Osmoregulated periplasmic glucans of *Salmonella enterica* serovar Typhimurium are required for optimal virulence in mice

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We purified osmoregulated periplasmic glucans (OPGs) from *Salmonella enterica* serovar Typhimurium and found them to be composed of 100% glucose with 2-linked glucose as the most abundant residue, with terminal glucose, 2,3-linked and 2,6-linked glucose also present in high quantities. The two structural genes for OPG biosynthesis, opgG and opgH, form a bicistronic operon, and insertion of a kanamycin resistance gene cassette into this operon resulted in a strain devoid of OPGs. The *opgGH* mutant strain was impaired in motility and growth under low osmolarity conditions. The *opgGH* mutation also resulted in a 2 log increase in the LD50 in mice compared to the wild-type strain SL1344. Inability to synthesize OPGs had no significant impact on the organism’s lipopolysaccharide pattern or its ability to survive antimicrobial peptides-, detergent-, pH- and nutrient-stress conditions. We observed that the *opgGH*-defective strain respired at a reduced rate under acidic growth conditions (pH 5.0) and had lower ATP levels compared to the wild-type strain. These data indicate that OPGs of *S. Typhimurium* contribute towards mouse virulence as well as growth and motility under low osmolarity growth conditions.

INTRODUCTION

Osmoregulated periplasmic glucans (OPGs) are a heterogeneous group of soluble glucans primarily located in the periplasmic space of Gram-negative bacteria (Kennedy, 1996). In *Escherichia coli*, OPGs are composed of 8–10 glucose units per molecule in a highly branched structure, and the backbone is extensively substituted with sn-1-phosphoglycerol, phosphoethanolamine, and O-succinyl ester residues (Kennedy et al., 1976). OPGs of *E. coli* and the cyclic glucans of the *Rhizobiaceae*, as well as other Gram-negative plant pathogens, are localized in the periplasmic space, have glucose as a major constituent and in most cases their synthesis is osmotically regulated (Bohin & Lacroix, 2007; Kennedy, 1996).
OPGs have a critical biological function because mutants of \( E. \ coli \) deficient in OPG biosynthesis show altered chemotaxis and motility (Fiedler & Rotering, 1988; Weissborn et al., 1992). OPGs have also been demonstrated to play a significant role in establishing successful pathogenic or symbiotic associations with plant hosts (Arellano-Reynoso et al., 2005; Bhagwat & Keister, 1995; Page et al., 2001). Among animal pathogens, the role of OPGs has been examined in \( Brucella \) abortus (Arellano-Reynoso et al., 2005), where synthesis of glucans is not osmoregulated (Briones et al., 1997). Despite the fact that the OPGs are postulated to play a significant role in many plant–pathogen interactions, the importance of OPGs in enteric human pathogens has not been studied (Coburn et al., 2005; Galan & Cossart, 2005). To our knowledge, neither the structure nor function of \( Salmonella \) OPGs has been reported. In \( E. \ coli \), genes for OPGs biosynthesis map to the \( opgGH \) operon and encode a glycosyltransferase (OpgH) and OpgG, a periplasmic protein presumed to be involved in polymerization of the OPG backbone (Bohin & Lacroix, 2007; Hanoulle et al., 2004). Similar gene sequence homologues are found in the \( Salmonella \) genome (Parkhill et al., 2001). In this study, we mutated the \( opgGH \) operon of \( Salmonella \) enterica serovar Typhimurium SL1344. We also determined the glucosyl composition of wild-type OPGs and observed glucose to vary in different growth media such as LB, LB with no salts (LBNs) and minimal E medium supplemented with 1.0 % Casamino acids (osmolarity of 407 ± 4, 85 ± 4 and 310 ± 9 mosmol l\(^{-1} \), respectively) (Lin et al., 1995). LBNS broth diluted 1:8 in distilled water was used as low nutrient no salt (LNNS) medium (36 ± 3 mosmol l\(^{-1} \)). Growth was measured using a Bioscreen C automatic turbidimetric analyser (GrowthCurves USA). Starter cultures were prepared by inoculating a single colony of the appropriate strain into LB followed by overnight incubation at 37 °C. This culture was diluted 1:10 000 into fresh media of varying osmolarity and 250 μl per well was transferred into a 100-well honeycomb Bioscreen plate. Growth was assessed at 37 °C with continuous shaking. To assess the effect of osmotic stress on growth, media were supplemented with varying amounts of salt (NaCl or KCl) or buffered with HEPES (50 mM, pH 7.1). Swarming motility was determined 12 h after spot inoculation on media containing 0.35 % Difco agar (Bhagwat et al., 1996; Chen et al., 2003) with varying amounts of NaCl (0–0.155 M (36 ± 3–242 ± 11 mosmol l\(^{-1} \)).

**Table 1.** Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td><strong>S. Typhimurium</strong></td>
<td>LT2 rpoS’</td>
<td>SGSC*</td>
</tr>
<tr>
<td>S. Typhimurium</td>
<td>Wild-type, NaI’</td>
<td>SGSC*</td>
</tr>
<tr>
<td>FG111</td>
<td>FIRN opgGH, Km’</td>
<td>This study</td>
</tr>
<tr>
<td>SL1344</td>
<td>Wild-type, his Sm’</td>
<td>Hoiseth &amp; Stocker (1981)</td>
</tr>
<tr>
<td>SG111</td>
<td>SL1344 opgGH, Km’</td>
<td>This study</td>
</tr>
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<td><strong>E. coli</strong></td>
<td>endA1 recA1 gyrA96 thi hsdR17 relA1 supE44 Δ(lac-proAB) mcrA (F’ traD36 proAB’ lacPZAM15)</td>
<td>Promega</td>
</tr>
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<td>JM109</td>
<td>recA thi pro hsdR M’ RP4: 2-Tc: MuKm Tn7 Δpir, Tp’</td>
<td>Kim et al. (2002)</td>
</tr>
<tr>
<td>S17-1(Δpir)</td>
<td>recA thi pro hsdR M’ RP4: 2-Tc: MuKm Tn7 Δpir, Tp’</td>
<td>Kim et al. (2002)</td>
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<td><strong>Plasmids</strong></td>
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<td>Promega</td>
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<tr>
<td>pUC4K</td>
<td>Source of the Km cassette</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pKNG101</td>
<td>oriR6K mobRK2 sacB, suicide vector, Sm’</td>
<td>Kim et al. (2002)</td>
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<td>pOPGG1</td>
<td>pGEM-T derivative harbouring 1924 bp opgG PCR fragment</td>
<td>This study</td>
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<tr>
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<td>882 bp HpaI–Bsu36I fragment containing the opgG coding region from pOPGG1 removed and replaced by Km cassette</td>
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<td>4.51 kbp insert fragment flanking opgGH cloned into pGEM-T</td>
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<tr>
<td>pBK16</td>
<td>4.51 kbp insert from pBP16 cloned into a low-copy-number vector (pPQSL2.0)</td>
<td>This study</td>
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</table>

*SGSC; Salmonella Genetic Stock Center, Alberta, Canada.

**METHODS**

**Bacterial strains and culture conditions.** The strains used in this study (Table 1) were grown in Luria–Bertani (LB) medium at 37 °C in a shaking incubator at 220 r.p.m. When required, the medium was supplemented with antibiotics at the following concentrations: ampicillin (100 μg ml\(^{-1} \)), kanamycin (50 μg ml\(^{-1} \); Km), nalidixic acid (10 μg ml\(^{-1} \); NaI), streptomycin (50 μg ml\(^{-1} \); Sm). \( Salmonella \)-\( Shigella \) agar and bismuth sulfite agar (Difco) \( Salmonella \) semi-selective indicator media with appropriate antibiotics were used to isolate \( Salmonella \) from mouse tissue. Osmolarity of growth media was measured with a Wescor vapour pressure osmometer (model 5500).

The growth rates of wild-type and the \( opgGH \) mutant were determined in different growth media such as LB, LB with no salts (LBNs) and minimal E medium supplemented with 1.0 % Casamino acids (osmolarity of 407 ± 4, 85 ± 4 and 310 ± 9 mosmol l\(^{-1} \), respectively) (Lin et al., 1995). LBNS broth diluted 1:8 in distilled water was used as low nutrient no salt (LNNS) medium (36 ± 3 mosmol l\(^{-1} \)). Growth was measured using a Bioscreen C automatic turbidimetric analyser (GrowthCurves USA). Starter cultures were prepared by inoculating a single colony of the appropriate strain into LB followed by overnight incubation at 37 °C. This culture was diluted 1:10 000 into fresh media of varying osmolarity and 250 μl per well was transferred into a 100-well honeycomb Bioscreen plate. Growth was assessed at 37 °C with continuous shaking. To assess the effect of osmotic stress on growth, media were supplemented with varying amounts of salt (NaCl or KCl) or buffered with HEPES (50 mM, pH 7.1). Swarming motility was determined 12 h after spot inoculation on media containing 0.35 % Difco agar (Bhagwat et al., 1996; Chen et al., 2003) with varying amounts of NaCl (0–0.155 M (36 ± 3–242 ± 11 mosmol l\(^{-1} \)).

**Statistical analysis.** For statistical analyses, SigmaStat 3.0 software (Aspire Software International) was used. Data were analysed by a
Construction of the opgGH mutant. opgGH is a bicistronic operon and the transcriptional start site of the opgH gene overlaps the C-terminal end of opgG by 7 nt (www.ncbi.nlm.nih.gov/genomes/lproks.cgi?view=1). The opgGH mutants of strain LT2 and FIRN were created in stepwise fashion. opgG with a 400 bp flanking region was amplified from LT2 genomic DNA using PCR primers OPGF1 (5′-ACACAAACTCGACAACCT-3′) and OPGR1 (5′-CCCTTGCAGG- AATGAATC-3′). The 1.9 kb amplified fragment was cloned into pGEM-T Easy vector (Promega), giving pOPGG1. The cloned opgG gene and the flanking region on pOPGG1 were confirmed by determining the DNA sequence in both orientations. An 882 bp portion from the opgG structural gene (aa 13–306) from pOPGG1 was deleted using restriction endonucleases BssHII and Hpal, and replaced with the 1.1 kbp kanamycin structural gene from pUC4K to yield pOPGG1. The insert was further cloned at an EcoRI site in pKNG101 to yield pOPGG11. pOPGG11 was mobilized to LT2 and FIRN by triparental mating (Bhagwat & Keister, 1995). After sucrose selection (Kim FIRN by triparental mating (Bhagwat & Keister, 1995). After sucrose selection (Kim
et al., 1985). For glycosyl linkage analysis, the samples were permethylated, hydrolyzed, and reduced with sodium borohydride. The products were subjected to SDS-PAGE using a 15 % acrylamide gel containing 0.1 % sodium dodecyl sulfate (SDS) and 0.01 % Bromophenol Blue in 1 M Tris/HCl buffer, pH 6.8. The samples were incubated at 10 °C for 10 min and then cooled on ice for 5 min. Samples were mixed with 25 μg Proteinase K, incubated for 1 h at 60 °C, and then mixed with 50 μl 90 % phenol for 15 min at 70 °C. Samples were centrifuged (12 500 g) for 10 min, and the aqueous phase was transferred to a new tube and extracted once with 500 μl ethyl ether to remove traces of phenol. The upper ether phase was aspirated and the lower phase was mixed with an equal volume of SDS-PAGE lysis buffer. Ten microliters of each sample was incubated at 100 °C for 5 min, and then subjected to SDS-PAGE using a 15 % acrylamide gel containing 4 M urea (Sprott et al., 1994). LPS was visualized by silver staining (Tsai & Frasch, 1982).

Mouse virulence studies. Five-week-old male BALB/c mice were purchased from the Small Animals Division of the National Cancer Institute (Frederick, MD, USA). Mice were housed in an AllenTown Caging Biocontainment isolator rack, 4–5 mice per cage, and provided with Harland–Teklad rodent chow and deionized water ad libitum. Mice were fasted 1 week prior to use and all animal protocols were approved by the Institutional Animal Care and Use Committee. Animals were fasted for approximately 12 h prior to being inoculated with 0.2 ml of an S. Typhimurium suspension (in 0.9 % NaCl) by oral gavage. Bacterial strains were grown in LB medium at 37 °C without shaking for 16–18 h, suspended in saline and adjusted to an appropriate cell density before oral infection. Viable cell counts from individual oral dose dilutions were confirmed by retrospective spread-plating onto LB agar plates and incubating the plates for overnight at 37 °C.

To determine the effect of opgGH mutation on S. Typhimurium virulence, groups of mice (6–12 mice per group) were inoculated and monitored twice daily for signs of morbidity or mortality. Moribund mice were sacrificed and counted as dead. Survival curves were analysed using the Kaplan–Meier method with post hoc analysis for statistical significance. A value of P<0.05 was considered significant. Data from two experiments were combined and a probit model was fitted to observed proportions surviving for each (strain, days, group) using SAS Proc PROBIT (SAS Institute, 1999) with the INVERSECL option to obtain LD50 estimates and confidence intervals (Finney, 1971; Hubert et al., 1988).

To analyse colonization of individual organs by each bacterial strain, mice were sacrificed 6 days post-infection. Individual organs (liver, spleen and entire intestine) were dissected, weighed and homogenized in LB. Cell counts were determined by spread-plating appropriate dilutions onto Brilliant green agar plates (Difco) containing streptomycin (50 μg ml⁻¹) or Km (25 μg ml⁻¹). Individual colonies were counted after overnight incubation at 37 °C and statistical

Lipopolysaccharide analysis by PAGE. Bacterial cultures for analysis of LPS were grown overnight in LB at 37 °C, diluted 1:100 in the same medium and grown at 37 °C for 3 h to late-exponential phase. A 10 ml sample was collected and centrifuged as described previously (Bhagwat et al., 1999; Johnson & Perry, 1976). The cell pellet was resuspended in PBS to an OD₆₀₀ of 0.5–0.6. The cell suspension (1.5 ml) for LPS extraction was centrifuged and the pellet was resuspended in 50 μl SDS-PAGE lysis buffer (2 % SDS, 4 % 2-mercaptoethanol, 10 % glycerol, 0.002 % Bromophenol Blue in 1 M Tris/HCl buffer, pH 6.8). Samples were incubated at 100 °C for 10 min and then cooled on ice for 5 min. Samples were mixed with 25 μg Proteinase K, incubated for 1 h at 60 °C, and then mixed with 50 μl 90 % phenol and 15 min at 70 °C. Samples were centrifuged (12 500 g) for 10 min, and the aqueous phase was transferred to a new tube and extracted once with 500 μl ethyl ether to remove traces of phenol. The upper ether phase was aspirated and the lower phase was mixed with an equal volume of SDS-PAGE lysis buffer. Ten microliters of each sample was incubated at 100 °C for 5 min, and then subjected to SDS-PAGE using a 15 % acrylamide gel containing 4 M urea (Sprott et al., 1994). LPS was visualized by silver staining (Tsai & Frasch, 1982).

Glycosyl composition and glycosyl linkage methylation analysis. Glycosyl composition analysis was performed at the Complex Carbohydrate Research Center (Athens, GA, USA). Composition was determined by combined GC/MS of the per-O-trimethylsilyl derivatives of the monosaccharide methylglycosides produced from the samples by acidic methanolysis (Merkle & Poppe, 1994; York et al., 1985). For glycosyl linkage analysis, the samples were permethylated, depolymerized, reduced and acetylated; and the resultant partially methylated alditol acetate samples were analysed by GC/MS (York et al., 1985).

Determination of succinate content from OPGs. Succinate content from OPGs was estimated as described earlier by Lacnix et al. (1999). Briefly, 1 mg OPG was dissolved in 200 μl 0.5 M NaOH and incubated at 100 °C for 30 min to liberate the succinyl residues from OPG. Glucosidic backbones were removed by adsorption on 50 mg charcoal, and the charcoal was then washed three times with 0.5 ml water. The four supernatants were pooled, hydrolyzed, dissolved in 200 μl water and desalted on a minicolumn of Dowex AG50 × 8, 20- to 50-mesh (H⁻ form; Bio-Rad). After lyophilization, samples were dissolved in 1 ml water, and succinic acid content was determined with a succinic acid kit (Roche R-Biopharma), according to the manufacturer’s instructions.
analysis was performed using ANOVA with post hoc analysis for multiple comparisons or the Mann–Whitney non-parametric test. A value of \( P < 0.05 \) was considered significant.

**Determination of cellular ATP contents.** To determine the ATP content of cells during growth under acidic conditions, inoculum was prepared as described above for the Biolog pH panel, except that cells were inoculated in IF-10 media containing triethanolamine and glutaric acid (30 mM each, pH 5.0). Samples (100 \( \mu l \) cell suspension) were withdrawn in triplicate at various times and mixed with 100 \( \mu l \) luciferin-luciferase/lysis reagent (Promega), and the luminescent signal was measured after 5 min with a Berthold LB 9501 luminometer (Berthold Technologies).

**RESULTS**

Isolation and characterization of OPGs

OPGs from wild-type *S. Typhimurium* SL1344 were obtained as a single major peak of apparent molecular mass \(~1300\) Da from a BioGel P4 gel filtration column (Fig. 1a). No hexose-containing polymers eluted in the void volume. OPGs from SL1344 and from strains LT2 and FIRN had identical elution patterns (data not shown). The BioGel P4 elution pattern of OPG preparations from *opgGH* mutants was devoid of the corresponding peak (Fig. 1a). A significant proportion of the OPGs synthesized was anionic, with only \( \sim 3\% \) OPGs being neutral and did not bind to DEAE-cellulose (data not shown). Also, the succinic acid content of the OPG samples was estimated to be \( 0.95 \pm 0.23 \) \( \mu \)mol (\( \mu \)mol OPG\(^{-1} \)) (assuming 8 glucose residues per molecule of OPG). Total OPGs were further analysed for their hexosyl composition, and glucose was found to be the only component in *S. Typhimurium* strain SL1344 (Fig. 1c) as well as strains LT2 and FIRN (data not shown). Glycosyl linkage methylation analysis of OPGs from SL1344 indicated that 2-linked glucose was the most abundant residue, with terminal glucose, 2,3-linked and 2,6-linked glucose also present in high quantities (Fig. 1c). Synthesis of total OPGs [measured as \( \mu \)g glucose equivalents (\( \mu \)g cellular protein\(^{-1} \)] was influenced by the osmolality of the external growth medium (Fig. 1b). Increasing growth medium osmolarity beyond 250 mosmol l\(^{-1} \) adversely affected OPG synthesis. Practically no OPGs were synthesized at medium osmolarity levels greater than 440 mosmol l\(^{-1} \).

Growth and motility characteristics

Growth rates of *opgGH* mutants in LB or LBNS broth, as well as in EG medium (see Supplementary Methods for a definition, available with the online version of this paper) with 1% Casamino acids (osmolality levels of 407 \( \pm \) 4, 85 \( \pm \) 4 and 310 \( \pm \) 9 mosmol l\(^{-1} \), respectively) were indistinguishable from wild-type parental strains (Fig. 2a; data not shown for EG medium). Compared to their growth
rates in LB broth (407 ± 4 mosmol l⁻¹), both SL1344 and SG111 strains were delayed by 89 and 85 min, respectively, in entering mid-exponential growth phase (time to reach an OD₆₀₀ of 0.6) when grown in a lower osmolarity medium such as LBNS (85 ± 4 mosmol l⁻¹) (Fig. 2a, open versus closed symbols). With further reduction in medium osmolarity such as low-nutrient low-salt medium (LNNS, 36 ± 3 mosmol l⁻¹; Fig. 2b), SG111 exhibited a much longer lag time in comparison with SL1344 (559 vs 445 min, respectively). The lag period of SG111 was gradually reduced with increasing medium osmolarity by addition of either NaCl (Fig. 2c), KCl or HEPES buffer (data not shown).

Similarly, motility swarms of SG111 were significantly smaller in LNNS medium supplemented with 0.35 % agar (Fig. 3), and this strain continued to exhibit reduced swarm motility even after adjusting the medium osmolarity up to 125 mosmol l⁻¹. There were no significant differences between SL1344 and SG111 in their swarm motility at osmolarity levels greater than 200 mosmol l⁻¹. An opgGH mutant strain carrying pBK16 exhibited normal motility swarms, indistinguishable from the wild-type strain (Fig. 3), and also had identical growth characteristics as observed for the wild-type (data not shown).

**Mouse virulence studies**

To determine if the lack of OPGs had any influence on pathogenesis and virulence of *Salmonella* strains, mice were orally infected with 10⁶ cells per animal and mortality was monitored over the following 15 days (Fig. 4). The *opgGH* mutant strain exhibited reduced virulence compared to the wild-type strain and the *opgGH* mutant carrying complementing plasmid pBK16. In an independent study, we also performed mice inoculation experiments in which mice were given an oral dose in the range of 10³–10⁷ cells per animal. The results of these experiments showed that log₁₀4.7 cells per animal were needed to achieve an LD₅₀.
within 10–12 days post-challenge with the wild-type strain SL1344 (data not shown). The LD$_{50}$ for the opgGH mutant had a substantially higher LD$_{50}$ (log$_{10}$6.9, $P<0.001$ at $10^5$ cells per animal), indicating that the strain expressed reduced virulence compared to SL1344. Bacterial colonization of individual organs was examined 6 days after the oral dose of $10^7$ cells per animal. Colonization of the intestine, spleen and liver, as measured by c.f.u. (g organ wt)$^{-1}$ was approximately 100- to 1000-fold lower for mice receiving the opgGH mutant strain compared to the wild-type parent (Fig. 5).

**LPS analysis of opgGH mutant**

Possible involvement of opgGH in the synthesis of other hexosyl-containing polymers prompted us to examine comparatively the expression of LPS in the opgGH mutant with that of parental strain SL1344. Electrophoresis of LPS on SDS-PAGE gels revealed no significant differences between wild-type SL1344 and the opgGH mutant (data not shown). Silver-staining of the gel showed that the LPS from all of the strains fractionated into approximately 25 bands.

**OPGs and stress-tolerance phenotypes**

The methods used to analyse stress-tolerance phenotypes are described in supplementary data, available with the online version of this paper. The ability to withstand acid and alkali stress was examined by exposing cells to extreme pH (pH 3.0 and 9.8) for 3 h at 37°C (Fig. S2a, available with the online version of this paper). Although acid-shock of pH 3.0 generated a 3-log reduction, there was no significant difference between the mutant and wild-type strain, and 0.1% of the original cell population survived in both strains. Alkaline stress conditions resulted in only a 1-log reduction in both the wild-type and the opgGH mutant strain. Likewise, no differences were observed in opgGH mutant strain FIRN in comparison with the wild-type parental strain (data not shown). Further analysis of additional stress conditions, such as exposure to heat shock (5 min at 58°C), bile salts (24 h in 15% ox bile at 37°C), polymyxin (1 µg ml$^{-1}$ for 1 h at 37°C), and oxidative stress (20 mM hydrogen peroxide for 2 h at 37°C) resulted in no discernible differences between wild-type and the opgGH mutant strain (Fig. S2a). Lastly, exposure to detergent (5% SDS, w/v in LB broth) in liquid cultures generated identical growth patterns over 24 h in shake cultures (37°C, 220 r.p.m.) (Fig. S2b). There were no apparent differences in the colony morphology of the wild-type and the mutant strain.

**Phenotypic microarray analysis**

The methods used for phenotypic microarray analysis are described in supplementary data, available with the online version of this paper. Based on tetrazolium redox chemistry that produces a colour change in response to cell growth and respiration (Bochner, 2003; Zhou et al., 2003), high-throughput analysis of ionic and pH tolerance was studied. In three independent experiments where the opgGH mutant strain was compared with the parental wild-type strain for 72 h, the mutant strain had a long lag period in acidic growth medium (pH 5.0) (Fig. S3). Phenotypic microarray
analysis showed that, comparatively, wild-type SL1344 cells under growth conditions of pH 4.5 could utilize various amino acids such as proline, threonine and aspartic acid more efficiently to alter the growth medium pH than could the opgGH mutant. The differences in the dye reduction (measured as area units; Fig. S3) between SL1344 and SG111 for some amino acids, was greater than twofold (i.e. arginine, asparagine and proline). We followed the high-throughput observation of extended lag under acidic growth conditions by performing individual tests and monitored viable cell counts. There was no discernible difference in the growth patterns of the two strains when growth was monitored by viable cell numbers (data not shown), but there was a significant difference in cellular ATP content in the two strains. At pH 5.0, the mutant strain had lower ATP levels after 12, 15 and 18 h incubation (Table 2). The differences in ATP levels were less prominent after 24 h incubation in acidic growth medium, and both strains had comparable levels of ATP. No differences in ATP content were observed at pH 5.5 and above (data not shown).

**DISCUSSION**

In this study, we have described the isolation and compositional analysis of OPGs from *S. Typhimurium* SL1344. Mutagenesis of the *opgGH* operon resulted in the total loss of OPG biosynthesis, and the mutant strain, SG111, was less virulent in mice. Many factors are involved in the virulence of pathogenic bacteria, and OPGs appear to be among them. In comparison with the wild-type strain, the *opgGH* mutant had lower ATP levels during growth initiation in low-pH medium. Biolog-based analysis of cell phenotypes is based on measuring overall cellular metabolic rates, resulting in a change in the redox potential which is monitored by a dye (Bochner et al., 2001). In light of an apparent paradoxical situation where viable cell numbers could not corroborate high throughput Biolog data (which indicated lower growth potential for the *opgGH* mutant), we examined cellular ATP contents of cells grown under acidic conditions (pH 5.0). The observation that the *opgGH* mutant had lower ATP levels compared to the wild-type cells (Table 2) may suggest that the mutant may be more susceptible to antimicrobial peptides in a low-pH environment, which occurs in phagosomes, and may result in reduced virulence (Alpuche Aranda et al., 1992; Rathman et al., 1996). An increase in LD50 for the *opgGH* mutant (Fig. 4) and poor recovery of this strain in mouse intestine (Fig. 5) could indicate that fewer bacteria survive oral inoculation or the increased ability of the host to kill the *opgGH* mutant. In addition, we observed reduced cellular respiration (Biolog-based dye reduction) when the *opgGH* mutant was exposed to pH 4.5 in the presence of proline, threonine and aspartic acid (Table S1 and Fig. S3). Amino acid decarboxylase systems are known to protect enteric pathogens from gastric acidity (Bhagwat et al., 2005; Foster, 2004). In this context, growth and respiration at low pH in the presence of different amino acid pools mimicking the conditions of phagosomes needs to be examined (Alpuche Aranda et al., 1992; Rathman et al., 1996).

Several studies have been conducted to elucidate the function of OPGs in Gram-negative bacteria, and a number of distinctive features specific to *Salmonella* sp. have emerged from the present study. For example, *opgGH* mutants of *Erwinia chrysanthemi* showed increased capsular polysaccharide synthesis (giving rise to mucoid colonies) as well as hypersensitivity towards bile salts (Coge et al., 2001; Page et al., 2001). No change in colony morphology or change in sensitivity towards bile salts was observed for strain SG111 (Fig S1a). OPG synthesis in *Brucella* sp., which are non-motile, is not under osmotic control, but a defect in the *opgG* gene results in increased sensitivity to surfactants (SDS and deoxycholic acid) (Arellano-Reynoso et al., 2005; Briones et al., 2001), indicating cell-surface alterations, a phenotype also not observed in strain SG111 (Fig S2b). The other significant component of Gram-negative bacteria, the enterobacterial common antigen, has been proposed to play an important role in virulence by protecting the pathogen from bile salts (Ramos-Morales et al., 2003). Nonetheless, the data showing that bile salt resistance of the *opgGH* mutant strain was comparable to the wild-type strain (Fig. S2a), coupled with the fact that the lipopolysaccharide gel pattern was unchanged (data not shown), may indicate that there may be no major alterations in this cell envelope component in the *opgGH* mutant. Stress-tolerance studies involving oxidative stress, pH, starvation, bile salts and heat challenge were unable to distinguish between survival responses of the wild-type and *opgGH* mutant strains (Fig. S2a).

Despite leading to exceedingly different outcomes in symbiotic and pathogenic interactions with their respective eukaryotic hosts, the possible involvement of OPGs in the

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**Table 2. ATP content of *S. enterica* serovar Typhimurium strains SL1344 and SG111**

ATP levels were monitored by luminescence enzyme assay and measured as relative light units (RLU). The RLU (luminescence) data were converted to mol ATP (using a standard ATP reference curve) and normalized for viable cell count (c.f.u.). Mean values (n=3) in each row that are not followed by the same letter in parenthesis indicate significant (P<0.05) differences.

<table>
<thead>
<tr>
<th>Time after inoculation in IF-10 medium (pH 5.0) (h)</th>
<th>10^18×ATP content (mol c.f.u.⁻¹)</th>
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<tr>
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<td>SL1344</td>
</tr>
<tr>
<td>12</td>
<td>1.01±0.04 (a)</td>
</tr>
<tr>
<td>15</td>
<td>0.6±0.02 (a)</td>
</tr>
<tr>
<td>18</td>
<td>0.43±0.01 (a)</td>
</tr>
<tr>
<td>24</td>
<td>0.37±0.02 (a)</td>
</tr>
</tbody>
</table>
respective interactions of many bacteria shows striking parallels (Arellano-Reynoso et al., 2005; Galan & Cossart, 2005; LeVier et al., 2000). For example, the hrpM mutant of *Pseudomonas syringae* pv. *syringae* does not synthesize 2005; LeVier (1,3)-

*Mol Plant Microbe Interact* contains gene homologues for linear and cyclic OPGs (Stover et al., 2000) and it has been shown that transcripts for (cyclic) OPG synthesis are stimulated in *P. aeruginosa* cells grown in biofilms (Mah et al., 2003). Although a mutation in an ndvB-like gene (Bhagwat & Keister, 1995) enabled cells to form biofilms with the characteristic wild-type architecture, it did not result in the development of high-level biofilm-specific antibiotic resistance. It has been further demonstrated that cyclic OPGs protect the cells by sequestering antibiotics and thereby increase survival of the pathogen inside the host. On the other hand, linear OPGs of *P. aeruginosa* are encoded by opgGH and it appears that this locus is not involved in the resistance of biofilms to antibiotics (Lequette et al., 2007). Further studies will be needed to define the precise role played by linear OPGs of *S. Typhimurium* in the mouse virulence model.

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