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Concentration of osmoregulated periplasmic glucans (OPGs) modulates the activation level of the RcsCD RcsB phosphorelay in the phytopathogen bacteria *Dickeya dadantii*

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1 **Concentration of Osmoregulated Periplasmic Glucans (OPGs)**
2 **modulates the activation level of the RcsCD-RcsB phosphorelay in**
3 **the phytopathogen bacteria *Dickeya dadantii*.**

4
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1 **Summary**

2 **Osmoregulated periplasmic glucans (OPGs) are general constituents of many**
3 **Proteobacteria. Synthesis of these oligosaccharides is repressed by increased osmolarity**
4 **of the medium. OPGs are important factors required for full virulence in many zoo- or**
5 **phytopathogens including *Dickeya dadantii*. The phytopathogen enterobacterium *D.***
6 ***dadantii* causes soft-rot disease on a wide range of plant species. The total loss of**
7 **virulence of *opg*-negative strains of *D. dadantii* is linked to the constitutive activation of**
8 **the RcsCD RcsB phosphorelay highlighting relationship between this phosphorelay and**
9 **OPGs. Here we show that OPGs control the RcsCD RcsB activation in a concentration**
10 **dependent manner, are required for proper activation of this phosphorelay by medium**
11 **osmolarity, and a high concentration of OPGs *in planta* is maintained to achieve the low**
12 **level of activation of the RcsCD RcsB phosphorelay required for full virulence in *D.***
13 ***dadantii*.**

14

1 **Introduction**

2 Osmoregulated periplasmic glucans (OPGs) are a family of periplasmic oligosaccharides
3 found in the envelope of most Proteobacteria. Their two common features are that glucose is
4 the sole constitutive sugar and their abundance in the periplasm increases as the osmolarity of
5 the medium decreases (Bohin *et al.*, 2006). Numerous studies indicate that these glucans
6 belong to the common virulence factors for zoopathogens and phytopathogens Proteobacteria,
7 since mutants devoid of OPGs exhibit a reduced or a non-virulent phenotype (Loubens *et al.*,
8 1993; Mahajan-Miklos *et al.*, 1999; Page *et al.*, 2001; Bhagwat *et al.*, 2009). In Enterobacteria,
9 the glucose backbone synthesis of OPGs is catalyzed by the *opgGH* operon products, and
10 *opgG* or *opgH* mutant strains are completely devoid of OPGs. OpgG and OpgH together
11 catalyze the synthesis of linear glucans containing 5 to 12 glucose units joined by β ,1-2
12 linkages and branched by β ,1-6 linkages (Bohin *et al.*, 2006).

13 *Dickeya dadantii*, an Enterobacterium, causes soft rot disease in a wide range of plant species,
14 including many economically important crops (Perombelon *et al.*, 2002). This opportunistic
15 pathogen devastates crops in storage facilities or in growing plants. In *D. dadantii*, *opgG* or
16 *opgH* mutant strains, completely devoid of OPGs, show a pleiotropic phenotype including
17 increased synthesis of exopolysaccharides, loss of motility, induction of a general stress
18 response and complete loss of virulence on potato tubers and chicory leaves. These
19 phenotypes were confirmed by a proteomic analysis and suggest that strains devoid of OPGs
20 are impaired in the perception of their environment (Page *et al.*, 2001; Bouchart *et al.*, 2007).
21 The isolation of revertant mutant strains in the *rscCDB* locus encoding a phosphorelay system,
22 restoring several of the wild-type phenotypes, indicated that OPGs are required in the
23 perception of environmental changes and that these glucans are needed to achieve a low level
24 of activation of the RcsCD RcsB signaling pathway (Bouchart *et al.*, 2010).

1 Two component systems (also called phosphorelay) are the main systems used by bacteria to
2 sense and respond to variations of the environment. Under stimuli essentially unknown, a
3 sensor histidine kinase autophosphorylates and transfers its phosphate group to its cognate
4 response regulator which in turn regulates the expression of different target genes (Buelow *et*
5 *al.*, 2010).

6 The RcsCD RcsB phosphorelay is restricted to enterobacteria. RcsC is the transmembrane
7 kinase, RcsB the phosphorylated regulator, and phosphorylation occurs via the intermediate
8 transmembrane protein RcsD. In several enterobacteria, it was shown that activation of the
9 RcsCD RcsB system enhanced exopolysaccharides synthesis but also cell division, by
10 increasing expression of the *ftsAZ* operon, (Carballès *et al.*, 1999; Bouchart *et al.*, 2010) and
11 decreased virulence but also motility by decreasing expression of the *flhDC* operon (Francez-
12 Charlot *et al.*, 2003; Garcia-Calderon *et al.*, 2005; Majdalani *et al.*, 2005; Bouchart *et al.*,
13 2010). The RcsCD RcsB system is induced by different stimuli including high osmolarity
14 (Sledjeski *et al.*, 1996; Hagiwara *et al.*, 2003; Mouslim *et al.*, 2003). In addition, the RcsCD
15 RcsB phosphorelay activity is enhanced by the outer membrane lipoprotein RcsF in *E. coli*
16 (Majdalani *et al.*, 2005) and repressed by the transmembrane IgaA protein in *S. enterica*
17 (Cano *et al.*, 2002).

18 Data presented in this paper indicate that OPGs concentration modulate activity of the RcsCD
19 RcsB phosphorelay. OPGs are present in bacteria throughout the virulence process *in planta*
20 and are required at a minimal concentration for full virulence.

21 **Results**

22 *Periplasmic OPGs concentration depends on arabinose concentration in the NFB3835 strain*
23 Previously, we showed that OPGs control the RcsCD RcsB phosphorelay in *D. dadantii* by
24 preventing its activation and that total loss of OPGs (*opgG* strains) leads to a constitutive
25 activation of this phosphorelay (Bouchart *et al.*, 2010). Thus, it was tempting to speculate that

1 variations of OPGs concentration affect activation of the RcsCD RcsB phosphorelay. To
2 unravel the relationship between OPGs and the RcsCD RcsB phosphorelay, we decided to test
3 the impact of variation of OPGs concentration on the activation of the RcsCD RcsB
4 phosphorelay.. In *Escherichia coli*, the *araBAD* operon is known to be tightly regulated by the
5 concentration of L-arabinose in the medium by the AraC protein (Guzman *et al.*, 1995). OPGs
6 concentration synthesized by the NFB3835 strain (*araC-P_{BAD}opgGH*, see experimental
7 procedures) was measured in M63 medium containing various L-arabinose concentrations
8 ranging from 0 g l⁻¹ to 1 g l⁻¹ (Fig. 1). No OPGs could be detected without L-arabinose. OPGs
9 concentration increased in cells as the L-arabinose concentration increased in the medium to
10 reach a maximal concentration of 20 µg mg⁻¹ of proteins when 1 g l⁻¹ arabinose was added to
11 the medium. OPGs concentration remained the same when higher L-arabinose concentrations
12 (2 g l⁻¹ or 5 g l⁻¹) were added to the medium (data not shown). This strain can be used to test
13 relationship between OPGs and the RcsCD RcsB phosphorelay, since OPGs concentration of
14 this strain depends on L-arabinose concentration of the medium up to 1 g l⁻¹.

15 *Increasing concentrations of OPGs decrease the activation of the RcsCD RcsB phosphorelay*
16 Relationship between OPGs and the RcsCD RcsB phosphorelay was followed by expression
17 of transcriptional fusions derived from two operons known to be regulated in an opposite way
18 by the RcsCD RcsB phosphorelay in *D. dadantii* (Bouchart *et al.*, 2010). The RcsCD RcsB
19 system regulates motility by down-regulation of the *flhDC* master operon encoding activators
20 required for expression of the flagellar apparatus genes. The *ftsAZ* operon is needed for cell
21 division. One of the promoters regulating this operon is up-regulated by the RcsCD RcsB
22 phosphorelay and it was cloned in the *ftsA-uidA* fusion (Bouchart *et al.*, 2010). The *flhD-uidA*
23 and the *ftsA-uidA* fusions were transduced into the NFB3835 strain and expressions of both
24 fusions were measured after growth in M63 medium with increasing concentrations of L-
25 arabinose. Motility was evaluated in the NFB3835 strain on M63 medium swimming plates

1 with increasing concentrations of L-arabinose. As a control, the respective motility levels of
2 the EC3937 (wild-type) and the NFB3723 (*opgG*) strains were measured in M63 medium
3 with or without various concentrations of L-arabinose. Motility levels remained constant
4 whatever the concentration of L-arabinose for both strains and were 2.2 fold higher for the
5 wild-type strain as compared to the *opgG* mutant strain (Fig. 2A). Motility of the NFB3835
6 strain increased as the concentration of L-arabinose increased (Fig. 2A). The lower motility
7 level was observed in the NFB3835 strain grown in M63 medium and was similar to the
8 motility level observed in the NFB3723 (*opgG*) strain grown in the same medium. The higher
9 motility level was observed in the NFB3835 strain grown in M63 medium with 1 g l⁻¹
10 arabinose added and was similar to the motility level observed in the EC3937 wild-type strain
11 grown in the same medium (Fig. 2A). Expression of the *flhD-uidA* fusion was measured after
12 growth of the NFB7300 strain in M63 medium containing the same increasing concentrations
13 of L-arabinose (Fig. 2B). As a control, the respective β -glucuronidase activity levels of the
14 NFB3800 (wild-type) and the NFB7301 (*opgG*) strains were measured in M63 medium with
15 or without various concentrations of L-arabinose. Glucuronidase activity remained constant
16 for both strains whatever the concentration of L-arabinose and was 2.3 fold higher in the wild-
17 type strain as compared to the *opgG* mutant strain (Fig. 2B). The lower level of β -
18 glucuronidase expression was measured after growth of the NFB7300 in M63 medium
19 without L-arabinose and was similar to the expression level measured in the *opgG* strain
20 (NFB7301) harboring the same fusion. The highest level of expression was measured after
21 growth in M63 medium containing 1 g l⁻¹ of L-arabinose and was similar to the expression
22 level observed in the wild-type strain harboring the same fusion (NFB3800). Increase in the
23 motility was correlated with an increased expression of the *flhD-uidA* fusion. These data
24 indicate that the concentration of OPGs modulates motility in *D. dadantii*. Expression of the
25 *ftsA-uidA* fusion was measured in the same conditions (Fig. 2C). As a control, the respective

1 glucuronidase activities levels of the NFB3809 (wild-type) and the NFB7274 (*opgG*) strains
2 were measured in M63 medium with or without various concentrations of L-arabinose.
3 Glucuronidase activity remained constant for both strains whatever the concentration of L-
4 arabinose and was 6 fold higher in the *opgG* strain as compared to the wild-type strain. The
5 lower level of expression was measured after growth of the NFB3837 strain in M63 medium
6 containing 1 g l⁻¹ L-arabinose and was similar to the expression level measured in the wild-
7 type strain (NFB3809) harboring the same fusion. The highest level was measured after
8 growth in M63 medium without L-arabinose and was similar to the expression level observed
9 in the *opgG* strain harboring the same fusion (NFB7274). These data indicate that
10 concentration of OPGs modulates *ftsA* expression in *D. dadantii*. To ensure that the
11 regulation observed occurs through the RcsCD RcsB phosphorelay, an *rscB* or an *rscC* null
12 mutations were introduced into NFB3835, NFB3837 and NFB7300 (NFB7368, NFB7371 and
13 NFB7369 respectively and NFB7400, NFB7406, NFB7405, respectively). Whatever the *rsc*
14 genetic context, motility and glucuronidase activities of both fusions remained constant
15 whatever the L-arabinose concentration and similar to the one observed for the wild-type
16 strain harboring the same fusions, respectively (Fig 2A, B and C). A recent study revealed
17 that the unphosphorylated form of RcsB is required for proper regulation of biofilm synthesis
18 (Latasa et al. 2012). Thus, the observed phenotypes in the *rscB* null mutant strains could be
19 the result of the lack of the RcsB protein itself rather than the loss of phosphorylation. An
20 *rscCDB*_{D56N} locus encoding an unphosphorylatable form of *rscB* was constructed (see
21 experimental procedures) and introduced into the *rscB* derivative strains NFB7368, NFB7369
22 and NFB7371 (NFB7401, NFB7403, NFB7402, respectively). Results were similar to those
23 observed with *rscB* null mutant strains for motility and glucuronidase activities (Fig. 2A, B
24 and C). To ensure that the RcsB cytoplasmic concentration was independent of the L-
25 arabinose concentration, a western blot analysis was performed in M63 medium with various

1 concentrations of L-arabinose added in the EC3937, NFB3723, NFB3835, NFB7112 and
2 NFB3682 strains. Similar amount of the RcsB protein was observed whatever the strain and
3 L-arabinose concentration tested (data not shown) indicating that regulation occurs through
4 variation of the phosphorylation level of the RcsB transcriptional regulator. Taken together,
5 these results indicate that OPGs control the RcsCD RcsB phosphorelay activity in a
6 concentration dependent manner.

7 *A minimal concentration of OPGs is required for D. dadantii throughout the virulence*
8 *process*

9 The RcsCD RcsB phosphorelay activity is severely repressed during virulence (Garcia-
10 Calderon *et al.*, 2005) and OPGs are required for this repression (Bouchart *et al.*, 2010). Thus,
11 OPGs concentration must affect virulence. To test the impact of OPGs concentration on
12 virulence of *D. dadantii* during pathogenesis, NFB3835 was grown in M63 medium with
13 various concentrations of L-arabinose prior to inoculation. Bacteria were centrifuged and
14 washed twice with M63 medium without a carbon source to remove arabinose to avoid OPG
15 synthesis *in planta*. Bacteria (10^7) were inoculated on chicory leaves and virulence was
16 monitored 24 h and 48 h later (Fig. 3). When no arabinose was provided to NFB3835
17 bacterial cells before inoculation (fig. 3), no maceration of chicory leaves was detected. This
18 indicates that no free arabinose is available *in planta*. A moderate maceration of chicory
19 leaves was observed at a concentration of 0.01 g l^{-1} of L-arabinose while maceration similar to
20 the wild-type strain was observed at an L-arabinose concentration of 1 g l^{-1} . Thus, because no
21 OPG could be synthesized *in planta*, severity of the disease depends on OPGs concentration
22 at time 0 and stops when OPGs concentration in bacteria falls down below the lower
23 concentration required for virulence because of dilution of OPGs during growth of bacteria.
24 This concentration could be estimated around 0.01 g l^{-1} of arabinose added (Fig. 3). This L-
25 arabinose concentration corresponds to $10 \text{ }\mu\text{g}$ of OPGs per mg of protein (Fig. 1). When

1 arabinose (1 g l^{-1}) was added to the bacterial inoculum on chicory leaves or 24 h after
2 inoculation, no virulence was observed when bacteria were grown in 63 medium before
3 inoculation while full virulence was observed whatever the arabinose concentration provided
4 in the 63 medium before inoculation (data not shown). These data indicate that a minimal
5 level of OPGs is required during all the pathogenesis process for full virulence of *D. dadantii*
6 and suggest that *D. dadantii* is able to adjust its OPG concentration *in planta*.

7 *D. dadantii* synthesizes a high concentration of OPGs in planta

8 To test this hypothesis, the concentration of OPGs was determined after growth of the wild-
9 type strain *in planta*. The EC3937 strain was grown in M63 medium and 10^7 bacteria were
10 inoculated on several chicory leaves. OPGs were extracted and purified from bacteria isolated
11 from culture in M63 medium before inoculation (0 h), and 24 h and 48 h after growth *in*
12 *planta*. For each time, CFU were determined. After 24 h of maceration, OPGs concentration
13 increased more than twofold as compared to the concentration observed at 0 h and remained
14 constant after 48 h (Fig. 4A). Because OPGs were purified on P4 gel filtration column, one
15 can argue that OPGs extracted from bacteria grown *in planta* were contaminated by
16 oligosaccharides of similar weight resulting from degradation of plant polysaccharides by
17 hydrolytic enzymes secreted by *D. dadantii* during infection. MALDI TOF mass spectrometry
18 was used to determine the sizes of the OPG backbones. Spectrum of OPGs extracted from
19 bacteria grown in M63 medium was used as a control and compared with spectra of OPGs
20 extracted from bacteria grown *in planta* after 24 h and 48 h of infection. As shown in Fig. 4B,
21 no difference in the sizes of OPGs was observed, whatever growth conditions. Each of them
22 was composed of 4 to 11 residues of glucose. This was clearly seen in mass spectra with the 8
23 $[\text{M}+\text{Na}]^+$ molecular ions at m/z 689, 851, 1013, 1175, 1337, 1499, 1661 and 1823 recovered
24 in each spectrum. For several molecular ions, an increment of 42Da was observed and
25 corresponds to the substitution of OPGs by acetyl residues, a characteristic feature of OPGs

1 isolated from *D. dadantii* (Cogez *et al.*, 2001). Thus, oligosaccharides extracted from bacteria
2 grown *in planta* were only constituted of OPGs. When bacteria were grown in media of
3 higher (0.1 or 0.2M NaCl added) osmolarity or in medium of lower osmolarity (half-strength
4 M63), no difference in the severity of the disease nor in the apparition of the symptoms were
5 observed (data not shown). Taken together, these results indicate that *in planta*, *D. dadantii*
6 adjust its OPG concentration to a higher level than the minimal concentration required to
7 reach a sufficient repression level of the RcsCD RcsB phosphorelay needed for full virulence
8 of *D. dadantii*.

9 *The RcsCD RcsB phosphorelay exhibits a low activity level during virulence*

10 This high concentration of OPGs must be related to a low activity level of the RcsCD RcsB
11 phosphorelay required for virulence. Expression of the *flhD-uidA* and *ftsA-uidA* fusions was
12 measured just before inoculation (0 h) and 24 h and 48 h after inoculation *in planta* in wild-
13 type and *rscB* genetic background (Fig. 4C). The *ftsA-uidA* fusion was expressed at a similar
14 and very low level while the *flhD-uidA* fusion was expressed at a similar and high level
15 during all the virulence process, in both genetic backgrounds and at each time tested. A
16 western blot analysis was performed with bacteria grown *in planta*. Similar amount of the
17 RcsB protein was observed after 0 h, 24 h and 48 h of maceration (data not shown). Thus,
18 these results indicate that the RcsCD RcsB phosphorelay activity is severely repressed during
19 the virulence process in *D. dadantii*.

20 *OPGs are one of the actors required for modulation of the RcsCD RcsB phosphorelay activity* 21 *by medium osmolarity*

22 OPGs synthesis and RcsCD RcsB activation are regulated by medium osmolarity in an
23 opposite way: OPG concentration decrease (Cogez *et al.*, 2001) while the RcsCD RcsB
24 phosphorelay activation occurs as the medium osmolarity increases. Thus, the RcsCD RcsB
25 activation could be modulated by osmolarity via variation of OPGs concentration. To test this

1 hypothesis, motility was evaluated on swimming plates and the *flhD-uidA* and the *ftsA-uidA*
2 transcriptional fusion activities were determined in different genetic backgrounds at various
3 osmolarities in M63 medium containing as a carbon source. In the wild-type strain (EC3937),
4 motility remained constant in half strength M63 medium and in M63 medium then decreased
5 as the osmolarity of the medium increased (Fig 5A). In the different mutant strains, motility
6 levels remained unaffected whatever the osmolarity: as expected, a loss of motility was
7 observed for the *opgG* strain (NFB3723) and motility similar to the wild-type strain was
8 observed for the *rcsB* (NFB7279), the *rcsB_{D56N}* (NFB7112) and the *rcsC* (NFB3682) strains
9 (Fig. 5A). The expression of the *flhD-uidA* fusion harbored by the wild-type strain (NFB3800)
10 remained constant in half strength M63 medium and in M63 medium then decreased as the
11 osmolarity of the medium increased (Fig. 6B). As expected, expression of the *flhD-uidA*
12 fusion in the *opgG* (NFB7301) strain remained low and constant whatever the osmolarity
13 while the expression level of the *flhD-uidA* fusion harbored by the *rcsB* (NFB7202), the
14 *rcsB_{D56N}* (NFB7399) and the *rcsC* (NFB7128) strains was similar to the wild-type strain
15 grown in M63 medium and remained unaffected when osmolarity was increased (Fig. 5B).
16 These results indicate that repression of motility by medium osmolarity depends on variation
17 of concentration of OPGs via activation of the RcsCD RcsB phosphorelay. For the *ftsA-uidA*
18 transcriptional fusion activity (Fig. 7A), a low level of expression in the *rcsB* (NFB7205), the
19 *rcsB_{D56N}* (NFB7408) and the *rcsC* (NFB7127) strains was observed, whatever the osmolarity.
20 Surprisingly, no increase in the expression of the *ftsA-uidA* fusion was observed in the wild-
21 type strain (NFB3809). In the *opgG* strain (NFB7274), expression of the *ftsA-uidA* fusion was
22 strongly increased as compared to the wild type strain and a 4 fold decrease was observed
23 between the medium of lower osmolarity (half strength M63 medium) as compared to the
24 medium of higher osmolarity (M63 full strength medium with 0.2 M NaCl added). This
25 suggests that in addition to OPGs, additional factors are required for regulation of the *ftsAZ*

1 operon by the RcsCD RcsB phosphorelay. As a control, OPGs concentrations were
2 determined (Fig. 6B). As previously shown (Cogez *et al.*, 2001), OPGs concentration
3 decreased as the osmolarity increased from 36 μ g of OPGs per mg of protein in half strength
4 M63 medium to 6 μ g of OPGs per mg of protein in M63 medium with 0.2 M NaCl added. A
5 western blot analysis was performed in M63 medium with (0.2 M) or without NaCl added in
6 an *opgG*, *rscC*, *rscB*_{D56N} and in the wild-type strains (Fig. 3C). Similar amounts of the RcsB
7 protein were observed whatever the strain and the osmolarity tested (data not shown). These
8 results indicate that in *D. dadantii*, variation of OPGs concentration is required for proper
9 activation of the RcsCD RcsB phosphorelay by osmolarity, but osmoregulation is a complex
10 multifactor phenomenon (Wood, 1999) where OPGs are needed but are only one of the actors
11 required for regulation of the RcsCD RcsB regulon.

12 **Discussion**

13 OPGs are known to be an important virulence factor for many Gram-negative bacteria (Bohin,
14 2006) and we show in a previous paper (Bouchart *et al.*, 2010) that OPGs control the RcsCD
15 RcsB phosphorelay. In this paper we demonstrate that in addition concentration of OPGs in
16 the periplasm modulates activation level of the RcsCD RcsB phosphorelay in *D. dadantii*.

17 In *D. dadantii*, motility and the *flhDC* operon are repressed by the activation of the RcsCD
18 RcsB phosphorelay while the *ftsA* gene is induced (Bouchart *et al.*, 2010) and reflect the
19 RcsCD RcsB phosphorelay activation level. When OPG concentration depends on L-
20 arabinose concentration of the medium, we showed that motility and expression of both
21 fusions genes tested depends on L-arabinose in an RcsB dependent manner and that activation
22 of the RcsCD RcsB phosphorelay increase as the concentration of OPGs decrease. OPGs
23 control the RcsCD RcsB activity in a concentration dependent manner. One of the stimuli
24 activating the RcsCD RcsB phosphorelay is osmolarity increased (Majdalani *et al.*, 2005)
25 while in the same conditions, OPGs concentration decreased (Cogez *et al.*, 2001; Bohin *et al.*,

1 2006). Decreased motility and expression of the *flhD-uidA* gene fusion observed when
2 osmolarity of the medium increased are in agreement with an increased activation of the
3 RcsCD RcsB phosphorelay and this activation depends both on a decreased OPG
4 concentration and on RcsB (Fig. 5 and Fig. 6). No increase in the *ftsA-uidA* expression was
5 observed when osmolarity increase while expression of this gene is known to be enhanced
6 under activation of the RcsCD RcsB phosphorelay. One explanation is the presence, in *D.*
7 *dadantii*, of a still unknown co-regulator of RcsB affecting interaction between RcsB and its
8 DNA regulatory sequences. This hypothesis was strengthened by the decrease expression of the
9 *ftsA-uidA* fusion gene in the *opgG* mutant strain since without additional regulatory
10 component, the constitutive activation of the RcsCD RcsB phosphorelay would lead to a
11 constant and high level of expression of this fusion. In *E. coli*, RcsA, GadE or BglJ form
12 heterodimer with RcsB and co-regulate different subset of genes of the RcsCD RcsB regulon
13 (Majdalani *et al.*, 2005; Krin *et al.*, 2010; Stratmann *et al.*, 2012) but we did not find
14 ortholog of these genes in the *D. dadantii* genome.

15 Variation in motility and in expression of target fusion genes is directly related the
16 modulation of the RcsB phosphorylation level itself by RcsC since i/ no variation in the
17 expression of both target genes was observed whatever the *rscB* or *rscC* mutant strains mutant
18 strains and ii/ no difference in RcsB amount was observed in western blot analysis (see Fig. 3)
19 whatever the growth conditions (data not shown). No difference in expression of the *rscB*
20 gene was also reported in *Erwinia amylovora* grown either in minimal medium or *in planta*
21 (Wang *et al.*, 2012). Thus, the activation level of the RcsC sensor and subsequently the
22 phosphorylation level of RcsB depend on periplasmic concentration of OPG.

23 A minimal concentration of OPGs is required for initiating virulence (Fig. 3). This
24 concentration could be estimated at 10 μ g of OPGs per mg of protein (Fig. 1) and was
25 measured in M63 medium containing 0.01 g L⁻¹ arabinose corresponding to the amount found

1 in M63 medium supplemented with 0.15 M NaCl (fig. 6). This concentration of OPGs may
2 correspond to the higher level of phosphorylated RcsB allowing virulence. Full virulence was
3 observed until 20 μg per mg of proteins and was measured in M63 medium containing 1 g L^{-1}
4 arabinose or in M63 medium (fig. 6). No difference was observed in virulence (timing or
5 severity of the disease) whatever the osmolarity of the medium before inoculation *in planta*.
6 Our data indicate that *D. dadantii* may adjust its concentration of OPGs within the 24 first
7 hours of infection. This concentration of OPGs was two times higher than the minimum
8 required to ensure a low level of phosphorylated RcsB (Fig. 4). This high OPGs concentration
9 may be explained by the low osmolarity of the plant apoplast even if this low osmolarity
10 varies depending on the plant tissue (Boyer *et al.*, 2001). Another explanation is that high
11 OPGs are required for other cellular processes (Delangle *et al.*, 2007) during virulence and
12 that additional regulation enhances OPGs synthesis. In *E. coli*, the *opgGH* operon is part of
13 the envelope stress sigma E factor regulon (Dartigalongue *et al.*, 2001). In *Yersinia*
14 *enterocolitica*, it was reported that the *opgGH* operon was induced within the host (Young
15 and Miller, 1997). Thus, because OPGs are needed for virulence in several mammalian
16 pathogenic enterobacteria, it would be of interest to measure OPG concentration in these
17 bacterial species since in the gut high osmolarity environmental condition, high concentration
18 of OPGs cannot be achieved by osmoregulation.

19

20 **Experimental procedures**

21 *Bacterial strains, media, antibiotics and growth condition*

22 Bacterial strains and plasmids are described in Table 1. Bacteria were grown at 30°C (*D.*
23 *dadantii*) or 37°C (*E. coli*) in Lysogeny broth (LB) (Bertani, 2004), or in minimal medium
24 M63 supplemented with glycerol as a carbon source at a concentration of 2 g l^{-1} (Miller, 1992).
25 Solid media were obtained by adding agar at 15 g l^{-1} . Osmolarity of the M63 medium

1 increased by adding 0.1 M or 0.2 M NaCl or decreased by a two-fold dilution. When
2 necessary L(+)-arabinose (Calbiochem) was added in the M63 medium at indicated
3 concentrations. X-Gluc (5-bromo-4-chloro-3-indolyl- β -D-glucuronide) was used in solid
4 media at a concentration of 40 $\mu\text{g ml}^{-1}$. Motility was tested on M63 agar plates containing 4 g
5 l^{-1} agar and swim diameters were measured after 30 h of incubation.

6 Antibiotics were used at the following concentrations: gentamicin at 3 $\mu\text{g ml}^{-1}$ (*E. coli*) or 2
7 $\mu\text{g.ml}^{-1}$ (*D. dadantii*), chloramphenicol and tetracycline at 25 $\mu\text{g ml}^{-1}$ (*E. coli*) or 12.5 $\mu\text{g ml}^{-1}$
8 (*D. dadantii*), ampicillin and kanamycin at 50 $\mu\text{g ml}^{-1}$ (*E. coli*) or 25 $\mu\text{g ml}^{-1}$ (*D. dadantii*),
9 streptomycin and spectinomycin at 100 $\mu\text{g ml}^{-1}$ (*E. coli*) or 50 $\mu\text{g ml}^{-1}$ (*D. dadantii*).

10 *Cloning of the opgGH operon under control of the araBAD promoter*

11 Plasmids and primers designed for PCR are listed in Table 1. The beginning of the operon
12 *opgGH* (without the *opgGH* promoter) was amplified by PCR (*opgGH*nhelF and *opgGH*kpnI
13 primers) from pNFW32 (Page *et al.*, 2001). This PCR product was digested by NheI and KpnI
14 and cloned into pBAD18 digested by the same enzymes (pNFW208). pNFW32 was digested
15 by EcoRI and KpnI and the DNA fragment containing the remaining of the *opgGH* operon
16 was cloned into pNFW208 digested by the same enzymes. The resulting plasmid (pNFW212)
17 contains the *opgGH* operon controlled by the *araBAD* promoter ($P_{BADopgGH}$). Plasmid
18 pNFW212 was digested by AclI and Bstz17I and the DNA fragment containing the *araC* gene
19 and $P_{BADopgGH}$ operon was inserted in the SmaI site of pUC18Not. The resulting plasmid
20 (pNFW219) was digested by NotI and the DNA fragment containing the *araC* gene and
21 $P_{BADopgGH}$ operon was inserted into the NotI site of pUTminiTn5-Spe. The resulting plasmid
22 (pNFW233) was introduced in *D. dadantii* by conjugation to allow integration, after
23 transposition of the miniTn5, of a single ectopic copy of the *araC* gene and $P_{BADopgGH}$
24 operon.

25 *Construction of the mutations*

1 The *rcsB::Gm* allele was constructed by excision of the Cml^r cassette from the pNFW397
2 (Bouchart *et al.*, 2010) digested by HpaI and replaced by the Gm^r cassette digested by the
3 same restriction enzyme after PCR amplification (Gm_{hpaI}f and Gm_{hpaI}r, Table 1). The
4 resulting plasmid (pNFW402) was introduced in *D. dadantii* cells by electroporation.

5 The *rcsB*_{D56N} allele was constructed by SOE (splicing by overlap extension) PCR. The
6 upstream region was amplified using primers RcsBDN3 and RcsBDNsoe1 (table 1), whereas
7 the downstream region was amplified using primers RcsBDNsoe2 and RcsBDN4 (Table 1).
8 The two PCR products were spliced together using the flanking primers RcsBDN3 and
9 RcsBDN4. The mutagenesis PCR product was purified, digested with HpaI and AscI, cloned
10 into pNFW257 digested by the same enzymes (pNFW374) and sequenced to confirm
11 mutation. The NotI fragment from pNFW374, containing the *rcsCB*_{D56N}*D* locus, was
12 subcloned into pUTmini-Tn5-Tet or pUTmini-Tn5-Spe digested by NotI. The resulting
13 plasmids (pNFW379 and pNFW380 respectively) were introduced into *D. dadantii* cells by
14 conjugation.

15 *Transduction, conjugation and transformation.*

16 Transformation of *E. coli* cells was carried out by the rubidium chloride technique (Miller,
17 1992). Plasmids were introduced in *D. dadantii* by electroporation (Sambrook *et al.*, 1989) or
18 conjugation (Miller, 1992). The insertions were integrated into the *D. dadantii* chromosome
19 by marker exchange recombination in the presence of the appropriate antibiotic, after
20 successive cultures in low phosphate medium (Roeder *et al.*, 1985). Transduction with phage
21 ϕ EC2 was carried out according to Resibois *et al.* (Resibois *et al.*, 1984).

22 *Transposon mutagenesis*

23 To allow integration, after transposition of the miniTn5, of a single ectopic copy of the *araC*
24 gene and *P*_{BAD}*opgGH* operon or the *rcsCDB*_{D56N} locus, transposon mutagenesis by mini-Tn5
25 was performed as described in (Bouchart *et al.*, 2010). Briefly, after conjugation between an *E.*

1 *coli* strain harboring the plasmid carrying the appropriate mini-Tn5 and a *D. dadantii* strain,
2 mutants were selected on M63 plates containing sucrose as a unique carbon source and the
3 required antibiotic.

4 *Determination of enzyme activities*

5 β -glucuronidase assays were performed on crude extracts obtained from bacteria disrupted by
6 sonication 2x 20 s (Sonifier cell disruptor B-30, Branson, 70% duty cycle, 7 microtip limit,
7 Hold time, continuous, appropriate probe). β -glucuronidase activity was determined by
8 spectrometric monitoring the hydrolysis of PNPU (4-nitrophenyl- β -D-glucuronide) at 405 nm.
9 The protein concentration was determined by the Bradford assay with bovine serum albumin
10 as standard (Bradford, 1976).

11 *OPGs analysis*

12 Bacteria were grown *in planta* or in M63 medium (200 ml) supplemented either with various
13 L-arabinose concentrations or with various concentrations of NaCl until mid-log phase.
14 Bacteria were collected by centrifugation at 4°C for 15 min at 8,000 \times g. Cell pellets were
15 resuspended in 20 ml of distilled water and extracted with 5% trichloroacetic acid (TCA). The
16 TCA extracts were neutralized with ammonium hydroxide 10% and concentrated by rotary
17 evaporation. The resulting material (2 ml) was then fractionated by gel filtration on a Bio-Gel
18 P-4 (Bio-Rad) column (55 by 1.6 cm) equilibrated with 0.5% acetic acid. The column was
19 eluted in the same buffer at a flow rate of 15 ml h⁻¹, and fractions of 1.5 ml were collected.
20 Presence of sugar in each fraction was determined colorimetrically by the anthrone reagent
21 procedure. Fractions containing OPGs were pooled and content was determined by the same
22 procedure (Spiro, 1966). For mass spectrometry analysis, OPGs were subsequently desalted in
23 water on a Bio-Gel P-2 (Bio-Rad) column (70 by 1.6 cm) and fractions containing OPGs were
24 lyophilized.

25 *Preparation of polyclonal antibodies against RcsB*

1 A DNA fragment encoding the *rcsB* gene of *D. dadantii* was amplified by PCR using the
2 primers *rscBacf* and *rscBacr*. The PCR product was cloned into a His₆ tag expression vector,
3 pET100/D-Topo® (Invitrogen Life Technologies) to give pNFW410. The resulting His-
4 tagged RcsB was expressed in *E. coli* BL21(DE3) and the protein was purified by affinity
5 chromatography according to the manufacturer's procedure (Ni-nitrilotriacetic acid [NTA]
6 agarose; Qiagen). The purified RcsB was used to immunize rabbit.

7 *Western blot hybridization*

8 Strains of *D. dadantii* were grown at 30°C to mid-log phase in M63 or after various time of
9 infection in planta. Bacteria were collected by centrifugation and immediately lysed with
10 SDS-PAGE buffer (2% SDS, 2.5% DTT, 25 mM Tris-HCl pH 7.5, 15% glycerol), heated to
11 100°C for 5 min and loaded onto a 10% SDS-polyacrylamide gel (34). Proteins from the gel
12 were transferred onto a nitrocellulose membrane (Whatman). Western blotting was performed
13 using standard protocols with the rabbit anti-RcsB polyclonal antibodies at a dilution of
14 1:1000 and anti-rabbit secondary antibody coupled to horseradish peroxidase (dilution
15 1:10,000). Blots were imaged by chemiluminescent detection (ECL kit, GE healthcare
16 Pharmacia Biotech).

17 *Pathogenicity test*

18 Chicory leaves were inoculated as previously described (Delangle *et al.*, 2007; Bouchart *et al.*,
19 2010).

20 *Bacteria isolation from infected chicory leaves*

21 Infected chicory leaves were introduced into tubes (one leave per tube) containing 20 mL of
22 minimal medium 63 without carbon source added. Bacteria were suspended in the medium by
23 vigorous shaking of the tubes during 15 min. Leaves were then carefully removed from the
24 tube. The medium was filtered through a paper filter (previously sterilized) to separate few
25 macerated tissue release during the shaking step from bacteria (no more than 6% bacteria

1 were lost during this step). Bacteria were then collected by centrifugation at 4°C for 15 min at
2 8,000 × g. Cell pellets were resuspended in 20 mL of minimal medium 63 for OPGs analysis
3 or in 2 mL of the same medium for β-glucuronidase assays. Before analysis or assays, an
4 aliquot of 0.1 mL of each bacterial suspension was taken up, diluted and counted. A total of
5 10⁸ to 10¹⁰ CFU were recovered per chicory leave depending on the incubation time.

6 *Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)*

7 All mass spectra were acquired on a Voyager Elite (DE-STR) reflectron time-of-flight (TOF)
8 mass spectrometer (Perseptive Biosystems, Framingham, MA), equipped with a pulsed
9 nitrogen laser (337 nm) and a gridless delayed extraction ion source. Oligosaccharide samples
10 were analyzed in delayed extraction mode using an accelerating voltage of 20 kV, a pulse
11 delay time of 200 ns and a grid voltage of 66%. Detector bias gating was used to reduce the
12 ion current for masses below 500 Da. Between 100 and 200 scans were averaged for each
13 mass spectrum. Oligosaccharide alditols were co-crystallized with 2,5-dihydroxybenzoic acid
14 (DHB) as matrix [10 mg ml⁻¹ of DHB in methanol/water (50/50) containing 0.1% Trifluoro
15 Acetic acid (TFA)]. For all measurements, the "dried droplet" preparation technique was used.
16 Typically, 1 μl of the matrix was mixed on-target with 1 μl of water-dissolved
17 oligosaccharides and allowed to dry under an air stream. They were analyzed in positive ion
18 mode.

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1
2
3
4
5

Table

Table 1. Bacterial Strains, plasmids and primers

Strain, plasmids or primer	Relevant genotype and/or phenotype ^{a,b}	Source or reference
<i>D. dadantii</i>		
EC3937	Wild type	Laboratory collection
NFB3723	<i>opgG</i> ::Cml	Laboratory collection
NFB3682	<i>rscC</i> ::Cml	Bouchart <i>et al.</i> , 2010
NFB3800	<i>flhD-uidA</i> -Kan ^r	Bouchart <i>et al.</i> , 2010
NFB3809	<i>ftsA-uidA</i> -Kan ^r	Bouchart <i>et al.</i> , 2010
NFB3835	<i>opgG</i> ::Cml, P _{BAD} <i>opgGH</i> -Spe ^r	This study
NFB3837	<i>opgG</i> ::Cml, P _{BAD} <i>opgGH</i> -Spe ^r , <i>ftsA-uidA</i> -Kan ^r	This study
NFB7112	<i>rscB</i> ::Cml, <i>rscCB</i> _{D56N} <i>D</i> -Spe ^r	This study
NFB7127	<i>rscC</i> ::Cml, <i>ftsA-uidA</i> -Kan ^r	Bouchart <i>et al.</i> , 2010
NFB7128	<i>rscC</i> ::Cml, <i>flhD-uidA</i> -Kan ^r	Bouchart <i>et al.</i> , 2010
NFB7202	<i>rscB</i> ::Cml, <i>flhD-uidA</i> -Kan ^r	Bouchart <i>et al.</i> , 2010
NFB7205	<i>rscB</i> ::Cml, <i>ftsA-uidA</i> -Kan ^r	Bouchart <i>et al.</i> , 2010
NFB7274	<i>opgG</i> ::Cml, <i>ftsA-uidA</i> -Kan ^r	This study
NFB7279	<i>rscB</i> ::Gm	This study
NFB7300	<i>opgG</i> ::Cml, P _{BAD} <i>opgGH</i> -Spe ^r , <i>flhD-uidA</i> -Kan ^r	This study
NFB7301	<i>opgG</i> ::Cml, <i>flhD-uidA</i> -Kan ^r	This study
NFB7368	<i>opgG</i> ::Cml, <i>rscB</i> ::Gm, P _{BAD} <i>opgGH</i> -Spe ^r	This study
NFB7369	<i>opgG</i> ::Cml, <i>rscB</i> ::Gm, P _{BAD} <i>opgGH</i> -Spe ^r , <i>flhD-uidA</i> -Kan ^r	This study
NFB7371	<i>opgG</i> ::Cml, <i>rscB</i> ::Gm, P _{BAD} <i>opgGH</i> -Spe ^r , <i>ftsA-uidA</i> -Kan ^r	This study
NFB7399	<i>rscB</i> ::Cml, <i>rscCB</i> _{D56N} <i>D</i> -Spe ^r , <i>flhD-uidA</i> -Kan ^r	This study
NFB7400	<i>opgG</i> ::FRT, <i>rscC</i> ::Cml, P _{BAD} <i>opgGH</i> -Spe ^r	This study
NFB7401	<i>opgG</i> ::Cml, <i>rscB</i> ::Gm, P _{BAD} <i>opgGH</i> -Spe ^r , <i>rscCB</i> _{D56N} <i>D</i> -Tet ^r	This study

NFB7402	<i>opgG::Cml, rcsB::Gm, P_{BAD}opgGH-Spe^r, rcsCB_{D56N}D-Tet^r, ftsA-uidA-Kan^r</i>	This study
NFB7403	<i>opgG::Cml, rcsB::Gm, P_{BAD}opgGH-Spe^r, rcsCB_{D56N}D-Tet^r, flhD-uidA-Kan^r</i>	This study
NFB7405	<i>opgG::FRT, rcsC::Cml, P_{BAD}opgGH-Spe^r, ftsA-uidA-Kan^r</i>	This study
NFB7406	<i>opgG::FRT, rcsC::Cml, P_{BAD}opgGH-Spe^r, ftsA-uidA-Kan^r</i>	This study
NFB7408	<i>rcsB::Cml, rcsCB_{D56N}D-Spe^r, ftsA-uidA-Kan^r</i>	This study
<i>E. coli</i>		
BL21 (DE3)	<i>ompT, hsdSB, gal, dcm</i>	Invitrogen
JM83	<i>ara Δ(lac-proAB), rpsL, thi, (φ80ΔlacZ-M15)</i>	Vieira <i>et al.</i> , 1982
S17-λpir	<i>recA1, thi, pro, hsdR-M+, RP4:2-Tc::Mu-Kan::Tn7, λpir</i>	De Lorenzo <i>et al.</i> , 1994
NFB3276	S17-λpir, pNFW233	This study
Plasmids		
pUC18Not	Amp ^r	Yanisch-Perron <i>et al.</i> , 1985
pUTminiTn5-Spe	mini-Tn5Spe, <i>oriR6K</i> , Spe ^r , Amp ^r	De Lorenzo <i>et al.</i> , 1990
pUTminiTn5-Tet	mini-Tn5tet, <i>oriR6K</i> , Tet ^r , Amp ^r	De Lorenzo <i>et al.</i> , 1990
pBAD18	Amp ^r , <i>araC</i> , promoter P _{BAD}	Guzman <i>et al.</i> , 1995
pNFW32	Amp ^r , <i>opgGH</i>	Page <i>et al.</i> , 2001
pNFW208	pBAD18, <i>opgG'</i>	This study
pNFW212	pBAD18, <i>opgGH</i>	This study
pNFW219	pUC18Not, <i>araC</i> , P _{BAD} <i>opgGH</i>	This study
pNFW257	pUC18Not, <i>rcsCBD</i>	Bouchart <i>et al.</i> , 2010
pNFW233	pUTminiTn5-Spe, <i>araC</i> , P _{BAD} <i>opgGH</i>	This study
pNFW374	pUC18Not, <i>rcsCB_{D56N}D</i>	This study
pNFW379	pUTminiTn5-Tet, pUC18Not, <i>rcsCB_{D56N}D</i>	This study
pNFW380	pUTminiTn5-Spe, pUC18Not, <i>rcsCB_{D56N}D</i>	This study
pNFW397	pUC18Not, <i>rcsCB::CmlD</i>	Bouchart <i>et al.</i> , 2010
pNFW402	pNFW397, Gm instead of Cml	This study

pNFW410	pET100/D-topo <i>rcsB</i>	This study
Primers	Sequence^{c,d}	
opgGHnhelf	GGGT <u>GCTAGCT</u> GATTTAGGCACACATACAGGGGG	
opgGHkpnlr	AACT <u>GGTACCAGAC</u> GGCAACGCCGTGTCGATAGCC	
gmhpalf	GTGT <u>GTTAAC</u> CCCCATCCCCCTGTTGAC	
gmhpalr	GTGT <u>GTTAAC</u> CGCAAGCTAGCTTGGCTGC	
rcsBacf	CACCATGAGCAATCTAAACGTAATTATTGCAG	
rcsBacr	CGCCGGCAAGAGCATTACTC	
rcsBDN3	CTGAAAGGTGTCTTTGCCATGCTGAATCTTCATCCC	
rcsBDNsoe1	GCATGGATAGATTGGTGATCAGGAC	
rcsBDNsoe2	GTCCTGATCACCAATCTATCCATGC	
rcsBDN4	TTCCAGCACTTTGCTGACGCTTTCC	

1 ^a-*uidA* fusions are carried by a Kan^r mini-Tn5, *P_{BAD}opgGH* fusion is carried by a Spe^r mini-Tn5, *rcsCB_{D56N}D* is
2 carried by a Tet^r mini-Tn5.

3 ^bCml^r, chloramphenicol resistance; Amp^r, ampicillin resistance, Kan^r, kanamycin resistance, Spe^r,
4 spectinomycin resistance, Str^r, streptomycin resistance, Gm^r, gentamicin resistance, Tet^r, tetracycline.

5 ^cRestriction sites are underlined. ^dThe mutation is indicated in bold.

6

7 **Figure legends**

8 **Fig. 1.** OPGs concentration of the NFB3835 (*P_{BAD}opgGH*) at various concentrations of L-
9 arabinose. Bacteria were grown until mid-log phase in M63 medium supplemented with
10 indicated concentrations of L-arabinose. OPGs were extracted, purified and quantified as
11 described in procedures. Results reported are the average of 3 independents experiments.

12

13

14 **Fig. 2.** Effect of the arabinose concentration on motility (A) and expression of the *flhD-uidA*
15 (B) or the *ftsA-uidA* (C) gene fusions in various strains. Motility was measured in M63
16 semisolid plates supplemented with indicated L-arabinose concentrations. Swim diameters
17 were measured after 30 h of incubation at 30°C. For fusion expression measurements, bacteria

1 were grown until mid log phase in the same media and broken by passing through a French
2 pressure cell. The β -glucuronidase activities were measured with PNPU as a substrate.
3 Specific activity is expressed as the change in optical density at 410 nm per min and per mg
4 of protein. Results reported are the average of 3 independent experiments.

5

6 **Fig. 3** Effect of arabinose concentration on pathogenicity of the NFB3835 ($P_{BAD}OPgGH$)
7 strain. Bacteria were grown until mid log phase in M63 medium supplemented with indicated
8 L-arabinose concentrations, washed twice and 10^7 bacteria were inoculated into scarified
9 chicory leaves. Evolution of the disease symptoms were observed after 24 and 48h.

10

11 **Fig. 4.** Effect of OPG concentration *in planta* on RcsCD RcsB activation. For determination
12 of OPG concentration, mass spectra or fusion activities, bacteria were collected after growth
13 to mid log phase in 63 medium (0 h), or after growth *in planta* during 24 h, or after growth *in*
14 *planta* during 48 h. For each time, CFU were determined. A/ concentration of OPGs purified
15 from the wild-type strain (EC3937) as described in experimental procedures. B/ MALDI mass
16 spectra acquired in positive ion mode on purified OPGs extracted from the wild-type strain.
17 The $[M+Na]^+$ molecular ions are indicated on the top of each peak. Their respective $[M+K]^+$
18 molecular ions are identified by an asterisk and have an increment of 16 Da in comparison to
19 the corresponding $[M+Na]^+$ ions. C/ Expression of the *flhD-uidA* or the *ftsA-uidA* fusion
20 genes in the wild-type (NFB3800 and NFB3809 respectively) or the *rscB* (NFB7202 and
21 NFB7205 respectively) strains. The β -glucuronidase activities were measured with PNPU as a
22 substrate. Specific activity is expressed as the change in optical density at 410 nm per min and
23 per 10^9 bacteria. Results reported are the average of 3 independent experiments.

24

1 **Fig. 5** Effect of medium osmolarity on motility (A) and expression of the *flhD-uidA* (B) gene
2 fusion in various strains. Motility was measured in half strength M63, M63, M63
3 supplemented with 0.1 M or M63 supplemented with 0.2M NaCl semisolid plates. For fusion
4 expression measurements, bacteria were grown until mid log phase in the same media and
5 broken by passing through a French pressure cell. The β -glucuronidase activities were
6 measured with PNPU as a substrate. Specific activity is expressed as the change in optical
7 density at 410 nm per min and per mg of protein. Results reported are the average of 3
8 independents experiments.

9
10 **Fig. 6** A/ Effect of medium osmolarity on expression of the *ftsD-uidA* (A) gene fusion in
11 various strains. For fusion expression measurements, bacteria were grown in half strength
12 M63, M63, M63 supplemented with 0.1 M or M63 supplemented with 0.2M NaCl until mid-
13 log phase and broken by passing through a French pressure cell. The β -glucuronidase
14 activities were measured with PNPU as a substrate. Specific activity is expressed as the
15 change in optical density at 410 nm per min and per mg of protein. Results reported are the
16 average of 3 independents experiments. B/ OPGs concentration of the wild-type strain
17 (EC3937) at various osmolarities. Bacteria were grown until mid log phase in the same media.
18 OPGs were extracted, purified and quantified as described in experimental procedures.
19 Results reported are the average of 3 independents experiments.

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