The EnvZ-OmpR Two-Component Signaling System Is Inactivated In A Mutant Devoid Of Osmoregulated Periplasmic Glucans In *Dickeya dadantii*

Caby Marine[1], Sébastien Bontemps-Gallo[1‡], Peggy Gruau[1], Brigitte Delrue[2], Edwige Madec[1], Jean-Marie Lacroix[1*]

[1] Unité de Glycobiologie Structurale et Fonctionnelle, UMR CNRS 8576, Université des Sciences et Technologies de Lille, Université de Lille, F-59655 Villeneuve d'Ascq, France

[2] Université de Lille, F-59655 Villeneuve d'Ascq, France

*corresponding author.

E-mail: jean-marie.lacroix@univ-lille.fr.

[‡]present address: Center for Infection and Immunity of Lille, U1019-UMR8204, Institut Pasteur de Lille, Inserm, CNRS, Université de Lille, France.

Deleted: [‡]present address : Laboratory of Bacteriology, National Institute of Allergy and Infectious Diseases, National Institute of Health, Hamilton, MT, United States of America.¶

Running title: Relationship EnvZ/OmpR & OPG

Number of Figures: 8

Number of words: 5,145

Deleted: 5545

Keywords

EnvZ/OmpR, osmoregulated periplasmic glucans, osmotic stress, plant pathogen, *D. dadantii*

Abstract

Osmoregulated periplasmic glucans (OPGs) are general constituents of alpha-, beta- and gamma-Proteobacteria. This polymer of glucose is required for full virulence of many pathogens including *Dickeya dadantii*. The phytopathogenic enterobacterium *D. dadantii* causes soft-rot disease in a wide range of plants. An OPG-defective mutant is impaired in environment sensing. We previously demonstrated that i) fluctuation of OPG concentration controlled the activation level of the RcsCDB system, and ii) RcsCDB, along with EnvZ/OmpR controlled the mechanism of OPG succinylation. These previous data lead us to explore whether OPGs are required for other two-component systems. In this study, we demonstrate that inactivation of the EnvZ/OmpR system in an OPG-defective mutant restores full synthesis of pectinase but only partial virulence. Unlike for the RcsCDB system, the EnvZ-OmpR system is not controlled by OPG concentration but requires OPGs for proper activation.

Deleted: phytopathogen

Deleted: on

Deleted: plant

Deleted:

Deleted: previously

Deleted: controlled

Deleted: mechanism

Deleted: the

Deleted: system

Deleted: demonstrated

Deleted: restored

Deleted: partially

Deleted: the

Deleted: required

Introduction

Osmoregulated periplasmic glucans (OPGs), β-D-glucans oligosaccharides, are major envelope components found in the periplasm of almost all proteobacteria. Their concentration increases as the osmolarity of the medium decreases (Kennedy, 1996; Bohin and Lacroix, 2006; Bontemps-Gallo et al., 2017). In enterobacteria, the gene products of the *opgGH* operon synthesize the OPG glucose backbone, which is composed of 5-12 glucose units joined by β,1-2 linkages and branched by β,1-6 linkages. *opgG* and *opgH* mutant strains are completely devoid of OPGs (Bontemps-Gallo et al., 2017). These glucans are well described as virulence factors of animal and plant pathogens including *Dickeya dadantii* (Bontemps-Gallo and Lacroix, 2015).

D. dadantii, the agent of soft rot disease, is directly responsible for 5 to 25% of potato crop loss in Europe and Israel (Toth et al., 2011). This phytopathogen is listed as an A2 quarantine organism by the European and Mediterranean Plant Protection Organization (EPPO, 1982; 1988; 1990). Maceration is the result of the synthesis and secretion of plant cell wall-degrading enzymes (PCWDEs), in particular, pectinases (Collmer and Keen, 1986). However, additional factors, such as motility, are required for full virulence (Charkowski et al., 2012; Reverchon and Nasser, 2013; Leonard et al., 2017). During infection, *D. dadantii* must overcome several stresses including osmotic stress. Previous studies suggest that bacteria encounter hypoosmotic stress at the early stage of infection and hyperosmotic stress later due to plant maceration (Reverchon and Nasser, 2013; Jiang et al., 2016; Reverchon et al., 2016).

In our model, OPG concentration dramatically increases during the first hour of infection (Bontemps-Gallo et al., 2013). Mutants devoid of OPGs show a pleiotropic phenotype including a loss of motility, decreased synthesis and secretion of PCWDEs, increased synthesis of exopolysaccharide, induction of a general stress response and complete loss of virulence on potato tubers or chicory leaves (Page et al., 2001; Bouchart et al., 2007). These phenotypes suggest that strains lacking OPGs are impaired in the sensing of their environment. Previously, our laboratory demonstrated a strong relationship between OPGs and the RcsCDB two-component system.

Two-component systems are key regulators of gene expression plasticity in response to environmental changes. Under stimuli, often unknown, a transmembrane sensor histidine kinase (HK) autophosphorylates on a histidine residue. This phosphate group is subsequently transferred to an aspartate residue on a cognate cytoplasmic response regulator (RR), which in turn regulates the expression of a set of target genes (Hoch, 2000; Groisman, 2016).

Deleted: of the envelope
Deleted: periplasm. The
Deleted: products
Deleted: to be a
Deleted: for
Deleted: pathogen
Deleted: the
Deleted: from
Deleted: the losses of
Deleted: crops
Deleted: a
Deleted: a set of
Deleted: particularly
Deleted: , but many
Deleted: like the motility
Deleted: the
Deleted: has to
Deleted: first a
Deleted: prior a
Deleted: OPGs
Deleted: increased
Deleted: the
Deleted: devoid of
Deleted: perception
Deleted: Last decade
Deleted: the
Deleted: the
Deleted: , transfers, directly or not, this
Deleted: of its
Deleted:)
Deleted: of its regulon

Inactivation of the RcsCDB system in an OPG-defective mutant restores several of the *D. dadantii* wild-type phenotypes (motility, mucoidy, virulence) (Bouchart et al., 2010), indicating that OPGs are involved in the perception of environmental changes. We have also shown that RcsCDB and OPG are tightly connected: i) fluctuation of OPG concentration controls the activation level of the RcsCDB system (Bontemps-Gallo et al., 2013), ii) RcsCDB, along with the two-component system EnvZ/OmpR, controls the mechanism of OPG succinylation (Bontemps-Gallo et al., 2016). These facts lead us to wonder whether the link between OPGs and the RcsCDB system is a unique feature.

Thirty years ago, Fiedler and Rotering isolated revertants in OPG-defective mutants of *E. coli* (Fiedler and Rotering, 1988). The mutation was localized to the *ompB* locus now known as the *envZ-ompR* operon. EnvZ-OmpR, the paradigm of two-component systems, regulates the balance between OmpF (large pore diameter) and OmpC (small pore diameter) to control the diffusion rate of nutrients (Cowan et al., 1992; Forst and Roberts, 1994; Egger et al., 1997; Castillo-Keller et al., 2006; Barbieri et al., 2013). This system is also known to control motility in several bacteria (Barker et al., 2004; Clemmer and Rather, 2007; Raczkowska et al., 2011; Lee and Park, 2013; Li et al., 2014; Tipton and Rather, 2016; Pruss, 2017) and is required for full virulence in *Yersinia pestis* (Gao et al., 2011; Reboul et al., 2014). In *D. dadantii*, the EnvZ/OmpR system regulates *ompF* expression (no *ompC* homolog is present) but also *kdgN*, which is required for transport of oligosaccharides arising from pectin degradation during plant infection (Condemine and Ghazi, 2007). Recently, in a global *in vitro* transcriptomic analysis of various stresses encountered during the infectious process, Jiang *et al.* showed that the EnvZ-OmpR system was up-regulated during osmotic stress (Jiang et al., 2016).

In this study, we demonstrate that EnvZ-OmpR system is not involved in virulence. Instead, inactivation of *envZ* or *ompR* in an OPG-defective mutant restores full synthesis of pectinase and partial virulence. We also show that EnvZ-OmpR is involved in regulation of motility. Finally, we demonstrate that *ompF* and *kdgN* are osmoregulated by EnvZ-OmpR and are required for proper regulation of OPGs.

Materials and Methods

Bacterial strains, media and growth conditions

Bacterial strains are described in Table 1. Bacteria were grown at 30°C in lysogeny broth (LB) (Bertani, 2004), or in minimal medium M63 glycerol (15mM $(NH_4)_2SO_4$, 1.8µM $FeSO_4$, 1mM $MgSO_4$ and 100mM K_2PHO_4) supplemented with 0.2% glycerol as a carbon

Deleted: The inactivation
Deleted: ,
Deleted:
Deleted: demonstrated
Deleted: controlled
Deleted: controlled
Deleted: mechanism
Deleted: All these
Deleted: the
Deleted: In *E. coli*
Deleted:
Deleted: thirty years ago
Deleted: in
Deleted: now
Deleted: the
Deleted: system, is described to regulate
Deleted: rate
Deleted: resulting
Deleted: pectins
Deleted: encounter
Deleted: show
Deleted: during osmotic stress
Deleted: demonstrated
Deleted: was
Deleted: but
Deleted: restored
Deleted: partially the
Deleted: showed
Deleted: was
Deleted: motility
Deleted: demonstrated
Deleted: were
Deleted: to be properly regulated

source (Miller, 1992). Solid media were obtained by adding agar at 15 $g.L^{-1}$. Motility tests were performed on LB plates containing agar at 4 $g.L^{-1}$.

Osmolarity (mOsM) was measured with a vapor pressure osmometer (Advanced Instruments, USA). M63 osmolarity was 330 mOsM. Osmolarity was decreased by diluting two-fold M63 with H_2O to 170 mOsM. Addition of 0.1M and 0.2M NaCl increased the osmolarity to 500 and 700 mOsM, respectively. Glycerol was added after dilution with water or addition of NaCl.

The solid media used to test the pectinase (M63 supplemented with 0.4% polygalacturonate (PGA) and 0.2% glycerol), cellulases (M63 supplemented with 0.2% carboxymethycellulose (CMC), 0.2% glycerol and 7mM $MgSO_4$) and proteases (LB complemented with 1% of Fat milk) activities have been described previously (Page et al., 2001).

Antibiotics were used at following concentrations: spectinomycin, 2.5µg.mL^{-1}; chloramphenicol, 12.5µg.mL^{-1} and gentamycin, 2µg.mL^{-1}.

Transduction, conjugation and transformation.

Construction of strains was performed by transferring genes from one strain of *D. dadantii* to another by generalized transduction with phage ΦEC2 as described previously (Resibois et al., 1984). Plasmids were introduced in *D. dadantii* by conjugation or electroporation.

Expression analysis

Bacteria were grown up to exponential phase at various osmolarities. RNAs were extracted using Nucleospin RNA Plus Kit (Macherey Nagel) following the manufacturer's instruction. RNAs were treated with DNase I (BioLabs). RNA qualities were checked by gel and nanodrop.

cDNAs were retrotranscribed using the Superscript IV First-Strand Synthesis (Invitrogen) according to the manufacturer's instruction.

qPCR was performed using SYBR method as described previously by Hommais *et al.* (Hommais et al., 2011). Primers used are listed in Table 2. *ipxC*, an UDP-N-acetylglycosamine deacetylase, was used as a reference gene (Hommais et al., 2011).

Phenotypic evaluation

Deleted: made

Deleted: in media

Formatted: Justified

Moved down [1]: Swim diameters were measured after 48 hours of incubation.¶
¶

Deleted: ¶
Motility assay¶
10^7 bacteria in 5µL were spotted into the motility plate, incubated at 30°C.

Deleted: **Proteases, cellulases and proteases assays** ¶
10^7 bacteria in 5µL were spotted onto pectinase (PGA), cellulase (CMC) or protease plate.

Deleted: indication of pectinase production. After 48h incubation, CMC plates were flooded with a 1mg/ml red Congo solution and washed several time with 1M NaCl, allowing the formation of a red complex with the CMC. Diameters of the clear haloes around the colony were measured as indication of cellulase production.

Moved down [2]: After 48h incubation, PGA plates were flooded with a 10% copper acetate solution, which forms a blue complex with the PGA. Diameters of the clear haloes around the colony were measured as

Moved down [3]: After 48h incubation, abilities of the strain to degrade milk protein were observed.

Deleted: ¶

10^7 bacteria in 5µL were spotted onto pectinase (PGA), cellulase (CMC), protease or motility plates. After 48h incubation, PGA plates were flooded with a 10% copper acetate solution, which forms a blue complex with the PGA. Diameters of the clear haloes around the colony were measured as an indication of pectinase production. After 48h incubation, CMC plates were flooded with a 1mg/ml red Congo solution and washed several time with 1M NaCl, allowing formation of a red complex with the CMC. Diameters of the clear haloes around the colony were measured as an indication of cellulase production. After 48h incubation, abilities of the strain to degrade milk protein were observed. Swim diameters were measured after 48 hours of incubation.

Moved (insertion) [2]

Moved (insertion) [3]

Moved (insertion) [1]

Formatted: Left

Pathogenicity test.

Potato tubers and chicory leaves were inoculated as previously described (Page et al., 2001). Bacteria from an overnight culture in LB medium were recovered by centrifugation and diluted in water. For potato tubers, sterile pipette tips containing a bacterial suspension of 10^7 cells in 5µL were inserted into the tuber (Amandine variety). After 72h of incubation in a dew chamber, tubers were sliced vertically through the inoculation point, and the weight of the maceration was measured. For chicory leaves, leaves were wounded prior inoculation of 10^7 bacteria and incubated in a dew chamber at 30°C until 48h.

Transmission electron microscopy

Samples were analyzed by the Bio Imaging Center of University of Lille (France). Wild-type and *opgG* strains were grown until mid-log phase. Cells were spun for 5 min at 7,000 x g at 4°C. Bacteria were fixed with 3.125% glutaraldehyde, washed in 0.1M phosphate buffer pH 7.4 and postfixed with 1% OsO_4. Samples were dehydrated with graded acetone series, embedding in EMBED resin, and air dried at 60°C. Thin and ultrathin sections were prepared using an ultramicrotome (Reichert OM U3) or an ultramicrotome (LKB Ultrotome III 8800) and stained with uranyl acetate. Microscopy was performed with a Hitachi H600 microscope at 75keV electron energy. The periplasm length was measured using ImageJ software.

Deleted: The samples

Deleted: strain

Statistical Analysis

For statistical analyses, Graph-prism6 software was used to analyze data using One-Way ANOVA.

Results

Characterization of *envZ* and *ompR* deletion in wild-type and *opgG* background

To determine whether the EnvZ-OmpR system interacts with OPG, we inactivated *envZ* or *ompR* in wild-type and *opgG* mutant backgrounds (Figure 1A). We then looked at *envZ* and *ompR* expression at various osmolarities (Figure 1B). As expected, expression of both genes was low in wild-type and not affected by osmolarity. In an *opgG* mutant, the expression level was similar to that observed in the wild-type strain. No expression of *envZ* or *ompR* was observed in their respective mutant strain. Interestingly, in the *ompR* background, a low but measurable expression of *envZ* was observed. Based on the locus organization, we would expect the *ompR* mutation to be polar. Expression of *envZ* in an *ompR* deletion background suggests the presence of a secondary promoter.

Inactivation of *envZ* or *ompR* restores the synthesis of pectinase in an OPG-defective strain

Strains devoid of OPGs are impaired in their ability to synthesize virulence factors, leading to total loss of virulence. We first assayed plant cell-degrading enzyme activity (Figure 2, Supplementary Figure 1), which is required for full virulence. Pectinase production and secretion were evaluated on minimal medium containing polygalacturonate, a substrate for pectinase, and, after 48h of incubation, haloes of degradation were measured (Figure 2A). As expected, the *opgG* mutant showed a 40% decrease in pectinase production compared to the wild-type. While inactivation of *envZ* or *ompR* did not decrease synthesis of pectinases, *envZ opgG* and *ompR opgG* double mutants showed full restoration of pectinase production to levels similar to wild-type.

Cellulase production and secretion were evaluated on minimal medium containing carboxymethylcellulose, the substrate for cellulase, and haloes of degradation were measured after 48 h of incubation (Figure 2B). As previously shown, *opgG* inactivation decreased production of cellulase by 30% (Page et al., 2001). *envZ* or *ompR* null mutants exhibited similar cellulase levels as the wild-type. *envZ opgG* and *ompR opgG* double mutants displayed a reduction in cellulase production similar to the *opgG* strain.

We also assayed for production of protease on plates containing 1% milk fat (Table 3). The ability of each strain to degrade milk protein was evaluated after 48 h. No restoration of protease activity was observed in any of the double-mutant strains.

Taken together, our data show that EnvZ-OmpR is not involved in regulation of PCWDEs. However, disruption of either *envZ* or *ompR* is enough to restore full pectinase production in an OPG-defective strain, but not cellulase or protease synthesis.

Deleted: delection
Deleted: In order to
Deleted: interact
Deleted: ,
Deleted: in wild-type and *opgG* background
Deleted: the
Deleted: as
Deleted: relative
Deleted: strains
Deleted: thought that
Deleted: will
Deleted: The expression
Formatted ... [1]
Deleted: restored
Deleted: OPG-
Deleted: strains
Deleted: the
Deleted: the
Deleted:)
Deleted: plates with
Deleted: : polygalacturonate. After
Deleted: the
Deleted: decreased of
Deleted: of the pectinases
Deleted: the
Deleted: a
Deleted: the pectinases at a
Deleted: level than the
Deleted: plates with
Deleted: : carboxymethylcellulose. After 48h of incubation, the
Deleted: by 30% the
Deleted: as showed previously
Deleted: a
Deleted: level of
Deleted: than
Deleted: of
Deleted: like
Deleted: the
Deleted: plate
Deleted: of Fat
Deleted: After 48h of incubation, abilities
Deleted: were
Deleted: activities
Deleted:
Deleted: showed
Deleted: PCWDEs
Deleted: was
Deleted:

The EnvZ-OmpR system is involved in motility regulation

Motility is known to be an important virulence factor (Reverchon and Nasser, 2013). Furthermore, by screening motility in OPG-defective mutants of *E. coli*, Fiedler and Rotering isolated revertants in the *envZ-ompR* operon (Fiedler and Rotering, 1988). To determine whether disruption of *envZ-ompR* could restore the loss of motility in the *opgG* mutant, we assayed for motility by measuring swim diameters on 0.4% agar plates (Figure 3A, Supplementary Figure 1). As described previously, the *opgG* mutant showed a reduction in motility (one third of wild-type levels). Inactivation of *envZ* or *ompR* resulted in a 40% reduction in motility compared to the wild-type strain. However, the same mutation in the *opgG* background did not restore motility.

The regulatory cascade for motility is separated into three classes of promoter (Figure 3B). Under motility-inducing conditions, *flhDC*, the master regulator, is up-regulated to modulate expression of genes under the control of a class II promoter. Finally, class II genes regulate genes with class III promoters (*e.g. fliC*, the flagellin). We next tested the effect of the EnvZ-OmpR system on regulation of motility. In wild-type background, expression of the master regulator *flhD*, and consequently *fliC*, decreased ten-fold from low (170 mOsM) to high (700 mOsM) osmolarity (Figure 3C, D). This data agrees with our previous observation of a two-fold decrease in wild-type motility in the same osmolarity range (Bontemps-Gallo et al., 2013). Inactivation of *envZ* or *ompR* lead to a decrease in *flhD* expression, but, save for 170 mOsM, this decrease was not statistically significant (Figure 3C). *fliC* expression decreased 1.5-fold at 170 and 330 mOsM in the *envZ* and *ompR* mutants compared to the wild-type (Figure 3C, D). Disruption of *opgG* resulted in low expression of both *flhD* and *fliC* regardless of the genetic background and osmolarity (Figure 3C, D). Our results show that EnvZ-OmpR are involved in the regulation of motility but not as a main regulator of this cascade. Inactivation of this system cannot rescue motility in the *opgG* background.

Inactivation of EnvZ-OmpR systems partially restores virulence in an OPG -defective strain

Previously, we demonstrated that restoration of pectinase production in an OPG-defective strain is enough to restore virulence in potato tubers but not in chicory leaves (Bontemps-Gallo et al., 2014). We observed that inactivation of the EnvZ-OmpR system in an *opgG* mutant lead to restoration of full pectinase synthesis (Figure 2A). We therefore determined whether inactivation of this system could restore virulence in both potato tubers

Deleted: The motility
Deleted: described
Deleted: in *E.*
Deleted: in OPG defective mutants by screening motility thirty years ago
Deleted: In order to
Deleted: the
Deleted: the
Deleted: motility.
Deleted: in the wild-type displayed a reduction of
Deleted: of the
Deleted: the
Deleted: of
Deleted: in
Deleted: class
Deleted: environmental
Deleted: gene
Deleted: with
Deleted: those
Deleted: gene
Deleted: promoter
Deleted: the motility
Deleted: . Expressions
Deleted: were decreasing
Deleted: osmolarities
Deleted: agreed
Deleted: of the
Deleted: ranged of
Deleted: leaded
Deleted: slight
Deleted: (
Deleted: except at 170 mOsM) of *flhD* expression
Deleted: However,
Deleted: of
Deleted: at 170 and 330 mOsM
Deleted: a
Deleted: osmolarities
Deleted: showed
Deleted: the
Deleted: the regulatory
Deleted: the
Deleted: **restored the**
Deleted: the
Deleted: of pectinase
Deleted:
Deleted: restore
Deleted: assayed if the
Deleted: the

(Figure 4, Supplementary Figure 1) and chicory leaves (Figure 5). Following inoculation of bacteria in both vegetables and incubation at 30°C, we analyzed virulence levels. Inactivation of *envZ* or *ompR* in a wild-type background had no effect on virulence levels regardless of the infection model used (Figures 4, 5). Interestingly, when the system was inactivated in an OPG-defective strain, macerations were observed in the tubers (Figure 4). However, severity of disease was not as strong as for the wild-type strain (only a third of the average maceration weight of the wild-type). No restoration of virulence was observed for *envZ opgG* or *ompR opgG* double mutants in chicory leaves (Figure 5). Our data demonstrate that EnvZ-OmpR is not involved in virulence in *D. dadantii*. Furthermore, restoration of pectinase synthesis in the double mutants allows for maceration but only in potato tubers.

Deleted: After
Deleted: the
Deleted: severity
Deleted: the
Deleted: or the severity of the virulence
Deleted: infectious
Deleted:
Deleted: the
Deleted: the
Deleted: the
Deleted: mutant
Deleted: demonstrated
Deleted: the
Deleted: the
Deleted: the pectate-lyase
Deleted: allowed the formation of
Deleted: a
Deleted: tuber
Deleted: were
Deleted: required
Deleted: to be regulated

ompF and *kdgN* are osmoregulated through EnvZ-OmpR and require OPG for regulation

In *D. dadantii*, EnvZ-OmpR regulates at least two genes involved in transport: *ompF* and *kdgN* (Condemine and Ghazi, 2007). KdgN transports oligosaccharides arising from pectin-mediated degradation during plant infection. OmpF is a porin with a pore diameter of 1.12 nm that allows non-specific import of hydrophilic metabolites of less than 600 Da. We analyzed expression of these two genes at 170, 330, 500 and 700 mOsM in a wild-type background (Figure 6A, B). Expression increased sixteen-fold for *ompF* and twenty-two-fold for *kdgN* between 170 and 330 mOsM. Subsequently, expression decreased two-fold for both genes between 330 and 500 mOsM, and two-fold for *ompF* when osmolarity increased to 700 mOsM. In *envZ* or *ompR* single mutants, regulation was completely lost showing that *ompF* and *kdgN* are part of the regulon (Figure 6A, B). Both genes followed a classic bell curve observed for gene regulation by EnvZ-OmpR in *E. coli* (Lan and Igo, 1998). Interestingly, in the *opgG* mutant, regulation was completely lost (Figure 6A, B). At 170 mOsM, the expression level of *ompF* or *kdgN* in the OPG-defective strain was at a similar level to wild-type, regardless of medium osmolarity. These data indicate that the EnvZ-OmpR system regulates expression of *ompF* and *kdgN* in an OPG-dependent manner.

Deleted: the
Deleted: the
Deleted: the
Deleted: Expressions
Deleted: Then
Deleted: only
Deleted: the
Deleted: regulated
Deleted: the
Deleted: The
Deleted:
Deleted: than at 170 mOsM for the
Deleted: the
Deleted: of the medium.
Deleted: regulate

OPGs are not required for the activation of the CpxAR two-component system

To show whether two-component system dysfunction is a general feature of bacteria lacking OPGs, we investigated the potential relationship between another two-component system and OPGs. Among the thirty-two two-component systems in *D. dadantii*, three systems are involved in sensing stress:RcsCDB, EnvZ-OmpR and CpxAR. CpxAR is involved in perception of envelope stress (Bontemps-Gallo et al., 2015). Inactivation of this system in an

Deleted: if
Deleted: systems
Deleted: OPG-devoid

opgG background does not restore any phenotype (Bontemps Gallo, 2013). CpxAR regulates *spy*, encoding for a periplasmic chaperon, and *degP*, a periplasmic protease (Bontemps-Gallo et al., 2015). As previously observed, expression of *spy* (Figure 6C) and *degP* (Figure 6D) were up-regulated in a *cpxA* background and down-regulated for *spy* or similar to wild-type for *degP* in a *cpxR* background (Figure 6C, D) (Bontemps-Gallo et al., 2015). Disruption of *opgG* does not affect the regulation of *spy* or *degP* by the CpxAR system (Figure 6C, D). Taken together, our data shows that OPGs have a specific relationship with certain two-component systems.

Periplasmic size is maintained in an OPG-defective mutant.

Periplasmic size is subject to fluctuations during osmotic stress (Bohin and Lacroix, 2006) and loss of OPGs, major periplasmic components representing up to 5% of the dry weight of a cell, could affect this size. Recently, Asmar *et al.* demonstrated that activation of two-component systems also relies on the distance between the two membranes (Asmar et al., 2017). To determine whether change in periplasm width may be one of the consequences of a lack of EnvZ-OmpR system activation in the *opgG* mutant, we grew bacteria until mid-log phase in low and high osmolarities and analyzed cell ultrastructure using transmission electron microscopy (Figure 7). At low osmolarity (Figure 7A, B), cells exhibited an altered cytoplasmic content with small dense granules being observed. Since poly-phosphate granules, often accumulated by *D. dadantii*, typically appear white by TEM (Ogawa et al., 2000; Ayraud et al., 2005; Stumpf and Foster, 2005), we suspect that the black granules are filled with ferrous poly-phosphates (Lechaire et al., 2002). This cytoplasmic modification had no effect on the growth of *D. dadantii*. At high osmolarity (Figure 7), the cell displayed a classic rod-shaped form. Despite the strong structural difference observed for bacteria grown in low and high osmolarities, no significant difference was observed in bacterial structure between the wild-type and the *opgG* mutant strains at any osmolarity. In addition, no relevant difference in periplasmic size was observed between the wild-type and the *opgG* mutant. Both strains displayed an equivalent periplasmic space: 23.99 nm +/- 3.26 for wild-type and 22.92 nm +/- 3.04 for the OPG-defective strain at low osmolarity and 22.23 nm +/- 3.21 for wild-type and 24.28 nm +/- 3.41 for the OPG-defective strain at high osmolarity (Figure 7E). This suggests that OPGs are not involved in control of periplasmic size. These periplasmic space measurements are similar to those observed by Asmar *et al.* for the closely related *E. coli* Enterobacterium in LB medium (around 350 mOsM) (Asmar et al., 2017). Taken together, the gene expression experiments and the microscopy observations strongly suggest that EnvZ-

Deleted: regulate

Deleted: before

Deleted: showed

Deleted: has

Deleted: particular

Deleted: was

Deleted:

Deleted: subjected

Deleted: fluctuation

Deleted: component

Deleted: demonstrate

Deleted: the

Deleted: the

Deleted: activation of the

Deleted: the cell

Deleted: by

Deleted: the cell ultrastructure

Deleted: the cell

Deleted: unusual

Deleted: aspect displaying a

Deleted: granule. Because

Deleted: appears classically

Deleted: on

Deleted: one can imagine

Deleted: by

Deleted: ,C,D

Deleted: rot

Deleted: in structure

Deleted: strain

Deleted: each

Deleted: could be measured

Deleted: the

Deleted:

Deleted: the

Deleted:

Deleted:) suggesting

Deleted: measurements of

Deleted: in

OmpR requires OPGs in the periplasm to be able to sense the osmolarity but that this sensing is not based on periplasmic size.

Increasing concentrations of OPGs do not affect the level of EnvZ-OmpR system activation

Previously, we demonstrated that the level of RcsCDB activation is controlled by the concentration of OPGs (Bontemps-Gallo et al., 2013). Therefore, we examined whether the concentration of OPGs could also modulate the level of EnvZ-OmpR activation (Figure 8). For this, we used a system in which the *opgGH* operon is under the control of the P_{BAD} promoter from *E. coli*. Control of L-arabinose concentration, enables tight regulation of the *opgGH* operon (Guzman et al., 1995). We grew the P_{BAD}-*opgGH*, *envZ* P_{BAD}-*opgGH, ompR* P_{BAD}-*opgGH*, as well as the wild-type and *opgG* strains, in M63 medium at various L-arabinose concentrations ranging from 0 - 1 g/L. We first confirmed that expression of the *opgG* and *opgH* genes increased in line with increasing concentration of L-arabinose (Figure 8A, B). As shown previously, without L-arabinose, no OPG is detected. OPG concentration increased in accordance with L-arabinose concentration, as described previously (Bontemps-Gallo et al., 2013). We then analyzed the expression of *ompF* and *kdgN* in the same strains under the same conditions. (Figure 8C, D). Without L-arabinose, expression of *ompF* and *kdgN* in the P_{BAD}-*opgGH* strain was similar to that measured for the *opgG* mutant (Figures 6, 8C, D). In the presence of L-arabinose, regardless of the concentration, the expression of both genes was similar to expression in the wild-type strain (Figures 6, 8C, D). Inactivation of either *envZ* or *ompR* in the P_{BAD}-*opgGH* strain lead to a low expression level regardless of the presence of L-arabinose. Our data show that OPGs are required for transmission of the sensing signal but that they do not control the level of EnvZ-OmpR activation.

Discussion

Since their first characterization in 1973 by E.P. Kennedy's group at Harvard Medical School, the osmoregulated periplasmic glucans have been described to play an important role in osmoprotection (Kennedy, 1982; Lacroix, 1989; Breedveld and Miller, 1994; Cayley et al., 2000; Bontemps-Gallo et al., 2017), in envelope structure (Delcour et al., 1992; Banta et al., 1998; Bontemps-Gallo et al., 2017), in virulence (Bhagwat et al., 2009) as well as in cell signaling (Fiedler and Rotering, 1988; Ebel et al., 1997; Bouchart et al., 2010). Among the different models used to study the biological function of this carbohydrate, *D. dadantii* is the most developed model for understanding their role in virulence and cell signaling.

Deleted: required
Deleted: via the
Deleted: did
Deleted: activation
Deleted: the
Deleted: level
Deleted: OPGs
Deleted: We
Deleted: if
Deleted: concentrations
Deleted: level
Deleted: We
Deleted: were
Deleted: This promoter allowed a
Deleted: by the concentration of L-arabinose through the AraC protein
Deleted: containing
Deleted: g/L to
Deleted: measured the
Deleted: and observed that the expression of the *opgGH* operon
Deleted: concomitantly
Deleted: concentrations
Deleted:) leading to an increase of the OPGs concentration as
Deleted: those
Deleted: and
Deleted: the
Deleted: what was
Deleted: in
Deleted: amount
Deleted: Inactivating
Deleted: *OmpR*
Deleted: showed
Deleted: the
Deleted: did
Deleted: of EnvZ-OmpR
Deleted: but also
Deleted: model
Deleted: actually
Deleted: the

The mutant devoid of OPG is described as having a complex pleiotropic phenotype: increased mucoid appearance (Breedveld and Miller, 1994; Ebel et al., 1997; Page et al., 2001), a decrease in motility (Fiedler and Rotering, 1988; Page et al., 2001; Bhagwat et al., 2009) and a loss of virulence (Bontemps-Gallo and Lacroix, 2015). The mucoid appearance of bacterial colonies is the consequence of activation of the RcsCDB two-component system (Bouchart et al., 2010; Bontemps-Gallo et al., 2013). This activation leads to up-regulation of the *eps* operon (Ebel et al., 1997; Bouchart et al., 2010), the genes of which are responsible for synthesis of exopolysaccharides. The dramatic decrease in motility is also demonstrated to be a consequence of inactivation of the RcsCDB two-component system (Bouchart et al., 2010; Bontemps-Gallo et al., 2013; Bontemps-Gallo and Lacroix, 2015). Here, we showed that if EnvZ-OmpR is involved in co-regulation of motility, inactivation of the system cannot restore motility in a strain lacking OPGs (Figure 3).

Loss of virulence, certainly the most investigated phenotype, is more complex to explain. Several mutations have now been described that partially (in genes encoding RcsCDB (Bouchart et al., 2010), KdgR, PecT (Bontemps-Gallo et al., 2014)) or fully restore virulence (in the gene encoding PecS (Bontemps-Gallo et al., 2014)) in *D. dadantii*. Restoration of virulence in potato tubers, reserve organs, depends only on the ability to restore full production of pectinase (Bontemps-Gallo et al., 2014). Restoration of virulence in non-reserve organs requires restoration of more factors, as bacteria will encounter several plant defense mechanisms (e.g. the oxidative burst) (Reverchon and Nasser, 2013; Bontemps-Gallo et al., 2014). In this study, we showed that inactivation of the EnvZ-OmpR system partially restores virulence in potato tubers (Figure 4) but not in chicory leaves (Figure 5). The result matched with the restoration of the pectinase production (Figure 1).

Finally, the second major finding of this study is the requirement for OPGs for activation of the EnvZ-OmpR system. In *E. coli*, the EnvZ-OmpR system senses osmolarity in an unknown manner and modulates expression of genes necessary for adaptation to the new conditions (Forst and Roberts, 1994; Castillo-Keller et al., 2006). This system is characterized both as a repressor (high osmolarity) and as an activator (low osmolarity) of *ompF* in *E. coli* (Lan and Igo, 1998). Surprisingly, in *D. dadantii*, the EnvZ-OmpR system only acts as an activator (Figure 6). This activation required OPGs in the periplasm (Figure 6). In contrast to RcsCDB (Bontemps-Gallo et al., 2013), periplasmic OPG concentration does not affect the level of activation of the EnvZ-OmpR system (Figure 8). The relationship between EnvZ-OmpR and OPGs is most likely indirect yet specific, since the CpxAR system was not affected by OPGs (Figure 6).

Deleted: OPG
Deleted: mutant
Deleted: to have
Deleted: aspect of the cell
Deleted: decreased of the
Deleted: aspect
Deleted: the colony
Deleted: the
Deleted: the
Deleted: synthesis
Deleted: of the
Deleted: activation
Deleted: the
Deleted: the
Deleted: the
Deleted: the
Deleted: an OPG devoid
Deleted: to restore
Deleted: virulence
Deleted: the
Deleted: a
Deleted: organ, depend
Deleted: of
Deleted: on
Deleted: organ required to restore
Deleted: because the
Deleted: have to
Deleted: restored the
Deleted: pectate-lyase
Deleted: of OPG
Deleted: systems sense
Deleted: on yet
Deleted: modulate
Deleted: the adequate gene
Deleted: adapt to
Deleted: condition
Deleted: The
Deleted: OPG
Deleted: of
Deleted: OPGs
Deleted: but is
Deleted: Several questions remain open and will required more investigations. Do other two-component systems require OPGs to be functional in *D. dadantii*? Do the specific relationship between RcsCDB or EnvZ-OmpR system and the OPGs also exist in neighboring bacterial species? Furthermore, the more intriguing feature is by which mecanism(s) OPGs modulate two-component system activation.

Several questions remain and require further investigations. Do other two-component systems need OPGs to be functional in *D. dadantii*? Preliminary data from our laboratory suggests that, among the thirty-two two-component systems, only RcsCDB and EnvZ-OmpR activation is affected by OPG presence/concentration. Does the specific relationship between the RcsCDB or EnvZ-OmpR system and OPGs also exist in phylogenetically closely-related bacterial species? In non-pathogenic *E. coli*, inactivation of RcsCDB or EnvZ-OmpR restores motility in an *opgG* mutant (Fiedler and Rotering, 1988; Girgis et al., 2007). In *Salmonella enterica* Serovar Typhimurium, inactivation of RcsCDB restores motility but not virulence in mice (Kannan et al., 2009). However, the relationship between OPGs and two-component systems has not been investigated in other bacteria. Finally, the more intriguing feature is the mechanism(s) by which OPGs modulate two-component system activation.

Table

Table 1: Strains used in the study

Strain	Relevant Genotype and/or phenotype[a]	Source or Reference
EC3937	wild-type	Laboratory collection
NFB3723	*opgG*::Cml	(Bontemps-Gallo et al., 2013)
NFB3835	*opgG*::Cml miniTn5 P_{BAD}-*opgGH*-Spe	(Bontemps-Gallo et al., 2013)
NFB7422	*ompR*::Gm	(Bontemps-Gallo et al., 2016)
NFB7423	*ompR*::Gm *opgG*::Cml	This study
NFB7440	*ompR*::Gm *opgG*::Cml miniTn5 P_{BAD}-*opgGH*-Spe	This study
NFB7515	*cpxA::Gm*	(Bontemps-Gallo et al., 2015)
NFB7521	*envZ*::Gm	(Bontemps-Gallo et al., 2016)
NFB7524	*envZ*::Gm *opgG*::Cml	This study
NFB7532	*cpxR::Gm*	(Bontemps-Gallo et al., 2015)
NFB7534	*cpxR opgG::Cml*	This study
NFB7632	*cpxA::Gm opgG::Cml*	This study
NFB7731	*envZ*::Gm *opgG*::Cml miniTn5 P_{BAD}-*opgGH*-Spe	This study

[a]: Cml: chloramphenicol resistance, Gm: Gentamicin resistance, Spe: spectinomycin resistance. P_{BAD}-*opgGH* fusion is carried by a mini-Tn5.

Table 2: qPCR primers

Deleted: Page Break

Deleted: Strain

Formatted Table

Deleted: primer

Primer	Sequence	Efficiency	Reference
ompF-F	CGT AAC TCT GGT GTT GCT ACT T	1.843	This study
ompF-R	AGT CGC TAT GTG CTG ATT GG		
kdgN-F	CCT GCG TTA TCG TCC TTT CTA C	1.428	This study
kdgN-R	CAG CAC GCT GGT AAT GGT ATA G		
ompR-F	GCT CGA TTG ATG TGC AGA TTT C	1.904	This study
ompR-R	ACA AAG ACG TAG CCC AAC C		
envZ-F	CTG GCG GAG TCG ATC AAT AA	1.652	This study
envZ-R	GCC ACT TCC ATC TGC ATT TC		
spy-F	CGG AAG GCG TAG TCA ATC AA	1.943	This study
spy-R	TTT CTG TTC CGG CGT CAA		
degP-F	CCA GAT TGT CGA ATA CGG AGA G	1.733	This study
degP-R	GCA TCC ACT TTC ATG GCT TTA G		
opgG-F	CCG GAA CAG GCT TAT GTG AT	1.774	This study
opgG-R	AAT CGA CCA GGA ATG CAG TAG		
opgH-F	GGA ACT GGC GAT AGC TTT GT	1.547	This study
opgH-R	CCA CTC CGC CGT ATG ATT TAG		
flhD-F	TCG GTT GGG TAT CAA TGA AGA A	1.815	This study
flhD-R	TCA CTG AAG CGG AAA TGA CAT A		
fliC-F	CAC GGC TCA TGT TGG ATA CT	1.676	This study
fliC-R	CA TTG ACA ACC TGA GCA ACA C		
ipxC-F	AAA TCC GTG CGT GAT ACC AT	1.862	(Hommais et al., 2011)
ipxC-R	CAT CCA GCA GCA GGT AGA CA		

Table 3: Protease activity

Protease activities were observed on plates by the presence of a clear halo and marked as '+'. Data represent observations from three independent experiments.

Strain	
Wild-type	+
opgG	-
envZ	+
envZ opgG	-

Formatted Table

Deleted: plate

Deleted: observation

Formatted Table

ompR	+
ompR opgG	-

Figure Legend

Figure 1: Characterization of the *envZ* and *ompR* deletion in wild-type and *opgG* background.
(A) Schematic of the *envZ-ompR* locus in the wild-type strain and genetic organization of the mutant strains. (B) Expression of *envZ* and *ompR* was analyzed by qPCR. Bacteria were grown at 170, 330, 500 and 700 mOsM. Relative gene expression was calculated using *ipxC* as a reference (Hommais et al., 2011). Data represent mean +/- standard deviation of three independent experiments. An asterisk indicates a significant difference with $p < 0.0001$.

Figure 2: Pectinase (A) and cellulase (B) activities
Exoenzyme activities were estimated on plates by measurement of halo diameters, expressed in cm of substrate degradation. Data represent mean +/- standard deviation of twenty independent experiments. An asterisk indicates a significant difference with $p < 0.0001$

Figure 3: Effect of EnvZ-OmpR on motility in wild-type and *opgG* background.
(A) Motility of wild-type, *opgG*, *envZ*, *envZ opgG*, *ompR*, *ompR opgG* strains. Motility was measured in M63 semisolid plates. Swim diameters were measured after 48h of incubation at 30°C. (B) Schematic of the regulatory cascade of motility. FlhDC, a master regulator and a class I promoter, modulate gene expression with a class II promoter (e.g. *fliA*). In return, the products of those genes regulate genes with a class III promoter (e.g. *fliC*). (C-D). Expression of (C) *flhD* and (D) *fliC* in wild-type, *opgG*, *envZ*, *envZ opgG*, *ompR* and *ompR opgG*, strains Bacteria were grown at 170, 330, 500 and 700 mOsM. The expression of (C) *flhD*, (D) *fliC* was analyzed by qPCR. Relative gene expression was calculated using *ipxC* as a reference (Hommais et al., 2011). Data represent mean +/- standard deviation of ten independent experiments. An asterisk indicates a significant difference with $p < 0.0001$ for ****, $p < 0.001$ for ***, $p < 0.01$ for ** and $p < 0.05$ for *.

Figure 4: Weight of maceration on potato tubers for wild-type, *opgG*, *envZ*, *envZ opgG*, *ompR*, *ompR opgG* strains.
Bacteria were inoculated into holes on potato tubers. Maceration (g) was weighed after 72h of incubation at 30°C. Data represent mean +/- standard deviation of at least ten independent experiments.

Figure 5: Pathogenicity of wild-type, *opgG*, *envZ*, *envZ opgG*, *ompR*, *ompR opgG* strains on chicory leaves.
Bacteria were inoculated into scarified chicory leaves. Disease symptoms were observed after 48h of incubation at 30°C. The results presented are one of the three independent experiments performed.

Figure 6: Expression of (A) *ompF* and (B) *kdgN* in wild-type, *opgG*, *envZ*, *envZ opgG*, *ompR* and *ompR opgG*, strains and of (C) *spy* and (D) *degP* in wild-type, *opgG*, *cpxA*, *cpxA opgG*, *cpxR*, *cpxR opgG* strains at various osmolarities
Bacteria were grown at 170, 330, 500 and 700 mOsM. The expression of (A) *ompF*, (B) *kdgN* (C) *spy* and (D) *degP* were analyzed by qPCR. Relative gene expression was calculated using

Deleted: .
Deleted: were
Deleted: Asterisk indicate
Deleted: Pectate-lyase
Deleted: the
Deleted: Asterisk indicate
Deleted: gene
Deleted: gene
Deleted: II
Deleted: were
Deleted: Asterisk indicate

ipxC as a reference (Hommais et al., 2011). Data represent mean +/- standard deviation of three independent experiments. An asterisk indicates a significant difference with $p < 0.0001$ for **** and $p < 0.01$ for **.

Figure 7: Transmission electron microscopy images of wild-type (A, C) and *opgG* mutant (B, D) at low osmolarity (A, B) and high osmolarity (C, D). Images show similar architecture for both strains when grown in the same medium but differences when osmolarity is varied. (E) Periplasm size (nm) from TEM images.

Figure 8: Effect of OPG concentration on expression of *opgG*, *opgH*, *ompF*, and *kdgN*. Bacteria were grown in M63 medium (330 mOsM) with increasing L-arabinose concentration ranging from 0 - 1 g/L. The expression of (A) *opgG*, (B) *opgH*, (C) *ompF*, and (D) *kdgN* was analyzed by qPCR. Relative gene expression was calculated using *ipxC* as a reference (Hommais et al., 2011). Data represent mean +/- standard deviation of three independent experiments. An asterisk indicates a significant difference with $p < 0.0001$ for ****, $p < 0.001$ for ***, $p < 0.01$ for ** and $p < 0.05$ for *.

Acknowledgements

We thank Loic Brunet for the electronic microscopy. We are grateful to Dr Ciaran Finn for the manuscript improvements.

This study was supported by the Centre National de la Recherche Scientifique (CNRS), the Université de Lille Sciences et Technologies and the Ministère de l'Enseignement Supérieur et de la Recherche. MC and SBG were funded by a doctoral fellowship of Lille University. The funding bodies were not involved in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author contributions

SBG and JML conceived, designed the study and wrote the manuscript. MC and SBG performed all experiments with the assistance of EM, PG, BD, MC, SBG, EM, and JML analyzed the data.

References

Asmar, A.T., Ferreira, J.L., Cohen, E.J., Cho, S.H., Beeby, M., Hughes, K.T., et al. (2017). Communication across the bacterial cell envelope depends on the size of the periplasm. *PLoS Biol* 15(12), e2004303. doi: 10.1371/journal.pbio.2004303.

Ayraud, S., Janvier, B., Labigne, A., Ecobichon, C., Burucoa, C., and Fauchere, J.L. (2005). Polyphosphate kinase: a new colonization factor of Helicobacter pylori. *FEMS Microbiol Lett* 243(1), 45-50. doi: 10.1016/j.femsle.2004.11.040.

Deleted: Asterisk indicate

Deleted: Picture revealed

Deleted: in

Deleted: between both

Deleted: g/L to

Deleted: were

Deleted: Asterisk indicate

Formatted: Justified

Deleted: CM

Deleted: ¶

Deleted: funders

Deleted: this

Deleted: CM

Deleted: carried out

Deleted: the

Deleted: . CM

880 Banta, L.M., Bohne, J., Lovejoy, S.D., and Dostal, K. (1998). Stability of the Agrobacterium
881 tumefaciens VirB10 protein is modulated by growth temperature and
882 periplasmic osmoadaption. *J Bacteriol* 180(24)**,** 6597-6606.
883 Barbieri, C.M., Wu, T., and Stock, A.M. (2013). Comprehensive analysis of OmpR
884 phosphorylation, dimerization, and DNA binding supports a canonical model for
885 activation. *J Mol Biol* 425(10)**,** 1612-1626. doi: 10.1016/j.jmb.2013.02.003.
886 Barker, C.S., Pruss, B.M., and Matsumura, P. (2004). Increased motility of Escherichia coli
887 by insertion sequence element integration into the regulatory region of the flhD
888 operon. *J Bacteriol* 186(22)**,** 7529-7537. doi: 10.1128/JB.186.22.7529-
889 7537.2004.
890 Bertani, G. (2004). Lysogeny at Mid-Twentieth Century: P1, P2, and Other Experimental
891 Systems. *J. Bacteriol.* 186(3)**,** 595-600. doi: 10.1128/jb.186.3.595-600.2004.
892 Bhagwat, A.A., Jun, W., Liu, L., Kannan, P., Dharne, M., Pheh, B., et al. (2009).
893 Osmoregulated periplasmic glucans of Salmonella enterica serovar Typhimurium
894 are required for optimal virulence in mice. *Microbiology* 155(Pt 1)**,** 229-237. doi:
895 10.1099/mic.0.023747-0.
896 Bohin, J.P., and Lacroix, J.M. (2006). "Osmoregulation in the periplasm," in *The Periplasm,*
897 ed. M. Ehrmann. (Washington, DC, USA: American Society for Microbiology), pp.
898 325–341.
899 Bontemps Gallo, S. (2013). *Dickeya dadantii: Towards the understanding of the biological*
900 *role of the osmoregulated periplasmic glucans (in French).* PhD thesis, Université
901 de Lille.
902 Bontemps-Gallo, S., Bohin, J.P., and Lacroix, J.M. (2017). Osmoregulated Periplasmic
903 Glucans. *EcoSal Plus* 7(2). doi: 10.1128/ecosalplus.ESP-0001-2017.
904 Bontemps-Gallo, S., and Lacroix, J.M. (2015). New insights into the biological role of the
905 osmoregulated periplasmic glucans in pathogenic and symbiotic bacteria. *Environ*
906 *Microbiol Rep* 7(5)**,** 690-697. doi: 10.1111/1758-2229.12325.
907 Bontemps-Gallo, S., Madec, E., Dondeyne, J., Delrue, B., Robbe-Masselot, C., Vidal, O., et al.
908 (2013). Concentration of osmoregulated periplasmic glucans (OPGs) modulates
909 the activation level of the RcsCD RcsB phosphorelay in the phytopathogen
910 bacteria Dickeya dadantii. *Environ Microbiol* 15(3)**,** 881-894. doi: 10.1111/1462-
911 2920.12054.
912 Bontemps-Gallo, S., Madec, E., and Lacroix, J.M. (2014). Inactivation of pecS restores the
913 virulence of mutants devoid of osmoregulated periplasmic glucans in the
914 phytopathogenic bacterium Dickeya dadantii. *Microbiology* 160(Pt 4)**,** 766-777.
915 doi: 10.1099/mic.0.074484-0.
916 Bontemps-Gallo, S., Madec, E., and Lacroix, J.M. (2015). The two-component system
917 CpxAR is essential for virulence in the phytopathogen bacteria Dickeya dadantii
918 EC3937. *Environ Microbiol* 17(11)**,** 4415-4428. doi: 10.1111/1462-2920.12874.
919 Bontemps-Gallo, S., Madec, E., Robbe-Masselot, C., Souche, E., Dondeyne, J., and Lacroix,
920 J.M. (2016). The opgC gene is required for OPGs succinylation and is
921 osmoregulated through RcsCDB and EnvZ/OmpR in the phytopathogen Dickeya
922 dadantii. *Sci Rep* 6**,** 19619. doi: 10.1038/srep19619.
923 Bouchart, F., Boussemart, G., Prouvost, A.F., Cogez, V., Madec, E., Vidal, O., et al. (2010).
924 The virulence of a Dickeya dadantii 3937 mutant devoid of osmoregulated
925 periplasmic glucans is restored by inactivation of the RcsCD-RcsB phosphorelay. *J*
926 *Bacteriol* 192(13)**,** 3484-3490. doi: 10.1128/JB.00143-10.
927 Bouchart, F., Delangle, A., Lemoine, J., Bohin, J.P., and Lacroix, J.M. (2007). Proteomic
928 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(Pt 3), 760-767. doi: 10.1099/mic.0.2006/000372-0.
Breedveld, M.W., and Miller, K.J. (1994). Cyclic beta-glucans of members of the family Rhizobiaceae. *Microbiol Rev* 58(2), 145-161.
Castillo-Keller, M., Vuong, P., and Misra, R. (2006). Novel mechanism of Escherichia coli porin regulation. *J Bacteriol* 188(2), 576-586. doi: 10.1128/JB.188.2.576-586.2006.
Cayley, D.S., Guttman, H.J., and Record, M.T., Jr. (2000). Biophysical characterization of changes in amounts and activity of Escherichia coli cell and compartment water and turgor pressure in response to osmotic stress. *Biophys J* 78(4), 1748-1764.
Charkowski, A., Blanco, C., Condemine, G., Expert, D., Franza, T., Hayes, C., et al. (2012). The role of secretion systems and small molecules in soft-rot Enterobacteriaceae pathogenicity. *Annu Rev Phytopathol* 50, 425-449. doi: 10.1146/annurev-phyto-081211-173013.
Clemmer, K.M., and Rather, P.N. (2007). Regulation of flhDC expression in Proteus mirabilis. *Res Microbiol* 158(3), 295-302. doi: 10.1016/j.resmic.2006.11.010.
Collmer, A., and Keen, N.T. (1986). The Role of Pectic Enzymes in Plant Pathogenesis. *Annual Review of Phytopathology* 24(1), 383-409. doi: doi:10.1146/annurev.py.24.090186.002123.
Condemine, G., and Ghazi, A. (2007). Differential regulation of two oligogalacturonate outer membrane channels, KdgN and KdgM, of Dickeya dadantii (Erwinia chrysanthemi). *J Bacteriol* 189(16), 5955-5962. doi: 10.1128/JB.00218-07.
Cowan, S.W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R.A., et al. (1992). Crystal structures explain functional properties of two E. coli porins. *Nature* 358(6389), 727-733. doi: 10.1038/358727a0.
Delcour, A.H., Adler, J., Kung, C., and Martinac, B. (1992). Membrane-derived oligosaccharides (MDO's) promote closing of an E. coli porin channel. *FEBS Lett* 304(2-3), 216-220.
Ebel, W., Vaughn, G.J., Peters, H.K., 3rd, and Trempy, J.E. (1997). Inactivation of mdoH leads to increased expression of colanic acid capsular polysaccharide in Escherichia coli. *J Bacteriol* 179(21), 6858-6861.
Egger, L.A., Park, H., and Inouye, M. (1997). Signal transduction via the histidyl-aspartyl phosphorelay. *Genes Cells* 2(3), 167-184.
EPPO (1982). "Data sheets on quarantine organisms, Erwinia chrysanthemi Burkholder.", in: *Bull OEPP.*).
EPPO (1988). A1 and A2 Lists of Quarantine Pests. Specific Quarantine Requirements. *EPPO/EPPO Publications Series B no. 92*.
EPPO (1990). Specific Quarantine Requirements. *EPPO Technical Documents no. 1008*.
Fiedler, W., and Rotering, H. (1988). Properties of Escherichia coli mutants lacking membrane-derived oligosaccharides. *J Biol Chem* 263(29), 14684-14689.
Forst, S.A., and Roberts, D.L. (1994). Signal transduction by the EnvZ-OmpR phosphotransfer system in bacteria. *Res Microbiol* 145(5-6), 363-373.
Gao, H., Zhang, Y., Han, Y., Yang, L., Liu, X., Guo, Z., et al. (2011). Phenotypic and transcriptional analysis of the osmotic regulator OmpR in Yersinia pestis. *BMC Microbiol* 11, 39. doi: 10.1186/1471-2180-11-39.
Girgis, H.S., Liu, Y., Ryu, W.S. and Tavazoie, S. (2007) A comprehensive genetic characterization of bacterial motility. *PLoS Genet* 3(9):1644-1660.

977 Groisman, E.A. (2016). Feedback Control of Two-Component Regulatory Systems. *Annu*
978 *Rev Microbiol* 70**,** 103-124. doi: 10.1146/annurev-micro-102215-095331.
979 Guzman, L.M., Belin, D., Carson, M.J., and Beckwith, J. (1995). Tight regulation,
980 modulation, and high-level expression by vectors containing the arabinose PBAD
981 promoter. *J Bacteriol* 177(14)**,** 4121-4130.
982 Hoch, J.A. (2000). Two-component and phosphorelay signal transduction. *Curr Opin*
983 *Microbiol* 3(2)**,** 165-170.
984 Hommais, F., Zghidi-Abouzid, O., Oger-Desfeux, C., Pineau-Chapelle, E., Van Gijsegem, F.,
985 Nasser, W., et al. (2011). lpxC and yafS are the most suitable internal controls to
986 normalize real time RT-qPCR expression in the phytopathogenic bacteria Dickeya
987 dadantii. *PLoS One* 6(5)**,** e20269. doi: 10.1371/journal.pone.0020269.
988 Jiang, X., Zghidi-Abouzid, O., Oger-Desfeux, C., Hommais, F., Greliche, N., Muskhelishvili,
989 G., et al. (2016). Global transcriptional response of Dickeya dadantii to
990 environmental stimuli relevant to the plant infection. *Environ Microbiol* 18(11)**,**
991 3651-3672. doi: 10.1111/1462-2920.13267.
992 Kannan, P., Dharne, M., Smith, A., Karns, J, and Bhagwatt, A.A. (2009) Motility revertants
993 of *opgGH* mutants of *Salmonella enterica* serovar Typhimurium remain defective
994 in mice virulence. *Curr Microbiol* 59(6), 641-645.
995 Kennedy, E.P. (1982). Osmotic regulation and the biosynthesis of membrane-derived
996 oligosaccharides in Escherichia coli. *Proc Natl Acad Sci U S A* 79(4)**,** 1092-1095.
997 Kennedy, E.P. (1996). "Membrane derived oligosaccharides (periplasmic beta-D-
998 glucans) of Escherichia coli," in *Escherichia coli and Salmonella. Cellular and*
999 *Molecular Biology,* eds. F.C. Neidhardt, R. Curtiss III, J.L. Ingraham, E.C.C. Lin, K.B.
1000 Low, B. Maganasik, W.S. Reznikoff, M. Riley, M. Schaechter & H.E. Umbarger. 2nd
1001 ed (Washington, D.C.: ASM Press), 1064-1074.
1002 Lacroix, J.-M. (1989). *Etude génétique et physiologique de la régulation osmotique de la*
1003 *biosynthèse du MDO chez Escherichia coli.* PhD thesis, Université de Paris-Sud.
1004 Lan, C.Y., and Igo, M.M. (1998). Differential expression of the OmpF and OmpC porin
1005 proteins in Escherichia coli K-12 depends upon the level of active OmpR. *J*
1006 *Bacteriol* 180(1)**,** 171-174.
1007 Lechaire, J.P., Shillito, B., Frebourg, G., and Gaill, F. (2002). Elemental characterization of
1008 microorganism granules by EFTEM in the tube wall of a deep-sea vent
1009 invertebrate. *Biol Cell* 94(4-5)**,** 243-249.
1010 Lee, C., and Park, C. (2013). Mutations upregulating the flhDC operon of Escherichia coli
1011 K-12. *J Microbiol* 51(1)**,** 140-144. doi: 10.1007/s12275-013-2212-z.
1012 Leonard, S., Hommais, F., Nasser, W., and Reverchon, S. (2017). Plant-phytopathogen
1013 interactions: bacterial responses to environmental and plant stimuli. *Environ*
1014 *Microbiol* 19(5)**,** 1689-1716. doi: 10.1111/1462-2920.13611.
1015 Li, W., Ancona, V., and Zhao, Y. (2014). Co-regulation of polysaccharide production,
1016 motility, and expression of type III secretion genes by EnvZ/OmpR and
1017 GrrS/GrrA systems in Erwinia amylovora. *Mol Genet Genomics* 289(1)**,** 63-75. doi:
1018 10.1007/s00438-013-0790-4.
1019 Miller, J.H. (1992). A short course in bacterial genetics: a laboratory manual and
1020 handbook for *Escherichia coli* and related bacteria. *Cold Spring Harbor Laboratory*
1021 *Press, New York*.
1022 Ogawa, N., Tzeng, C.M., Fraley, C.D., and Kornberg, A. (2000). Inorganic polyphosphate in
1023 Vibrio cholerae: genetic, biochemical, and physiologic features. *J Bacteriol*
1024 182(23)**,** 6687-6693.

Page, F., Altabe, S., Hugouvieux-Cotte-Pattat, N., Lacroix, J.M., Robert-Baudouy, J., and Bohin, J.P. (2001). Osmoregulated periplasmic glucan synthesis is required for Erwinia chrysanthemi pathogenicity. *J Bacteriol* 183(10)**,** 3134-3141. doi: 10.1128/JB.183.10.3134-3141.2001.

Pruss, B.M. (2017). Involvement of Two-Component Signaling on Bacterial Motility and Biofilm Development. *J Bacteriol* 199(18). doi: 10.1128/JB.00259-17.

Raczkowska, A., Skorek, K., Bielecki, J., and Brzostek, K. (2011). OmpR controls Yersinia enterocolitica motility by positive regulation of flhDC expression. *Antonie Van Leeuwenhoek* 99(2)**,** 381-394. doi: 10.1007/s10482-010-9503-8.

Reboul, A., Lemaitre, N., Titecat, M., Merchez, M., Deloison, G., Ricard, I., et al. (2014). Yersinia pestis requires the 2-component regulatory system OmpR-EnvZ to resist innate immunity during the early and late stages of plague. *J Infect Dis* 210(9)**,** 1367-1375. doi: 10.1093/infdis/jiu274.

Resibois, A., Colet, M., Faelen, M., Schoonejans, E., and Toussaint, A. (1984). phiEC2, a new generalized transducing phage of Erwinia chrysanthemi. *Virology* 137(1)**,** 102-112.

Reverchon, S., Muskhelisvili, G., and Nasser, W. (2016). Virulence Program of a Bacterial Plant Pathogen: The Dickeya Model. *Prog Mol Biol Transl Sci* 142**,** 51-92. doi: 10.1016/bs.pmbts.2016.05.005.

Reverchon, S., and Nasser, W. (2013). Dickeya ecology, environment sensing and regulation of virulence programme. *Environ Microbiol Rep* 5(5)**,** 622-636. doi: 10.1111/1758-2229.12073.

Stumpf, J.D., and Foster, P.L. (2005). Polyphosphate kinase regulates error-prone replication by DNA polymerase IV in Escherichia coli. *Mol Microbiol* 57(3)**,** 751-761. doi: 10.1111/j.1365-2958.2005.04724.x.

Tipton, K.A., and Rather, P.N. (2016). An ompR/envZ Two-Component System Ortholog Regulates Phase Variation, Osmotic Tolerance, Motility, and Virulence in Acinetobacter baumannii strain AB5075. *J Bacteriol*. doi: 10.1128/JB.00705-16.

Toth, I., Van der Wolf, J., Saddler, G., Lojkowska, E., Hélias, V., Pirhonen, M., et al. (2011). Dickeya species: an emerging problem for potato production in Europe. *Plant Pathology* 60**,** 385–399. doi: 10.1111/j.1365-3059.2011.02427.x.

Page 7: [1] Formatted	sébastien bontemps	9/16/18 11:49:00 PM

Font: Italic