

Increased phosphorylation of the RcsB regulator of the RcsCDB phosphorelay in strains of *Dickeya dadantii* devoid of osmoregulated periplasmic glucans revealed by Phos-tag gel analysis

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Osmoregulated periplasmic glucans (OPGs) are general constituents of many proteobacteria. OPGs are important factors required for full virulence in many pathogens including *Dickeya dadantii*. *D. dadantii* causes the soft-rot disease in a wide range of plant species. The pleiotropic phenotype of *opg*-negative strains includes total loss of virulence and motility, and is linked to the constitutive activation of the RcsCDB phosphorelay, deduced from expression analysis of genes of the RcsCDB regulon. The constitutive activation of the RcsCDB phosphorelay in an *opg*-negative strain was demonstrated by direct analysis of the phosphorylation level of the RcsB regulator protein *in vivo* by using a Phos-tag retardation gel approach, and was correlated with the phenotype and the expression of motility genes. Data revealed a low level of RcsB phosphorylated form in the wild-type strain and a slight increase of phosphorylation in *opgG* mutant strains sufficient to induce the pleiotropic phenotype observed.

INTRODUCTION

Osmoregulated periplasmic glucans (OPGs) are oligosaccharides found in the envelope of most proteobacteria. Their two common features are that the backbone of OPGs is only constituted of glucose and that OPG concentration increases as the osmolarity of the medium decreases (Bohin & Lacroix, 2006). These glucans belong to the common virulence factors of many zoopathogenic and phytopathogenic proteobacteria such as *Pseudomonas aeruginosa* (Mahajan-Miklos *et al.*, 1999), *Brucella abortus* (Arellano-Reynoso *et al.*, 2005) or *Dickeya dadantii* (Page *et al.*, 2001).

Dickeya dadantii is a phytopathogenic enterobacterium that causes soft-rot disease in a wide range of plant species (Perombelon, 2002). This opportunistic pathogen devastates economically important crops in storage facilities or in growing plants. In *D. dadantii*, the linear backbone is constituted by a family of 5 to 12 glucose residues linked by β ,1-2 linkages and branched by β ,1-6 linkages. This backbone is synthesized by the two glucosyl-transferases encoded by the *opgGH* operon. The transmembrane OpgH

enzyme catalyses the β ,1-2 linear glucose backbone while the periplasmic OpgG enzyme adds β ,1-6 glucose linkage to this linear β ,1-2 linear glucose backbone (Bohin & Lacroix, 2006). *opgG* or *opgH* mutant strains are completely devoid of OPGs. These *opgG* or *opgH* mutant strains show the same pleiotropic phenotype including loss of motility, increased synthesis of exopolysaccharides (Page *et al.*, 2001), induction of a general stress response (Bouchart *et al.*, 2007) and complete loss of virulence on potato tubers and chicory leaves (Page *et al.*, 2001). All these phenotypes suggest an impairment in the perception of the environment. This hypothesis was confirmed by the restoration of motility, exopolysaccharide synthesis and virulence on potato tubers by inactivation of the RcsCDB phosphorelay regulatory system in strains devoid of OPGs. In addition, we showed that the activation level of the RcsCDB phosphorelay was controlled by the periplasmic amount of OPGs. The activation of the RcsCDB phosphorelay is related to the OPG concentration in the periplasm. The activation level of RcsCDB increased as the OPG concentration decreased (Bontemps-Gallo *et al.*, 2013). Perturbation of the regulation process was not restricted to this phosphorelay since restoration of virulence never occurred on chicory leaves. More recently, we showed that the three main repressors of virulence of *D. dadantii* (namely: KdgR, PecT and PecS) cannot be derepressed in *opg* deficient mutant strains (Bontemps-Gallo *et al.*, 2014). Loss of OPGs affects the perception of

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Abbreviations: OPG, osmoregulated periplasmic glucans; PA, phosphoramidate.

the environment of bacteria in such a way that bacteria were unable to recognize the environment properly, including its various plant hosts (Bouchart *et al.*, 2010; Bontemps-Gallo *et al.*, 2013, 2014).

Phosphorelays (also called two-component systems) are the key of gene expression plasticity in response to environmental variations. The RcsC sensor has both kinase and phosphatase activities (Clarke, 2010). Under various stimuli, the transmembrane RcsC sensor activates, its kinase activity increases, and RcsC autophosphorylates and transfers its phosphate group to its cognate cytoplasmic response regulator, RcsB, via the intermediate transmembrane protein RcsD. In turn, RcsB regulates the expression of numerous target genes affecting various cellular processes, including activation of exopolysaccharide synthesis, repression of virulence and repression of motility (Cano *et al.*, 2002; Majdalani & Gottesman, 2005). In contrast, when the RcsC sensor phosphatase activates and dephosphorylates the RcsB regulator, virulence and motility occur while exopolysaccharide synthesis decreases.

Different mutations in the *rcsCDB* locus were able to restore wild-type phenotypes in the *opgG* strains, such as the *opgG rcsC2*, the *opgG rcsC* and the *opgG rcsB_{D56N}* double mutant strains. In these strains, the secondary mutation encoded a mutated RcsC2 sensor with an increased phosphatase activity, no RcsC sensor or a non-phosphorylatable RcsB_{D56N} regulator, respectively (Bouchart *et al.*, 2010; Bontemps-Gallo *et al.*, 2013). Phosphorylation of the RcsB_{D56N} protein is impossible since its phosphorylatable aspartic acid residue at position 56 was changed to an asparagine residue. These conclusions about the activation level of the RcsCDB phosphorelay system in the wild-type and the different mutant strains were classically deduced from expression analysis of genes regulated by this phosphorelay, but a direct effect on the RcsB phosphorylation level was never observed.

In the present study, we showed the variation of the activation level of the RcsCDB phosphorelay system in different genetic backgrounds by direct observation of the RcsB phosphorylated and non-phosphorylated protein forms, separated with a Phos-tag acrylamide gel retardation approach (Barbieri & Stock, 2008). Phenotypes associated with the RcsCDB phosphorelay and expression of genes controlled by this phosphorelay correlated with RcsB phosphorylation level. A low level of phosphorylation was observed in the wild-type strain and a moderate increase of the phosphorylation level of the RcsB protein is responsible for the pleiotropic phenotype observed in strains devoid of OPGs.

METHODS

Bacterial strains, media and growth conditions. Bacterial strains and plasmids are described in Table 1. Bacteria were grown in Lysogeny broth (LB) (Bertani, 2004) at 30 °C (*D. dadantii*) or 37 °C (*Escherichia coli*). Solid media were obtained by adding agar at 15 g

l⁻¹. Antibiotics were used at the following concentrations for *D. dadantii*: chloramphenicol at 12.5 µg ml⁻¹, kanamycin at 25 µg ml⁻¹ and spectinomycin at 50 µg ml⁻¹. Ampicillin was used at 50 µg ml⁻¹ for *E. coli*.

Motility was tested on LB agar plates containing 4 g l⁻¹ agar and swim diameters were measured after 30 h of incubation.

Plasmid constructs and protein purification. The plasmid used for the expression of the His-tagged RcsB, pNF410, was previously described by Bontemps-Gallo *et al.* (2013). For the construction of the His-tagged RcsB_{D56N}, a DNA fragment encoding the mutated *rcsB_{D56N}* gene was amplified by PCR using pNFW374 as template, with the *rcsBacf* (CACCATGAGCAATCTAAACGTAATTATTGCAG) and *rcsBacr* (CGCCGGCAAGAGCATTACTC) primers, cloned into the His-tag expression vector pET100/D-Topo (Invitrogen Life Technologies) to give pNFW491. The His-tagged RcsB and RcsB_{D56N} proteins were expressed in *E. coli* BL21(DE3) and purified according to Bontemps-Gallo *et al.* (2013).

Production of the phosphoramidate. The phosphoramidate (PA) used to phosphorylate the RcsB protein *in vitro* was synthesized by reaction of phosphoryl chloride with ammonium as described by Sheridan *et al.* (1972).

Analysis of RcsB phosphorylation *in vitro*. Phosphorylation reactions of purified RcsB or RcsB_{D56N} were performed with 5 µg of protein in 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂ and 2 mM β-mercaptoethanol. PA was added to a final concentration of 15 mM to initiate the reaction. After 0, 15 or 30 min of incubation at room temperature, the reactions were stopped by addition of SDS-PAGE loading buffer (final concentration: 50 mM Tris/HCl pH 6.8, 2%, w/v, SDS, 10%, w/v, glycerol, 20 mM DTT, 0.02% bromophenol blue). The mixtures were resolved using phosphoprotein affinity gel electrophoresis as described in Barbieri & Stock (2008), with minor modifications. Briefly, Phos-tag acrylamide gels were composed of a 10% resolving solution [10% (w/v) 37.5:1 acrylamide/N,N'-methylenebisacrylamide, 375 mM Tris (pH 8.8) and 0.1% (w/v) SDS, 125 µM Phos-tag acrylamide and 250 µM MnCl₂] and a 4% stacking solution [4% (w/v) 37.5:1 acrylamide/N,N'-methylenebisacrylamide, 125 mM Tris (pH 6.8) and 0.1% (w/v) SDS]. The gels were run at 4 °C under constant voltage (150 V) with standard running buffer (0.1%, w/v, SDS, 25 mM Tris and 192 mM glycine) and stained with Coomassie blue.

Analysis of RcsB phosphorylation *in vivo*. At mid-exponential phase, the equivalent of 1 ml of 0.15 OD_{620 nm} of *D. dadantii* cells was harvested by centrifugation and pellets were immediately lysed with 12.7 µl of 1M formic acid, solubilized by 5 µl of 4× SDS-PAGE loading buffer and neutralized by 2.8 µl 5 N NaOH. Samples were quickly loaded onto gels containing 35 µM Phos-tag acrylamide and 70 µM MnCl₂ prepared and run as described before. After a 10 min wash with transfer buffer (25 mM Tris and 192 mM glycine) supplied with 1 mM EDTA, followed by a 10 min wash with transfer buffer without EDTA, gels were transferred to nitrocellulose membranes using a Trans-Blot Turbo Blotting system (Bio-Rad) with a pre-programmed protocol (2.5 A, up to 25 V, 7 min). Western blotting against RcsB was then performed (see below). When required, purified RcsB or RcsB_{D56N} protein (2.5 µg) was phosphorylated by a 30 min incubation at room temperature with PA, as described previously. RcsB or phosphorylated RcsB proteins were added to bacteria prior to lysis.

Quantification of the phosphorylated RcsB protein amount. Phosphorylated RcsB and RcsB were quantified by determination of the area intensity of each band with the software Quantity One (Bio-Rad) after staining with Coomassie blue or detection by Western blot. Quantification of phosphorylated RcsB was expressed as the ratio of

Table 1. Strains and plasmids

| Strain | Genotype*, † | Source or reference |
|-------------------------|--|-------------------------------------|
| <i>Dickeya dadantii</i> | | |
| NFB3800 | <i>flhD-uidA-Kan^r</i> | Bouchart <i>et al.</i> (2010) |
| NFB3805 | <i>opgG::Cml^r, flhD-uidA-Kan^r</i> | Bouchart <i>et al.</i> (2010) |
| NFB3806 | <i>opgG::Cml^r, rcsC2, flhD-uidA-Kan^r</i> | Bouchart <i>et al.</i> (2010) |
| NFB3807 | <i>rcsC2, flhD-uidA-Kan^r</i> | Bouchart <i>et al.</i> (2010) |
| NFB7202 | <i>rcsB::Cml^r, flhD-uidA-Kan^r</i> | Bouchart <i>et al.</i> (2010) |
| NFB7128 | <i>rcsC::Cml^r, flhD-uidA-Kan^r</i> | Bouchart <i>et al.</i> (2010) |
| NFB7399 | <i>rcsB::Cml^r, rcsCB_{D56N}D-Spe^r, flhD-uidA-Kan^r</i> | Bontemps-Gallo <i>et al.</i> (2013) |
| A 5009 | <i>fliC::uidA-Kan^r</i> | Hommais <i>et al.</i> (2008) |
| <i>Escherichia coli</i> | | |
| BL21(DE3) | <i>ompT, hsdSB, gal, dcm</i> | Invitrogen |
| Plasmids | | |
| pNFW374 | pUC18Not- <i>rcsCB_{D56N}D</i> , Amp ^r | Bontemps-Gallo <i>et al.</i> (2013) |
| pNFW410 | pET100/D-topo <i>rcsB</i> , Amp ^r | Bontemps-Gallo <i>et al.</i> (2013) |
| pNFW491 | pET100/D-topo <i>rcsB_{D56N}</i> , Amp ^r | This study |

**rcsCB_{D56N}D* is carried by an Spe^r mini-Tn5; *flhD-uidA* is carried by a Kan^r mini-Tn5.

†Amp^r, Ampicillin resistance; Cml^r, chloramphenicol resistance; Kan^r, kanamycin resistance; Spe^r, spectinomycin resistance.

the phosphorylated RcsB amount divided by the sum of the RcsB and the phosphorylated RcsB amounts as described by Barbieri & Stock (2008).

Detection of FliC amount. Strains of *D. dadantii* were grown at 30 °C to mid-exponential phase in LB. Bacteria were collected by centrifugation and immediately lysed with SDS-PAGE buffer, heated to 100 °C for 5 min and loaded onto 10 % SDS-polyacrylamide gel (Bio-Rad) (Laemmli, 1970). Proteins from the gel were transferred onto a PVDF membrane (Bio-Rad) before Western blotting (see below).

Western blot hybridization. Western blotting was performed using either the rabbit anti-RcsB polyclonal antibodies at a dilution of 1 : 1000 (obtained as described in Bontemps-Gallo *et al.*, 2013) for the RcsB phosphorylation analysis or the rabbit anti-FliC polyclonal antibodies at a dilution of 1 : 25 000 (Abcam) for the FliC amount analysis, and anti-rabbit secondary antibody coupled to horseradish peroxidase (dilution 1 : 10 000) for both. Blots were imaged by chemiluminescent detection (ECL kit, GE healthcare).

Determination of enzymes 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 by the hydrolysis of PNPU (4-nitrophenyl- β -D-glucuronide) at 405 nm. The protein concentration was determined by the Bradford assay with BSA as a standard (Bradford, 1976).

RESULTS AND DISCUSSION

Phosphorylated RcsB (RcsB-P) migrate slower than RcsB

The method to observe the phosphorylated form of cytoplasmic regulator proteins is available when the phosphate group is linked to a serine, threonine or tyrosine because the

linkage is relatively stable. In the case of bacterial phosphorelays, the phosphate group is linked to an aspartate residue for the cytoplasmic regulator. Unfortunately, in this last case, linkage is highly unstable, and until recently, observation of these kinds of phosphorylated proteins was impossible.

In 2008, Stock's laboratory published a method allowing separation of the phosphorylated and the non-phosphorylated forms of phosphorelay regulators (Barbieri & Stock, 2008). This method relies on Phos-tag: a dinuclear metal complex that acts as a specific phosphate-binding agent. In a Phos-tag acrylamide gel, the Phos-tag linked to acrylamide molecules interacts with the phosphorylated form of the protein, which migrates slower than the non-phosphorylated protein allowing the separation of both forms of the protein. Before *in vivo* analyses of RcsB phosphorylation, we first used the method for separation of *in vitro* phosphorylated RcsB since the method must be adapted to each regulator (Barbieri & Stock, 2008). Purified RcsB was phosphorylated *in vitro* by the phosphodonor phosphoramidate (PA) (Lukat & Stock, 1993). Five micrograms of RcsB was incubated for different times with or without PA (see Methods), samples were loaded onto a Phos-tag acrylamide gel and RcsB was revealed with Coomassie blue after migration. Without PA added, a single band, corresponding to the non-phosphorylated RcsB form (called RcsB) was observed (lanes 1 and 2, Fig. 1a). In contrast, a retardation of migration of 57 % and 61 % of the band was observed (lanes 3 and 4, Fig. 1a) when PA was added during 15 min and 30 min, respectively. This strongly suggested that the shifted band corresponded to the phosphorylated RcsB form (called RcsB-P) but indicating that in our conditions, PA was unable to phosphorylate all RcsB. The same experiment was performed with the non-phosphorylatable RcsB_{D56N} protein (lanes 5 to 8, Fig. 1a). In these

lanes, a single band migrating as the single band of lanes 1 and 2 was observed. These data indicate that the lower band corresponded to RcsB, that the upper band corresponded to RcsB-P and that PA phosphorylates the proper aspartic residue of RcsB. These conditions enabled us to analyse the stability of the phosphorylation of RcsB *in vivo*.

Lysis with formic acid has no effect on the RcsB-P amount

The *in vivo* analysis required rapid lysis of bacteria with formic acid before loading onto the gel (Barbieri & Stock, 2008). Because the phospho-aspartate bond is very unstable, it was important to check both the effect of the formic acid and the lysis of cell on the stability of RcsB and of the RcsB-P phospho-aspartate bond. To do this, 5 µg of purified RcsB or RcsB-P, phosphorylated by PA, was added to an *rcsB* null mutant strain of *D. dadantii* prior lysis with formic acid. Lysates were then loaded onto a Phos-tag gel. As controls, the same amount of RcsB and RcsB-P without bacteria was loaded onto the same gel. The same experiments and controls were performed with the RcsB_{D56N}. After running the gel, RcsB was revealed by Western blot. When the lysate of the *rcsB* null mutant strain was loaded onto the gel alone, RcsB was not detected

(lane 1, Fig. 1b). A similar amount of RcsB was revealed when RcsB was added to the *rcsB* null mutant strain before lysis (lane 2, Fig. 1b) or when RcsB was loaded alone onto the gel (lane 6, Fig. 1b). Two bands, corresponding to RcsB (53 %, lower band) and RcsB-P (47 %, upper band), were revealed when RcsB phosphorylated by PA was added to the *rcsB* null mutant strain before lysis (lane 3, Fig. 1b). The same two bands in similar amounts, corresponding to RcsB (52 %, lower band) and RcsB-P (48 %, upper band), were revealed when RcsB phosphorylated by PA was loaded alone on the gel (lane 7, Fig. 1b). The same conclusions can be drawn when RcsB_{D56N} was used instead of RcsB except that only one band was observed corresponding to the non-phosphorylated form of RcsB (lanes 4, 5, 8, 9, Fig. 1b). Taken together, these results indicate that neither cell lysate nor formic acid affected the RcsB stability or the RcsB-P phospho-aspartate bond stability and that the two forms of RcsB are separated within a cell lysate. These results enabled us to analyse the RcsB phosphorylation *in vivo*.

Phosphorylation of RcsB increases in a strain devoid of OPGs

The RcsCDB phosphorelay was constitutively activated in an *opgG* strain devoid of OPGs and constitutive activation

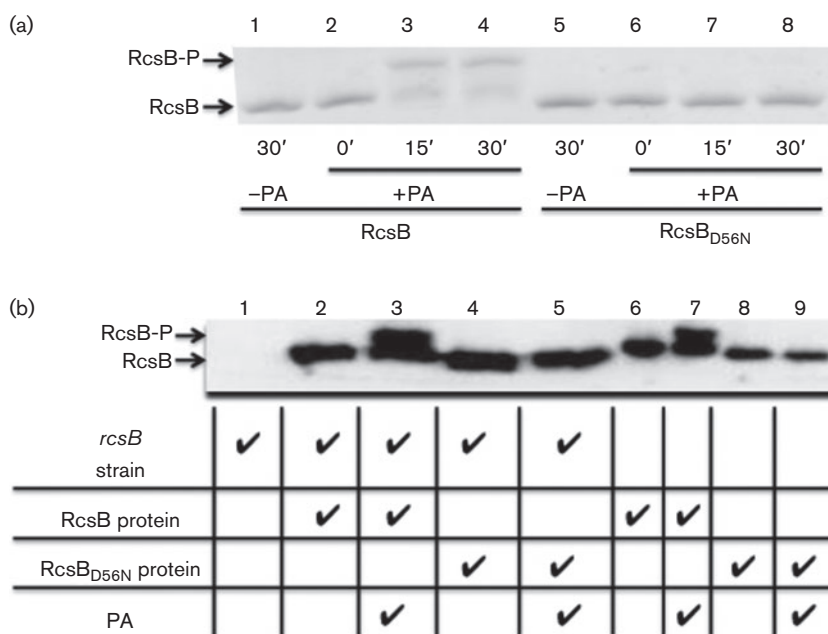


Fig. 1. Separation of RcsB and RcsB-P by Phos-tag PAGE after phosphorylation *in vitro*. (a) Five micrograms of either purified wild-type RcsB (lanes 1 to 4) or RcsB_{D56N} (lanes 5 to 8) incubated in the presence of 15 mM PA, during 0 min (lanes 2, 6), 15 min (lanes 3, 7) and 30 min (lanes 4, 8) or in the absence of PA (lanes 1, 5) before loading onto the gel. After migration, RcsB and RcsB-P proteins were visualized by Coomassie-stained Phos-tag gel (125 µM). (b) Purified RcsB (lane 2), RcsB-P with PA (lane 3) or unphosphorylatable RcsB_{D56N} (lanes 4-5) were mixed with cells of *rcsB* mutant strain before treatment with formic acid as described in the Methods. As controls, *rcsB* cell lysate (lane 1), RcsB protein (lanes 6) RcsB-P (lane 7) and RcsB_{D56N} protein (lanes 8, 9) were also loaded on the gel. Revelation of RcsB was performed by Western blot analysis after electrophoresis on Phos-tag gel (35 µM). Lanes where PA is noted indicated that RcsB (or RcsB_{D56N}) was treated 30 min with PA prior to lysis. The results presented are one of the three independent experiments performed.

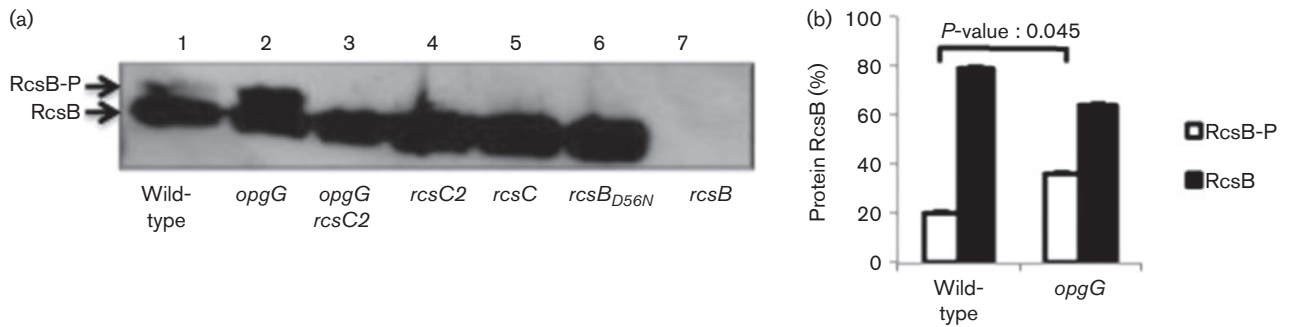


Fig. 2. Separation of RcsB and RcsB-P by Phos-tag PAGE after extraction from bacteria *in vivo*. (a) Cell lysate of wild-type (lane 1), *opgG* (lane 2), *opgG rcsC2* (lane 3), *rcsC2* (lane 4), *rcsC* (lane 5), *rcsBD56N* (lane 6) and *rcsB* (lane 7) mutant strains of *D. dadantii* were subjected to Phos-tag acrylamide gel electrophoresis and revelation of RcsB was performed by Western blot analysis. The results presented are one of the three independent experiments performed. (b) Quantification of RcsB and RcsB-P extracted from the wild-type and the *opgG* strains. Results reported are the average of three independent experiments.

was resumed by secondary mutations reducing the activity of the RcsCDB phosphorelay (see Introduction). The level of *in vivo* phosphorylation of RcsB was performed using the Phos-tag approach (Fig. 2a) in various mutant strains.

As expected, no RcsB was detected in the *rcsB* strain (lane 7, Fig. 2a). In the wild-type strain (lane 1, Fig. 2a, b), a low amount of RcsB-P (20%) was observed as compared to the RcsB amount. In the *opgG* strain (lane 2, Fig. 2a, 2b), an

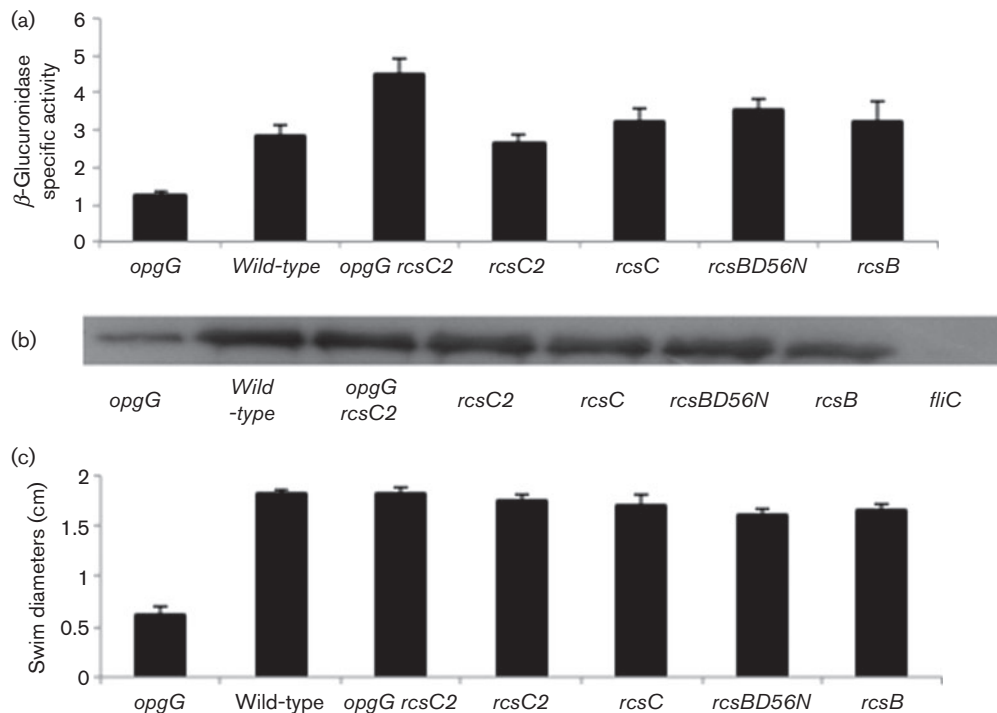


Fig. 3. Expression of the *flhD-uidA* fusion (a), the FliC (b) protein and motility (c) in various strains. For fusion expression measurements and Western blot analysis, bacteria were grown until mid-exponential phase in LB medium and broken by sonication or by boiling, respectively. The β -glucuronidase activity was measured with PNPU as a substrate. Specific activity is expressed as the change in optical density at 410 nm min^{-1} and per mg of protein. Motility was measured in LB semisolid plates. The FliC protein was revealed by Western blot analysis. Swim diameters were measured after 30 h of incubation at 30 °C. The results presented are one of the three independent experiments performed and results reported are the average of these three independent experiments.

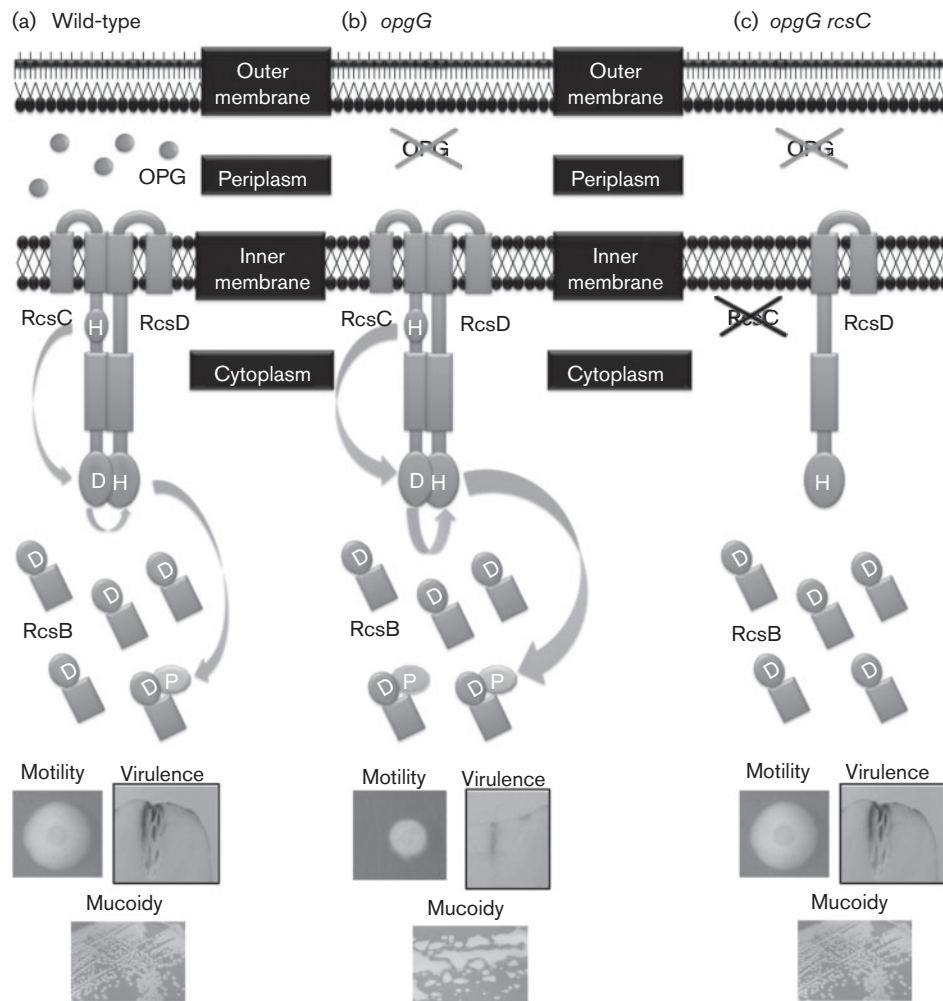


Fig. 4. Working model of relationship between OPGs and the RcsCDB phosphorelay. (a) The activation of the RcsCD sensor begins with phosphorylation of a histidine residue of the RcsC sensor moiety followed by transfer to an aspartate of the same RcsC sensor moiety (first arrow). The phosphate group is then transferred to a histidine of the RcsD sensor moiety (second arrow). Finally, a phosphate group is transferred to the RcsB regulator (third arrow). In the wild-type strain, phosphorylated RcsB regulator level allows motility and virulence on potato tuber and no mucoidy. (b) The increased activation of the RcsCD sensor in an *opgG* mutant strain leads to an increase of the phosphorylated RcsB repressing motility and virulence on potato tuber and increasing mucoidy. (c) The loss of the RcsCD activation in an *opgG rcsC* double mutant strain leads to the total loss of phosphorylated RcsB restoring motility and virulence on potato tuber and no mucoidy.

increased amount of RcsB-P (36%) as compared to RcsB was observed as compared to the wild-type, but the non-phosphorylated form of RcsB remained more abundant. In the *opgG rcsC2* (lane 3, Fig. 2a), in the *rcsC2* (lane 4, Fig. 2a), in the *rcsC* null mutant (lane 5, Fig. 2a) and in the *rcsB_{D56N}* (lane 6, Fig. 2a) strains, RcsB-P could not be detected. The same result was observed for the *opgG rcsC* and the *opgG rcsB_{D56N}* double mutant strains (data not shown).

These data indicated that in wild-type cells, the RcsB-P form is present in basal but low amounts and that the slight increase of RcsB-P amount observed in *opgG* mutant strains of *D. dadantii* is sufficient to display the pleiotropic phenotype.

Expression and phenotype of motility genes correlate with the phosphorylation of RcsB

In order to link the RcsB phosphorylated level to the expression and phenotype of genes controlled by the RcsCDB phosphorelay, we decided to compare expression of the *flhDC* operon encoding the master regulator of motility genes, directly repressed by phosphorylated RcsB, and *fliC*, an essential motility gene encoding the flagellin, a constituent of flagella. Bacterial strains were grown and subsequently divided in three for concomitant analyses of the phosphorylation of RcsB (see before), the expression of the *flhD::uidA* fusion and the expression of *fliC* gene product by Western blot. One can imagine that three

patterns of gene expression must be observed. The first one corresponds to the wild-type strain (basal RcsB-P level), the second one corresponds to the *opgG rcsC2*, the *rcsC2*, the *rcsC*, the *rcsB_{D56N}* and the *rcsB* strains (no detected RcsB-P), and the third one corresponds to the *opgG* strain (increased RcsB-P level). Surprisingly, despite the difference in phosphorylation level, similar expression levels of the *flhD* gene (Fig. 3a) and similar production of the FliC protein (Fig. 3b) were observed in the *opgG rcsC2*, the *rcsC2*, the *rcsC*, the *rcsB_{D56N}*, the *rcsB* strains (no RcsB-P) and the wild-type strain (20 % RcsB-P). As expected with the increased level of RcsB-P (36 %), a severe reduction of both the *flhD* gene expression (Fig. 3a) and the production of the FliC protein (Fig. 3b) was observed for the *opgG* strain (Fig. 3a, b). These results were in agreement with the phenotype of motility (Fig. 3c) because swimming diameters were similar for all the strains except for the *opgG* strain displaying a swimming diameter severely reduced.

These data strongly suggest that regulation by the RcsCDB phosphorelay occurred only above a minimum RcsB-P amount but that a slight increase was sufficient for important variation of expression of genes of the regulon as shown with *opg*-negative strains.

Finally, a working model of the impact of OPGs on the RcsCDB phosphorelay was drawn (Fig. 4) taking into account both the phosphorylation level of RcsB, the pleiotropic phenotypes (see Introduction) and the expression of genes of the *rcsCDB* regulon. The phosphorylation level of the RcsB regulator is probably the result of both kinase and phosphatase activities of the RcsCD sensor, but because only activation of this phosphorelay creates disorder, only activation was shown in Fig. 4.

The activation of the RcsCD sensor (Fig. 4a, b) begins with phosphorylation of a histidine residue of the RcsC sensor moiety followed by a transfer to an aspartate of the same RcsC sensor moiety (first arrow). The phosphate group is then transferred to a histidine of the RcsD sensor moiety (second arrow). Finally, a phosphate group is transferred to the RcsB regulator (third arrow). In the wild-type strain (Fig. 4a) and at a classical osmolarity of the medium (LB or minimal medium), OPGs are abundant in the periplasm and the activation level of the RcsCDB phosphorelay is low. In these conditions, 20 % of the RcsB regulator is phosphorylated allowing virulence on potato tubers, motility and no mucoidy. The absence of OPGs in an *opgG* mutant strain (Fig. 4b) leads to the increased activation of the RcsCD sensor. In these conditions and despite the low level of increased phosphorylation of RcsB (from 20 % to 36 %), it is sufficient to repress virulence on potato tubers and motility, and to increase mucoidy. In an *opgG rcsC* double mutant strain, no activation of the RcsCD sensor occurs in the absence of RcsC despite the absence of OPGs (Fig. 4c). This leads to the total loss of phosphorylated RcsB restoring virulence on potato tubers, motility and no mucoidy.

In a wild-type strain, OPGs could be seen as an intermediate between osmolarity and the RcsCDB phosphorelay. One of the major stimuli sensed by the RcsCDB phosphorelay is the increase of the osmolarity of the medium leading to increased mucoidy and decreased motility. It remains to be established if the activation of this phosphorelay is the result of a direct interaction between OPGs molecules and the RcsCD proteins, both transmembrane proteins displaying one periplasmic domain, or if this phosphorelay senses more complex modifications of the envelope resulting from variation of OPGs concentration in the periplasm.

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