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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 LD_{50} 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).

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Abbreviation: OPG, osmoregulated periplasmic glucan.

Supplementary methods (describing stress-tolerance experiments and phenotypic microarray analysis), figures and tables are available with the online version of this paper.

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 be the only constituent. Disruption of the *opgGH* operon resulted in a strain that lacked OPGs. The *opgGH* mutant had a longer lag period, reduced motility in hypo-osmotic growth conditions and had attenuated virulence in mice.

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}; Nal), 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 turbidometric 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 analysed 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

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
***S.* Typhimurium**		
S. Typhimurium	LT2 *rpoS′*	SGSC*
FIRN	Wild-type, Nal^r	SGSC*
FG111	FIRN *opgGH*, Km^r	This study
SL1344	Wild-type, *his* Sm^r	Hoiseth & Stocker (1981)
SG111	SL1344 *opgGH*, Km^r	This study
E. coli		
JM109	*endA1 recA1 gyrA96 thi hsdR17 relA1 supE44* Δ(*lac-proAB*) *mcrA* (F′ *traD36 proAB*$^+$ *lacI*^{q}*Z*ΔM15)	Promega
S17-1(λ*pir*)	*recA thi pro hsdR*$^-$*M*$^+$ *RP4*: *2-Tc*: *MuKm* Tn*7* λ*pir*, Tp^r	Kim *et al.* (2002)
Plasmids		
pGEM-T	Vector for cloning of PCR products	Promega
pUC4K	Source of the Km cassette	Stratagene
pKNG101	*oriR6K mobRK2 sacB*, suicide vector, Sm^r	Kim *et al.* (2002)
pOPGG1	pGEM-T derivative harbouring 1924 bp *opgG* PCR fragment	This study
pOPGG11	882 bp *Hpa*I–*Bsu*36I fragment containing the *opgG* coding region from pOPGG1 removed and replaced by Km cassette	This study
pOPGG111	2.1 kbp *Eco*RI fragment from pOPGG11 cloned into pKNG101 at *Sma*I site	This study
pBP16	4.51 kbp insert fragment flanking *opgGH* cloned into pGEM-T	This study
pBK16	4.51 kbp insert from pBP16 cloned into a low-copy-number vector (pPQSL2.0)	This study

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

one-way ANOVA test to determine statistical differences between means of treatments.

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′-ACACCAACTCCGACAACCT-3′) and OPGR1 (5′-CCCTTCGCC-AATGAATC-3′). The 1.9 kbp 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 *Bsu*36I and *Hpa*I, and replaced with the 1.1 kbp kanamycin structural gene from pUC4K to yield pOPGG11. The insert was further cloned at an *Eco*RI site in pKNG101 to yield pOPGG111. pOPGG111 was mobilized to LT2 and FIRN by triparental mating (Bhagwat & Keister, 1995). After sucrose selection (Kim *et al.*, 2002), homologous recombinants (Km^r and Sm^s) were selected and confirmed by Southern blot hybridization (Fig. S1, available with the online version of this paper). The *opgGH* mutant strain of SL1344 was obtained by P22 transduction and confirmed by Southern blot analysis.

To clone the *opgGH* genes from *S.* Typhimurium SL1344 for complementation analysis, a 4.51 kbp region, including 426 bp upstream and 22 bp downstream, at the *opgGH* locus was amplified with forward primer G10 (5′-TTAAACAGGGTTAGCCACCA-3′) and reverse primer C12 (5′-CTCACTCCAGACCTGAAGACC-3′) using a Phusion Fidelity PCR kit (New England Biolabs), according to the manufacturer's instructions. The 4.51 kbp fragment was cloned into pGEM-T to yield pBP16. The *opgGH* genes were cloned into a low-copy-number pACYC177-based vector (pPQSL_2.0) to obtain pBK16. pBK16 was mobilized into *opgGH* mutants by electroporation for complementation studies.

Large-scale isolation and purification of OPGs. To maximize the yield of OPGs, cells were grown in LBNS medium (85 ± 4 mosmol l^{-1}), and OPGs were extracted by the charcoal adsorption procedure and eluted with aqueous pyridine as described by Lequette *et al.* (2004). After lyophilization, crude OPGs were subjected to gel filtration using a BioGel P-4 column (1.5×100 cm). The column was eluted at room temperature with 7 % 1-propanol, and OPG-containing fractions were monitored by determining the presence of hexose using the phenol/sulfuric acid assay (Dubois *et al.*, 1956). The peak fractions containing the highest amounts of hexose were pooled and lyophilized to isolate total OPGs. The charged OPGs were further fractionated from total OPGs by ion-exchange chromatography using a DEAE-cellulose (Whatman DE53; Sigma) column and eluted in a step-wise gradient from 0 to 0.5 M NaCl. The unbound fraction was referred to as neutral OPGs, and the charged OPGs were eluted with 0.5 M NaCl.

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 were estimated as described earlier by Lacroix *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, lyophilized, 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.

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 OD_{600} 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 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 microlitres 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 acclimated 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 fit to observed proportions surviving for each (strain, days, group) using SAS Proc PROBIT (SAS Institute, 1999) with the INVERSECL option to obtain LD_{50} 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

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 µl cell suspension) were withdrawn in triplicate at various times and mixed with 100 µl luciferin-luciferase/lysis reagent (Promega), and the luminescent signal was measured after 5 min with a Berthold LB 9501 luminometer (Berthold Technologies).

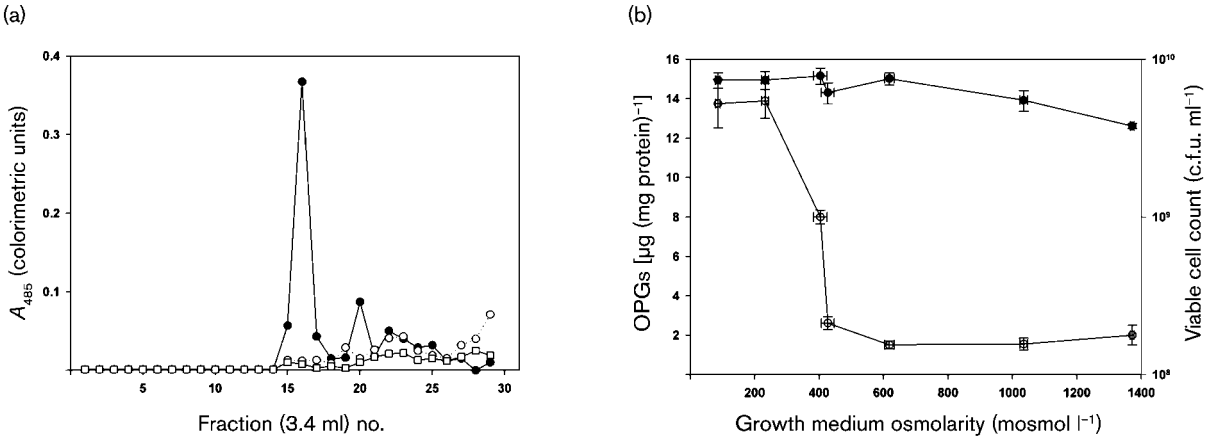
RESULTS

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 ~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 ± 0.23 µmol (µmol 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 µg glucose equivalents (mg cellular protein)$^{-1}$] was influenced by the osmolarity 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 (osmolarity levels of 407 ± 4, 85 ± 4 and 310 ± 9 mosmol l^{-1}, respectively) were indistinguishable from wild-type parental strains (Fig. 2a; data not shown for EG medium). Compared to their growth



(c)

Glucose linkages (mol%)

t	-3-linked	-2-linked	-6-linked	-2,3-linked	-3,6-linked	-2,6-linked	-2,3,6-linked
17.13±1.1	4.27±1.7	35.60±4.0	4.80±1.6	9.23±1.1	1.37±1.0	14.90±2.6	3.40±2.9

Fig. 1. Characterization of OPGs from *S. enterica* serovar Typhimurium SL1344. (a) BioGel P4 gel filtration chromatography of OPGs from SL1344 (●) and *opgGH* mutant strain SG111 (□). Crude OPGs obtained after pyridine extraction were lyophilized and fractionated using 7 % 1-propanol on a BioGel P4 column (1.5×100 cm). Fractions (3.5 ml) were collected and analysed for total hexose by the phenol/sulfuric acid method. Cells were grown in LBNS [85 mosmol l^{-1}; SL1344 (●) and *opgGH* (□)] or in LBNS + 0.5 M NaCl (1038 mosmol l^{-1}; SL1344, ○). (b) Osmoregulation of OPGs. SL1344 cells were grown in LBNS with varying concentrations of NaCl (85–1370 mosmol l^{-1}). OPGs obtained after BioGel P4 chromatography were estimated and plotted as µg OPGs (mg cellular protein)$^{-1}$ (○). Viable cell count was determined by spread-plating serial 10-fold dilutions onto LB agar (●). (c) Linkage analysis of OPGs obtained after BioGel P4 chromatography.

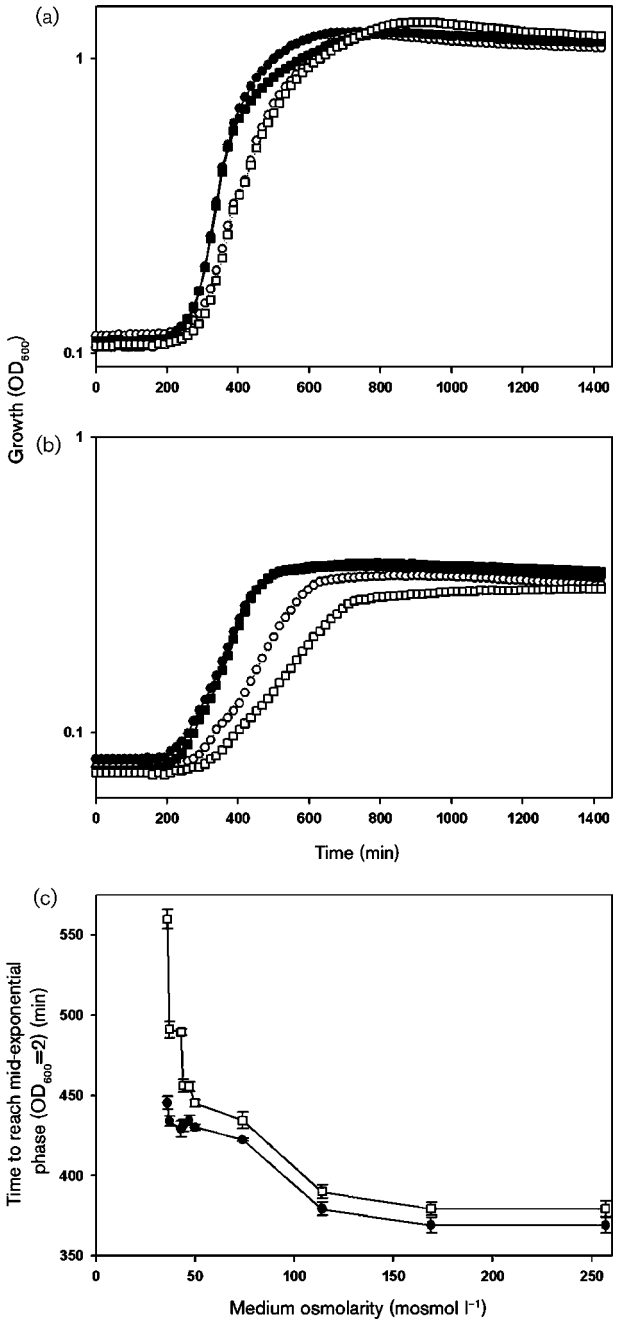


Fig. 2. Growth characteristics of wild-type (SL1344 ○, ●) and the *opgGH* mutant strain (□, ■) in media with different osmolarities. (a) Growth curves in LBNS (84 mosmol l^{-1}; ○, □) and LB (407 mosmol l^{-1}; ●, ■) media. (b) Growth curves in LNNS (36 mosmol l^{-1}; ○, □) and LNNS + 0.155 M NaCl (240 mosmol l^{-1}; ●, ■). (c) Effect of medium osmolarity on time required to reach mid-exponential growth phase for SL1344 (●) and *opgGH* mutant strain SG111 (□). LNNS medium was supplemented with varying amounts of NaCl to adjust the osmolarity.

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 OD_{600} 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 10^6 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 10^3–10^7 cells per animal. The results of these experiments showed that $\log_{10}4.7$ cells per animal were needed to achieve an LD_{50}

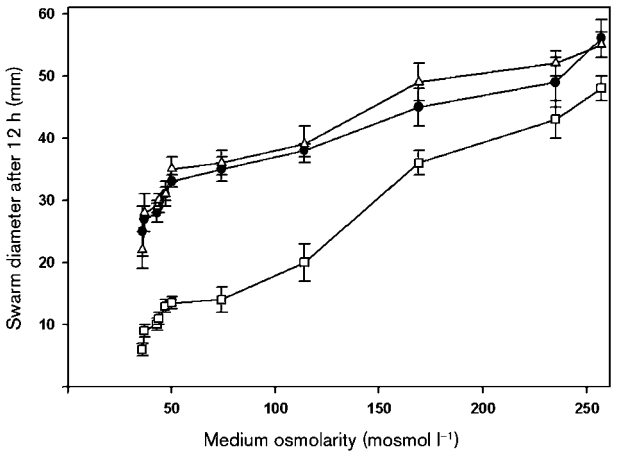


Fig. 3. Effect of the *opgGH* mutation on swarm motility. The graph shows swarm motility diameters (mm) of SL1344 (●), *opgGH* (□) and *opgGH*(pBK16) (△) 12 h after inoculation in LNNS medium with varying osmolarity.

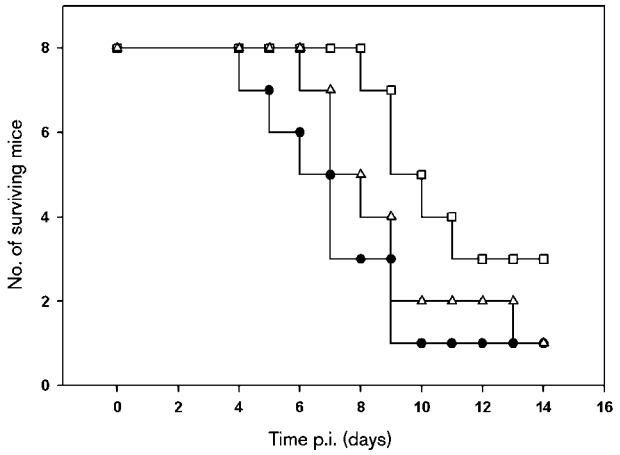


Fig. 4. Mouse virulence study of *S.* Typhimurium strains. BALB/c mice were orally infected with 10^6 c.f.u. of *S.* Typhimurium wild-type SL1344 (●), the *opgGH* mutant strain (□) or *opgGH*(pBK16) (△). Cells were suspended in 0.2 ml saline and mortality was determined over a period of 15 days. Lines represent the number of animals surviving in each experimental group over time (n=8 per group). The experiment was repeated three times; survival curves from a representative experiment are shown.

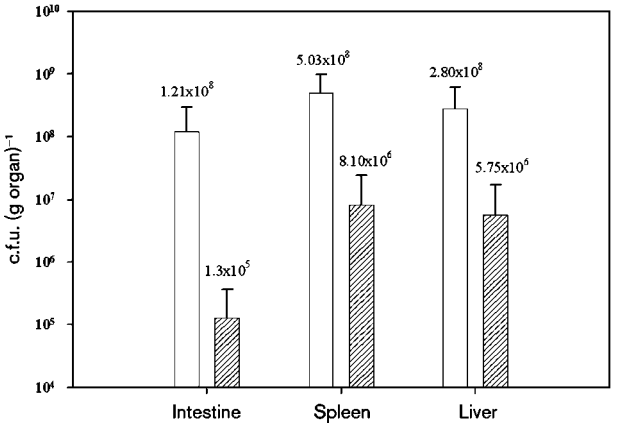


Fig. 5. Effect of the *opgGH* mutation on tissue colonization. Groups of five mice were infected orally with *S.* Typhimurium wild-type strain SL1344 (open bars) or the mutant strain SG111 (shaded bars), each at 1.5×10^7 organisms per mouse. The number of bacteria present in different organs was determined 6 days after infection. Each bar represents the mean for five mice; error bars are SEM. Data shown are representative of three independent experiments. $P<0.0001$ for all tissues.

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 of 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, available with the online version of this paper). 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 LD_{50} 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 (Bearson *et al.*, 1997; 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 (Cogez *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

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.

Time after inoculation in IF-10 medium (pH 5.0) (h)	$10^{18}\times$ATP content (mol c.f.u.$^{-1}$)	
	SL1344	SG111
12	1.01 ± 0.04 (a)	0.31 ± 0.01 (b)
15	0.6 ± 0.02 (a)	0.25 ± 0.03 (b)
18	0.43 ± 0.01 (a)	0.30 ± 0.01 (b)
24	0.37 ± 0.02 (a)	0.36 ± 0.03 (a)

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 OPGs and loses its ability to cause brown spot disorder on common bean (*Phaseolus vulgaris*) (Loubens *et al.*, 1993). *P. aeruginosa* 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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