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► **To cite this version:**

Sébastien Bontemps-Gallo, Jean-Pierre Bohin, Jean-Marie Lacroix. Osmoregulated Periplasmic Glucans. *EcoSal Plus*, 2017, 7 (2), pp.0001-2017. 10.1128/ecosalplus.ESP-0001-2017 . hal-02475319

HAL Id: hal-02475319

<https://hal.science/hal-02475319v1>

Submitted on 24 Sep 2020

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Osmoregulated Periplasmic Glucans

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Abstract

Among all the systems developed by Enterobacteria to face osmotic stress, only osmoregulated periplasmic glucans (OPGs) were found to be modulated during osmotic fluxes. First detected in 1973 by E.P. Kennedy's group in a study of phospholipid turnover in *Escherichia coli*, OPGs have been shown across alpha, beta and gamma subdivision of the Proteobacteria. Discovery of OPG-like compounds in the epsilon subdivision strongly suggested that the presence of periplasmic glucans is essential for almost all Proteobacteria. This chapter offers an overview of the different classes of OPGs. Then, the biosynthesis of OPGs and their regulation in *E. coli* and other species are discussed. Finally, the biological role of OPGs is developed. Beyond structural function, OPGs are involved in pathogenicity, in particular by playing a role in signal transduction pathways. Recently, OPG synthesis proteins have been suggested to control cell division and growth rate.

Introduction

Escherichia coli and *Salmonella enterica* are found in many environments with a wide range of osmolarity: intestinal tract of mammalian host (> 300 mosM), urinary tract (500/800 mosM for an adult with a normal diet, but may vary from 40 to 1,400 mosM)(1-3), in sea (1,000 mosM)(4) or freshwater (10 mosM)(5). To adapt to these osmotic fluxes, Enterobacteria have developed many tools to maintain the osmotic pressure inside of the cell (see EcoSal chapter Osmotic Stress (6)). Among these systems/molecules involved in osmoadaptation, the only periplasmic components whose synthesis was found to be

modulated by osmotic pressure in various bacterial species are osmoregulated periplasmic glucans (OPGs).

OPG structures found in different bacterial species share several common characteristics: i) D-glucose is the only constituent sugar; ii) glucose units are linked, at least partially, by β -glycosidic bonds; iii) there are a limited number of glucose units (5 to 24); iv) in most cases, but with a few exceptions, OPG concentration in the periplasm increases in response to a decrease in environmental osmolarity. In many but not all, species, the OPG backbones can be decorated to various extents with a variety of substituents. These substituents appear to belong to two classes: i) residues originating from membrane phospholipids like phosphoglycerol, phosphoethanolamine, or phosphocholine; ii) residues originating from intermediary metabolism like acetyl, succinyl and methylmalonyl. For the latter, acyl-coenzymes A are the likely donor molecules but this has not been demonstrated (7).

OPGs were detected during the study of phospholipid turn-over in *E. coli* by E. P. Kennedy's group (8). In this bacterium, rapid phosphatidylglycerol turn-over is associated with the transfer of *sn*-1-phosphoglycerol to a new class of oligosaccharides, accordingly named "membrane-derived oligosaccharides" (MDOs, reviewed in (9)). The homology between the linear β -glucans found in the periplasm of *E. coli* and the cyclic β -glucans found in the periplasm of *A. tumefaciens* was established in 1986 with the demonstration that the syntheses of both kinds of molecules are osmoregulated (10). We now know that these compounds are found in many Proteobacteria and most do not contain any substituent derived from membrane lipids. The term 'MDO' is confusing because, despite being part of the envelope, these compounds may not be derived from the membrane. For

this reason, the term OPGs was preferred and the gene and protein nomenclatures were changed accordingly (11).

OPG structures

OPG structures were described in several species of the alpha, beta and gamma subdivisions of the Proteobacteria. Recently, Szymanski's group demonstrated the presence of OPG-like compounds, called free oligosaccharide (fOS), in the epsilon subdivisions of the Proteobacteria (12, 13). No information is available for other Gram-negative bacteria. Beyond their common features, OPGs show an unexpected structural diversity. Four families (I to IV) have been defined on the basis of backbone organization (Fig. 1) (7).

Family I

OPGs of family I are found in almost all γ -Proteobacteria such as *E. coli* ((9, 14); and references therein), , *Salmonella enterica* serovar Typhimurium (unpublished data) *Dickeya dadantii* (15), *Pseudomonas aeruginosa* (16), *Pseudomonas syringae* (17). The structures found in these bacteria are very similar. In *E. coli*, OPGs are heterogeneous in size and appear to range from 5 to 12 glucose residues, with the principal species containing 8 or 9 glucose residues. The sugar backbone consisting of β -1,2-linked glucose units is highly branched by the irregular addition of one glucose units attached by β -1,6 linkages (Fig. 1). In *E. coli* and probably in *S. enterica*, a heterogeneous substitution by phosphoglycerol, succinyl and phosphoethanolamine residues is superimposed to this heterogeneity of backbone structures (9, 11, 18, 19). A specific

pattern of substitution was found depending on bacterial species (20). As example, in *D. dadantii*, OPGs are substituted by succinyl and acetyl residues (15, 21).

Family II

OPGs of family II are found in various members of the family Rhizobiaceae ((22) and references therein). *Agrobacterium*, *Rhizobium*, *Sinorhizobium*, *Brucella* and *Mesorhizobium* species synthesize periplasmic glucans with similar structures (23, 24). In these genera, OPGs are composed of a cyclic β -1,2-glucan backbone containing 17 to 25 glucose residues (Fig. 1). Much larger molecules (up to 40 glucose units) were detected within cultures of a particular strain of *Sinorhizobium meliloti* (22). The predominant substituent of OPGs of *S. meliloti* and *A. tumefaciens* strains is phosphoglycerol, but substitutions by succinyl and methylmalonyl residues were also found in other related species or strains. The degree of substitution may vary greatly among different strains and the stage of growth also influences the degree of substitution.

Family III

OPGs of family III were first described in *Bradyrhizobium japonicum* as β -1,6- and β -1,3-cyclic glucans containing 10 to 13 glucose units per ring (22, 25). Highly similar OPGs were found in *Azorhizobium caulinodans* (26) and in *Azospirillum brasilense* as judged from NMR analysis (27). For the latter, high-performance anion-exchange chromatography allowed for the separation of three distinct structures: glucan I, comprised of 12 glucose units linked by 3 β -1,3, 8 β -1,6 and one β -1,4 linkages; glucan II derived from glucan I by the addition of a glucose linked by an α -1,3 linkage; glucan III derived from glucan II by the addition of a 2-O-methyl group onto the α linked glucose unit (Fig. 1). Thus, the OPGs of family III differ from those of the family II not only by

the nature of the glycosidic linkage but also by a strict control of the ring size. While OPGs of family III are predominantly uncharged, OPGs of *B. japonicum* also demonstrate substitutions with phosphocholine (25).

Family IV

OPGs of family IV have very similar structural features. They were found in *Ralstonia solanacearum* (28), *Xanthomonas campestris* (28, 29) and *Rhodobacter sphaeroides* (30). These OPGs are cyclic and have a unique degree of polymerization (DP = 13, 16, and 18, respectively). One linkage is α -1,6 whereas all the other glucose residues are linked by β -1,2 linkages (Fig. 1). The presence of this α -1,6 linkage induces structural constraints in these kind of molecules, which contrast with the very flexible structures of the cyclic all β -1,2 OPGs of family II (31). While OPGs found in *X. campestris* and *R. solanacearum* are not substituted, those found in *R. sphaeroides* can be substituted by one to seven succinyl esters residues, and by one or two acetyl residues.

Thus, OPG diversity is very high not only among different bacterial species, due to different genomic capacity (see below), but also in a particular bacterial strain, because control of the backbone structure is often not stringent and because backbone substitution (if any) is heterogeneous. Consequently, OPGs may be neutral or moderately to highly anionic molecules.

Mechanisms of OPG biosynthesis in *E. coli*

OPG backbone enzymes

In *E. coli*, two specific proteins are necessary for glucan backbone biosynthesis (Fig. 2). They are encoded by the *opgGH* operon (32). *In vitro*, the presence of OpgH in inner membrane vesicles is necessary to obtain the production of linear β -1,2 polyglucose chains from the precursor UDP-glucose. For unknown reasons, the acyl carrier protein, which normally functions in fatty acid synthesis, is also necessary for this activity (33). OpgH (97 kDa) is composed of three large cytoplasmic regions linked by eight transmembrane segments (34). The central cytoplasmic region shows structural features of a glucosyltransferase of family 2 where several aspartic acid residues are necessary for OPG synthesis (35). It is postulated that the eight transmembrane segments in OpgH could form a channel for OPG translocation to the periplasm during synthesis (34). OpgG (56 kDa) is a periplasmic protein whose function has not been established. In the *in vitro* test of OpgH activity, OpgG was absent and the product showed a higher degree of polymerization and no branching (9, 33). However, OpgG defective mutants form neither mature OPG molecules nor any precursor forms (32). The 2.5Å crystal structure of OpgG has been reported (36). The protein is composed of two distinct β -sandwich domains. The N-terminal domain shares some similarities with carbohydrate-active enzymes and the C-terminal domain could interact with other molecules. Modeling analyses suggest interaction between both proteins (37). These data support the hypothesis that OpgG interacts with OpgH for the translocation of nascent molecules and catalyzing the addition of branches.

In *E. coli*, a third protein, OpgD, was described (11), which is encoded by a paralog of *opgG*. When *opgD* was inactivated, normal amounts of OPGs were recovered

but backbone structure was altered, showing a higher degree of polymerization. Thus, OpgD seems to control (or interfere with) the OPG biosynthetic machinery (Fig. 2).

The functions of genes similar to the *E. coli* *opgG* and *opgH* genes have been demonstrated in *P. syringae* (38) and *D. dadantii* (39), which produce linear OPGs of the family I, and also in *R. sphaeroides* (40) and (very likely) in *X. campestris* (41), which produce cyclic OPGs of the family IV. The *opgD* gene can be absent from certain genomes, while in some cases, *opgG* is absent but *opgD* is present (see above). Therefore, one can envisage that interactions between OpgH, on the one hand, and OpgG/OpgD, on the other hand have evolved differently in different species.

Highly similar OPG genes were found in *A. tumefaciens* (*chvB* and *chvA*), *S. meliloti* (*ndvB* and *ndvA*) and *B. abortus* (*cgs* and *cgt*) (22, 42-45). These genes are strict homologues; genes from one species complementing OPG biosynthetic defects in other species. While the two genes are adjacent to each other in *A. tumefaciens* and *S. meliloti*, they are separated by 857 bp in *B. abortus*. The biosynthetic protein of *B. abortus* (Cgs) is a large cytoplasmic membrane protein (316 to 319 kDa). It is composed of six transmembrane segments, which determine four large cytoplasmic domains and three very small periplasmic regions (46). The biosynthetic enzymatic activity can be assayed efficiently *in vitro*. Unpurified membrane preparations are able to catalyze the formation of cyclic β -1,2 glucans from UDP-glucose and a high molecular-weight membrane protein is labeled when radioactive UDP-glucose is present (22, 47). The second protein (Cgt) is a 66- or 67-kDa inner membrane protein which shares amino-acid sequence similarity with several ATP-binding cassette transporters. When the protein is absent, cyclic glucans are formed but they are not substituted. Since substitution occurs in the

periplasm (see below), it can be concluded that the function of Cgt is the translocation of the cyclic molecules towards the periplasm.

Using the hypothesis that structurally different OPGs could functionally compensate for OPG defect in a *S. meliloti ndvB* mutant, Bhagwat *et al.* identified a gene they named *ndvB* from *B. japonicum* (48). Later, two others genes were characterized, *ndvC* (49) and *ndvD* (50), which form a locus of three monocistronic genes with *ndvB*. NdvB (102 kDa), NdvC (62 kDa) and NdvD (26 kDa) are predicted to be membrane-bound with NdvB and NdvC showing several transmembrane segments and NdvD only one. When *ndvB* was inactivated, no OPGs were synthesized *in vivo* and mutant membrane preparations failed to produce glucans *in vitro*. In contrast, when *ndvC* was inactivated normal amounts of OPGs were produced, but their structures contained almost only β ,1-3 linkages. Inactivation of *ndvD* abolished OPG synthesis *in vivo* but did not affect the glucan synthesis by membrane preparations *in vitro*. Thus, NdvB and NdvC are most likely two membrane-bound biosynthetic enzymes, while the function of NdvD remains elusive.

OPG substitution enzymes

In *E. coli*, the OPGs produced by the biosynthetic enzymes are very heterogeneous, and backbone variety can be further modified by 1, 2, or 3 residues of phosphoglycerol, and/or succinyl, and/or phosphoethanolamine. Thus, a majority of OPG molecules have a high anionic character (up to 5 negative charges), while a minority are neutral (Fig. 2). To date, only three genes have been recognized to participate in OPG substitution: *opgB* (51), *opgC* (19), *opgE* (18). Phosphoglycerol transfer was shown to occur in the periplasmic compartment (52). Two phosphoglycerol transferase (PGT)

activities have been measured *in vitro*: the membrane bound PGT I and the periplasmic PGT II. Soluble OPGs behave as phosphoglycerol acceptors for the latter but not for the former enzyme. Jackson and Kennedy have proposed a two-step model to account for phosphoglycerol substitution: first, PGT I transfers residues from the membrane phosphatidylglycerol to nascent OPG molecules; second, PGT II swaps residues from one OPG molecule to another (53). OpgB (85 kDa) consists of a large periplasmic sulfatase domain anchored in the inner membrane by three putative transmembrane segments and corresponds to PGT I. This protein was found to be highly sensitive to proteolytic cleavage and PGT II (OpgB') is actually a soluble form of OpgB (54).

OpgE (60 kDa), like OpgB, consists of a periplasmic sulfatase domain anchored in the inner membrane by four putative transmembrane domains. OpgE was proposed to transfer phosphoethanolamine residues from phosphatidylethanolamine to OPG molecules (18).

If one considers the probable viscosity of the periplasm, OpgB and OpgE should greatly facilitate the transfer of phosphoglycerol or phosphoethanolamine residues from the surface of the inner membrane toward OPGs molecules located in the external part of the periplasm (Fig. 2).

OpgC (44 kDa) is a polytopic protein with ten putative transmembrane segments. OpgC was proposed to catalyze the transfer of succinyl residues from the cytoplasmic side of the membrane to the nascent glucan backbones on the periplasmic side of the membrane (19).

In *R. sphaeroides*, OpgC_{Rsp} (44 kDa) exhibits stretches of hydrophobic amino acids over its entire length and eleven transmembrane segments are predicted. In *D.*

dadantii, OpgC_{Dda} (48 kDa) is also an inner membrane protein with 10 putative transmembrane domains (55). Thus, OpgC_{Rsp}, OpgC_{Dda} and OpgC_{Eco} share several common characteristics; however they do not show any significant sequence similarities (55).

In conclusion, despite the fact that they carry out very similar functions, OPG substitution enzymes described to date are not phylogenetically related but appear to result from convergent evolution.

Genomic overview of OPG biosynthesis

Genetic analyses of OPG synthesis were done in a limited number of bacterial species and OPG genes were defined with unrelated names: *mdo* in *E. coli* (56) and *chv* in *A. tumefaciens* (for chromosomal virulence) (44). Later, mutants were also obtained in other genera: *R. sphaeroides* (40) and *X. campestris* (41) where the genes were named *opg*. Complementation experiments were used to identify homologous genes in related organisms of the Rhizobiales group, essentially *S. meliloti* (42) and *B. japonicum* (48) where the genes were named *ndv*, for nodule development. This strategy also allowed the isolation of a locus similar to *chvB*, *cviB*, in *Azospirillum brasilense* while the *chv* and *ndv* nucleotide sequences of *A. tumefaciens* and *S. meliloti* were highly similar, they did not share any significant similarity with the *opg* genes. Moreover, the *ndv* genes found in *B. japonicum* share some similarities with some *opg* genes but no similarity with the *chv* genes. Thus, three distinct sets of genes were described and similar genes can be recognized in various completely sequenced genomes of Proteobacteria (Fig. 3). The first set of genes includes *opgG*, *opgH*, *opgD*, *opgI*, which synthesize linear OPGs. *opgG*,

opgH and *opgD* were described initially in *E. coli* and *opgI* in *R. sphaeroides*. Phylogenetic analyses were previously published for OpgH (40, 55), OpgG (11, 55) and OpgD (11). The second set of genes includes *ndvB*, *ndvC* and *ndvD*, initially described in *B. japonicum*, and the third includes *chvA* and *chvB*, initially described in *A. tumefaciens*. Both the second and third gene sets are involved in cyclic OPG synthesis.

Several conclusions can be drawn from the data shown in Fig. 3. Several bacterial species do not possess any of the OPG genes already described, as exemplified by *Yersinia pestis*. *Yersinia* species do not synthesize OPGs regardless of the presence and the expression of the *opgGH* operon (58). The *opgH* gene which encodes a protein belonging to the glycosyltransferase family 2 (59-61) is present in many bacteria of the alpha, beta, and gamma subdivisions. The *opgG* and *opgD* genes are diversely distributed with many possible combinations; *opgG* alone, *opgG* and *opgD*, or only *opgD*. In some cases, two copies of *opgH*, *opgG*, or *opgD* are detected. The *opgI* gene encodes another accessory protein only present in *Rhodobacter*. The combination of all three *ndvB*, *ndvC* and *ndvD* genes is found only in related alpha Proteobacteria in addition to *opgH* and *opgG* while *ndvB* and/or *ndvC* encoding proteins belonging to the glycosyl hydrolases family 17 (61-66) can also be found in *Pseudomonas*. The *chvB* gene encodes a protein belonging to the glycoside hydrolase of family 94 (61-66) and the *chvA* gene encodes an ABC transporter. These genes are found together among Rhizobiaceae and *Burkholderia*. The *chvA* gene is found in association with the *ndvB*, *ndvC* and *ndvD* genes for the transport of cyclic glucans. The *chvB* gene is found in various other bacteria. However, until the functions of such genes are demonstrated, their assignments to OPG synthesis should be approached with caution since they can encode biosynthetic as well as

degradative enzymes. For example, an *ndvB* defective mutant of *R. sphaeroides* synthesizes normal OPGs (40).

Since OpgB and OpgE are only found in *E. coli*, it's currently not possible to analyze the origin of these genes. OpgC was found and characterized in three different bacteria (19, 40, 55). In a phylogenetic analysis, two separate origins were demonstrated between *E. coli* and *D. dadantii*, while OpgC_{Rsp} was outside of these two groups. Both groups also had a specific synteny. Interestingly, a functional complementation occurred between *opgC_{Dda}* and *opgC_{Eco}* (55).

There is no simple correlation between the natures of the OPG genes present and the structures and substitution of OPG produced by a particular bacterial species and sequence-based predictions are not possible. Likely, the need for substitutions on OPGs may reflect the lifestyle of bacteria.

Regulation of OPG biosynthesis

OPG synthesis was shown to be osmotically regulated in a wide range of Proteobacteria (7, 14) except for *B. abortus* (23) and strains of *R. leguminosarum*. The observations in *R. leguminosarum* were complicated because these strains secrete large amounts of OPG when grown in media of high osmolarity, suggesting outer membrane modifications (see below) (22).

OPG amount increases as the osmolarity of the medium decreases to an extent depending on the species. In *E. coli*, periplasmic glucans represent up to 5% of the dry weight of the cell in a medium of low osmolarity (50 to 100 mosM), while it decreases to 0.5% in a medium of high osmolarity (600 to 700 mosM).

Osmotic regulation takes place at an early stage of OPG backbone biosynthesis at the transcriptional level and/or at the post translational level depending on the species considered. *In vitro*, the membrane bound activities found in extracts isolated from bacteria of *S. meliloti*, *R. leguminosarum*, *A. tumefaciens* and *E. coli* grown in media of low or high osmolarity were very similar and were very sensitive to elevation of ionic strength. In *E. coli*, the rate of OPG synthesis measured in a strain (*zwf*, *pgi* – Fig. 4) unable to synthesize UDP-glucose in the absence of glucose, falls abruptly when osmolarity increases suddenly (67). Increase of medium osmolarity correlates with an increase in the cytoplasm ionic strength. OPGs are necessary for hypoosmotic adaptation of most Rhizobiaceae, since mutants devoid of OPGs are impaired for growth in media of low osmolarity. This is consistent with an OPG synthesis regulated solely at the enzymatic level. Nevertheless, in *E. coli*, growth of mutants devoid of OPG remains unaffected in media of low osmolarity and when the osmolarity of the medium decreases suddenly, almost one generation time is necessary to adjust the level of OPG. This observation is inconsistent with a regulation limited to an enzymatic modulation. Thus, transcription of the *opgGH* operon of *E. coli* was found to be osmoregulated (32), and membrane bound activity of *A. brasilense* was reduced in extracts of cells grown in a medium of high osmolarity (47).

Independently of this osmotic regulation, a feedback regulation exerted by the end product was observed whatever the osmolarity is. After addition of glucose to the *E. coli zwf, pgi* strain, OPGs are accumulated rapidly until they reach the concentration observed in the wild type strain. *In vitro*, glucosyltransferase membrane bound activity of *R. leguminosarum* was severely reduced when 15 mM OPG was added. Thus, in the

secretory strains of this species, secreted OPGs escape from feedback control, explaining the large accumulation of OPG in the medium (68).

However, factors other than osmolarity affect the regulation of OPG biosynthesis in several bacterial species. OPG synthesis was found to be upregulated by a diffusible signal factor in *X. campestris* pv. *campestris* (69). The *opgH* gene was downregulated in *Xanthomonas axonopodis* pv. *citri* grown *in planta* (70), but *opgH* was found to be induced in *Xylella fastidiosa* during biofilm formation (71), and *opgG* is one of the 153 genes induced during growth of *Ralstonia solanacearum* in tomatoes (72). In *D. dadantii*, OPG biosynthesis is only regulated at the enzymatic level by an as yet unknown signal *in planta* (21) and osmolarity *in vitro* (15, 39). A previous study demonstrated that the amount of OPGs increased abruptly *in planta*, while the amount of both OpgG and OpgH protein remained unchanged (21, 73). Finally, the *opgGH* operon of *E. coli* was found to be activated by σ^E , the sigma factor which responds to misfolding of proteins in the envelope (74).

The *opgD* gene of *E. coli* is regulated by growth phase because OpgD was preferentially observed in stationary growth phase (75). This gene contains a cytosine base in position -13 of its promoter (one important characteristic of σ^S dependence), and is partially transcribed by the σ^S RNA polymerase (35). Factor σ^S is activated when growth slows down, particularly in stationary growth phase (76). However, *opgD* was not observed amongst the forty-one genes whose expression was reduced in a σ^S defective mutant (77). The *opgD* homolog of *X. axonopodis* pv. *citri* was shown to be overexpressed *in planta* (77).

In *E. coli*, none of the substitution genes were demonstrated to be osmoregulated (19, 52, 55). In *D. dadantii*, only succinylation was demonstrated to be osmoregulated, while acetylation of OPGs wasn't affected by the osmolarity or *in planta* (15, 55). *opgC_{Dda}* gene expression occurred at high osmolarity and required activation of both EnvZ-OmpR and RcsCDB phosphorelays (55). In *S. meliloti*, the *cgmB* gene, encoding the phosphoglyceroltransferase, was shown to be osmoregulated (78).

Regulation of OPG synthesis is complex not only because osmoregulation does not follow common features among species but it is complicated by the existence of additional regulatory factors.

Connection with trehalose synthesis

Trehalose is a non-reducing disaccharide of glucose. It is the most widespread disaccharide in nature, occurring in bacteria, fungi, insects, and plants (79). It serves as a carbon source and/or can be accumulated as a protectant against high osmolarity, heat, desiccation (80). In *E. coli*, externally supplied trehalose is only used as a carbon source and not as an osmoprotectant because of the synthesis of a high activity periplasmic trehalase (the product of *treA*) upon exposure to high osmolarity media and a PTS system synthesized at low osmolarity and repressed at high osmolarity (81). Thus, osmoprotective trehalose must be endogenously synthesized for accumulation in media of high osmolarity. Trehalose is synthesized from UDP-glucose (like OPGs) and glucose-6-Phosphate and accumulated up to 400 mM (Fig. 4) (80). Because large accumulation of OPGs and trehalose occurs in media of low and high osmolarity respectively, no competition for UDP-glucose availability exists for the synthesis of both kinds of

molecules. Since OPGs are never degraded within cells, they never serve as a glucose source for trehalose synthesis. On the contrary, one could imagine that trehalose serves as a glucose donor for OPG biosynthesis after hydrolysis by the cytoplasmic trehalase, the *treF* product, in media of low osmolarity despite its low affinity for trehalose (Km: 1.9 mM) (82). In *E. coli*, little is known about what becomes of trehalose once cells are in media of low osmolarity. Release of osmoprotectants such as betaine and trehalose is known to occur through mechanosensitive channels in response to osmotic downshock (83), thus it is possible to hypothesize the hydrolysis of trehalose by TreF. In *B. japonicum*, *in vivo* NMR studies on cells in stationary growth phase clearly reveal that no connection exists between OPGs and trehalose (84). After a hypoosmotic shock, OPGs are synthesized from glycogen in stationary growth phase and from glucose in exponential growth phase. When no glucose was present in the medium, synthesis of OPGs does not occur. Nevertheless, trehalose was synthesized in media of high osmolarity but was released into the medium during the hypoosmotic shock. Thus, the disaccharide cannot serve as a precursor for OPG biosynthesis. Because osmoprotectants are often released into the medium after an osmotic downshock and because OPGs cannot be degraded, one can consider that in a majority of bacterial species, trehalose and OPGs cannot serve as a precursor to each other.

Pleiotropic phenotypes of OPG defective mutants

OPG defective mutants characterized in various bacterial species have in common a highly pleiotropic phenotype, which is indicative of a global alteration of their envelope properties. In *E. coli* and *S. enterica* but also in most species tested, Opg⁻ colonies

generally exhibit a mucous phenotype and a defect in motility and chemotaxis. This defect was shown to be the consequence of a reduced number of flagella in *E. coli* (85, 86). A proteomic analysis of Opg⁻ mutants of *D. dadantii* confirmed several years later by the same approach in *S. enterica* indicated a constitutive induction of a general stress response and an altered envelope structure (87, 88). In a similar way, Opg⁻ mutants showed increased sensitivity to hydrophobic antibiotics in *S. meliloti* (89), biliary salts in *D. dadantii* (39), sodium dodecyl sulfate in *E. coli* (90), or increased resistance to endogenously produced lysis protein of phage MS2 in *E. coli* (91). Depending upon medium composition, other phenotypic changes were found in envelope proteins in *E. coli* (85) and *A. tumefaciens* (92). Moreover, Opg⁻ mutants of *D. dadantii* produce and secrete lower amounts of plant cell wall-degrading enzymes (proteases, cellulases and pectate lyases) (39).

The doubling times for growth in hypoosmotic media were twice as higher for Opg⁻ mutants of *A. tumefaciens* (92) and *S. meliloti* (89) compared to the wild type, and increasing the medium osmolarity compensated for that defect. In contrast, growth of Opg⁻ mutants in *E. coli* (52), *D. dadantii* (39) or *B. Japonicum* (50) is only slightly affected.

Several groups have isolated suppressor mutations capable of restoring a wild-type phenotype for Opg⁻ mutants. Some *E. coli* chemotactic pseudorevertants mainly involved in osmoregulation of the outer membrane porins (85) were mapped by transduction in the *envZ-ompR* locus. However, this locus is not involved in regulation of OPG synthesis. Similarly, pseudorevertants of *S. meliloti ndv* mutants were selected for restoration of osmotolerance, motility, or symbiosis. Pseudorevertants for vegetative

properties regained only minor symbiotic ability while symbiotic pseudorevertants were unrestored for vegetative properties and remained highly impaired in the first steps of the symbiotic interaction (89).

Thus, in some cases, OPGs may serve in osmoadaptation but they also appear to play other roles unrelated to osmoadaptation. This is not surprising since OPG synthesis is regulated by additional factors in several bacteria.

OPGs and pathogenicity

Mutants defective in OPG synthesis were primarily obtained during the screening or the selection of attenuated or avirulent mutants of plant or animal pathogens. Thus, mutants of *A. tumefaciens* (*chvA* and *chvB*, (44)), *P. syringae* pv. *syringae* (*hrpM*, an OpgH homolog (93)) and *X. campestris* pv. *vesicatoria* (*opgH*, (41)) are completely or severely impaired in their virulence toward their host plants. The *P. syringae* mutant is severely impaired in its ability to grow in the plant host and failed to elicit a nonhost hypersensitive response when inoculated on tobacco. Similarly, mutants of *S. meliloti* (42) form defective nodules where tissue differentiation occurs in the absence of bacterial invasion. As mentioned earlier, OPGs can be recovered in the external medium under certain conditions. However, addition of purified OPGs to the inoculum cannot restore attachment and virulence or symbiotic ability of Rhizobiaceae Opg⁻ mutants.

Random mutations affecting OPG biosynthesis were also recovered during investigation of animal pathogens. *opgH* was found among chromosomal loci expressed by *Y. enterocolitica* at an early stage of the infection process (94). However, inactivation of the *opgGH* operon in *Y. pseudotuberculosis* didn't affect the virulence (58).

Furthermore, *Y. pestis* expressing *opgGH* grew normally and was able to complete its infectious cycle (flea-host) (58). Knowing that OPGs cannot be detected in other *Yersinia* species, even if the *opgGH* operon is expressed, , suggests that the role of OPGs was lost in an ancestral *Yersinia* strain.

One of several *P. aeruginosa* PA14 mutants severely impaired in virulence toward *Caenorhabditis elegans* possesses transposon insertion in *opgH* (95). This mutant also has a dramatic effect in a mouse model, causing no mortality, and is severely affected in its ability to grow in *Arabidopsis* leaves.

A mutant of *P. aeruginosa* was identified that, while still capable of forming biofilms with normal architecture, does not develop high-level biofilm-specific resistance to antibiotics (96). The mutation was identified in the *ndvB* gene (see Fig. 3) and the mutant is impaired in the synthesis of cyclic glucan. Is this glucan corresponding to OPGs? While the authors accept this hypothesis, and discuss the possibility that its cyclic nature allows sequestration of antibiotics, we can raise objections. OPGs of the closely related species, *P. syringae*, belong to family I and both species have highly similar *opgGH* genes. Moreover, in *R. sphaeroides*, genes similar to *opgGH* and to *ndvB* coexist (see Fig. 3), and only the former is involved in the synthesis of a cyclic OPG. Thus, further structural investigations are necessary. Nonetheless, one can imagine that *P. aeruginosa* can produce two kinds of OPGs, either simultaneously or independently in response to different environmental signals.

The implication of OPGs in pathogenicity was further confirmed by directed inactivation of *opg* genes in various species. Thus, nodule development was severely impaired in *ndvB* mutants of *B. japonicum* which are devoid of OPGs, and to a lesser

degree in *ndvC* mutants which produce OPGs with altered structures (97). Arellano-Reynoso *et al.* have reported that Opg⁻ mutants of *B. abortus*, an intracellular pathogen, are unable to evade phagosome-lysosome fusion (98). In pathogenic strains of *E. coli* devoid of OPG, virulence is affected (unpublished data) and in *S. enterica*, virulence of Opg⁻ mutants is severely reduced (86). Similarly, Opg⁻ mutants of *D. dadantii* are non-virulent on potato tubers, chicory leaves, and carrots, and this lack of virulence was correlated with their inability to grow and to survive in the plant host (39, 99).

In conclusion, every time their role in host-bacteria interactions was tested, OPGs appeared to be essential component of the bacterial envelope necessary for virulence or symbiosis.

OPGs and stress signaling

OPGs appear to be important intrinsic components of the Gram-negative bacterial envelope, which can be essential in extreme conditions found in nature, especially when bacteria must interact with a eukaryotic host. But, what is the fundamental function of these compounds?

A Periplasmic osmoprotectant?

Stock *et al.*, in their seminal study of the enterobacterial periplasm, have shown this compartment to comprise 20 to 40% of the total cell volume (100) and that a Gibbs-Donnan equilibrium exists between the periplasm and the extracellular medium, allowing isoosmolality between periplasm and cytoplasm (101). Thus, E. P. Kennedy proposed that OPGs function as periplasmic osmoprotectants on the basis of their anionic character, where OPGs would participate in the Gibbs-Donnan equilibrium through the

outer membrane, and he found, accordingly to this hypothesis, that OPG synthesis is osmoregulated in *E. coli* (102).

However, conflicting data and interpretations have been published concerning the volume and concentration of periplasm (84, 103-106). It is necessary to make a distinction between transient states following rapid upshift or downshift of external osmolarity, and steady-state adaptation to a particular medium where the bacterial cell can grow. Only the first situations can be considered as stress conditions essentially because fluxes of water are extremely rapid, as are those of small molecules in the case of abrupt downshift in osmolarity. Cayley *et al.* have calculated the OPG concentrations in steady-state cells of *E. coli* grown at different external osmolarities (103). The obtained values, from 50mM at 70 mosM to 3 mM at 800 mosM, are in the same range as the estimation of 15 mM reported for cells of the family Rhizobiaceae (22). The amount of OPGs is sufficient to quantitatively explain the observed Gibbs-Donnan potential maintained across the outer membrane (107). However, even in the case of *E. coli*, the anionic character of OPGs is only effective when ionic strength is high (104), and OPGs produced by several other bacteria are not charged molecules. Moreover, *E. coli* mutants defective in OPG substitution (*opgB*, *opgC*, *opgE*), as well as mutants defective in OPG backbone synthesis (*opgH* or *opgD*), do not show a particular osmosensitive phenotype (56) (unpublished data). More intriguing is the fact that during osmotic stress OPG concentration is only slowly adjusted to that found in steady-state: after abrupt downshift it takes at least half a generation time for *E. coli* cells to accumulate OPGs up to the ten-fold-higher steady-state level (67), and following upshift, OPG content decreases to a ten-fold-lower level by dilution due to successive cell divisions, because degradation of

OPGs was never observed (14, 108). Thus, osmoregulation of OPG synthesis, when it exists, appears more likely to be a way to detect diluted media rather than a true osmoadaptation.

A structural role?

OPGs may have a structural role in the envelope organization similar to carbohydrate molecules interacting with other structural components like phospholipids and/or peptidoglycan (109). The periplasm is generally described as a highly viscous gel-like compartment where peptidoglycan, protein junctions between inner and outer membranes, periseptal and polar annuli would drastically limit molecular movements. However, evidence of free movement of the jellyfish protein within the periplasm have been reported (110). Similarly, the use of high-resolution NMR spectroscopy under magic angle spinning revealed that the major fraction of OPGs detected *in vivo* in *R. solanacearum* undergoes significant rotational diffusion (111); they are not associated with macromolecules.

OPGs could be seen as molecular agents necessary to maintain a minimal periplasmic space at low medium osmolarity. However, *E. coli* Opg⁻ mutant cells exhibited larger periplasmic space than wild type cells when subjected to gentle plasmolysis (91). Thus, they seem necessary to maintain interactions between the outer and inner membranes. OPGs induce the porin channels to close (112), while changing the Gibbs-Donnan potential has no effect on the permeability of porin channels (107).

A role in protein folding, association and function?

OPGs by their presence may participate in a macromolecular crowding determinant for protein folding, association and function (113). From this point of view,

it is noteworthy that *opgGH* transcription is controlled, at least in part, by the sigma E factor whose synthesis is induced following accumulation of unfolded proteins in the periplasm. An *opgH* mutant exhibits partially increased expression of *osmY* (114). Similarly, in a *dsbA* mutant that does not form disulfide in the envelope proteins, a stress is perceived that results in a reduced amount of OpgG and a higher amount of OsmY , two proteins which do not contain disulfide bonds (115). The *dsbA* cells behave as if they perceived a high external osmolarity.

The feedback regulation of OPG synthesis observed in *E. coli* cannot be explained by a simple hypothesis. A similar control is exerted at low and high external osmolarity at different OPG periplasmic concentrations. Since the membrane-bound glucosyl transferase OpgH is the ultimate target of this control, one can envisage that OpgH directly or indirectly senses a particular subfraction of the OPGs with similar properties, whatever the external conditions. Alternatively, OpgH, as other membrane-bound proteins, could sense mechanical deformation or alterations in the physicochemistry of the membrane bilayer (116) due to OPG accumulation.

A role in cell signaling?

OPGs may also have a function as informational molecules sensed by specific proteins present in the periplasmic compartment or bound to one or the other membrane. As discussed previously, when bacteria are shifted from a diluted medium to a concentrated environment, OPGs are slowly diluted. During the first steps of infection, having been exposed previously to media of low osmolarities, bacteria would possess, in their periplasmic space, OPG concentrations higher than those characteristic of their new environment. If OPG concentration could be monitored by one or several sensor proteins,

it could be used as a kind of internal counter of the number of cell division, and, indirectly, of bacteria at low population densities. This could control the expression of a set of genes in response to an increase in cell numbers and it would be, in some way, the counterpart of the quorum sensing systems involved in the control of gene expression at high cell density.

OPGs were proposed as a possible signal for two *E. coli* inner membrane sensor proteins: EnvZ (85) and RcsC (117). Several phenotypic changes observed in OPG defective cells (e.g., increased synthesis of exopolysaccharides and decreased number of flagella) can result from activation of the RcsCDB sensor/regulator system (118). Genes belonging to the RcsCDB regulon appear essential to control the timing of infection processes and formation of biofilms. According to the proposed model, RcsC (a histidine kinase) and RcsD sense a signal and cooperate in a phosphorelay to phosphorylate the response regulator RcsB. In an *opgH* mutant this system is constitutively activated, and RcsC or/and RcsD could sense directly the OPGs present in the periplasm (117).

Recently, the concentration of OPGs was demonstrated to directly modulate the activation level of the RcsCDB system in *D. dadantii* (21, 119, 120). In this bacterium, RcsCDB activation is deleterious for virulence. Thus, OPG concentration dramatically increases at the early step of the infectious process to strongly repress the histidine kinase activity of RcsC (21). Moreover, RcsCDB and EnvZ-OmpR (37) controlled the succinylation of OPGs in *D. dadantii* (55). This data strengthen the interconnection between OPGs and the RcsCDB system in Enterobacteria. A relationship between OPGs and the RcsCDB phosphorelay was not demonstrated in *E. coli* or *S. enterica* but Opg⁻

phenotypes observed for these bacteria strongly suggest a similar control of the RcsCDB phosphorelay by OPGs in these species.

Interestingly, *Y. pestis*, closely related to *D. dadantii*, lost the *opgGH* operon during the evolution from *Y. pseudotuberculosis* but the RcsCDB system of *Y. pestis* is also altered by a mutated *rcsD* gene (121). Taken together, both events - *i.e.* inactivation of the two sides of the same system - could be linked to ensure new virulence attributes of *Y. pestis*.

OPGs and cell division

Finally, OPGs may also be involved in cell size control. Recently, Hill *et al.* demonstrated that OpgH interacts with the FtsZ protein, one of the main components of the cell division ring (122). OpgH sequestered FtsZ to modulate the division and size of the cell depending on the nutrient availability, as sensed by OpgH through UDP-glucose concentration. An identical role is suggested in *Y. pseudotuberculosis*. An *opgH* mutant of *Y. pseudotuberculosis* was smaller than the wild-type (58). Interestingly, Hill *et al.* compared OpgH and UgtP from *Bacillus subtilis*. UgtP is an enzyme required for synthesis of the diglucosyl-diacylglycerol anchor for lipoteichoic acid (LTA), one of the major components of the Gram-positive cell wall (123, 124). UgtP was shown to inhibit FtsZ assembly and control cell size and growth rate (125). Even if both OpgH and UgtP don't share homology or similar enzymatic properties, they are functional homologs (122). It was speculated that OPGs and LTA could play similar roles in Gram negative and Gram positive bacteria, respectively (122). All these data suggest a more complex role for OPGs and proteins involved in their biosynthesis than expected 45 years ago...

Acknowledgments

Jean-Marie Lacroix' group is funded by the University of Lille, the French National Center for Scientific Research, the French National Research Agency. S. Bontemps-Gallo is supported by the Intramural Research Program of the National Institute for Allergy and Infectious Diseases, National Institutes of Health. We thank Ryan Kissinger for help with graphics. We thank Dr. Ciaran Finn for English corrections.

Figure 1. Backbone structure of the four families of OPGs from Bohin, 2000 (7)

Figure 2. Working model of the OPG biosynthetic complex of *E. coli*. The variety of backbone structures and patterns of substitution are schematically represented (see text for details). Ptd-Gro: phosphatidylglycerol, Ptd-Etn: phosphatidylethanolamine, DG: diacylglycerol, Suc-CoA: succinyl-coenzyme A, CoA: coenzyme A, and UDP: uridine-diphosphate.

Figure 3. Occurrences of OPG genes in various representative genomes. Protein-protein BLAST program was used to search in the non-redundant databases sequences highly similar to OpgG, OpgH and OpgD from *E. coli*, OpgI from *R. sphaeroides*, NdvB, NdvC and NdvD from *B. japonicum*, and ChvB and ChvA from *A. tumefaciens*, OpgB and OpgE from *E. coli*, OpgC from *E. coli* and *D. dadantii*, and CgmB from *S. meliloti*.

Figure 4. Schematic of UDP-Glucose production, utilization and connection with trehalose and OPG biosynthesis in *E. coli*. The Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to establish the metabolic pathway (126, 127). Glucose is transported into the cell via the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS). During uptake, glucose is activated and transformed into Glucose-6-P. Glucose-6-P is used by Pgm and GalU enzymes to produce Uridine-diphosphate (UDP)-Glucose (in red). The UDP-Glucose can be used either to produce OPGs via the OpgH/OpgG complex or Trehalose-6-P via OtsA. Trehalose-6-P is transformed into Trehalose by OtsB enzyme. Then, it can either be excreted via the stretch-activated proteins (SAP) into the periplasm or maintained inside the cell. In both

cases, the trehalose will be degraded in 2 glucoses by TreF in the cytoplasm or TreA in the periplasm. P: Phosphate, OPG: Osmoregulated Periplasmic Glucan.

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