The *Salmonella typhi* melittin resistance gene *pqaB* affects intracellular growth in PMA-differentiated U937 cells, polymyxin B resistance and lipopolysaccharide

Sarah J. Baker,¹ John S. Gunn² and Renato Morona¹

Author for correspondence: Sarah J. Baker. Tel: +61 88303 5827. Fax: +61 88303 4362.
e-mail: sbaker@microb.adelaide.edu.au

¹ Department of Microbiology and Immunology, University of Adelaide, Adelaide, South Australia, Australia 5005

² Department of Microbiology, University of Texas Health Science Centre at San Antonio, 7703 Floyd Curl Dr, San Antonio, TX 78284-7758, USA

***Salmonella typhi* is the causative agent of typhoid fever in humans. A cell-culture based assay involving the human monocyte macrophage cell line U937 has been developed to examine *S. typhi* invasion and survival. An *S. typhi* PhoP⁻ (null) mutant was shown to be restricted in net growth in phorbol myristate acetate (PMA) differentiated U937 (PMA-U937) cells, and an *S. typhi* PhoP^c (constitutive) mutant showed a defect in invasion. Neither of the *phoP/Q* mutants were growth impaired in HeLa cells, however the PhoP^c mutant was impaired in invasion. As opposed to what was found for *S. typhi*, *Salmonella typhimurium* wild-type, PhoP⁻ and PhoP^c mutants grew equally well in PMA-U937 cells, indicating that the PhoP⁻-mediated net growth restriction in the PMA-U937 cells was *S. typhi* specific. An *S. typhi* mutation, *pqaB::MudJ*, recently shown to be a PhoP-activated locus, was shown to have a net growth defect in PMA-U937 cells. Sequencing of the *S. typhi* *pqaB* gene revealed it had 98% identity to the fifth gene in a *S. typhimurium* PmrA/B regulated operon necessary for 4-aminoarabinose lipid A modification and polymyxin B resistance. The *pqaB* locus was regulated by PmrA/B (whose activity is modulated by PhoP-PhoQ) and the *pqaB* transposon mutant was sensitive to polymyxin B. The lipopolysaccharides (LPS) of *S. typhi* and *S. typhimurium* wild-type, PhoP⁻ and PhoP^c mutants, were compared by SDS-PAGE and silver staining. Differences in the LPS profile between the two *Salmonella* species were observed, and shown to be affected differently by the PhoP^c mutation. Additionally, the *pqaB::MudJ* mutation affected *S. typhi* LPS. The effects on LPS may have ramifications for the difference between *S. typhi* and *S. typhimurium* infection of hosts.**

Keywords: virulence, PhoP/Q regulon, *pqa/pqr*, PmrA/B, polymyxin B

INTRODUCTION

Salmonella typhi, the causative agent of typhoid fever in humans, remains a significant health problem throughout the world, especially in developing nations. This species is highly host adapted and is non-pathogenic in animals (Miller *et al.*, 1995). An important part of *Salmonella* pathogenesis is the ability of the bacterium to survive and multiply in macrophages (Fields *et al.*,

1986; Buchmeier & Heffron, 1989). It has been suggested that the adaptation of *Salmonellae* to a particular host species is related to their relative ability to survive and replicate in the reticulo-endothelial system (Barrow *et al.*, 1994).

Salmonella typhimurium survival in macrophages has been shown to be dependent on the PhoP/Q two-component regulatory system, which has been extensively reviewed (Groisman & Saier, 1990; Miller, 1991; Vescovi *et al.*, 1994). The PhoP/Q regulon both activates and represses numerous unlinked genes in response to environmental signals: extracellular Mg²⁺ levels and pH (Garcia Vescovi *et al.*, 1996; Bearson *et al.*, 1998; Soncini & Groisman, 1996). A large number of PhoP-

Abbreviations: 4AA, 4-aminoarabinose; GI, growth index; LPS, lipopolysaccharide; PMA, phorbol myristate acetate; PmB, polymyxin B.

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is AF0718082.

activated genes (*pag/pqa*) and PhoP-repressed genes (*prg/pqr*) have been identified in *S. typhimurium*, for example genes involved in macrophage survival (*pagC*) (Pulkkinen *et al.*, 1991), invasion of mammalian cells and protein secretion (*prgHIJK*) (Behlau & Miller, 1993; Johnston *et al.*, 1996), *phoN*, which encodes a non-specific acid phosphatase (Kasahara *et al.*, 1991; Groisman *et al.*, 1992), and *mgtA* and *mgtCB* genes encoding high-affinity magnesium transporters (Soncini & Groisman 1996). PhoP/Q has also been found to regulate genes encoding the PmrA/B two-component regulatory system, providing a regulatory cascade which regulates genes including *pmrE/ugd* (Groisman *et al.*, 1997; Gunn & Miller, 1996) and the recently identified *pmrF* operon, which is involved with polymyxin B (PmB) resistance and lipid A modifications (Gunn & Miller, 1996; Guo *et al.*, 1997; Gunn *et al.*, 1998). PhoP null (PhoP⁻) *S. typhimurium* mutants (in which PhoP-repressed genes are upregulated and PhoP-activated genes downregulated) have been shown to be defective in macrophage survival (Miller *et al.*, 1989), attenuated for virulence in mice (Fields *et al.*, 1989), sensitive to host antimicrobial peptides (Groisman *et al.*, 1992; Selsted *et al.*, 1992; Porter *et al.*, 1997), and recent studies have shown that an *S. typhi* Δ *phoPQ* mutant is attenuated in humans (Hohmann *et al.*, 1996a, b). This clearly demonstrated that the PhoP-activated genes are involved with *S. typhi* pathogenesis. *S. typhimurium* PhoP constitutive (PhoP^c) mutants (in which PhoP-activated genes are upregulated and PhoP-repressed genes are downregulated) have been shown to be deficient in invasion of mammalian cells (Behlau *et al.*, 1993; Pegues *et al.*, 1995), formation of spacious phagosomes within professional and non-professional phagocytes (Alpuche-Aranda *et al.*, 1994, 1995), defective for macrophage survival and are attenuated for virulence in mice (Miller & Mekalanos, 1990). Therefore, it has been suggested that a temporal shift from PhoP-repressed genes to PhoP-activated genes may be necessary for invasion and survival within macrophages.

We have previously isolated and characterized a number of mutants in *S. typhi* PhoP/Q regulated *pqa/pqr* genes (Baker *et al.*, 1997); the *pqaA* and *pqaB* mutants were found to have increased sensitivity to the antimicrobial peptide melittin. In this work we screened these mutants for defects in invasion and macrophage survival by using PMA- (phorbol myristate acetate) differentiated U937 cells (PMA-U937 cells), which are macrophage-like cells. U937 is a human monocytic suspension cell line (Sundstrom & Nilsson, 1976), and the cells express many of the normal mononuclear phagocyte characteristics, including adherence, when differentiated by PMA (Harris & Ralph, 1985; Hass *et al.*, 1989; Minta & Pambrun, 1995; Pedrinaci *et al.*, 1989). We identified the *S. typhi* PhoP/Q-regulated *pqaB::MudJ* fusion mutant as being defective in net growth in the PMA-U937 cells. Cloning and sequence analysis of the *pqaB* gene showed it to be almost identical to a gene within the *S. typhimurium* PmrA/B-regulated *pmrF* operon affecting modification of lipid A. Finally, we showed by SDS-

PAGE analysis that PhoP and PhoQ have different effects on the lipopolysaccharide (LPS) of *S. typhi* and *S. typhimurium*.

METHODS

Bacterial strains, mammalian cell lines and chemicals. Bacterial strains used in this study are described in Table 1. Bacteria were grown at 37 °C with aeration in Luria-Bertani broth (LB) (Morona *et al.*, 1995), unless noted otherwise. Bacteria were plated out on Luria Agar (LA) plates consisting of LB containing 1.5% (w/v) Bacto agar (Difco). Colour indicator plates were X-pho plates (MM or LA with the addition of 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim) or X-Gal (LA with the addition of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (Boehringer Mannheim) with both X-pho and X-Gal freshly dissolved in dimethyl formamide at a final concentration of 40 μ g ml⁻¹). Tetracycline (Sigma) was used at 10 μ g ml⁻¹. The U937 monocyte cell line (obtained from Professor L. Ashman, Hanson Cancer Research Centre, Adelaide) was maintained in RPMI-1640 media containing 10% (v/v) foetal bovine serum, 1% (w/v) glutamine with 100 U penicillin ml⁻¹ and 100 μ g streptomycin ml⁻¹ (Gibco-BRL) (RPMI maintenance media) at 37 °C with 5% CO₂. HeLa cells (human epithelial carcinoma cells – ATCC CCL2) were maintained in MEM (Minimal Eagles salt medium) containing 10% (v/v) foetal bovine serum, 1% (w/v) glutamine with 100 U penicillin ml⁻¹ and 100 μ g streptomycin ml⁻¹ (Gibco-BRL) (MEM maintenance media). Tissue culture assay medium was the same as the maintenance medium for each cell type but did not contain antibiotics. PMA (Sigma) was dissolved in DMSO and stored at -20 °C at 10 μ g ml⁻¹. Stocks of PmB (Sigma) were freshly made each time by dissolving appropriate amounts of solid in PBS.

Bacterial and tissue cell culture. The PMA-differentiated cell assay was modified from the assay used by Hone *et al.* (1992). Differentiation of U937 cells with PMA to obtain monocyte-derived macrophages involved washing U937 cells grown to a high density, and seeding in 24 multi-well plates (Corning, 25820) at approximately 1 $\times$ 10⁶ cells per well in RPMI maintenance media containing 6 ng PMA ml⁻¹. Cells were differentiated with PMA for 72 h before being washed three times with PBS prior to the assay. HeLa cells were grown to semi-confluency and seeded in a 24 well tray. After incubation for 24 h (cells were now ~1 $\times$ 10⁶ cells per well), the cells were washed three times with PBS prior to the assay.

All bacterial strains were grown for 16 h in LB at 37 °C with aeration, subcultured 1/20 into 10 ml LB and grown for 2.5 h until bacteria had reached 1 $\times$ 10⁹ cells ml⁻¹. Bacteria were then washed and diluted in the appropriate tissue culture media (RPMI or MEM assay media) to add to the tissue culture cells.

Salmonella PhoP⁺, PhoP⁻ and PhoP^c strains can be differentiated on LA + X-pho (Baker *et al.*, 1997). All strains used in the bacterial invasion and survival assays were tested after 24 h growth on colour-indicator plates; all strains gave correct-coloured colonies, indicating that the mutations were stable throughout the assay.

Bacterial infection of HeLa cells and PMA-U937 cells. After bacteria were added to the HeLa and PMA-U937 cells (*S. typhi* strains were added at a ratio of 10:1 and *S. typhimurium* strains at 1:1 bacteria:tissue-culture cells), invasion was allowed to occur for 2 h, the bacteria were removed and the cells washed once with PBS, and the appropriate media containing 0.2 mg gentamicin ml⁻¹ (Gibco-BRL) was added

Table 1. *Escherichia coli* and *Salmonella* strains used in this study

Strain	Characteristics	Source/construction
<i>E. coli</i> K-12		
DH5 α	F- ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) <i>supE</i> -44 <i>thi</i> -1 <i>gyrA</i> -96 <i>recA</i> -1 <i>endA</i> -1 <i>relA</i> -1 <i>hsdR</i> 17(<i>r_k⁻ m_k⁺</i>)	BRL
RMA292	DH5 α (pRMSB50)	This work
RMA295	DH5 α (pRMSB53)	This work
<i>S. typhimurium</i>		
C5	Wild-type	Laboratory collection
RMA1010	C5 <i>phoP</i> 12, PhoP ⁻	Baker <i>et al.</i> (1997)
RMA1024	C5 <i>phoP</i> 24, PhoP ^c	Baker <i>et al.</i> (1997)
JSG421	<i>pmrA</i> ::Tn10	Gunn & Miller (1996)
<i>S. typhi</i>		
Ty2	Wild-type	Hone <i>et al.</i> (1988)
RMA1030	Ty2 <i>phoP</i> 12, PhoP ⁻	Baker <i>et al.</i> (1997)
RMA1090	Ty2 <i>phoP</i> 24, PhoP ^c	Baker <i>et al.</i> (1997)
RMA1126	RMA1030 <i>pqaB</i> ::MudJ	Baker <i>et al.</i> (1997)
RMA1180	Ty2 <i>pqrA</i> ::MudJ	Baker <i>et al.</i> (1997)
RMA2310	Ty2 <i>pqaA</i> ::MudJ	Baker <i>et al.</i> (1997)
RMA2312	Ty2 <i>pqaD</i> ::MudJ	Baker <i>et al.</i> (1997)
RMA2316	Ty2 <i>pqrB</i> ::MudJ	Baker <i>et al.</i> (1997)
RMA2326	Ty2 <i>pqaB</i> ::MudJ	Baker <i>et al.</i> (1997)
RMA2526	RMA1090 <i>pqaB</i> ::MudJ	Baker <i>et al.</i> (1997)
RMA1307	RMA2326 <i>pmrA</i> ::Tn10	P22 on JSG421*
RMA1309	RMA2526 <i>pmrA</i> ::Tn10	P22 on JSG421*

* Transduction with P22HTint105 propagated on *S. typhimurium* JSG421, selected for Tc^r.

for 1 h. Cells were then washed three times with PBS and the 0 h timepoint taken by lysing cells with 0.5 ml 0.1% (v/v) Triton X-100 in PBS. All remaining wells were incubated in the appropriate assay media containing 10 μ g gentamicin ml⁻¹ for the remainder of the assay. Subsequent timepoints were taken at 6, 18 and 24 h. All bacteria (including the initial inoculum) were diluted in saline and plated out for counts on LA plates. After 18 h incubation, the colonies were counted and the c.f.u. ml⁻¹ at each timepoint was calculated.

Lactate dehydrogenase assay. Survival of the tissue culture cells during the bacterial-infection assays was determined using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). We measured the amount of lactate dehydrogenase remaining in the cells as it may break down in the supernatant over 24 h. Infected and control cells were washed three times with PBS, as for lysis for bacterial counts, but were then lysed with 200 μ l lysis buffer according to the manufacturer's instructions and assayed accordingly. A standard lactate dehydrogenase curve was performed with each assay to calculate the amount of lactate dehydrogenase units per well present at 0 and 24 h after bacterial infection.

Statistical analysis. Statistical analysis was performed using Student's two-tailed *t*-test for independent means.

DNA and Southern-blotting methods. The preparation of plasmid DNA, restriction enzyme digestion, ligation and agarose gel electrophoresis were performed as described previously (Morona *et al.*, 1995). Southern blotting was performed as recently described (Morona *et al.*, 1995). Hybridization and detection were performed with digoxigenin labelling and detection kits (Boehringer Mannheim), according to the manufacturer's instructions.

Cloning the *S. typhi* Ty2 *pqaB* gene. The 0.85 kb *Hind*III/*Cla*I fragment was isolated by a method based on the single specific primer (SSP)-PCR (Shyamala & Ames, 1993). This method allows the amplification of a piece of DNA for which sequence is only available at one end. *S. typhi* *pqaB*::MudJ (RMA2326) chromosomal DNA was digested with *Hind*III/*Cla*I and ligated to similarly digested pBluescript KS+; this ligation mix was then used as a template for a PCR reaction. The PCR was performed with oligonucleotides 2308 (5' TCGCATTTA-TCGTGAAACGCTTTCG 3'; anneals to the MuR right end of MudJ before the *Hind*III site) and the M13 reverse primer for pBluescript KS+ (Stratagene). The 0.85 kb PCR product was cloned into pGEMT (Progen) according to the manufacturer's instructions, to create pRMSB50. After transformation into *E. coli* DH5 α , plasmid DNA was prepared and used for DNA sequencing on an Applied Biosystems 373A sequencer using the Applied Biosystems DNA Dye Terminator kit as recommended by the manufacturer. The 0.85 kb fragment was then labelled by digoxigenin-PCR and used to probe *S. typhi* Ty2 chromosomal DNA digested with a number of restriction enzymes. The smallest fragment detected was an *Eco*RV fragment (approx. 3 kb), and inverse PCR (Ochman *et al.*, 1988) was used to obtain the fragment. This involved digesting 10 μ g *S. typhi* Ty2 chromosomal DNA with *Eco*RV, circularization and religation of the restriction fragments, and amplification by PCR using oligonucleotides 2512 (5' CGATTCGCCATGAGCGATG 3') and 2513 (5' CACGATCCAGTCGCCAGAC 3') which anneal to nucleotides 735–753 and 152–134, respectively, of the *pqaB* sequence. This PCR fragment was cloned into pGEMT to create pRMSB53, and transformed into DH5 α to enable sequencing. The *Eco*RV fragment was fully sequenced on both strands by Dye

Terminator sequencing and was found to be 2.83 kb in size (GenBank AF0718082). Sequence analysis of *S. typhi* *pqaB* and adjacent genes, and the *S. typhimurium* *pmrF* operon (ORF1–7) (Gunn *et al.*, 1998) was performed by using BLASTX/P (Altschul *et al.*, 1990). Sequence alignments are available from the authors by request.

P22 bacteriophage propagation and transduction. P22HT-int105 (P22) bacteriophage propagation and transduction were performed according to the method described by Davis *et al.* (1980).

β -Galactosidase assays. These were performed as described by Baker *et al.* (1997) on cultures grown in LB at 37 °C with aeration for 16 h.

PmB assays. Bacteria grown for 16 h in LB at 37 °C with aeration were subcultured (1:20) in LB and grown to mid-exponential phase. These cultures were then diluted to approximately 1×10^4 bacteria ml⁻¹ and chilled. Aliquots (100 μ l) of diluted culture were added to a 96 well microtitre plate (Corning wells catalogue no. 250850-96). One hundred microlitres of appropriately diluted PmB in PBS, pH 7.5, were added to each well. Controls consisted of bacteria (100 μ l) and PBS (100 μ l). The microtitre plates were incubated at 37 °C for 1 h, after which they were placed on ice. Plates (LA) were spread with 50 μ l from each well; the plates were incubated overnight at 37 °C for 18 h, and the colonies counted. The data are presented as percentage survival relative to the control with no added PmB.

LPS and SDS-PAGE silver staining. The *S. typhimurium* C5, PhoP⁻ and PhoP^c strains and all the *S. typhi* strains (Ty2, PhoP⁻, PhoP^c and *pqa/pqr::MudJ* fusion strains) were serotyped by the Salmonella Reference Laboratory (The Institute of Medical and Veterinary Science, Adelaide) and found to be identical to the wild-type strains. All *Salmonella* strains were grown for 16–18 h at 37 °C in LB with aeration. Small-scale LPS preparations were made by proteinase K treatment of whole-cell lysates (Hitchcock & Brown, 1983). After electrophoresis on SDS-20% polyacrylamide gels, LPS was detected by silver staining as described previously (Morona *et al.*, 1991).

RESULTS

Invasion and net growth of *S. typhi* strains in PMA-differentiated U937 cells

As *S. typhimurium* PhoP⁻ and PhoP^c mutants have previously been shown to have decreased survival in macrophage cell lines, because of the host specificity of these typhoid-fever causing *Salmonella* species, we wished to assess *S. typhi* Ty2 and *phoP/Q*-mutant derivatives in a human macrophage cell line for their ability to invade and grow. *S. typhi* strain Ty2 and the isogenic PhoP⁻ (RMA1030) and PhoP^c (RMA1090) strains were assessed for their ability to invade and grow in the macrophage-like PMA-U937 cells (Tables 2 and 3; Fig. 1a). The *S. typhi* PhoP⁻ mutant invaded as well as the wild-type Ty2 strain; however, an approximate 10-fold decrease in invasion for PhoP^c was noted (Fig. 1a, Table 2). This correlated with data from an *S. typhimurium* PhoP^c mutant which had previously been shown to have reduced invasion (Behlau & Miller, 1993). The net growth index (GI) at 24 h for Ty2 in the differentiated U937 cells was 15–25, and the PhoP^c strain

Table 2. Invasion of PMA-U937 cells by *S. typhi* and *S. typhimurium* strains

Invasion percentages were calculated by dividing the number of bacteria at time 0 after gentamicin treatment by the number of bacteria initially added, multiplied by 100. Results were calculated from data in Figs 1 and 2 and are presented as the mean $\pm$ SD.

Strain	Percentage invasion
<i>S. typhi</i>	
Ty2 (PhoP ⁺)	6.3 $\pm$ 2.5
RMA1030 (PhoP ⁻)	3.3 $\pm$ 1.3
RMA1090 (PhoP ^c)	0.9 $\pm$ 0.69
RMA2326 (<i>pqaB::MudJ</i>)	4.9 $\pm$ 2.5
<i>S. typhimurium</i>	
C5 (PhoP ⁺)	95.7 $\pm$ 4.7
RMA1010 (PhoP ⁻)	82.7 $\pm$ 4.8
RMA1024 (PhoP ^c)	14.5 $\pm$ 2.6

Table 3. Growth index of *S. typhi* and *S. typhimurium* in PMA-U937 cells

ND, Not done.

Bacterial strain	Growth index	
	Cell line ...	HeLa PMA-U937
<i>S. typhi</i>		
Ty2 (PhoP ⁺)	6*	15–25†
RMA1030 (PhoP ⁻)	6*	2–3†
RMA1090 (PhoP ^c)	6*	42*
RMA2326 (<i>pqaB::MudJ</i>)	5*	3*
<i>S. typhimurium</i> ‡		
C5 (PhoP ⁺)	ND	7
RMA1010 (PhoP ⁻)	ND	32
RMA1024 (PhoP ^c)	ND	10

* Growth index for these strains was calculated by the number of bacteria at 24 h divided by the number of bacteria at 0 h.

† Growth index for these strains was calculated from data in Fig. 1.

‡ Growth index for *S. typhimurium* strains was calculated by the number of bacteria at 18 h divided by the number of bacteria at 0 h and was calculated from data in Fig. 2.

(RMA1090) had a GI of 42 (Fig. 1; Table 3); a possible explanation for this increased net growth of the *S. typhi* PhoP^c strain compared to Ty2 is discussed later. The PhoP⁻ strain (RMA1030) was limited in intracellular net growth (GI 2–3) when compared to the wild-type strain. As the difference in net growth between Ty2 and RMA1030 was significant ($P < 0.02$ at 24 h, Student's *t*-test), we assayed previously identified *S. typhi* *pqa/pqr* mutants (Baker *et al.*, 1997) for invasion and intracellular net growth in PMA-U937 cells. All five *S. typhi* *pqa/pqr::MudJ* mutant strains (*pqaA::MudJ*, *pqaB*

Fig. 1. Net growth of *S. typhi* strains in PMA-U937 cells. (a) Net growth characteristics of *S. typhi* Ty2 (■), RMA1030 (PhoP⁻) (●) and RMA1090 (PhoP⁺) (▲) strains after invasion of PMA-differentiated U937 cells. (b) Net growth characteristics of *S. typhi* Ty2 (■), RMA1030 (PhoP⁻) (●) and RMA2326 (*pqaB::MudJ*) (▼) strains after invasion of PMA-differentiated U937 cells. *S. typhi* strains were added at a ratio of 10:1 (bacteria:cells). Each point represents the arithmetic mean of four assays expressed as c.f.u. ml⁻¹ ± SD. Data for (a) and (b) are from separate experiments.

::MudJ, *pqaD::MudJ*, *pqrA::MudJ* and *pqrB::MudJ*) tested were unaffected in their ability to invade PMA-U937 cells. Only the *pqaB::MudJ* mutant (RMA2326) showed a decrease in intracellular net growth (GI 3) compared to the wild-type strain ($P < 0.02$ at 24 h) (Fig. 1b, Table 3).

Growth of *S. typhi* strains in HeLa cells

To demonstrate that the intracellular growth deficiencies of the *S. typhi* PhoP⁻ and *pqaB::MudJ* mutants were specifically related to the nature of the macrophage-like PMA-U937 cells, we tested these strains in an epithelial (non-macrophage) cell line. The *S. typhi* Ty2 wild-type, isogenic PhoP⁻ (RMA1030), PhoP⁺ (RMA1090) and *pqaB::MudJ* (RMA2326) strains were assessed for their ability to grow inside HeLa cells (Table 3). All four strains grew to give a GI of 5–6, which correlated with data previously reported for *S. typhi* intracellular-growth levels in HeLa cells (Mills & Finlay, 1994), and indicated that the PhoP⁻ and *pqaB* mutations selectively affected *S. typhi* intra-macrophage net growth inside PMA-U937 cells. *S. typhi* Ty2, PhoP⁻ (RMA1030) and *pqaB::MudJ* (RMA2326) strains gave similar levels of

Fig. 2. Net growth of *S. typhimurium* strains in PMA-U937 cells. This figure shows the net growth characteristics of *S. typhimurium* C5 (□), RMA1010 (PhoP⁻) (○) and RMA1024 (PhoP⁺) (▽) strains after invasion of PMA-differentiated U937 cells. *S. typhimurium* strains were added at a ratio of 1:1 (bacteria:cells). Each point represents the arithmetic mean of three assays expressed as c.f.u. ml⁻¹ ± SD.

invasion, with a 10-fold invasion defect for the PhoP⁺ strain (RMA1090) (data not shown). The GI results for the *S. typhi* PhoP⁻ and PhoP⁺ strains agree with data reported for *S. typhimurium* PhoP⁻ and PhoP⁺ strains; the latter showed no intracellular-growth deficiency compared to wild-type in an epithelial cell line (Miller & Mekalanos, 1990).

Invasion and net growth of *S. typhimurium* strains in PMA-U937 cells

As the *S. typhi* PhoP⁻ mutant was defective in net growth in the PMA-U937 cell line compared to the wild-type Ty2 strain, we wanted to know whether the *S. typhimurium* PhoP⁻ mutant would show a similar defect in net growth compared to the parent C5 strain. Therefore we investigated the ability of *S. typhimurium* C5 and isogenic PhoP⁻ (RMA1010) and PhoP⁺ (RMA1024) mutants, to invade and grow within PMA-U937 cells. We found that after 24 h in the PMA-U937 cells, the *S. typhimurium* cells caused the macrophage-like cells to die and lyse, as indicated by lack of trypan blue exclusion (data not shown). Therefore, the *S. typhimurium* PMA-U937 assay was only taken to 18 h rather than the 24 h used for *S. typhi*. We observed that the *S. typhimurium* strains had a greatly increased level of invasion (approx. 10- to 20-fold) compared to *S. typhi* strains (Table 2); this has been noted before for HeLa cell invasion (Mills & Finlay, 1994). Therefore the m.o.i. for *S. typhimurium* was adjusted to 1:1 instead of 10:1 used for *S. typhi* so the numbers of bacteria invading the PMA-differentiated U937 cells were similar for both species. The *S. typhimurium* PhoP⁺ mutant (RMA1024) showed the expected invasion defect compared to the wild-type and PhoP⁻ derivative (Table 2) (Behlau & Miller, 1993). The *S. typhimurium* PhoP⁻ mutant had no net growth limitations compared to the *S. typhimurium* wild-type strain (Fig. 2), and actually gave a higher GI

Table 4. Lactate dehydrogenase (LDH) assay for *S. typhi* infection of PMA-U937 cells

Bacterial strain	Units LDH per well (0 h)*	Fold decrease (0 h)†	Units LDH per well (24 h)*	Fold decrease (24 h)†
None	$4.03 \times 10^{-3} \pm 1.47 \times 10^{-3}$	1.00	$3.50 \times 10^{-3} \pm 9.22 \times 10^{-4}$	0.87
Ty2 (PhoP ⁺)	$2.03 \times 10^{-3} \pm 9.25 \times 10^{-4}$	0.50	$1.09 \times 10^{-3} \pm 9.47 \times 10^{-4}$	0.27
RMA1030 (PhoP ⁻)	$1.96 \times 10^{-3} \pm 1.15 \times 10^{-3}$	0.49	$1.16 \times 10^{-3} \pm 9.55 \times 10^{-4}$	0.29
RMA1090 (PhoP ^c)	$2.98 \times 10^{-3} \pm 1.55 \times 10^{-3}$	0.74	$2.41 \times 10^{-3} \pm 1.25 \times 10^{-3}$	0.60
RMA2326 (<i>pqaB</i> ::MudJ)	$2.92 \times 10^{-3} \pm 1.27 \times 10^{-3}$	0.72	$2.30 \times 10^{-3} \pm 1.50 \times 10^{-3}$	0.57

* Units LDH per well are presented as the mean of four assays $\pm$ SD.

† Fold decrease is the decrease in units LDH per well compared to uninfected cells at time 0.

than wild-type (Table 3). Hence the net growth restriction for *S. typhi* in PMA-U937 cells caused by the PhoP⁻ mutation appeared to be specific for *S. typhi*.

Lactate dehydrogenase assay

The PMA-U937 assay appeared to differentiate between *S. typhi* Ty2, PhoP⁻ and *pqaB*::MudJ mutant strains by the bacterial GI. However, we needed to know whether the differences in the *Salmonella* c.f.u. ml⁻¹ at the timepoints (particularly 0 and 24 h) for the PMA-U937 cell assay were actually due to net growth differences between *Salmonella* strains and not because the bacteria had a cytotoxic effect on the infected macrophage-like cells, therefore resulting in a lower c.f.u. ml⁻¹ count. By visual inspection, the majority of the infected cells still adhered to the bottom of the 24 well trays at 24 h and most of these excluded trypan blue, indicating that they were viable. As a further test, we assayed lactate dehydrogenase, which is a stable cytosolic enzyme released during cells lysis, and can therefore be used to evaluate the amount of PMA-U937 cells remaining viable over the 24 h assay. We tested the amount of lactate dehydrogenase contained within the PMA-U937 cells during infection of various *Salmonella* strains (Table 4). The data shows a 25–35% variation in the number of PMA-U937 cells per well before bacterial infection (Table 4) which is consistent with data for other adherent cells lines (Monack *et al.*, 1996). This difference in the number of PMA-U937 cells may explain the large error bars seen in Figs 1 and 2. Both *S. typhi* Ty2 and PhoP⁻ (RMA1030) strains caused some cytolysis of the U937 cells during invasion but at 24 h, the damage was approximately the same, indicating that the difference in GI (Table 3) between the two strains is not due to differences in PMA-U937 cytolysis by the bacteria. Both the *S. typhi* PhoP^c (RMA1090) and *pqaB*::MudJ (RMA2326) strains also caused some cytolysis of PMA-U937 cells, although less than wild-type. PMA-U937 cells infected with *S. typhimurium* strains were also tested for release of lactate dehydrogenase and at 18 h the bacteria gave a similar level of cytolysis of the PMA-U937 cells to the *S. typhi* Ty2 strain at 24 h (data not shown).

Fig. 3. The location of the sequenced *EcoRV* fragment (AF0718082) containing the *S. typhi* *pqaB* gene and surrounding genes. The *S. typhimurium* *pmrF* operon (AF036677) and homologous *E. coli* (AE000315) region are also shown. The site of the *MudJ* insertion between nucleotides 847 and 848 of the *S. typhi* *pqaB* gene is shown. Relevant restriction sites are also indicated. Arrows indicate direction of transcription.

Cloning and sequencing of the *S. typhi* *pqaB* gene

Our experiments suggested that the *S. typhi* *pqaB* gene was required for wild-type level of net growth/survival in PMA-U937 cells. Therefore we isolated and sequenced this gene from *S. typhi*. Southern analysis of the *pqaB*::MudJ insertion mutant chromosome with a probe to the right hand side of *MudJ* (MuR), which would contain part of the transposon and chromosomal DNA corresponding to *pqaB*, enabled us to identify a *pqaB*::MudJ 0.85 kb *HindIII*/*ClaI* end fragment (data not shown). The 0.85 kb fragment was isolated, cloned into pGEMT to create pRMSB50 and sequenced (Methods). The fragment was found to have 70% homology with an *E. coli* K-12 ORF (AE000315, ORF o550) and ~98% homology with the fifth gene of an *S. typhimurium* PmrA/B-regulated *pmrF* operon (AF036677; Gunn *et al.*, 1998). A larger 2.83 kb *EcoRV* fragment was then isolated (Methods), sequenced (AF0718082) and found to contain the whole *pqaB* gene plus adjacent sequence with strong homology to the *S. typhimurium* *pmrF* operon ORF4, ORF6 and ORF7 (Fig. 3) (Gunn *et al.*, 1998). The *MudJ* insertion was

Fig. 4. Putative pathway for the biosynthesis of L-deoxy-4AA and modification of lipid A. The predicted functions of individual *pmrF* operon gene products are described in the text.

found to be between nucleotides 847 and 848 of the *pqaB* gene (Fig. 3).

Putative biosynthesis pathway for 4AA

The *S. typhimurium pmrF* operon was recently identified as being both PhoP/Q and PmrA/B regulated and highly likely to encode the enzymes required for the biosynthesis of the 4-aminoarabinose (4AA) modification of the lipid A component of LPS (Gunn *et al.*, 1998). The *pmrF* mutation was shown to be in the second ORF of the operon and we have shown that *pqaB::MudJ* is a mutation in ORF5 of this operon. To obtain an understanding of the possible role of the *pmrF* and ORF5/*pqaB* gene products in 4AA-lipid A modification, we analysed the ORFs in the operon by BLASTX/BLASTP computer homology analysis to obtain a speculative pathway for biosynthesis of 4AA and the modification of lipid A by 4AA.

The *de novo* biosynthesis of C₅ monosaccharides has been extensively studied in plants (Feingold & Barber, 1990; Tenhaken & Thulke, 1996). UDP-glucuronic acid is the key precursor, and is obtained from the metabolic pool by the action of UDP-glucose dehydrogenase on UDP-glucose (Fig. 4, step I). UDP-glucose dehydrogenase is predicted to be encoded by the remotely located *pmrE* (*ugd*) gene (Gunn *et al.*, 1998; Groisman *et al.*, 1997; Morona *et al.*, 1995; Valdivia & Falkow, 1996). The enzymes encoded by the *pmrF/pqaB* operon are then predicted to act on UDP-glucuronic acid. Initially, an oxido-reductase encoded by ORF3 converts UDP-glucuronic acid to UDP-4-keto-glucuronic acid (Fig. 4, step II). This activity is encoded by the carboxy-terminal half of ORF 3, which has approximately 26% identity with a variety of dehydratases, and in common with this class of enzymes has an NAD-binding motif (GXXGXXG) near the start of the dehydratase domain of the 660 aa protein. The next step is the removal of the

carboxyl group in a decarboxylation reaction (Fig. 4, step III). The nature of the enzymes which perform this step are poorly characterized and no sequences have been reported. The only ORF which could potentially be assigned as encoding this function is ORF4, which encodes a hydrophilic protein. The decarboxylation reaction results in a C₅ keto intermediate, which could be stereo-specifically reduced at the C-4 position by an amino-transferase encoded by ORF1 (Fig. 4, step IV) and results in UDP-4AA. The ORF1 protein shows approximately 34% identity with other proteins predicted to perform this reaction, thereby resulting in a variety of 4-amino monosaccharides, e.g. perosamine (Stroehrer *et al.*, 1995). The following two steps result in lipid A modification by 4AA. The PmrF protein has both amino acid sequence and hydropathy profile similarity with the Bgt protein of *Shigella flexneri* (Mavris *et al.*, 1997). In reactions analogous to those recently described for glucosylation of the *S. flexneri* O-antigen (Mavris *et al.*, 1997), 4AA is transferred to bactoprenol to form 4AA-P-bactoprenol by the *pmrF*-encoded glycosyl transferase (Fig. 4, step V), and from this intermediate, 4AA is transferred to a free phosphate on lipid A by a specific transferase encoded by ORF5 (Fig. 4, step VI). The 550 aa ORF5 protein is highly hydrophobic with multiple potential membrane-spanning regions, and little similarity to other proteins. In this regard, it is similar to the specific glucosyl transferases (GtrX, GtrI, GtrII, GtrV) which modify the *S. flexneri* O-antigen (Verma *et al.*, 1993; Bastin *et al.*, 1997; Huan *et al.*, 1997a, b; Mavris *et al.*, 1997). The remaining unassigned ORFs 6 and 7 (ORF6 in the *E. coli* K-12 operon) encode small, highly hydrophobic proteins with no discernible similarity with other proteins or ORFs. An interesting aside is that the amino-terminal half of the ORF3 protein has approximately 28% identity with proteins able to formylate a variety of molecules. We speculate that the amino group of 4AA can be formylated by this domain of ORF3 (Fig. 4, step VII). This type of modification has been reported for the 4-amino sugar component (perosamine) of the O-antigen of *Brucella* sp. (Moreno *et al.*, 1987). The presence of this modification on 4-AA has not been reported.

PmrA/B regulation of the *S. typhi* *pqaB* gene

The *S. typhimurium* *pmrF* operon has been shown to be strongly regulated by PmrA/B (Gunn *et al.*, 1998). To test whether the *S. typhi* *pqaB* gene was also regulated by PmrA/B, a *pmrA* null mutation (*pmrA*::Tn10d) from the *S. typhimurium* strain JSG421 was transduced by phage P22 into *S. typhi* *pqaB*::MudJ, PhoP⁺ (RMA2326), and *pqaB*::MudJ, PhoP^c (RMA2356) strains, to create RMA1307 and RMA1309, respectively. These strains produced white colonies on LA + X-Gal plates compared to the intense blue colonies of their *pmrA*⁺ parent strains. This indicated that the *S. typhi* *pqaB*::MudJ fusion was strongly regulated by the PmrA/B operon. β -Galactosidase assays were performed on overnight cultures grown in LB, as described in Methods. The results are given for each *S. typhi* strain,

Fig. 5. PmB resistance of *S. typhi* strains. This figure shows the percentage survival of *S. typhi* RMA1030 (PhoP⁻), Ty2, RMA2326 (*pqaB*::MudJ), RMA1090 (PhoP^c) and RMA2526 (*pqaB*::MudJ, PhoP^c) strains after exposure for 1 h to four concentrations (0.1, 0.2, 0.4 and 0.6 µg ml⁻¹) of PmB at 37 °C. Each bar represents the arithmetic mean of three assays, expressed as percentage survival $\pm$ SD. Arrows indicate survival $\leq$ 1%.

and presented as β -galactosidase units from the mean of two duplicate assays $\pm$ SD: RMA1126 (PhoP⁻, *pqaB*::MudJ), 17 $\pm$ 1; RMA2326 (PhoP⁺, *pqaB*::MudJ), 395 $\pm$ 1; RMA2526 (PhoP^c, *pqaB*::MudJ), 996 $\pm$ 35; RMA1307 (PhoP⁺, *pqaB*::MudJ *pmrA*::Tn10), 6 $\pm$ 1 and RMA1309 (PhoP^c, *pqaB*::MudJ *pmrA*::Tn10), 8 $\pm$ 4. These results showed that *pqaB* expression was PmrA/B dependant, as the β -galactosidase activity of *S. typhi* RMA1307 and RMA1309 strains were greatly down-regulated (66- and 125-fold, respectively) compared to their *pmrA*⁺ parental strains.

PmB sensitivity of *S. typhi* *pqaB* mutant

Since the *S. typhimurium* *pmrF* mutant is sensitive to PmB, compared to the wild-type strain (Gunn *et al.*, 1998), we tested the sensitivity of the *S. typhi* *pqaB* mutant to this antimicrobial agent. Previous assays with other antimicrobial agents such as melittin and protamine (Baker *et al.*, 1997) indicated that *S. typhi* was approximately 10-fold more sensitive to these agents than *S. typhimurium*. In preliminary experiments, we found that *S. typhi* Ty2 was approximately 10-fold more sensitive to PmB than the *S. typhimurium* C5 strains. The survival of *S. typhi* Ty2, RMA1030 (PhoP⁻), RMA1090 (PhoP^c), RMA2326 (*pqaB*::MudJ), RMA2526 (*pqaB*::MudJ, PhoP^c), RMA1307 (*pqaB*, *pmrA*::Tn10) and RMA1309 (*pqaB*, *pmrA*::Tn10, PhoP^c) strains were tested with four concentrations of PmB (0.1, 0.2, 0.4 and 0.6 µg ml⁻¹) and the results are shown in Fig. 5. The difference between percentage survival of *S. typhi* Ty2 and RMA1030 (PhoP⁻) was small, however RMA1090 (PhoP^c) showed increased resistance to PmB (discussed below); this correlates with previous *S. typhimurium* data (Gunn *et al.*, 1998; Groisman *et al.*, 1997). Little or no difference was seen

Fig. 6. Comparison of *S. typhi* and *S. typhimurium* lipopolysaccharides. Samples (representing 10^8 cells) were prepared from 18 h cultures and electrophoresed on an SDS-20% polyacrylamide gel, then subjected to silver staining to detect LPS. The strains in each lane are: *S. typhimurium* RMA1010 (PhoP⁻), *S. typhimurium* C5 (PhoP⁺), *S. typhimurium* RMA1024 (PhoP^c), *S. typhi* RMA1030 (PhoP⁻), *S. typhi* Ty2 (PhoP⁺), *S. typhi* RMA1090 (PhoP^c), *S. typhi* RMA2326 (*pqaB::MudJ*, PhoP⁺) and *S. typhi* RMA2526 (*pqaB::MudJ*, PhoP^c). *** indicates that the LPS chains lack discrete banding. Smooth LPS O-antigen chains and lipid A + core sugar molecules are also indicated.

between *S. typhi* Ty2 and the *pqaB::MudJ* insertion mutant (RMA2326), but a large difference was observed between the RMA2526 (*pqaB::MudJ*, PhoP^c) and RMA1090 (PhoP^c) strains. These data indicate that the modifications to the LPS by this operon affect PmB resistance in both *S. typhi* and *S. typhimurium*.

Comparison between *S. typhi* and *S. typhimurium* LPS

Since the *S. typhimurium* *pmrF* operon affects lipid A modifications and both *pqaB* in *S. typhi* and *pmrF* in *S. typhimurium* affect PmB resistance, we decided to examine the LPS produced by *S. typhi* and *S. typhimurium* wild-type, PhoP⁻ and PhoP^c strains, and *S. typhi* strains with the *pqaB* mutation in PhoP⁺ and PhoP^c backgrounds. As shown in Fig. 6, the LPS profile of *S. typhi* Ty2 and *S. typhimurium* C5 wild-type strains showed minor differences compared to that of their respective isogenic PhoP⁻ mutants. However the LPS of the *S. typhimurium* PhoP^c (RMA1024) strain had a

dramatically shorter O-antigen chain compared to the wild-type and PhoP⁻ strains (Fig. 6). There was little difference between the LPSs of the *S. typhi* PhoP^c (RMA1090), wild-type (Ty2) and PhoP⁻ (RMA1030) strains. A subtle difference between the LPS of the *S. typhi* *pqaB* mutants (RMA2326 and RMA2356) and their wild-type (Ty2) and PhoP^c (RMA1090) counterparts could be seen as the lack of discrete LPS bands in the high molecular mass range (marked by *** in Fig. 6). The lack of discrete bands was more apparent with RMA2526 (*pqaB::MudJ*, PhoP^c), which also seemed to have LPS with a reduced number of O-antigen chains; this result was reproduced on three separate occasions (data not shown). Fig. 6 also shows that the LPS of *S. typhi* Ty2 has O-antigen chains with a larger modal chain length than the LPS of *S. typhimurium* C5.

DISCUSSION

Salmonella and the PMA-U937 tissue-culture model

In this study, we used a tissue-culture model (PMA-differentiated U937 cells) to study the invasion and intracellular net growth of *S. typhi* wild-type, PhoP⁻, PhoP^c and *pqa/pqr* mutants in macrophage-like cells. The invasion and net growth of *S. typhimurium* wild-type, PhoP⁻ and PhoP^c derivatives in PMA-U937 cells was also investigated. The *S. typhi* strains mentioned above were also assayed in an epithelial (HeLa) cell line to test for general defects in intracellular net growth.

The PMA-U937 cell assay was able to differentiate between *S. typhi* Ty2 and its PhoP⁻ derivative (Fig. 1b, Table 3), indicating that it was a suitable assay to test the *S. typhi* *pqa/pqr* mutants. The *S. typhi* PhoP⁻ and PhoP^c mutants did not show a net growth defect compared to wild-type *S. typhi* Ty2, in the HeLa cell assay (Table 1), indicating that any net growth defects in the PMA-U937 cell assay were specific to the macrophage-like cells. The assay also appeared to be specific for *S. typhi* strains, as there was no intracellular net growth defect for the *S. typhimurium* PhoP⁻ (RMA1010) strain, which was comparable to that of wild-type *S. typhimurium* C5 (Fig. 2, Table 3). Possible reasons for this will be discussed below. The data for the *S. typhi* PhoP^c mutant do not correlate with published observations with an *S. typhimurium* PhoP^c mutant, which was defective in intracellular net growth within macrophages (Miller & Mekalanos, 1990). The observed difference may be due to the use of a human macrophage-like cell line for our experiments, as opposed to the mouse macrophage cell line used by Miller & Mekalanos (1990). We also observed no net growth restriction for the *S. typhimurium* PhoP^c (RMA1024) mutant in the PMA-U937 cells, where growth was comparable to that of *S. typhimurium* C5 (Fig. 2, Table 3).

The *S. typhi* *pqaB* (RMA2326) mutant had a significantly lower intracellular net-growth level at 24 h compared to *S. typhi* Ty2 in PMA-U937 cells ($P < 0.02$), however its net growth was not affected compared to the wild-type strain in HeLa cells (Table 3), indicating that the

intracellular net growth defect was specific to the macrophage-like PMA-U937 cell line. Strain RMA2326 was found to be defective in net growth in PMA-U937 cells during the last 6 h of infection but overall was not as deficient in intracellular net growth as the *S. typhi* PhoP⁻ mutant (RMA1030) (Fig. 1b). This indicates that other *pqa* genes are involved with *S. typhi* intramacrophage survival.

S. typhi *pqaB* gene

The *S. typhi* *pqaB* gene was cloned and sequenced along with adjacent genes, and was found to have a high (~98%) level of homology to ORF5 of the *S. typhimurium* *pmrF* operon (Fig. 3). The *S. typhimurium* *pmrF* operon has been identified as being involved with 4AA-lipid A modification and PmB resistance (Gunn *et al.*, 1998). A putative pathway for the biosynthesis of 4AA based on sequence alignments and predicted biochemistry is described in Fig. 4 and strongly suggests that *pqaB*/ORF5 encodes a bactoprenol-4AA-lipid A transferase. The *pmrF* operon was found to be strongly regulated by the PmrA/B operon. A *pmrA*::Tn10d mutation was transduced into our *pqaB*::MudJ mutant and expression of the *S. typhi* *pqaB*::MudJ fusion was strongly reduced. Hence *pqaB*/ORF5 is also regulated by PmrA/B, as expected from its location in the *pmrF* operon.

As the *S. typhimurium* *pmrF* operon is involved with PmB resistance, we tested our *S. typhi* strains for PmB resistance. The *S. typhi* Ty2 wild-type strain was approximately 10-fold more sensitive to PmB than *S. typhimurium* C5 (data not shown), and the *S. typhi* PhoP^c (RMA1090) mutant had an increased resistance to PmB compared to the wild-type Ty2 strain (Fig. 5). The *pqaB*::MudJ PhoP^c (RMA2326) strain was more sensitive to PmB than RMA1090 (PhoP^c), indicating that the *S. typhi* *pqaB* gene is also involved with PmB resistance. The sensitivity of *pmrF* and *pqaB* mutants to PmB is consistent with these genes affecting the same pathway for LPS modifications (ORFs 1–7, functions outlined in Fig. 4).

It has been suggested that since LPS is negatively charged and most antimicrobial peptides (including PmB and melittin) are cationic at physiological pH, mutations which alter the phosphate content or LPS substitutions may change the negative charge of the LPS and therefore affect the ability of the cationic peptides to bind to the LPS (Groisman, 1994). An increase in both ethanolamine and 4-deoxyaminoarabinose substitution results in a lower surface negative charge of the lipid A and inner core parts of the LPS, and therefore give a greater resistance to cationic peptides (Helander *et al.*, 1994). As both *S. typhi* and *S. typhimurium* PhoP^c mutants have their *pqa/pag* genes constitutively up-regulated, this would explain why they have a higher resistance to antimicrobial agents such as PmB, as seen for the *S. typhi* PhoP^c (RMA1090) strain compared to the wild-type strain (Fig. 5), and for *S. typhimurium* (Gunn *et al.*, 1998).

Compared to *S. typhi*, *S. typhimurium* strains have been previously shown to have an approximately 10-fold higher resistance level to a number of antimicrobial agents (unpublished data; Baker *et al.*, 1997). This higher level of resistance may be due to either different *pag/pqa* genes or greater/different modifications for *S. typhimurium* LPS compared to *S. typhi*. Modifications such as the 4-deoxyaminoarabinose and ethanolamine may be regulated/added to give a higher modification level in *S. typhimurium* than *S. typhi*. Alternatively there may be other modifications, as yet undetected, that may be different between the two *Salmonella* species, such that *S. typhi* LPS may have a naturally higher negative charge than *S. typhimurium* LPS. Our PMA-U937 assay may be sensitive enough to detect these differences in *S. typhi* strains but not *S. typhimurium*. This may explain why, unlike the *S. typhi* PhoP⁻ mutant, the *S. typhimurium* PhoP⁻ mutant was not limited in net growth in the PMA-U937 cells compared to the wild-type strain.

Effects on *Salmonella* LPS

The results mentioned above strongly indicate that *S. typhi* and *S. typhimurium* LPS may be different, although they have some of the same PhoP/Q- and PmrA/B-regulated lipid A modifications. We examined the LPS of *S. typhi* Ty2, PhoP⁻, PhoP^c, *pqaB*::MudJ and *pqaB*::MudJ/PhoP^c on an SDS-PAGE gel, and the LPS of *S. typhimurium* C5 and PhoP⁻, PhoP^c derivatives. Based on chemical analysis showing that *S. typhimurium* PhoP^c LPS had shorter O-antigen chains (Guo *et al.*, 1997), we expected to see a difference between the LPS of the *S. typhimurium* wild-type and its PhoP^c derivative. As can be seen in Fig. 6, there was a remarkable difference between *S. typhimurium* C5 and PhoP^c LPS, with much shorter O-antigen chains being evident for the PhoP^c strain. This is in direct contrast to the *S. typhi* Ty2, PhoP⁻ and PhoP^c strains, for which no difference in O-antigen chain length could be detected (Fig. 6). The lack of discrete high-molecular-mass bands is the only subtle difference seen between the *S. typhi* *pqaB* mutants (RMA2326 and RMA2356) and their wild-type (Ty2) and PhoP^c (RMA1090) counterparts. The LPS of strain RMA2356 also appeared to have fewer O-antigen chains. The LPS of both *S. typhi* Ty2 and *S. typhimurium* C5 strains showed no dramatic differences compared to their PhoP⁻ derivatives; this was reported previously for *S. typhimurium* wild-type and PhoP⁻ strains by Galan & Curtiss (1989). Interestingly, the modal chain length of the LPS for *S. typhi* is much longer than that of the LPS of *S. typhimurium*. This suggests that they have different *wzz* genes. We have been able to confirm this as we were unable to amplify the *S. typhi* *wzz* gene by PCR using *S. typhimurium* *wzz* gene-specific oligonucleotides (C. Daniels & R. Morona, unpublished data), which suggests significant nucleotide differences of these two genes in *S. typhi* and *S. typhimurium*. The differences between *S. typhi* and *S. typhimurium* LPS described above correlate with differential susceptibility to PmB, antimicrobial peptides and net growth in PMA-U937 cells. Given the importance of LPS in *Salmonella*

virulence, these differences may impact on the relative virulence of *Salmonella* species in different hosts.

Currently, we are studying the structural modifications of LPS from *S. typhi* Ty2, PhoP⁻, PhoP^e and *pqaB::MudJ* derivatives by mass spectrometry to confirm and extend the known differences between *S. typhi* and *S. typhimurium* LPS.

ACKNOWLEDGEMENTS

This study was funded by a Project Grant from the National Health and Medical Research Council of Australia. Mr Chris Murray of the Salmonella Reference Laboratory is thanked for serotyping our *Salmonella* strains.

REFERENCES

- Alpuche-Aranda, C. M., Racoosin, E. L., Swanson, J. A. & Miller, S. I. (1994). *Salmonella* stimulate macrophage macropinocytosis and persist within spacious phagosomes. *J Exp Med* 179, 601–608.
- Alpuche-Aranda, C. M., Berthiaume, E. P., Mock, B., Swanson, J. A. & Miller, S. I. (1995). Spacious phagosome formation within mouse macrophages correlates with *Salmonella* serotype pathogenicity and host susceptibility. *Infect Immun* 63, 4456–4462.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* 215, 403–410.
- Baker, S. J., Daniels, C. & Morona, R. (1997). PhoP/Q regulated genes in *Salmonella typhi* identification of melittin sensitive mutants. *Microb Pathog* 22, 165–179.
- Barrow, P. A., Huggins, M. B. & Lovell, M. A. (1994). Host specificity of *Salmonella* infection in chickens and mice is expressed in vivo primarily at the level of the reticuloendothelial system. *Infect Immun* 62, 4602–4610.
- Bastin, D. A., Lord, A. & Verma, N. K. (1997). Cloning and analysis of the glucosyl transferase gene encoding type I antigen in *Shigella flexneri*. *FEMS Microbiol Lett* 156, 133–139.
- Bearson, B. L., Wilson, L. & Foster, J. W. (1998). A low pH-inducible, PhoPQ-dependent acid tolerance response protects *Salmonella typhimurium* against inorganic acid stress. *J Bacteriol* 180, 2409–2417.
- Behlau, I. & Miller, S. I. (1993). A PhoP-repressed gene promotes *Salmonella typhimurium* invasion of epithelial cells. *J Bacteriol* 175, 4475–4484.
- Buchmeier, N. A. & Heffron, F. (1989). Intracellular survival of wild-type *Salmonella typhimurium* and macrophage-sensitive mutants in diverse populations of macrophages. *Infect Immun* 57, 1–7.
- Davis, R. W., Botstein, P. & Roth, J. R. (1980). *Advanced Bacterial Genetics; a Manual for Genetic Engineering*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Feingold, D. S. & Barbar, G. A. (1990). Nucleotide sugars. In *Carbohydrates*, pp. 39–78. Edited by P. M. Dey. London: Academic Press.
- Fields, P. I., Swanson, R. V., Haidaris, C. G. & Heffron, F. (1986). Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc Natl Acad Sci USA* 83, 5189–5193.
- Fields, P. I., Groisman, E. A. & Heffron, F. (1989). A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. *Science* 243, 1059–1062.
- Galan, J. E. & Curtiss, R. D. (1989). Virulence and vaccine potential of *phoP* mutants of *Salmonella typhimurium*. *Microb Pathog* 6, 433–443.
- Garcia Vescovi, E., Soncini, F. C. & Groisman, E. A. (1996). Mg²⁺ as an extracellular signal: environmental regulation of *Salmonella* virulence. *Cell* 84, 165–174.
- Groisman, E. A. (1994). How bacteria resist killing by host-defense peptides. *Trends Microbiol* 15, 30–33.
- Groisman, E. A. & Saier, M. H., Jr (1990). *Salmonella* virulence: new clues to intramacrophage survival. *Trends Biochem Sci* 15, 30–33.
- Groisman, E. A., Saier, M. H., Jr & Ochman, H. (1992). Horizontal transfer of a phosphatase gene as evidence for mosaic structure of the *Salmonella* genome. *EMBO J* 11, 1309–1316.
- Groisman, E. A., Kayser, J. & Soncini, F. C. (1997). Regulation of polymyxin resistance and adaptation to low-Mg²⁺ environments. *J Bacteriol* 179, 7040–7045.
- Gunn, J. S. & Miller, S. I. (1996). PhoP-PhoQ activates transcription of PmrAB, encoding a two-component regulatory system involved in *Salmonella typhimurium* antimicrobial peptide resistance. *J Bacteriol* 178, 6857–6864.
- Gunn, J. S., Lim, K. B., Krueger, J., Kim, K., Guo, L., Hackett, M. & Miller, S. I. (1998). PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. *Mol Microbiol* 27, 1171–1182.
- Guo, L., Lim, K. B., Gunn, J. S., Bainbridge, B., Darveau, R. P., Hackett, M. & Miller, S. I. (1997). Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes *phoP-phoQ*. *Science* 276, 250–253.
- Harris, P. & Ralph, P. (1985). Human leukemic models of myelomonocytic development: a review of the HL-60 and U937 cell lines. *J Leukoc Biol* 37, 407–422.
- Hass, R., Bartels, H., Topley, N., Hadam, M., Kohler, L., Goppelt Strube, M. & Resch, K. (1989). TPA-induced differentiation and adhesion of U937 cells: changes in ultrastructure, cytoskeletal organization and expression of cell surface antigens. *Eur J Cell Biol* 48, 282–293.
- Helander, I. M., Kilpelainen, I. & Vaara, M. (1994). Increased substitution of phosphate groups in lipopolysaccharides and lipid A of the polymyxin-resistant *pmrA* mutants of *Salmonella typhimurium*: a 31P-NMR study. *Mol Microbiol* 11, 481–487.
- Hitchcock, P. J. & Brown, T. M. (1983). Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J Bacteriol* 154, 269–277.
- Hohmann, E. L., Oletta, C. A., Killeen, K. P. & Miller, S. I. (1996a). *phoP/phoQ*-deleted *Salmonella typhi* (Ty800). is a safe and immunogenic single-dose typhoid fever vaccine in volunteers. *J Infect Dis* 173, 1408–1414.
- Hohmann, E. L., Oletta, C. A. & Miller, S. I. (1996b). Evaluation of a *phoP/phoQ*-deleted, *aroA*-deleted live oral *Salmonella typhi* vaccine strain in human volunteers. *Vaccine* 14, 19–24.
- Hone, D. M., Tacket, C. O., Harris, A. M., Kay, B., Losonsky, G. & Levine, M. M. (1992). Evaluation in volunteers of a candidate live oral attenuated *Salmonella typhi* vector vaccine. *J Clin Invest* 90, 412–420.
- Huan, P. T., Bastin, D. A., Whittle, B. L., Lindberg, A. A. & Verma, N. K. (1997a). Molecular characterization of the genes involved in O-antigen modification, attachment, integration and excision in *Shigella flexneri* bacteriophage SfV. *Gene* 195, 217–227.
- Huan, P. T., Whittle, B. L., Bastin, D. A., Lindberg, A. A. & Verma, N. K. (1997b). *Shigella flexneri* type-specific antigen V: cloning, sequencing and characterization of the glucosyl transferase gene of temperate bacteriophage SfV. *Gene* 195, 207–216.

- Johnston, C., Pegues, D. A., Hueck, C. J., Lee, A. & Miller, S. I. (1996). Transcriptional activation of *Salmonella typhimurium* invasion genes by a member of the phosphorylated response-regulator superfamily. *Mol Microbiol* **22**, 715–727.
- Kasahara, M., Nakata, A. & Shinagawa, H. (1991). Molecular analysis of the *Salmonella typhimurium* *phoN* gene, which encodes nonspecific acid phosphatase. *J Bacteriol* **173**, 6760–6765.
- Mavris, M., Manning, P. A. & Morona, R. (1997). Mechanism of bacteriophage Sfil-mediated serotype conversion in *Shigella flexneri*. *Mol Microbiol* **26**, 939–950.
- Miller, S. I. (1991). PhoP/PhoQ: macrophage-specific modulators of *Salmonella* virulence? *Mol Microbiol* **5**, 2073–2078.
- Miller, S. I. & Mekalanos, J. J. (1990). Constitutive expression of the *phoP* regulon attenuates *Salmonella* virulence and survival within macrophages. *J Bacteriol* **172**, 2485–2490.
- Miller, S. I., Kukral, A. M. & Mekalanos, J. J. (1989). A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. *Proc Natl Acad Sci USA* **86**, 5054–5058.
- Miller, S. I., Hohmann, E. L. & Pegues, D. A. (1995). *Salmonella* (including *Salmonella typhi*). In *Principles and Practice of Infectious Diseases*, pp. 2013–2033. Edited by G. L. Mandell, J. E. Bennett & R. Dolen. New York: Churchill Livingstone.
- Mills, S. D. & Finlay, B. B. (1994). Comparison of *Salmonella typhi* and *Salmonella typhimurium* invasion, intracellular growth and localization in cultured human epithelial cells. *Microb Pathog* **17**, 409–423.
- Minta, J. O. & Pambrun, L. (1985). In vitro induction of cytologic and functional differentiation of the immature human monocyte-like cell line U-937 with phorbol myristate acetate. *Am J Pathol* **119**, 111–126.
- Monack, D. M., Raupach, B., Hromockyj, A. E. & Falkow, S. (1996). *Salmonella typhimurium* invasion induces apoptosis in infected macrophages. *Proc Natl Acad Sci USA* **93**, 9833–9838.
- Moreno, E., Mayer, H. & Moriyon, I. (1987). Characterization of a native polysaccharide hapten from *Brucella melitensis*. *Infect Immun* **55**, 2850–2853.
- Morona, R., Brown, M. H., Yeaton, J., Heuzenroeder, M. W. & Manning, P. A. (1991). Effect of lipopolysaccharide core synthesis mutations on the production of *Vibrio cholerae* O-antigen in *Escherichia coli* K-12. *FEMS Microbiol Lett* **66**, 279–285.
- Morona, R., van den Bosch, L. & Manning, P. A. (1995). Molecular, genetic and topological characterization of O-antigen chain length regulation in *Shigella flexneri*. *J Bacteriol* **177**, 1059–1068.
- Ochman, H., Gerber, A. S. & Hartl, D. L. (1988). Genetic applications of an inverse polymerase chain reaction. *Genetics* **120**, 621–623.
- Pedrinaci, S., Huelin, C., Patarroyo, M., Ruiz Cabello, F. & Garrido, F. (1989). Studies on CD11a and CD18 molecules with two new monoclonal antibodies: differential myelomonocytic antigen expression of PMA treated HL60 and U937 cell lines. *Hybridoma* **8**, 13–23.
- Pegues, D. A., Hantman, M. J., Behlau, I. & Miller, S. I. (1995). PhoP/PhoQ transcriptional repression of *Salmonella typhimurium* invasion genes: evidence for a role in protein secretion. *Mol Microbiol* **17**, 169–181.
- Porter, E. M., van Dam, E., Valore, E. V. & Ganz, T. (1997). Broad-spectrum antimicrobial activity of human intestinal defensin 5. *Infect Immun* **65**, 2396–2401.
- Pulkkinen, W. S. & Miller, S. I. (1991). A *Salmonella typhimurium* virulence protein is similar to a *Yersinia enterocolitica* invasion protein and a bacteriophage lambda outer membrane protein. *J Bacteriol* **173**, 86–93.
- Selsted, M. E., Miller, S. I., Henschen, A. H. & Ouellette, A. J. (1992). Enteric defensins: antibiotic peptide components of intestinal host defense. *J Cell Biol* **118**, 929–936.
- Shyamala, V. & Ames, G. F. (1993). Single specific primer-polymerase chain reaction (SSP-PCR) and genome walking. In *PCR Protocols: Current Methods and Applications*, pp. 339–348. Edited by B. A. White. Totowa: Humana Press.
- Soncini, F. C. & Groisman, E. A. (1996). Two-component regulatory systems can interact to process multiple environmental signals. *J Bacteriol* **178**, 6796–6801.
- Stroeher, U. H., Karageorgos, L. E., Brown, M. H., Morona, R. & Manning, P. A. (1995). A putative pathway for perosamine biosynthesis is the first function encoded within the *rfb* region of *Vibrio cholerae* O1. *Gene* **166**, 33–42.
- Sundstrom, C. & Nilsson, K. (1976). Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int J Cancer* **17**, 565–577.
- Tenhaken, R. & Thulke, O. (1996). Cloning of an enzyme that synthesizes a key nucleotide-sugar precursor of hemicellulose biosynthesis from soybean: UDP-glucose dehydrogenase. *Plant Physiol* **112**, 1127–1134.
- Verma, N. K., Verma, D. J., Huan, P. T. & Lindberg, A. A. (1993). Cloning and sequencing of the glucosyl transferase-encoding gene from converting bacteriophage X (SFX) of *Shigella flexneri*. *Gene* **129**, 99–101.
- Vescovi, E. G., Sancini, F. C. & Groisman, E. A. (1994). The role of *phoP/Q* regulon in *Salmonella* virulence. *Res Microbiol* **145**, 473–480.
- Valdivia, R. H. & Falkow, S. (1996). Bacterial genetics by flow cytometry: rapid isolation of *Salmonella typhimurium* acid-inducible promoters by differential fluorescence induction. *Mol Microbiol* **22**, 367–378.

Received 27 July 1998; revised 29 September 1998; accepted 3 November 1998.