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

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*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 (PMA4937) cells, and an *S. typhi* PhoPc (constitutive) mutant showed a defect in invasion. Neither of the *phoPIQ* mutants were growth impaired in HeLa cells, however the PhoPc mutant was impaired in invasion. As opposed to what was found for *S. typhi*, *Salmonella typhimurium* wild-type, PhoP’ and PhoPc 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 PhoPc 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 PhoPc 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-
activated genes (pag/pqa) and PhoP-repressed genes (prg/prq) 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 (prg/H1JK) (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/sgd (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 (Alpuce-Aranda et al., 1994, 1995), defective for macrophage survival and are attenuated for virulence in mice (Miller & Meekalons, 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 (Baker et al., 1997), 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; Pedrini 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 (Boehminger Mannheim) or X-Gal (LA with the addition of 5-bromo-4-chloro-3-indolyl-p-D-galactopyranoside (Boehminger 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) glucose 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) glucose 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 monocytederived macrophages involved washing U937 cells grown to a high density, and seeding in 24 multi-well plates (Corning, 25820) at approximately 1 × 10⁸ 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 × 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 LA at 37 °C with aeration, subcultured 1/20 into 10 ml LB and grown for 2.5 h until bacteria had reached 1 × 10⁸ 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° 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 1:1 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, and the remaining in the cells as it may break down in the supernatant. 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.

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/Clal 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/Clal 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-TCGTGAAGCCGTTCG 3’; anneals to the MuR right end of MudJ before the HindIII site) and the M13 reverse primer of MudJ before the HindIII site) and the M13 reverse primer. The PCR product was cloned into pGEMT (Progen) 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 digestion with 10 µg S. typhi Ty2 chromosomal DNA with EcoRV, circularization and religation of the restriction fragments, and amplification by PCR using oligonucleotides 2512 (5’ CGATTCGCCATGACGGATG 3’) and 2513 (5’ CACGATCCAG-TCGCAAGAC 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Characteristics</th>
<th>Source/construction</th>
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<tbody>
<tr>
<td>E. coli K-12</td>
<td></td>
<td></td>
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<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>BRL</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>
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<tr>
<td>S. typhi</td>
<td></td>
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<tr>
<td>Ty2</td>
<td>Wild-type</td>
<td>Hone et al. (1988)</td>
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<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>
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<tr>
<td>RMA1180</td>
<td>Ty2 pqrA::MudJ</td>
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<td>Baker et al. (1997)</td>
</tr>
<tr>
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<td>Ty2 pgaB::MudJ</td>
<td>Baker et al. (1997)</td>
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<tr>
<td>RMA2316</td>
<td>Ty2 pqrB::MudJ</td>
<td>Baker et al. (1997)</td>
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<tr>
<td>RMA2326</td>
<td>Ty2 pgaB::MudJ</td>
<td>Baker et al. (1997)</td>
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<tr>
<td>RMA2526</td>
<td>RMA1090 pqaB::MudJ</td>
<td>Baker et al. (1997)</td>
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<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 P22HTint105 propagated on S. typhimurium JSG421, selected for Tc′.

**Table 1. Escherichia coli and Salmonella strains used in this study**
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 × 10^8 bacteria ml^-1 and chilled. Aliquots (100 µl) of diluted culture were added to a 96 well microtitre plate (Corning well 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 µl) and PBS (100 µl). The microtitre plates were incubated at 37 °C for 1 h, after which they were placed on ice. Plates (LA) were spread with 50 µl 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^+ strains and all the S. typhi strains (Ty2, PhoP^-, PhoP^+ 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^+ 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^+ (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

<table>
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<tr>
<th>Table 2. Invasion of PMA-U937 cells by S. typhi and S. typhimurium strains</th>
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<tr>
<td><strong>Strain</strong></td>
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<tr>
<td><strong>S. typhi</strong></td>
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<tr>
<td>Ty2 (PhoP^+)</td>
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<tr>
<td>RMA1030 (PhoP^-)</td>
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<td>RMA1090 (PhoP^+)</td>
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<tr>
<td>RMA2326 (pqaB::MudJ)</td>
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<tr>
<td><strong>S. typhimurium</strong></td>
</tr>
<tr>
<td>C5 (PhoP^-)</td>
</tr>
<tr>
<td>RMA1010 (PhoP^+)</td>
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<td>RMA1024 (PhoP^-)</td>
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<tr>
<th>Table 3. Growth index of S. typhi and S. typhimurium in PMA-U937 cells</th>
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<tr>
<td><strong>Bacterial strain</strong></td>
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<tr>
<td><strong>Cell line</strong></td>
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<tr>
<td><strong>S. typhi</strong></td>
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<tr>
<td>Ty2 (PhoP^+)</td>
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<td>RMA1010 (PhoP^+)</td>
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<td>RMA1024 (PhoP^-)</td>
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* 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.
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 ± s.d. 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⁺ (RMA-1090) 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 × 10^{-3} ± 1.47 × 10^{-3}</td>
<td>1.00</td>
<td>3.50 × 10^{-3} ± 9.22 × 10^{-4}</td>
<td>0.87</td>
</tr>
<tr>
<td>Ty2 (PhoP⁻)</td>
<td>2.03 × 10^{-3} ± 9.25 × 10^{-4}</td>
<td>0.50</td>
<td>1.09 × 10^{-3} ± 9.47 × 10^{-4}</td>
<td>0.27</td>
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<tr>
<td>RMA1030 (PhoP⁻)</td>
<td>1.96 × 10^{-3} ± 1.15 × 10^{-3}</td>
<td>0.49</td>
<td>1.16 × 10^{-3} ± 9.55 × 10^{-4}</td>
<td>0.29</td>
</tr>
<tr>
<td>RMA1090 (PhoP⁻)</td>
<td>2.98 × 10^{-3} ± 1.55 × 10^{-3}</td>
<td>0.74</td>
<td>2.41 × 10^{-3} ± 1.25 × 10^{-3}</td>
<td>0.60</td>
</tr>
<tr>
<td>RMA2326 (pqaB::MudJ)</td>
<td>2.92 × 10^{-3} ± 1.27 × 10^{-3}</td>
<td>0.72</td>
<td>2.30 × 10^{-3} ± 1.50 × 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.

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

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 cell 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/ClaI end fragment (data not shown). The 0.85 kb fragment was isolated, cloned into pGEMT to create pRMSB.50 and sequenced. 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
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 ORFS 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 C9 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 (Strooher et al., 1995). The following two steps result in lipid A modification by 4AA. The PmrF protein has both amino acid sequence and hydrophy 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 4AAP-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 glucosyltransferases (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.

Pmr/A/B regulation of the S. typhi pqaB gene

The S. typhimurium pmrF operon has been shown to be strongly regulated by Pmr/A/B (Guen et al., 1998). To test whether the S. typhi pqaB gene was also regulated by Pmr/A/B, a pma null mutation (pmaA::Tn10d) from the S. typhimurium strain JSG421 was transduced by phage P22 into S. typhi Ty2, PhoP+, pmaB::MudJ, PhoP+ (RMA2326), and pmaB::MudJ, PhoP+ (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 pmaA+ parental strains. This indicated that the S. typhi pqaB::MudJ fusion was strongly regulated by the Pmr/A/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+, pgaB::MudJ), 17 ± 1; RMA2326 (PhoP+, pgaB::MudJ), 395 ± 1; RMA2526 (PhoP+, pgaB::MudJ), 996 ± 35; RMA1307 (PhoP+, pgaB::MudJ), 6 ± 1 and RMA1309 (PhoP+, pgaB::MudJ), 8 ± 4. These results showed that pgaB 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 pmaA+ 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 protease (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 CS strains. The survival of S. typhi Ty2, RMA1030 (PhoP+), RMA1090 (PhoP-), RMA2326 (pgaB::MudJ), RMA2526 (pgaB::MudJ), PhoP+, RMA1307 (pgaB::MudJ), PhoP+, RMA1309 (pgaB::MudJ), PhoP+ strains were tested with 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 RMA1303 (PhoP+), Ty2, RMA2326 (pgaB::MudJ), RMA1090 (PhoP+) and RMA2526 (pgaB::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%.

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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 (PhoP*) 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* strains, and *S. typhi* strains with the *pqaB* mutation in PhoP* and PhoP* 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 LPSs 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* and *pqa/pqr* mutants in macrophage-like cells. The invasion and net growth of *S. typhimurium* wild-type, PhoP* and PhoP* 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* 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* mutant do not correlate with published observations with an *S. typhimurium* PhoP* 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* (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

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**Fig. 6. Comparison of *S. typhi* and *S. typhimurium* lipopolysaccharides.** Samples (representing 10⁶ 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*), *S. typhi* RMA1030 (PhoP*), *S. typhi* Ty2 (PhoP*), *S. typhi* RMA1090 (PhoP*), *S. typhi* RMA2326 (*pqaB*: :MudJ, PhoP*) and *S. typhi* RMA2326 (*pqaB*: :MudJ, PhoP*). *** indicates that the LPS chains lack discrete banding. Smooth LPS O-antigen chains and lipid A + core sugar molecules are also indicated.
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 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::Tnl10 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+ (RMA2326) strain was more sensitive to PmB than RMA1090 (PhoP0), 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-deoxyaminobaroarbose 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-deoxyaminarabinose 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+, PhoP0, pqaB::MudJ and pqaB::MudJ/PhoP0 on an SDS-PAGE gel, and the LPS of S. typhimurium C5 and PhoP+, PhoP0 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 (RMA2326 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 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-, PhoO- and pgaB-: MudJ derivatives by mass spectrometry to confirm and extend the known differences between S. typhi and S. typhimurium.

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