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

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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 (Grosman & 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 & Grosman, 1996). A large number of PhoP-
activated genes (pag/pqa) and PhoP-repressed genes (prg/prq) have been identified in *S. typhimurium*, for example genes involved in macrophage survival (pagC) (Pukkinnen 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/wgd* (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* ΔphoPQ 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+) 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, 1983; Hass et al., 1989; Minta & Pambrun, 1995; Pedrinacci 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 onto 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-p-D-galactopyranoside (Boehringer Mannheim) with both X-pho and X-Gal freshly dissolved in dimethyl formamide at a final concentration of 40 μg ml⁻¹). Tetracycline (Sigma) was used at 10 μg 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 μg 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 μg 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 μg 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 1.25 x 10⁶ cells ml⁻¹. Bacteria were washed three times with PBS prior to the assay. HeLa cells were grown to semi-confluence and seeded in a 24 well tray. After incubation for 24 h (cells were now ~1 x 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 x 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.

**Salmoneilla PhoP₄, PhoP₅ and PhoP₆ 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
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 μg 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 μl 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 HindIII/ClaI 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 HindIII/ClaI 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-TCGTGAACGCTTTG 3'; anneals to the MuR right end of MudJ before the HindIII site) and the M13 reverse primer (Stratagene). The 0.85 kb PCR product was cloned into pGEMT (Promega) according to the manufacturer’s instructions, to create pRMSB50. After transformation into *E. coli* DH5α, 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 with a number of restriction enzymes. The smallest fragment detected was an EcoRV fragment (approx. 3 kb), and inverse PCR (Ochman et al., 1988) was used to obtain the fragment. This involved digesting 10 μg *S. typhi* Ty2 chromosomal DNA with EcoRV, circularization and religation of the restriction fragments, and amplification by PCR using oligonucleotides 2512 (5' CGAT-TCGCCATGACGGTCG 3') and 2513 (5' CACGGATCCG-TCGGCAACG 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 DH5α to enable sequencing. The EcoRV fragment was fully sequenced on both strands by Dye

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics</th>
<th>Source/construction</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli K-12</td>
<td></td>
<td>BRL</td>
</tr>
<tr>
<td>DH5α</td>
<td>F-80 lacZAM15 Δ(lacZYA-argF) supE44 thi-1 gyrA-96 rec-A1 end-A1 rel-A1 hsd-R17(rk- m-)</td>
<td></td>
</tr>
<tr>
<td>RMA292</td>
<td>DH5α(pRMSB50)</td>
<td>This work</td>
</tr>
<tr>
<td>RMA295</td>
<td>DH5α(pRMSB53)</td>
<td>This work</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C5</td>
<td>Wild-type</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>RMA1010</td>
<td>C5 phoP12, PhoP⁻</td>
<td>Baker et al. (1997)</td>
</tr>
<tr>
<td>RMA1024</td>
<td>C5 phoP24, PhoP⁺</td>
<td>Baker et al. (1997)</td>
</tr>
<tr>
<td>JSG421</td>
<td>pmrA::Tn10</td>
<td>Gunn &amp; Miller (1996)</td>
</tr>
<tr>
<td>S. typhi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ty2</td>
<td>Wild-type</td>
<td>Hone et al. (1988)</td>
</tr>
<tr>
<td>RMA1030</td>
<td>Ty2 phoP12, PhoP⁺</td>
<td>Baker et al. (1997)</td>
</tr>
<tr>
<td>RMA1090</td>
<td>Ty2 phoP24, PhoP⁺</td>
<td>Baker et al. (1997)</td>
</tr>
<tr>
<td>RMA1126</td>
<td>RMA1030 pqaB::MudJ</td>
<td>Baker et al. (1997)</td>
</tr>
<tr>
<td>RMA1180</td>
<td>Ty2 pqrA::MudJ</td>
<td>Baker et al. (1997)</td>
</tr>
<tr>
<td>RMA2310</td>
<td>Ty2 pgaA::MudJ</td>
<td>Baker et al. (1997)</td>
</tr>
<tr>
<td>RMA2312</td>
<td>Ty2 pgaD::MudJ</td>
<td>Baker et al. (1997)</td>
</tr>
<tr>
<td>RMA2316</td>
<td>Ty2 pgrB::MudJ</td>
<td>Baker et al. (1997)</td>
</tr>
<tr>
<td>RMA2326</td>
<td>Ty2 pgaB::MudJ</td>
<td>Baker et al. (1997)</td>
</tr>
<tr>
<td>RMA2526</td>
<td>RMA1090 pqaB::MudJ</td>
<td>Baker et al. (1997)</td>
</tr>
<tr>
<td>RMA1307</td>
<td>RMA2326 pmrA::Tn10</td>
<td>P22 on JSG421*</td>
</tr>
<tr>
<td>RMA1309</td>
<td>RMA2526 pmrA::Tn10</td>
<td>P22 on JSG421*</td>
</tr>
</tbody>
</table>

* Transduction with P22HTinti105 propagated on *S. typhimurium* JSG421, selected for Tc⁺.
RESULTS

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

As S. typhimurium PhoP- and PhoP+ 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 Ty2 and the isogenic PhoP- (RMA1030) and PhoP+ (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- was noted (Fig. 1a, Table 2). This correlated with data from an S. typhimurium PhoP+ 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+ strain (RMA1090) had a GI of 42 (Fig. 1; Table 3); a possible explanation for this increased net growth of the S. typhi PhoP+ 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...
**S. typhi** PhoP/Q regulated genes and U937 cells

Fig. 7. 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 ± so. 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 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 CS strain. Therefore we investigated the ability of *S. typhimurium* CS 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

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

* Units LDH per well are presented as the mean of four assays ± 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⁺ (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).

### 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/Clal end fragment (data not shown). The 0·85 kb fragment was isolated, cloned into pGEMT to create pRMSB.50 and sequenced (Methods). The fragment was found to have 70% homology with an *E. coli* K-12 ORF (AE000315) region and also had 95% homology with the fifth gene of an *S. typhimurium* pmrF operon (AF035677) 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.
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 hydrophathy 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 S50 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 (Guen 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, PhoPE (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+ parental 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, and presented as β-galactosidase units from the mean of two duplicate assays ±SD: RMA1126 (PhoP+, pqaB::MudJ), 17 ± 1; RMA2326 (PhoP+, pqaB::MudJ), 395 ± 1; RMA2526 (PhoP+, pqaB::MudJ), 996 ± 35; RMA1307 (PhoP+, pqaB::MudJ, pmrA::Tn10), 6 ± 1 and RMA1309 (PhoP+, pqaB::MudJ, pmrA::Tn10), 8 ± 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 (Guen 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-), Ty2, RMA2326 (pqaB::MudJ), RMA1090 (PhoP- and RMA2525 (pqaB::MudJ, PhoP- 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 ±SD. Arrows indicate survival ≤1 %.

**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-) and RMA2525 (pqaB::MudJ, PhoP-) 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 ±SD. Arrows indicate survival ≤1 %.
between S. typhi Ty2 and the pqaB::MudJ insertion mutant (RMA2326), but a large difference was observed between the RMA2526 (pqaB::MudJ, PhoP*) and RMA1090 (PhoE*) 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 PhoE* strains, and S. typhi strains with the pqaB mutation in PhoP* and PhoE* 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* (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 LPS of the S. typhi PhoP* (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* (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*), 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*, PhoE* and pqa/pqr mutants in macrophage-like cells. The invasion and net growth of S. typhimurium wild-type, PhoP* and PhoE* 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 PhoE* 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 PhoE* mutant do not correlate with published observations with an S. typhimurium PhoE* 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 PhoE* (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 RMA2236 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 pga 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⁻ (RMA1090) mutant had an increased resistance to PmB compared to the wild-type Ty2 strain (Fig. 5). The pqaB::MudJ PhoP⁺ (RMA2236) strain was more sensitive to PmB than RMA1090 (PhoP⁻), 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 (Grosman, 1994). An increase in both ethanolamine and 4-deoxyxymAnarabinose 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⁺ 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⁺ (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⁻, pqaB::MudJ and pqaB::MudJ/PhoP⁺ on an SDS-PAGE gel, and the LPS of S. typhimurium C5 and PhoP⁺, PhoP⁻ derivatives. Based on chemical analysis showing that *S. typhimurium* PhoP⁺ 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⁻ derivative. As can be seen in Fig. 6, there was a remarkable difference between S. typhimurium C5 and PhoP⁻ LPS, with much shorter O-antigen chains being evident for the PhoP⁻ strain. This is in direct contrast to the S. typhi Ty2, PhoP⁻ and PhoP⁺ 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 (RMA2236 and RMA2356) and their wild-type (Ty2) and PhoP⁺ (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 & Curtis (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 suggest 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<sup>e</sup> and pgaB::MudJ derivatives by mass spectrometry to confirm and extend the known differences between *S. typhi* and *S. typhimurium* LPS.

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**REFERENCES**


