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1 **The inactivation of *pecS* restores the virulence of mutant devoid of Osmoregulated**
2 **Periplasmic Glucans (OPGs) in the phytopathogen bacteria *Dickeya dadantii***

3
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1 **Summary**

2
3 *Dickeya dadantii* is a phytopathogen Enterobacterium that causes soft rot disease in a wide
4 range of plant species. The maceration, apparent symptoms of the disease, is the result of
5 the synthesis and secretion of a set of plant cell wall degrading enzymes but many
6 additional factors are required for full virulence. Among them, osmoregulated periplasmic
7 glucans (OPGs) and the PecS transcriptional regulator are one of these essential virulence
8 factors. Several cellular functions are controlled by both OPGs and PecS. Strains devoid of
9 OPGs display a pleiotropic phenotype including total loss of virulence, loss of motility and
10 severe reduction in plant cell wall degrading enzymes synthesis. PecS is one of the major
11 regulator of virulence in *D. dadantii* acting mainly as a repressor. It repress various cellular
12 functions including virulence, motility and plant cell wall degrading enzymes synthesis. In
13 this paper, we show that inactivation of the *pecS* gene restored virulence in a *D. dadantii*
14 strain devoid of OPGs indicating that PecS cannot be derepressed in strains devoid of OPGs.

1 **Introduction**

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

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

19 In *D. dadantii*, mutants devoid of OPGs show a pleiotropic phenotype including a loss of motility, a
20 decreased synthesis and secretion of PCWDE linked to a reduced synthesis of the Out system, an
21 increased exopolysaccharide synthesis, an induction of general stress response and a complete loss
22 of virulence on potato tubers or chicory leaves (Bouchart *et al.*, 2007; Page *et al.*, 2001). In strains of
23 *D. dadantii* devoid of OPGs, inactivation of the RcsCDB phosphorelay regulatory system restored
24 PCWDE synthesis and secretion, motility, normal exopolysaccharide synthesis and virulence in
25 potato tubers indicating that OPGs control the activation level of this phosphorelay (Bontemps-Gallo
26 *et al.*, 2013; Bouchart *et al.*, 2010). Relationship between this phosphorelay and OPGs highlight the
27 role of these glucans as intermediate in the perception of environmental changes since
28 phosphorelays are the main systems used by bacteria to sense and adapt to environmental
29 variations (Clarke, 2010). Repression of the activation level of the RcsCDB phosphorelay by OPGs is
30 not sufficient to explain the pleiotropic phenotype of strains devoid of OPGs since virulence
31 restoration was observed only on potato tubers but not on chicory leaves. This suggests relationship
32 between OPGs and additional regulatory proteins linked to virulence.

1 The transcriptional regulatory protein PecS, acting mainly as a repressor, is a complex global
2 regulator of major importance for the virulence of *D. dadantii*. As it was observed for the RcsCDB
3 phosphorelay, several cellular functions are controlled by both this regulator and OPGs. Among the
4 numerous genes of the PecS regulon are found PCWDE genes, oxidative stress response genes such
5 as the antioxidant blue pigment indigoidine, and motility genes (Hommais *et al.*, 2008; Rouanet *et*
6 *al.*, 2004). Actually, no inducer is known to inactivate the PecS transcriptional repressor.
7 In this study, we investigate the relationship between OPGs and the PecS regulator. Inactivation of
8 this regulator restores virulence in potato tubers but also in chicory leaves in strain devoid of OPGs.
9 This suggests a constitutive repression state of the PecS transcriptional regulator in strains devoid
10 of OPGs. In addition, we showed that restoration of PCWDE secretion and synthesis was sufficient to
11 explain restoration of virulence of strains devoid of OPGs on potato tubers but not on chicory leaves.
12

1 **Methods**

2 **Bacterial strains, media and growth conditions**

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

7 Motility tests were made on LB plates containing agar at 0.4 g.l⁻¹. 10⁷ bacteria in 5µL were spotted
8 onto the plate, incubated at 30°C and swim diameters were measured after 30 hours of incubation.

9 Antibiotics in media were used at following concentrations: kanamycin, 25 µg.ml⁻¹ (*D. dadantii*) or
10 50 µg.ml⁻¹ (*E. coli*) chloramphenicol, 25 µg.ml⁻¹ (*D. dadantii*); spectinomycin, 50 µg.ml⁻¹ (*D. dadantii*),
11 gentamycin, 2 µg.ml⁻¹ (*D. dadantii*) tetracycline 12.5µg.ml⁻¹ (*D. dadantii*) and ampicillin 50 µg.ml⁻¹ (*E.*
12 *coli*).

13 **Construction of transcriptional fusions.**

14 For construction of the *pecS::uidA* and *pecM::uidA*, coding sequence of the *uidA* gene was amplified
15 by PCR (**Gus-SphI-F** forward CACGCATGCATTCCGGACCAGTATTATTATC and **Gus-HindIII-R** reverse
16 CACAAAGCTTATCCGCCGCTCACAATTCCAC primers). The *uidA* DNA fragment was digested by SphI
17 and HindIII and cloned into pUC18Not digested by the same enzymes to give pUC18Not-uidA.
18 Promoter and the beginning of the coding sequences of the *pecS* and *pecM* genes were amplified by
19 PCR (**pecSfusF** forward CACTCTAGACAGGGAGCGTAAAAAGCGA and **pecSfusR** reverse
20 CAGGCATGCATACTTCCAGGTAGCGTGCC for *pecS* and **pecMfusF** forward
21 CACTCTAGACTTCCAGGTAGCGTGCCATT and **pecMfusR** reverse
22 CAGGCATGCACAGGGAGCGTAAAAAGCGA for *pecM*). The *pecS* and *pecM* DNA fragments were
23 digested by XbaI and SphI and cloned into pUC18Not-uidA digested by the same enzymes to give
24 pNFW518 and pNFW520 respectively. Both plasmids were digested by NotI and cloned in
25 pUTminiTn5 to give pNFW519 and pNFW521 respectively. These two last plasmids were
26 introduced into *D. dadantii* by conjugation.

27 **Transduction and Conjugation.**

28 Construction of strains were performed by transferring genes from one strain of *D. dadantii* to
29 another by generalized transduction with phage ΦEC2 according to Resibois *et al.* (Resibois, 1984).

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

32 **Determination of enzyme activities**

1 β -glucuronidase assays were performed on crude extracts obtained from bacteria disrupted by
2 sonication 2 x 20 s (Sonifier cell disruptor B-30, Branson, 70% duty cycle, 7 microtip limit, Hold
3 time, continuous, appropriate probe). β -glucuronidase activity was determined by spectrometric
4 monitoring the hydrolysis of PNPU (4-nitrophenyl- β -D-glucuronide) at 405 nm.

5 The protein concentration was determined by the Bradford assay with bovine serum albumin as a
6 standard (Bradford, 1976).

7 **Assays of pectate-lyase**

8 Strains were grown overnight in M63 supplemented with glycerol with or without the inducer PGA.
9 Cultures were centrifuged at 2500g and supernatants were used for pectate-lyase activity assays.
10 Pectate-lyase activity was determined by spectrometric monitoring the degradation of PGA to
11 unsaturated products at 235 nm (Moran *et al.*, 1968) at 37°C in the PL buffer (Tris-HCl 50 mM pH
12 8,5, CaCl₂ 0,1 mM, PGA 0,05%).

13 **Quantification of blue pigment indigoidine production**

14 Strains were grown overnight in M63 medium supplemented with glycerol . The culture was
15 centrifuge at 3000g to harvest the cells. After centrifugation, the bacterial pellet was suspended in
16 20% of the initial culture volume of dimethylformamide (99%) to extract the blue pigment. Cells
17 were discarded after centrifugation at 3000g and the supernatant was used to quantify the blue
18 pigment. The quantity of the blue pigment was measured in a spectrophotometer at 615 nm.

19 **Susceptibility to oxidative stress and antimicrobial peptides.**

20 The susceptibility to oxidative stress (H₂O₂) and to antimicrobial peptides (polymixin B) was
21 previously described (Haque *et al.*, 2009). H₂O₂ at 6 mM or polymixin B at 1 mg.ml⁻¹ was added to
22 cells grown until stationary-phase in M63 glycerol medium. After different times of incubation,
23 aliquot of bacteria were taken up, and the survival were determined by numeration of CFU after
24 plating serial dilutions on LB plates.

25 **Pathogenicity test.**

26 Chicory leaves and potato tubers were inoculated as previously described (Page *et al.*, 2001).
27 Briefly, bacteria from an overnight culture in LB medium were recovered by centrifugation and
28 diluted in physiological water. After wounding, 10⁷ bacteria were inoculated and incubated in a dew
29 chamber until 48h at 28°C.

30 To measure the severity of the disease on potato tubers, the whole maceration was weighted. To
31 determine the number of bacteria within infected plants, whole maceration of potato tubers or
32 chicory leaves was suspended in 10 ml of sterile water. Number of CFU was determined after
33 plating serial dilutions on LB plates.

1 **Statistical Analysis.**

2 For statistical analyses, XLstat software (Addinsoft) was used. Data were analysed by paired
3 Wilcoxon Mann Whitney test, a value of $p < 0.05$ was considered significant.

4

5 **Results**

6 **The inactivation of the *pecS* gene in strains devoid of OPGs restores the virulence on potato** 7 **tubers and on chicory leaves**

8 Relationship between the *pecS* gene, encoding one of the main repressors of virulence of *D. dadantii*
9 (see introduction) and OPGs was tested by construction by transduction and analysis of a double
10 mutant strain *opgG pecS* (see methods). The virulence of the wild-type, the *opgG*, the *pecS* and the
11 *opgG pecS* double mutant strains, were assessed by measuring the severity of the disease for 48h
12 following inoculation of 10^7 bacteria on potato tubers and on chicory leaves. On potato tubers,
13 virulence was observed after 24h, and 48h of incubation. For the wild-type strain, growth occurred
14 from 7×10^8 cells and 0.5g of maceration after 24h incubation until 3×10^9 cells and 1.5g of maceration
15 after 48h of incubation (Fig. 1a). As expected, no maceration nor growth of bacteria were observed
16 for the *opgG* mutant strain on potato tubers (Fig. 1b). For the single mutant strain *pecS*, maceration
17 amount and bacterial growth were increased by a factor of 1.7 after 24h incubation as compared to
18 the wild-type strain (Fig. 1c). After 48h of incubation, growth was 3.4 higher for the *pecS* strain as
19 compared to the wild-type strain whereas maceration increases by a factor of 2.2 as compared to
20 the wild-type strain (Fig. 1c). Virulence was restored for the *opgG pecS* double mutant strain as
21 compared to the *opgG* strain. Maceration amount and bacterial growth were similar to those
22 observed for the wild-type strain at 24h and 48h (Fig. 1d). On chicory leaves, virulence was
23 observed after 20h, 24h, 30h, 40h and 48h of incubation. For the wild-type strain, maceration area
24 increased as the incubation time increased (Fig. 2). As expected, no maceration occurred for the
25 *opgG* mutant strain (Fig. 2). The maceration area was severely increased for the *pecS* single mutant
26 strain as compared to the wild-type strain at each time tested (Fig. 2). Virulence was restored in the
27 *opgG pecS* double mutant strain as compared to the *opgG* strain. The maceration area was similar for
28 the *opgG pecS* double mutant and for the wild-type strains whatever the incubation time suggesting
29 that colonization speed of chicory leaves was similar for both strains (Fig. 2). These results strongly
30 suggest that in strains devoid of OPGs, repression by the PecS negative transcriptional regulator
31 cannot be resumed.

32

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

1 Pectinases are essential factors required for full virulence (see introduction). Synthesis and
2 secretion of these exoenzymes are cellular functions strictly controlled directly by many
3 transcriptional regulators particularly by PecS and indirectly by OPGs. The global pectate-lyase
4 activity was quantified after growth with or without the well known inducer polygalacturonate
5 (PGA). As expected, in the wild-type strain, pectinase activity was 3 time higher after growth in
6 medium containing PGA. In the *opgG* single mutant strain, pectinase activity remained inducible by
7 PGA since activity was 3 time higher after growth in medium containing PGA but pectinase activities
8 were decreased by a factor of 2 as compared to the wild-type strain (Fig. 3) as previously described
9 (Page *et al.*, 2001). For the *pecS* single mutant strain, pectinase activity remained inducible by PGA
10 since activity was 3 time higher after growth in medium containing PGA but pectinase activity was
11 increased by a factor of 2 as compared to the wild-type strain (Fig. 3). For the *opgG pecS* double
12 mutant strain, pectinase activity was restored as compared to the *opgG* single mutant strain.
13 Pectinase activity remained inducible by PGA since activity was two time higher after growth in
14 medium containing PGA. When grown in medium containing PGA, pectinase activity of this double
15 mutant strain was similar to the pectinase activity measured in the wild-type strain. When grown in
16 medium without PGA, pectinase activity of this double mutant strain was 1.5 time higher than the
17 pectinase activity measured in the wild-type strain (Fig. 3). These results suggest that restoration of
18 pectinase synthesis and secretion are required for restoration of virulence in strains devoid of OPGs
19 and one can imagine that restoration of pectinase synthesis and secretion may be sufficient to
20 restore virulence in strains devoid of OPGs.

21
22 **Restoration of pectinase activity is sufficient to restore virulence of the *opgG* strain in potato**
23 **tubers but not in chicory leaves**

24 To test this hypothesis, inactivation of the *kdgR* and the *pecT* genes was performed, introduced by
25 transduction in an *opgG* mutant strain and pectinase and virulence activity were assessed in the
26 conditions described for the *pecS* and derivative mutants strains (see before). The *KdgR*
27 transcriptional regulator is known to repress only the synthesis of pectinases (Reverchon *et al.*,
28 1991). This repression is likely to be suppressed *in planta* or when PGA was added to the medium
29 resulting in the accumulation in bacteria of the 2-keto-3-deoxygluconate (KDG) a pectin degradation
30 product and *in planta* inducer of *KdgR* (Nasser *et al.*, 1992). The *PecT* transcriptional regulator is
31 also known to repress the synthesis of pectinases (Surgey *et al.*, 1996). In the *kdgR* single mutant
32 strain, pectinases were constitutively expressed since pectinase activities were similar after growth
33 with or without PGA added and was similar to the pectinase activity observed for the wild-type

1 strain grown with PGA added (Fig. 3) (Nasser *et al.*, 1991). For the *opgG kdgR* double mutant
2 strain, pectinase activity was restored as compared to the *opgG* mutant strain. Pectinase activities
3 were constitutively expressed since pectinase activities were similar after growth with or without
4 PGA added, to a level similar to the pectinase activity observed in the wild-type strain grown with
5 PGA added (Fig. 3). Virulence was assessed on potato tubers. For the single mutant strains *kdgR*,
6 maceration amount and bacterial growth were increased by a factor of 4 and 3.4 after 24h and 48h
7 incubation respectively as compared to the wild-type strain (Fig. 1e). For the double mutant strain
8 *opgG kdgR*, virulence was restored as compared to the *opgG* strain but maceration amount and
9 bacterial growth were decreased by a factor of 1.7 and 1.4 after 24h and 48h incubation respectively
10 as compared to the wild-type strain (Fig. 1f). On chicory leaves, maceration occurred to a similar
11 level for the single mutant *kdgR* strain and the wild-type strain while no maceration occurred for
12 the *opgG kdgR* double mutant strain (Fig. 2). For the *pecT* single mutant strain, pectinase activity
13 remained inducible by PGA since activity was 3 time higher after growth in medium containing PGA
14 but pectinase activity was increased by a factor of 2 as compared to the wild-type strain (Fig. 3). For
15 the *opgG pecT* double mutant strain, pectinase activity was restored as compared to the *opgG* single
16 mutant strain without PGA added but was no longer inducible by PGA (Fig. 3). For the single mutant
17 strain *pecT*, maceration amount and bacterial growth were increased by a factor of 3.6 and 2 after
18 24h and 48h incubation respectively as compared to the wild-type strain (Fig. 1g). For the double
19 mutant strain *opgG pecT*, virulence was restored as compared to the *opgG* strain but maceration
20 amount and bacterial growth were decreased by a factor of 25 and 25 after 24h and 48h incubation
21 respectively as compared to the wild-type strain (Fig. 1h). On chicory leaves, maceration occurred to
22 a similar level for the single mutant *pecT* strain and the wild-type strain while no maceration
23 occurred for the *opgG pecT* double mutant strain (Fig. 2). Secreted pectinases were extracted and
24 concentrated from a supernatant of wild-type culture (see methods). The *opgG* mutant and the wild-
25 type strains were incubated with these purified pectinases on potato tubers (Fig. 1 i, j). Virulence
26 was restored in the *opgG* strain grown with pectinases added as compared to the same mutant
27 strain grown without pectinase. In contrast, no virulence was observed when an *opgG* mutant strain
28 was incubated with purified pectinases on chicory leaves since maceration occurred but growth of
29 bacteria never resumed (data not shown). Taken together, these results indicated that restoration of
30 pectinase synthesis is sufficient to restore virulence of strains devoid of OPGs on potato tubers but
31 that additional characters must be restored to restore virulence on chicory leaves.

32

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

1 OPGs and PecS control motility which is required for full virulence and was thought to be required
2 for extension of the disease throughout the plant. The sigma factor σ^{28} , encoded by the *fliA* gene, is
3 required for expression of the flagella apparatus genes including the *fliC* gene encoding flagellin.
4 Expression of the *fliA* gene is repressed by PecS and activated by FlhC₂D₂ (Hommais *et al.*, 2008)
5 encoded by the master operon *flhDC* requiring OPGs for expression (Bontemps-Gallo *et al.*, 2013).
6 Thus, restoration of motility may be one of the factors required for restoration of virulence in
7 strains devoid of OPGs. Motility was measured on soft agar plates. The *opgG* single mutant strain
8 showed a severe decrease in motility as compared to the wild-type strain while the *pecS* single
9 mutant strain showed a slight increase in motility (Fig. 4a). Surprisingly, the *opgG pecS* double
10 mutant strain was completely non motile. Expression of the *flhD-uidA* and the *fliC-uidA* gene fusions
11 in the same strains correlated with these results. No expression of both genes was observed for the
12 *opgG pecS* double mutant strain while a decrease of expression was observed for the *opgG* single
13 mutant strain as compared to the wild-type strain (Fig. 4b, c). No difference of expression for the
14 *flhD* gene was observed for the *pecS* single mutant strain as compared to the wild-type strain
15 confirming that the *flhDC* operon is not regulated by PecS while an important increase of the *fliC*
16 expression was observed as compared to the wild-type strain confirming that regulator role of PecS
17 in flagella synthesis (Fig. 4b, c). These data indicated that motility is not required for virulence in
18 our conditions.

19

20 **Potato tubers are unable to eliminate the *opgG* strain.**

21 One can imagine that one of the most important differences between potato tubers and chicory
22 leaves submitted to bacterial pathogens injury resulted in the more important capability of chicory
23 leaves to induce defense mechanisms against these bacterial pathogens (see introduction). Thus, we
24 decided to compare survival of the *opgG* strain inoculated into potato tubers and chicory leaves with
25 wild-type strain as a control. 10⁷ bacteria were inoculated into potato tubers and chicory leaves.
26 Bacteria were recovered after 24h and 48h, of incubation and numeration was performed on LB
27 plates. On potato tubers, no growth occurred but bacterial amount remained constant whatever the
28 incubation time (Fig. 5a). On chicory leaves, no growth occurred but bacterial amount decreased
29 regularly over time. In addition, death of the entire bacterial inoculum was observed in less than
30 72h (fig. 5a). As a control, the same experiments were performed on the wild-type strain. As
31 expected, bacterial amount increased regularly over time in both vegetable (Fig. 5b). These results
32 strongly suggested that the *opgG* strain is impaired in resistance against defence of plant host and
33 that potato tubers are unable to induce an effective defence mechanism if any against an *opgG* strain.

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The resistance to major stresses remained unaffected in the *opgG pecS* double mutant strain

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

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

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 0.1M NaCl and full strength with 0.2M NaCl. For each osmolarity tested, no significant growth rate difference was observed between the different strains ranging from 0,40 h⁻¹, 0,69 h⁻¹, 0,70 h⁻¹ and 0,24 h⁻¹ at increased osmolarity respectively.

Resistance to acid stress, encountered by bacteria during penetration in the host plant, was monitored by comparison of growth rate between the different mutants strains in 63 media of increasing pH: pH5, pH6, pH7 and pH8. For each pH tested, no significant growth rate difference was observed between the different strains ranging from 0,61 h⁻¹, 0,68 h⁻¹, 0,70 h⁻¹ and 0,69 h⁻¹ at pH 5, 6, 7 and 8 respectively.

Resistance of plants hosts are also associated to secretion of antimicrobial peptides.. Resistance of the wild-type strain, the *opgG* and *pecS* single mutant strains and the *opgG pecS* double mutant strains was followed by numeration of the survival bacteria in the presence of 1 mg/mL of polymixin B during 1h. No significant difference in survival was observed for the different strains tested.

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

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

Finally, the oxidative stress was tested. In the *pecS* strains, the *indABC* operon, encoding the products catalysing the synthesis of the blue pigment indigoidine, is up-regulated (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.

1 6a). No detectable indigoidine was measured for the wild-type and the *opgG* mutant strains. As
2 expected, a high level of indigoidine was secreted by the *pecS* mutant as well as the double *opgG*
3 *pecS* mutant (Fig. 6a). Expression of an *indA-uidA* transcriptional fusion was measured after
4 introduction in the same strains and correlated with indigoidine secretion. In the wild-type strain
5 and the *opgG* single mutant strain, the *indA* gene expression level was similarly low (Fig. 6b). In the
6 *pecS* single mutant and the *opgG pecS* double mutant strains, the *indA* gene expression level was
7 similar and 10 times higher than in the wild-type strain (Fig. 6b).
8 Thus, the virulence of the *pecS opgG* double mutant strain *in planta* may be explained by a
9 restoration of the defences against oxidative stress induced by the plant. To test this hypothesis, the
10 resistance to the ROS (Reactive Oxygen Species), generating the oxidative stress, was tested by
11 measuring the hydrogen peroxide resistance of the four strains. Resistance of the wild-type strain,
12 the *opgG* and *pecS* single mutant strains and the *opgG pecS* double mutant strain different strains
13 was followed by numeration of the survival bacteria in the presence of 6 mM H₂O₂ during 1h (Fig.
14 6c). The survival percentage of the wild-type strain, the single *pecS* and the double *opgG pecS*
15 mutants decreased rapidly to reach 20% of the initial population after 20 min. of incubation then
16 stabilize for the *pecS* and the *opgG pecS* strains while the survival reach to 5% of the initial
17 population for the wild-type strain. Surprisingly, the *opgG* mutant was more resistant since 40% of
18 the initial population was still alive after 60 min. of incubation. These data indicated that despite the
19 increased synthesis of indigoidine of the *opgG pecS* double mutant strain and a ROS resistance
20 increased as compared to the wild-type strain, this double mutant strain remains less resistant than
21 the *opgG* single mutant strain. Thus, restoration of oxidative stress cannot explain restoration of
22 virulence of the *opgG pecS* double mutant strain in chicory leaves.

23
24 **Increased synthesis of exopolysaccharides is responsible for increased resistance to ROS in**
25 ***opgG* strain.**

26 It was recently shown that in the symbiotic bacterium *Sinorhizobium meliloti*, exopolysaccharides
27 had a protective effect against ROS (Lehman & Long, 2013) and exopolysaccharides are much more
28 abundant around bacteria in *opg* strains as compared to the wild-type strain (Page *et al.* 2001).
29 Second, in *Escherichia coli*, it was shown that the closing of the TCA cycle generated ROS synthesis
30 within bacteria thus inducing the ROS stress response (Kohanski *et al.*, 2008) and in a previous
31 proteomic analysis (Bouchart *et al.*, 2007), it was shown that in an *opgG* strain, increasing energy
32 level was required as compared to a wild-type strain and resulted in a closed tricarboxylic acid
33 (TCA) cycle. To test these hypothesis, an *opgG eps* double mutant strain was constructed by

1 transduction. Exopolysaccharides are synthesized by the *eps* operon products (Condemine *et al.*,
2 1999). While the *opgG* strain displays a mucoid phenotype, the *eps opgG* strain restored a non
3 mucoid phenotype. Resistance to ROS was performed as described before. The survival percentage
4 of the single *eps* and the double *opgG eps* mutants decreased rapidly to reach 15% of the initial
5 population after 20 min. of incubation then stabilize for the *eps* strain while the survival reach to
6 10% of the initial population for the *opgG eps* double mutant strain after 60 min. of incubation (Fig.
7 6c). Thus, the ROS resistance decreased severely in the *opgG eps* double mutant strain as compared
8 to the *opgG* single mutant strain. These data indicated that the ROS resistance of the *opg* negative
9 strains is the result of the increased exopolysaccharide synthesis and secretion. One can imagine
10 that increased exopolysaccharides synthesis and secretion are one of the reasons of the full survival
11 of *opgG* strains in potato tubers and the 72h survival on chicory leaves. Thus, we decided to
12 compare survival of the *opgG eps* strain inoculated into potato tubers and chicory leaves with the
13 *opgG* strain. 10^7 bacteria were inoculated into potato tubers and chicory leaves. Bacteria were
14 recovered after 24h and 48h, of incubation and numeration was performed on LB plates. On potato
15 tubers, no growth occurred but bacterial amount remained constant whatever the incubation time
16 as observed for the *opgG* strain (Fig. 5c). On chicory leaves, no growth occurred but bacterial
17 amount decreased regularly over time. In addition, death of the entire bacterial inoculum occurred
18 more rapidly for the *opgG eps* strain (48h, Fig. 5c) than in the *opgG* strain (72h, Fig. 5 a). As a control,
19 the same experiments were performed on the *eps* mutant strain. Bacterial amount increased
20 regularly over time in both vegetable (Fig. 5d) but to a less extend than observed for the wild-type
21 strain (Fig. 5b). These data strongly suggest that exopolysaccharides display a protective but
22 insufficient effect for *opg* negative strains in chicory leaves.

23

24 **Discussion**

25 In this paper, we describe the relationship between the transcriptional regulator PecS and OPGs.
26 Inactivation of the *pecS* gene leads to the restoration of virulence in an *opgG* strain both on potato
27 tubers and on chicory leaves. In contrast, the other known suppressor of the Opg phenotype, i.e. the
28 *rscC* gene (Bouchart *et al.*, 2010), the *kdgR* and the *pecT* genes (this study) allow restoration only on
29 potato tubers. All these suppressors restore pectinase synthesis and secretion and data presented
30 here showed that this restoration is sufficient to restore virulence in potato tubers in strains devoid
31 of OPGs. This result could be explained by the incapability of this reserve tissue to fight efficiently
32 against the bacterial pathogen. This hypothesis was strengthened by the observation that strains
33 devoid of OPGs do not grow but survive for days in potato tubers. Other kind of plant tissues such as

1 leaves possessed several defence mechanisms to respond to bacterial injury. One can imagine that
2 in strains devoid of OPGs, response to stresses caused by plant tissues in response to bacterial
3 pathogen entry are severely impaired. This hypothesis was strengthened by the observation that
4 strains devoid of OPGs die within 72 hours in chicory leaves.
5 Surprisingly, response and resistance to major stresses known to be encountered by bacteria *in*
6 *planta* (changes in the pH and the osmolarity of the environment, antimicrobial peptide or ROS
7 secretion) were neither decreased in the *opgG* single mutant strain nor increased in the *opgG pecS*
8 double mutant strain. On the contrary, in the *opgG* strain, ROS resistance was increased as
9 compared to the wild-type strain. We showed that this is the result of the protective effect of
10 exopolysaccharides against ROS as observed recently in the symbiotic bacterium *Sinorhizobium*
11 *meliloti*, (Lehman & Long, 2013) because exopolysaccharides are much more abundant around
12 bacteria in *opg* strains as compared to the wild-type strain (Page *et al.* 2001). Data presented here
13 strongly suggest that still unknown additional virulence factors are required for restoration of
14 virulence in plant for the *opgG pecS* mutant strain. Data also strongly suggested that these virulence
15 factors are repressed by PecS. The role of OPGs remains unclear and is probably indirect. In a
16 previous study, relationship between OPGs and the phosphorelay RcsCDB was demonstrated
17 (Bouchart *et al.*, 2010). Direct interaction between the RcsC and/or RcsD sensor proteins and OPGs
18 cannot be excluded since periplasmic domain of the both proteins may interact with these
19 periplasmic glucans. In contrast, despite the fact that in an *opgG* strain, the transcriptional regulator
20 PecS cannot be derepressed, the relationship between the cytoplasmic PecS protein and periplasmic
21 glucans is probably indirect. It remains very surprising that the loss of such a simple oligosaccharide
22 leads to such an important deregulation of several cellular pathways, two of them resulting from the
23 absence of derepression of the PecS regulator and the constitutive activation of the RcsCDB
24 phosphorelay. The result is a severe alteration in the interactions with their environment of these
25 *opg* negative strains. This leads to a global disorder of the internal control systems of the bacteria
26 that we can qualify of "bacterial autism".

27

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43 strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene*, **33**, 103-119.

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45

1 **Table 1**

Strain or plasmid	Relevant description/genotype ^a	Source or reference
<i>Dickeya dadantii</i>		
EC 3937	wild-type strain	Laboratory collection
A2148	<i>pecT::Cml</i>	G. Condemine
A2575	<i>eps::lacZ-Tet</i>	Condemine <i>et al.</i> , 1999
A 3953	<i>pecS::MudIIPR13-Cml</i>	Reverchon <i>et al.</i> , 1994
A 3954	<i>indA::uidA-Kan</i>	Reverchon <i>et al.</i> , 1994
A 3956	<i>pecS::MudIIPR13-Cml indA::uidA-Kan</i>	Hommais <i>et al.</i> , 2008
A 4115	<i>kdgR::Spe</i>	G. Condemine
A 5009	<i>fliC::uidA-Kan</i>	Hommais <i>et al.</i> , 2008
NFB 3500	<i>opgG::uidA-Kan</i>	Laboratory collection
NFB 3723	<i>opgG::Cml</i>	Laboratory collection
NFB 3800	<i>flhD::uidA-Kan</i>	Bouchart <i>et al.</i> , 2010
NFB 3848	<i>opgG::FRT</i>	Laboratory collection
NFB 7196	<i>opgG::uidA-Kan, pecS::MudIIPR13-Cml</i>	This work
NFB 7234	<i>opgG::Cml indA::uidA-Kan</i>	This work
NFB 7268	<i>opgG::FRT pecS::MudIIPR13-Cml indA::uidA-Kan</i>	This work
NFB 7283	<i>opgG::FRT pecS::MudIIPR13-Cml flhD::uidA-Kan</i>	This work
NFB 7284	<i>pecS::MudIIPR13-Cml flhD::uidA-Kan</i>	This work
NFB 7299	<i>opgG::Cml kdgR::Spe</i>	This work
NFB 7301	<i>opgG::Cml flhD::uidA-Kan</i>	Bontemps-Gallo <i>et al.</i> , 2013
NFB 7362	<i>pecT::Cml opgG::uidA-Kan</i>	This work
NFB 7363	<i>fliC::uidA-Kan opgG::Cml</i>	This work
NFB 7495	<i>fliC::uidA-Kan opgG::FRT pecS::MudIIPR13-Cml</i>	This work
NFB 7500	<i>fliC::uidA-Kan pecS::MudIIPR13-Cml</i>	This work
NFB7527	<i>eps::lacZ-Tet opgG::uidA-Kan</i>	This work
NFB 7528	<i>pecS::uidA-Kan</i>	This work
NFB 7529	<i>pecS::uidA-Kan opgG::Cml</i>	This work
NFB 7530	<i>pecM::uidA-Kan</i>	This work
NFB 7531	<i>pecM::uidA-Kan</i>	This work
<i>Escherichia coli</i>		
Top 10 F'	<i>F'[lacI^qTn10] mcrA, Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15, ΔlacX74, deoR, nupG, recA1, araD139, Δ(ara-leu)7697, galU, galK, rpsL, endA1</i>	Invitrogen
S17-1λpir	<i>recA, thi, pro, hsdR-M+, RP4-2-Tet::Mu-Kan::Tn7, λpir</i>	Simon <i>et al.</i> , 1989
Plasmids		
pUC18Not	AmpR	Yanisch-Perron <i>et al.</i> , 1995
pUC18Not-uidA	AmpR	This work
pUTminiTn5-Kan	Mini-Tn5-Kan, oriR6K, KanR, AmpR	De Lorenzo <i>et al.</i> , 1990

pNFW518	pUC18Not- <i>pecS::uidA</i>	This work
pNFW519	pUTminiTn5- <i>pecS::uidA-Kan</i>	This work
pNFW520	pUC18Not- <i>pecM::uidA</i>	This work
pNFW521	pUTminiTn5- <i>pecM::uidA-Kan</i>	This work

1
2 ^aCml, chloramphenicol resistance ; Kan, kanamycin resistance ; Spe, spectinomycin resistance ; Tet,
3 tetracyclin resistance; Amp, Ampicillin resistance.
4

1 **Figure legends**

2
3 **Fig. 1.** Virulence of the wild-type without (a) or with pectate-lyases added (i), the *opgG* without (b)
4 or with pectate-lyases added (j) , the *pecS* (c), the *opgG pecS* (d), the *kdgR* (e), the *opgG kdgR* (f), the
5 *pecT* (g) and the *opgG pecT* (h)strains inoculated on potato tubers. 10^7 bacteria were inoculated into
6 holes on potato tubers. Weight of macerated tissue (bars with the scale on the right) and number of
7 CFU within macerated tissue (curves with the scale on the left) were determined after 24h and 48h
8 of incubation at 28°C. Pectate-lyase enzymes were purified from *D. dadantii* grown in vitro. The
9 results reported are an average of three independent experiments.

10
11 **Fig. 2.** Time course of symptoms development for the wild-type, the *opgG*, the *pecS* mutants, the
12 *opgG pecS* , the *kdgR*, the *opgG kdgR*, the *pecT* and the *opgG pecT* strains inoculated into chicory
13 leaves. Virulence was monitored for 2 days.

14
15 **Fig. 3.** Pectate-lyase activity of the wild-type, the *opgG*, the *pecS*, the *opgG pecS*, the *kdgR* the *opgG*
16 *kdgR*, the *pecT* and the *opgG pecT* strains. Cultures were grown in minimal medium overnight with
17 (black bars) or without (white bars) the inducer polygalacturonate (PGA). Cells were pelleted and
18 the pectate-lyases activity in the culture supernatant was determined (see Methods). The results
19 reported are an average of four independent experiments.

20
21 **Fig. 4.** Motility (a), *flhD* gene expression (b), and *fliC* (c) gene expression of the wild-type, the *opgG*,
22 the *pecS* mutants, and the *opgG pecS* strains. For the expression of the *flhD::uidA* and *fliC::uidA* gene
23 fusions, bacteria were grown until mid-log phase and broken by sonication. The β -glucuronidase
24 activity was measured with PNPU as a substrate. Specific activity was expressed as the change in
25 optical density at 405 nm per min and per mg of protein. Results are the average of three
26 independent experiments.

27
28 **Fig. 5.** Survival of the *opgG* (a), the wild-type (b), the *opgG eps* (c) and the *eps* (d) strains in potato
29 tubers and on chicory leaves. 10^7 bacteria were inoculated into holes on potato tubers or into
30 chicory leaves. The number of bacteria was counted each 24h for 72h. Results are the average of
31 four independent experiments.

32

1 **Fig. 6.** Indigoidine secretion (a), expression of the *indA-uidA* gene fusion (b), and resistance to H₂O₂
2 for the wild-type, the *opgG*, the *pecS* mutants the *opgG pecS*, the *eps* and the *opgG eps* strains (c).
3 Secreted indigoidine was assayed by measuring optical density at 615 nm (see Methods). The
4 indigoidine quantity is expressed as the OD_{615nm}/OD_{610nm}. For expression of the *indA-uidA* gene
5 fusion, bacteria were grown until mid-log phase and broken by sonication. The β-glucuronidase
6 activity was measured with PNPU as a substrate. Specific activity is expressed as the change in
7 optical density at 410 nm per min and per mg of protein. For resistance to H₂O₂, bacteria were
8 grown until mid-log phase. The culture was incubated with 6 mM H₂O₂ and the survival were
9 enumerated. The survival rate was expressed as the number of CFU counted at each time/the
10 number of CFU before addition of H₂O₂. The asterisks indicated that a significant difference exists
11 only between the value of the *opgG* strain and the values of the other strains. Results are an average
12 of three independent experiments.

13