. The second group contained IFB0123 and IFB0223, with growth rates of 0.24 and 0.18 per hour, respectively.

The production of PCWDE was then analyzed. The total pectate lyase activity produced by the strains differed (Fig. 4A and B). In noninduced conditions, the most virulent strain, IFB0099, showed a high basal pectate-lyase activity, approximately fourfold higher than *D. dadantii* 3937. The pectate lyase activities of the other *D. solani* strains were not significantly different from that of *D. dadantii* 3937 (Fig. 4A). When polygalacturonate was added to induce pectinase production (induced conditions), pectate-lyase activity increased for all strains and IFB0099 showed significantly higher activity than the other strains (Fig. 4B).

The protease production of IFB0099 was also higher than that of the tested strains (Fig. 4C). The protease production

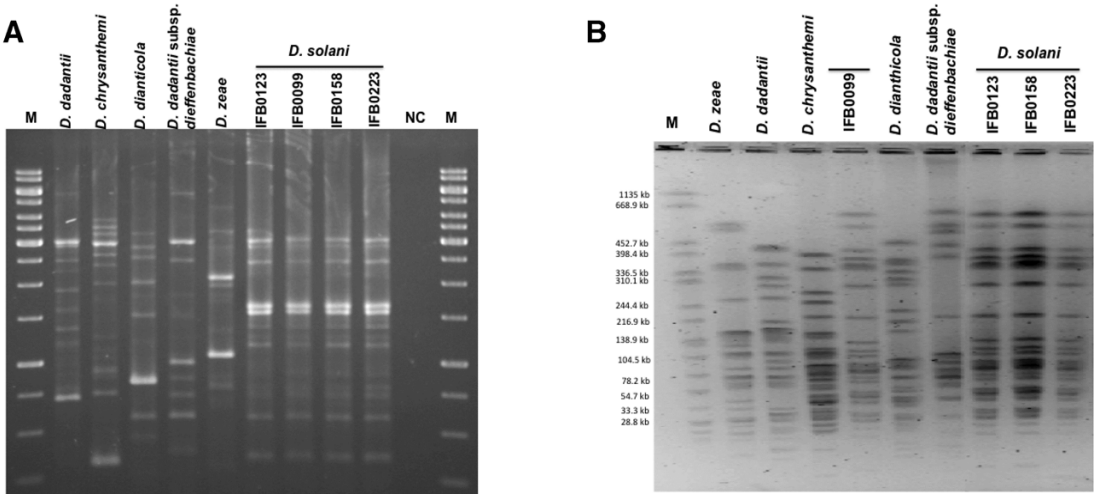

Fig. 2. Repetitive extragenic palindromic polymerase chain reaction (REP-PCR) and pulsed-field gel electrophoresis (PFGE) profiles of *Dickeya* strains. **A,** REP-PCR profiles obtained with the use of REP1 RI and REP 2I primers. **B,** PFGE profiles obtained by digestion of genomic DNA with XbaI. Strains used are *Dickeya dadantii* IFB0010, *D. chrysanthemi* IFB0055, *D. dianthicola* IFB0103, *D. dadantii* subsp. *dieffenbachiae* IFB0113, *D. zeae* IFB0119, and the four *D. solani* strains IFB0099, IFB0123, IFB0158, and IFB0223. NC = negative control, M = DNA size marker (1-kb ladder; Fermentas) for A and genomic DNA of *Salmonella enterica* ser. *braenderup* digested with *Xba*I for B.

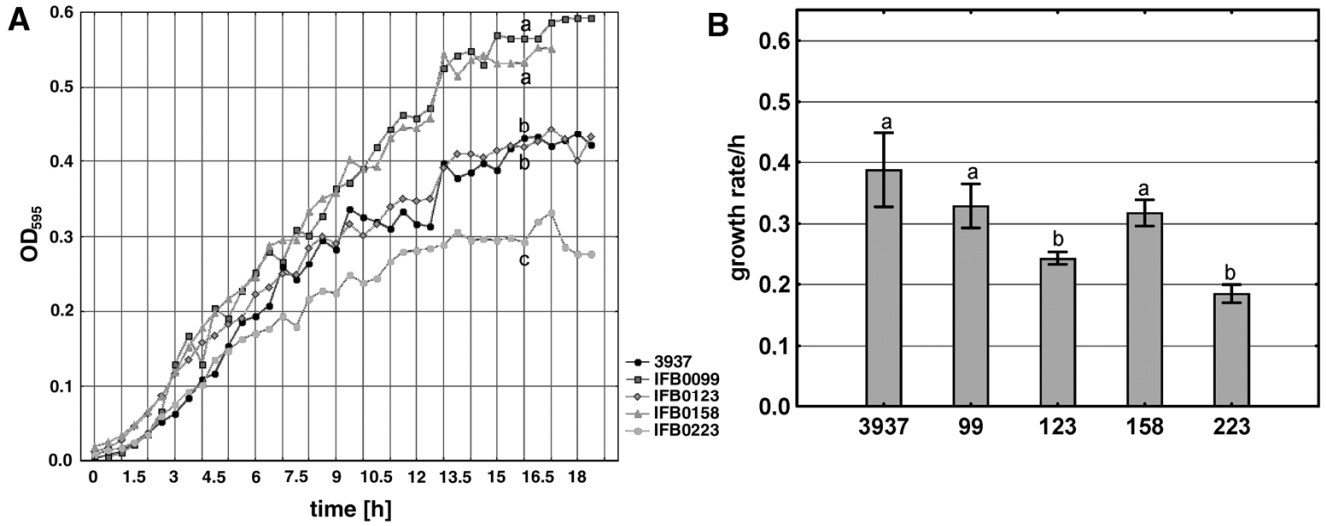

Fig. 3. Growth of *Dickeya solani* and *D. dadantii* wild-type strains. Bacteria were grown in Luria-Bertani medium at 28°C and growth was measured by optical density at 595 nm (OD_{595}). **A,** Growth curve is given as OD_{595} values in the function of time (h) ($n = 2$, $F = 49.4$). For simplicity and clarity of the graph, standard errors were excluded. OD_{595} values at 16 h were compared. **B,** Growth rate of the bacterial strains ($n = 2$, $F = 4.3$). Strains used are *D. dadantii* 3937 and the *D. solani* strains IFB0099, IFB0123, IFB0158, and IFB0223. Experiments were repeated twice. Within B, columns with different letters are significantly different when assayed with analysis of variance followed by Fisher test at $P < 0.05$, with the F values indicated above for each test.

was observed for other strains, except for IFB0223, for which no protease activity was detected. A strong difference between *D. solani* strains and *D. dadantii* was observed for cellulase production. The four *D. solani* strains showed a significantly higher cellulase production than *D. dadantii* 3937 (Fig. 4D). In contrast, the ability of *D. solani* strains to chelate Fe^{3+} ions was significantly lower than that of *D. dadantii* 3937 (Fig. 4E). None of the strains demonstrated biosurfactant production in the tested conditions (data not shown). Their swarming ability was low and no difference was observed between the *D. solani* and *D. dadantii* strains (data not shown). However, the swimming ability of *D. solani* IFB0099 was approximately 25% higher than that of the other strains (Fig. 4F).

The production of signaling molecules belonging to the AHL family was investigated using different *Escherichia coli* biosensor strains (Andersen et al. 2001; Winson et al. 1998). For all the *D. dadantii* 3937 and *D. solani* strains, the production of C6-HSL and C8-HSL was detected with *E. coli* HB101/pSB401. Use of two other biosensor strains, *E. coli* HB101/pSB534 and *E. coli* HB101/pSB1075, indicated that none of the tested *Dickeya* strains were able to produce C4-HSL, C10-HSL, C12-HSL, and C14-HSL (data not shown).

Infection of the *D. solani* strains with the bacteriophage phi-EC_2.

D. dadantii 3937 is known to be sensitive to the bacteriophage phi-EC_2 (Résibois et al. 1984); therefore, the sensitivity of *D. solani* strains to this bacteriophage was analyzed. Using stocks prepared by infection of *D. dadantii* 3937, the *D. solani* strains appeared to be less sensitive to phage phi-EC_2 than *D. dadantii* 3937 and they showed different degrees of sensitivity to infection in the serial dilution test. With strains IFB0123 and IFB0223, the number of phage plaques was similar to that obtained with *D. dadantii* 3937. Compared with these three strains, approximately 100-fold lower numbers of plaques were observed with strains IFB0099 and IFB0158.

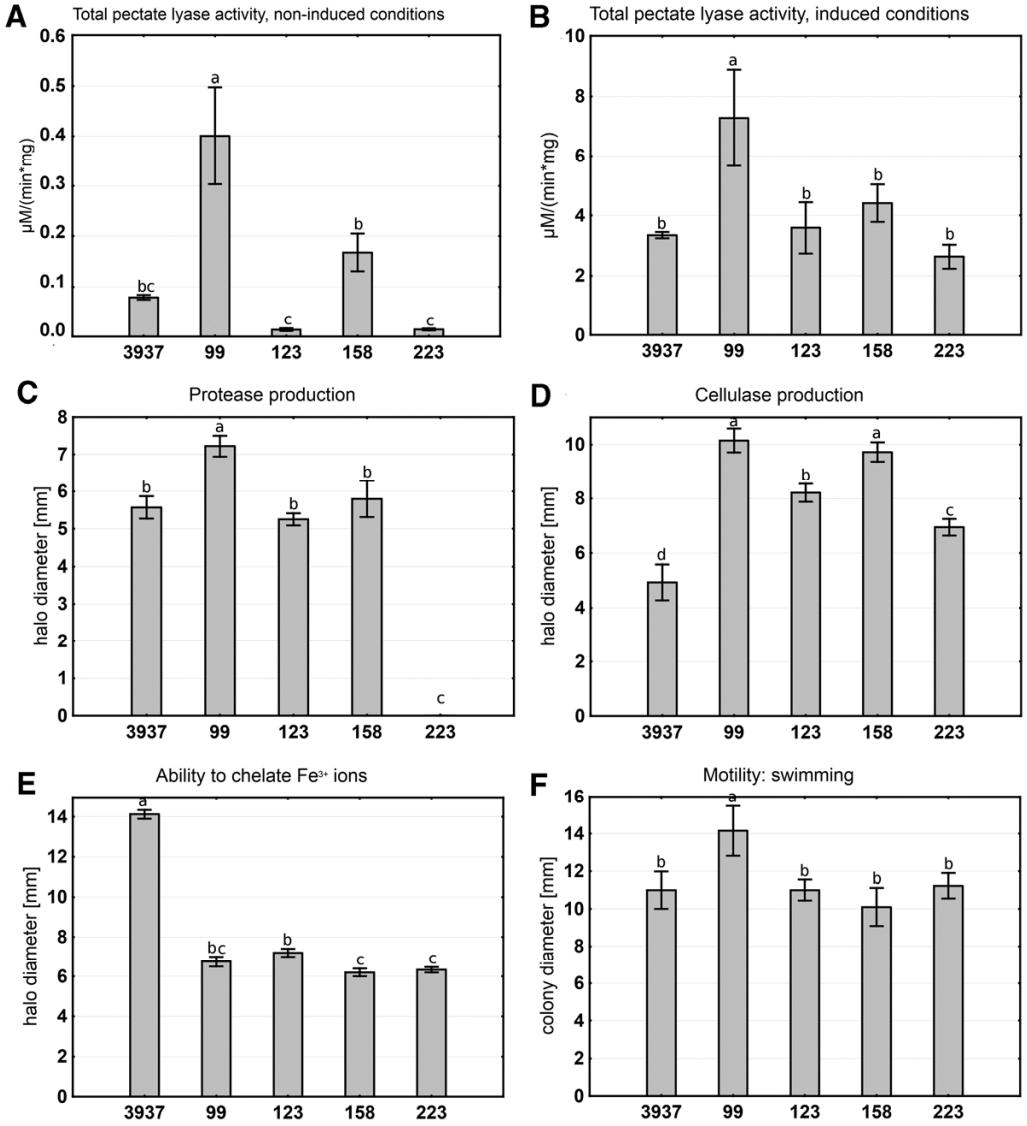

Fig. 4. Phenotypic characterization of four wild-type *Dickeya solani* strains and comparison with *D. dadantii* 3937. **A,** Total pectate lyase specific activity (µmol min^{-1} mg^{-1}) in noninduced conditions ($n = 4$; $F = 11.5$). **B,** Total pectate lyase specific activity (µmol min^{-1} mg^{-1}) in induced conditions (induction by addition of polygalacturonate) ($n = 3$; $F = 4.1$). **C**, Protease production was estimated by the diameter (mm) of the haloes observed on the detection plates ($n = 4$; $F = 66.3$); **D,** Cellulase production was estimated by the diameter (mm) of the haloes observed on the detection plates ($n = 7$; $F = 23.0$). **E,** Iron chelating capacity was estimated by the diameter (mm) of the haloes observed around colonies ($n = 4$; $F = 254$). **F,** Motility was estimated by the diameter (mm) of the spread of the colonies ($n = 5$; $F = 2.7$). All of the experiments were performed twice. Strains used were *D. dadantii* 3937 (3937) and the *D. solani* strains IFB0099 (99), IFB0123 (123), IFB0158 (158), and IFB0223 (223). Within each figure, columns with different letters are significantly different when assayed with analysis of variance followed by Fisher test at $P < 0.05$, with the F values indicated above for each test.

The bacteriophage phi-EC_2 is classically used to transfer selectable mutations by transduction in *D. dadantii* 3937 (Résibois et al. 1984). Thus, transduction of a chloramphenicol (Cm) resistance marker (Cm^R) on the *D. solani* chromosome was tested using phi-EC_2 stocks obtained by infection of different *D. dadantii* mutants containing a Cm insertion in a regulatory gene (A1077, A2174, A3009, A3148, and A3494) (Table 1). Compared with the number of transductants obtained for strain 3937, used as the control (100%), the levels of Cm^R transductants obtained for the *D. solani* strains corresponded to 57 ± 17, 26 ± 9, 16 ± 5, and 7 ± 4% for IFB0223, IFB0123, IFB0099, and IFB0158, respectively. The statistical analysis using analysis of variance (ANOVA) (F = 99.6) followed by Fisher post-hoc test showed that the differences in the efficiencies of transduction between the strains are significant and divided them into two groups: the most-sensitive strains IFB0223 and IFB0123 and the less-sensitive strains IFB0099 and IFB0158. Thus, the efficiency of transduction correlates well with the level of sensitivity observed for each strain using plaque formation in the serial dilution test.

Conservation of virulence regulatory genes and construction of mutants.

One aim of this study was to analyze the role of some of the regulatory genes previously identified in *D. dadantii* 3937 in the virulence of the *D. solani* strains. First, we checked, using PCR, for the presence of the genes *kdgR*, *pecS*, *pecT*, *expR*, and *expI* in the four *D. solani* strains. The five genes were present in the four *D. solani* strains. Sequencing of the *kdgR* PCR products demonstrated 100% identity between the *kdgR* genes of the four *D. solani* strains. The availability of the genome sequence of IPO2222 (IFB0123), (Pritchard et al 2013) allowed us to evaluate the sequence of the different genes in this strain. These sequences were also obtained by searching the draft genome sequences of IFB0099 and IFB0223 (*unpublished data*). Again, the sequences of *kdgR*, *pecS*, *pecT*, *expR*, and *expI* were found to be 100% identical among the strains examined.

We compared the sequences of the regulatory genes of the four different *D. solani* strains with those of other *Dickeya* spp.: *D. dadantii* 3937, *D. chrysanthemi* 1591, *D. zeae* Ech586, and *D. paradisiaca* Ech703 (GenBank, with corrections for

Table 1. Bacterial strains used in this study

Strain[a]	Species	Description[b]	Source, reference[c]
Wild-type strains			
3937	*Dickeya dadantii*	Wild-type	Kotoujansky et al. 1982
IFB0010	*D. dadantii*	Wild-type, type strain	CFBP1269
IFB0055	*D. chrysanthemi*	Wild-type, type strain	CFBP2048
IFB0103	*D. dianthicola*	Wild-type, type strain	CFBP1200
IFB0113	*D. dadantii* subsp. *dieffenbachiae*	Wild-type, type strain	CFBP2051
IFB0119	*D. zeae*	Wild-type, type strain	CFBP2052
IFB0099	*D. solani*	Wild-type, Poland	Slawiak et al. 2009a
IFB0123	*D. solani*	Wild-type, The Netherlands	IPO2222, Van der Wolf et al. 2014
IFB0158	*D. solani*	Wild-type, Poland	This work
IFB0223	*D. solani*	Wild-type, Germany	Weinert et al. 2010
Mutant strains			
A1077	*D. dadantii*	3937 *rafR lacB kdgR*::Cm	Rodionov et al. 2004
A2174	*D. dadantii*	3937 *rafR lacB pecT*::Cm	Surgey et al. 1996
A3009	*D. dadantii*	3937 *rafR lacB expR*::Cm	Nasser et al. 1998
A3148,	*D. dadantii*	3937 *rafR lacB expI*::Cm	Nasser et al. 1998
A3494	*D. dadantii*	3937 *rafR lacB pecS*::Cm	Nasser et al. 1999
3937R	*D. dadantii*	3937 *kdgR*::Cm	This work
3937S	*D. dadantii*	3937 *pecS*::Cm	This work
3937T	*D. dadantii*	3937 *pecT*::Cm	This work
3937E	*D. dadantii*	3937 *expR*::Cm	This work
IFB0099R	*D. solani*	IFB0099 *kdgR*::Cm	This work
IFB0123R	*D. solani*	IFB0123 *kdgR*::Cm	This work
IFB0158R	*D. solani*	IFB0158 *kdgR*::Cm	This work
IFB0223R	*D. solani*	IFB0223 *kdgR*::Cm	This work
IFB0099S	*D. solani*	IFB0099 *pecS*::Cm	This work
IFB0123S	*D. solani*	IFB0123 *pecS*::Cm	This work
IFB0158S	*D. solani*	IFB0158 *pecS*::Cm	This work
IFB0223S	*D. solani*	IFB0223 *pecS*::Cm	This work
IFB0099T	*D. solani*	IFB0099 *pecT*::Cm	This work
IFB0123T	*D. solani*	IFB0123 *pecT*::Cm	This work
IFB0158T	*D. solani*	IFB0158 *pecT*::Cm	This work
IFB0223T	*D. solani*	IFB0223 *pecT*::Cm	This work
IFB0099E	*D. solani*	IFB0099 *expR*::Cm	This work
IFB0123E	*D. solani*	IFB0123 *expR*::Cm	This work
IFB0158E	*D. solani*	IFB0158 *expR*::Cm	This work
IFB0223E	*D. solani*	IFB0223 *expR*::Cm	This work
IFB0099I	*D. solani*	IFB0099 *expI*::Cm	This work
IFB0123I	*D. solani*	IFB0123 *expI*::Cm	This work
IFB0158I	*D. solani*	IFB0158 *expI*::Cm	This work
IFB0223I	*D. solani*	IFB0223 *expI*::Cm	This work
Harboring plasmids (AHL)			
HB101/pSB401	*Escherichia coli*	Tet^R *luxRI'::luxCDABE* (pACYC184-derived)	Winson et al. 1998
HB101/pSB534	*E. coli*	Amp^R *lasRI::luxBCDAE*	Andersen et al. 2001
HB101/pSB1075	*E. coli*	Tet^R *lasRI::luxBCDAE*	Winson et al. 1998

[a] IFB = Intercollegiate Faculty of Biotechnology UG&MUG collection numbers, A = Microbiologie Adaptation et Pathogénie collection numbers, and AHL = acyl-homoserine lactone biosensors.
[b] Cm = chloramphenicol; Tet^R and Amp^R = resistant to tetracycline and ampicillin.
[c] IPO = International Plant Protection collection numbers and CFBP = French Collection of Plant-Associated Bacteria.

strain classification by Pritchard and associates [2013]) and *D. solani* IPO2222 (Pritchard et al. 2013). For each gene studied, the *D. solani* sequences presented the highest percentage of identity with sequences of *D. dadantii* 3937, with 94% identity for *kdgR*, 95% for *pecS*, 92% for *pecT*, 95% for *expR*, and 95% for *expI*. The percent identity varied from 75 to 88% between *D. solani* and the three other *Dickeya* spp. (*D. chrysanthemi, D. zeae*, and *D. paradisiaca*), depending on the gene. A major difference was observed for the *D. paradisiaca* Ech703 genome, which lacks sequences similar to *expR* and *expI*. These sequence homology data suggest a high conservation of the genes studied among the *D. solani* strains and a greater similarity between *D. solani* and *D. dadantii* genomes than between *D. solani* and any other sequenced *Dickeya* sp.

D. solani mutants affected in each of the regulatory genes were constructed by transduction (Table 1) in order to analyze the effect of gene inactivation. The *D. dadantii* 3937 mutants used in this study were prepared by transduction in the same conditions as the *D. solani* mutants to facilitate a comparison between the strains. Only the phenotypes for which we observed differences between the mutants and the wild-type strains are described. For example, cellulase production and swarming ability are not discussed because they did not show statistically significant differences between the mutants and the corresponding wild-type strains (data not shown).

Phenotypes of the *D. solani kdgR* mutants.

The *kdgR* mutation in both the *D. solani* strains and *D. dadantii* 3937, did not affect the ability to macerate potato tubers (Fig. 5A). For all the strains, the pectate-lyase-specific activity of the *kdgR* mutants measured in noninduced conditions was unregulated compared with the corresponding wild-type strain (Fig. 5B). The differences were significant, except for IFB0158. In the presence of polygalacturonate as inducer, the pectate-lyase-specific activity in the *kdgR* mutants was not significantly affected compared with the activity measured in the corresponding parental strain (Fig. 5C). Protease production was unregulated in the *kdgR* mutants of *D. dadantii* 3937 and IFB0223 (Fig. 6A). The ability to chelate Fe^{3+} ions slightly increased in the *kdgR* mutants of 3937 and IFB0158 (Fig. 6B).

Phenotypes of the *D. solani pecS* mutants.

A characteristic of all the *pecS* mutants was the overproduction of a blue pigment that was examined visually. This production was the highest in IFB0099, intermediate in IFB0158 and 3937, and very low in the IFB0123 and IFB0223 *pecS* mutants. The *pecS* mutation increased the ability of strains IFB0123 and IFB0158 to macerate potato tubers, whereas there was no significant change in the other strains (Fig. 5A). The basal pectate-lyase-specific activity significantly increased in the *pecS* mutants of *D. dadantii* 3937 and IFB0123 (Fig. 5B). Pectate lyase activity in induced conditions was unregulated in most of the *pecS* mutants (Fig. 5C). This up regulation in induced conditions was significant for 3937, IFB0123, and IFB0158. Protease production increased only in the *pecS* mutant of *D. dadantii* 3937 and IFB0223 (Fig. 6A). The ability to chelate Fe^{3+} ions was unregulated in all *pecS* mutants but IFB0223 (Fig. 6B). Swimming ability significantly increased in the *pecS* mutants of *D. dadantii* 3937, IFB0099, and IFB0123 (Fig. 6C). In addition, the production of biosurfactant became visible in the *pecS* mutants of *D. dadantii* 3937 and of the *D. solani* strains, except IFB0223 (data not shown).

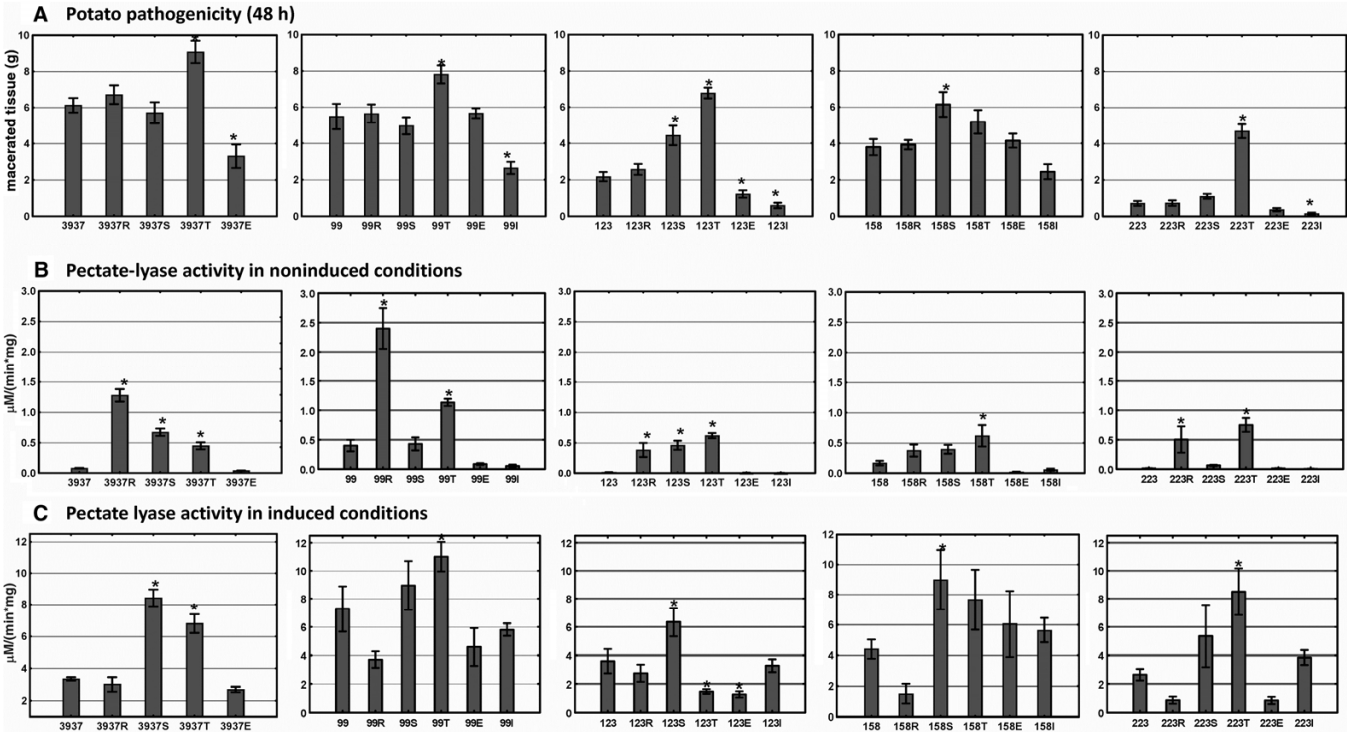

Fig. 5. Phenotypic characteristics of the mutants compared with the parental strains. **A,** Virulence on potato tubers, in grams of macerated tissue 48 h postinoculation (n = 9; for each graph, F = 13.7, 14.6, 50.8, 5.7, and 87.7, respectively). **B,** Total pectate lyase specific activity (µmol min^{-1} mg^{-1}) in noninduced conditions; (n = 3; for each graph, F = 82.9, 33.5, 26.1, 5.5, and 10.7, respectively). **C,** Total pectate lyase specific activity (µmol min^{-1} mg^{-1}) in induced conditions (induction by addition of polygalacturonate) (n = 3; for each graph, F = 38.9, 4.3, 7.2, 3.5, and 6.8, respectively). Presented experiments were performed twice. Strains were *Dickeya dadantii* 3937 (3937); the *D. solani* strains IFB0099 (99), IFB0123 (123), IFB0158 (158) and IFB0223 (223); and the derived mutants indicated with R for the *kdgR*::Cm mutants (e.g., 99R), S for the *pecS*::Cm mutants (e.g., 99S), T for the *pecT*::Cm mutants (e.g., 99T), E for the *expR*::Cm mutants (e.g., 99E), and I for the *expI*::Cm mutants (e.g., 99I). For each figure, the F values resulting from analysis of variance followed by a Fisher test are indicated above, with asterisks indicating mutants that differed significantly from the corresponding wild-type strain ($P < 0.05$).

Phenotypes of the *D. solani pecT* mutants.

The *pecT* mutants of all the *Dickeya* strains showed higher virulence on potato tubers than the wild-type strains (Fig. 5A). The maceration augmentation was significant for all strains except IFB0158. An increase of approximately 1.5-fold of maceration, compared with the wild-type strains, was observed after potato tuber infection with the *pecT* mutants of *D. dadantii* 3937 and IFB0099. The highest increase was observed in the *pecT* mutants of the less-virulent strains, IFB0223 (6.5-fold) and IFB0123 (3.1-fold). The *pecT* mutation had a stronger effect on the virulence on potato than mutations in the other negative regulators tested. The basal pectate lyase activity of all the *pecT* mutants was significantly elevated (Fig. 5B). These results are consistent with the increased ability of the *pecT* mutants to macerate potato tubers. The induced pectate lyase activity was also unregulated in most of the *pecT* mutants but was not statistically significant for IFB0158 (Fig. 5C). Protease activity did not change in most of the *pecT* mutants (Fig. 6A) but it was down regulated for IFB0158 and unregulated for IFB0223. As with *kdgR* and *pecS* mutants, *pecT* mutants of IFB0223 exhibited a protease production not observed in the wild-type strain. The ability to chelate Fe^{3+} ions increased twofold for most of the *D. solani pecT* mutants but there was no change in the *pecT* mutants of *D. dadantii* 3937 and IFB0158 (Fig. 6B). Swimming capabilities significantly increased in all of the *D. solani pecT* mutants and were highest in the IFB0099 *pecT* mutants, where high biosurfactant production was also noticed (Fig. 6C; data not shown).

Phenotypes of the *D. solani expR* and *expI* mutants.

We analyzed the ability of the mutants to produce signaling molecules of the AHL family. The production of AHL was not affected in the *kdgR*, *pecS*, or *pecT* mutants; like the wild-type strains, the mutants were able to produce only C6-HSL and C8-HSL. As expected, the *expI* mutants did not show any AHL production (data not shown). The *expI* and *expR* mutations, which influence QS, appeared to decrease the virulence of *D. dadantii* 3937 and *D. solani* strains on potato tubers (Fig. 5A; not shown for the 3937 *expI* mutant). The *expI* mutation had a greater influence on virulence, and the maceration decrease was significant for IFB0099, IFB0123, and IFB0223 *expI* mutants, with a reduction of two- to threefold compared with the corresponding wild-type strains. Considering the *expR* mutants, the virulence on potato was significantly decreased only for 3937 and IFB0123. The basal pectate lyase activity was not significantly altered by *expR* and *expI* mutations (Fig. 5B). The induced pectate lyase activity was significantly downregulated only in the IFB0123 *expR* mutant (Fig. 5C). Protease production was significantly downregulated in all the *expR* and *expI* mutants, except in IFB0158 *expI* and in IFB0223, which has no protease activity (Fig. 6A). Swimming motility was significantly increased in *D. dadantii* 3937 *expR* mutants and *D. solani* IFB0158 *expI* mutants.

DISCUSSION

Plant-pathogenic pectinolytic bacteria are associated with the increasing economic losses in potato production due to the black-leg and soft-rot symptoms that develop in the field and during storage. Because they are able to survive at a low inoculum level (10^1 to 10^6 cells g^{-1} of peel) as saprophytes on the plant surface and in the intracellular spaces, they are difficult to detect and eradicate (Pérombelon 2002). A new group of strains from the *Dickeya* genus, *D. solani*, has emerged on potato cultivars in temperate climate (Degefu et al. 2013; Slawiak et al. 2009a and b; Toth et al. 2011; Van der Wolf et al. 2014).

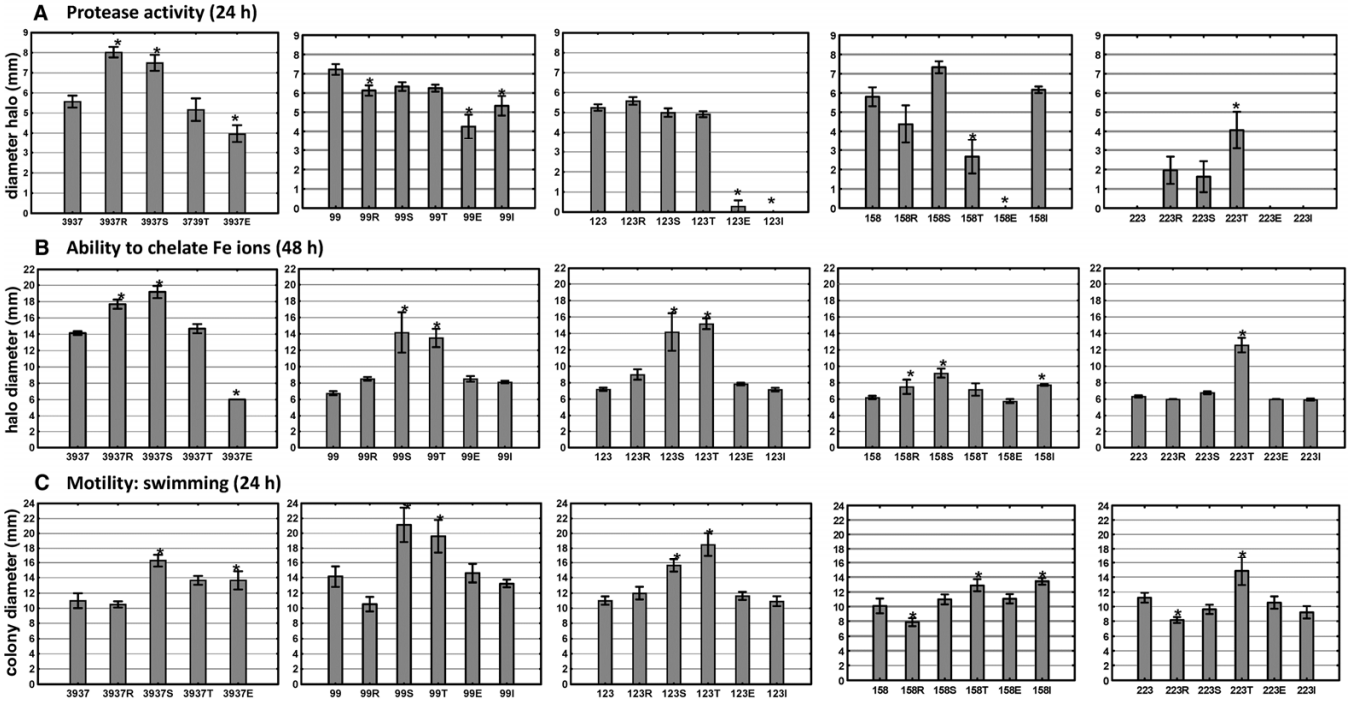

Fig. 6. Phenotypic characteristics of the mutants compared with the parental strains. **A,** Protease production was estimated by the diameter (mm) of the haloes observed on the detection plates (n = 3; for each graph, F = 19.4, 7.3, 135.5, 11.4, and 7.2, respectively). **B,** Iron chelating capacity was estimated by the diameter (mm) of the haloes observed around colonies (n = 3; for each graph, F = 109.8, 12.4, 22.4, 7.8, 3 and 8.8, respectively). **C,** Motility was estimated by the diameter (mm) of the spread of the colonies (n = 6, for each graph, F = 7.1, 7, 10.8, 7.3, and 5.5, respectively). All the experiments were performed twice. Strains were *Dickeya dadantii* 3937 (3937); the *D. solani* strains IFB0099 (99), IFB0123 (123), IFB0158 (158) and IFB0223 (223); and the derived mutants indicated with R for the *kdgR*::Cm mutants (e.g., 99R), S for the *pecS*::Cm mutants (e.g., 99S), T for the *pecT*::Cm mutants (e.g., 99T), E for the *expR*::Cm mutants (e.g., 99E), and I for the *expI*::Cm mutants (e.g., 99I). For each figure, the F values resulting from analysis of variance followed by a Fisher test are indicated above, with asterisks indicating mutants that differed significantly from the corresponding wild-type strain ($P < 0.05$).

In recent years, most of the black-leg cases in The Netherlands were caused by the newly emerged *D. solani* (Czajkowski et al. 2009, 2012). In 2010, Scotland introduced a "zero tolerance" policy for the presence of *Dickeya* spp. in seed potato tubers (Toth et al. 2011). The same policy is gradually being applied in other countries, especially in North Africa (e.g., Egypt), where *Dickeya* spp. can cause much more severe losses in potato production than in Europe (Tsror et al. 2009).

Although it has been shown that *D. solani* can more easily spread through the plant vascular system and initiate the development of the disease from lower inoculum levels, and has a higher range of optimal temperature for disease development than the other potato pathogen, *D. dianthicola* (Czajkowski et al. 2010, 2013), little is known about the molecular mechanisms responsible for these characteristics. Because it has been shown from draft bacterial genome sequences (Pritchard et al 2013), DNA-DNA hybridization, multilocus sequence typing analysis, and matrix-assisted laser desorption ionization time-of-flight mass spectrometry protein mass fingerprints (Van der Wolf et al. 2014) that *D. solani* and *D. dadantii* species are close relatives, we wanted to investigate whether the regulators involved in the control of *D. dadantii* 3937 virulence are also important for *D. solani* virulence. The objective of this study was to examine the importance in *D. solani* virulence of three major negative transcriptional regulators (KdgR, PecS, and PecT) and of the QS system mediated by ExpR and ExpI. Because the virulence regulation is well documented in *D. dadantii* 3937, this strain and its mutants were used for comparison. To acquire a better understanding of the regulation of *D. solani* virulence, we used four strains that exhibit significant differences in the ability to cause disease symptoms. Mutants in each of the selected regulatory genes were constructed in the four *D. solani* strains. An extensive characterization of the phenotypic traits of the four *D. solani* wild-type strains and of the derived mutants was performed.

Phenotypes of the wild-type strains.

A study of several *D. solani* strains from Poland, different European countries, and Israel that exhibit differences in their ability to cause disease symptoms (Lojkowska et al. 2012) may reveal general effects, rather than strain-specific effects, and also indicate the extent of interstrain variation. Thus, four strains showing significant differences in virulence on potato and chicory were selected for this investigation. Despite the similarity in their genomic profiles (Fig. 2), the *D. solani* strains showed certain dissimilarities in many of the phenotypes tested (Figs. 3 and 4). Not surprisingly, the most striking differences were observed between IFB0099, the most virulent strain, and IFB0223, the least virulent strain.

The basal and induced levels of pectate lyase activity globally correlated with the level of maceration caused by the tested *D. solani* strains on chicory and potato, with maximum activity for the most virulent strains, IFB0099 and IFB0158, and lowest activity for the least virulent strain, IFB0223 (Fig. 4A and B). This is not surprising because pectate lyases are regarded as the main virulence factor of *Dickeya* spp. A high level of production in noninduced conditions may enable the strains to provoke symptoms more rapidly at the beginning of infection, before induction becomes really efficient (Hugouvieux-Cotte-Pattat et al. 1996). Together with a high growth rate, a high basal production of pectate lyases may be crucial in the initiation of the disease. As regards the production of proteases (Fig. 4C), slight differences were observed among the *D. solani* strains, with the exception of IFB0223, where no protease production was observed. Together with its low growth rate and low pectate-lyase level, the absence of protease production and a weak ability to chelate Fe^{3+} may contribute to the low virulence of strain IFB0223. In addition, strain IFB0223 was more sensitive to the phage phi-EC_2 infection than the other *D. solani* strains.

It is noticeable that, among the *D. solani* strains tested, the most virulent strain, IFB0099, showed the highest growth rate, basal and induced pectate lyase activity, cellulase and protease production, and swimming ability (Fig. 4F), a capacity that could promote the spread of bacteria in the plant tissues. All these features are also known to contribute to the fitness of the bacteria in their environment, and IFB0099 seems to be the best "equipped" strain in this respect.

Phenotypes of the regulatory mutants.

Prior to the construction of the mutants, the presence of genes coding for the chosen regulators was verified using both PCR and analysis of the draft genome sequences of IFB0099 and IFB0223 (*unpublished data*). Gene conservation was complete in the two *D. solani* strains and all the orthologous genes were found to be 100% identical. Moreover, the orthologous genes of *D. solani* and *D. dadantii* 3937 shared the highest level of identity (92 to 95%) in comparison with the genes of other *Dickeya* spp. whose genomes are available (75 to 88% identity between orthologous genes). The high degree of identity between *D. dadantii* and *D. solani* regulatory genes enabled us to construct *D. solani* mutants using a transduction procedure with phage phi-EC_2 previously described for *D. dadantii* 3937 (Résibois et al. 1984). In addition, this approach allowed for a subsequent comparison of the *D. dadantii* 3937 and *D. solani* mutants.

The phenotypic characterization of *D. solani* and *D. dadantii* 3937 mutants suggested that, overall, PecS, PecT, and KdgR proteins act in a similar manner in both species. These regulators repress the synthesis of different virulence factors (Hugouvieux-Cotte-Pattat et al 1996). The most important regulator of *D. solani* virulence appeared to be PecT. A clear increase in virulence was observed in the *pecT* mutants compared with the wild-type strains, and the maceration symptoms increased from 1.37- to 6-fold, depending on the *D. solani* strain. The smallest increase was observed for the most virulent strain (IFB0099) whereas the highest increase was observed for the weakly virulent strain (IFB0223). Similarly, basal and induced pectate lyase activity, the ability to chelate Fe^{3+} ions, and swimming motility were all upregulated in the *D. solani pecT* mutants. Although no protease production was detected in wild-type strain IFB0223, it become apparent after PecT, PecS, or KdgR inactivation, suggesting that the genes coding for protease production and secretion are present in the IFB0223 genome but they are strongly repressed. It is noticeable that *pecT* is the least well-conserved gene between *D. solani* and *D. dadantii* 3937 among those analyzed, with 92% identity for *pecT*, compared with 94 to 95% identity for *kdgR*, *pecS*, *expI*, or *expR*. PecT was recently shown to be involved in the thermoregulation of pectate lyase genes (Hérault et al. 2014). It would be interesting to determine whether the PecT regulator plays a role in conferring the large optimal temperature range observed for *D. solani* virulence.

The repressor PecS also affects various virulence factors in *D. solani*. As previously shown in *D. dadantii* (Hommais et al. 2008), blue pigment production was observed in all the *pecS* mutants of *D. solani*. A correlation was observed between the level of blue pigment production and the virulence of the corresponding wild-type strain: the IFB0099 *pecS* mutant produced the largest amount of pigment and the IFB0223 *pecS* mutant produced the lowest amount of pigment. This blue pigment, indigoidin, may facilitate bacterial survival in plants because it is thought to act as a scavenger of free radicals, protecting the bacteria from the plant defense response (Hommais et al. 2008).

Another interesting aspect of the regulation of *D. solani* virulence is the potential effect of QS. QS has been shown to have an important influence on the virulence of a vast number of plant-pathogenic bacteria (Mansfield et al. 2012). To analyze the role of QS in *D. solani,* we constructed mutants with a disruption of *expR* or *expI*, the two genes responsible for QS in *D. dadantii* (Nasser et al. 1998). This system participates in pectate lyase regulation in *D. dadantii* 3937 but is considered to be of little importance for the virulence of this strain (Mhedbi-Hajri et al. 2011). However, QS is thought to play a role in *D. zeae* EC1 virulence (Hussain et al. 2008). Our results also suggest that QS is involved in the regulation of the *D. solani* virulence factors, although to a different level for each strain tested. The virulence of the *D. solani expI* mutants on potato decreased two- to threefold compared with the corresponding wild-type strains. Surprisingly, the *expR* mutant of *D. dadantii* 3937 showed a similar reduction in virulence on potato tubers. The discrepancies between our results and those previously reported (Mhedbi-Hajri et al. 2011) may be due to the use of different plants to study the virulence of the strain 3937. We used potato tubers, whereas Mhedbi-Hajri and associates (2011) infected *Saintpaulia ionantha* and *Arabidopsis thaliana*. It is possible that QS is more important for potato infection. In *D. solani*, the *expI* mutation has a greater effect on virulence than the *expR* mutation, suggesting that AHL produced by ExpI may affect other receptors. No increase in swimming motility was observed in most of the QS mutants of *D. solani* but this effect was reported for *D. zeae* EC1 (Hussain et al. 2008). Moreover, no *expR* and *expI* homologs were found in the *D. paradisiaca* Ech703 genome. Thus, the effects of the ExpI-ExpR QS system seem to vary considerably depending on the *Dickeya* sp.

In conclusion, our study indicates that the major transcriptional regulators KdgR, PecS, and PecT play a similar role in the regulation of virulence in *D. dadantii* 3937 and in *D. solani* (i.e., they act as negative regulators of various factors involved in bacterial virulence). However, some disparities concerning the degree of control exerted by each regulator were observed between the two species and among the *D. solani* strains, with significant differences between the strongly and weakly virulent strains. In addition, this study indicates that the thermoregulator PecT is an essential regulator of *D. solani* virulence. Finally, this work demonstrates the role of QS in the modulation of *Dickeya* virulence, with clear variations depending on the *Dickeya* species and, perhaps, depending on the strains and on the host plants.

MATERIALS AND METHODS

Bacterial strains.

The wild-type *Dickeya* strains used in this study are *D. dadantii* 3937; four *D. solani* strains isolated in Poland, Germany, and The Netherlands; and other *Dickeya* strains listed in Table 1. Mutants in the genes coding for either one of the main virulence negative regulators (*kdgR, pecS*, and *pecT*) or the synthesis and perception of signaling molecules (*expI* and *expR*) are listed in Table 1. Three *E. coli* strains harboring plasmids were used for AHL detection (Table 1). The phi-EC_2 generalized transducing phage was used for transduction of the *Dickeya* strains (Résibois et al. 1984).

Media and growth conditions.

Bacteria were grown in LB (Sambrook et al. 2000) or M63 (Miller 1992) medium supplemented with glycerol (2 g $liter^{-1}$) or polygalacturonic acid (4 g $liter^{-1}$). When required, the media were solidified with agar (15 g $liter^{-1}$). *D. dadantii* or *D. solani* cells were incubated at 30 or 28°C, whereas *E. coli* cells were grown at 37°C. When required, Cm was added at a concentration of 20 or 5 μg ml^{-1}.

Genomic analyses.

The genomic DNA from all strains was purified using a Genomic Mini AX Bacteria Kit (A&A Biotechnology, Gdynia, Poland). The *D. solani* strains were analyzed using the repetitive-sequence-based REP-PCR with a pair of primers (REP 1 R-I and REP 2-I), as described by Versalovic and associates (1991). After electrophoresis, the band patterns of the different strains were compared.

The strains were also analyzed using PFGE after digestion of the genomic DNA with the *Xba*I restriction enzyme (Degefu et al. 2013).

For *dnaX* gene amplification, the PCR conditions and primers were as previously described (Slawiak et al. 2009b). PCR products were purified with a Clean-Up kit (A&A Biotechnology) and sequenced from both primers by Genomed (Warsaw, Poland).

Pathogenicity tests.

Chicory leaves and potato tubers were inoculated as previously described (Hugouvieux-Cotte-Pattat 2004). Three chicory leaves were infected for each strain, using 10^6 CFU per inoculation site. After incubation in a dew chamber for 24 h at 30°C, the length of rotted tissue was measured to estimate the disease severity. Six to nine potato tubers ('Charlotte') were inoculated with 5 μl of bacterial suspension (5×10^6 CFU) using a sterile pipette tip. Following incubation in a dew chamber for 48 h at 30°C, the weight of rotted tissue was measured to estimate the disease severity. The experiments were performed twice, with the number of replicates indicated in the figure legends.

Growth curves.

After inoculation with 50 μl of the bacterial overnight culture, the growth of the *Dickeya* strains was followed in 2 ml of LB medium at 28°C for approximately 20 h. The cultures were performed in 24-well plates (Sarstedt, Nümbrecht, Germany) covered with an adhesive, transparent PCR seal to prevent evaporation of the medium. The optical density (OD) at 595 nm was measured automatically every 30 min in a Beckman Counter DTX800 multimode detector under the control of Multimode Analysis Software 3.1.0.1. For shaking between the measurements, three alternate methods were used: orbital, linear, and squared. After incubation, the cultures were diluted and plated on LB agar plates to determine the number of viable bacteria. The count results agreed with the results obtained from OD measurements. The experiments with two replicates were performed twice.

Enzyme assays and detection.

Pectate lyase activity was determined by monitoring spectrophotometrically the formation of unsaturated products from polygalacturonate at 235 nm (Tardy et al. 1997). Specific activity is expressed as micromoles of unsaturated products liberated per minute per milligram of bacterial dry weight. The pectate lyase activity was measured in bacterial cultures grown for 24 h at 28°C.

Protease production was measured on medium containing skim milk (12.5 g $liter^{-1}$) after incubation for 24 h at 30°C (Ji et al. 1987). The diameter of the clear halo around the colonies was measured.

The ability to produce cellulases was analyzed on M63 agar plates supplemented with glycerol at 2 g $liter^{-1}$ and carboxymethylcellulose at 10 g $liter^{-1}$, as described by Wood (1980). Bacterial suspension (2 μl of 0.5 McFarland) was spotted onto this medium and incubated for 24 h at 28°C. After incubation,

the plates were flooded with Congo Red solution at 10 mg ml^{-1} for 10 min and washed for 5 min with 1 M NaCl. The diameter of the clear halo around the colonies was measured. All experiments were performed twice, with the number of replicates indicated in the figure legends.

Siderophore and biosurfactant production.

Siderophore production was determined on chrome azurol S–agar plates (Schwyn and Neilands 1987) as described by Krzyzanowska and associates (2012). To visualize the production of biosurfactant, bacterial strains were inoculated by stabbing 0.7% semisolid agar plates, and the halo size of surfactant production was examined after growth for 24 h at 30°C (Hommais et al. 2008). The experiment was performed twice, with the number of replicates indicated in the figure legends.

Motility assays.

To assess the swarming motility on the surface of a solid medium, bacterial strains were inoculated onto 0.8% semisolid agar plates which were incubated at 28°C for 24 h (Harshey 2003). To assay the swimming motility inside the medium, the bacterial strains were inoculated into 0.3% semisolid agar plates and incubated at 28°C for 24 h (Harshey 2003). Inoculations were performed with 2 µl of bacterial suspension (10^4 CFU ml^{-1}). The experiment was performed twice, with the number of replicates indicated in the figure legends.

AHL production.

Bacteria were grown on LA plates (Sambrook et al. 2000). After 24 h of growth, the plates were covered with an overlay of 5 ml of soft-top agar (4 g $liter^{-1}$) and 100 µl of one of the *E. coli* indicator strains (HB101/pSB401, HB101/pSB534, or HB101/pSB1075) (Table 1). After overnight incubation at 28°C, the plates were analyzed for chemiluminescence in ChemiDoc (Bio-Rad, Hercules, CA, U.S.A.).

For AHL isolation (Shaw et al. 1997), 5 ml of an overnight culture (morpholinepropanesulfonic acid–buffered LB, pH 6.5) was extracted twice with ethyl acetate and the extract was dried under nitrogen gas. The pellet was resuspended in 50 µl of ethyl acetate and 10-fold dilutions were used for further analysis. Thin-layer chromatography was performed on TLC RP18 plates; 1 µl of a 10-fold dilution of each AHL extract was spotted and allowed to dry, and then an overlay was poured with soft-top agar containing one of the biosensor *E. coli* strain (Andresen et al. 2001; Winson et al. 1998). The experiments were performed twice, with three replicates for each strain.

Sensitivity to bacteriophage phi-EC_2.

The sensitivity of the *D. solani* strains to phi-EC_2 was examined. Bacteriophage stocks were prepared by infecting *D. dadantii* 3937 and these stocks were used to infect each *Dickeya* strain. Bacterial lawns were prepared with 0.1 ml of an overnight culture mixed with 5 ml of soft agar (4 g $liter^{-1}$), poured onto LB supplemented with glucose at 2 g $liter^{-1}$ and 2 mM $CaCl_2$, a medium that improved the multiplication of the phages. Serial dilutions (10 µl; up to 10^{-5}) of the phage stocks were spotted onto the bacterial lawns. The plates were incubated for 24 h at 30°C. The transparent plaques under the drops of phage stock indicated the lysis of the tested bacterial strain by phage phi-EC_2. The experiment was performed three times.

Construction of *D. solani kdgR*, *pecS*, *pecT*, *expR*, and *expI* mutants.

Because *D. solani* strains IFB0099, IFB0128, IFB0158, and IFB0223 are sensitive to phi-EC_2, this phage was used to transfer mutations from *D. dadantii* to *D. solani* strains. First, phage stocks were prepared by infection of each *D. dadantii* mutant containing a regulatory mutation constructed by insertion of a cassette encoding Cm^R. Then, the phage stocks were used to infect *D. solani* strains or *D. dadantii* 3937 and the mutations were selected using LB + Cm solid medium. In most cases, three different Cm^R colonies were recovered from each transduction (Table 1). The correct insertion of the Cm cassette was verified using PCR with specific primers (Table 2). PCR reactions were carried out in a 25-µl reaction mixture containing 2 µl of bacterial lysate. The reaction mixture contained 1× reaction buffer (Fermentas, Vilnius, Lithuania), 2.5 mM $MgCl_2$, 80 µM each dNTP, 0.4 µM each primer pair, and 1 U of recombinant DNA *Taq* polymerase (Fermentas). PCR reactions were performed using a TGradient Biometra thermocycler at the following settings: a denaturation step (94°C, 5 min); 30 cycles of denaturation (94°C, 60 s), annealing (50°C, 60 s), and extension (72°C, 120 s); with a final extension step (72°C, 10 min). The amplified products were stained with 1 µl of GelRed Nucleic Acid Stain at 0.5 µg ml^{-1} and analyzed on 1.5% agarose (Prona, Madrid) gels in 0.5× Tris-borate-EDTA buffer. Gels were run at 100 V for approximately 40 min at room temperature. A 100-bp or 1-kb ladder (Fermentas) was used as a size marker.

Data analysis.

Comparisons were conducted between the phenotypic characteristics of the different wild-type strains as well as between the phenotypes of the mutants and that of corresponding wild-type strains. Statistical analysis (ANOVA followed by Fisher post-hoc test) was carried out using Statistica 10.0 StatSoft Polska. Data representing the mean values and error bars representing standard deviations are shown in the figures. The significance level used was $P < 0.05$.

ACKNOWLEDGMENTS

We thank K. Smalla for the gift of the strain IFB0223 isolated in Germany. Appreciation is expressed to G. Condemine, V. Shevchik, and V.

Table 2. Primers designed and used in this study

Gene	Name	Sequence 5′–3′	Product size (bp)[a]
kdgR	kdgRf	GCTCTAGACCGACAGACAATTCGACAGTC	
	kdgRr	GCGGATCCTGTTTACGTCTGATGTCGATGG	1,200
pecS	pecSf	GACGTATCACATCCTGTGAC	
	pecSr	AAACCGGAACGTCTGACG	1,021
pecT	pecTf	AACATCAACACCAGGTATG	
	pecTr	TAATGATTCATCTGTCGG	991
expR	expRf	CAATTTGGCTTTCCGATC	
	expRr	CATTACACAGATAACAGACGC	971
expI	expIf	ATGTCAAATAACAAGCTGGATG	
	expIr	ATGTCAAATAACAAGCTGGATG	591

[a] Primers were used to check the presence of the genes in the *Dickeya solani* genomes and their inactivation in the mutants constructed by transduction. Product size is given without the inserted antibiotic-resistance cassette (1.1 kb long).

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