Linear osmoregulated periplasmic glucans are encoded by the opgGH locus of *Pseudomonas aeruginosa*

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Osmoregulated periplasmic glucans (OPGs) are produced by many proteobacteria and are important for bacterial–host interactions. The *opgG* and *opgH* genes involved in the synthesis of OPGs are the most widely distributed genes in proteobacterial genomes. Two other non-homologous genes, both named *ndvB*, are also involved in OPG biosynthesis in several species. The *Pseudomonas aeruginosa* genome possesses two ORFs, PA5077 and PA5078, that show similarity to *opgH* and *opgG* of *Pseudomonas syringae*, respectively, and one ORF, PA1163, similar to *ndvB* of *Sinorhizobium meliloti*. Here, we report that the *opgGH* locus of *P. aeruginosa* PA14 is involved in the synthesis of linear polymers with $\beta$-1,2-linked glucosyl residues branched with a few $\beta$-1,6 glucosyl residues. Succinyl residues also substitute this glucose backbone. Transcription of *opgGH* is repressed by high osmolarity. Low osmolarity promotes the formation of highly structured biofilms, but biofilm development is slower and the area of biomass is reduced under high osmolarity. Biofilm development of an *opgGH* mutant grown under low osmolarity presents a similar phenotype to the wild-type biofilm grown under high osmolarity. These results suggest that OPGs are important for biofilm formation under conditions of low osmolarity. A previous study suggested that the *P. aeruginosa ndvB* gene is involved in the resistance of biofilms to antibiotics. We have shown that *ndvB* is not involved in the biosynthesis of the OPG described here, and *opgGH* do not appear to be involved in the resistance of *P. aeruginosa* PA14 biofilms to antibiotics.

INTRODUCTION

Osmoregulated periplasmic glucans (OPGs) occur in a wide variety of Gram-negative bacterial species. The only sugars found in the backbone of OPGs are glucosyl residues, which are bound with $\beta$-glucosidic linkages. Generally, OPG synthesis is activated by conditions of low osmolarity (Bohin & Lacroix, 2006). OPGs vary from 5 to 25 glucosyl residues per molecule and the glucose backbones show structural diversity among different species. There are four families of OPG: Family I consists of heterogeneous-sized OPGs with a linear backbone of $\beta$-1,2-linked glucosyl residues to which branches of glucosyl units are linked by $\beta$-1,6 bonds (Bohin & Lacroix, 2006); Family II consists of heterogeneous-sized OPGs with cyclic $\beta$-1,2-glucan backbones; Family III consists of homogeneous-sized OPGs with a cyclic backbone containing glucosyl residues linked by $\beta$-1,3, $\beta$-1,6 bonds and sometimes by $\beta$-1,4 bonds; Family IV members consist of homogeneous-sized OPGs with cyclic $\beta$-1,2-glucan backbones and one $\alpha$-1,6 bond. Additionally, substituents derived from membrane phospholipids (phosphoglycerol, phosphoethanolamine, phosphocholine residues) or from intermediary metabolism (succinate, acetate residues) can decorate the backbones independent of backbone structure (Bohin & Lacroix, 2006).

Studies of OPG synthesis in different species have revealed three distinct glycosyl transferases involved in the biosynthesis of the glucosyl backbone. Genomic sequencing suggests that the *opgH*-encoded glucosyl transferase is the most common among the three. The OpgH protein was initially named MdoH (membrane-derived oligosaccharide) (Lacroix *et al.*, 1991) in *Escherichia coli* and HrpM...
(hypersensitive reaction and pathogenicity) in Pseudomonas syringae (Mukhopadhyay et al., 1988). OpgH homologues, which show a typical glycosyl transferase 2 domain, can catalyse the synthesis of linear Family I OPGs, as in P. syringae (Talaga et al., 1994), or cyclic Family IV OPGs, as in Rhodobacter sphaeroides (Talaga et al., 2002). opgH is the second gene of the opgGH operon. opgG encodes the periplasmic protein OpgG that is necessary for OPG biosynthesis, although at present the function of this protein is unknown. OpgG has been proposed to be involved in the formation of β,1-6 linkages and/or in periplasmic release of newly synthesized OPG (Bohin & Lacroix, 2006). Synthesis of Family II and III OPGs is dependent on proteins named NdvB in Sinorhizobium meliloti and Bradyrhizobium japonicum. However, the two NdvB polypeptides do not show significant sequence similarity.

The importance of OPGs in pathogenesis has been shown for many human, animal and plant pathogens (Bohin & Lacroix, 2006). Mutants defective in OPG biosynthesis display pleiotropic phenotypes, often including hyperproduction of exopolysaccharides, motility defects and hyper-sensitivity to antibiotics. An explanation for this pleiotropy is that OPGs are critical for normal organization of the cell envelope.

The opportunistic pathogen Pseudomonas aeruginosa can be found in a variety of moist environments, including natural and man-made environments (Hardalo & Edberg, 1997; Schwartz et al., 2006; Spiers et al., 2000). This bacterium can cause infections in a variety of animals and plants, and can cause acute infections or chronic biofilm infections in the lungs of cystic fibrosis patients (Burns et al., 1993; Costerton, 1995; Costerton et al., 1999; Hoiby, 1993; Singh et al., 2000; Smith & Iglewski, 2003). A report by Mah et al. (2003) demonstrated that the P. aeruginosa ORF PA1163 plays a role in the tolerance of biofilm cells to antibiotics. The protein encoded by PA1163 shows 58% identity to NdvB of B. japonicum. The NdvB protein synthesizes cyclic β-glucans; however, the identity of the compound synthesized by this protein in P. aeruginosa has not been determined. Two other P. aeruginosa ORFs, PA5077 and PA5078, encode putative polypeptides showing sequence similarity with OpgH and OpgG, respectively. The polypeptide encoded by PA5077 in P. aeruginosa shows 74 and 76% similarity to the OpgH polypeptides of P. syringae and E. coli, respectively. The polypeptide encoded by PA5078 shows 82 and 81% similarity to the OpgG polypeptides of P. syringae and E. coli, respectively. An opgH mutant (PA5077) of P. aeruginosa PA14, obtained by screening a random transposon insertion library, showed impaired virulence in Caenorhabditis elegans, mouse and Arabidopsis virulence models (Mahajan-Miklos et al., 1999).

Here, we report that PA5077 and PA5078 in P. aeruginosa PA14 are responsible for the synthesis of OPGs similar to those present in a variety of bacterial species (Bohin & Lacroix, 2006). The glucans are linear with β-1,2-linked glucose units branched with β-1,6 glucose units. Succinyl residues substitute the glucose backbone. We also show that opgGH and ndvB do not co-operate in the biosynthesis of these linear OPGs. Although strains with opgG and opgH defects form abnormal biofilms, the linear glucans do not appear to participate in the tolerance of biofilm cells to antibiotics.

METHODS

**Bacterial strains, plasmids and media.** All bacterial strains and plasmids used are listed in Table 1. The parent strain was P. aeruginosa PA14.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics*</th>
<th>Source or reference</th>
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<tr>
<td><strong>P. aeruginosa</strong></td>
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<tr>
<td>PA14</td>
<td>Wild-type</td>
<td>Rahme et al. (2000)</td>
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<tr>
<td>YL119</td>
<td>Gm&lt;sup&gt;+&lt;/sup&gt;, ΔopgGH::Gm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td><strong>E. coli</strong></td>
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<tr>
<td>DH5&lt;sup&gt;x&lt;/sup&gt;</td>
<td>F&lt;sup&gt;+&lt;/sup&gt; endA1 hisD17 supE44 thi-1 recA1 gyrA relA1 Δ(lacZYA-argF) U169 deoR φ80 ΔlacZ-M15 recA1</td>
<td>Woodcock et al. (1989)</td>
</tr>
<tr>
<td>SM10</td>
<td>Km&lt;sup&gt;+&lt;/sup&gt;, thi-1 thr leu tonA lacY supE recA1::RP4-2Tc::Mu</td>
<td>de Lorenzo &amp; Timmis (1994)</td>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pUCP18</td>
<td>Cl&lt;sup&gt;+&lt;/sup&gt;, pUCP18 vector containing a P. aeruginosa origin of replication</td>
<td>Schweizer (1991)</td>
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<tr>
<td>pMRP9</td>
<td>pUCP18 with constitutive gfp</td>
<td>Davies et al. (1998)</td>
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<tr>
<td>pKV69</td>
<td>Gm&lt;sup&gt;+&lt;/sup&gt; Tc&lt;sup&gt;+&lt;/sup&gt;, vector carrying mobRP4 origin</td>
<td>K. Visick, University of Chicago, USA</td>
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<tr>
<td>pYL177</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, Gm cassette flanked with 1.58 kb region upstream and 1.89 kb region downstream of opgGH locus</td>
<td>This study</td>
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<tr>
<td>pYL178</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt;, mobilization cassette from pKV69 cloned into KpnI restriction site of pYL177</td>
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<td>pYL205</td>
<td>Plasmid containing opgGH locus</td>
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<tr>
<td>pYL205-G</td>
<td>pYL205 containing a constitutively expressed gfp gene</td>
<td>This study</td>
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</table>

*Ap<sup>+</sup>, ampicillin resistance; Cl<sup>+</sup>, carbenicillin resistance; Gm<sup>+</sup>, chloramphenicol resistance; Gm<sup>+</sup>, gentamicin resistance; Tc<sup>+</sup>, tetracycline resistance.
**Isolation and purification of OPGs.** *P. aeruginosa* strains were grown overnight in 350 ml LOS medium. Cells were harvested by centrifugation and the OPGs were extracted from cell pellets with 5% trichloroacetic acid followed by charcoal adsorption and elution with aqueous pyridine as described by Lacroix *et al.* (1989). OPGs present in the aqueous pyridine extracts were purified by gel filtration chromatography on a Bio-Rad Biogel P6 column (1.8 × 62 cm, flow rate 10 ml h⁻¹). The column was pre-equilibrated with 0.05% acetate solution and OPGs were eluted with 0.05% acetate solution in fractions of 1 ml. Sugar content was determined by a colorimetric procedure using phenol/sulfuric acid reagent (Dubois *et al.*, 1956). Fractions containing sugar were pooled and concentrated by rotary evaporation. Where indicated, fractions of OPG were treated with 0.05 M KOH at 37 °C for 1 h to remove O-ester-bound substituents. After neutralization with AG 50W-X8 (Bio-Rad; H⁺ form), samples were desalted on a Bio-Gel P2 column (Bio-Rad). Native and KOH-treated oligosaccharides were lyophilized.

**Mass spectrometry.** Matrix-assisted laser desorption-ionization (MALDI)-MS experiments were done with a Vision 2000 (Finnigan MAT) time-of-flight mass spectrometer equipped with a nitrogen laser (337 nm wavelength and 3 ns pulse width). A 2.5-dihydroxybenzoic acid matrix was used for carbohydrate analysis (10 g l⁻¹ in water) (Stahl *et al.*, 1991). Lyophilized oligosaccharide samples were dissolved in double-distilled water at a concentration of 0.1 µg µl⁻¹, and then diluted with an appropriate volume of the matrix solution (1:2, v/v). One microtitre of the resulting solution was deposited on a stainless steel target, and the solvent was evaporated under a gentle stream of warm air. After microscope-assisted selection of the appropriate site on the target, laser light was focused onto the sample/matrix mixture at an angle of 15° and a power level of 106–107 W cm⁻². Positive ions were extracted by a 5–10 keV acceleration potential and focused with a lens. Masses were separated by a reflectron time-of-flight instrument. Ions were post-accelerated to 20 keV for maximum detection efficiency. Resulting signals were recorded by using a fast transient digitizer with a maximum of 2.5 ns channel resolution. All MALDI mass spectra are the result of 20 single-shot accumulations.

**Methylation analysis.** Oligosaccharides were treated with sodium borodeuteride to reduce the reducing glycosyl termination. Glucosidic linkage analysis was performed by the methylation method of Parente *et al.* (1985). Methyl ether compounds were hydrolysed (4 M trifluoroacetic acid at 100 °C for 4 h), reduced with sodium borodeuteride and peracetylated. The partially methylated and acetylated glycosides were analysed by GLC-MS using a Delsi apparatus with a capillary column (25 m × 0.2 mm) coated with DB-1 (0.5 µm film thickness). We applied a temperature gradient of 110–240 °C at 2 °C min⁻¹ and a helium pressure of 40 kPa. Mass spectra were recorded on a Nermag 10-10B mass spectrometer (Ruell Malmaison) using an electron energy of 70 eV and an ionizing current of 0.2 mA. Specific standards of glucosyl residues were used to calibrate the GLC column and identify the eluted glycosides. The identity of each eluted glycoside was confirmed by MS coupled to GLC (Fournet *et al.*, 1981).

**RESULTS**

The *opgGH* locus is required for synthesis of linear OPGs

*opgGH*-encoded products are involved in the synthesis of cyclic or linear OPGs in bacteria (Bohin & Lacroix, 2006).

Osmoregulated periplasmic glucans in *P. aeruginosa*
To determine the function of \(\text{OpgH (PA5077)}\) and \(\text{OpgG (PA5078)}\) in \(P. \text{aeruginosa}\), we generated a deletion mutant, YL119, which is devoid of the \(\text{opgGH}\) locus, in a strain PA14 background. Extracts from the parent strain PA14 and YL119 were separated by gel filtration. A large peak containing sugar residues was eluted in fractions 80–100 from PA14 extracts (Fig. 1). This peak was absent in extracts from the \(\text{opgGH}\) mutant strain YL119. For each strain, fractions 80–100 were pooled for further analyses. MS of the pooled fractions from PA14 extracts revealed the presence of 8 molecular ions (Fig. 2). Five molecular ions, \(m/z=1013, 1175, 1337, 1499\) and 1661, correspond to five distinct linear polymers containing 6–10 glucose residues, respectively. Three molecular ions with \(m/z=1275, 1437\) and 1599 correspond to linear polymers of 7–9 glucosyl residues, respectively, with a mass increment of 100 Da each (Fig. 2). A mild alkaline treatment of OPGs eliminates the substituent responsible of this increment. The mass increment of 100 Da may correspond to a succinate residue or an isomer. MS of the pooled fractions 80–100 from strain YL119 did not reveal any polymers of glucose (data not shown). To confirm that the \(\text{opgGH}\) locus is necessary for the biosynthesis of OPGs, we complemented the \(\text{opgGH}\) mutation in strain YL119 with pYL205, which contains \(\text{opgGH}\) and 1.58 kb upstream of the \(\text{opgG}\) translation start codon. Strain YL119(pYL205) produced 14 times more OPGs than wild-type PA14 (27 \(\times\) \(10^{15}\) and \(2 \times 10^{-15}\) g equivalent glucosyl residues per cell, respectively, taking into account only the second peak in Fig. 1). The overexpression of OPGs caused by the presence of \(\text{opgGH}\) on a multicopy plasmid did not influence the growth of planktonic culture (supplementary Fig. S1, available with the online version of this paper).

OPGs were desubstituted with mild alkaline treatment for further structural characterizations. After reduction, methanolysis and peracetylation (see Methods), products were analysed by GLC-MS to determine the types of glucosyl linkages. The analysis revealed four methylglucose species: 3,4-di-, 3,4,6-tri-, 2,3,4,6-tetra- and 1,3,4,6-tetra-O-methylglucoses with respective proportions of 12, 40, 34 and 14 %. According to these proportions, an average OPG molecule containing 7 glucosyl units is formed of one 1,2,6-linked glucosyl residue (3,4-di-O-methylglucose, point of branch), three 1,2-linked glucosyl residues (3,4,6-tri-O-methylglucose), two 1-linked glucosyl residues (2,3,4,6-tetra-O-methylglucosyl, non-reducing termination) and one 2-linked glucosyl residue (1,3,4,6-tetra-O-methylglucose, reducing termination). The glucose backbones of OPG from the complemented mutant YL119 were indistinguishable from the parent OPG.

In other bacteria the expression of \(\text{opgGH}\) homologues is generally repressed by high environmental osmolarity (Bohin & Lacroix, 2006). To determine if this is true of \(\text{opgGH}\) in \(P. \text{aeruginosa}\), we measured \(\text{opgGH}\) transcript levels by real-time PCR in \(P. \text{aeruginosa}\) PA14 grown in LOS medium (low osmolarity medium, 70 mOsm) and LOS medium supplemented with 300 mM sucrose or 150 mM NaCl (370 mOsm). In LOS medium, the level of \(\text{opgGH}\) RNA reached 54 \(\pm\) 10 fg \(\text{opgGH}\) RNA (ng RNA)\(^{-1}\), while in LOS medium with added sucrose or NaCl, \(\text{opgGH}\) RNA levels were only 13 \(\pm\) 2 or 10 \(\pm\) 3 fg \(\text{opgGH}\) RNA (ng RNA)\(^{-1}\), respectively. The expression of \(\text{opgGH}\) in LOS medium was four times higher \((P<0.01)\) than in LOS medium with added sucrose and five times higher \((P<0.002)\) than in LOS medium supplemented with...
NaCl. These results confirm that OPG biosynthesis in *P. aeruginosa* is repressed under high osmolarity conditions.

**Involvement of linear OPGs in biofilm formation**

An earlier report indicated that cyclic OPGs were critical for the tolerance of *P. aeruginosa* biofilm cells to antibiotics (Mah *et al.*, 2003). To determine whether the *opgGH*-dependent linear OPG influenced biofilm formation or biofilm tolerance to antibiotics, we first measured biofilm formation in LOS medium by using a microtitre dish assay (O’Toole & Kolter, 1998) and we detected no significant difference in the amount of biofilm formed by the mutant *opgGH* and the parent using LOS medium with or without added NaCl (150 mM) or sucrose (300 mM), or M63 medium (data not shown). We then measured biofilm sensitivity to tobramycin using the microtitre dish assay described by Mah *et al.* (2003). We also detected no difference in sensitivity of the *opgGH* mutant and the parent to tobramycin. The minimal bactericidal concentration (MBC) was 400 µg ml⁻¹ for both strains. As a control we tested a mutant *ndvB* strain, described by Mah *et al.* (2003) as sensitive to tobramycin during biofilm growth. This mutant showed a reduced MBC in the biofilm mode of growth (50–100 µg ml⁻¹), but was unaffected in planktonic growth (MBC for all three strains was 4–8 µg ml⁻¹). Based on these results, the OPGs described here do not appear to play a significant role in attachment of cells to a substrate or in *P. aeruginosa* biofilm resistance to tobramycin.

All of the previously described biofilm experiments involve early biofilm development under static growth conditions. Thus we decided to study the influence of *opgGH* on biofilm development under a flow of culture medium by using scanning confocal microscopy (Fig. 3). Twenty-four hours after inoculation with the parent PA14, individual cells and some small cell clusters were evident on the glass surface of the flow cell. A similar pattern was observed with the *opgGH* mutant at 24 h. At 48 h, microcolonies of the parent had developed, but the mutant bacterial cells remained as individuals. At 72 h, microcolonies of the parent were larger than those observed at 48 h with a mean thickness of 25 µm and a maximum thickness of 65 µm (Table 2). The biofilm covered the entire glass surface. During the next 4 days, the biofilm formed the mushroom-like structures characteristic of mature *P. aeruginosa* biofilms grown under the conditions we used (Fig. 3).

**Motility and rhamnolipid production are not affected by *opgGH***

Motility mediated by type IV pili and the polar monotrichous flagellum, respectively (Harshey, 2003), and rhamnolipid production are known to be involved in microcolony and mushroom-like structure development (Davey *et al.*, 2003; Klausen *et al.*, 2003a, b; Lequette & Greenberg, 2005). To determine whether abnormal biofilm development in the *opgGH* mutant resulted from altered motility, we compared flagellar and twitching motility of the mutant and the parent. We used LOS medium as the base medium with 0.3, 0.5 and 1% agar for swimming, swarming and twitching motility measurements, respectively (Bradley, 1980; Kohler *et al.*, 2000; Taylor & Koshland, 1974). The mutant and parent were indistinguishable (data not shown). We monitored rhamnolipid gene expression by using an *rhlA-gfp* fusion, and rhamnolipid production by a rhamnolipid plate assay (Kohler *et al.*, 2000; Lequette & Greenberg, 2005). As with motility, we could not distinguish the parent and mutant (data not shown).

**DISCUSSION**

A previous screen for virulence mutations in *P. aeruginosa* indicated that *opgH* is involved in pathogenesis (Mahajan-Miklos *et al.*, 1999). Similarly, an *opgH* mutant of the plant pathogen *P. syringae* *opgH* was shown to be non-pathogenic (Mukhopadhyay *et al.*, 1988). Genomic sequencing has shown that *opgH* is often located immediately downstream of *opgC* in a putative operon (Bohin & Lacroix, 2006). In *P. aeruginosa* the *opgC* (PA5078) and *opgH* (PA5077) homologues are contiguous and show a three-codon overlap. Thus, the two genes are probably co-transcribed as an operon.

To confirm that the biofilm defect in mutant YL119 was a result of the *opgGH* defect, the complemented strain YL119(pYL205-G), with a constitutively expressed *gfp* gene, was used for comparison with wild-type PA14. Biofilms formed by strain YL119(pYL205-G) were similar to those of the parent strain PA14 (Table 2 and supplementary Fig. S2, available with the online version of this paper). Apparently, OPG molecules have subtle effects on biofilm development. The mutant is slow to develop microcolonies and does not develop into mature biofilms with mushroom-like structures. We studied biofilm formation of wild-type PA14 and the *opgGH* mutant under conditions of high osmolarity by adding sucrose (300 mM) or NaCl (150 mM) to LOS medium. Both wild-type PA14 and the *opgGH* mutant biofilms showed the same phenotype as the biofilm of the YL119 mutant grown under low osmolarity conditions (Table 2 and Fig. 3). No difference in growth rate was detected when planktonic growth of strains YL119 and PA14 under different conditions of osmolarity was studied (supplementary Fig. S1). This result shows that the biofilm growth defect in YL119 is specific to the biofilm growth mode.
Fig. 3. Biofilm formation of the parent and the opgGH mutant in a flow chamber. Biofilm development was followed over 7 days and three-dimensional reconstructions of biofilms are shown for wild-type PA14 and the opgGH mutant (YL119) grown in LOS medium with or without added sucrose. Scales are indicated on each image. The gfp gene was constitutively expressed from the self-maintained plasmid pMRP9.
We have shown that, in *P. aeruginosa*, opgGH are involved in the biosynthesis of linear glucans of heterogeneous size, ranging from 6 to 10 glucosyl units per molecule. Expression of opgGH is repressed by elevating the osmolality of the culture medium. Our analysis of opgGH-dependent glucans revealed that the glucosyl backbone is a linear chain of glucosyl units linked at positions 1 and 2, and branched with a few glucosyl residues linked at position 6. All OpgH homologues share positions 1 and 2, and branched with a few glucosyl residues linked at position 6. All OpgH homologues share motifs conserved in Family II glucosyl transferases that catalyze β anomer glycosyl linkages (Coutinho et al., 2003) (Carbohydrate-Active Enzymes server at http://afmb.cnrs-mrs.fr/CAZY/). OpgH homologues catalyze the formation of β-1,2 linkages using UDP-α-glucose as a substrate (Loubens et al., 1993; Therisod et al., 1986). One can assume that like other OpgHs, the *P. aeruginosa* OpgH catalyses the synthesis of a β-1,2-linked linear glucan chain by using UDP-α-glucose as well. Unlike OPG in *P. syringae*, our analysis indicates the glucosyl backbones of *P. aeruginosa* OPG are substituted, probably with succinate residues. We do not know what physiological significance such decoration might confer. OPG synthesis was not affected by inactivation of ndvB, and no cyclic glucans could be detected after extraction of the samples with 5% trichloroacetic acid (data not shown), a procedure known to allow purification of cyclic OPG (Talaga et al., 2002).

Periplasmic glucans synthesized by *P. aeruginosa* OpgGH can represent 0.75% of the dry cell weight in parent strain PA14. Moreover, in the *opgGH* overexpression strain, OPGs constituted nearly 10% of the total cell dry weight. OPGs can represent 5–20% of the total cell dry weight depending on the species and growth conditions (Bohin & Lacroix, 2006; Breedveld & Miller, 1994). Low osmolality and high levels of available nutrients are two of the critical conditions for large amounts of glucans. In several species, the synthesis of large amounts of glucans does not disturb growth when cultures are grown under optimal conditions, such as in the laboratory (Bohin & Lacroix, 2006; Breedveld & Miller, 1994). However, we should be cautious about the significance of these quantities, considering the harsh conditions encountered by bacteria in the environment, where growth rate is low and nutrient availability is restricted.

We investigated the involvement of opgGH in biofilm development because a previous report suggested that OPGs might be involved in the innate tolerance of cells in *P. aeruginosa* biofilms to antibiotics (Mah et al., 2003). This earlier study involved a genetic screen for mutations that increased the sensitivity of biofilms to the clinically relevant antibiotic tobramycin. The screen revealed that mutations in ndvB decreased biofilm resistance to tobramycin. Using an assay similar to that used previously, we showed that, unlike ndvB, the opgGH locus is not involved in tolerance of biofilm-grown bacteria to tobramycin, even though the OPGs synthesized by OpgGH were significant in their quantity. This line of investigation led us to explore the potential relationship between opgGH and biofilm development more thoroughly. Our experiments indicate that initiation of biofilm development is not altered by an opgGH mutation, but there is a delay in the development of microcolonies which subsequently do not develop into the normal mushroom-like structures characteristic of wild-type biofilms under low osmolality conditions. Increasing the medium osmolality also affected the biofilm growth rate and the development of mushroom-like structures in wild-type PA14 (Table 2). These results suggest that *P. aeruginosa* PA14 develops preferentially larger and more structured biofilms under low osmolality conditions, and that OPGs are important for biofilm development under these conditions.

In summary, previous investigations have shown that opgH is involved in virulence of *P. aeruginosa*. We show here that, like in other bacteria, the *P. aeruginosa* opgGH operon is required for synthesis of abundant osmotically regulated

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total biomass (μm² μm⁻²)</th>
<th>Mean thickness (μm)</th>
<th>Roughness coefficient</th>
<th>10⁻⁶×Surface area of biomass (μm²)</th>
<th>Surface to biovolume ratio (μm² μm⁻³)</th>
<th>Maximum thickness (μm)</th>
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<tbody>
<tr>
<td>LOS medium</td>
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<td></td>
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<tr>
<td>PA14</td>
<td>41 ± 16</td>
<td>70 ± 34</td>
<td>0.59 ± 0.20</td>
<td>4.42 ± 0.28</td>
<td>1.99 ± 0.70</td>
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<td>YL119</td>
<td>21 ± 9</td>
<td>25 ± 10</td>
<td>0.42 ± 0.10</td>
<td>1.21 ± 0.67</td>
<td>1.17 ± 0.65</td>
<td>62 ± 14</td>
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<td>PA14 (day 3)</td>
<td>21 ± 8</td>
<td>24 ± 9</td>
<td>0.54 ± 0.29</td>
<td>1.19 ± 0.33</td>
<td>1.21 ± 0.49</td>
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<td>YL119(pYL205-G)</td>
<td>37 ± 7</td>
<td>51 ± 11</td>
<td>0.51 ± 0.10</td>
<td>3.67 ± 1.53</td>
<td>2.08 ± 0.71</td>
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<td>PA14</td>
<td>15 ± 3</td>
<td>16 ± 4</td>
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<td>0.62 ± 0.17</td>
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<td>LOS medium with added NaCl (150 mM)</td>
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<td>12 ± 3</td>
<td>0.52 ± 0.10</td>
<td>0.59 ± 0.10</td>
<td>1.0 ± 0.21</td>
<td>48 ± 7</td>
</tr>
<tr>
<td>YL119</td>
<td>9 ± 3</td>
<td>9 ± 3</td>
<td>0.49 ± 0.14</td>
<td>0.61 ± 0.08</td>
<td>1.1 ± 0.31</td>
<td>45 ± 6</td>
</tr>
</tbody>
</table>

**Table 2.** Quantitative analyses of biofilm structures

The quantitative analysis of biofilm architecture was done using COMSTAT v.1. Results are the means of 10 representative images stacks ± SD. Biofilm architectures of strains PA14 and YL119 with or without plasmid pYL205-G in the medium indicated were measured at day 5. Biofilm architecture of strain PA14 was also quantified at day 3 where indicated.
glucans, which we assume to be periplasmic. We have characterized the structures of these glucans, but how they function in virulence remains to be determined.

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REFERENCES


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