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

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17
18 **Abstract**
19 Osmoregulated periplasmic glucans (OPGs) are general constituents of many
20 Proteobacteria. OPGs are important factors required for full virulence in many pathogens
21 including *Dickeya dadantii*. *D. dadantii* causes the soft-rot disease on a wide range of plant
22 species. The total loss of virulence and motility of *opg*-negative strains of *D. dadantii* is
23 linked to the constitutive activation of the RcsCDB phosphorelay deduced from expression
24 analysis of genes of the RcsCDB regulon. Here, the *in vivo* level of constitutive activation of
25 the RcsCDB phosphorelay *opg*-negative strain was demonstrated by direct analysis of the
26 phosphorylation level of the RcsB regulator protein by using a Phos-tag™ retardation gel
27 approach and was correlated with the phenotype and the expression of gene of motility.

28
29 **Keywords** : Osmoregulated periplasmic glucans, RcsCDB phosphorelay, *Dickeya dadantii*,
30 phosphorylation

31

32

33 **1. Introduction**

34 Osmoregulated periplasmic glucans (OPGs) are oligosaccharides found in the envelope of
35 most Proteobacteria (Bohin and Lacroix, 2006). The backbone is synthesized by the two
36 products of the *opgGH* operon and consists of β ,1-2 linked glucose units to which the
37 branches are attached by β ,1-6 linkages. These glucans belong to the common virulence
38 factors of many Proteobacteria zoopathogens and phytopathogens such as *Pseudomonas*
39 *aeruginosa* (Mahajan-Miklos *et al.*, 1999), *Brucella abortus* (Arellano-Reynoso *et al.*, 2005)
40 or *Dickeya dadantii* (Page *et al.*, 2001).

41 *Dickeya dadantii* is a phytopathogen Enterobacteria that causes soft rot disease in a wide
42 range of plant species (Perombelon, 2002). This opportunistic pathogen devastates
43 economically important crops in storage facilities or in growing plants. In *D. dadantii*, *opgG*
44 or *opgH* mutant, completely devoid of OPGs, show a pleiotropic phenotype including loss of
45 motility (Page *et al.*, 2001), induction of a general stress response (Bouchart *et al.*, 2007)
46 and complete loss of virulence on potato tubers and chicory leaves (Page *et al.*, 2001). All
47 these phenotypes suggest an impairment in the perception of the environment. This
48 hypothesis was confirmed by the restoration of motility and virulence on potato tubers by
49 inactivation of the RcsCDB phosphorelay regulatory system in strains devoid of OPGs.

50 Phosphorelays (also called two component systems) are the key of gene expression
51 plasticity in response to environmental variations. The RcsC sensor has both kinase and
52 phosphatase activities (Clarke, 2010). Under various stimuli, the transmembrane RcsC
53 sensor activates, its kinase activity increased, RcsC autophosphorylates and transfers its
54 phosphate group to its cognate cytoplasmic response regulator, RcsB, via the intermediate
55 transmembrane protein RcsD. In turn, RcsB regulates the expression of numerous target
56 genes affecting various cellular processes including repression of virulence and motility
57 (Cano *et al.*, 2002, Majdalani and Gottesman, 2005). In contrast, when the RcsC sensor
58 phosphatase activates and dephosphorylates the RcsB regulator, virulence and motility
59 occurs.

60 Loss of OPGs leads to a constitutive activation of the RcsCDB phosphorelay system.
61 Restoration of virulence and motility was obtained in the *opgG* by secondary mutations
62 decreasing the activation level of the RcsCDB phosphorelay. This was the case for the *opgG*

63 *rscC2*, the *opgG rscC* or the *opgG rscB_{D56N}* double mutant strains. In these strains, the
64 secondary mutation encoded a mutated RcsC2 protein with an increased phosphatase
65 activity, no RcsC protein or a non phosphorylatable RcsB_{D56N} protein respectively (Bouchart
66 et al., 2010, Bontemps-Gallo et al. 2013). The phosphorylation of the RcsB_{D56N} protein is
67 impossible since its phosphorylatable aspartic acid residue at position 56 was changed in
68 an asparagine residue. These conclusions about the activation level of the RcsCDB
69 phosphorelay system in the wild-type and the different mutant strains were classically
70 deduced from expression analysis of genes regulated by this phosphorelay.

71 In the present study, we showed the variation of activation level of the RcsCDB
72 phosphorelay system in different genetic background by direct observation of the RcsB
73 phosphorylated and non phosphorylated protein forms separated with a Phos-tag™
74 acrylamide gel retardation approach(ref phos tag). We demonstrated that the loss of OPGs
75 severely increases the phosphorylation level of the RcsB protein and this constitutive
76 phosphorylation repress motility .

77

78 **2. Materials and Methods**

79 *2.1 Bacterial strains, media and growth conditions*

80 Bacterial strains and plasmids are described in Table 1. Bacteria were grown in Lysogeny
81 broth (LB) (Bertani, 2004) at 30°C (*D. dadantii*) or 37°C (*Escherichia coli*). Antibiotics were
82 used at the following concentrations for *D. dadantii*: gentamicin at 2 µg.ml⁻¹,
83 chloramphenicol at 12.5 µg ml⁻¹, and kanamycin at 25 µg ml⁻¹, spectinomycin at 50 µg ml⁻¹.
84 Ampicillin was used at 50 µg ml⁻¹ for *E. coli*.

85

86 *2.2 Plasmid constructs and protein purification*

87 Plasmid used for the expression of the His tagged-RcsB, pNF410, was previously described
88 in Bontemps-Gallo *et al.*, 2013. For the construction of the His tagged-RcsB_{D56N}, a DNA
89 fragment encoding the mutated *rscB_{D56N}* gene was amplified by PCR using pNFW374 as
90 template, with the *rscBacf* (CACCATGAGCAATCTAAACGTAATTATTGCAG) and *rscBacr*
91 (CGCCGGCAAGAGCATTACTC) primers, cloned into the His-tag expression vector

92 pET100/D-Topo[®] (Invitrogen Life Technologies) to give pNFW491. The His-tagged RcsB
93 and RcsB_{D56N} proteins were expressed in *E. coli* BL21(DE3) and purified according to
94 Bontemps-Gallo *et al.*, 2013.

95

96 *2.3 Production of the phosphoramidate*

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

100

101 *2.4 Analysis of RcsB phosphorylation in vitro*

102 Phosphorylation reactions of purified RcsB or RcsB_{D56N} were performed with 5 µg of
103 proteins in 50 mM Tris-HCl, pH7.5, 100 mM NaCl, 10 mM MgCl₂ and 2 mM β-
104 mercaptoethanol. PA was added to a final concentration of 15 mM to initiate the reaction.
105 After 0, 15 or 30 minutes of incubation at room temperature, the reactions were stopped by
106 addition of SDS-PAGE loading buffer (final concentration: 50 mM Tris-HCl pH6.8, 2% (w/v)
107 SDS, 10% (w/v) glycerol, 20 mM DTT, 0.02% bromophenol blue). The mixtures were
108 resolved using phosphoprotein affinity gel electrophoresis as described in Barbieri and
109 Stock, 2008, with minor modifications. Briefly, Phos-tag[™] acrylamide gels were composed
110 of a 10% resolving solution [10% (w/v) 37.5:1 acrylamide/N,N'methylenebisacrylamide,
111 375 mM Tris (pH8.8) and 0.1% (w/v) SDS, 125µM Phos-tag[™] acrylamide and 250 µM
112 MnCl₂] and a 4% stacking solution [4% (w/v) 37.5:1
113 acrylamide/N,N'methylenebisacrylamide, 125 mM Tris (pH6.8) and 0.1% (w/v) SDS]. The
114 gels were run at 4 °C under constant voltage (150 V) with standard running buffer (0.1%
115 (w/v) SDS, 25 mM Tris and 192 mM glycine) and stained with Coomassie Blue.

116

117 *2.5 Analysis of RcsB phosphorylation in vivo*

118 At mid-log phase, equivalent of 1 ml of 0.15 OD_{620nm} of *D. datantii* cells was harvested by
119 centrifugation and pellets were immediately lysed with 12.7 µl of 1M formic acid,
120 solubilised by 5 µl of 4X SDS-PAGE loading buffer and neutralized by 2.8 µl 5 N NaOH.
121 Samples were quickly loaded onto gels containing 35 µM Phos-tag[™] acrylamide and 70 µM
122 MnCl₂ prepared and run as described before. After 10 min wash with transfer buffer (25

123 mM Tris and 192 mM glycine) supplied with 1 mM EDTA, followed by 10 min wash with
124 transfer buffer without EDTA, gels were transferred to nitrocellulose membranes using a
125 Trans-Blot® Turbo™ Blotting system (Bio-Rad) with a pre-programmed protocol (2.5 A, up
126 to 25 V, 7 min). Western blotting against RcsB were performed as described in Bontemps-
127 Gallo *et al.*, 2013. When required, purified RcsB or RcsB_{D56N} protein (2.5 µg) were
128 phosphorylated by a 30 min incubation at room temperature with PA, as described
129 previously. RcsB or phosphorylated RcsB proteins were added to bacteria prior lysis.

130
131 *2.6 Quantification of the phosphorylated RcsB protein amount.*
132 Phosphorylated RcsB and RcsB were quantified by determination of the area intensity of
133 each band with the software Quantity One (Bio Rad) after staining with coomassie blue or
134 detection by Western blot. Quantification of phosphorylated RcsB was expressed as the
135 ratio of the phosphorylated RcsB amount divided by the sum of the RcsB and the
136 phosphorylated RcsB amounts as described elsewhere (Barbieri and Stock, 2008).

137
138 *2.7 Dosage fusion*

139
140 *2.8 Analyse motility nage et fliC antibodies*

141
142 **3. Results and Discussion**

143 *3.1 Phosphorylated RcsB (RcsB-P) migrate slower than RcsB*

144 The method to observe the phosphorylated form of cytoplasmic regulators proteins is
145 available when the phosphate group is linked to a serine, threonine or tyrosine because the
146 linkage is relatively stable. In the case of bacterial phosphorelays, the phosphate group is
147 linked to an aspartate residue for the cytoplasmic regulator. Unfortunately, in this last case,
148 linkage is highly unstable and since recently, observation of thse kind of phosphorylated
149 proteins was impossible.

150 In 2008, Stock's lab published a method allowing separation of the phosphorylated and the
151 non-phosphorylated forms of phosphorelay regulators (Barbieri and Stock, 2008). This
152 method relies on Phos-tag™: a dinuclear metal complex acts as a specific phosphate-
153 binding agent. In a Phos-tag™ acrylamide gel, the Phos-tag™ linked to acrylamide

154 molecules interact with the phosphorylated form of the protein which migrates slower than
155 the non-phosphorylated protein allowing the separation of both forms of the protein. The
156 method was first applied *in vitro* on RcsB since method must be adapted to each regulator
157 (dixit Stock ref). Purified RcsB was phosphorylated *in vitro* by the phosphodonor
158 phosphoramidate (PA) (Lukat *et al.*, 1992). 5 µg of RcsB was incubated different times with
159 or without PA (see materials and methods), samples were loaded onto a Phos-tag™
160 acrylamide gel and RcsB was revealed with coomassie blue after migration. Without PA
161 added, a single band, corresponding to the non-phosphorylated RcsB form (called RcsB)
162 was observed (lanes 1 and 2, Fig. 1A). In contrast, a retardation of migration of 57% and
163 61% of the band was observed (lanes 3 and 4, Fig. 1A) when PA was added during 15 min
164 and 30 min respectively. This strongly suggests that the shifted band corresponds to the
165 phosphorylated RcsB form (called RcsB-P) but indicating that in our conditions, PA was
166 unable to phosphorylate all RcsB. The same experiment was performed with the non
167 phosphorylatable RcsB_{D56N} protein (lanes 5 to 8, Fig. 1A). In these lanes, a single band
168 migrating as the single band of lanes 1 and 2 was observed. These data indicate that the
169 lower band corresponded to RcsB, that the upper band corresponded to RcsB-P and that PA
170 phosphorylates the proper aspartic residue of RcsB. These conditions allowed us to analyze
171 the stability of the phosphorylation of RcsB *in vivo*.

172 Fin de la correction

173 XXX

174

175 3.2 The lysis with formic acid had no effect on the RcsB-P amount

176 The *in vivo* analysis required rapid lyse of bacteria with formic acid before loading onto the
177 gel (Barbieri and Stock, 2008). Because the phospho-aspartate bond is very unstable, it was
178 important to check both the effect of the formic acid and the lysis of cell on the stability of
179 RcsB and of the RcsB-P phospho-aspartate bond. To do this, 5 µg of purified RcsB or RcsB-P,
180 phosphorylated by PA, was added to an *rcsB* null mutant strain of *D. dadantii* (NFB7279)
181 prior lysis with formic acid. Lysates were then loaded onto a Phos-tag™ gel. As controls, the
182 same amount of RcsB and RcsB-P without bacteria were loaded onto the same gel. The same
183 experiments and controls were performed with the RcsB_{D56N}. After run, RcsB was revealed
184 by Western blot. When the lysate of the NFB7279 strain was loaded onto the gel alone, RcsB

185 was not detected (lane 1, Fig. 1B). A similar amount of RcsB was revealed when RcsB was
186 added to the NFB7279 strain before lysis (lane 2, Fig. 1B) or when RcsB was loaded alone
187 onto the gel (lane 6, Fig. 1B). The same two bands in similar amount, corresponding to RcsB
188 (53%, lower band) and RcsB-P (47%, upper band), were revealed when RcsB
189 phosphorylated by PA was added to the NFB7279 strain before lysis (lane 3, Fig. 1B). The
190 same two bands in similar amount, corresponding to RcsB (52%, lower band) and RcsB-P
191 (48%, upper band), were revealed when RcsB phosphorylated by PA was loaded alone on
192 the gel (lane 7, Fig. 1B). The same conclusions can be drawn when RcsB_{D56N} was used
193 instead of RcsB except that only one band was observed corresponding to the non
194 phosphorylated form of RcsB (lane 4, 5, 8, 9, Fig. 1B). Taken together, these results indicate
195 that neither cell lysate nor formic acid affect the RcsB stability or the RcsB-P phospho-
196 aspartate bond stability and that the two forms of RcsB are separated within a cell lysate.
197 These results allowed us to analyse the RcsB phosphorylation *in vivo*.

198

199 3.3 The phosphorylation of RcsB is increased in a strain devoid of OPGs

200 We previously showed that the RcsCDB phosphorelay was constitutively activated in an
201 *opgG* strain devoid of OPGs and constitutive activation was resumed by secondary
202 mutations reducing the activity of the RcsCDB phosphorelay (see introduction). These
203 conclusions were deduced from expression analysis of genes regulated by the RcsCDB
204 phosphorelay. To confirm these results, the level of *in vivo* phosphorylation of RcsB was
205 performed using the Phos-tag™ approach in the same strains (Fig. 1C). As expected, no
206 RcsB was detected in the *rscB* strain (lane 7, Fig. 1C). As expected, in the *rscB*_{D56N}, only one
207 band was observed corresponding to the RcsB form (lane 6, Fig. 1C). In the wild-type strain
208 (lane 1, Fig. 1C), a low amount of RcsB-P (11% upper band) was observed as compared to
209 the RcsB amount (89%, lower band). In the *opgG* strain (lane 2, Fig. 1C), an increased
210 amount of RcsB-P (23%, upper band) as compared to RcsB (77%, lower band) was
211 observed as compared to the wild-type but RcsB remained more important. In the *opgG*
212 *rscC2* (lane 3, Fig. 1C), in the *rscC2* (lane 4, Fig. 1C), in the *rscC* null mutant (lane 5, Fig. 1C),
213 and in the *rscB*_{D56N} (lane 6, Fig. 1C) strains, RcsB-P cannot be detected. These data
214 confirmed the results observed by Bouchart *et al.* (2010). In addition, they showed that in
215 wild-type cells, the RcsB-P form is present in very limited amount and that a moderate

216 increase in RcsB-P (*opgG* strain) is sufficient to confer the pleiotropic phenotype observed
217 in *D. dadantii*. The direct analysis of phosphorylation level of regulators of phosphorelays is
218 of a great interest particularly for determination of the phosphorylated to non
219 phosphorylated ratio of regulator impossible to do with analysis of gene expression but
220 remains difficult to use systematically because adaptation of the method is required for
221 each new phosphorelay studied is time consuming.

222

223

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229

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272 Legends to figures

273 **Figure 1:** Separation of RcsB and RcsB-P by Phos-tagTM polyacrylamide gel electrophoresis
274 and detection *in vivo* by Western analysis.

275 A. Phosphorylation of RcsB *in vitro*. Each lane contains 5µg of either purified wild-type
276 RcsB (lanes 1 to 4) or RcsB_{D56N} (lanes 5 to 8) incubated in presence of 15mM PA, during
277 0 min (lanes 2, 6), 15 min (lanes 3, 7) and 30 min (lanes 4, 8) or in absence of PA (lanes
278 1, 5) before loading onto the gel. After migration, RcsB and RcsB-P proteins were
279 visualized by coomassie-stained Phos-tagTM gel.

280 B. Stability of RcsB and phospho-aspartate of RcsB-P during cell lysate processing. Purified
281 RcsB (lane 2), RcsB-P (lane 3) or unphosphorylatable RcsB_{D56N} (lanes 4-5) were mixed
282 with *rscB* mutant strain before treatment with formic acid as described in Materials and
283 Methods. As controls, *rscB* cell lysate (lane 1), RcsB protein (lanes 6) RcsB-P (lane 7)
284 and RcsB_{D56N} protein (lanes 8, 9) were also loaded on the gel. Revelation of RcsB was
285 performed by Western blot analysis after electrophoresis on Phos-tagTM gel. Lanes were
286 PA is noted indicated that RcsB (or RcsB_{D56N}) was treated 30 min with PA prior to lysis.

287 C. Phosphorylation of RcsB *in vivo*. Cell lysates of wild-type (lane 1), *opgG* (lane 2), *opgG*
288 *rscC2* (lane 3), *rscC2* (lane 4), and *rscC* (lane 5), *rscB*_{D56N} (lane 6) and *rscB* (lane 7)
289 mutant strains of *D. dadantii* were subjected to Phos-tagTM acrylamide gel
290 electrophoresis and Western blot analysis.

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303 **Table 1**

Strain	Genotype ^{a, b}	Source or Reference
<i>Dickeya dadantii</i>		
EC3937	Wild-type	Laboratory collection
NFB3591	<i>opgG::uidA-Kan^r rcsC2</i>	Bouchart <i>et al.</i> , 2010
NFB3611	<i>rcsC2</i>	Bouchart <i>et al.</i> , 2010
NFB3682	<i>rcsC::Cml^r</i>	Bouchart <i>et al.</i> , 2010
NFB3723	<i>opgG::Cml^r</i>	Laboratory collection
NFB7112	<i>rcsB::Cml^r rcsCB_{D56N}D-Spe^r</i>	Bontemps-Gallo <i>et al.</i> , 2013
NFB7279	<i>rcsB::Gm^r</i>	Bontemps-Gallo <i>et al.</i> , 2013
<i>Escherichia coli</i>		
BL21(DE3)	<i>ompT, hsdSB, gal, dcm</i>	Invitrogen
Plasmids		
pNFW374	pUC18Not- <i>rcsCB_{D56N}D, Amp^r</i>	Bontemps-Gallo <i>et al.</i> , 2013
pNFW410	pET100/D-topo <i>rcsB, Amp^r</i>	Bontemps-Gallo <i>et al.</i> , 2013
pNFW491	pET100/D-topo <i>rcsB_{D56N}, Amp^r</i>	This study

304 ^a*rcsCB_{D56N}D* is carried by a Spe^r mini-Tn5305 ^bCml^r, chloramphenicol resistance; Amp^r, ampicillin resistance, Kan^r, kanamycin resistance,
306 Spe^r, spectinomycin resistance, Gm^r, gentamicin resistance.

307