The enzyme phosphoglucosyltransferase (Pgm) is required by *Salmonella enterica* serovar Typhimurium for O-antigen production, resistance to antimicrobial peptides and *in vivo* fitness

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The enzyme phosphoglucosyltransferase (Pgm) catalyses the interconversion of glucose 1-phosphate and glucose 6-phosphate and contributes to glycolysis and the generation of sugar nucleotides for biosynthesis. To assess the role of this enzyme in the biology of the pathogen *Salmonella enterica* serovar Typhimurium we have characterized a *pgm* deletion mutant in strain SL1344. Compared to SL1344, SL1344 *pgm* had impaired growth *in vitro*, was deficient in the ability to utilize galactose as a carbon source and displayed reduced O-antigen polymer length. The mutant was also more susceptible to antimicrobial peptides and showed decreased fitness in the mouse typhoid model. The *in vivo* phenotype of SL1344 *pgm* indicated a role for *pgm* in the early stages of infection, most likely through deficient O-antigen production. Although *pgm* mutants in other pathogens have potential as live attenuated vaccine strains, SL1344 *pgm* was not sufficiently attenuated for such use.

INTRODUCTION

The enzyme phosphoglucosyltransferase (Pgm, EC 5.4.2.2) catalyses the interconversion of glucose 1-phosphate and glucose 6-phosphate. Glucose 6-phosphate enters the glycolysis pathway while glucose 1-phosphate serves as a precursor for the generation of sugar nucleotides used in biosynthesis. *pgm* mutants in *Escherichia coli* are defective in their ability to utilize galactose as a carbon source because this carbohydrate enters glycolysis via its conversion to glucose 1-phosphate, which is then converted to glucose 6-phosphate by Pgm (Adhya & Schwartz, 1971). The enzyme is important in the virulence of a number of Gram-positive and Gram-negative pathogens, including *Bordetella bronchiseptica* (West *et al.*, 2000), *Neisseria meningitidis* (Plant *et al.*, 2006), *Stenotrophomonas maltophilia* (McKay *et al.*, 2003), *Brucella abortus* (Ugalde *et al.*, 2000), *Streptococcus gordonii* (Bizzini *et al.*, 2007), *Streptococcus pneumoniae* (Hardy *et al.*, 2001) and *Streptococcus iniae* (Buchanan *et al.*, 2005). These attenuated virulence phenotypes are typically associated with alterations in LPS, cell wall and/or polysaccharide capsule production due to the depletion of the sugar nucleotides needed for the biosynthesis of these structures. Furthermore, *pgm* mutants in *B. abortus* and *S. iniae* have potential as live attenuated vaccine strains (Buchanan *et al.*, 2005; Ugalde *et al.*, 2003). Despite the apparent importance of *pgm* in the virulence of several pathogens and its

potential exploitation through the development of live attenuated vaccine strains, few data are available on the role and importance of this enzyme in the biology of *Salmonella enterica*. *S. enterica* is a diverse pathogen divided into six subspecies and further classified into a total of >2400 serovars based on the antigenicity of the H (flagella)- and O (LPS)-antigens. It is responsible for a range of infections in different hosts, including humans. Of particular note is *S. enterica* serovar Typhi, the cause of typhoid fever, a disease with an estimated annual worldwide incidence of 22 million cases with 200 000 deaths (Crump *et al.*, 2004). *S. enterica* serovar Typhimurium, itself an important pathogen as a cause of gastroenteritis in humans, has added significance due to its use in the mouse to produce a model of typhoid fever. The *S. enterica* serovar Typhimurium Pgm enzyme (GenBank accession no. AAL19642) and its *E. coli* counterpart (AAC73782) are both 546 aa in length and share 97 % identity at the protein level. It has been demonstrated that *pgm* contributes to the ability of *S. Typhimurium* to grow under conditions of high temperature and osmolarity through the production of trehalose derived from UDP-glucose, with trehalose being suggested to aid protein stabilization or turgor maintenance under these conditions (Canovas *et al.*, 2001). However, no data are available on the contribution of *pgm* to virulence-related phenotypes of *S. enterica*, so we have characterized a *pgm* deletion mutant in *S. Typhimurium*. We found that *pgm* in *S. Typhimurium* contributed to resistance to antimicrobial peptides and to *in vivo* fitness in

Abbreviation: AMP, antimicrobial peptide.

a mouse model of typhoid fever. These phenotypes are likely to be due, at least in part, to defects in LPS biosynthesis in the *pgm* mutant.

METHODS

Generation of *pgm* deletion mutant and complementation. The strains used in this study are shown in Table 1. A *S. Typhimurium* SL1344 *pgm* deletion mutant was constructed by a modification of the ET-cloning procedure (Mo *et al.*, 2006), replacing *pgm* (gene designation STM0695 in LT2, GenBank accession no. AAL19642) with the kanamycin-resistance gene cassette from pUC4Kan (Amersham). PCR was used to amplify the antibiotic-resistance cassette with 5' and 3' 60 bp homology arms complementary to the flanking regions of *pgm* (see Table 2 for primer sequences). Electrocompetent *S. Typhimurium* LB5010 carrying plasmid pBAD λ red was grown in LB broth to an OD₅₉₅ of 0.25. Arabinose was added to 0.2% (final w/v) to induce expression of the phage lambda genes *exo*, *bet* and *gam* encoded by pBAD λ red and the culture was grown to OD₅₉₅ 0.5. The bacteria were then electroporated with the PCR product containing the kanamycin-resistance cassette prepared using Qiagen PCR Clean-up. Mutant colonies were selected on LB agar plates supplemented with 50 μ g kanamycin ml⁻¹. Allelic replacement of *pgm* on the bacterial chromosome was confirmed by PCR and sequencing of the product.

The *pgm* mutation in *S. Typhimurium* LB5010 was then transduced by bacteriophage P22 (Schmiegler, 1972) into strain SL1344. Transductants were selected on 50 μ g kanamycin ml⁻¹ and the *pgm* deletion was confirmed by PCR and sequencing, as described above, and by Southern blot using the kanamycin-resistance cassette as a probe. LPS serotype was confirmed by agglutination with anti-O4 serotype antisera using anti-O9 antisera as negative control (Remel Europe/Oxoid).

For complementation, *pgm* from SL1344 was amplified by PCR (Table 2) and cloned into pBR322 via *Hind*III and *Bam*HI (New England Biolabs). It was confirmed by DNA sequencing that the construct was correct. The resultant plasmid was designated pBR322-*pgm*+. Empty pBR322 was used as negative control and both were electroporated into SL1344 *pgm* with selection on LB containing ampicillin at 100 μ g ml⁻¹. The presence of the correct plasmid was confirmed by PCR using primers flanking the cloning site (Table 2).

Growth *in vitro* and galactose utilization. Overnight cultures were prepared in 10 ml LB broth by inoculation with three colonies from freshly streaked LB agar plates and incubated with shaking (180 r.p.m.) at 37 °C for 17.5 h. These cultures were diluted 1:100 000 into 100 ml fresh, pre-warmed LB broth and incubated with shaking at 37 °C with samples taken at the times indicated for OD₅₉₅ readings and viable counts onto LB agar plates. Exponential

generation times were calculated from growth rates between 4 and 6 h. To assess the ability to utilize galactose, SL1344 and SL1344 *pgm* were grown in M9 minimal medium (plus histidine at 40 μ g ml⁻¹) supplemented with either glucose (0.2% w/v), as positive control, or galactose (0.2% w/v). Growth was assessed visually.

Examination of bacterial morphology. Exponential- and stationary-phase cultures grown in LB broth were viewed with a Philips XL30 FEG scanning electron microscope at $\times$ 5000–25 000 magnification as described previously (Paterson *et al.*, 2009).

Examination of LPS. Stationary-phase cultures were harvested and resuspended in 1 ml PBS and 250 μ l LPS buffer 1 (0.1875 M Tris HCl pH 6.8, 6% w/v SDS, 30% w/v glycerol), and 20 μ l of this resuspended cell pellet was boiled for 5 min. Samples were allowed to cool and 70 μ l LPS buffer 2 (0.0625 M Tris/HCl pH 6.8, 0.1% w/v SDS, 10% w/v glycerol, 0.1% w/v bromophenol blue) was added along with proteinase K to a final concentration of 1.8 mg ml⁻¹. Samples were digested at 55 °C overnight, then separated by electrophoresis using Novex 16% Tricine gels (Invitrogen) and visualized by silver staining based on the method of Tsai & Frasch (1982). Equal loading was based on the viable count of the original culture.

Susceptibility to antimicrobial peptides. Susceptibility to melittin (5 μ g ml⁻¹), cecropin P (3 μ g ml⁻¹) and polymyxin B (10 μ g ml⁻¹) (all Sigma) was tested essentially as described by Fields *et al.* (1989). Results were expressed as percentage survival compared to survival in PBS, which was taken as the 100% value. Incubations were for 1 h with polymyxin B and melittin and 3 h with cecropin P. Bacterial counts below the detection limits were ascribed a value just below that limit for the purpose of statistical analysis.

Susceptibility to oxidative stress. Overnight LB cultures were diluted 1:1000 in H Top agar and 3 ml was poured onto LB agar plates. Once set, 6 mm filter discs (Oxoid) to which 10 μ l 2% paraquat was applied (Sigma) were plated on top and the diameter of growth inhibition measured after 24 h incubation at 37 °C.

Mouse infection model. Bacteria from fresh LB plates were grown statically in LB broth at 37 °C for ~17.5 h. Cultures were centrifuged at 4300 g for 10 min at 15 °C, resuspended in PBS and adjusted to the required concentration. Female BALB/c mice (Harlan), 6–8 weeks old, were inoculated intravenously into the lateral tail vein with 10³ c.f.u. in a 200 μ l volume. The dose was confirmed by viable count in LB agar. At the time points indicated, mice were killed and their spleens and livers were removed into sterile water and homogenized using a Stomacher 80 Lab System (Seward). Bacteria were enumerated by viable counts in LB agar.

All work was licensed in compliance with UK Home Office regulations.

Table 1. *S. Typhimurium* strains used in this study

Name	Description	Source/reference
LB5010	Restriction-deficient derivative of LT2	Bullas & Ryu (1983)
SL1344	Wild-type for this study	Hoiseth & Stocker (1981)
SL1344 <i>pgm</i>	<i>pgm</i> deletion mutant in SL1344.	This study
SL1344 <i>pgm</i> (pBR322- <i>pgm</i> +))	<i>pgm</i> mutant complemented with <i>pgm</i> cloned into pBR322	This study
SL1344 <i>pgm</i> (pBR322)	<i>pgm</i> carrying empty pBR322	This study
SL3261	<i>aroA</i> mutant of SL1344; well-characterized live attenuated vaccine strain.	Hoiseth & Stocker (1981)
SL1344 <i>waaB</i>	<i>waaB</i> deletion in SL1344	This study

Table 2. Primers used in this study

Name	Sequence (5'–3')	Use
<i>pgm</i> ODM For	GCGGAATTGATTGAAAAGTTTGC GGAACAATTTAATCCTTG-CATTAGAAGGACCAGGCAGGGAAAGCCACGTTGTGTCTC	Generation of construct to delete <i>pgm</i> ; kanamycin resistance cassette sequence underlined
<i>pgm</i> ODM Rev	GCGCAGCGCCGTTAATACAAAAAGGAGCCGTAAGGCTCCT-TTTTCTTCCATATCGCCCAGCCGCGTCCCGTCAAGTCAGCG	Generation of construct to delete <i>pgm</i> ; kanamycin resistance cassette sequence underlined
<i>pgm</i> For	ACTAAACCTAAGCACGTTCC	Confirmation of <i>pgm</i> deletion
<i>pgm</i> Rev	GTGCGTATCGAATGCGACGC	Confirmation of <i>pgm</i> deletion
<i>pgm</i> Comp For	GCTAGCTGATTAGAAGGACCAGGCAATGG	Cloning of <i>pgm</i> into pBR322; <i>NheI</i> site underlined
<i>pgm</i> Comp Rev	GGATCCTCCATATCGCCCATCAGG	Cloning of <i>pgm</i> into pBR322; <i>Bam</i> HI site underlined
pBRseqIII R	GATCTTCCCATCGGTGATGTCCGC	Confirmation of complementation constructs
pBRseqIII L	ACCATTATTATCATGACATTAACCT	Confirmation of complementation constructs

Bacterial growth in whole mouse blood. Fresh heparinized whole blood from female BALB/c mice (Harlan) was inoculated with each bacterial strain to 2×10^4 c.f.u. ml⁻¹ and incubated with shaking at 37 °C. Viable counts were taken on LB agar at the time points indicated.

Statistical analysis. Data were analysed using the unpaired Student's *t*-test or one-way ANOVA as appropriate using GraphPad Version 4, *P* < 0.05 being considered significant.

RESULTS

Growth *in vitro* and carbon utilization

The growth in LB broth of the *S. Typhimurium* SL1344 *pgm* deletion mutant was compared to that of the wild-type parent strain. Deletion of *pgm* caused slightly impaired growth in LB as shown by viable counts and OD₅₉₅ (Fig. 1). To quantify this defect, generation times were calculated from viable counts between 4 and 6 h. From four separate experiments, the mean ($\pm$ SEM) generation time of SL1344 was 23.24 ± 0.79 min while for SL1344 *pgm* it was 32.46 ± 1.20 min, which was significantly different (*P* = 0.0007). *pgm* mutants in *E. coli* are impaired, but not completely deficient, in their ability to utilize galactose as a carbon source (Adhya & Schwartz, 1971). To test if this is also the case for *S. Typhimurium*, SL1344 and SL1344 *pgm* were grown in M9 minimal medium supplemented with galactose as the sole carbon source and growth assessed visually. M9 minimal media with and without glucose were included as positive and negative controls respectively. As expected, both SL1344 and SL1344 *pgm* were able to utilize

glucose as carbon source while neither grew in M9 minimal medium alone. However, when galactose was the sole carbon source, while SL1344 grew normally, the growth of SL1344 *pgm* was impaired but not completely inhibited.

Deletion of *pgm* alters LPS production in *S. Typhimurium*

Glucose 1-phosphate produced by Pgm is a key precursor in the generation of sugar nucleotides, which in turn are required for LPS biosynthesis. Indeed, defects in LPS production have been shown for *pgm* mutants in *Neisseria meningitidis* (Plant *et al.*, 2006) *Bordetella bronchiseptica* (West *et al.*, 2000), *Vibrio furnissi* (Kim *et al.*, 2003), *Brucella abortus* (Ugalde *et al.*, 2000) and *Stenotrophomonas maltophilia* (McKay *et al.*, 2003). Examination of proteinase K-digested lysates by silver staining revealed that SL1344 *pgm* had lower levels of O-antigen and a reduced average O-antigen polymer length compared to SL1344 (Fig. 2). This phenotype was complemented by provision of *pgm* on pBR322 but not by the empty vector control, confirming that the defect in O-antigen production was due specifically to the deletion of *pgm* (Fig. 2). SL1344 *pgm* did, however, produce small amounts of O-antigen as evidenced by the banding pattern on the silver-stained gel and positive reactions with O-antigen-specific antisera in a slide agglutination assay. SL1344 *pgm* is therefore not a rough mutant but rather displays a reduction in the production of O-antigen and in the average length of the O-antigen polymers. Alterations to the LPS core in SL1344 *pgm* are also apparent on the silver-stained gel (Fig. 2),

Fig. 1. Growth of SL1344 *pgm* in LB broth. Overnight cultures of SL1344 wild-type (■) and SL1344 *pgm* (□) were inoculated into fresh pre-warmed LB and grown with shaking at 37 °C at 180 r.p.m. Growth was measured at the times indicated by viable count (a) and OD₅₉₅ (b). Data representative of two experiments giving similar results.

Fig. 2. Analysis of LPS from *S. Typhimurium* SL1344 wild-type and SL1344 *pgm*. Proteinase K-digested cell lysates from approximately 5×10^7 c.f.u. were separated by SDS-PAGE and silver stained. Lanes: 1, SL1344; 2, SL1344 *pgm*; 3, SL1344 *pgm* (pBR322-*pgm*⁺); 4, SL1344 *pgm* (pBR322).

which may reflect an increased abundance of core lacking O-antigen relative to SL1344.

Analysis of bacterial morphology

Alterations in bacterial cell morphology have been noted previously in *pgm* mutants of several species, including *E. coli* (Lu & Kleckner, 1994), *Streptococcus gordonii* (Bizzini *et al.*, 2007), *Bacillus subtilis* (Lazarevic *et al.*, 2005) and *Streptococcus iniae* (Buchanan *et al.*, 2005). We therefore examined exponential- and stationary-phase cultures of SL1344 and SL1344 *pgm* by scanning electron microscopy, but there was no gross difference evident in the morphology of the bacteria (data not shown).

SL1344 *pgm* is hypersusceptible to antimicrobial peptides

Antimicrobial peptides (AMPs) are important in innate host defence against diverse pathogens, including salmonellae (Jenssen *et al.*, 2006). Given the key role of LPS in bacterial resistance to AMPs (Banemann *et al.*, 1998; Ernst *et al.*, 2001; Gunn, 2008), we tested the susceptibility of SL1344 *pgm* to three AMPs: melittin, cecropin P and polymyxin B (Fig. 3). Melittin is found in bee venom, cecropin P is produced in the porcine intestine and polymyxin B is produced by the soil bacterium *Bacillus polymyxa*. In each case, the resistance to the AMPs of SL1344 *pgm* was significantly reduced compared to SL1344 (Fig. 3). Resistance was restored to wild-type levels by the plasmid pBR322-*pgm*⁺ but not by pBR322, the empty vector control (Fig. 3). Pgm therefore makes an important contribution to the resistance of *S. Typhimurium* to

antimicrobial peptides. SL1344 *pgm* was also more susceptible to the aminoglycoside antibiotic gentamicin (MIC < 1 µg ml⁻¹ compared to an MIC of 10 µg ml⁻¹ for SL1344). This phenotype precluded analysis of the ability of the *pgm* mutant to infect and survive in mammalian cells using the standard gentamicin protection assay because the antibiotic can enter into host cells at low levels and would influence survival of SL1344 *pgm* (Hamrick *et al.*, 2003).

Deletion of *pgm* does not increase susceptibility to oxidative stress

Deletion of *pgm* in *B. bronchiseptica* led to increased susceptibility of the mutant to the superoxide-radical-generating compound paraquat (West *et al.*, 2000). To test if such an effect is seen in *S. Typhimurium*, SL1344 and SL1344 *pgm* were exposed to paraquat in a disc diffusion assay. The mean zone of inhibition was identical for the two strains (14 mm). Deletion of *pgm* in *S. Typhimurium* therefore does not heighten susceptibility to killing by superoxide. The decreased resistance to paraquat shown by *B. bronchiseptica* *pgm* was proposed to result from absence of O-antigen that was shielding the bacterial cell from oxygen radicals through its high charge (West *et al.*, 2000). To test if the O-antigen is important in resistance to oxidative stress in *S. Typhimurium*, we examined the effects of paraquat on an SL1344 *waaB* mutant. Its susceptibility was identical to that of SL1344 and SL1344 *pgm* (mean zone of inhibition 14 mm). *S. Typhimurium* O-antigen is therefore not an important determinant of resistance to oxidative stress, at least under the conditions tested.

SL1344 *pgm* has reduced fitness in a mouse model of typhoid fever

The phenotypes displayed by SL1344 *pgm* *in vitro* suggested that Pgm may have an important role in the ability of *S. Typhimurium* to infect and grow in a mammalian host. To examine the contribution of *pgm* to *S. Typhimurium* fitness *in vivo*, wild-type SL1344 and SL1344 *pgm* were administered to BALB/c mice intravenously. The ability of bacteria to survive and grow was assessed by determining viable counts from spleens and livers for 4 days after infection (Fig. 4a, b). In both the spleen and the liver, and at each time point examined, the viable count was significantly lower for SL1344 *pgm* compared to SL1344. However, SL1344 *pgm* was still able to grow in the liver and spleen with a rate of growth between days 1 and 4 similar to that of SL1344. *pgm* therefore contributes to the *in vivo* fitness of *S. Typhimurium* in this model, particularly in the early stages of infection ($\leq$ day 1). The provision of *pgm* on pBR322 restored SL1344 *pgm* bacterial counts to a level similar to those of SL1344 while the empty vector had no such effect, confirming that the attenuated colonization was due to the specific deletion of *pgm* (Fig. 4c, d).

Deletion of *pgm* in *Brucella abortus* and *Streptococcus iniae* generated attenuated strains that conferred protection

Fig. 3. Susceptibility to antimicrobial peptides. Strains were exposed to melittin (5 µg ml⁻¹) (a), cecropin P (3 µg ml⁻¹) (b) or polymyxin B (10 µg ml⁻¹) (c) and viable counts after 1 h (for melittin and polymyxin B) or 3 h (for cecropin P) expressed as the percentage of the count in PBS in the absence of these antimicrobial peptides. Data pooled from three experiments giving similar results, each performed in duplicate. *, $P < 0.05$ compared to wild-type.

against subsequent virulent infection (Buchanan *et al.*, 2005; Ugalde *et al.*, 2003). To assess if SL1344 p_gm may have utility as a novel live attenuated vaccine strain, two groups of mice were immunized intravenously with either 10⁵ or 10⁶ c.f.u. The prototype attenuated vaccine strain SL3261, an *aroA* mutant, was included as a control. At these doses SL3261 induces robust protection against subsequent wild-type challenge but does not induce significant clinical signs (data not shown). However, at both doses all SL1344 p_gm-infected mice displayed clinical signs and were killed when they were deemed to have irreversibly succumbed to infection. This was in contrast to SL3261, which was tolerated by the mice with minimal clinical effects. On the basis of these data, SL1344 p_gm is not sufficiently attenuated for use as a live attenuated vaccine strain of *S. Typhimurium*.

SL1344 p_gm has impaired growth in whole mouse blood

From the *in vivo* growth curves of SL1344 and SL1344 p_gm, it appears that events during the early stages of infection (<1 day) are critical in determining the attenuated fitness of SL1344 p_gm (Fig. 4). Given that survival in blood is a key event in the early stages of this systemic infection (Roantree, 1967), we compared the growth of SL1344 and SL1344 p_gm in whole mouse blood. The net growth of SL1344 p_gm in whole mouse blood was significantly lower than that of SL1344 after 6 h incubation (Fig. 5). This phenotype was complemented by provision of pBR322-p_gm⁺ but not pBR322 (Fig. 5).

DISCUSSION

We have investigated the effects of deleting the p_gm gene in *S. Typhimurium*. Our data demonstrate a contribution of

p_gm to a number of important phenotypes; in particular, deletion of p_gm resulted in reduced O-antigen polymer length, decreased resistance to antimicrobial peptides and reduced fitness in a mouse model of typhoid fever, as assessed by the ability to colonize infected organs. In addition, SL1344 p_gm was impaired for growth *in vitro* and in the ability to utilize galactose. This deficiency, but not complete inability, in the utilization of galactose has been seen for p_gm mutants in *E. coli* (Adhya & Schwartz, 1971; Lu & Kleckner, 1994). In contrast to p_gm mutants in other bacterial species, we failed to detect an effect of p_gm deletion on gross bacterial cell morphology or resistance to the superoxide radical-generating compound paraquat. Such effects may be species-specific or too subtle in *S. Typhimurium* to be identified under our experimental conditions. In the case of susceptibility to oxidative stress, it was proposed that a p_gm mutant of *Bordetella bronchiseptica* showed reduced resistance to paraquat due to loss of the shielding effect of the O-antigen charge (West *et al.*, 2000). This is not likely to apply to the O-antigen of *S. Typhimurium*, which, unlike the galacturonic acid O-antigen of *B. bronchiseptica*, is composed of neutral sugars and will not be charged under physiological conditions. Indeed, we confirmed that O-antigen is not involved in the resistance of *S. Typhimurium* to paraquat using SL1344 *waaB*.

Defects in LPS biosynthesis have been seen with p_gm mutants in several pathogens, and this phenotype is likely to contribute to their attenuation in models of infection (Kim *et al.*, 2003; Ugalde *et al.*, 2000; West *et al.*, 2000). We extend these findings to *S. Typhimurium* by showing that SL1344 p_gm has reduced O-antigen production and truncated O-antigen polymer compared to SL1344. This is probably due to the role of UDP-glucose as the glucose donor in LPS biosynthesis in salmonellae and other organisms. It should be noted that the alterations in LPS

Fig. 4. SL1344 *pgm* is attenuated for colonization of host organs in the mouse typhoid model. (a, b) Bacterial counts in spleens (a) and livers (b) following intravenous infection with 10^3 c.f.u. *S. Typhimurium* SL1344 wild-type (■) and SL1344 *pgm* (□). (c, d) Complementation with pBR322-*pgm*⁺ restores SL1344 *pgm* bacterial counts to SL1344 levels in spleens (c) and livers (d) at day 3 post-infection. Mean $\pm$ SEM, $n=4$, representative of two experiments giving similar results. *, $P<0.05$ compared to wild-type.

in the absence of *pgm* differ between bacterial species due to the different composition of their LPS. In particular, SL1344 *pgm* was not a smooth mutant as seen with *pgm* mutants in other bacterial species and did produce some O-antigen, albeit with a shorter average O-antigen polymer length than SL1344.

Fig. 5. SL1344 *pgm* is impaired for growth in whole mouse blood. Strains were inoculated into fresh mouse blood at $\sim 2 \times 10^4$ c.f.u. ml⁻¹ and grown with shaking at 37 °C. Bacterial viable counts were taken at the time points indicated. ■, SL1344; □, SL1344 *pgm*; ●, SL1344 *pgm*(pBR322-*pgm*⁺); ○, SL1344 *pgm*(pBR322). *, $P<0.05$ compared to SL1344.

Antimicrobial peptides are an important component of host defence against pathogens, including *S. Typhimurium*. For example, matrilysin-deficient mice, which are unable to activate α -defensin precursors in the intestine, are more susceptible to oral *S. Typhimurium* infection than wild-type mice (Wilson *et al.*, 1999). Deletion of *pgm* markedly decreased the resistance of SL1344 to three AMPs: melittin, cecropin P and polymyxin B. A *B. bronchiseptica* *pgm* mutant also displays heightened susceptibility to the AMP cecropin P (West *et al.*, 2000). Given the role of LPS in protecting bacteria from environmental assaults, the altered LPS production by *pgm* mutants is likely to contribute to this susceptibility to AMPs. Indeed, confirming a role for LPS in protecting *S. Typhimurium* from AMPs, we have found that the rough mutant, SL1344 *waaB*, has decreased resistance to melittin, cecropin P and polymyxin B compared to the wild-type.

The *pgm* mutant was significantly attenuated for colonization of host organs in a mouse model of typhoid fever. Bacterial counts in spleens and livers from mice infected with SL1344 *pgm* were significantly lower compared to those infected with SL1344. *Pgm* therefore contributes to the *in vivo* fitness of *S. Typhimurium*. This is consistent with the attenuated phenotypes shown by *pgm* mutants in several other bacterial pathogens including *Bordetella bronchiseptica* (West *et al.*, 2000), *Neisseria meningitidis* (Plant *et al.*, 2006), *Stenotrophomonas maltophilia* (McKay

et al., 2003), *Brucella abortus* (Ugalde *et al.*, 2000), *Streptococcus gordonii* (Bizzini *et al.*, 2007), *Streptococcus pneumoniae* (Hardy *et al.*, 2001) and *Streptococcus iniae* (Buchanan *et al.*, 2005). Although bacterial counts were lower for SL1344 *pgm*-infected mice compared to those infected with SL1344, the rate of net growth of these strains was similar over days 1–4. This suggests that the contribution of Pgm to *in vivo* fitness manifested in the early stages ($\leq$ day 1) of this infection and Pgm is subsequently dispensable. Indeed, we found SL1344 *pgm* was impaired for growth in whole mouse blood, which is a critical early event in infection. This impaired growth of SL1344 *pgm* in mouse blood is likely to cause reduced bacterial seeding to the spleen and liver following infection and this probably contributes to the lower bacterial counts we describe at day 1 in these organs following infection of mice.

Similar early infection defects *in vivo* have been described previously for *Salmonella* rough and semi-rough LPS mutants, which are more rapidly cleared by phagocytosis (Roantree, 1967). This similarity between SL1344 *pgm* and LPS mutants suggests that reduced O-antigen production and polymer length is likely to be a key cause of the reduced fitness of SL1344 *pgm*. Our data indicating a limited role, if any, for *pgm* after the initial stages of infection are compatible with work showing that O-antigen is not required for intracellular replication of *S. Typhimurium* (Bjur *et al.*, 2006). Finally, *pgm* mutants in other pathogens have shown potential as live attenuated vaccine strains (Buchanan *et al.*, 2005; Ugalde *et al.*, 2003). The potential of SL1344 *pgm* as a vaccine strain was examined in comparison with the prototype attenuated and protective *aroA* mutant SL3261. However, SL1344 *pgm* was considerably more virulent than SL3261 and deemed not significantly attenuated for further consideration as a vaccine strain. This finding probably relates to the fact that, although SL1344 *pgm* is impaired in the early stages of colonizing host organs, once there, it is still able to grow in these organs at a similar rate to wild-type, resulting in disease.

To conclude, we have examined a *pgm* deletion mutant in *S. Typhimurium* and found it to have reduced O-antigen production and average O-antigen polymer length, increased susceptibility to AMPs and reduced fitness in a mouse typhoid fever model. This reduced fitness *in vivo* appears, in large part, due to a particular role for *pgm* in the early stages of infection ($\leq$ day 1) such as survival in blood.

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