A chromosomal region surrounding the \textit{ompD} porin gene marks a genetic difference between \textit{Salmonella typhi} and the majority of \textit{Salmonella} serovars

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

Keywords: \textit{Salmonella}, outer-membrane protein, \textit{smvA}, genetic variability, host specificity

INTRODUCTION

\textit{Salmonella typhi} causes the systemic disease typhoid fever only in its human host. The closely related \textit{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 \textit{S. typhi} and \textit{S. typhimurium} have been suggested to contribute to their respective host specificity and infectivity. The relative order of genes on the \textit{S. typhimurium} and \textit{S. typhi} chromosomes is different, as is the relative stability of their genomes. In addition, the \textit{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 (\textit{rrn}) operons. Thus, it has been postulated that genomic
rearrrangements, 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, 1995a; 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 homologous (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* clinical 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 1⁻¹; Bacto yeast extract, 5 g 1⁻¹; NaCl, 5 g 1⁻¹) and aerated by shaking. When required, LB was supplemented with ampicillin (100 mg 1⁻¹) and tetracycline (10 mg 1⁻¹). Media were solidified by the addition of agar (15 g 1⁻¹).

**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*: Tn10 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*: Tn10 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 MgCl₂, 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 MgCl₂, 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'-GAC ACA TAC CCA CCC CCG CTA TGA TTT-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 *Tn10*. 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 *PstI* (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 Na₂HPO₄ 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

<table>
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<tr>
<th>Strain*</th>
<th>Genotype/phenotype</th>
<th>Source†</th>
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<tr>
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<td>WHO</td>
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<tr>
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<td>LT2 <em>ompD</em>159::Tn10</td>
<td>This work</td>
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<td>S. Maloy</td>
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<td><em>hspS</em>29 <em>metE</em>551 <em>xyl</em>404 <em>rpsL</em>120 <em>fla</em>66 <em>metA</em>22</td>
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<td></td>
<td><em>trpC</em>2 <em>H1-b</em> <em>H2-c,n,x</em> <em>nml</em></td>
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<td>Wild-type</td>
<td>ISP</td>
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*SARB, Salmonella Reference Collection B (Selander et al., 1996).*
† S. Maloy, University of Illinois, USA; ISP, Instituto de Salud Pública (Public Health Institute), Santiago, Chile.

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

\textbf{S. typhi does not produce the OmpD porin}

Previously, we have reported that \textit{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 \textit{S. typhimurium} outer membrane (Lee & Schnaitman, 1980; Nikaido & Vaara, 1985; Nikaido, 1996). To investigate the presence of OmpD among the OMPs of a variety of \textit{Salmonella} serovars most frequently associated with systemic infection of vertebrate hosts, the OMPs of a variety of \textit{Salmonella} serovars were analysed by SDS-PAGE. Electrophoretic profiles of OMPs produced by \textit{Salmonella} enteritidis, \textit{Salmonella} paratyphi A, \textit{Salmonella} paratyphi B, \textit{Salmonella} paratyphi C, \textit{Salmonella} choleraesuis, \textit{Salmonella} gallinarum, \textit{Salmonella} pullorum, \textit{Salmonella} dublin, \textit{Salmonella} saintpaul, \textit{Salmonella} muenchen, \textit{Salmonella} heidelberg and \textit{Salmonella} montevideo showed that all of these serovars produce a major protein migrating as \textit{S. typhimurium} OmpD porin (Fig. 1a).

The same protein band was observed when this analysis was extended to include \textit{Salmonella} agona, \textit{Salmonella} anatum, \textit{Salmonella} brandenburg, \textit{Salmonella} decatur, \textit{Salmonella} derby, \textit{Salmonella} duisberg, \textit{Salmonella} emek, \textit{Salmonella} infantis, \textit{Salmonella} miami, \textit{Salmonella} newport, \textit{Salmonella} panama, \textit{Salmonella} reading, \textit{Salmonella} rubislaw, \textit{Salmonella} schwarmengrund, \textit{Salmonella} sendai, \textit{Salmonella} senftenberg, \textit{Salmonella} stanley, \textit{Salmonella} thompson, \textit{Salmonella} typhus and \textit{Salmonella} wen (data not shown). This protein band disappeared from the SDS-PAGE OMP profiles when the ompD::Tn10 allele was transduced from \textit{S. typhimurium} strain MST2944 to \textit{S. enteritidis}, \textit{S. paratyphi} A, \textit{S. paratyphi} B, \textit{S. pullorum} and \textit{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 \textit{Salmonella} serovars included in this study. On the other hand, the analysis of OMPs from \textit{S. typhi} strain Ty2 (Fig. 1a) and 26 independent clinical isolates of \textit{S. typhi} (data not shown) indicated that OmpD is not produced by these strains.

The finding that \textit{S. typhi} strains do not produce OmpD can be explained by one of two alternative hypotheses. \textit{S. typhi} may have an ompD gene that is not expressed, or there may be no homologous ompD gene in \textit{S. typhi}. Because the current sequence data produced by the \textit{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 \textit{ompD} gene is absent from the \textit{S. typhi} genome.

\textbf{PCR and DNA hybridization analyses indicate that the \textit{ompD} gene is absent from the \textit{S. typhi} genome}

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

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{SDS-PAGE analysis of porins from several \textit{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.}
\end{figure}
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 template (see Fig. 2b for example). In contrast, a product from S. typhi was evaluated directly by DNA hybridization. Therefore, the 740 bp obtained from S. typhi strain Ty2 (Fig. 3a). This small band indicates a DNA fragment containing the ompD gene, the presence of the ompD gene from several 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. baija, 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. typhimurium ompD gene into S. typhi using P1 mediated transduction. The S. typhimurium ompD gene was transduced to S. typhi strain Ty2, and the transductants were selected on LB agar plates containing 100 μg/mL chloramphenicol. The transductants were then screened for the presence of the S. typhimurium ompD gene by Southern blot hybridization using the S. typhimurium ompD probe. The results showed that the transductants had the expected band of approximately 740 bp, confirming the successful transduction of the S. typhimurium ompD gene into S. typhi.
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::Tn10 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. 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 PstI. 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 PstI, 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 Tn10 tetAR genes (a).
the ompD gene from *S. typhimurium* strain LT2 ompD::Tn10 and *S. typhi* strain Ty2 ompD::Tn10. DNA templates yielded the expected 740 bp product, revealing that the amplified fragment of ompD does not include the site of the ompD::Tn10 insertion (Fig. 2a). The presence of the ompD::Tn10 allele in the chromosome of these interspecific hybrids was confirmed by Southern hybridization, using a probe specific to the tetRA genes of Tn10. As shown in Fig. 4(a), the Tn10 probe hybridizes with a band of approximately 14.3 kb in *Pst*I-restricted DNA obtained from *S. typhi* strain Ty2 ompD::Tn10 and *S. typhimurium* strains MST2944 ompD::Tn10 and LT2 ompD::Tn10. A comparison of the OMP profiles by SDS-PAGE clearly showed that *S. typhimurium* strain LT2 ompD::Tn10 and *S. typhi* strain Ty2 ompD::Tn10 do not express the OmpD porin (Fig. 5).

In a second cross, the *S. typhi* strain Ty2 ompD::Tn10 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::Tn10, as well as *S. typhi* strains Ty2 ompD::Tn10 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.
demonstrate that, due to the absence of the serovars, with the exception of S. typhi, in the outer membrane of many different Salmonella strains. The OmpD porin is one of the most abundant proteins in the outer membrane of Salmonella typhimurium LT2. The smaller band represents a fragment carrying 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 PstI 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: Tn10 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) showed that the OmpD porin was one of the most abundant proteins in the outer membrane of many different Salmonella serovars, with the exception of S. typhi.
& 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+: Tn10 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 (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 (narGHJI) 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 Tn10 insertion in ompD has a slightly greater LD<sub>50</sub> than its otherwise isogenic wild-type parent. However, Meyer et al. (1998) found that there was no statistically significant difference between the LD<sub>50</sub> 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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