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Inactivation of *pecS* restores the virulence of mutants devoid of osmoregulated periplasmic glucans in the phytopathogenic bacterium *Dickeya dadantii*

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Dickeya dadantii is a phytopathogenic enterobacterium that causes soft rot disease in a wide range of plant species. Maceration, an apparent symptom of the disease, is the result of the synthesis and secretion of a set of plant cell wall-degrading enzymes (PCWDEs), but many additional factors are required for full virulence. Among these, osmoregulated periplasmic glucans (OPGs) and the PecS transcriptional regulator are essential virulence factors. Several cellular functions are controlled by both OPGs and PecS. Strains devoid of OPGs display a pleiotropic phenotype including total loss of virulence, loss of motility and severe reduction in the synthesis of PCWDEs. PecS is one of the major regulators of virulence in *D. dadantii*, acting mainly as a repressor of various cellular functions including virulence, motility and synthesis of PCWDEs. The present study shows that inactivation of the *pecS* gene restored virulence in a *D. dadantii* strain devoid of OPGs, indicating that PecS cannot be de-repressed in strains devoid of OPGs.

INTRODUCTION

Osmoregulated periplasmic glucans (OPGs) are a family of periplasmic oligosaccharides found in the envelope of most *Proteobacteria*. Their two common features are that glucose is the sole constitutive sugar and their abundance in the periplasm increases as the osmolarity of the medium decreases (Bohin & Lacroix, 2006). In *Enterobacteria* and related bacteria, both products of the *opgGH* operon are required for synthesis of the glucose backbone of OPGs. *opgG* or *opgH* mutant strains are completely devoid of OPGs and exhibit a reduced or non-virulent phenotype, indicating that these glucans belong to the common virulence factors of many zoopathogens and phytopathogens (Loubens *et al.*, 1993; Mahajan-Miklos *et al.*, 1999; Page *et al.*, 2001).

Dickeya dadantii is a phytopathogenic enterobacterium that causes soft rot disease in a wide range of plant species. This pathogen devastates economically important crops in storage facilities or growing plants (Glasner *et al.*, 2011). Maceration, an apparent symptom of the disease, is the result of the synthesis and secretion of a set of plant cell wall-degrading enzymes (PCWDEs), particularly pectinases (Barras *et al.*, 1994; Collmer & Keen, 1986), but many additional factors are required for full virulence. Expression of these factors during pathogenesis is strictly controlled by a complex regulatory network required for adaptation, resulting in bacterial response to host injury after entry into the host. In particular, bacteria must respond to acid stress upon penetration of host cells, oxidative stress during invasion and osmotic stress during maceration (Plessis *et al.*, 2011).

In *D. dadantii*, mutants devoid of OPGs show a pleiotropic phenotype including a loss of motility, decreased synthesis and secretion of PCWDEs linked to reduced synthesis of the Out system, increased synthesis of exopolysaccharide, induction of a general stress response and complete loss of virulence on potato tubers or chicory leaves (Bouchart *et al.*, 2007; Page *et al.*, 2001). In strains of *D. dadantii* devoid of OPGs, inactivation of the RcsCDB phosphorelay regulatory system restored PCWDE synthesis and secretion, motility, normal exopolysaccharide synthesis and virulence in potato tubers, indicating that OPGs control the activation level of this phosphorelay (Bontemps-Gallo *et al.*, 2013; Bouchart *et al.*, 2010). The relationship between this phosphorelay and OPGs highlights the role of these glucans as intermediates in the perception of environmental changes, given that phosphorelays are the main systems used by bacteria to sense and adapt to environmental variations (Clarke, 2010). Repression of the activation level of the RcsCDB phosphorelay by OPGs is not sufficient to explain the pleiotropic phenotype of strains devoid of OPGs, as virulence restoration was observed only on potato tubers but not on chicory leaves.

Abbreviations: OPG, osmoregulated periplasmic glucan; PCWDE, plant cell wall-degrading enzyme; PGA, polygalacturonate; PNPU, 4-nitrophenyl β-D-glucuronide; ROS, reactive oxygen species; TCA, tricarboxylic acid.

This suggests that the relationship between OPGs and additional regulatory proteins is linked to virulence.

The transcriptional regulatory protein PecS, acting mainly as a repressor, is a complex global regulator of major importance for the virulence of *D. dadantii*. As was observed for the RcsCDB phosphorelay, several cellular functions are controlled by both this regulator and OPGs. Among the numerous genes of the PecS regulon are PCWDE genes, oxidative stress response genes such as the antioxidant blue pigment indigoidine, and motility genes (Hommais *et al.*, 2008; Rouanet *et al.*, 2004). No inducer is known to inactivate the PecS transcriptional repressor.

In this study, we investigated the relationship between OPGs and the PecS regulator. Inactivation of this regulator restores virulence in potato tubers, but also in chicory leaves in strains devoid of OPGs. This suggests a constitutive repression state of the PecS transcriptional regulator in strains devoid of OPGs. In addition, we show that

Table 1. Strains and plasmids

Strain or plasmid	Relevant description/genotype*	Source or reference
Dickeya dadantii		
EC 3937	Wild-type strain	Laboratory collection
A 2148	*pecT*::*Cml*	G. Condemine
A 2575	*eps*::*lacZ–Tet*	Condemine *et al.* (1999)
A 3953	*pecS*::MudIIPR13–*Cml*	Reverchon *et al.* (1994)
A 3954	*indA*::*uidA–Kan*	Reverchon *et al.* (1994)
A 3956	*pecS*::MudIIPR13–*Cml indA*::*uidA–Kan*	Hommais *et al.* (2008)
A 4115	*kdgR*::*Spe*	G. Condemine
A 5009	*fliC*::*uidA–Kan*	Hommais *et al.* (2008)
NFB 3500	*opgG*::*uidA–Kan*	Laboratory collection
NFB 3723	*opgG*::*Cml*	Laboratory collection
NFB 3800	*flhD*::*uidA–Kan*	Bouchart *et al.* (2010)
NFB 3848	*opgG*::FRT	Laboratory collection
NFB 7196	*opgG*::*uidA–Kan pecS*::MudIIPR13–*Cml*	This work
NFB 7234	*opgG*::*Cml indA*::*uidA–Kan*	This work
NFB 7268	*opgG*::FRT *pecS*::MudIIPR13–*Cml indA*::*uidA–Kan*	This work
NFB 7283	*opgG*::FRT *pecS*::MudIIPR13–*Cml flhD*::*uidA–Kan*	This work
NFB 7284	*pecS*::MudIIPR13–*Cml flhD*::*uidA–Kan*	This work
NFB 7299	*opgG*::*Cml kdgR*::*Spe*	This work
NFB 7301	*opgG*::*Cml flhD*::*uidA–Kan*	Bontemps-Gallo *et al.* (2013)
NFB 7362	*pecT*::*Cml opgG*::*uidA–Kan*	This work
NFB 7363	*fliC*::*uidA–Kan opgG*::*Cml*	This work
NFB 7495	*fliC*::*uidA–Kan opgG*::FRT *pecS*::MudIIPR13–*Cml*	This work
NFB 7500	*fliC*::*uidA–Kan pecS*::MudIIPR13–*Cml*	This work
NFB 7527	*eps*::*lacZ–Tet opgG*::*uidA–Kan*	This work
NFB 7528	*pecS*::*uidA–Kan*	This work
NFB 7529	*pecS*::*uidA–Kan opgG*::*Cml*	This work
NFB 7530	*pecM*::*uidA–Kan*	This work
NFB 7531	*pecM*::*uidA–Kan opgG*::*Cml*	This work
Escherichia coli		
Top 10 F′	F′[*lacI*^{q}Tn*10*] *mcrA* Δ(*mrr–hsdRMS–mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74* *deoR nupG recA1 araD139* Δ(*ara–leu*)*7697 galU galK rpsL endA1*	Invitrogen
S17-1λpir	*recA thi pro hsd* (r^- m^+) RP4-2-*Tet*::Mu-*Kan*::Tn*7* λ*pir*	Simon *et al.* (1989)
Plasmids		
pUC18Not	AmpR	Yanisch-Perron *et al.* (1985)
pUC18Not-uidA	AmpR	This work
pUTminiTn5-Kan	Mini-Tn*5*-Kan, oriR6K, KanR, AmpR	de Lorenzo *et al.* (1990)
pNFW518	pUC18Not-*pecS*::*uidA*	This work
pNFW519	pUTminiTn*5*-*pecS*::*uidA–Kan*	This work
pNFW520	pUC18Not-*pecM*::*uidA*	This work
pNFW521	pUTminiTn*5*-*pecM*::*uidA–Kan*	This work

*Cml, chloramphenicol resistance; Kan, kanamycin resistance; Spe, spectinomycin resistance; Tet, tetracyclin resistance; Amp, ampicillin resistance.

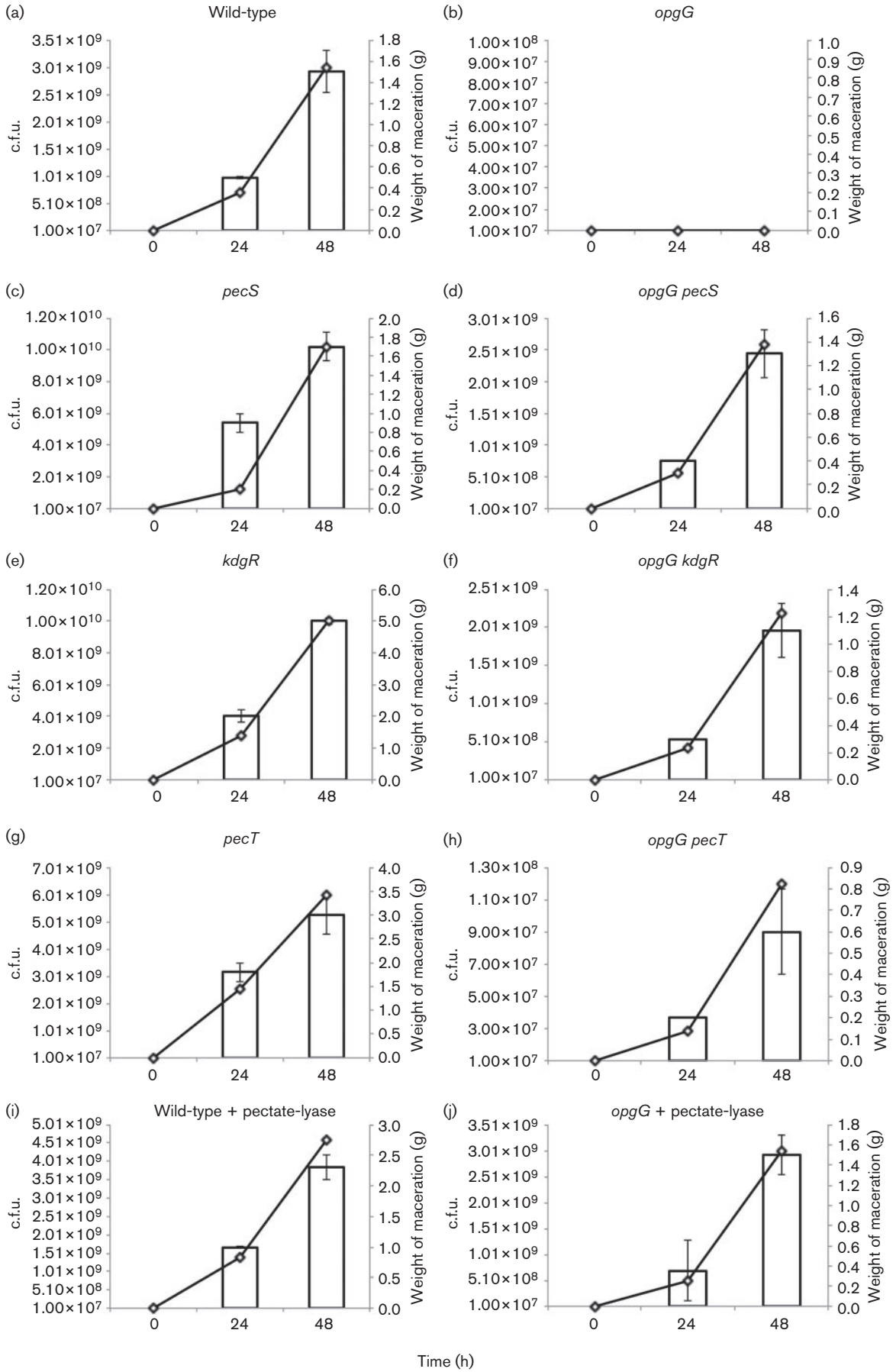
(a)
Wild-type
(b)
opgG
(c)
pecS
(d)
opgG pecS
(e)
kdgR
(f)
opgG kdgR
(g)
pecT
(h)
opgG pecT
(i)
Wild-type + pectate-lyase
(j)
opgG + pectate-lyase
c.f.u.
Weight of maceration (g)
Time (h)

Fig. 1. Virulence of the wild-type without (a) or with pectate-lyases added (i), the *opgG* without (b) or with pectate-lyases added (j), and the *pecS* (c), *ogpG pecS* (d), *kdgR* (e), *opgG kdgR* (f), *pecT* (g) and *opgG pecT* (h) strains inoculated on potato tubers. Bacteria (10^7) were inoculated into holes on potato tubers. Weight of macerated tissue (bars with scale to the right) and number of c.f.u. within macerated tissue (curves with scale to the left) were determined after 24 and 48 h of incubation at 28 °C. Pectate-lyase enzymes were purified from *D. dadantii* grown *in vitro*. The results reported are the mean of three independent experiments.

restoration of PCWDE secretion and synthesis was sufficient to explain restoration of virulence in strains devoid of OPGs on potato tubers but not on chicory leaves.

METHODS

Bacterial strains, media and growth conditions. Bacterial strains are described in Table 1. Bacteria were grown at 30 °C in either lysogeny broth (LB) (Bertani, 2004), minimal medium M63 supplemented with glycerol as a carbon source at a concentration of 2 g l^{-1} (Miller, 1992) or with polygalacturonate (PGA) as an inducer of pectate-lyase synthesis at a concentration of 4 g l^{-1} (Surgey *et al.*, 1996). Solid media were obtained by adding 15 g agar l^{-1}.

Motility tests were made on LB plates containing 0.4 g agar l^{-1}. Bacteria (10^7 in 5 µl) were spotted onto the plate, incubated at 30 °C and swim diameters were measured after 30 h of incubation.

Antibiotics in media were used at the following concentrations: kanamycin, 25 µg ml^{-1} (*D. dadantii*) or 50 µg ml^{-1} (*Escherichia coli*); chloramphenicol, 25 µg ml^{-1} (*D. dadantii*); spectinomycin, 50 µg ml^{-1} (*D. dadantii*); gentamicin, 2 µg ml^{-1} (*D. dadantii*); tetracycline, 12.5 µg ml^{-1} (*D. dadantii*); and ampicillin, 50 µg ml^{-1} (*E. coli*).

Construction of transcriptional fusions. For construction of *pecS*::*uidA* and *pecM*::*uidA*, the coding sequence of the *uidA* gene was amplified by PCR (forward CACGCATGCATTCCGGACCAGTATTATTATC and reverse CACAAAGCTTATCCGCCGCTCACAATTCCAC primers). The *uidA* DNA fragment was digested by *Sph*I and *Hin*dIII and cloned into pUC18Not digested by the same enzymes to yield pUC18Not-uidA. Promoter and the beginning of the coding sequences of the *pecS* and *pecM* genes were amplified by PCR (forward CACTCTAGACAGGGAGCGTAAAAAGCGA and reverse CAGGCATGCATACTTCCAGGTAGCGTGCC for *pecS* and forward CACTCTAGACTTCCAGGTAGCGTGCCATT and reverse CAGGCATGCACAGGGAGCGTAAAAAGCGA for *pecM*). The *pecS* and *pecM* DNA fragments were digested by *Xba*I and *Sph*I and cloned into pUC18Not-uidA digested by the same enzymes to yield pNFW518 and pNFW520, respectively. Both plasmids were digested by *Not*I and cloned in pUTminiTn*5* to yield pNFW519 and pNFW521, respectively. These two latter plasmids were introduced into *D. dadantii* by conjugation.

Transduction and conjugation. Construction of strains was performed by transferring genes from one strain of *D. dadantii* to another by generalized transduction with phage ϕEC2 according to Resibois *et al.* (1984).

Plasmids were introduced into *D. dadantii* by conjugation. The fusions were integrated into the *D. dadantii* chromosome by transposition events selected in the presence of the appropriate antibiotic.

Determination of enzyme activities. β-Glucuronidase assays were performed on crude extracts obtained from bacteria disrupted by sonication (2 × 20 s; Sonifier cell disruptor B-30, Branson; 70 % duty cycle; 7 microtip limit; hold time, continuous; appropriate probe). β-Glucuronidase activity was determined by spectrometric monitoring of the hydrolysis of 4-nitrophenyl β-D-glucuronide (PNPU) at 405 nm. The protein concentration was determined by Bradford assay with BSA as a standard (Bradford, 1976).

Assays of pectate-lyase. Strains were grown overnight in M63 supplemented with glycerol with or without the inducer PGA. Cultures were centrifuged at 2500 ***g*** and supernatants were used for pectate-lyase activity assays. Pectate-lyase activity was determined by spectrometric monitoring of the degradation of PGA to unsaturated products at 235 nm (Moran *et al.*, 1968) at 37 °C in PL buffer (50 mM Tris/HCl, pH 8.5, 0.1 mM $CaCl_2$, 0.05 % PGA).

Quantification of blue pigment indigoidine production. Strains were grown overnight in M63 medium supplemented with glycerol. The culture was centrifuged at 3000 ***g*** to harvest the cells. After centrifugation, the bacterial pellet was suspended in 20 % of the initial culture volume of dimethylformamide (99 %) to extract the blue pigment. Cells were discarded after centrifugation at 3000 ***g*** and the supernatant was used to quantify the blue pigment. The quantity of blue pigment was measured in a spectrophotometer at 615 nm.

Susceptibility to oxidative stress and antimicrobial peptides. Susceptibility to both oxidative stress (H_2O_2) and antimicrobial peptides (polymixin B) was determined as described by Haque *et al.* (2009). H_2O_2 at 6 mM or 1 mg polymixin B ml^{-1} was added to cells grown to stationary phase in M63 glycerol medium. After different incubation times, aliquots of bacteria were taken up and survival was determined by numeration of c.f.u. after plating serial dilutions on LB plates.

Pathogenicity test. Chicory leaves and potato tubers were inoculated as previously described (Page *et al.*, 2001). Briefly, bacteria from an overnight culture in LB medium were recovered by centrifugation and diluted in physiological water. After wounding, 10^7 bacteria were inoculated and incubated in a dew chamber for 48 h at 28 °C.

To measure the severity of the disease on potato tubers, total maceration material was weighed. To determine the number of bacteria within infected plants, maceration of potato tubers or chicory leaves was suspended in 10 ml sterile water. The number of c.f.u. was determined after plating serial dilutions on LB plates.

Statistical analysis. For statistical analyses, XLstat software (Addinsoft) was used. Data were analysed using a paired Wilcoxon Mann–Whitney test; $P<0.05$ was considered significant.

RESULTS

Inactivation of *pecS* in strains devoid of OPGs restores virulence in potato tubers and chicory leaves

The relationship between the *pecS* gene, encoding one of the main repressors of virulence of *D. dadantii* (see Introduction), and OPGs was tested by construction, with transduction and analysis of a double mutant strain *opgG pecS* (see Methods). Virulence of the wild-type, *opgG*, *pecS* and *opgG pecS* double mutant strains was assessed by

measuring the severity of the disease for 48 h following inoculation of 10^7 bacteria on both potato tubers and chicory leaves. On potato tubers, virulence was observed after 24 and 48 h of incubation. For the wild-type strain, growth occurred from 7×10^8 cells and 0.5 g of maceration after 24 h of incubation to 3×10^9 cells and 1.5 g of maceration after 48 h of incubation (Fig. 1a). As expected, no maceration or growth of bacteria was observed for the *opgG* mutant strain on potato tubers (Fig. 1b). For the single mutant strain *pecS*, maceration and bacterial growth were increased by a factor of 1.7 after 24 h of incubation as compared with the wild-type strain (Fig. 1c). After 48 h of incubation, growth was 3.4-fold higher for the *pecS* strain as compared with the wild-type strain whereas maceration increased by a factor of 2.2 as compared with the wild-type strain (Fig. 1c). Virulence was restored for the *opgG pecS* double mutant strain as compared with the *opgG* strain. Maceration and bacterial growth were similar to those observed for the wild-type strain at 24 and 48 h (Fig. 1d). On chicory leaves, virulence was observed after 20, 24, 30, 40 and 48 h of incubation. For the wild-type strain, maceration area increased as the incubation time increased (Fig. 2). As expected, no maceration occurred for the *opgG* mutant strain (Fig. 2). The maceration area was severely increased for the *pecS* single mutant strain as compared with the wild-type strain at each time point (Fig. 2). Virulence was restored in the *opgG pecS* double mutant strain as compared with the *opgG* strain. The maceration area was similar for the *opgG pecS* double mutant and the wild-type strains irrespective of incubation time, suggesting that the colonization speed of chicory leaves was similar for both strains (Fig. 2) In addition, no difference in *pecS* expression was observed between wild-type and *opgG* mutant strains, indicating that the constitutive repression of the PecS regulon is not the result of over-production of PecS in an *opgG* strain (data not shown). These results suggest strongly that in strains devoid of OPGs, repression by the PecS negative transcriptional regulator cannot be derepressed.

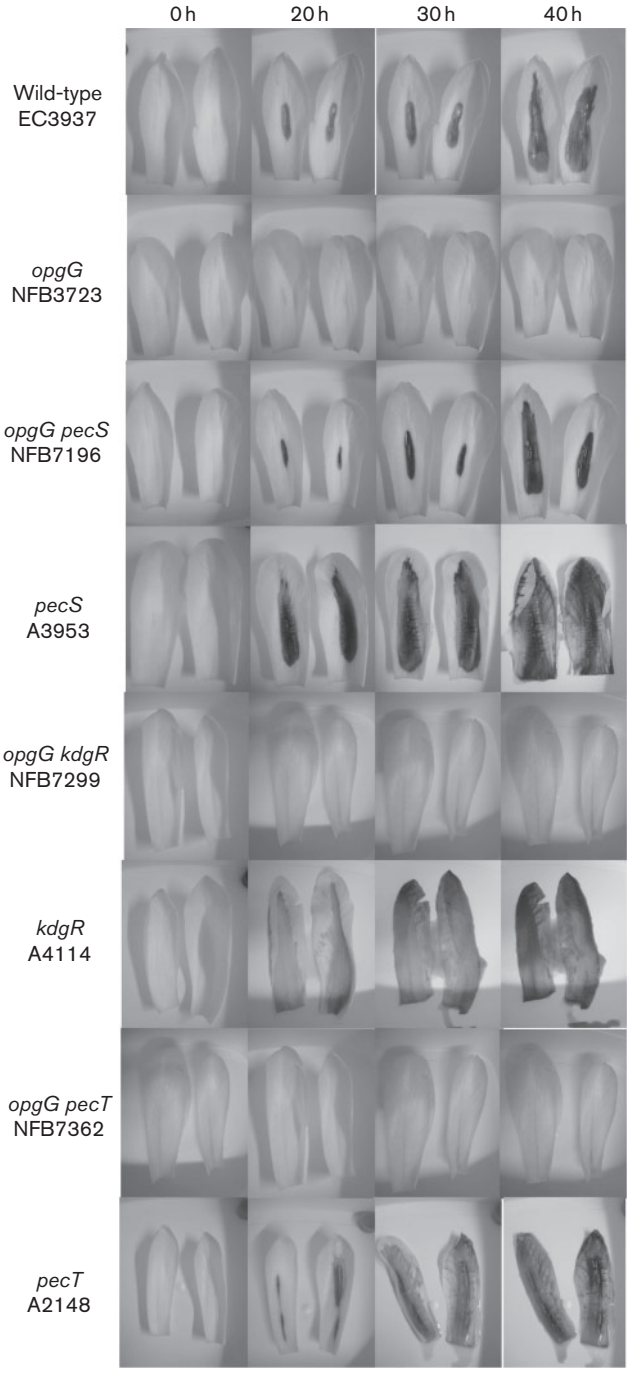

Fig. 2. Time-course of symptom development for the wild-type, *opgG*, *pecS*, *opgG pecS*, *kdgR*, *opgG kdgR*, *pecT* and *opgG pecT* strains inoculated into chicory leaves. Virulence was monitored for 2 days.

Synthesis and secretion of pectinases are restored in the *opgG pecS* double mutant strain

Pectinases are essential factors required for full virulence (see Introduction). Synthesis and secretion of these exoenzymes are cellular functions strictly controlled directly by many transcriptional regulators, particularly PecS, and indirectly by OPGs. Global pectate-lyase activity was quantified after growth with or without the well-known inducer PGA. As expected, in the wild-type strain, pectinase activity was threefold higher after growth in medium containing PGA. In the *opgG* single mutant strain, pectinase activity remained inducible by PGA, given that activity was three fold higher after growth in medium containing PGA, but pectinase activities were decreased by a factor of two as compared with the wild-type strain (Fig. 3), as previously described (Page *et al.*, 2001). For the *pecS* single mutant strain, pectinase activity remained inducible by PGA as activity was three times higher after growth in medium containing PGA, but pectinase activity was increased by a factor of two as compared with the wild-type strain (Fig. 3). For the *opgG pecS* double mutant strain, pectinase activity was restored as compared with the *opgG* single mutant strain. Pectinase activity remained inducible by PGA, with activity twofold higher after growth

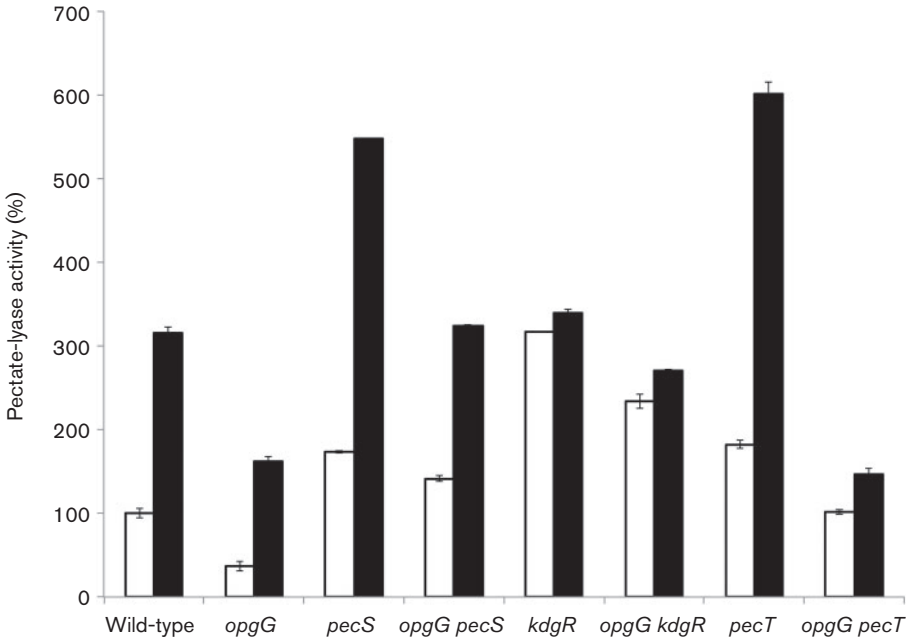

Fig. 3. Pectate-lyase activity of the wild-type, *opgG*, *pecS*, *opgG pecS*, *kdgR*, *opgG kdgR*, *pecT* and *opgG pecT* strains. Cultures were grown in minimal medium overnight with (filled bars) or without (open bars) the inducer PGA. Cells were pelleted and pectate-lyase activity in the culture supernatant was determined (see Methods). The results reported are the mean of four independent experiments.

in medium containing PGA. When grown in medium containing PGA, pectinase activity of this double mutant strain was similar to that measured in the wild-type strain. When grown in medium without PGA, pectinase activity of this double mutant strain was 1.5 times higher than that measured in the wild-type strain (Fig. 3). These results suggest that restoration of pectinase synthesis and secretion are required for restoration of virulence in strains devoid of OPGs, and restoration of pectinase synthesis and secretion, may be sufficient to restore virulence in strains devoid of OPGs.

Restoration of pectinase activity is sufficient to restore virulence of the *opgG* strain in potato tubers but not in chicory leaves

To test this hypothesis, inactivation of the *kdgR* and *pecT* genes was performed, introduced by transduction in an *opgG* mutant strain, and pectinase and virulence activity were assessed under the conditions described for the *pecS* and derivative mutants. The KdgR transcriptional regulator is known to repress only the synthesis of pectinases (Reverchon *et al.*, 1991). This repression is likely to be suppressed *in planta* or when PGA is added to the medium, resulting in the accumulation in bacteria of 2-keto-3-deoxygluconate (KDG), a pectin degradation product and *in planta* inducer of KdgR (Nasser *et al.*, 1992). The PecT transcriptional regulator is also known to repress the synthesis of pectinases (Surgey *et al.*, 1996). In the *kdgR* single mutant strain, pectinases were constitutively expressed given that pectinase activities were similar after growth with or without PGA added and were similar to the pectinase activity observed for the wild-type strain grown with PGA added (Fig. 3) (Nasser *et al.*, 1991). For the *opgG kdgR* double mutant strain, pectinase activity was restored as compared with the *opgG* mutant strain. Pectinase activities were constitutively expressed as pectinase activities were similar after growth with or without PGA added, to a level similar to the pectinase activity observed in the wild-type strain grown with PGA added (Fig. 3). Virulence was assessed on potato tubers. For the *kdgR* single mutant strain, maceration amount and bacterial growth were increased by a factor of 4 and 3.4 after 24 and 48 h of incubation, respectively, as compared with the wild-type strain (Fig. 1e). For the double mutant strain *opgG kdgR*, virulence was restored as compared with the *opgG* strain but maceration amount and bacterial growth were decreased by a factor of 1.7 and 1.4 after 24 and 48 h of incubation, respectively, as compared with the wild-type strain (Fig. 1f). On chicory leaves, maceration occurred at a similar level for the single mutant *kdgR* strain and the wild-type strain while no maceration occurred for the *opgG kdgR* double mutant strain (Fig. 2). For the *pecT* single mutant strain, pectinase activity remained inducible by PGA, being threefold higher after growth in medium containing PGA; however, pectinase activity was increased by a factor of two as compared with the wild-type strain (Fig. 3). For the *opgG pecT* double mutant strain, pectinase activity was

restored as compared with the *opgG* single mutant strain without PGA added but was no longer inducible by PGA (Fig. 3). For the single mutant strain *pecT*, maceration amount and bacterial growth were increased by a factor of 3.6 and 2.0 after 24 and 48 h of incubation, respectively, as compared with the wild-type strain (Fig. 1g). For the double mutant strain *opgG pecT*, virulence was restored as compared with the *opgG* strain but maceration amount and bacterial growth were decreased by a factor of 25 after 24 and 48 h of incubation, respectively, as compared with the wild-type strain (Fig. 1h). On chicory leaves, maceration occurred at a similar level for the single mutant *pecT* strain and the wild-type strain, while no maceration occurred for the *opgG pecT* double mutant strain (Fig. 2). Secreted pectinases were extracted and concentrated from a supernatant of wild-type culture (see Methods). The *opgG* mutant and the wild-type strains were incubated with these purified pectinases on potato tubers (Fig. 1i, j). Virulence was restored in the *opgG* strain grown with pectinases added as compared with the same mutant strain grown without pectinase. In contrast, no virulence was observed when an *opgG* mutant strain was incubated with purified pectinases on chicory leaves, as maceration occurred but growth of bacteria did not resume (data not shown). Taken together, these results indicate that restoration of pectinase synthesis is sufficient to restore the virulence of strains devoid of OPGs on potato tubers but that additional characteristics must be re-establish to restore virulence in chicory leaves.

Motility is not restored in the *opgG pecS* double mutant strain

OPGs and PecS control motility, which is required for full virulence and was thought to be required for extension of the disease throughout the plant. The sigma factor σ^{28}, encoded by the *fliA* gene, is required for expression of the flagella apparatus genes (including the *fliC* gene) encoding flagellin. Expression of the *fliA* gene is repressed by PecS and activated by $FlhC_2D_2$ (Hommais *et al.*, 2008) encoded by the master operon *flhDC*, requiring OPGs for expression (Bontemps-Gallo *et al.*, 2013). Thus, restoration of motility may be one of the factors required for restoration of virulence in strains devoid of OPGs. Motility was measured on soft agar plates. The *opgG* single mutant strain showed a severe decrease in motility as compared with the wild-type strain, while the *pecS* single mutant strain showed a slight increase in motility (Fig. 4a). Surprisingly, the *opgG pecS* double mutant strain was completely non-motile. Expression of the *flhD–uidA* and *fliC–uidA* gene fusions in the same strains correlated with these results. No expression of either gene was observed for the *opgG pecS* double mutant strain, while a decrease in expression was observed for the *opgG* single mutant strain as compared with the wild-type strain (Fig. 4b, c). No difference in expression for the *flhD* gene was observed for the *pecS* single mutant strain as compared with the wild-type strain, confirming that the *flhDC* operon is not regulated by PecS, while a marked increase in *fliC* expression was observed as compared with the wild-type strain, confirming a regulatory role of PecS in flagella synthesis (Fig. 4b, c). These data indicate that motility is not required for virulence under the conditions tested here.

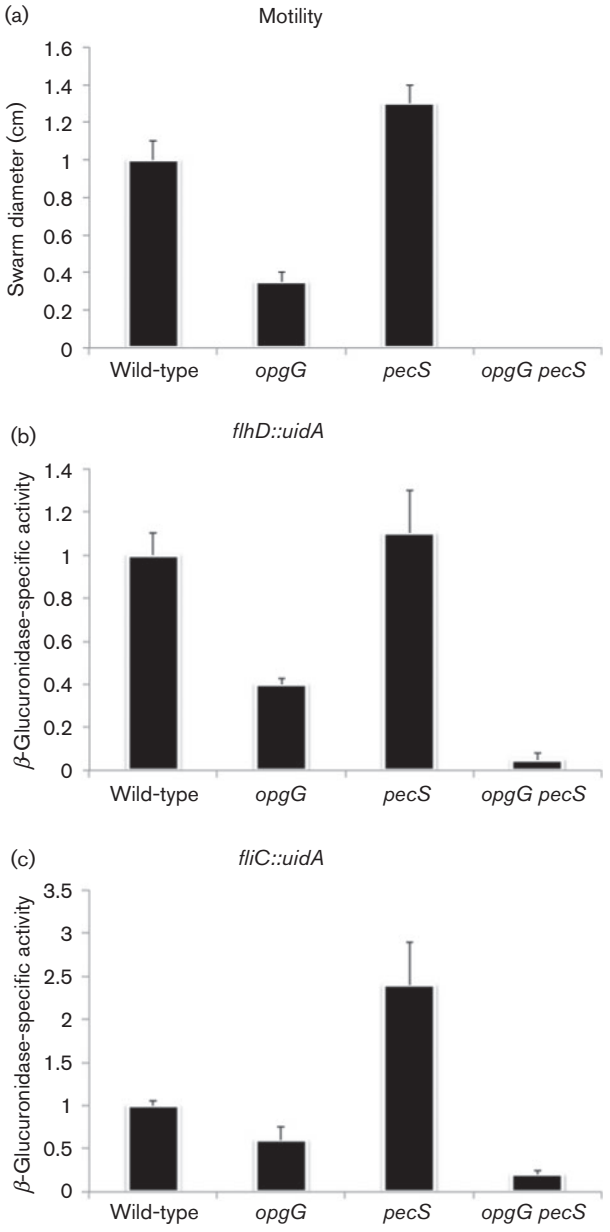

Fig. 4. Motility (a), *flhD* gene expression (b) and *fliC* (c) gene expression of the wild-type, *opgG*, *pecS* and *opgG pecS* strains. For expression of the *flhD*::*uidA* and *fliC*::*uidA* gene fusions, bacteria were grown to the mid-exponential phase and broken by sonication. β-Glucuronidase activity was measured with PNPU as a substrate. Specific activity was expressed as the change in OD_{405} per minute and per milligram of protein. Results are the mean of three independent experiments.

Potato tubers are unable to eliminate the *opgG* strain

The differences between potato tubers and chicory leaves submitted to bacterial pathogen injury may have resulted from the enhanced capability of chicory leaves to induce defence mechanisms against these bacterial pathogens (see Introduction). Thus, we decided to compare the survival of the *opgG* strain inoculated into potato tubers and chicory leaves with the wild-type strain as a control. Bacteria (10^7) were inoculated into potato tubers and chicory leaves. Bacteria were recovered after 24 and 48 h of incubation, and numeration was performed on LB plates. On potato tubers, no growth occurred but bacterial amount remained constant whatever the incubation time (Fig. 5a). On chicory leaves, no growth occurred but bacterial amount decreased regularly over time. In addition, death of the entire bacterial inoculum was observed in less than 72 h (Fig. 5a). As a control, the same experiments were performed on the wild-type strain. As expected, bacterial amount increased regularly over time in both potato and chicory (Fig. 5b). These results suggest strongly that the *opgG* strain is impaired in resistance against the plant host's defences and that potato tubers are unable to induce an effective (or any) defence mechanism against an *opgG* strain.

Resistance to major stresses remains unaffected in the *opgG pecS* double mutant strain

The essential resistance mechanisms required by *D. dadantii* to counteract the defence generated by plant hosts are resistance to acid, oxidative molecules, osmotic stress and antimicrobial peptides (Plessis *et al.*, 2011).

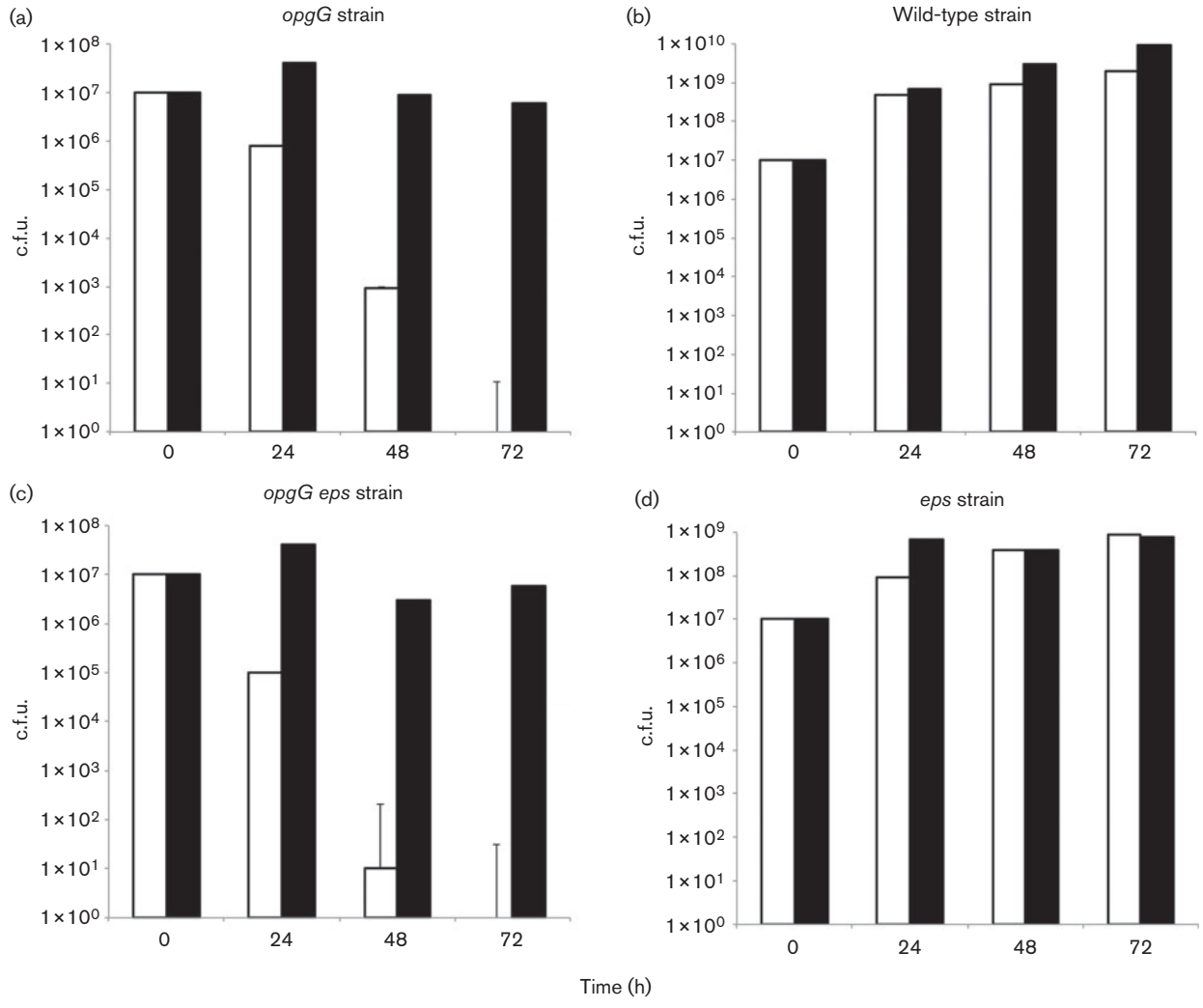

Fig. 5. Survival of the *opgG* (a), wild-type (b), *opgG eps* (c) and *eps* (d) strains in potato tubers (filled bars) and in chicory leaves (open bars). Bacteria (10^7) were inoculated into holes on potato tubers or into chicory leaves. The number of bacteria was counted every 24 h for 72 h. Results are the mean of four independent experiments.

Resistance of the wild-type strain, the single *opgG* and *pecS* mutant strains, and the *opgG pecS* double mutant strain was compared under these various stress conditions.

Resistance to osmotic stress, encountered by bacteria during maceration, was monitored by comparison of growth rate between the different mutant strains in 63 media of increasing osmolarity: half strength, full strength, full strength with 0.1 M NaCl and full strength with 0.2 M NaCl. For each osmolarity tested, no significant growth rate difference was observed between the different strains, with values of 0.40, 0.69, 0.70 and 0.24 h^{-1}, respectively.

Resistance to acid stress, encountered by bacteria during penetration of the host plant, was monitored by comparison of growth rate between the different mutant strains in 63 media of increasing pH: pH 5, 6, 7 and 8. For each pH tested, no significant growth rate difference was observed between the different strains, with values of 0.61, 0.68, 0.70 and 0.69 at pH 5, 6, 7 and 8, respectively.

Resistance of plants hosts is also associated with secretion of antimicrobial peptides. Resistance of the wild-type strain, the *opgG* and *pecS* single mutant strains and the *opgG pecS* double mutant strain was observed by numeration of the survival of bacteria in the presence of 1 mg polymixin B ml^{-1} over a 1 h period. No significant difference in survival was observed for the different strains tested.

These data indicate that resistance to the stresses tested cannot explain the restoration of virulence observed in chicory leaves for the *pecS opgG* double mutant strain.

Resistance to oxidative stress is increased in the *opgG* strain but not in the *opgG pecS* strain

Finally, oxidative stress was tested. In the *pecS* strains, the *indABC* operon, encoding the products catalysing the synthesis of the blue pigment indigoidine, is upregulated (Reverchon *et al.*, 2002). This pigment is known to be one of the responses to the oxidative stress induced by the host plant during invasion. The amount of indigoidine secreted by the wild-type strain, the *opgG* and *pecS* single mutant strains and the *opgG pecS* double mutant strain was quantified (see Methods) (Fig. 6a). No detectable indigoidine was measured for the wild-type and *opgG* mutant strains. As expected, a high level of indigoidine was secreted by both the *pecS* mutant and the *opgG pecS* double mutant (Fig. 6a). Expression of an *indA–uidA* transcriptional fusion was measured after introduction in the same strains and correlated with indigoidine secretion. In the wild-type and *opgG* single mutant strains, *indA* gene expression was similarly low (Fig. 6b). In the *pecS* single mutant and *opgG pecS* double mutant strains, *indA* gene expression was similar and 10 times higher than in the wild-type strain (Fig. 6b).

Thus, the virulence of the *pecS opgG* double mutant strain *in planta* may be explained by a restoration of the defences against oxidative stress induced by the plant. To test this hypothesis, resistance to reactive oxygen species (ROS), generating the oxidative stress, was tested by measuring the hydrogen peroxide resistance of the four strains. Resistance of the wild-type strain, the *opgG* and *pecS* single mutant strains and the *opgG pecS* double mutant strain was observed by numeration of the survival of bacteria in the presence of 6 mM H_2O_2 over a 1 h period (Fig. 6c). The survival percentage of the wild-type strain, the single *pecS* and the double *opgG pecS* mutants decreased rapidly to reach 20 % of the initial population after 20 min of incubation, then stabilized for the *pecS* and the *opgG pecS* strains while survival reached 5 % of the initial population for the wild-type strain. Surprisingly, the *opgG* mutant was more resistant, with 40 % of the initial population still extant after 60 min of incubation. These data indicate that despite the increased synthesis of indigoidine of the *opgG pecS* double mutant strain and increased ROS resistance as compared with the wild-type strain, this double mutant strain remained less resistant than the *opgG* single mutant strain. Thus, restoration of oxidative stress cannot explain restoration of virulence of the *opgG pecS* double mutant strain in chicory leaves.

Increased synthesis of exopolysaccharides is responsible for increased resistance to ROS in the *opgG* strain

It was recently shown that in the symbiotic bacterium *Sinorhizobium meliloti*, exopolysaccharides had a protective effect against ROS (Lehman & Long, 2013) and exopolysaccharides are much more abundant around bacteria in *opg* strains as compared with the wild-type strain (Page *et al.*, 2001). Second, in *E. coli*, it was shown that closing of the tricarboxylic acid (TCA) cycle generated ROS synthesis within bacteria, thus inducing the ROS stress response (Kohanski *et al.*, 2008), and in a previous proteomic analysis (Bouchart *et al.*, 2007), it was shown that in an *opgG* strain, increasing energy level was required as compared with a wild-type strain and resulted in a closed TCA cycle. To test these hypotheses, an *opgG eps* double mutant strain was constructed by transduction. Exopolysaccharides are synthesized by the *eps* operon products (Condemine *et al.*, 1999). While the *opgG* strain displayed a mucoid phenotype, the *eps opgG* strain restored a non-mucoid phenotype. Resistance to ROS was performed as described before. The survival percentage of the single *eps* and double *opgG eps* mutants decreased rapidly to reach 15 % of the initial population after 20 min of incubation, then stabilized for the *eps* strain but reached 10 % of the initial population for the *opgG eps* double mutant strain after 60 min of incubation (Fig. 6c). Thus, ROS resistance decreased severely in the *opgG eps* double mutant strain as compared with the *opgG* single mutant strain. These data indicate that ROS resistance of the *opg* negative strains is the result of increased exopolysaccharide synthesis and secretion. Increased exopolysaccharide synthesis and secretion may explain the total survival of *opgG* strains in potato tubers and

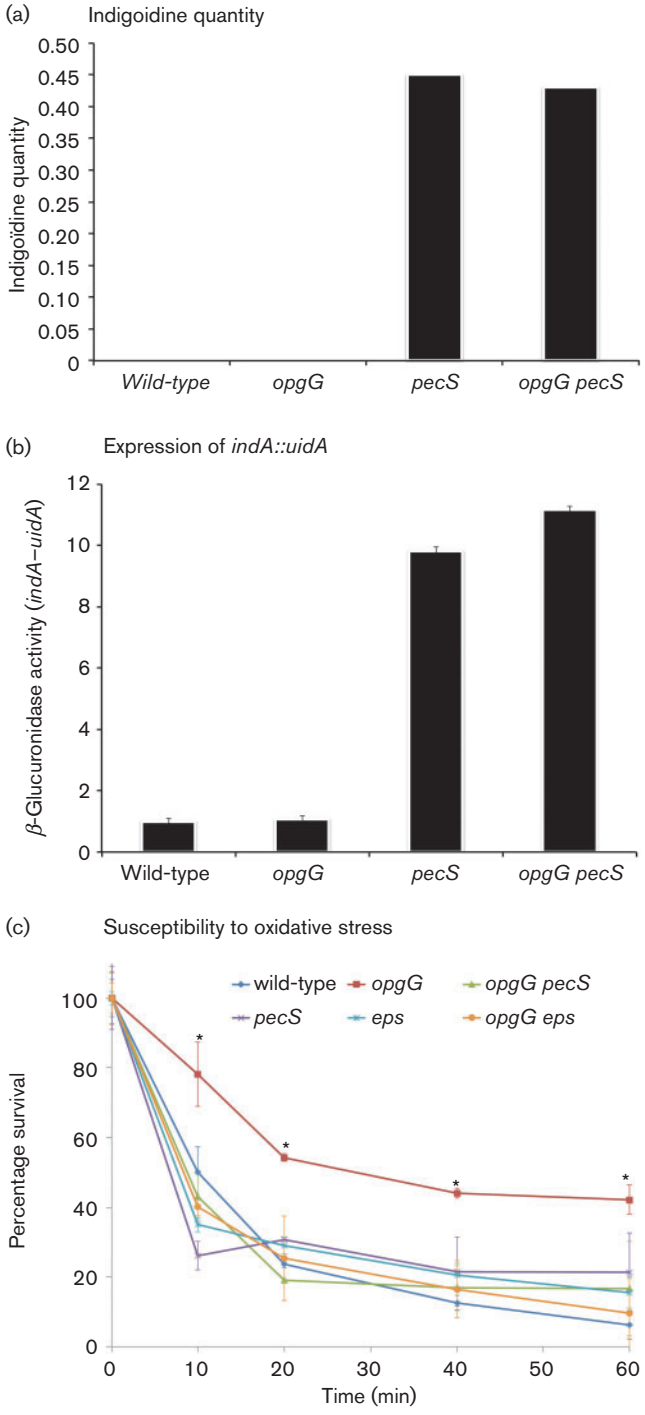

Fig. 6. Indigoidine secretion (a), expression of the *indA–uidA* gene fusion (b) and resistance to H_2O_2 for the wild-type, *opgG*, *pecS*, *opgG pecS*, *eps* and *opgG eps* strains (c). Secreted indigoidine was assayed by measuring optical density at 615 nm (see Methods). The quantity of indigoidine is expressed as OD_{615}/OD_{610}. For expression of the *indA–uidA* gene fusion, bacteria were grown to the mid-exponential phase and broken by sonication. β-Glucuronidase activity was measured with PNPU as a substrate. Specific activity is expressed as the change in OD_{410} per minute and per milligram of protein. For resistance to H_2O_2, bacteria were grown to the mid-exponential phase. The culture was incubated with 6 mM H_2O_2 and survival was numerated. Survival rate was expressed as the number of c.f.u. counted at each time/the number of c.f.u. before addition of H_2O_2. Asterisks indicate that a significant difference exists only between the value of the *opgG* strain and the values of the other strains. Results are the mean of three independent experiments.

the 72 h survival on chicory leaves. Thus, we compared survival of the *opgG eps* strain inoculated into potato tubers and chicory leaves with the *opgG* strain. Bacteria (10^7) were inoculated into potato tubers and chicory leaves. Bacteria were recovered after 24 and 48 h of incubation and numeration was performed on LB plates. On potato tubers, no growth occurred but bacterial counts remained constant whatever the incubation time, as observed for the *opgG* strain (Fig. 5c). On chicory leaves, no growth occurred but bacterial counts decreased regularly with time. In addition, death rate of the entire bacterial inoculum was slightly increased for the *opgG eps* strain (Fig. 5c) as compared with the *opgG* strain (Fig. 5a) but death of all the bacterial inoculum required the same time (72 h). As a control, the same experiments were performed on the *eps* mutant strain. Bacterial counts increased regularly with time in both potato and chicory (Fig. 5d) but to a lesser extent than observed for the wild-type strain (Fig. 5b). These data strongly suggest that exopolysaccharides display a protective but insufficient effect of *opg* negative strains in chicory leaves.

DISCUSSION

In this paper, we describe the relationship between the transcriptional regulator PecS and OPGs. Inactivation of the *pecS* gene led to the restoration of virulence in an *opgG* strain on both potato tubers and chicory leaves. By contrast, the other known suppressors of the Opg phenotype, i.e. the *rcsC* gene (Bouchart *et al.*, 2010), the *kdgR* and the *pecT* genes (this study), allowed restoration only on potato tubers. All these suppressors restore pectinase synthesis and secretion, and the data presented here show that this restoration is sufficient to restore virulence in potato tubers in strains devoid of OPGs. This result could be explained by the inability of this reserve tissue to fight efficiently against the bacterial pathogen. This hypothesis was strengthened by the observation that strains devoid of OPGs do not grow but survive for days in potato tubers. Other plant tissues such as leaves possess several defence mechanisms in response to bacterial injury. In strains devoid of OPGs, responses to stresses caused by plant tissues induced by bacterial pathogen entry may be severely impaired. This hypothesis was strengthened by the observation that strains devoid of OPGs died within 72 h in chicory leaves.

Surprisingly, response and resistance to major stresses known to be encountered by bacteria *in planta* (changes in the pH and osmolarity of the environment, antimicrobial

peptide or ROS secretion) were neither decreased in the *opgG* single mutant strain nor increased in the *opgG pecS* double mutant strain. By contrast, in the *opgG* strain, ROS resistance was increased as compared with the wild-type strain. We showed that this is the result of the protective effect of exopolysaccharides against ROS, as observed recently in the symbiotic bacterium *S. meliloti* (Lehman & Long, 2013), because exopolysaccharides are considerably more abundant around bacteria in *opg* strains as compared with the wild-type strain (Page *et al.* 2001). The data presented here strongly suggest that additional virulence factors as yet unknown are required for restoration of virulence *in planta* for the *opgG pecS* mutant strain. The data also strongly suggest that these virulence factors are repressed by PecS. The role of OPGs remains unclear and is probably indirect. In a previous study, the relationship between OPGs and the phosphorelay RcsCDB was demonstrated (Bouchart *et al.*, 2010). Direct interaction between the RcsC and/or RcsD sensor proteins and OPGs cannot be excluded, as the periplasmic domain of both proteins may interact with these periplasmic glucans. In contrast, despite the fact that in an *opgG* strain, the transcriptional regulator PecS cannot be derepressed, the relationship between the cytoplasmic PecS protein and periplasmic glucans is probably indirect. It remains surprising that the loss of such a simple oligosaccharide leads to such an important deregulation of several cellular pathways, two of these resulting from the absence of derepression of the PecS regulator and the constitutive activation of the RcsCDB phosphorelay. The result is severe alteration in the interactions of these *opg* negative strains with their environment. This leads to a global disorder of the internal control systems of the bacteria, which we term 'bacterial autism'.

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