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A chromosomal region surrounding the *ompD* porin gene marks a genetic difference between *Salmonella typhi* and the majority of *Salmonella* serovars

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In this work it is shown that the majority of *Salmonella* serovars most frequently associated with the systemic infection of vertebrate hosts produce a major outer-membrane porin, OmpD. However, OmpD is absent from the outer-membrane protein profiles of *Salmonella typhi* strain Ty2 and 26 clinical isolates of *S. typhi* examined by SDS-PAGE. To determine whether the *ompD* gene is present in *S. typhi*, primers internal to the *ompD* coding sequence were used to amplify the gene by PCR. With the exception of *S. typhi* strains, the *ompD* gene was amplified from the genomes of all *Salmonella* serovars tested. Consistently, a specific *ompD* probe did not hybridize with DNA isolated from the *S. typhi* strains. Taken together, these results demonstrate that *S. typhi* does not produce OmpD due to the absence of the *ompD* gene. Furthermore, it was investigated whether the deletion of *ompD* extended to *smvA*. This gene is adjacent to *ompD* in the *Salmonella typhimurium* chromosome and encodes a protein involved in the resistance to methyl viologen, a superoxide-generating agent. Although PCR failed to amplify the *smvA* gene from the *S. typhi* strain Ty2 genome, it was possible to amplify it from the chromosome of the clinical strains. On the other hand, hybridization analyses showed that the *smvA* gene is present in all the *S. typhi* strains tested. In contrast to the other *Salmonella* serovars, *S. typhi* strain Ty2 and the clinical isolates showed sensitivity to methyl viologen, suggesting that *smvA* gene is inactive in *S. typhi*. In conclusion, the *ompD–smvA* region is variable in structure among *Salmonella* serovars. It is hypothesized that the absence of *ompD* may suggest a role in host specificity.

Keywords: *Salmonella*, outer-membrane protein, *smvA*, genetic variability, host specificity

INTRODUCTION

Salmonella typhi causes the systemic disease typhoid fever only in its human host. The closely related *Salmonella typhimurium* causes a typhoid-like systemic disease in the mouse, but most frequently causes only a self-limited gastroenteritis in the human host. Several genetic variations between *S. typhi* and *S. typhimurium* have been suggested to contribute to their respective host specificity and infectivity. The relative order of genes on the *S. typhimurium* and *S. typhi* chromosomes is different, as is the relative stability of their genomes. In addition, the *S. typhi* genome is considered to be fluid because different chromosome structures are found among clinical isolates (Echeita & Usera, 1998; Liu & Sanderson, 1996). Many of these genomic rearrangements are thought to be due to homologous recombination events between pairs of the seven rRNA (*rrn*) operons. Thus, it has been postulated that genomic

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Abbreviation: OMP, outer-membrane protein.

rearrangements, rather than mutations, are responsible for producing the ribotype heterogeneity in *S. typhi* (Ng & Sanderson, 1999). In contrast, no *rrn*-mediated rearrangements have been detected among wild-type strains of *S. typhimurium* (Liu & Sanderson, 1995b; Liu & Sanderson, 1996) in spite of the high frequency of rearrangements observed for the *S. typhimurium* strain LT2 when grown in culture (Anderson & Roth, 1981). *S. typhi* appears to be exceptional among enteric bacteria because of its significant genome structure plasticity (Krawiec & Riley, 1990; Liu & Sanderson, 1996).

Because the sequences of conserved genes between *S. typhi* and *S. typhimurium* are nearly identical, they are said to be homeologous (Zahrt & Maloy, 1997; Zahrt *et al.*, 1994). However, it is unlikely that the minor fraction (1–2 %) of nucleotide base pair differences between genes conserved in these two serovars contributes significantly to their remarkably different host specificities. Rather, the striking differences in *S. typhi* and *S. typhimurium* host specificity is most likely due to the gross rearrangements one observes when comparing their structures at a more macroscopic level. The substitutions and insertions between *S. typhi* and *S. typhimurium* genomes range in size from islands comprised of more than 100 genes to smaller substitutions corresponding to single ORFs (McClelland & Wilson, 1998). In addition, about 20 % of the *S. typhi* genome is not present in *S. typhimurium* and vice versa (Lan & Reeves, 1996). Therefore, it is possible that as many as 900 *S. typhi* genes could be serovar-specific, a subset of which is likely to be involved in determining host range (Bäumler *et al.*, 1998).

In this work, we describe one of the smaller interserovar differences in genome structure between *S. typhi* and *S. typhimurium*. The region of the *S. typhimurium* genome including *smvA*, *ompD* and *yddG* genes is different in *S. typhi*. These genes encode proteins that confer resistance to methyl viologen, to an abundant outer-membrane porin and to a putative transmembrane permease, respectively. The primary structure of the region containing these functions is genetically variable among *Salmonella* serovars.

METHODS

Bacterial strains and growth conditions. The *Salmonella* strains used in this study are listed in Table 1. The *S. typhi* clinical strains (STH) were obtained from the Infectious Diseases Hospital (Hospital Lucio Córdova) in Santiago, Chile. Bacteria were grown routinely at 37 °C in Luria–Bertani broth (LB; Bacto tryptone, 10 g l^{-1}; Bacto yeast extract, 5 g l^{-1}; NaCl, 5 g l^{-1}) and aerated by shaking. When required, LB was supplemented with ampicillin (100 mg l^{-1}) and tetracycline (10 mg l^{-1}). Media were solidified by the addition of agar (15 g l^{-1}).

Genetic techniques. Transductional crosses were carried out at low m.o.i. using the high-frequency transducing phage P22 HT105/1 *int*-201 as described by Maloy (1990). A recombinant *S. typhi* Ty2 *ompD*$^+$ strain was obtained by transducing the wild-type *ompD* gene from *S. typhimurium* LT2 to *S. typhi* strain Ty2. In a first cross, the *ompD*::Tn*10* allele was transduced using phage P22 from *S. typhimurium* strain MST2944 to *S. typhi* strain Ty2, selecting for tetracycline resistance. In a second cross, the wild-type *ompD* allele was transduced from *S. typhimurium* strain LT2 to *S. typhi* strain Ty2 *ompD*::Tn*10* to produce the *S. typhi* strain Ty2 *ompD*$^+$. The latter was obtained by selecting for loss of tetracycline resistance on Bochner plates (Maloy & Nunn, 1981). Serotypes of the transductants were confirmed by agglutination using specific antisera against O-antigen groups D1 and B.

Preparation of outer-membrane proteins (OMPs). Outer-membrane fractions were prepared as described by Lobos & Mora (1991), based on a modification of the method of Schnaitman (1971). Bacteria were grown overnight at 37 °C and harvested by centrifugation at 13000 ***g*** for 15 min. Cell pellets were washed and resuspended in 1 ml 10 mM Tris/HCl pH 8·0 buffer. Bacteria were disrupted by sonication (Vibra Cell, Sonics & Materials) for 100 s and centrifuged at 8000 ***g*** for 5 min. Supernatants were collected and centrifuged at 13000 ***g*** for 30 min. Supernatants were then discarded and pellets were resuspended in 500 µl buffer containing 10 mM Tris/HCl pH 8·0, 10 mM $MgCl_2$, 2 % Triton X-100. After incubation at 37 °C for 30 min, mixtures were centrifuged at 13000 ***g*** for 30 min. The insoluble outer-membrane fraction was resuspended in buffer consisting of 100 mM Tris/HCl pH 8·0, 2 % SDS. SDS-PAGE was performed in 12·5 % polyacrylamide slabs as described by Lobos & Mora (1991).

PCR amplifications. All amplifications were conducted using a Perkin Elmer thermal cycler (GeneAmp PCR System 2400) and *Taq* polymerase (Gibco-BRL). The final volume in the tubes for amplification was 50 µl and consisted of 1 × *Taq* PCR buffer, 1·5 mM $MgCl_2$, each dNTP at a concentration of 200 µM, 50 pmol of each primer, 0·1 mg DNA and 1·25 U *Taq* polymerase. Standard conditions for amplification were 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, followed by a final extension step at 72 °C for 5 min. Primers OMPD1 (5′-GAC AAA GAC AAA ACC CGT T-3′) and OMPD2 (5′-CGT CCA GCA GGT TGA TTT T-3′) were used to amplify a 740 bp internal fragment of the *ompD* gene (Singh *et al.*, 1996). Primers SMVA1 (5′-CTT AAC CGC CCG CTA TGA T-3′) and SMVA2 (5′-GCT GAA CCA CAT CCC TAC C-3′) were designed from the reported *smvA* sequence (GenBank accession no. D26057) and used to amplify a 940 bp fragment internal to *smvA*.

Southern hybridizations. Plasmid pNK2883 (Kleckner *et al.*, 1991) was cleaved with *Bam*HI, and the 3 kb fragment with the *tetRA* genes was purified, labelled using the Bioprime DNA labelling system (Gibco-BRL) and used as the probe for Tn*10*. Hybridization probes for *ompD* and *smvA* were generated by PCR using a mixture of dNTPs containing biotin-14-dCTP and purified by using the Concert gel extraction system (Life Technologies). Genomic DNA of *Salmonella* serotypes was prepared as described by Sambrook *et al.* (1989), restricted with *Pst*I (Gibco-BRL) and the fragments were separated on a 0·8 % agarose gel. The DNA was then transferred onto a nylon membrane and cross-linked by UV irradiation. Hybridization was performed in solutions without formamide at 65 °C. Two 15 min washes were performed at 65 °C in a buffer consisting of 0·5 M Na_2HPO_4 pH 7·2, 2 % SDS, 1 mM EDTA. Hybridization was detected by using the nonradioactive Photogene nucleic acid detection system (Life Technologies) and XAR-5 Kodak films.

Table 1. *Salmonella* strains used in this study

Strain*	Genotype/phenotype	Source†
S. typhi		
Ty2	Vaccine strain, Vi minus	WHO
TYT2015	Ty2 *ompD159::Tn10*	This work
TYT2016	Ty2 *ompD*$^+$	This work
S. typhimurium		
LT2	Wild-type	S. Maloy
SC1	LT2 *ompD159::Tn10*	This work
MST2944	*ompD159*::Tn*10 galE496 his-6165 ilv-452 hspLT6 hspS29 metE551 xyl-404 rpsL120 fla-66 metA22 trpC2* H1-b H2-e,n,x nml	S. Maloy
Other salmonellae		
S. agona SARB 1	Wild-type	S. Maloy
S. anatum SARB 2	Wild-type	S. Maloy
S. brandenburg SARB 3	Wild-type	S. Maloy
S. choleraesuis SARB 4	Wild-type	S. Maloy
S. decatur SARB 8	Wild-type	S. Maloy
S. derby SARB 9	Wild-type	S. Maloy
S. dublin SARB 12	Wild-type	S. Maloy
S. duisberg SARB 15	Wild-type	S. Maloy
S. enteritidis SARB 16	Wild-type	S. Maloy
S. emek SARB 20	Wild-type	S. Maloy
S. gallinarum SARB 21	Wild-type	S. Maloy
S. haifa SARB 22	Wild-type	S. Maloy
S. heidelberg SARB 23	Wild-type	S. Maloy
S. infantis SARB 26	Wild-type	S. Maloy
S. miami SARB 28	Wild-type	S. Maloy
S. muenchen SARB 32	Wild-type	S. Maloy
S. newport SARB 36	Wild-type	S. Maloy
S. panama SARB 39	Wild-type	S. Maloy
S. paratyphi A SARB 42	Wild-type	S. Maloy
S. paratyphi B SARB 43	Wild-type	S. Maloy
S. paratyphi C SARB 48	Wild-type	S. Maloy
S. pullorum SARB 51	Wild-type	S. Maloy
S. reading SARB 53	Wild-type	S. Maloy
S. rubislaw SARB 54	Wild-type	S. Maloy
S. saintpaul SARB 56	Wild-type	S. Maloy
S. schwarzengrund SARB 57	Wild-type	S. Maloy
S. sendai SARB 58	Wild-type	S. Maloy
S. senftenberg SARB 59	Wild-type	S. Maloy
S. stanley SARB 60	Wild-type	S. Maloy
S. thompson SARB 62	Wild-type	S. Maloy
S. typhisuis SARB 70	Wild-type	S. Maloy
S. wien SARB 71	Wild-type	S. Maloy
S. montevideo 3799-98	Wild-type	ISP

*SARB, Salmonella Reference Collection B (Selander *et al*., 1996).

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Tests for resistance to methyl viologen (paraquat). Each of the *Salmonella* strains tested was isolated on MacConkey-lactose agar, and single colonies were streaked onto LB agar plates containing 0·5 mM methyl viologen, an agent that generates superoxide and oxygen radicals (Farr & Kogoma, 1991). Plates were incubated overnight at 37 °C and scored for bacterial growth. To determine the minimal dose of methyl viologen required to kill *Salmonella* strains, a lawn of the strain being tested was seeded on LB agar plates and 10 µl of serial dilutions of methyl viologen stock were added. Plates were incubated overnight at 37 °C and scored for bacterial growth.

RESULTS

S. typhi does not produce the OmpD porin

Previously, we have reported that *S. typhi* strain Ty2 does not produce the OmpD porin (Calderón *et al.*, 1984). In contrast, OmpD is one of the most abundant proteins in the *S. typhimurium* outer membrane (Lee & Schnaitman, 1980; Nikaido & Vaara, 1985; Nikaido, 1996). To investigate the presence of OmpD among the majority of *Salmonella* serovars most frequently associated with systemic infection of vertebrate hosts, the OMPs of a variety of *Salmonella* serovars were analysed by SDS-PAGE. Electrophoretic profiles of OMPs produced by *Salmonella enteritidis*, *Salmonella paratyphi* A, *Salmonella paratyphi* B, *Salmonella paratyphi* C, *Salmonella choleraesuis*, *Salmonella gallinarum*, *Salmonella pullorum*, *Salmonella dublin*, *Salmonella saintpaul*, *Salmonella muenchen*, *Salmonella heidelberg* and *Salmonella montevideo* showed that all of these serovars produce a major protein migrating as *S. typhimurium* OmpD porin (Fig. 1a).

The same protein band was observed when this analysis was extended to include *Salmonella agona*, *Salmonella anatum*, *Salmonella brandenburg*, *Salmonella decatur*, *Salmonella derby*, *Salmonella duisberg*, *Salmonella emek*, *Salmonella haifa*, *Salmonella infantis*, *Salmonella miami*, *Salmonella newport*, *Salmonella panama*, *Salmonella reading*, *Salmonella rubislaw*, *Salmonella schwarzengrund*, *Salmonella sendai*, *Salmonella senftenberg*, *Salmonella stanley*, *Salmonella thompson*, *Salmonella typhisuis* and *Salmonella wien* (data not shown). This protein band disappeared from the SDS-PAGE OMP profiles when the *ompD*::Tn*10* allele was transduced from *S. typhimurium* strain MST2944 to *S. enteritidis*, *S. paratyphi* A, *S. paratyphi* B, *S. pullorum* and *S. gallinarum* recipient strains, by using phage P22 (Fig. 1b). These data indicate that the protein band corresponds to the OmpD porin in these serovars, making it highly probable that it also represents OmpD in all the *Salmonella* serovars included in this study. On the other hand, the analysis of OMPs from *S. typhi* strain Ty2 (Fig. 1a) and 26 independent clinical isolates of *S. typhi* (data not shown) indicated that OmpD is not produced by these strains.

The finding that *S. typhi* strains do not produce OmpD can be explained by one of two alternative hypotheses. *S. typhi* may have an *ompD* gene that is not expressed, or there may be no homologous *ompD* gene in *S. typhi*. Because the current sequence data produced by the *S. typhi* Sequencing Group at the Sanger Center (ftp://ftp.sanger.ac.uk/pub/pathogens/st/ST.dbs) do not include an OmpD coding sequence, sequence data alone do not allow the distinction between these two hypotheses. Therefore, a combination of genetic and biochemical experiments was designed to determine whether the *ompD* gene is absent from the *S. typhi* genome.

PCR and DNA hybridization analyses indicate that the *ompD* gene is absent from the *S. typhi* genome

To determine whether the *ompD* gene is present in *S. typhi*, we first attempted to amplify a 740 bp fragment internal to *ompD* using PCR, with *ompD*-specific oligonucleotide primers (Singh *et al.*, 1996). With these primers, no product was obtained when DNA isolated

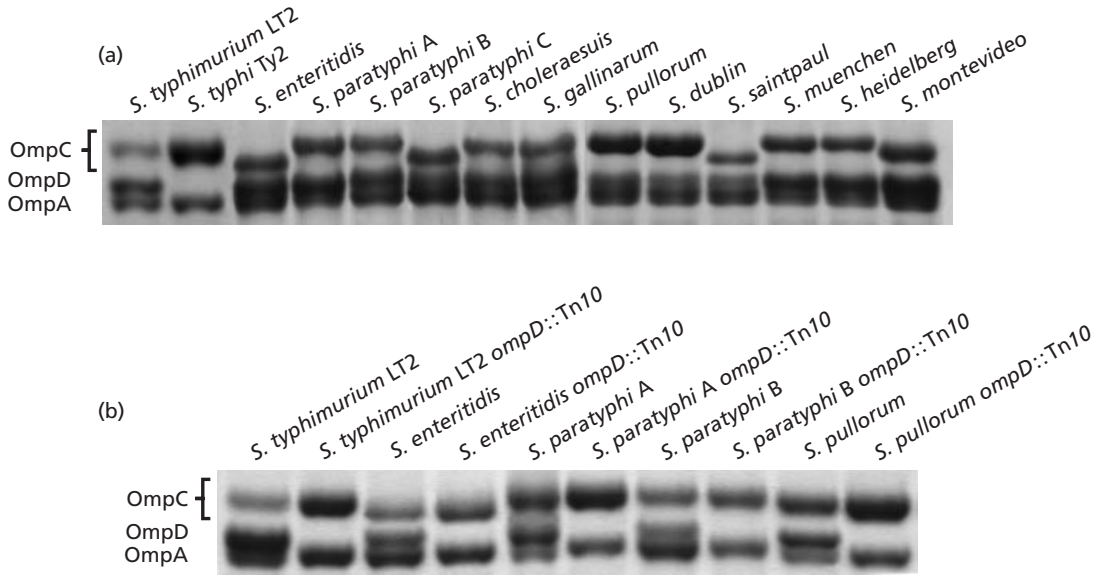

Fig. 1. SDS-PAGE analysis of porins from several *Salmonella* serovars. OMPs were prepared from bacteria grown aerobically at 37 °C in LB broth to stationary phase. Protein samples were resolved by electrophoresis through 12·5% acrylamide and stained with Coomassie blue. Under the bacterial growth conditions used, OmpF porin is not detected in the OMP profiles.

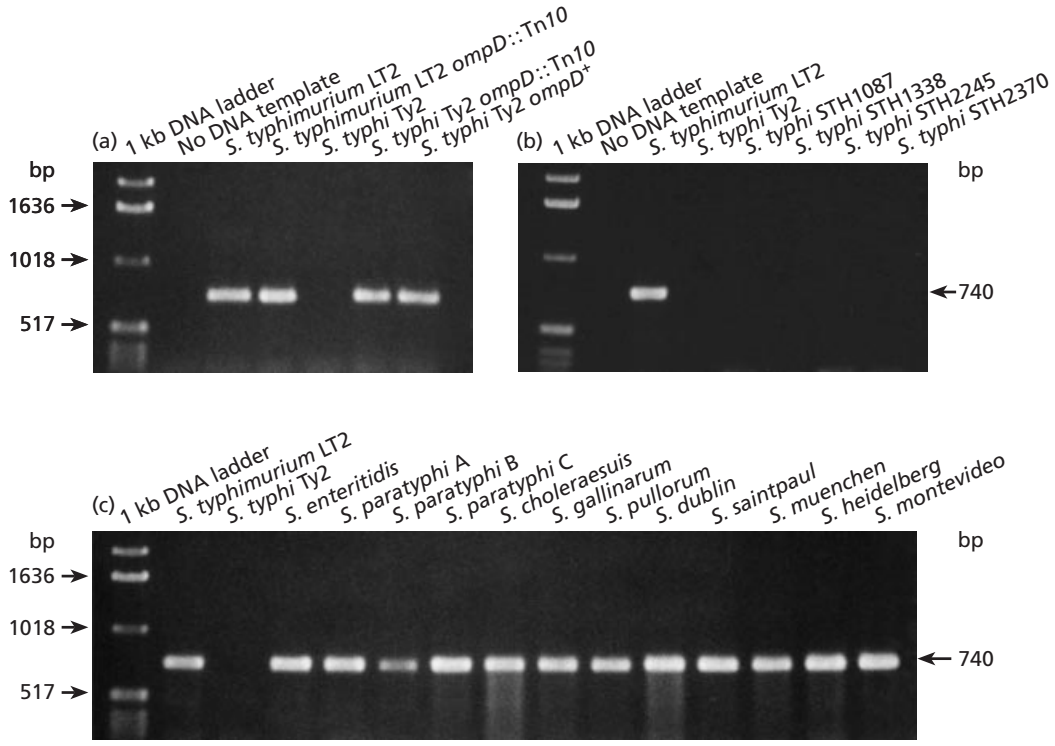

Fig. 2. Amplification of the *ompD* gene from several *Salmonella* serovars. PCR amplifications were performed as described in Methods, using primers internal to *ompD*. Chromosomal DNA templates were obtained from (a) interspecific hybrid strains between *S. typhimurium* and *S. typhi*, (b) *S. typhi* clinical strains and (c) several *Salmonella* serovars.

from *S. typhi* strain Ty2, or DNA isolated from each of 26 independent *S. typhi* clinical isolates, was used as template (see Fig. 2b for example). In contrast, a product of the predicted size was amplified using the same primers and DNA templates prepared from all the other *Salmonella* serovars tested (Fig. 2c).

Since deficient primer annealing due to low homology could account for the absence of PCR amplification of the *ompD* gene, the presence of the *ompD* gene in *S. typhi* was evaluated directly by DNA hybridization. This method is less dependent on the sequence homologies between the *S. typhi* and *S. typhimurium* serovars. Therefore, the 740 bp *ompD* fragment obtained from *S. typhimurium* was labelled and used to probe Southern blots of *S. typhi* DNA. The *ompD* probe hybridized neither with DNA obtained from *S. typhi* strain Ty2, nor with DNA from each of 10 randomly chosen *S. typhi* clinical isolates (Fig. 3a). In contrast, it did hybridize with a band of approximately 4·4 kb in DNA samples obtained from *S. typhimurium* strain LT2 (Fig. 3a). This small band indicates a DNA fragment containing the wild-type *ompD* gene. The DNAs of several *Salmonella* serovars were also examined by Southern blot hybridization using the highly specific probe to the *ompD* gene. Hybridization was observed in three patterns, one of which was identical to that observed using *S. typhimurium* DNA and included *S. brandenburg*, *S. emek*, *S. haifa*, *S. heidelberg*, *S. infantis*, *S. miami*, *S. panama*, *S. paratyphi* A, *S. pullorum*, *S. reading*, *S. schwarzengrund* and *S. wien* serovars. The second hybridization pattern revealed a band of approximately 14 kb in DNA samples obtained from *S. agona*, *S. anatum*, *S. choleraesuis*, *S. decatur*, *S. derby*, *S. dublin*, *S. duisberg*, *S. enteritidis*, *S. gallinarum*, *S. montevideo*, *S. muenchen*, *S. newport*, *S. paratyphi* C, *S. rubislaw*, *S. saintpaul*, *S. sendai*, *S. stanley*, *S. thompson* and *S. typhisuis* serovars. The third pattern of hybridization revealed a band of approximately 11·5 kb, and was only observed in DNA samples obtained from *S. paratyphi* B (Fig. 3b). These results strongly suggest that the *ompD* locus is widely distributed among *Salmonella* serovars, with the exception of *S. typhi*.

The *S. typhimurium ompD* gene can be transduced to and expressed in *S. typhi*

To understand the genetic variation of the *ompD* gene between *S. typhi* and *S. typhimurium*, we assessed the feasibility of constructing a derivative of *S. typhi* harbouring the *S. typhimurium ompD* gene (*S. typhi* strain Ty2 *ompD*$^+$). Previously, it has been shown that multiple sequence differences in genes conserved between these two serovars present a formidable barrier to the construction of interspecific hybrids. This is because both mismatch repair and the RecBCD recombination pathways inhibit homologous recombination events (Zahrt & Maloy, 1997; Zahrt *et al.*, 1994). Nonetheless, it is possible to introduce small regions of the *S.*

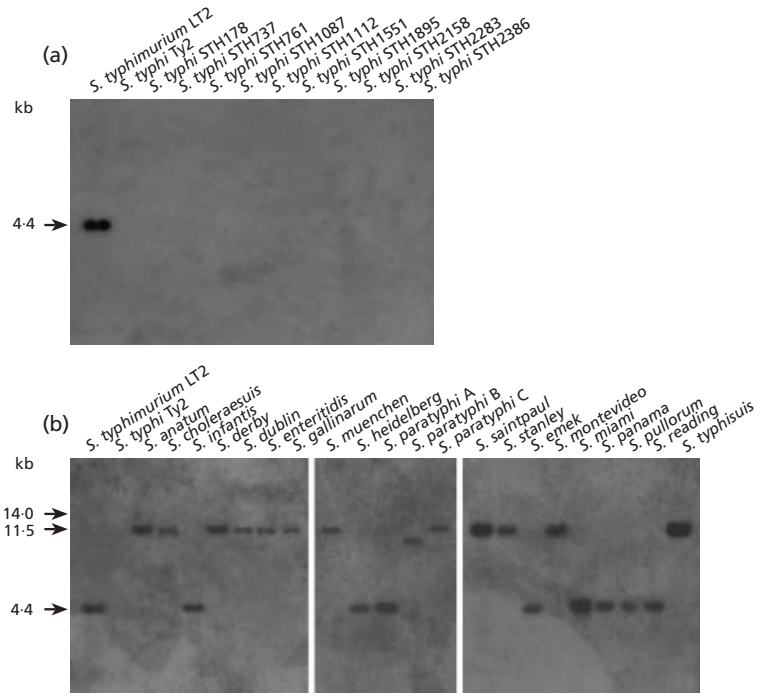

Fig. 3. Southern blot hybridization of genomic DNAs from *Salmonella* strains to an *ompD*-specific probe. DNA samples obtained from *S. typhi* clinical strains (a) or several *Salmonella* serovars (b) were digested with *Pst*I. The fragments were resolved by electrophoresis through 0·8% agarose gel and probed at high stringency with a PCR-amplified 740 bp fragment of *ompD*.

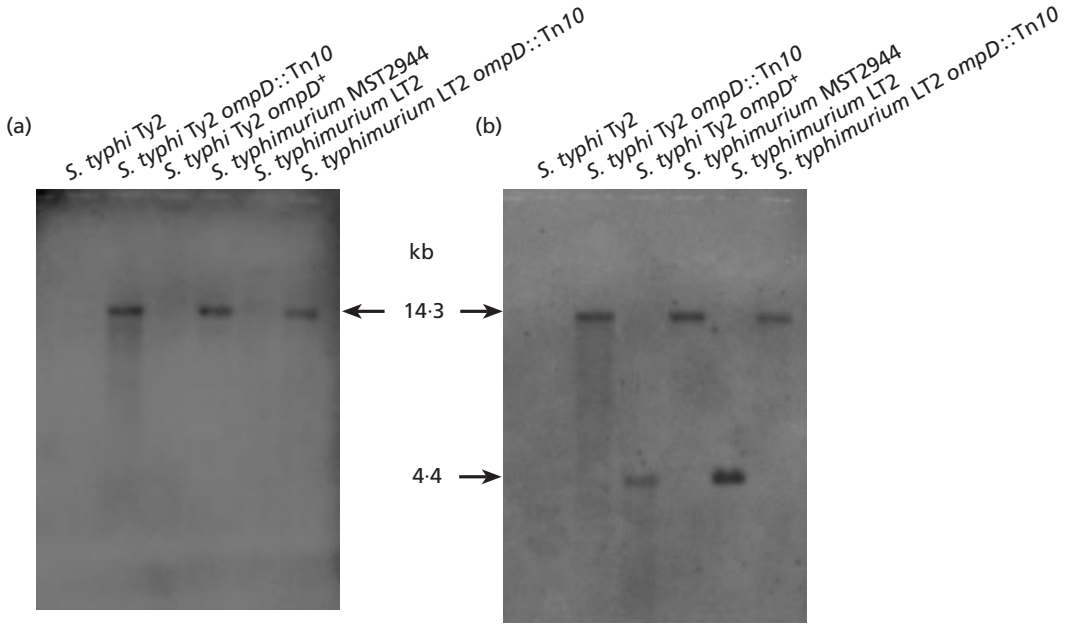

Fig. 4. Southern blot hybridization of genomic DNAs from interspecific hybrids between *S. typhimurium* and *S. typhi*. DNA samples were digested with *Pst*I, fragments were resolved by electrophoresis through 0·8% agarose gel, then probed at high stringency with a PCR-amplified 740 bp fragment of *ompD* (b) and with a DNA fragment internal to the Tn*10 tetAR* genes (a).

typhimurium genome into *S. typhi* by generalized transduction with temperate phage P22 with low but measurable efficiencies. Therefore, we designed a two-step genetic approach using P22-mediated transduction to construct the *S. typhi* strain Ty2 *ompD*$^+$.

In a first cross, we introduced an *ompD*::Tn*10* allele from donor *S. typhimurium* strain MST2944 into recipient *S. typhi* strain Ty2 by generalized transduction with phage P22 and selection for recombinants that had acquired resistance to tetracycline. PCR amplification of

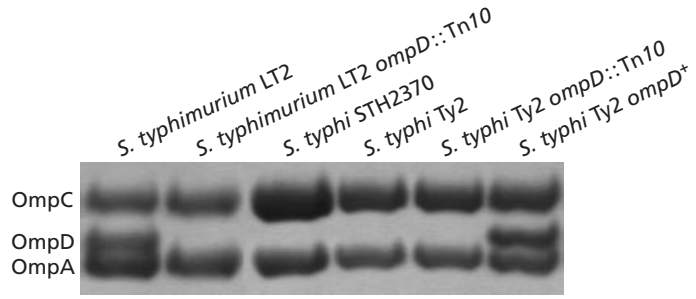

Fig. 5. SDS-PAGE analysis of porins from *S. typhimurium* and *S. typhi* interspecific hybrid strains. OMP preparations were obtained from bacteria grown aerobically at 37 °C in LB broth to stationary phase, resolved by electrophoresis through 12·5% acrylamide and stained with Coomassie blue. Under the bacterial growth conditions used, OmpF porin is not detected in the OMP profiles.

the *ompD* gene from *S. typhimurium* strain LT2 *ompD*::Tn*10* and *S. typhi* strain Ty2 *ompD*::Tn*10* DNA templates yielded the expected 740 bp product, revealing that the amplified fragment of *ompD* does not include the site of the *ompD*::Tn*10* insertion (Fig. 2a). The presence of the *ompD*::Tn*10* allele in the chromosome of these interspecific hybrids was confirmed by Southern hybridization, using a probe specific to the *tetRA* genes of Tn*10*. As shown in Fig. 4(a), the Tn*10* probe hybridizes with a band of approximately 14·3 kb in *Pst*I-restricted DNA samples obtained from *S. typhi* strain Ty2 *ompD*::Tn*10* and *S. typhimurium* strains MST2944 *ompD*::Tn*10* and LT2 *ompD*::Tn*10*. A comparison of the OMP profiles by SDS-PAGE clearly showed that *S. typhimurium* strain LT2 *ompD*::Tn*10* and *S. typhi* strain Ty2 *ompD*::Tn*10* do not express the OmpD porin (Fig. 5).

In a second cross, the *S. typhi* strain Ty2 *ompD*::Tn*10* was used as the recipient of the *ompD*$^+$ allele from *S. typhimurium* strain LT2, selecting for transductants that had lost the tetracycline resistance determinant (Maloy & Nunn, 1981). PCR amplification of genomic DNA isolated from the *S. typhi* strain Ty2 *ompD*$^+$ using the *ompD*-specific primers yielded a 740 bp product (Fig. 2a). Accordingly, a band of approximately 4·4 kb was observed when *Pst*I-digested DNA samples obtained from *S. typhi* Ty2 *ompD*$^+$ were hybridized with the *ompD* probe (Fig. 4b). A band of the same size was also observed when *S. typhimurium* DNA was probed, revealing the presence of the *ompD*$^+$ allele. Remarkably, we confirmed the presence of the OmpD porin in OMP preparations of the *S. typhi* strain Ty2 *ompD*$^+$ by SDS-PAGE (Fig. 5). These results show that the *ompD* gene can be introduced into the *S. typhi* genome, and can be expressed in this genetic background, suggesting that there are no species-specific factors required for *ompD* expression in *S. typhi*.

S. typhi is highly sensitive to methyl viologen

The observation that *S. typhi* strain Ty2, as well as the clinical strains, are missing the *ompD* gene is suggestive of a deletion in the *S. typhi* genome with respect to the *S. typhimurium* genome that may extend beyond the *ompD* gene. Analysis of the *S. typhimurium* chromosome reveals that *ompD* is adjacent to *smvA* (Hongo *et al*., 1994). It has been suggested that SmvA is an inner-membrane protein involved in the export of ammonium quaternary substrates, such as methyl viologen, by exporting them to the extracellular space (Hongo *et al*., 1994). Methyl viologen generates superoxide and oxygen radicals (Farr & Kogoma, 1991) and has been used *in vitro* to mimic the oxidative environment found within macrophages (Buchmeier *et al*., 1997).

Therefore, *S. typhi* strains, as well as other *Salmonella* serovars were tested for their sensitivity or resistance to methyl viologen following the same procedure used to identify the *S. typhimurium smvA* gene (Hongo *et al*., 1994). Among 35 *Salmonella* serovars tested, only *S. typhi* strain Ty2 was unable to grow on Luria agar plates supplemented with 0·5 mM methyl viologen. In addition, the 26 clinical isolates of *S. typhi* used in this study were also unable to grow in the presence of the same amount of methyl viologen. To determine the level of resistance to methyl viologen, some strains were tested for their ability to grow in the presence of a range of concentrations of this compound. The highest concentration that allowed the growth of *S. typhi* strain Ty2 and *S. typhimurium* strain LT2 were 0·4 mM and 50 mM, respectively. These results suggest either that *S. typhi* is missing the *smvA* gene, or that the *smvA* gene is not functional in this serovar.

To distinguish between these two alternatives, primers from the reported *smvA* sequence (GenBank accession no. D26057) were designed to amplify a portion of this gene. Amplification of a *S. typhi* strain Ty2 DNA template did not yield a product internal to *smvA*. In contrast, amplification of chromosomal DNA isolated from *S. typhimurium* strains LT2 *ompD*$^+$ and LT2 *ompD*::Tn*10*, as well as *S. typhi* strains Ty2 *ompD*::Tn*10* and Ty2 *ompD*$^+$, resulted in the expected 940 bp product (Fig. 6a). Remarkably, DNA obtained from four randomly chosen clinical isolates of *S. typhi*, which were sensitive to methyl viologen, yielded an amplification product of the expected size (Fig. 6b). Thus, although the SmvA function is absent from all tested *S. typhi* strains, the *smvA* gene appears to be present in *S. typhi* clinical strains.

In addition, the 940 bp *smvA* fragment from *S. typhimurium* was labelled and used to probe Southern blots of *S. typhi* DNA digested with *Pst*I. Since there is a single *Pst*I site within the *S. typhimurium smvA* sequence reported, and this site is present in the probe, the *smvA* probe hybridized with two fragments (approx. 4·4 and 11·8 kb) present in *S. typhimurium* strain LT2 DNA

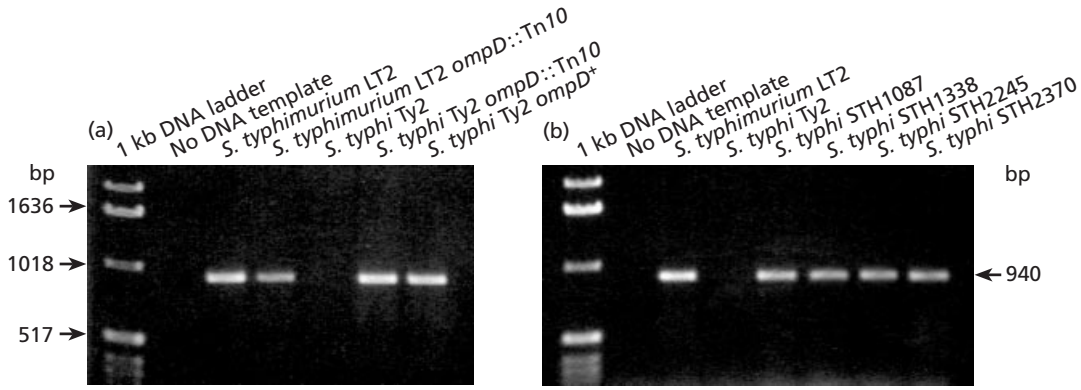

Fig. 6. Amplification of the *smvA* gene from *Salmonella* strains. PCR amplifications were performed as described in Methods, using primers internal to *smvA*. Chromosomal DNA templates were obtained from (a) interspecific hybrids between *S. typhimurium* and *S. typhi*, and (b) *S. typhi* clinical strains. RFLP analysis indicated that the *smvA* fragment amplified from *S. typhi* clinical strains does not have the *Pst*I site present in the *S. typhimurium smvA* gene (unpublished observation).

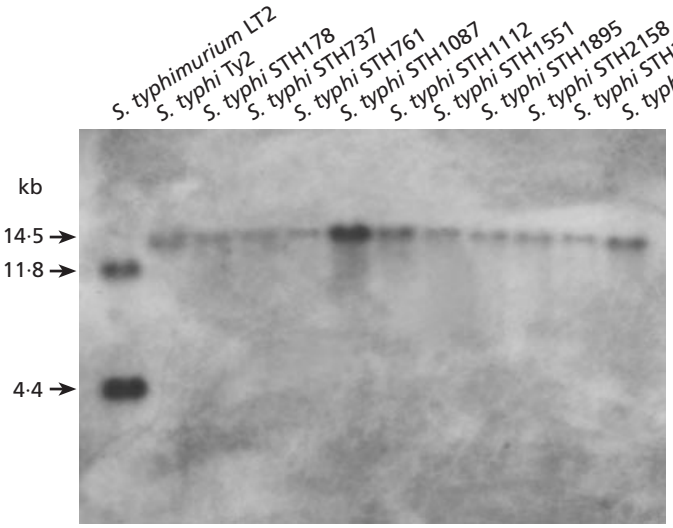

Fig. 7. Southern blot hybridization of genomic DNAs from *Salmonella* strains to a *smvA*-specific probe. DNA samples were digested with *Pst*I. The fragments were resolved by electrophoresis through 0·8 % agarose gel and probed at high stringency with a PCR-amplified 940 bp fragment of *smvA*. Only one band was observed when DNA isolated from *S. typhi* was probed because *S. typhi smvA* does not have the *Pst*I site present in the *S. typhimurium smvA* gene.

(Fig. 7). The smaller band represents a fragment containing both *ompD* and a portion of *smvA*, because it also hybridized with the *ompD* probe. On the other hand, in contrast to the PCR results suggesting that *smvA* is missing from *S. typhi* strain Ty2, the specific *smvA* probe hybridized with a single band of approximately 14·5 kb in DNA samples obtained from this strain (Fig. 7). The same hybridization band was observed when DNA isolated from 10 randomly chosen clinical isolates of *S. typhi* was probed (Fig. 7). This is because *S. typhi smvA* does not have the *Pst*I site present in the *S. typhimurium smvA* gene, as indicated by RFLP analysis (data not shown).

DISCUSSION

The OmpD porin is one of the most abundant proteins in the outer membrane of many different *Salmonella* serovars, with the exception of *S. typhi*. Our results demonstrate that, due to the absence of the *ompD* gene, *S. typhi* does not produce the OmpD porin. Both PCR and Southern analyses show that the OmpD coding sequence is absent from the *S. typhi* genome. Nonetheless, it was possible to construct a derivative of *S. typhi* carrying the *S. typhimurium ompD* gene which expressed the porin. Presumably, this interspecific hybrid carries only a small cassette of genes that includes *ompD*, and provides us with a control suggesting that there are no species-specific factors required for *ompD* expression in *S. typhi*.

To construct a recombinant strain with a single copy of *ompD* in the proper genetic position, we introduced an *ompD*::Tn*10* allele from *S. typhimurium* MST2944 donor strain into recipient *S. typhi* strain Ty2 by generalized transduction with *S. typhimurium* phage P22. This transduction was originally performed by Liu & Sanderson (1995a, b) as one of a systematic set of transductions used to assign genes on the *S. typhi* strain Ty2 genome to positions corresponding to those on the *S. typhimurium* genetic map. Using this approach, Liu

& Sanderson (1995a, b) concluded that the *ompD* gene is located in the centre of a 500 kb inversion which includes the replication terminus of the *S. typhi* strain Ty2 chromosome. The *ompD*::Tn*10* allele from *S. typhimurium* can be transduced into the *S. typhi* genome even though the recipient does not have the corresponding homeologous gene. This is because P22 packages about 44 kb of DNA, and P22-mediated generalized transduction permits homologous recombination events to occur in genes flanking a region of DNA that is not present in the recipient genome.

Our Southern blot analyses show that all *S. typhi* strains tested retain an *smvA* coding sequence, but they are nevertheless sensitive to methyl viologen, suggesting that the gene is not expressed in these strains. Alternatively, they may be lacking or be defective in additional genes required for methyl viologen resistance. In *Escherichia coli*, a related Gram-negative bacillus, at least three genes dispersed on the bacterial genome are required for methyl viologen resistance, and mutations in each of these genes confer sensitivity to methyl viologen (Morymio, 1988; Morymio *et al*., 1992). Transductants of *S. typhi* strain Ty2 carrying the $ompD^{+}$ allele and the cotransduced *smvA* gene express the OmpD porin in the outer membrane, and acquire resistance to methyl viologen (unpublished observation), suggesting that the *smvA* gene in clinical isolates of *S. typhi* may not be functional. Thus, the *ompD–smvA* region is genetically variable among different *Salmonella* serovars.

The *ompD* and *smvA* genes have been mapped at centisomes 33·7 and 38·6, respectively, in the *S. typhimurium* chromosome (Sanderson *et al*., 1995). Because both genes are cotransducible with phage P22, they cannot be that far apart. Instead, our results confirm those of Singh *et al*. (1996) that place three *S. typhimurium* genes, *ompD*, *smvA* and *narU*, at centisome 33·7 (GenBank accession no. D26057). Analysis of the *S. typhi* genome sequence data released by the Sanger Center (http://www.sanger.ac.uk/Projects/S_typhi/BLAST_server.shtml and ftp://ftp.sanger.ac.uk/pub/pathogens/st/ST.dbs) reveals that *S. typhi* has two *nar* operons, 85% and 81% identical at the nucleotide level to the *E. coli narGHJI* and *narZYWV* operons, respectively. Both *nar* operons are also present in *S. typhimurium* (Barrett & Riggs, 1982; Spector *et al*., 1999), but only one of them (*narGHIJ*) has been mapped, lying at centisome 38·6 (Casse *et al*., 1973; Sanderson *et al*., 1995). In *E. coli*, the *narU* gene is located immediately upstream of the *narZYWV* operon (Gennis & Stewart, 1996). Given the remarkable conservation of gene order between *E. coli* strain K12 and *S. typhimurium* strain LT2 (Krawiec & Riley, 1990), we can assign the *narZYWV* operon to a map position at centisome 33·7 on the *S. typhimurium* genome, adjacent to the *ompD–smvA–narU* region. Recently, the Genome Sequence Center from the Washington University School of Medicine (http://genome.wustl.edu/gsc/Projects/bacterial/salmonella.shtml) sequenced an ORF upstream of the *S. typhimurium ompD* gene. This ORF, designated *yddG*, encodes a putative transmembrane permease. Notably, *yddG* is not present in the *S. typhi* genome sequence data released by the Sanger Center, suggesting that the genetic variability in the *ompD–smvA* region of the *Salmonella* genome also includes the *yddG locus*.

Because *S. typhi* is the only *Salmonella* serovar missing the *ompD* gene, the determination of its presence in other *Salmonella* serovars has a clinical significance. This kind of assay allows us to distinguish *S. typhi* from *S. paratyphi* serovars that cause systemic human infections presenting nearly identical symptoms. Thus, a PCR assay could be developed by using primers hybridizing upstream and downstream of the presumed deletion in the *S. typhi* strains, where the length of the amplified segment should discriminate between the different *Salmonella* serovars responsible for clusters of typhoid-like fevers and thus allow a more accurate assessment of their epidemiology.

What is the role of the *ompD* gene in *Salmonella* infection? The *ompD* gene does not appear to play a major role in the virulence of *Salmonella* serovars, because the effects of mutations in the *ompD* gene on the virulence of *S. typhimurium* are subtle. Dorman *et al*. (1989) found that a strain with a Tn*10* insertion in *ompD* has a slightly greater LD_{50} than its otherwise isogenic wild-type parent. However, Meyer *et al*. (1998) found that there was no statistically significant difference between the LD_{50} of *S. typhimurium* wild-type and *ompD* mutant strains.

Although it seems likely that the *ompD* gene plays only a minor role in virulence, it may be important for determining host specificity. Many studies have characterized the genetic determinants of *Salmonella* virulence; however, very little is known about the genetic determinants of host specificity. Initial attempts to define the genetic basis of host specificity revealed that its determinants in *Salmonella* are likely to be multifactorial. In generalized transduction experiments with phage P22 grown in a *S. typhimurium* donor and with *S. typhi* recipients, no virulent recombinants that could kill mice were obtained (Zahrt, 1998). These negative results indicate that multiple genetic differences between these serovars must contribute to host specificity.

We hypothesize that the *ompD–smvA* region contributes to host specificity, because the presence of *ompD* is strongly correlated with the ability of *Salmonella* serovars to grow in alternative, non-human hosts. OmpD is present in all *Salmonella* serovars, such as *S. typhimurium* and *S. enteritidis*, that grow in multiple mammalian hosts. In contrast, OmpD is absent from *S. typhi*, which can grow only in a human host. Although the *S. paratyphi* A, B and C serovars express OmpD, and were once thought to cause systemic infections only in humans, several reports have shown that *S. paratyphi* serovars B and C are not restricted to a human host and can infect cattle and poultry (George *et al*., 1972; Thomas, 1978; Ojeniyi, 1984). The almost perfect correlation between the absence of *ompD* and a host range restricted to humans raises the important question

of whether *S. paratyphi* A also has an alternative vertebrate host.

In recent years, it has become clear that the outer-membrane porins are involved in the interactions of Gram-negative species, including *Helicobacter pylori* (Tufano *et al*., 1994), *Neisseria gonorrhoeae* (Bauer *et al*., 1999), *Shigella flexneri* (Bernardini *et al*., 1993), *S. typhi* (Blanco *et al*., 1997) and possibly *Pasteurella haemolytica* (Davies *et al*., 1997), with their specific hosts. The OmpD porin must play a critical role in the survival of *S. typhimurium* in certain environments, because OmpD facilitates the entry of some critical nutrients (Nikaido, 1996). Furthermore, OmpD may also play a critical role in the interactions between *S. typhimurium* and host cells, and is likely to be involved in the adhesion of *S. typhimurium* to murine macrophages (Negm & Pistole, 1998).

The characterization of this important genomic difference between *S. typhi* and other *Salmonella* serovars that includes *ompD* is significant for the following three reasons. First, the report of similar small differences in genome structure among different serovars will enable the development of additional PCR assays for a more robust assessment of the epidemiology of *Salmonella* outbreaks. Second, by demonstrating that generalized transduction can be used to construct interspecific hybrids between different *Salmonella* serovars, it is possible to take a genetic approach to assessment of the contributions of small genomic differences between two serovars to differences in host specificity. Third, the correlation between the presence of the *ompD* gene and the ability of *Salmonella* serovars to infect vertebrate, non-human hosts is the first reported correlation between an individual gene and the host range of *Salmonella* serovars.

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