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

Yannick Lequette,1† Eglantine Rollet,2 Aurélie Delangle,2 E. Peter Greenberg1 and Jean-Pierre Bohin2

1Department of Microbiology, University of Washington, Seattle, WA 98195, USA
2Unité de Glycobiologie Structurale et Fonctionnelle CNRS UMR 8576, Université des Sciences et Technologies de Lille, 59655 Villeneuve d’Ascq Cedex, France

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 β-1,2-linked glucosyl residues branched with a few β-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 β-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 β-1,2-linked glucosyl residues to which branches of glucosyl units are linked by β-1,6 bonds (Bohin & Lacroix, 2006); Family II consists of heterogeneous-sized OPGs with cyclic β-1,2-glucan backbones; Family III consists of homogeneous-sized OPGs with a cyclic backbone containing glucosyl residues linked by β-1,3, β-1,6 bonds and sometimes by β-1,4 bonds; Family IV members consist of homogeneous-sized OPGs with cyclic β-1,2-glucan backbones and one α-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 transfers involved in the biosynthesis of the glucosyl backbone. Genomic sequencing suggests that the opgH-encoded glycosyl transferase is the most common among the three. The OpgH protein was initially named MdoH (Bohin & Lacroix, 2006).

Abbreviations: LOS, low-osmolarity (medium); OPG, osmoregulated periplasmic glucan.
involved in the formation of protein is unknown. OpgG has been proposed to be OPG biosynthesis, although at present the function of this encodes the periplasmic protein OpgG that is necessary for infection in the lungs of cystic fibrosis patients (Burns et al., 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.

**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA14</td>
<td>Wild-type</td>
<td>Rahme et al. (2000)</td>
</tr>
<tr>
<td>YL119</td>
<td>Gm&lt;sup&gt;f&lt;/sup&gt;, ΔopgGH::Gm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SM10</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;, thi-1 thr leu tonA lacY supE recA1::RP4-2Tc::Mu</td>
<td>de Lorenzo &amp; Timmis (1994)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUCP18</td>
<td>Cb&lt;sup&gt;f&lt;/sup&gt;, pUCP18 vector containing a P. aeruginosa origin of replication</td>
<td>Schweizer (1991)</td>
</tr>
<tr>
<td>pMRP9</td>
<td>pUCP18 with constitutive gfp</td>
<td>Davies et al. (1998)</td>
</tr>
<tr>
<td>pkV69</td>
<td>Cm&lt;sup&gt;r&lt;/sup&gt;, Tc&lt;sup&gt;r&lt;/sup&gt;, vector carrying mobRP4 origin</td>
<td>K. Visick, University of Chicago, USA</td>
</tr>
<tr>
<td>pYL177</td>
<td>Ap&lt;sup&gt;r&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>
</tr>
<tr>
<td>pYL178</td>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;, mobilization cassette from pkV69 cloned into KpnI restriction site of pYL177</td>
<td>This study</td>
</tr>
<tr>
<td>pYL205</td>
<td>Plasmid containing opgGH locus</td>
<td>This study</td>
</tr>
<tr>
<td>pYL205-G</td>
<td>pYL205 containing a constitutively expressed gfp gene</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Ap<sup>r</sup>, ampicillin resistance; Cb<sup>f</sup>, carbenicillin resistance; Cm<sup>r</sup>, chloramphenicol resistance; Gm<sup>r</sup>, gentamicin resistance; Tc<sup>r</sup>, tetracycline resistance.
pseudomonas PA14. Strain YL119 contained a PA5077 and PA5078 deletion and was constructed as follows. The intermediate construct pYL177 was generated by flanking a gentamicin resistance cassette with a 1.58 kb DNA fragment containing the region upstream of the PA5078 start codon and a 1.89 kb DNA fragment containing the region downstream of the PA5077 stop codon. A KpnI fragment containing the mobilization cassette from pKV69 was inserted into the KpnI restriction site of pYL177 to create pYL178, a mobilizable suicide vector. We moved pYL178 from E. coli SM10 into P. aeruginosa YL119 by conjugation. To obtain P. aeruginosa YL119 we selected gentamicin-resistant colonies and then screened for carbencillin sensitivity. Strain YL119 has a deletion encompassing the entire operon PA5078-PA5077, from 250 bp upstream of the PA5078 start codon to 171 bp downstream of the PA5077 stop codon. The deletion was confirmed by PCR analysis. A DNA fragment containing 1.58 kb upstream of the PA5078 start codon, the two ORFs PA5078 and PA5077 and 171 bp downstream of the PA5077 stop codon was cloned into pUCP18 to generate pYL205.

Real-time PCR. The following primers were designed with Primer Express software (Taqman) and used for real-time PCR analyses: 5'-CGTTCACCCCCCGCTCAGAA-3' and 5'-ACCCGCGTCTCCTGGA-TCCT-3' for PA5078, and 5'-CTACGATGGTGTATCGAAG-3' and 5'-CGGGTTAGGTGTATCGCAGA-3' for nadB. Total RNA, extracted from cultures when they reached an OD600 of 0.8, was 9 pure and 5 treated with a 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 µA. 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).
To determine the function of OpgH (PA5077) and OpgG (PA5078) in *P. aeruginosa*, we generated a deletion mutant, YL119, which is devoid of the *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 *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 *opgGH* locus is necessary for the biosynthesis of OPGs, we complemented the *opgGH* mutation in strain YL119 with pYL205, which contains *opgGH* and 1.58 kb upstream of the *opgG* translation start codon. Strain YL119(pYL205) produced 14 times more OPGs than wild-type PA14 (27 ± 10² to 2 ± 10² 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 *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 peracylation (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 %.

In other bacteria the expression of *opgGH* homologues is generally repressed by high environmental osmolarity (Bohin & Lacroix, 2006). To determine if this is true of *opgGH* in *P. aeruginosa*, we measured *opgGH* transcript levels by real-time PCR in *P. 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 *opgGH* RNA reached 54 ± 10 fg *opgGH* RNA (ng RNA)⁻¹, while in LOS medium with added sucrose or NaCl, *opgGH* RNA levels were only 13 ± 2 or 10 ± 3 fg *opgGH* RNA (ng RNA)⁻¹, respectively. The expression of *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

---

**Fig. 1.** Purification of OPGs from *P. aeruginosa*. Extracts from wild-type PA14 (●), *opgGH* mutant strain YL119 (○) and YL119 with plasmid pYL205 (□) were eluted on a gel-filtration Biogel P6 column (see Methods). PA14 and YL119 data are plotted on the left-hand y-axis and YL119-pYL205 data are plotted on the right-hand y-axis.

**Fig. 2.** Positive ion mass spectrum of *P. aeruginosa* OPGs. Numbers over each peak are molecular masses of cationized species. Numbers in parentheses represent the degree of polymerization of the corresponding glucosyl polymer. Asterisks indicate the presence of a succinate residue in the glucosyl polymer.
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 nvdB 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 substratum 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). Mutant strain microcolonies were evident at 72 h. However, the mutant biofilm did not develop further during the next 4 days (Fig. 3). A statistical analysis with COMSTAT v.1 (Heydorn *et al.*, 2000) confirmed our qualitative analysis (Table 2). For example, the mean thickness of a 5-day mutant biofilm was 25% of the parent, and the total area of biomass was about half that of the parent. The mutation did not affect growth of *P. aeruginosa* in either LB broth or LOS medium (data not shown).

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.

**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 opgG in a putative operon (Bohin & Lacroix, 2006). In *P. aeruginosa* the opgG (PA5078) and opgH (PA5077) homologues are contiguous and show a three-codon overlap. Thus, the two genes are probably co-transcribed as an operon.
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 osmolarity 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 catalyse the formation of 1,2 linkages using UDP-α-glucose as a substrate. Unlike OPG in *P. syringae* (Loubens et al., 1993; Therisod et al., 1986). One can assume that like other OpgHs, the *P. aeruginosa* OpgH catalyses the synthesis of a 1,2-linked linear glucan backbone 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 (Talaqa 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 osmolarity 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 (Mahan 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 osmolarity conditions. Increasing the medium osmolarity 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 osmolarity 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 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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total biomass (μm³ μm⁻²)</th>
<th>Mean thickness (μm)</th>
<th>Roughness coefficient</th>
<th>10⁻⁴×Surface area of biomass (μm²)</th>
<th>Surface to biovolume ratio (μm² μm⁻³)</th>
<th>Maximum thickness (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOS medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<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>
<td>133 ± 26</td>
</tr>
<tr>
<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>
</tr>
<tr>
<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>
<td>65 ± 11</td>
</tr>
<tr>
<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>
<td>148 ± 29</td>
</tr>
<tr>
<td>LOS medium with sucrose (300 mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA14</td>
<td>15 ± 3</td>
<td>16 ± 4</td>
<td>0.47 ± 0.17</td>
<td>0.62 ± 0.17</td>
<td>0.85 ± 0.16</td>
<td>51 ± 8</td>
</tr>
<tr>
<td>YL119</td>
<td>15 ± 3</td>
<td>15 ± 4</td>
<td>0.49 ± 0.07</td>
<td>0.70 ± 0.18</td>
<td>0.91 ± 0.30</td>
<td>58 ± 9</td>
</tr>
<tr>
<td>LOS medium with NaCl (150 mM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA14</td>
<td>11 ± 2</td>
<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>
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.

ACKNOWLEDGEMENTS

We thank Yves Leroy for expert technical assistance for GLC-MS analyses, Sudha Chugani for helpful discussions, and G. O’Toole for providing strain SMC1759. This work was supported by grants from the W. M. Keck Foundation, the US National Institute of General Medical Sciences (GM59026), the French Centre National de Recherche Scientifique (UMR 8576), and the French Ministère de l’Enseignement Supérieur de la Recherche et de la Technologie.

REFERENCES


Edited by: P. Cornelis