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The Virulence of a *Dickeya dadantii* 3937 Mutant Devoid of Osmoregulated Periplasmic Glucans Is Restored by Inactivation of the RcsCD-RcsB Phosphorelay▽

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***Dickeya dadantii* is a pectinolytic phytopathogen enterobacterium that causes soft rot disease on a wide range of plant species. The virulence of *D. dadantii* involves several factors, including the osmoregulated periplasmic glucans (OPGs) that are general constituents of the envelope of proteobacteria. In addition to the loss of virulence, *opg*-negative mutants display a pleiotropic phenotype, including decreased motility and increased exopolysaccharide synthesis. A nitrosoguanidine-induced mutagenesis was performed on the *opgG* strain, and restoration of motility was used as a screen. The phenotype of the *opg* mutant echoes that of the Rcs system: high level activation of the RcsCD-RcsB phosphorelay is needed to activate exopolysaccharide synthesis and to repress motility, while low level activation is required for virulence in enterobacteria. Here, we show that mutations in the RcsCDB phosphorelay system restored virulence and motility in a *D. dadantii opg*-negative strain, indicating a relationship between the Rcs phosphorelay and OPGs.**

Osmoregulated periplasmic glucans (OPGs) are general periplasmic constituents of the envelope of most proteobacteria. Their common features are that glucose is the sole constituent sugar, and their abundance in the periplasm increases as the osmolarity of the medium decreases. In *Enterobacteriaceae* and related bacteria, the glucose backbone synthesis is catalyzed by both products of the *opgGH* operon (5). Studies of several bacterial pathogens, including *Dickeya dadantii*, showed the importance of OPGs for virulence (4, 5, 18, 25, 26).

Dickeya dadantii is a member of the pectinolytic erwiniae causing soft rot disease in a wide range of plant species (33). The virulence of *D. dadantii* is associated with the synthesis and the secretion of a set of plant cell wall-degrading enzymes (pectinases, cellulases, and proteases) causing maceration of the plant tissues (22). *D. dadantii* synthesize OPGs containing 5 to 12 glucose units joined by β,1-2 linkages and branched by β,1-6 linkages that are substituted with succinyl and acetyl residues (11). The *opgG* or *opgH* mutants unable to synthesize OPGs show a pleiotropic phenotype. They are nonvirulent on chicory leaves and potato tubers, and synthesis and secretion of pectate-lyases, cellulases, and proteases are reduced (32). Motility is severely reduced, while exopolysaccharide secretion is increased (mucoid phenotype) (32). Data suggest that the *opg* mutants are impaired in perception of the environment, which prevents *D. dadantii* from recognizing host cells, suggesting a possible dysfunction of phosphorelay signaling pathways, major systems required for environmental perception in bacteria (6). In these systems, upon stimuli, a kinase/phosphatase sensor autophosphorylates and transfers the phosphate group to a cytoplasmic regulator which modulates expression of target genes.

Here, we show that mutations in the *rcsC* and *rcsB* genes, encoding, respectively, the sensor and the cognate regulator of the RcsCD-RcsB phosphorelay, suppress several phenotypes of an *opgG* mutant, including the nonvirulent phenotype on potato tubers. This suggests interactions between the RcsCD-RcsB phosphorelay and OPG molecules and constitutes a first hint at the molecular role of these ubiquitous glycans in virulence.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *D. dadantii* and *Escherichia coli* strains are listed in Table 1. Bacteria were grown at 30°C (*D. dadantii*) or 37°C (*E. coli*) in Luria-Bertani broth (LB) or in minimal medium M63 supplemented with a carbon source at a concentration of 2 g/liter (29). Solid media were obtained by adding agar at 15 g/liter. When low-osmolarity medium was required, LB without NaCl was used. Motility was tested on LB agar plates containing 4 g/liter agar. The solid media used to test the pectinases, cellulases, and proteases activities have been described previously (32).

Antibiotics were used at the following concentrations: ampicillin and kanamycin (Kan) at 50 μg/ml (*E. coli*) or 25 μg/ml (*D. dadantii*), chloramphenicol and tetracycline at 25 μg/ml (*E. coli*) or 12.5 μg/ml (*D. dadantii*), and spectinomycin at 100 μg/ml (*E. coli*) or 50 μg/ml (*D. dadantii*). X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and X-GlcA (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) were used at a concentration of 40 μg/ml.

Recombinant DNA techniques. Standard procedures were performed for genomic and plasmid DNA extractions (36). DNA purification was performed with Nucleospin Extract II (Macherey-Nagel). Restriction enzymes (Biolabs), T4 DNA ligase (Biolabs), *Taq* polymerase (Eppendorf), and the large fragment of DNA polymerase I (Klenow fragment; Invitrogen) were used according to the manufacturer's recommendations.

Construction of the mutations. Plasmids and primers designed for PCR are listed in Tables 2 and 3, respectively. The chloramphenicol cassette used for gene inactivation was released from pNFCml after digestion by EcoRV. The *rcsCBD* locus was cloned by shotgun. The *D. dadantii* 3937 chromosomal DNA was

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TABLE 1. Bacterial strains

Strain	Relevant description/genotype	Source or reference
D. dadantii strains		
EC3937	Wild type	Laboratory collection
NFB3500	EC3937 *opgG*::*uidA* Kan^r	Laboratory collection
NFB3591	NFB3500 *rcsC2*	This study
NFB3602	EC3937 *opgG*::Cml^r *ura*	Laboratory collection
NFB3609	NFB3602 *rcsC2*	This study
NFB3611	EC3937 *rcsC2*	This study
NFB3682	EC3937 *rcsC*::Cml^r	This study
NFB3683	NFB3500 *rcsC*::Cml^r	This study
NFB3753	EC3937 Δ(*rcsCBD*)::Cml^r	This study
NFB3754	NFB3500 Δ(*rcsCBD*)::Cml^r	This study
NFB3800	EC3937 (mini-Tn*5*Kan *flhD-uidA*)	This study
NFB3805	NFB3602 (mini-Tn*5*Kan *flhD-uidA*)	This study
NFB3806	NFB3609 (mini-Tn*5*Kan *flhD-uidA*)	This study
NFB3807	NFB3611 (mini-Tn*5*Kan *flhD-uidA*)	This study
NFB3808	NFB3753 (mini-Tn*5*Kan *flhD-uidA*)	This study
NFB3809	EC3937 (mini-Tn*5*Kan *ftsA-uidA*)	This study
NFB3810	NFB3602 (mini-Tn*5*Kan *ftsA-uidA*)	This study
NFB3811	NFB3609 (mini-Tn*5*Kan *ftsA-uidA*)	This study
NFB3812	NFB3611 (mini-Tn*5*Kan *ftsA-uidA*)	This study
NFB3813	NFB3753 (mini-Tn*5*Kan *ftsA-uidA*)	This study
NFB3859	NFB3811 (mini-Tn*5*Spe *rcsCBD*)	This study
NFB3946	EC3937 *opgG*::FRT	Laboratory collection
NFB3948	NFB3810 (mini-Tn*5*Spe *rcsC2BD*)	This study
NFB3983	NFB3946 Δ(*rcsCBD*)::Cml^r	This study
NFB3991	NFB3500 (mini-Tn*5*Spe *rcsCBD*)	This study
NFB7007	NFB3983 (mini-Tn*5*Kan *ftsA-uidA*)	This study
NFB7107	NFB3983 (mini-Tn*5*Kan *flhD-uidA*)	This study
NFB7127	NFB3682 (mini-Tn*5*Kan *ftsA-uidA*)	This study
NFB7128	NFB3682 (mini-Tn*5*Kan *flhD-uidA*)	This study
NFB7154	NFB3946 *rcsC*::Cml^r	This study
NFB7191	NFB7154 (mini-Tn*5*Kan *ftsA-uidA*)	This study
NFB7192	NFB7154 (mini-Tn*5*Kan *flhD-uidA*)	This study
NFB7199	EC3937 *rcsB*::Cml^r	This study
NFB7200	EC3937 *rcsC2 rcsB*::Cml^r	This study
NFB7202	NFB7199 (mini-Tn*5*Kan *flhD-uidA*)	This study
NFB7203	NFB7200 (mini-Tn*5*Kan *flhD-uidA*)	This study
NFB7205	NFB7199 (mini-Tn*5*Kan *ftsA-uidA*)	This study
NFB7206	NFB7200 (mini-Tn*5*Kan *ftsA-uidA*)	This study
NFB7208	NFB3946 *rcsB*:Cml^r	This study
NFB7209	NFB3946 *rcsC2 rcsB*::Cml^r	This study
NFB7210	NFB3500 *rcsB*::Cml^r	This study
NFB7211	NFB3500 *rcsC2 rcsB*::Cml^r	This study
NFB7212	NFB7208 (mini-Tn*5*Kan *flhD-uidA*)	This study
NFB7213	NFB7208 (mini-Tn*5*Kan *ftsA-uidA*)	This study
NFB7214	NFB7209 (mini-Tn*5*Kan *flhD-uidA*)	This study
NFB7215	NFB7209 (mini-Tn*5*Kan *ftsA-uidA*)	This study
E. coli strains		
JM83	F′ *ara* Δ(*lac-proAB*) *rpsL* (ϕ80d*lacZ*ΔM15)	40
S17-1λpir	*recA thi pro hsdR* λ*pir* RP4-2-Tet::Mu-Kan::Tn*7* Tmp^r Str^r	30

TABLE 2. Plasmids

Plasmids	Genotype and/or phenotype[a]	Source or reference
pUC18	Amp^r	40
pUC18Not	Amp^r	21
pB21	pSUP102::Tn*5*-*B*21 Tet^r	37
pNFCml	Cml^r	Laboratory collection
pUIDK11	Cml^r *uidA*-Kan^r	3
pOK1	*sacB oriR6K* Spe^r	23
pCP20	Rep(Ts) *flp*$^+$ Amp^r Kan^r	9
pUTmini-Tn*5*Kan	mini-Tn*5*Kan *oriR6K* Kan^r Amp^r	13
pUTmini-Tn*5*Spe	mini-Tn*5*Spe *oriR6K* Spe^r Amp^r	13
pNFW190	pKO1 *opgG*::Kan^r	This study
pNFw198	pKO1 *opgG*::FRT	This study
pNFW138	pUC18 *rcsC*	This study
pNFW149	pUC18 *rcsC*::Cml^r	This study
pNFW161	pOK1 *rcsC*::Cml^r	This study
pNFW170	pUC18 *′gyrA* Δ*rcsCBD menF′*	This study
pNFW171	pUC18 *′gyrA* Δ*rcsCBD*::Cml^r *menF′*	This study
pNFW181	pUC18Not *flhD′*	This study
pNFW203	pUC18Not *flhD-uidA*-Kan^r	This study
pNFW204	pUC18Not *flhD-uidA*	This study
pNFW215	pUT(mini-Tn*5*Kan *flhD-uidA*)	This study
pNFW182	pUC18Not *ftsA′*	This study
pNFW193	pUC18Not *ftsA-uidA*-Kan^r	This study
pNFW201	pUC18Not *ftsA-uidA*	This study
pNFW220	pUT(mini-Tn*5*Kan *ftsA-uidA*)	This study
pNFW257	pUC18Not *rcsCBD*	This study
pNFW261	pUT(mini-Tn*5*Spe *rcsCBD*)	This study
pNFW336	pUC18Not *rcsC2BD*	This study
pNFW339	pUT(mini-Tn*5*Spe *rcsC2BD*)	This study
pNFW397	pUC18Not *rcsCB*::Cml^r *rcsD*	This study
pNFW398	pUC18Not *rcsC2B*::Cml^r *rcsD*	This study

[a] Cml^r, chloramphenicol resistance; Amp^r, ampicillin resistance; Kan^r, kanamycin resistance; Spe^r, spectinomycin resistance; Tet^r, tetracycline resistance.

digested by SacI and XbaI, and fragments from 6 kb to 8 kb were isolated by agarose gel electrophoresis, purified, and cloned into pUC18Not digested by the same restriction enzymes (21). Plasmids extracted from mucoid colonies were analyzed by restriction. The NotI fragment from pNFW257, containing the *rcsCBD* locus, was subcloned into pUTmini-Tn*5*Spe (13) digested by NotI. The resulting plasmid (pNFW261) was introduced into *D. dadantii* cells by conjugation.

To clone the *rcsC2BD* locus, a fragment of the *rcsC* DNA encompassing the *rcsC2* mutation was amplified by PCR (rcs5 and rcs6 primers) from the NFB3611 chromosomal DNA, digested by SspI and NheI, and cloned into pNFW257 digested by the same enzymes (pNFW336); the cloned sequence was sequenced. The SacI-AscI fragment of pNFW336 was cloned into pNFW261 digested by the same enzymes. The resulting plasmid (pNFW339) was introduced into *D. dadantii* cells by conjugation.

To inactivate *rcsC*, *rcsC* was amplified by PCR (rcs7 and rcs8 primers), digested by SacI and HindIII, and cloned into pUC18 cleaved by the same enzymes (pNFW138). The cloned DNA fragment was sequenced. A Cml^r cassette was inserted into pNFW138 digested by PshAI and SmaI (pNFW149). The SalI-HpaI fragment from pNFW149, containing the *rcsC* gene disrupted by the Cml^r cassette, was cloned into the pOK1 vector digested by SalI and SmaI. The resulting plasmid (pNFW161) was introduced into *D. dadantii* cells by conjugation.

The *rcsB*::Cml^r allele was obtained by the insertion of the Cml^r cassette into pNFW257 digested by HpaI. The resulting plasmid (pNFW397) was introduced in *D. dadantii* cells by electroporation.

The *rcsC2 rcsB*::Cml^r allele was obtained by the insertion of the Cml^r cassette into pNFW336 digested by HpaI. The resulting plasmid (pNFW398) was introduced in *D. dadantii* cells by electroporation.

To delete the *rcsCBD* locus, two DNA fragments (1 kb), located upstream and encompassing, for the first fragment, the beginning of the *rcsC* coding sequence (rcs1 and rcs2 primers) and encompassing, for the second fragment, the beginning of the *rcsD* coding sequence (rcs3 and rcs4 primers), were amplified by PCR. These fragments were digested by KpnI and SmaI and by SmaI and HindIII, respectively, and cloned into pUC18 cleaved by KpnI and HindIII (pNFW170). The cloned sequence was sequenced. The Cml^r cassette was inserted into pNFW170 digested by SmaI. The resulting plasmid, pNFW171, was introduced into *D. dadantii* cells by electroporation.

The *opgG*::FRT allele was obtained as described by Cherepanov and Wackernagel (9). An *opgG*::Kan^r allele was inserted into the pOK1 plasmid (pNFW190). The Kan^r cassette is flanked by two FLP recombination target

TABLE 3. Primer sequences

Primer	Sequence[a]
rcs1	AAGGTACCTTGCCGGAAGCGAGCC GCGGCGCTCGCGGT
rcs2	AACCCGGGTCAAAGGCATACCGTA AAGGTTGTCAGCAA
rcs3	AACCCGGGCGCAGGCATGAAATCA GGCTCCTGATGAAC
rcs4	AAAAGCTTTACCCCGTAATAACCTG CTTACCCACCCGC
rcs5	GCAATAGCCTCAGCCATTACCCGGA
rcs6	CGTCTGACAAAGAGTAATGC
rcs7	GCTGAACCGGTGGAAGACGATGAG CTCGACAGCGTG
rcs8	CTGAAAGGTGTCTTTGCCATGCTGA ATCTTCATCCC
flhD1	CACTGCGGGGTAAGGATCCGTGAA ATATTATG
flhD2	GCATCGAGCTCGATGCGTCTGAGG TGCCGGCTCTTCA
ftsA1	GATCGAGGATCCGCCCTGGATTAA ACAGGCCAGCG
ftsA2	CGGCATGAGCTCATTGCCGATGCGC TCAGCCGGC
Tn*5*-1	CTAGGCGGCCAGATCTGATCAA
Tn*5*-2	TCAAAAGGTCATCCACCGGATC
Tn5B21-1	CATGGAAGTCAGATCCTGG
Tn5B21-2	GTTCACTCCGTTCTCTTGC

[a] Restriction sites are underlined.

(FRT) sites recognized by the Flp recombinase expressed by the pCP20 plasmid. This recombinase was used to obtain a Kan^s mutant *opgG* allele by removing the Kan^r cassette. The resulting pOK1 plasmid harboring the *opgG*::FRT allele (pNFW198) was introduced into *D. dadantii* cells by conjugation.

Construction of the transcriptional fusions. The *uidA*-Kan^r cassette used for gene fusions was extracted from plasmid pUIDK11 (3). The *flhD′* and *ftsA′* DNA fragments were amplified by PCR (flhD1 and flhD2, and ftsA1 and ftsA2 primers, respectively). The *flhD′* fragment was digested by BamHI and SacI and cloned into pUC18Not digested by the same enzymes (pNFW181). The SacI DNA fragment containing the *uidA*-Kan^r cassette was inserted into pNFW181 digested by SacI (pNFW203). The *ftsA′* DNA fragment was digested by BamHI and SacI and cloned into pUC18Not digested by the same enzymes (pNFW182). The SmaI DNA fragment containing the *uidA*-Kan^r cassette was inserted into pNFW182 digested by NgoMIV and blunt ended with the Klenow enzyme (pNFW193).

The Kan^r cassette was removed from pNFW203 (*flhD′*) and pNFW193 (*ftsA′*) by NcoI and HpaI digestion, blunt ended, and annealed (pNFW204 and pNFW201, respectively). The NotI fragments of these plasmids were subcloned into pUTmini-Tn*5*Kan (13) to give pNFW215 and pNFW220, respectively (Table 2), and introduced into *D. dadantii* by conjugation.

Transduction, conjugation, and transformation. Transformation of *E. coli* cells was carried out by the rubidium chloride technique (29). Plasmids were introduced in *D. dadantii* by electroporation (36) or conjugation (30). The insertions were integrated into the *D. dadantii* chromosome by marker exchange recombination in the presence of the appropriate antibiotic after successive cultures in low-phosphate medium (35). Transduction with phage ΦEC2 was carried out according to the method of Resibois et al. (34).

Nitrosoguanidine and transposon mutagenesis. *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (nitrosoguanidine) mutagenesis was performed according to Adelberg et al. (1). Briefly, NFB3500 was grown in LB medium at 30°C to mid-log phase. One milliliter of culture was centrifuged, and the pellet was washed with TM buffer [50 mM Tris, 50 mM maleic acid, 8 mM $MgSO_4$, 15 mM $(NH_4)_2SO_4$, pH 6]. The washed bacterial pellet was resuspended in 1 ml TM buffer, nitrosoguanidine was added at a concentration of 100 μg/ml, and the suspension was incubated for 30 min at 30°C. The bacteria were then washed twice with TM buffer. The pellet was resuspended in 1 ml LB medium and divided into 10 independent aliquots of 0.1 ml. Each aliquot was diluted in 5 ml LB medium, grown overnight, and stored in 20% glycerol at −80°C. Nitrosoguanidine mutagenesis efficiency was estimated by the percentage of auxotroph mutant.

Transposon mutagenesis by Tn*5*-*B21* or mini-Tn*5* was performed by conjugation between the *E. coli* S17-1λpir harboring the suicide vector containing transposon and various *D. dadantii* EC3937 derivatives. Mutants were selected on minimal medium 63 plates containing sucrose as a unique carbon source (counterselection of the *E. coli* donor) and the appropriate antibiotic (13, 37).

Localization of transposon insertions and sequence data. Transposon insertions were localized on the *D. dadantii* chromosome by inverse PCR using Tn*5*-1 and Tn*5*-2 primers (mini-Tn*5* insertions) or Tn*5*B21-1 and Tn*5*B21-2 primers (Tn*5*-*B21* insertions) after digestion of the chromosomal DNA with TaqI. The sequence and annotations of the *D. dadantii* chromosome (J. D. Glasner, C.-H. Yang, S. Reverchon, N. Hugouvieux-Cotte-Pattat, G. Condemine, J.-P. Bohin, F. van Gijsegem, S. Yang, T. Franza, D. Expert, G. Plunkett, M. San Francisco, A. Charkowski, B. Py, L. Grandemange, K. Bell, L. Rauscher, P. Rodriguez-Palenzuela, A. Toussaint, M. Holeva, S.-Y. He, V. Douet, M. Boccara, C. Blanco, I. Toth, A. D. Anderson, B. Biehl, B. Mau, S. M. Flynn, F. Barras, M. Lindeberg, P. Birch, S. Tsuyumu, X. Shi, M. Hibbing, M.-N. Yap, U. Masahiro, J. F. Kim, P. Soni, G. F. Mayhew, D. Fouts, S. Gill, F. R. Blattner, N. T. Keen, and N. T. Perna, submitted for publication) are available at http://asap.ahabs.wisc.edu/asap/ASAP1.htm. Sequence accession numbers of the *rcsC*, *rcsB*, and *rcsD* genes are ABF-0017295, ABF-0017296, and ABF-0017297, respectively.

Determination of enzyme activities. β-Glucuronidase assays were performed on crude extracts obtained from bacteria disrupted by passage through a French pressure cell at 1.4 10^7 Pa (20,000 lb/in^2) as previously described (12). β-Glucuronidase activity was determined by monitoring spectrometrically at 410 nm the hydrolysis of PNPU (4-nitrophenyl-β-D-glucuronide).

The protein concentration was determined by the Bradford assay with bovine serum albumin as a standard (7).

OPG analysis. Measurement of OPG synthesis in *D. dadantii* was performed as previously described (32).

Pathogenicity test. Chicory leaves were inoculated as previously described (12). Bacteria from an overnight culture in LB medium were recovered by centrifugation and diluted in M63 medium. Leaves were slightly wounded in their center with a sterile razor blade and infected using 10^7 bacteria per inoculation site. After incubation in a dew chamber for 48 h at 30°C, the length of rotted tissue was measured to estimate the disease severity. Potatoes tubers were inoculated as previously described (24). Sterile pipette tips containing a bacterial suspension of 10^7 or 10^8 bacteria in 5 μl were inserted into the tuber parenchyma. After 72 h at 30°C, tubers were sliced vertically through the inoculation point and the weight of decayed tissues was measured. Each measure was repeated in three independent experiments.

RESULTS

Screening and characterization of the *rcsC2* mutation. Nitrosoguanidine-induced mutations were isolated from the *opgG* strain NFB3500. Mutagenized bacteria were screened for motility by spotting 10^6 bacteria on soft agar plates. The swarm diameter obtained for the *opgG* mutagenized strain was higher than the swarm diameter observed for the *opgG* strain. Bacteria from the periphery circumference of the halo were purified and screened for motility. Motile clones were tested for mucoidy and virulence on chicory leaves and potato tubers. None of the tested clones were virulent on chicory leaves, but two of them were virulent on potato tubers (Fig. 1) and display a nonmucoid phenotype. One of the suppressive mutations (NFB3591) was further studied because these two clones were isolated from the same mutagenesis pool.

Random transposon mutagenesis with Tn*5*-*B21* (Tet^r) was performed on wild-type strain EC3937 and used to map the suppressive mutation. Transposon insertions were then introduced into the suppressor strain (NFB3591) by generalized transduction using the ΦEC2 phage, and the resulting Tet^r colonies were screened for mucoidy and motility. One insertion of Tn*5*-*B21*, located in the gene ABF-0020432, was 24% cotransducible with the mucoid and the nonmotile phenotypes. The distance between the Tn*5* insertion and the suppressive mutation is about 20 kb according to the equation of Wu (39). The distance, the phenotypes observed, and the isolation of a

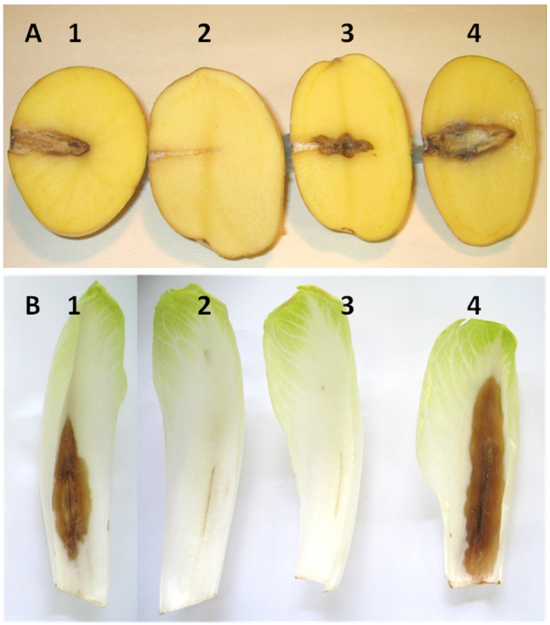

FIG. 1. Pathogenicity of the wild-type (EC3937) strain and the *opgG* (NFB3500), *opgG rcsC2* (NFB3591), and *rcsC2* (NFB3611) mutant strains of *D. dadantii* on potato tubers and chicory leaves. (A) Bacteria (10^7) of the wild-type strain (EC3937) (spot 1), the *opgG* strain (NFB3500) (spot 2), the *opgG rcsC2* strain (NFB3591) (spot 3), and the *rcsC2* strain (NFB3611) (spot 4) were inoculated into holes on potato tubers. Disease symptoms were observed after 72 h of incubation at 30°C. (B) Bacteria (10^7) of the wild-type strain (EC3937) (spot 1), the *opgG* strain (NFB3500) (spot 2), the *opgG rcsC2* strain (NFB3591) (spot 3), and the *rcsC2* strain (NFB3611) (spot 4) were inoculated into scarified chicory leaves. Disease symptoms were observed after 48 h of incubation at 30°C.

null mutation in the *rcsC* gene abolishing the mucoid phenotype of an *opgH* null mutation in *E. coli* (15), suggest that the suppressor mutation lies within the *rcsCBD* locus. DNA sequencing revealed a unique C/T transition located in the *rscC* gene. This mutation, named *rcsC2*, led to an alanine-to-valine substitution at position 463. This A463V substitution is located in the cytoplasmic linker domain of the protein RcsC (in the vicinity of phosphorylatable H479) between the second transmembrane domain and the histidine kinase domain (http://smart.embl-heidelberg.de/). RcsC is the transmembrane sensor component of the RcsCD-RcsB signaling pathways phosphorylating the RcsB regulator via the intermediate RcsD protein (27). These data suggest that a modification of RcsCD-RcsB signaling suppresses *opg* mutation phenotypes.

Decreased activation of the RcsCD-RcsB phosphorelay signaling pathway restores motility and the nonmucoid aspect in the *opgG* strain. To test this hypothesis, null *rcsC*, *rcsB*, and Δ*rcsCBD* mutations were introduced in the *opgG* mutant strain. Motility, mucoid aspect, plant cell-degrading enzyme activity, and virulence of the double mutants were evaluated. The nonmucoid aspect of colonies was restored in the *opgG rcsC*, *opgG rcsB*, and the *opgG* Δ*rcsCDB* double mutant strains (Table 4). Motility was determined on swarming plates. Restoration of motility occurred in all the *opgG rcs* mutant strains compared to the *opgG* strain (Table 4), while motility levels were similar for the wild-type and the various *rcs* strains (Table 4). These data suggest that loss of activation of RcsCD-RcsB signaling suppresses the *opg* mutation phenotype.

Decreased activation of the RcsCD-RcsB phosphorelay signaling pathway is required for restoration of virulence in the *opgG* strain. Secretion of plant cell wall-degrading enzymes is required for full virulence of *D. dadantii*. Global pectinase, cellulase, and protease activities were estimated on plates. The halo diameters of degraded substrates indicated that the global exoenzyme activities were restored in the *opgG rcsC2*, *opgG rcsC*, *opgG rcsB*, and *opgG* Δ*rcsCBD* double mutant strains compared to the *opgG* single mutant strain, while activities observed for the corresponding single *rcs* mutant strains remained unaffected compared to the wild-type strain (Table 4). Thus, in *D. dadantii*, activation of the RcsCD-RcsB phosphorelay represses exoenzyme synthesis, as observed with *Pectobacterium carotovorum* (2).

Virulence of the *opgG rcsC2*, *opgG rcsC*, *opgG rcsB*, and *opgG* Δ*rcsCBD* double mutant strains was assessed by measuring the severity of the disease on potato tubers and on chicory leaves. Virulence was restored in potato tubers for all the double mutant strains compared to the *opgG* single mutant strain. The weights of macerated tissues were similar for the wild-type and for all the double mutant strains (Fig. 2). On chicory leaves, no restoration of virulence was observed for any of the double mutant strains (data not shown). In contrast, for the *rcsC*, *rcsB*, and Δ*rcsCBD* single mutant strains, virulence levels were similar to those observed for the wild-type strain for both potato tubers (Fig. 2) and chicory leaves (data not shown). A slight but reproducible increase of the disease severity was observed for the *rcsC2* strain (Fig. 1 and 2). This increase depends on the RcsCD-RcsB phosphorelay since the maceration observed with the *rcsB rcsC2* double mutant was reduced to the level observed for the *rcsB* single mutant strain (Fig. 2). These data demonstrate that the restoration of virulence in the *opgG* strain is the result of decreased activation of the RcsCD-RcsB phosphorelay.

Absence of OPGs induces the Rcs regulon. The RcsCD-RcsB phosphorelay regulates motility by negatively regulating the *flhDC* master operon (19, 20). The *flhD* and *flhC* genes

TABLE 4. Phenotypes observed in various *opg* and *rcs* mutant strains compared to the wild-type strain[a]

Main strain or genotype (strain name)	Motility	Pectinase activity	Cellulase activity	Protease activity	Mucoidy
Wild-type (EC3937)	2.6	2.5	1.6	+	−
opgG (NFB3500)	1.1	1.6	1	−	+
rcsC2 (NFB3611)	2.6	2.7	1.8	+	−
opgG rcsC2 (NFB3591)	2.4	2.4	1.8	+	−
rcsC (NFB3682)	2.9	2.3	1.4	+	−
opgG rcsC (NFB3683)	2.8	2.3	1.8	+	−
Δ*rcsCBD* (NFB3753)	2.8	2.5	1.7	+	−
opgG Δ*rcsCBD* (NFB3754)	1.7	2.3	1.8	+	−
rcsB (NFB7199)	2.7	2.7	1.7	+	−
opgG rcsB (NFB7210)	1.6	2.3	1.5	+	−
rcsB rcsC2 (NFB7200)	2.9	2.9	1.8	+	−
opgG rcsB rcsC2 (NFB7211)	2.1	2.2	1.6	+	−

[a] For motility, the swarm diameters are expressed in cm. Exoenzyme activities: for pectinase activity, the halo diameters of substrate degradation are expressed in cm; for cellulase activity, the halo diameters of substrate degradation are expressed in cm; for protease activity, + or − indicates presence or absence of halo of degradation. For mucoidy, − indicates a nonmucoid phenotype, while + indicates a mucoid phenotype.

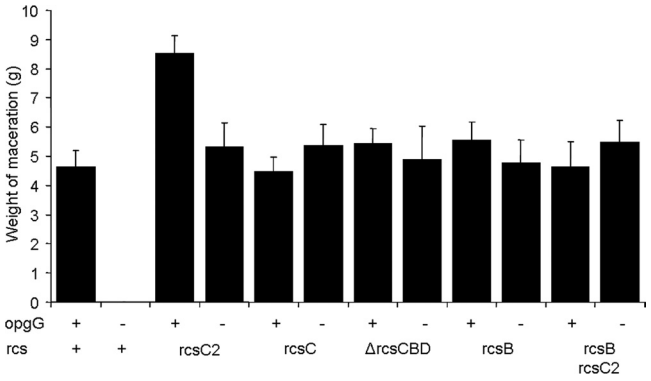

FIG. 2. Weight of maceration on potato tubers for various *opgG* and *rcs* mutant strains of *D. dadantii* measured. Bacteria (10^7) were inoculated into holes on potato tubers. Maceration (g) was weighed after 72 h of incubation at 30°C.

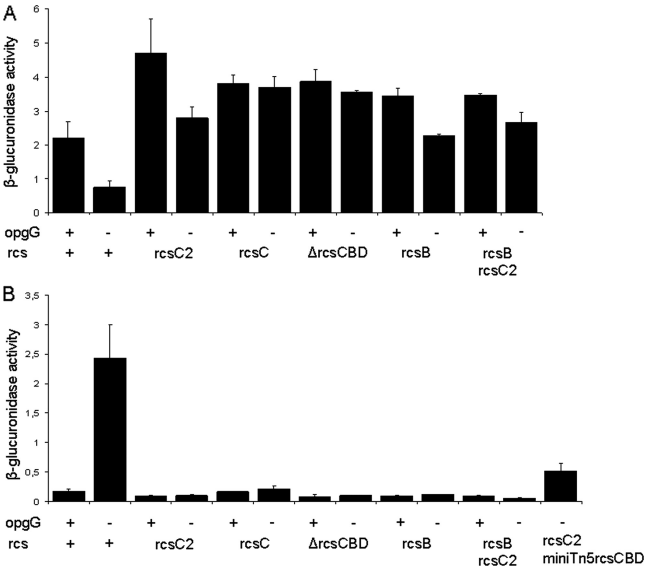

FIG. 3. Expression of the *flhD-uidA* (A) or the *ftsA-uidA* (B) gene fusions in various *opg* and *rcs* mutant strains of *D. dadantii*. Bacteria were grown in LB without NaCl at 30°C until mid-log phase and broken by passing through a French press cell, and β-glucuronidase activity was measured with PNPU as a substrate (see Materials and Methods). Results reported are the average of 3 independent experiments. Specific activity is expressed as the change in optical density at 410 nm (ΔOD_{410}) per min and per mg of protein.

encode regulators activating expression of the flagellar apparatus genes. An *flhD-uidA* transcriptional fusion was constructed and introduced in the various *opgG* and *rcs* single and double mutant strains, and its expression was evaluated. In the four strains harboring the different *rcs* alleles (the *rcsC2*, *rcsC*, *rcsB*, and Δ*rcsCDB* mutants), expression of the *flhD-uidA* fusion that was 2-fold higher than that of the wild-type strain was observed (Fig. 3A), indicating that the expression of *flhDC* is repressed by activation of the RcsCD-RcsB phosphorelay in *D. dadantii*. In the *opgG rcsC2*, *opgG rcsC*, *opgG rcsB*, and *opgG* Δ*rcsCBD* double mutant strains, expression of the *flhD-uidA* fusion that was 3- to 4-fold higher than that of the *opgG* strain was observed (Fig. 3A). Repression of the *flhDC* operon was severely diminished in the *opgG* strains harboring the *rcs* mutations. To analyze the regulation of other genes of the Rcs regulon in the absence of OPGs, an *ftsA-uidA* transcriptional fusion was constructed and introduced in the various *opgG* and *rcs* single and double mutant strains, and its expression was evaluated. The *ftsAZ* operon, needed for cell division, is activated by the RcsCD-RcsB phosphorelay (8). The *ftsAZ* operon is transcribed by several promoters, one of them, activated by the RcsB regulator, was cloned in the *ftsA-uidA* fusion. In the four strains harboring the different *rcs* alleles inactivating the RcsCD-RcsB phosphorelay, a low-level expression of the *ftsA-uidA* fusion in the different *rcs* strains was observed (Fig. 3B), indicating that the *ftsAZ* operon is positively regulated by activation of the RcsCD-RcsB phosphorelay in *D. dadantii*. The *ftsA-uidA* expression of the *opgG* strain was 13- to 14-fold higher than in the *opgG rcsC2*, *opgG rcsC*, *opgG rcsB*, and *opgG* Δ*rcsCBD* double mutant strains (Fig. 3B). Thus, activation was diminished in the *opgG* strains harboring the different *rcs* mutations. These results indicate an increased activation level of the RcsCD-RcsB phosphorelay in the *opgG* mutant strain. Thus, OPGs regulate genes under the control of the Rcs phosphorelay system.

The *rcsC2* allele is dominant over the wild-type allele of *rcsC*. The simplest explanation for the observed phenotype of the *rcsC2* allele is that this mutant gene encodes a mutant protein decreasing the kinase activity or increasing the phosphatase activity of the RcsCD-RcsB phosphorelay. These two possibilities could be distinguished genetically by phenotypic analysis of a merodiploid strain harboring both the wild-type *rcsC* allele and the *rcsC2* one. If the kinase activity is decreased, the *rcsC2* mutation will be recessive, and if the phosphatase activity is increased, the *rcsC2* mutation will be, at least in part, dominant. The *rcsCBD* locus, cloned into a minitransposon Tn*5*, was introduced in the *opgG rcsC2* double mutant strain. Two merodiploid clones, isolated from independent transposon mutagenesis, were tested, and both present similar phenotypes. Motility of the merodiploid strain (NFB3859) was restored, but the halo diameter was 25% lower than that in the parental *opgG rcsC2* strain (NFB3811). The expression of the *ftsA-uidA* fusion in the NFB3859 strain showed an intermediate level between the NFB3810 (*opgG*) and the NFB3811 (*opgG rcsC2*) strains (Fig. 3B). Maceration occurred in 15/30 and in 10/10 potato tubers inoculated with, respectively, 10^7 and 10^8 bacteria of the merodiploid strain. Similar results were observed when the *rcsC2* mutation was located on the transposon. A merodiploid for the wild-type *rcsCBD* locus in an *opgG* strain displayed nonvirulent and mucoid phenotypes. Taken together, these results indicate that wild-type *rcsC* and *rcsC2* alleles are codominant and suggest that the *rcsC2* mutation increases the phosphatase activity of the RcsCD-RcsB phosphorelay. However, the *rcsC* null mutation and the *rcsC2* mutation display similar phenotypes because both of them lower RcsB phosphorylation.

DISCUSSION

In this paper, we characterized a suppressor of the Opg phenotype in *D. dadantii* lying within the *rcsC* gene. The RcsCD-RcsB phosphorelay was first identified in *E. coli* as a positive regulator of exopolysaccharide synthesis (27). Further

studies demonstrated the role of this phosphorelay in regulation of motility, cell division, and virulence in various enterobacterial species, such as *E. coli*, *Salmonella enterica*, and *P. carotovorum* (2, 8, 19, 20, 28, 31). In *D. dadantii*, this phosphorelay also controls these cellular processes.

Restoration of virulence in the *opgG rcs* double mutant strains occurs only on potato tubers, despite the restoration of essential virulence factors, such as motility and exoenzyme secretion. Infection of chicory leaves by *D. dadantii* is a more demanding process than infection of potato tubers. Absence of restoration of virulence in chicory leaves may be explained in part by a more efficient defense host response in chicory leaves than in potato tubers. In addition, absence of restoration of virulence in chicory leaves may be explained by an inappropriate expression of additional bacterial virulence genes needed for infection in chicory leaves remaining in strains devoid of OPGs. This suggests that this wide-host-range phytopathogenic bacterium requires expression of different sets of genes to achieve virulence, depending on the colonized host.

The *rcsC2* mutation is dominant and results in constitutive, low-level activation of the phosphorelay compatible with constitutive phosphatase activity of RcsC2. This is in agreement with the low level of RcsCD-RcsB activation needed for full virulence in *S. enterica* (14, 20, 31) and with the restoration of virulence in *D. dadantii* for *opgG* strains harboring the null mutation *rcsC* or *rcsB*. Three dominant mutations were isolated in the *barA* gene encoding the sensor protein of the BarA-UvrY phosphorelay system of *E. coli*. Like the *rcsC2* mutation, these mutations lie within the linker domain of BarA located just upstream of the histidine kinase domain and harbor constitutive phosphatase activity (38). Constitutive kinase activity-dominant mutations were isolated in the same region of the *rcsC* gene of *S. enterica* (20). These mutations suggest that this domain is important for sensor proteins to finely tune the phosphorylated level of regulators.

Isolation of the suppressor of the Opg phenotype found in the *envZ-ompR* locus in *E. coli* (17) or in the *pigX* gene in *Serratia* sp., encoding a cyclic dimeric GMP phosphodiesterase (18), the alteration of the proteome in an *opgG* mutant strain (6), and the fact that the RcsCD-RcsB phosphorelay is specific to enterobacteria (16, 27) could not be explained only by inaccurate expression of the RcsCD-RcsB phosphorelay-regulated genes. Expression of additional genes, whose expression is independent of this phosphorelay, may be affected in *opg* mutant strains. The requirement of several phosphorelay systems and the requirement of periplasmic glucans in virulence are now well established (5). The relationship between OPGs and the RcsCD-RcsB phosphorelay was shown for the regulation of colanic acid capsular polysaccharide synthesis in *E. coli* (15), but to our knowledge, this is the first study connecting these glucans and a phosphorelay system with virulence.

The constitutive activation of the RcsCD-RcsB phosphorelay could be the result of the alteration of envelope integrity in *opg* strains (6, 10, 32). This hypothesis cannot be excluded, but in this case, it is surprising that in the various *rcs opgG* double mutant strains virulence is restored and that no additional deleterious phenotypes are associated with this mutant strain. The relationship between OPGs and the RcsCD-RcsB phosphorelay may be direct or indirect and remains to be elucidated. One of the stimuli activating the RcsCD-RcsB phosphorelay is an upshift in osmolarity (15, 41). These are physiological conditions under which the OPG level is low, since OPG levels decrease as the medium osmolarity increases (5). Thus, one can imagine that variation of the OPG level in the periplasm modulates the RcsCD-RcsB phosphorelay activation. This modulation could occur indirectly via the IgaA protein, an attenuator of RcsCD-RcsB phosphorelay activation (14), since an *igaA* mutant and an *opgG* mutant display similar phenotypes. This hypothesis will be further investigated.

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REFERENCES

1. **Adelberg, E. A., M. Mandel, and G. Chein Ching Chen.** 1965. Optimal conditions for mutagenesis by *N*-methyl-*N′*-nitroso-*N*-nitrosoguanidine in *Escherichia coli* K12. Biochem. Biophys. Res. Commun. **18:**788–795.
2. **Andresen, L., V. Koiv, T. Alamae, and A. Mae.** 2007. The Rcs phosphorelay modulates the expression of plant cell wall degrading enzymes and virulence in *Pectobacterium carotovorum* ssp. *carotovorum*. FEMS Microbiol. Lett. **273:**229–238.
3. **Bardonnet, N., and C. Blanco.** 1992. *′uidA*-antibiotic-resistance cassettes for insertion mutagenesis, gene fusions and genetic constructions. FEMS Microbiol. Lett. **72:**243–247.
4. **Bhagwat, A. A., W. Jun, L. Liu, P. Kannan, M. Dharne, B. Pheh, B. D. Tall, M. H. Kothary, K. C. Gross, S. Angle, J. Meng, and A. Smith.** 2009. Osmoregulated periplasmic glucans of *Salmonella enterica* serovar Typhimurium are required for optimal virulence in mice. Microbiology **155:**229–237.
5. **Bohin, J.-P., and J.-M. Lacroix.** 2007. Osmoregulation in the periplasm, p. 325–341. *In* M. Ehrmann (ed.), The periplasm. ASM Press, Washington, DC.
6. **Bouchart, F., A. Delangle, J. Lemoine, J.-P. Bohin, and J.-M. Lacroix.** 2007. Proteomic analysis of a non virulent mutant of the phytopathogenic bacterium *Erwinia chrysanthemi* deficient in osmoregulated periplasmic glucans: change in protein expression is not restricted to the envelope, but affects general metabolism. Microbiology **153:**760–767.
7. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
8. **Carballès, F., C. Bertrand, J.-P. Bouché, and K. Cam.** 1999. Regulation of *Escherichia coli* cell division genes *ftsA* and *ftsZ* by the two-component system *rcsC-rcsB*. Mol. Microbiol. **34:**442–450.
9. **Cherepanov, P. P., and W. Wackernagel.** 1995. Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene **158:**9–14.
10. **Clavel, T., J.-C. Lazzaroni, A. Vianney, and R. Portalier.** 1996. Expression of the *tolQRA* genes of *Escherichia coli* K12 is controlled by the RcsC sensor protein involved in capsule synthesis. Mol. Microbiol. **19:**19–25.
11. **Cogez, V., P. Talaga, J. Lemoine, and J.-P. Bohin.** 2001. Osmoregulated periplasmic glucans of *Erwinia chrysanthemi*. J. Bacteriol. **183:**3127–3133.
12. **Delangle, A., A.-F. Prouvost, V. Cogez, J.-P. Bohin, J.-M. Lacroix, and N. Hugouvieux-Cotte-Pattat.** 2007. Characterization of the *Erwinia chrysanthemi gan* locus, involved in galactan catabolism. J. Bacteriol. **189:**7053–7061.
13. **de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. **172:**6568–6572.
14. **Dominguez-Bernal, G., M. G. Pucciarelli, F. Ramos-Morales, M. Garcia-Quintallina, D. A. Cano, J. Casadesus, and F. Garcia del Portillo.** 2004. Repression of the RcsC-YojN-RcsB phosphorelay by the IgaA protein is a requisite for *Salmonella* virulence. Mol. Microbiol. **53:**1437–1449.
15. **Ebel, W., G. J. Vaughn, H. K. Peters III, and J. E. Trempy.** 1997. Inactivation of *mdoH* leads to increased expression of colanic acid capsular polysaccharide in *Escherichia coli*. J. Bacteriol. **179:**6858–6861.
16. **Erickson, K. D., and C. S. Detweiler.** 2006. The Rcs phosphorelay system is specific to enteric pathogens/commensals and activates *ydeI*, a gene important for persistent *Salmonella* infection of mice. Mol. Microbiol. **62:**883–894.
17. **Fiedler, W., and H. Roterings.** 1988. Properties of *Escherichia coli* mu-

tants lacking membrane-derived oligosaccharides. J. Biol. Chem. **263:** 14684–14689.
18. **Fineran, P. C., N. R. Williamson, K. S. Lilley, and G. P. C. Salmond.** 2007. Virulence and prodigiosin antibiotic biosynthesis in *Serratia* are regulated pleiotropically by the GGDEF/EAL domain protein, PigX. J. Bacteriol. **189:**7653–7662.
19. **Francez-Charlot, A., B. Laugel, A. Van Gemert, N. Dubarry, F. Wiorowski, M. P. Castanié-Cornet, C. Gutierrez, and K. Cam.** 2003. RcsCDB His-Asp phosphorelay system negatively regulates the *flhDC* operon in *Escherichia coli*. Mol. Microbiol. **49:**823–832.
20. **García-Calderon, C. B., M. García-Quintanilla, J. Casadesus, and F. Ramos-Morales.** 2005. Virulence attenuation in *Salmonella enterica rcsC* mutants with constitutive activation of the Rcs system. Microbiology **151:** 579–588.
21. **Herrero, M., V. De Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. **172:**6557–6567.
22. **Hugouvieux-Cotte-Pattat, N., G. Condemine, W. Nasser, and S. Reverchon.** 1996. Regulation of pectinolysis in *Erwinia chrysanthemi*. Annu. Rev. Microbiol. **50:**213–257.
23. **Huguet, E., K. Hahn, K. Wengelnik, and U. Bonas.** 1998. hpaA mutants of *Xanthomonas campestris pv. vesicatoria* are affected in pathogenicity but retain the ability to induce host-specific hypersensitive reaction. Mol. Microbiol. **29:**1379–1390.
24. **Lojkowska, E., C. Masclaux, M. Boccara, J. Robert-Baudouy, and N. Hugouvieux-Cotte-Pattat.** 1995. Characterization of the *pelL* gene encoding a novel pectate lyase of *Erwinia chrysanthemi* 3937. Mol. Microbiol. **16:**1183–1195.
25. **Loubens, I., L. Debarbieux, A. Bohin, J.-M. Lacroix, and J.-P. Bohin.** 1993. Homology between a genetic locus (*mdoA*) involved in the osmoregulated biosynthesis of periplasmic glucans in *Escherichia coli* and a genetic locus (*hrpM*) controlling pathogenicity of *Pseudomonas syringae*. Mol. Microbiol. **10:**329–340.
26. **Mahajan-Miklos, S., M.-W. Tan, L. G. Rahme, and F. M. Ausubel.** 1999. Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa-Caenorhabditis elegans* pathogenesis model. Cell **96:**47–56.
27. **Majdalani, N., and S. Gottesman.** 2005. The Rcs phosphorelay: a complex signal transduction system. Annu. Rev. Microbiol. **59:**379–405.
28. **Mariscotti, J. F., and F. García-del Portillo.** 2009. Genome expression analyses revealing the modulation of the *Salmonella* Rcs regulon by the attenuator IgaA. J. Bacteriol. **191:**1855–1867.
29. **Miller, J. H.** 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
30. **Miller, V. L., and J. J. Mekalanos.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
31. **Mouslim, C., M. Delgado, and E. A. Groisman.** 2004. Activation of the RcsC/YojN/RcsB phosphorelay system attenuates *Salmonella* virulence. Mol. Microbiol. **54:**386–395.
32. **Page, F., S. Altabe, N. Hugouvieux-Cotte-Pattat, J.-M. Lacroix, J. Robert-Baudouy, and J.-P. Bohin.** 2001. Osmoregulated periplasmic glucan synthesis is required for *Erwinia chrysanthemi* pathogenicity. J. Bacteriol. **183:**3134–3141.
33. **Perombelon, M., and A. Kelman.** 1980. Ecology of the soft rot *Erwinia*. Annu. Rev. Phytopathol. **18:**361–387.
34. **Resibois, A., M. Colet, M. Faelen, T. Schoonejans, and A. Toussaint.** 1984. Phi-EC2, a new generalized transducing phage of *Erwinia chrysanthemi*. Virology **137:**102–112.
35. **Roeder, D. L., and A. Collmer.** 1985. Marker-exchange mutagenesis of a pectate lyase isozyme gene in *Erwinia chrysanthemi*. J. Bacteriol. **164:**51–56.
36. **Sambrook, J. E., F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
37. **Simon, R., J. Quandt, and W. Klipp.** 1989. New derivatives of transposon Tn*5* suitable for mobilization of replicons, generation of operons fusions and induction of genes in Gram-negative bacteria. Gene **80:**161–169.
38. **Tomenius, H., A.-K. Pernestig, C. F. Mendez-Catala, D. Georgellis, S. Normark, and O. Melefors.** 2005. Genetic and functional characterization of the *Escherichia coli* BarA-UvrY two-component system: point mutations in the HAMP linker of the BarA sensor give a dominant-negative phenotype. J. Bacteriol. **187:**7317–7324.
39. **Wu, T. T.** 1966. A model for a three point analysis of random general transduction. Genetics **54:**405–410.
40. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mpl8 and pUC19 vectors. Gene **33:**103–119.
41. **Zhou, L., X. H. Lei, B. R. Bochner, and B. L. Wanner.** 2003. Phenotype microarray analysis of *Escherichia coli* K-12 mutants with deletions of all two-component systems. J. Bacteriol. **185:**4956–4972.