Biosynthesis of osmoregulated periplasmic glucans in *Escherichia coli*: the membrane-bound and the soluble periplasmic phosphoglycerol transferases are encoded by the same gene

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In *Escherichia coli*, osmoregulated periplasmic glucans (OPGs) are highly substituted by phosphoglycerol, phosphoethanolamine and succinyl residues. A two-step model was proposed to account for phosphoglycerol substitution: first, the membrane-bound phosphoglycerol transferase I transfers residues from membrane phosphatidylglycerol to nascent OPG molecules; second, the periplasmic phosphoglycerol transferase II swaps residues from one OPG molecule to another. Gene *opgB* was reported to encode phosphoglycerol transferase I. In this study, we demonstrate that the periplasmic enzyme II is a soluble form of the membrane-bound enzyme I. In addition, timing of OPG substitution was investigated. OPG substitution by succinyl residues occurs rapidly, probably during the backbone polymerization, whereas phosphoglycerol addition is a very progressive process. Thus, both phosphoglycerol transferase activities appear biologically necessary for complete OPG substitution.

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INTRODUCTION

Escherichia coli, like other Gram-negative bacteria, accumulates in its periplasmic space large amounts of short glucans (5–12 glucose units) in response to low osmolarity of the growth medium (Bohin & Lacroix, 2006). In *E. coli*, osmoregulated periplasmic glucans (OPGs) consist of a linear backbone made of β -1,2-linked glucose units to which several β -1,6-linkage branches are added. This backbone is highly substituted by *sn*-1-phosphoglycerol, by phosphoethanolamine residues derived from membrane phospholipids, and by *O*-succinyl ester of unknown origin (Kennedy, 1996). OPGs were described for the first time in *E. coli* by E. P. Kennedy and his coworkers during their study of phospholipid turnover (Van Golde *et al.*, 1973), and they called them 'membrane-derived oligosaccharides' (MDOs) because of the membrane origin of the phosphoglycerol substituents.

Four genes are known to participate in OPG biosynthesis: two are needed for formation of the backbone (*opgGH*; Lacroix *et al.*, 1991) and two are needed for its substitution by phosphoglycerol (*opgB*; Jackson *et al.*, 1984; Lanfroy & Bohin, 1993) and succinyl residues (*opgC*; Lacroix *et al.*, 1999). Phosphatidylethanolamine, exogenously added to intact cells, was shown to be a source of phosphoethanolamine residues that are transferred to OPGs (Miller &

Kennedy, 1987), but this activity was not further characterized because attempts to develop an *in vitro* assay were unsuccessful, and its gene remained unidentified. Actually, only two enzyme activities have been accurately defined *in vitro*, both of them being involved in the phosphoglycerol substitution of OPGs.

The first described phosphoglycerol transferase was a soluble protein found in the periplasmic space (Goldberg *et al.*, 1981). This enzyme was partially purified and has an apparent molecular mass of 56 000 in gel permeation chromatography. *In vitro*, it catalyses the transfer of phosphoglycerol to unsubstituted OPG molecules or model β -glucosides in the presence of Mn^{2+} . However, the enzyme is unable to use phosphatidylglycerol as the donor substrate, but it catalyses the interchange of phosphoglycerol residues between OPG molecules. Moreover, at low concentration of acceptor, the enzyme acts as a cyclase with the liberation of cyclic phosphoglycerol.

The second described activity was a membrane-bound phosphoglycerol transferase. *In vitro*, the enzyme catalyses the transfer of phosphoglycerol residues from phosphatidylglycerol to OPGs or to synthetic β -glucoside acceptors such as arbutin (Jackson & Kennedy, 1983). *In vivo*, the transfer occurs also to arbutin, which is not transported or degraded by the bacterium. Thus, this enzyme should have its catalytic site present on the periplasmic side of the cytoplasmic membrane (Bohin & Kennedy, 1984).

Abbreviation: OPG, osmoregulated periplasmic glucan.

Jackson & Kennedy (1983) proposed a two-step model in which phosphoglycerol residues would be first transferred by the membrane-bound phosphoglycerol transferase I to newly synthesized glucans, still linked to the membrane. Then, the periplasmic phosphoglycerol transferase II would transfer those residues from one molecule of OPG (potentially still an acceptor in the first step) to another already liberated in the periplasmic space.

Mutants in the phosphoglycerol transfer (Jackson *et al.*, 1984) were isolated on the basis of the toxic accumulation of diacylglycerol resulting from addition of arbutin to the growth medium of a diacylglycerol kinase mutant (*dkg*). As expected from the model, the OPGs synthesized by these *opgB* mutants were devoid of phosphoglycerol. Moreover, crude extracts from an *opgB* strain still possessed the ability to remove phosphoglycerol residues from wild-type OPGs.

The *opgB* gene was cloned and localized immediately adjacent to the *dnaTC* operon (Lanfroy & Bohin, 1993). Further characterization of the *opgB* gene product was hindered by an unusual instability of the protein, most of which was recovered in a soluble form. Thus, we decided to re-examine the relationship between the two enzymes.

METHODS

Bacterial strains and media. The *E. coli* K-12 strains and plasmids used in this study are listed in Table 1. Bacteria were grown at 37 °C with vigorous shaking in Luria broth (LB) or in minimal medium 63 supplemented with the required metabolites and glucose as the carbon source (Miller, 1992). Solid media were obtained by adding agar (15 g l⁻¹). When low-osmolarity medium was required, low-osmolarity medium (LOS) or LB without NaCl was used (Lacroix *et al.*, 1989). Antibiotics in media were used at the following concentrations: ampicillin, 50 µg ml⁻¹; kanamycin, 50 µg ml⁻¹; and tetracycline, 25 µg ml⁻¹. X-Gal was used in media at the concentration of 20 µg ml⁻¹.

Transduction and transformation. Transduction by phage P1vir was carried out according to Miller (1992). *E. coli* cells were made competent by the rubidium chloride technique (Miller, 1992).

Recombinant DNA techniques. Standard procedures (Sambrook *et al.*, 1989) were used for λ DNA isolation, large-scale plasmid isolation and rapid analysis of recombinant plasmids. Restriction endonucleases (Biolabs), large (Klenow) fragment of DNA polymerase I, S1 nuclease and ligase of phage T4 (Gibco-BRL) were used according to the manufacturer's recommendations. Exonuclease III was used according to the method of Debarbieux *et al.* (1997).

DNA sequencing. Double-stranded *opgB-blaM* fusion plasmid DNAs were sequenced according to the Sequenase version 2.0 sequencing protocol (United States Biochemicals). The sequencing primer hybridizing at the 5' end of *blaM* (5'-CCCACTCGTGCAC-CCAACT-3') was produced by Eurogentec.

Plasmid construction. The *opgB* gene was cloned as follows. λ phage 8D1 DNA (Kohara *et al.*, 1987) was first digested by *Sall* and *KpnI* (Fig. 1) and the 3.6 kb DNA fragment containing the *opgB* gene was cloned into pBGS18 digested by *Sall* and *KpnI* to give pNF601. Then, the 2.6 kb *HindIII* DNA fragment of pNF601 was cloned into the *HindIII* site of pYZ4 (Debarbieux *et al.*, 1997) to give pNF604. For the construction of random fusion plasmids, the 3.2 kb *SphI* DNA fragment of pNF601 was cloned into the *SphI* site of pNF150 (Loubens *et al.*, 1993), thus placing the *opgB* gene upstream of a truncated version of *blaM* (pNF673). The resulting plasmid, pNF673, was digested by *SstI* and *Sall* before digestion by exonuclease III, allowing deletions of the *opgB* gene only. Two additional *opgB-blaM* fusions were generated as follows by direct cloning into restriction sites. For pNF710, plasmid pNF601 was digested by *KpnI* and *DraI* and the 1.2 kb DNA fragment was cloned into pNF150 digested by *KpnI* and *EcoRV*. For pNF717, plasmid pNF601 was digested by *EcoRI* and *BamHI*, blunt-ended by the large fragment of DNA polymerase I, and the 2.7 kb DNA fragment was cloned into pNF150 digested by *XbaI* and blunt-ended by the large fragment of DNA polymerase I. For the expression of a truncated version of OpgB deleted of its two first transmembrane segments, pNF604 was digested by *BamHI* and blunt-ended by S1 nuclease, thus deleting a 340 bp DNA fragment to give pNF721. Plasmid pNF596, in which *opgB* is fused to *blaM* after the codon for residue 164, was similarly engineered to give pNF723.

Table 1. Bacterial strains and plasmids

Strain	Genotype	Source or reference
BB2636	HfrC, <i>glpD3 glpK4 glpR2 plsB26 phoA fhuA22 rel-1</i>	Bell (1974)
DF214	<i>his pgi::Mu Δ(zwf-edd)1 eda-1 rpsL</i>	Vinopal <i>et al.</i> (1975)
JM83	<i>Δ(lac-pro) ara rpsL thi φ80(ΔlacZ-M15)</i>	Vieira & Messing (1982)
678	<i>proC trp tsx xyl mtl ΔphoA8 rpsL</i>	Laboratory collection
NFB758	BB2636, <i>opgB214::Tn10</i>	This work
NFB732	JM83, <i>opgB214::Tn10</i>	This work
NFB1814	678, <i>opgB214::Tn10</i>	This work
Plasmids	Relevant genotype	
pNF601	<i>opgB</i> gene	This work
pNF604	<i>opgB</i> gene under control of the <i>lac</i> promoter	This work
pNF673	<i>opgB</i> gene upstream of a truncated version of <i>blaM</i> (generating fusion P ₁₆₄ to P ₇₄₇)	This work
pNF710	<i>opgB-blaM</i> (fusion P ₄₁)	This work
pNF717	<i>opgB-blaM</i> (fusion P ₉₇)	This work
pNF721	Truncated form of <i>opgB</i> (OpgB deleted of its two first transmembrane segments)	This work
pNF723	Truncated form of <i>opgB-blaM</i> (P ₁₆₄ deleted of its two first transmembrane segments)	This work

Fig. 1. Left top: genetic organization of the *opgB* region. Genes are indicated by black arrows. Relevant restriction sites are indicated and transcription terminators are shown as stem-loop structures. Middle: organization of the OpgB protein. The three transmembrane segments are coloured black and numbered. The cytoplasmic domains are hatched. The periplasmic domains are coloured white except for the sulfatase Pfam domain (Bateman *et al.*, 2002), which is coloured grey. Each *opgB*–*blaM* gene fusion site is indicated. The location of the corresponding β -lactamase domain (Debarbieux *et al.*, 1997) is abbreviated as follows: mp, membrane-bound periplasmic; mc, membrane-bound cytoplasmic; p, soluble periplasmic. Bottom: nucleotide and amino acid sequences of the beginning of the *opgB*. The potential ribosome-binding site is coloured black. The three putative transmembrane segments are boxed and relevant restriction sites are indicated. Right: model of the OpgB protein based upon the fusion results. The three transmembrane segments are numbered.

Preparation of [2-³H]glycerol-labelled OPGs. The glycerol auxotrophic strain BB2636 was grown in LOS medium (5 ml) complemented with 0.45 mM [2-³H]glycerol (1850 MBq mmol⁻¹). OPGs were extracted as described previously (Lacroix *et al.*, 1989) and purified as follows. Pyridine extract was chromatographed on a Biogel P4 column (Bio-Rad). The column (1.5 cm × 65 cm) was equilibrated with 0.5% acetic acid and eluted at a rate of 15 ml h⁻¹ in the same buffer. Fractions (1.5 ml) containing OPGs were pooled, concentrated by rotary evaporation and desalted on a Biogel P2 column (Bio-Rad). Fractions containing OPGs were pooled and concentrated by rotary evaporation. These OPGs were used in the cyclic phosphoglycerol assay (see below) as a donor substrate of phosphoglycerol.

Assay of OPG substitution with [2-³H]glycerol. The glycerol auxotrophic strain BB2636 and its *opgB* derivative (NFB758) were grown in LOS medium (5 ml) complemented with 1.35 mM [2-³H]glycerol (296 MBq mmol⁻¹) and OPGs were extracted from overnight cultures as described previously (Lacroix *et al.*, 1989).

Determination of neutral and anionic characteristics of OPGs. Cultures (5 ml) of DF214 were grown in LOS medium; 0.24 mM D-[U-¹⁴C]glucose (125 MBq mmol⁻¹) was added in the exponential growth phase for various times and the reaction was stopped by adding 1 ml 30% TCA. OPGs were then extracted and purified as described above and desalted on a PD10 column (Pharmacia Biotech) equilibrated with Tris/HCl 10 mM (pH 7.4). Fractions containing OPGs were pooled and chromatographed on a DEAE-Sephacel column (1.5 cm × 38 cm, Pharmacia Biotech) equilibrated with Tris/

HCl 10 mM (pH 7.4) and eluted with the same buffer containing increasing concentrations of NaCl ranging from 0 M to 1 M by steps of 0.05 M. A volume of 60 ml was used for each NaCl concentration and the volume of each collected fraction was 4 ml. When necessary, fractions of one peak were pooled and the succinyl substituents of OPGs were removed by incubation in 100 mM KOH for 2 h at 30 °C. Treated OPGs were then desalted and chromatographed on a DEAE-Sephacel column as previously described.

Determination of ampicillin resistance of cells expressing β -lactamase fusion proteins. The ampicillin resistance of individual cells of NFB732 containing *opgB*–*blaM* fusion plasmids was determined according to Debarbieux *et al.* (1997).

Cellular location of fusion proteins. Soluble or membrane-bound location of fusion proteins was determined according to Loubens *et al.* (1993).

Partial purification of periplasmic proteins. Cells of strain 678 and derivatives were grown in LB without NaCl (100 ml). When cell densities reached 6×10^8 cells ml⁻¹, periplasmic protein release was performed according to Loubens *et al.* (1993). One hundred microlitres of BSA (50 mg ml⁻¹) was added to the periplasmic protein-containing supernatant as a tracer. This supernatant was chromatographed on Sephadex G-50 (1 cm × 18 cm) at 4 °C. Protein content of each fraction of 1 ml was measured as A₂₆₀. Fractions containing proteins (7 ml) were pooled, partially purified and concentrated in Macrosep 30K tubes (Pall Filtron) at 3000 g for 5 h at 4 °C to a final volume of 350 μ l.

Cyclic phosphoglycerol assay. The method was adapted from Goldberg *et al.* (1981), in which the activity of the enzyme was measured by cleavage and formation of cyclic phosphoglycerol from OPGs. The incubation buffer contained 50 mM Tris/HCl (pH 7.8), 0.25 mM MnCl_2 , 2.5 mg BSA ml^{-1} and 3.5 μM $[2\text{-}^3\text{H}]\text{glycerol}$ -labelled OPGs (285 000 d.p.m. nmol^{-1}). The final volume of reaction was 200 μl . After addition of periplasmic proteins (145 μl), samples were incubated at 37 °C for various times. The reactions were stopped by the addition of 1 ml charcoal suspension (Norit A, 20 mg ml^{-1} in distilled water). Samples were then vigorously agitated for 10 min. Unlike OPGs, cyclic phosphoglycerol is not adsorbed on charcoal. Thus, the charcoal-containing labelled OPGs was removed by centrifugation at 8000 g for 10 min. Radioactivity of the supernatant, containing $[2\text{-}^3\text{H}]\text{glycerol}$ -labelled cyclic phosphoglycerol liberated by the enzyme, was counted. Activity was calculated according to Goldberg *et al.* (1981).

RESULTS AND DISCUSSION

Nucleotide sequence analysis of the *opgB* gene

The open reading frame of the *opgB* gene encodes a 763 amino acid polypeptide beginning and ending at a TTG codon and a TAA codon respectively. Seven nucleotides upstream of the TTG codon is found a putative ribosome-binding site (Fig. 1). Eleven nucleotides downstream from the TAA is found a putative transcription terminator consisting of two inverted repeats of 12 nucleotides. Transcription analysis of the *dnaTCyjjA* operon, located upstream of the *opgB* gene, was reported (Masai & Arai, 1988). The majority of the transcripts pass through a terminator downstream of *dnaC* and terminate downstream of *yjjA* (Fig. 1). When introduced into strain NFB732 (*opgB214::Tn10*), plasmids pNF601 (*SphI*–*Sall*) and pNF604 (*HindIII*–*Sall*) (Fig. 1) complemented the phosphoglycerol transferase I defect (data not shown), indicating that the 2.6 kb insert of pNF604 contains the full-length *opgB* coding sequence. The *HindIII* site is located close to the ribosome-binding site, suggesting that in pNF604 the *opgB* promoter is missing and the open reading frame is transcribed from the *lac* promoter of the pYZ4 vector. For location of the promoter, two deletions of one *opgB*–*blaM* plasmid fusion (protein fusion point downstream of amino acid 164, see below) were performed. Strains harbouring plasmids with the *XmnI* deletion (removing the *lac* promoter of the plasmid vector) or plasmids with the *SphI*–*HindIII* deletion (see Fig. 1) remained ampicillin resistant. Strains harbouring plasmids with both deletions became ampicillin sensitive, indicating that the *opgB* promoter is located upstream of the *HindIII* site. Taken together, these data suggested that the *opgB* promoter is located between the *yjjA* terminator and the *HindIII* site (Fig. 1).

Amino acid sequence analysis of OpgB and prediction of its topology

OpgB is a protein with a deduced molecular mass of 85 494 Da. This is in agreement with the apparent

molecular mass of 84 000 Da calculated from SDS-PAGE during the analysis of gene products located at the vicinity of the *dnaTCyjjA* operon (Masai & Arai, 1988). The deduced protein OpgB was found to be a member of the alkaline phosphatase superfamily of metalloenzymes (Galperin *et al.*, 1998) and its amino acids 163–448 form a conserved sulfatase domain (Pfam00884, <http://pfam.jouy.inra.fr>, Bateman *et al.*, 2002). The dense alignment surface (DAS) method (<http://www.sbc.su.se/~miklos/DAS>, Cserzo *et al.*, 1997) and the TopPred II algorithm (<http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html>, Claros & von Heijne, 1994) allowed the prediction of the same three or four membrane-spanning segments in the first 124 amino acids (Fig. 1). We have no evidence for the existence of the putative transmembrane segment located in the vicinity of the N-terminal part of OpgB, which is not represented in Fig. 1. Four consecutive arginine residues are found in a 9 amino acid sequence between the two last transmembrane segments. Taking into account the ‘positive inside’ rule, one can deduce that the hydrophilic C-terminal part of the protein (amino acids 125–763) is responsible for its catalytic activity on the periplasmic face of the membrane (Bohin & Kennedy, 1984). To test the validity of this model (Fig. 1), β -lactamase was used as a topological reporter.

Analysis of *opgB*–*blaM* gene fusions

Twenty-three in-frame *opgB*–*blaM* fusions were obtained after exonuclease III digestion (see Methods). The position of the β -lactamase moiety (cytoplasmic or periplasmic) was determined for each fusion by streaking the colonies, up to individual cells, on LB agar plates containing 50 μg ampicillin ml^{-1} . All the fusions conferred resistance on individual cells, indicating that these strains have a β -lactamase moiety in the periplasmic space. The fusion point between the *opgB* and truncated *blaM* genes of the 23 fusions was determined by DNA sequencing. Then, nine of them, regularly spaced over the OpgB protein, were further characterized. Protein fusion points were downstream of amino acids 164 (P₁₆₄), 228 (P₂₂₈), 307 (P₃₀₇), 371 (P₃₇₁), 446 (P₄₄₆), 529 (P₅₂₉), 574 (P₅₇₄), 652 (P₆₅₂) and 747 (P₇₄₇) (i.e. after transmembrane segment 3, see Fig. 1). This random procedure did not give a fusion in the first 163 amino acids. Two *opgB*–*blaM* fusions were then generated by direct cloning into restriction sites (see Methods) with fusion points downstream of amino acids 41 (P₄₁) and 97 (P₉₇) (i.e. at the end of the predicted transmembrane segments 1 and 2 respectively, see Fig. 1). Ampicillin resistance conferred by these two fusions indicated that the β -lactamase moieties were periplasmic and cytoplasmic respectively (Fig. 1). To confirm the location of each fusion protein, crude extracts were prepared from cells of strain NFB732 harbouring the corresponding plasmids and the β -lactamase activity was determined in the supernatant (soluble activity) and the pellet (insoluble membrane-bound activity) after centrifugation at 150 000 g for 2 h as described previously (Loubens *et al.*, 1993). As expected, most of the β -lactamase activity was recovered in the pellet for the two first fusion proteins

(89 % and 83 % for P₄₁ and P₉₇ respectively). Surprisingly, most of the β -lactamase activity was recovered in the supernatant for the nine other fusion proteins (99 %, 78 %, 73 %, 78 %, 84 %, 84 %, 76 %, 69 % and 87 % for P₁₆₄, P₂₂₈, P₃₀₇, P₃₇₁, P₄₄₆, P₅₂₉, P₅₇₄, P₆₅₂ and P₇₄₇ respectively). Thus, only P₄₁ and P₉₇ were found to be membrane-bound. These results confirmed the topological orientation of the three membrane-spanning segments. All the fusions beyond amino acid 163 were found to be essentially soluble. These results suggested a proteolytic cleavage of OpgB.

Phosphoglycerol transferase II is encoded by *opgB*

Phosphoglycerol transferase II was initially characterized from soluble proteins extracted from 600 g (wet weight) of *E. coli* cells after several purification steps (Goldberg *et al.*, 1981). When *opgB* mutants were obtained, their phosphoglycerol transferase II activities were determined by the phosphoglycerol cyclase test on total soluble proteins in crude extracts without subsequent purification steps. The activity was found to be similar in the mutant strains and in the wild-type strain (0.33 and 0.31 U mg⁻¹ h⁻¹ respectively), suggesting that two different genes encode the two phosphoglycerol transferases (Jackson *et al.*, 1984). In this work, we developed a micro-method using partially purified periplasmic extracts (see Methods). Three strains were compared for their phosphoglycerol cyclase activities (see Methods): strain 678, from which periplasmic extracts are particularly easy to obtain (Loubens *et al.*, 1993), an *opgB* derivative of strain 678 (NFB1814) and strain 678 harbouring plasmid pNF604 (*opgB*⁺). Accumulation of tritiated cyclic phosphoglycerol increased linearly for at least 2 h in assays performed with extracts from strain 678 and strain 678(pNF604). The level of cyclase activity was 0.25 ± 0.01 U (mg protein)⁻¹ h⁻¹, 5.37 ± 0.50 U (mg protein)⁻¹ h⁻¹ and 0.02 ± 0.01 U (mg protein)⁻¹ h⁻¹ in periplasmic extracts from strain 678, strain 678(pNF604) and strain NFB1814 respectively. Whereas only a residual activity could be detected in NFB1814, a 25-fold increase in cyclase activity resulted from the introduction of the *opgB*⁺ plasmid in strain 678. Because of the difference between our data and those published by Jackson *et al.* (1984), crude extracts were also tested on our strains. Tritiated glycerol liberation activities were 0.70 ± 0.01 U (mg protein)⁻¹ h⁻¹ and 0.30 ± 0.01 U (mg protein)⁻¹ h⁻¹ for strain 678 and NFB1814 respectively. The apparent discrepancy between the data is most probably the result of a non-specific cytoplasmic activity present in crude extracts but absent from periplasmic extracts. Thus, the membrane-bound and the soluble phosphoglycerol transferase are encoded by the same gene.

Analysis of phosphoglycerol transferase II activity *in vivo*

From the model (Fig. 1) it is obvious that the cleavage should occur before the conserved sulfatase domain, just

downstream of the third transmembrane segment. A deletion of plasmid pNF604 allowed the fusion of the two first codons of *lacZ* (encoded by the vector) with the codon corresponding to the last residue of the second transmembrane segment of OpgB (pNF721). The SignalP program (Nielsen *et al.*, 1997, <http://www.cbs.dtu.dk/services/SignalP/>) allowed the prediction that signal peptidase I could recognize the remaining transmembrane segment as a signal sequence with a cleavage site between Ala₁₂₄ and Ser₁₂₅. A similar construction was done with OpgB fused to BlaM downstream of amino acid 164, and its ampicillin-resistance phenotype indicated that the hybrid protein was expressed as a soluble periplasmic protein.

Plasmid pNF721 was introduced into strain BB2636 (a glycerol auxotroph that enables a specific and quantitative labelling of OPGs with [2-³H]glycerol, Bohin & Kennedy, 1984) and into its *opgB* derivative NFB758. OPGs were extracted from overnight cultures. In the presence of the plasmid, the OPG radioactivity remained null in the *opgB* background but was increased by 79 % in the wild-type background. These OPGs were further analysed by DEAE-Sephacel chromatography (Fig. 2). As described previously, OPGs of *E. coli* can be separated by this method in five subfractions corresponding to increasing charge-to-mass ratios (Lacroix *et al.*, 1999, see Fig. 3). In strain BB2636(pNF721), the glycerol content of subfractions IV

Fig. 2. DEAE-Sephacel anion-exchange column chromatography profiles of [2-³H]glycerol-labelled OPGs from strains BB2636 (*opgB*⁺) harbouring plasmid pNF721 (a) or not (b). pNF721 allowed the expression of a truncated OpgB protein deleted of its first two transmembrane segments. Ionic strength was increased by steps of 0.05 M NaCl at fractions indicated by the arrows. Fractions (4 ml) were collected and radioactivity was measured in aliquots.

Fig. 3. DEAE-Sephacel anion-exchange column chromatography profiles of [U- ^{14}C]glucose-labelled OPGs from strain DF214. Labelling was done for 10 min (a), 3 h (b), or 6 h (c). Ionic strength was increased by steps of 0.05 M NaCl at fractions indicated by the arrows. Fractions (4 ml) were collected and radioactivity was measured in aliquots. Peaks: I, neutral glucans; II–V, anionic glucans.

and V was strongly increased (Fig. 2). In conclusion, when the two first transmembrane segments of OpgB are deleted, a periplasmic protein is expressed that cannot complement a defect of phosphoglycerol transferase I activity but can increase significantly the phosphoglycerol substitution of OPGs in a wild-type background. These results demonstrate allotropic properties (Racker, 1967) of OpgB, which can use phosphatidylglycerol as a donor substrate only when anchored in the cell membrane, in complete agreement with the two-step mechanism proposed by Jackson & Kennedy (1983).

Time-course of the phosphoglycerol substitution of OPGs

In the two-step model, OPGs still linked to or in the close vicinity of the membrane are substrates for the membrane-bound enzyme whereas OPGs liberated after a delay in the periplasmic space are substrates for the soluble enzyme. In order to test this model, [^{14}C]glucose incorporation was followed in OPGs of strain DF214. This strain is defective for both phosphoglucose isomerase and glucose-6-phosphate dehydrogenase activities and can synthesize UDP-glucose, and consequently OPGs, only when exogenous glucose is provided in the medium, thus allowing specific

labelling of OPGs. DF214 was grown in low-osmolarity medium without glucose added until mid-exponential phase. D-[U- ^{14}C]Glucose was then added to the medium and aliquots of the culture were taken up after 10 min, 3 h (exponential growth phase) and 6 h (stationary growth phase). OPGs were extracted and analysed by DEAE-Sephacel chromatography (see Methods). The elution profiles for the three kinds of OPG preparations were markedly different. Actually, the time-course of phosphoglycerol substitution was followed from extracts containing only newly synthesized OPGs (10 min), from extracts containing a mixture of new and mature OPG molecules (3 h) and from extracts containing only mature OPG molecules (6 h) since OPGs are no longer synthesized during stationary growth phase (J.-P. Bohin & J.-M. Lacroix, unpublished results). As previously observed (Lacroix *et al.*, 1999), OPGs extracted from cells in stationary phase had various mass-to-charge ratios and were separated into five subfractions, representing 2 % (I), 16 % (II), 38 % (III), 30 % (IV) and 14 % (V) of the total, respectively (Fig. 3c). OPGs extracted after 10 min of synthesis were much less anionic. Subfraction V was absent and subfractions I–IV represented 14 % (I), 40 % (II), 41 % (III) and 5 % (IV) of the total (Fig. 3a). OPGs extracted after 3 h showed an intermediate distribution (Fig. 3b). Newly synthesized OPGs (10 min) were further analysed. Subfractions II and III were submitted to an alkaline hydrolysis to remove succinyl residues linked to the backbone by ester linkages, then analysed once again by DEAE-Sephacel chromatography (amounts in subfraction IV were too low to allow accurate analysis). Sixty per cent of the treated subfraction II was now eluted in the neutral subfraction I while 40 % remained eluted in subfraction II; 60 % of the treated subfraction III was eluted in the neutral subfraction I and 40 % in subfraction II (data not shown). Thus, 14 % of the newly synthesized OPGs were neutral, 65 % were substituted by succinyl residues, and 33 % were substituted by phosphoglycerol residues (17 % being substituted by both substituents). Eighty per cent of the OPGs extracted from *opgB* cells in stationary phase are substituted by succinyl residues (Lacroix *et al.*, 1999) and this level is probably lower in wild-type cells, where OpgB and OpgC are in competition for the same acceptor substrate. Thus, one can consider that succinyl substitution was already achieved in 10 min while phosphoglycerol substitution was a much slower process.

Concluding remarks

The *opgB* gene encodes a protein which is anchored in the cytoplasmic membrane and has the properties of phosphoglycerol transferase I. After post-translational modification (probable cleavage between Ala₁₂₄ and Ser₁₂₅), this protein becomes a soluble, periplasmic protein whose enzymic activity changes into phosphoglycerol transferase II. The *cgmB* gene, encoding a phosphoglycerol transferase catalysing phosphoglycerol substitution of OPGs in *Sinorhizobium meliloti*, was characterized by Wang *et al.*

(1999). Because CgmB of *S. meliloti* and OpgB of *E. coli* showed no significant similarity, and because CgmB is a soluble periplasmic protein, the authors suggested that CgmB may be more similar to phosphoglycerol transferase II. But, since in *E. coli* both phosphoglycerol transferase activities are encoded by the same gene, it appears that this hypothesis was not correct. In addition, no significant similarity was observed between Cgm and OpgC, two enzymes catalysing the succinyl substitution of OPGs in *Brucella abortus* (Roset *et al.*, 2006) and in *E. coli* (Lacroix *et al.*, 1999) respectively, or between these enzymes and both kinds of phosphoglycerol transferases. Thus, the various OPG substitution enzymes, which catalyse very similar reactions, have probably emerged from independent evolutions.

In *E. coli*, OPG synthesis occurs essentially at the inner-membrane level in a complex of proteins including four membrane proteins, two periplasmic proteins and one cytoplasmic protein (Bohin & Lacroix, 2006). Succinyl substitution occurs very early in the synthetic process and probably during the glucose backbone synthesis itself. A primary substitution by phosphoglycerol residues occurs at the same time. However, supplementary substitution, facilitated by a soluble periplasmic enzyme, is needed to achieve complete anionic substitution of OPGs.

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