

Osmoregulated periplasmic glucans are needed for competitive growth and biofilm formation by *Salmonella enterica* serovar Typhimurium in leafy-green vegetable wash waters and colonization in mice

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Introduction

Salmonella are common agents of gastrointestinal-based diseases in humans and have been recognized as a major foodborne hazard. In humans, the organism is most commonly acquired following ingestion of contaminated food or water (D'Aoust *et al.*, 2001). Traditionally, poultry products have been documented as a major source of contamination in many developed countries (Hald *et al.*, 2004). However, in recent years, *Salmonella* infections

Abstract

Osmoregulated periplasmic glucans (OPGs) are major periplasmic constituents of Gram-negative bacteria. The role of OPGs has been postulated in symbiotic as well as pathogenic host–microorganism interactions. Here, we report the role of OPGs from *Salmonella enterica* serovar Typhimurium during growth and biofilm formation in leafy-green vegetable wash water. The *opgGH* mutant strain, which was defective in OPG biosynthesis, initiated the growth at a slower rate in wash waters obtained from spinach, lettuce and green collard and severely impaired biofilm formation. The lack of OPG synthesis did not influence biofilm formation by the *opgGH* mutant in low-nutrient low-osmolarity laboratory media. In coculture experiments initiated with equal proportions of cells, the *opgGH* mutant was outnumbered by the wild-type strain under the planktonic as well as the biofilm growth conditions. The *opgGH* mutant strain poorly colonized mouse organs when introduced orally along with the wild-type strain. This is the first report demonstrating the role of OPGs of *Salmonella* in competitive colonization of biofilms, planktonic cultures and mouse organs.

associated with raw vegetables have occurred with increased frequency, particularly involving fresh produce (Horby *et al.*, 2003; CDC, 2005; Singh *et al.*, 2007). While the specific sources of contamination have not been identified, fresh produce is grown in natural habitats for *Salmonella* reservoirs (such as birds, amphibians, reptiles and poultry). One possible source for foodborne infections is the quality of water, either in the liquid phase used to wash the produce or in the form of ice used in shipping or storage (Bhagwat, 2006). Irrigation water quality is also of significant

importance as it may be responsible for carrying microorganisms from the field to the fork (Wachtel *et al.*, 2002; Steele & Odumeru, 2004; Duffy *et al.*, 2005). In several instances, foodborne illnesses have been traced to poor or unsanitary postharvest practices, specifically to nonpotable cooling water and ice (Harris *et al.*, 2003; Steele & Odumeru, 2004). *Salmonella*, as a consequence of its lifestyle, endures extended periods of nutrient deprivation in natural aquatic and terrestrial environments while retaining its pathogenic potential (Foster & Spector, 1995; D'Aoust *et al.*, 2001). A number of environmental factors, including nutrient deprivation, osmolarity and availability of oxygen, have been implicated in modulating the virulence of *Salmonella*, implying an empirical relationship between survival in nature and survival in the host organism (Arricau *et al.*, 1998; Barak *et al.*, 2005).

Relatively little is known about how *Salmonella* survive in nutrient-deprived, low-osmolarity environments such as irrigation and vegetable wash waters (Barrow *et al.*, 1996; Casani *et al.*, 2005). Growth of cells requires that the cytoplasm contains essential constituents with a total osmolarity of about 300 mosmol L⁻¹ (Kennedy, 1996). When cells are grown in a low-osmolarity medium, swelling and rupturing of the cytoplasmic membrane is prevented by the osmolarity of the periplasmic space, which is mainly due to anionic short glucose chains, referred to in the literature as membrane-derived oligosaccharides (MDOs) or osmoregulated periplasmic glucans (OPGs) as per the new nomenclature (Miller *et al.*, 1986; Kennedy, 1996; Bohin, 2000; Bohin & Lacroix, 2007). When cells of *Escherichia coli* are grown in a low-osmolarity medium (*c.* 30 mosmol L⁻¹), OPGs may represent as much as 5–7% (dry weight) of the cells, and may constitute a considerable fraction of the fixed anions in the periplasm (Miller *et al.*, 1986). OPGs are thought to play an important, but poorly understood, role in host–microorganism interactions involving specific plant and animal hosts (Bhagwat *et al.*, 1996; Page *et al.*, 2001; Arellano-Reynoso *et al.*, 2005). In *E. coli*, the roles of OPGs in cell-to-cell signaling, chemotaxis, regulation of polysaccharide synthesis and resistance to sodium dodecyl sulfate (SDS) have been postulated (Ebel *et al.*, 1997; Bohin, 2000; Rajagopal *et al.*, 2003).

We recently observed that the *opgGH* (previously referred to as *mdoGH*) mutant of *Salmonella enterica* serovar Typhimurium was compromised in mouse virulence and required a 2-log higher oral dose to achieve a lethal dose 50% (LD₅₀) comparable to that of the wild-type strain (Bhagwat *et al.*, 2009). We wanted to investigate the role of OPGs in the growth and competence of *Salmonella* in environments critical for foodborne outbreaks such as wash waters obtained from leafy-green vegetables. Because bacteria often adhere to surfaces and form biofilm communities in their natural settings (Donlan & Costerton, 2002; Burmolle *et al.*,

2006), we investigated the biofilm-forming ability of the *opgGH* mutant strain under low-nutrient low-osmolarity conditions. Lastly, we checked whether the inability to synthesize OPGs would compromise the strain's competitive colonization potential in mouse tissue as well as under biofilm and planktonic conditions.

Materials and methods

Bacterial strains and culture conditions

Salmonella enterica serovar Typhimurium wild-type strain SL1344 (Nal-r) and the *opgGH* null mutant SG111 (Km-r) (Bhagwat *et al.*, 2009) 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 kanamycin (50 µg mL⁻¹), nalidixic acid (10 µg mL⁻¹) or streptomycin (50 µg mL⁻¹). *Salmonella–Shigella* agar and brilliant green agar (Difco, Franklin Lakes, NJ), *Salmonella* semi-selective indicator media with appropriate antibiotics, were used to isolate *Salmonella* from mouse tissue. The osmolarity of growth media (mosmol L⁻¹) was measured using a Wescor vapor pressure osmometer (model 5500, Wescor Inc., Logan, UT).

The growth rates of the wild type and *opgGH* mutant were determined in different growth media such as LB broth and low-nutrient no-salt (LNNS) media (which are 1 : 20 diluted LB broth without NaCl) having osmolarity values of 407 ± 4 and 31 ± 3 mosmol L⁻¹, respectively (+/- denote SD of mean). Growth was measured using a Bioscreen C automatic turbidometric analyzer (GrowthCurves USA, NJ). Starter cultures were prepared by inoculating a single colony of the appropriate strain into LB broth, followed by overnight incubation at 37 °C. This culture was diluted 1 : 10 000 into fresh media of varying osmolarities, and 250 µL per well was transferred into a 100-well honeycomb Bioscreen plate. Growth was analyzed at 37 °C with continuous shaking, and for each sample, data were collected from five replicate wells. In the initial experiments, growth was also measured by performing viable cell counts on LB agar media to ensure that the OD reflects viable cell numbers appropriately (Mellefont *et al.*, 2005). 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).

Biofilm formation

The overnight-grown cultures were diluted 1 : 10 000 in fresh media or vegetable wash waters, placed in sterile polystyrene microplates at 100 µL per well and incubated for 24 h static at 30 °C for use in biofilm studies (O'Toole & Kolter, 1998). Eight wells were inoculated for each sample. After a 24-h incubation, microplate cultures were aspirated

and washed five times with 450 μL sterile distilled water with an EL_x50 plate washer (BioTek Instruments Inc., Winooski, VT). The plates were air dried, and 150 μL of Protocol crystal violet solution (0.41% w/v dye, 12.0% ethanol and 0.1% phenol in water; Fisher Scientific Company, LLC, Kalamazoo, MI) was added per well and incubated at room temperature for 45 min. The wells were then aspirated and washed five times with 450 μL sterile distilled water. After allowing the plates to air dry, 200 μL of 95% ethanol was added to each well. A multichannel pipettor was used to mix the contents of the wells and to dissolve the crystal violet dye. $A_{600\text{ nm}}$ was then recorded for each well using a microquant microplate spectrophotometer (BioTek Instruments Inc.). The average absorbance of eight control wells (that had contained culture medium only) was subtracted from each sample well to determine the amount of biofilm present.

In order to determine viable counts, biofilms from individual wells (before crystal violet staining) were suspended in 100 μL of saline and 100 mg glass powder (Sigma Chemical Co., St. Louis, MO). The suspended biofilms were recovered in three washes with 100 μL saline. The suspension was vortexed vigorously for 1 min, and 10-fold serial dilutions were plated on selective media to determine viable cell counts. The detection limit was 10^3 cells per well.

Preparation of vegetable wash water

The vegetable rinse water was prepared as described previously (Bhagwat, 2004). Briefly, fresh spinach (*Spinacia oleracea*), lettuce (*Lactuca sativa*) and collard green (*Brassica oleracea*) vegetables were obtained from local grocery stores. The produce (250 g) was sliced to c. 5 cm \times 5 cm and washed for 30 min by gentle shaking in a plastic container (33 cm \times 22 cm \times 8 cm) containing 500 mL of deionized distilled water. The vegetable rinse solution was decanted from the tray and was filtered through a glass wool column placed in a 50-mL syringe. The filtered vegetable rinse-water was centrifuged for 10 min at 4000 g at room temperature. The supernatant was made bacteria-free by filtering through a 0.22- μm nylon filter and was used in biofilm experiments. The pH and osmolarity values were measured as described.

Mouse virulence studies

Five-week-old male BALB/c mice were purchased from the Small Animals Division of the National Cancer Institute (Frederick, MD). Mice were housed in an AllenTown Caging Biocontainment-isolator rack, four to five per cage, and provided with Harland-Teklad rodent chow and deionized water *ad libitum*. Mice were acclimated 1 week before use, and all animal protocols were approved by the Institutional Animal Care and Use Committee. Animals were fasted for c. 12 h before being inoculated with 0.2 mL of an *S. enterica* serovar Typhimurium suspension (in 0.9% NaCl) by an oral

gavage. Bacterial strains were grown in LB medium at 37 °C without shaking for 16–18 h, suspended in saline and adjusted to the appropriate cell density before oral infection. Viable cell counts were confirmed by retrospective spread plating onto LB agar plates and incubating the plates overnight at 37 °C.

To analyze colonization of individual organs by each bacterial strain, mice were sacrificed 6 days postinfection. Individual organs (liver, spleen and intestine) were dissected, weighed and homogenized in LB medium. Cell counts were determined by spread plating appropriate dilutions onto Brilliant green agar plates (Difco) containing streptomycin (50 $\mu\text{g mL}^{-1}$) or kanamycin (25 $\mu\text{g mL}^{-1}$). Individual colonies were counted after an overnight incubation at 37 °C, and statistical analysis was performed using ANOVA with *post hoc* analysis for multiple comparisons or the Mann–Whitney nonparametric test. A value of $P < 0.05$ was considered significant.

Statistical analysis

For statistical analyses, SIGMASTAT 3.0 software (Ashburn, VA) was used. Data were analyzed by the one-way ANOVA test to determine statistical differences between the means of treatments.

Results

OPGs are needed to achieve optimal growth rates in low-nutrient and low-osmolarity media

We examined the contributions of OPGs in growth and biofilm formation by *S. enterica* serovar Typhimurium strains in LNNS media (Fig. 1a). Upon 1:10 000-fold dilution of stationary-phase culture to a fresh LNNS medium, wild-type cells had a 'relative OD lag time' of 400 ± 22 min as compared with 585 ± 31 min required by the *opgGH* mutant (Fig. 1a, filled symbols). The delay in initiating growth was a phenomenon specific to low osmolarity of the medium, as increasing the osmolarity from 31 to 240 mosmol L^{-1} by addition of NaCl (0.155 M final concentration) restored normal growth in the *opgGH* mutant strain (Fig. 1a, open symbols).

We then compared the growth potential of wild-type and *opgGH* mutant strains in vegetable wash waters obtained from leafy-green vegetables such as spinach, collard green and lettuce (Fig. 1b–d). Similar to what was observed with LNNS growth medium, lack of OPG synthesis in the *opgGH* mutant severely affected its ability to initiate growth in vegetable wash waters that had osmolarity values of 25–29 mosmol L^{-1} (Table 1). The observed delay to initiate growth by the *opgGH* mutant does not appear to be related to its ability to utilize certain nutrients in vegetable wash waters. By the time strains reached the stationary growth

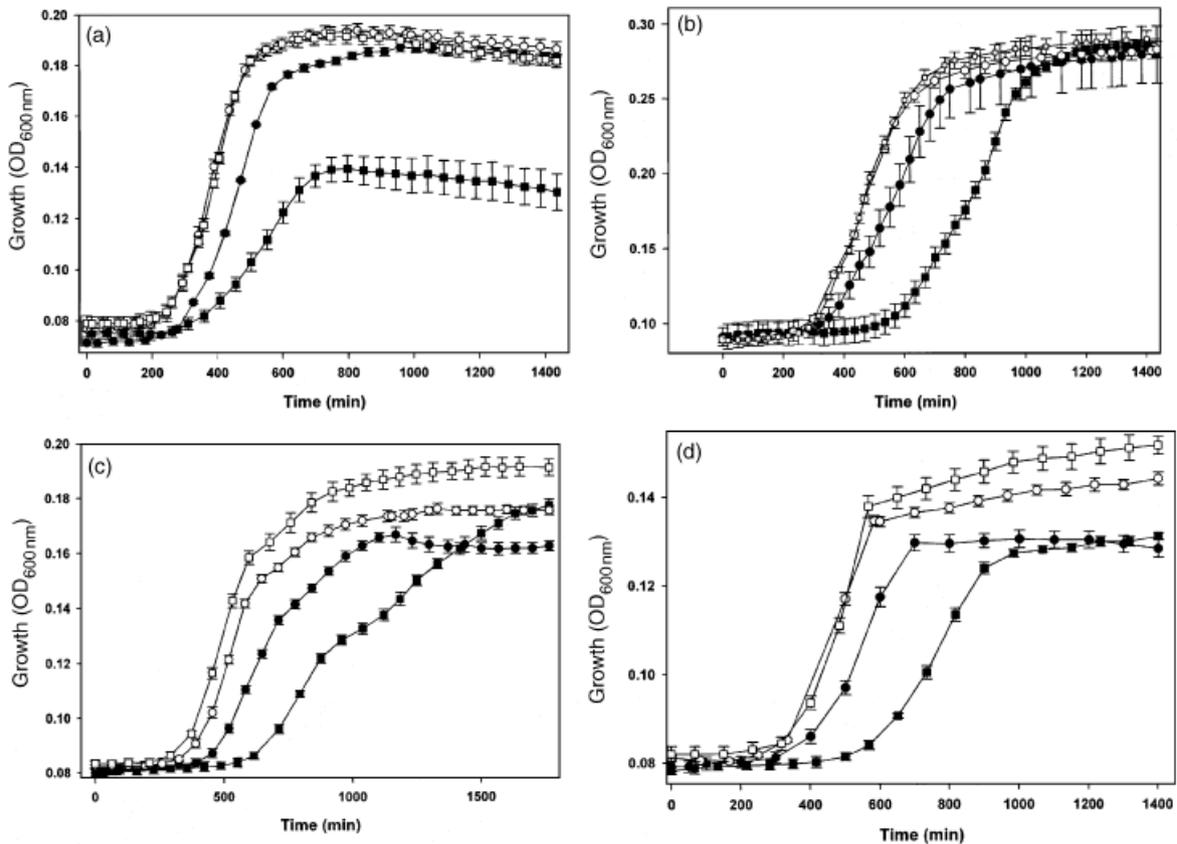


Fig. 1. Growth of *Salmonella enterica* serovar Typhimurium wild-type and *opgGH* mutant strains in (a) LNNS medium, (b) wash waters obtained from spinach, (c) green collard and (d) lettuce. Growth was measured every 15 min by measuring $A_{600\text{ nm}}$ using a BioScreen growth chamber either at low osmolarity (\bullet , \blacksquare ; 25–31 mosmol L^{-1}) or after addition of NaCl to 0.155 M (final concentration) (\circ , \square ; 240–255 mosmol L^{-1}). Wild type (\bullet , \circ) and *opgGH* mutant strain (\blacksquare , \square).

Table 1. Ability of various wash waters to support the growth of *Salmonella* with and without addition of external osmoticum

Growth medium	Addition of external osmoticum (NaCl, 0.15 M final concentration)*		Osmolarity (mosmol L^{-1})	Viable count after 24 h of growth in pure culture (CFU mL^{-1}) [†]	
		pH		Wild type	<i>opgGH</i>
LNNS	–	6.8 ± 0.1	31 ± 3	$5.48 \pm .03 \times 10^8$	$5.85 \pm 1.1 \times 10^8$
LNNS	+	6.8 ± 0.1	240 ± 11	$5.28 \pm 0.6 \times 10^8$	$5.3 \pm 0.6 \times 10^8$
Spinach wash water	–	6.0 ± 0.1	29 ± 4	$5.85 \pm 0.6 \times 10^8$	$6.6 \pm 0.7 \times 10^8$
Spinach wash water	+	6.0 ± 0.1	244 ± 6	$5.48 \pm .03 \times 10^8$	$7.22 \pm 1.1 \times 10^8$
Green collard wash water	–	6.6 ± 0.1	28 ± 5	$9.9 \pm 2.0 \times 10^8$	$5.3 \pm 3.0 \times 10^8$
Green collard wash water	+	6.6 ± 0.1	255 ± 7	$1.3 \pm 0.5 \times 10^8$	$1.1 \pm 0.27 \times 10^8$
Lettuce wash water	–	5.7 ± 0.1	26 ± 6	$1.9 \pm .07 \times 10^8$	$0.9 \pm .04 \times 10^8$
Lettuce wash water	+	5.7 ± 0.1	250 ± 4	$1.5 \pm 0.1 \times 10^8$	$1.4 \pm .2 \times 10^8$

*Osmolarity was adjusted by addition of 5 M NaCl to 0.15 M final concentration.

[†]Viable cell counts measured immediately after inoculation were $7.3 \pm 1.91 \times 10^4$ (wild type) and $7.29 \pm 1.98 \times 10^4$ (*opgGH* mutant).

phase, all three leafy-green wash waters supported *c.* 11 doublings of the wild type and *opgGH* mutant, and the cell densities measured as viable cell counts after 24 h of growth were not significantly different ($P > 0.05$). Moreover,

adjusting the osmolarity of vegetable wash waters by adding NaCl (0.155 M final concentration) to 240 mosmol L^{-1} restored the normal growth of the *opgGH* mutant in all three wash waters (Fig. 1b–d, open symbols).

OPGs and biofilm formation

Attachment of *Salmonella* to food-processing surfaces and subsequent development of biofilms may have significant economic and public health consequences (Donlan & Costerton, 2002; Burmolle *et al.*, 2006; Agle, 2007). It is suggested that *Salmonella* biofilms adapt structurally to changes in the medium osmolarity and nutrients (Lapidot *et al.*, 2006; Mangalappalli-Illathu *et al.*, 2008). We examined biofilm formation by wild-type and *opgGH* mutant strains in LNNS medium (Fig. 2). In general, the *opgGH* mutant strain appeared to form less biofilm than the wild type in LNNS medium, but the differences were not statistically significant ($P > 0.05$). However, the *opgGH* mutant formed significantly reduced or no biofilms in leafy-green wash waters (Fig. 2). Biofilm formation by the *opgGH* strain in wash waters obtained from green collard and lettuce was below the detection limit ($A_{600\text{ nm}}$ after crystal violet staining < 0.05). In spite of the fact that the *opgGH* mutant was able to support a number of cell divisions and was able to grow in vegetable wash waters, it formed significantly lower quantities of biofilm in spinach wash waters. In general, wild-type *Salmonella* cells formed reduced quantities of biofilm in vegetable wash waters in comparison with LNNS medium.

OPGs and competitive growth in free-living and biofilm states

We examined how the initial delay in resuming growth of the *mdoGH* mutant might affect the strains' ability to

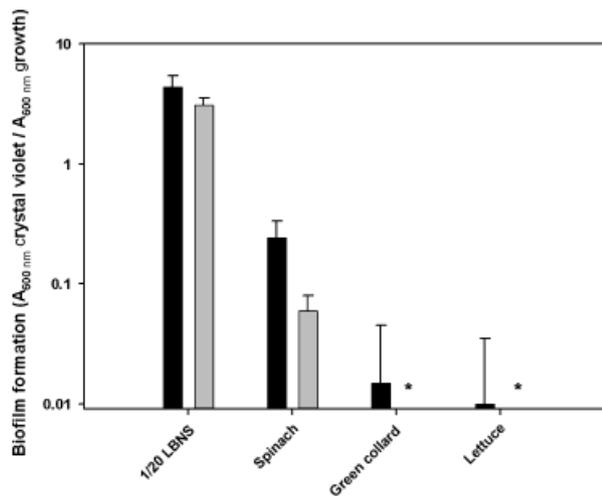


Fig. 2. Biofilm formation by *Salmonella enterica* serovar Typhimurium wild-type and *opgGH* mutant strains. Growth was measured by determining $A_{600\text{ nm}}$. Biofilm was measured after staining with crystal violet. The ratio of $A_{600\text{ nm}}$ after crystal violet staining to initial growth turbidity ($A_{600\text{ nm}}$) is plotted for the wild-type (black bars) and the *opgGH* strain (gray bars) after incubation in LNNS and vegetable wash waters. * $A_{600\text{ nm}}$ of crystal violet < 0.001 .

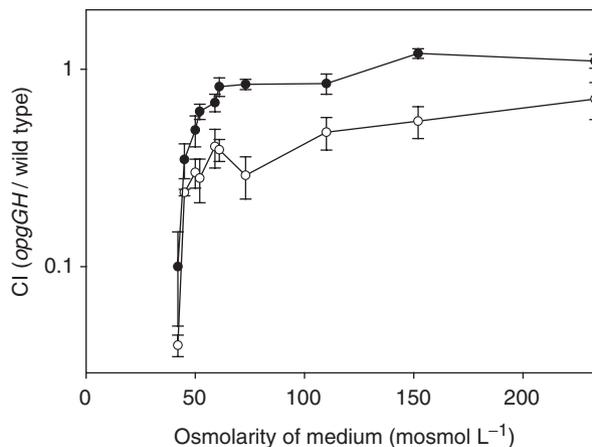


Fig. 3. Competitive growth of *Salmonella enterica* serovar Typhimurium wild-type and *opgGH* mutant strain in planktonic and biofilm states in LNNS medium at varying osmolarities. Wild-type and *opgGH* mutant strains were inoculated in equal proportions in LNNS media with varying osmolarities. Viable cell counts were determined from biofilms (○) and planktonic cells (●) on selective media containing antibiotics. CI is the ratio of viable cell counts for the *opgGH* mutant to that of the wild-type cells at the time of harvest.

compete in biofilm and free-living settings when coinoculated in equal proportions with the wild-type strain (Fig. 3). Biofilms were initiated by inoculating equal numbers of wild-type and *opgGH* mutant cells in LNNS medium of varying osmolarities. After 24 h of static growth in the medium of osmolarity < 40 mosmol L⁻¹, the *opgGH* mutant was outnumbered by the wild-type strain by *c.* 10-fold in the planktonic as well as in the biofilm state [competitive index (CI) ≤ 0.1]. Upon addition of NaCl to increase the medium osmolarity, although the total biofilm formation was negatively affected (data not shown), the competitiveness of the *opgGH* mutant was restored, and CI values closer to 1.0 were obtained at osmolarity > 100 mosmol L⁻¹ (Figs 3 and 4, gray bars). Likewise, in vegetable wash waters obtained from spinach and green collard (Fig. 4), the CI for the *opgGH* mutant was < 0.01 in biofilms, indicating that the OPG-lacking cells were outnumbered by the wild-type strain in biofilms. Viable count data from biofilms obtained from vegetable wash waters indicated that the biofilms were mainly composed of the wild-type strain ($< 1.0\%$ of the total viable cells carried the *opgGH* mutation). It may be noted that in pure cultures, very low or no biofilm formation by the *opgGH* mutant strain was observed in vegetable wash waters (Fig. 2) ($< 10^3$ cells mL⁻¹ were recovered from individual wells, data not shown).

Role of OPGs in mouse organ colonization

Recently, we observed that lack of OPG synthesis rendered *Salmonella* less virulent, and *c.* 2-log more cells were needed

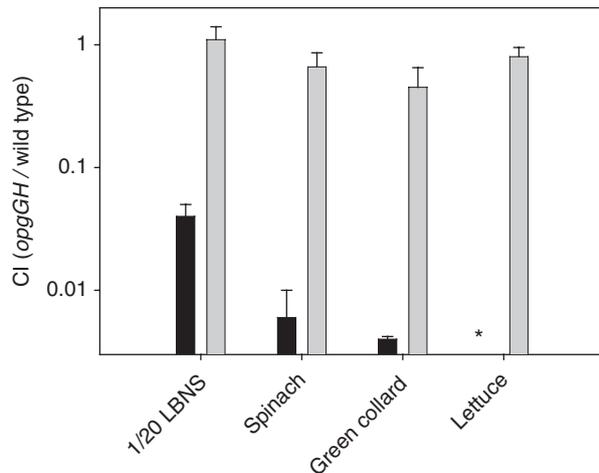


Fig. 4. CI during biofilm formation by *Salmonella enterica* serovar Typhimurium wild-type and *opgGH* mutant strains in vegetable wash waters. Wild-type and *opgGH* mutant strains were mixed in equal proportions and inoculated in LNNS or vegetable wash waters with (gray bars) or without (black bars) addition of NaCl to 0.15 M (final concentration). Viable cell counts were determined from biofilms by plating on selective media containing antibiotics. CI is the ratio of viable cells of the *opgGH* mutant to that of the wild type recovered 24 h postinoculation from biofilms. *Viable cell counts of the *opgGH* mutant strain were below the detection limit ($< 10^2$ cells mL⁻¹).

to achieve an LD₅₀ comparable to that of wild-type cells (Bhagwat *et al.*, 2009). We inoculated mice with equal proportions of wild-type and *opgGH* mutant strains and examined their colonization in the intestine, spleen and liver (Fig. 5). Although there was variation among individual mice, the average CI for the intestine was 0.0001, while the values for the spleen and the liver were 0.08 and 0.09, respectively. The data indicated that the *Salmonella* strain lacking OPGs was unable to colonize the intestinal track in competition with the wild-type strain. On the other hand, during the later stages of infection involving spleen and liver colonization, the *opgGH* mutant was outnumbered by the wild-type strain only by *c.* 10-fold.

Discussion

Water reuse is increasingly regarded as a necessity. Currently, reconditioned water is used for initial washing of vegetables, fluming of unprepared products and scalding water of meat and poultry (Rajkowski *et al.*, 1996; Rajkowski & Baldwin, 2003; Casani *et al.*, 2005). Agricultural and farm wash waters are likely to have low-nutrient low-osmolarity conditions. However, little is known about the microbial factors that are critical to growth, survival and biofilm formation by food-borne pathogens such as *Salmonella* under such conditions (Rychlik & Barrow, 2005). We have demonstrated here that the ability to synthesize OPGs is beneficial for maintaining

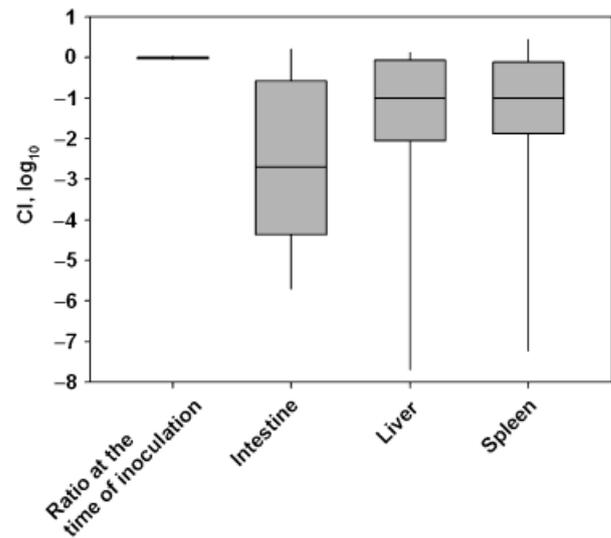


Fig. 5. Competitive colonization of mouse organs after coinoculation with *Salmonella enterica* serovar Typhimurium wild-type and *opgGH* mutant strain 6 days postinfection. CI is the ratio between the *opgGH* mutant strain and the wild type in the individual organs divided by the ratio of the two strains in the inoculum. The distribution of CI from individual animals is shown in a box and whisker format. Boxes range from the 25th to the 75th percentile and are intersected by the median line. Whiskers extending below and above the box range from the 10th to the 90th percentile, respectively.

the optimal growth potential. The longer time needed by *Salmonella* under low-osmotic conditions could be due to the instability of the membrane structures created due to the lack of OPGs. As the name indicates, OPGs are osmoregulated and their synthesis and accumulation is inversely proportional to the osmotic strength of the environment (Bohin, 2000). Thus, after increasing medium osmolarity from 31 to 240 mosmol L⁻¹, bacterial growth was relatively independent of OPG synthesis (Fig. 1 and Table 1). *Salmonella* strain lacking OPGs was a poor competitor in various mouse organs as well as in low-osmolarity low-nutrient media in planktonic and biofilm states (Figs 3–5).

OPGs were suggested to play a critical role in conferring resistance to SDS in *E. coli* (Rajagopal *et al.*, 2003). It was postulated that anionic detergents such as SDS would be repelled by the OPGs. In *E. coli*, lack of OPGs also resulted in increased colonic acid production and reduced motility (Kennedy, 1996; Ebel *et al.*, 1997). It was further demonstrated that secondary mutations in the *rsc* gene family (namely, *rscB*, *rscD* and *rscF*) restored motility in *opgGH* mutant strains of *E. coli* (Giris *et al.*, 2007). The authors suggested that the *rsc* pathway could be hyperactivated in *opgGH* mutants because *rscB* and *rscD* act as repressors of flagella synthesis (Majdalani & Gottesman, 2005). Based on what is observed in *E. coli*, the Rcs pathway might be overactivated under low-osmolarity conditions in the *opgGH* mutant strain of *Salmonella*. Hyperactivation of the

Rcs pathway in *Salmonella* has been shown to attenuate its virulence response (Mousslim *et al.*, 2004; Garcia-Calderon *et al.*, 2005). Thus, OPGs may be required to maintain the low level of stress response regulators such as Rcs B, RcsD and RcsF during mouse colonization.

Comparatively little is known about how *Salmonella* strains grow under low-nutrient, low-osmolarity conditions. In-depth growth analysis of enteric human pathogens under such challenging conditions may provide insights into strategies to reduce human health risk associated with farm waters. We have presented evidence to indicate that the ability to synthesize OPGs is of a significant advantage to bacteria in order to grow, form biofilms and compete in environments such as leafy-green wash waters as well as mouse colonization. It is possible that the presence of OPGs may confer structural stability, which is required for efficient nutrient uptake. Further studies are required to determine whether the *opgGH* mutant is impaired in nutrient uptake, especially under low-osmolarity conditions.

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