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

Carlos A. Santiviago, Cecilia S. Toro,[†] Sergio A. Bucarey and Guido C. Mora

Author for correspondence: Guido C. Mora. Tel: +56 2 6862849. Fax: +56 2 2225515.
e-mail: gmora@genes.bio.puc.cl

Laboratorio de Microbiología, Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Alameda no. 340, Santiago, Chile

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 (*rnn*) operons. Thus, it has been postulated that genomic

[†] **Present address:** Programa de Microbiología, Instituto de Ciencias Biomédicas, Universidad de Chile, Independencia no. 127, Santiago, Chile.

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*::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'-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 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 *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 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

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

* 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

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::Tn10* 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.*

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. dusseldorf*, *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 Tn10 *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*::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. 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*::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 samples 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

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::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) 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 (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 (*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 LD₅₀ than its otherwise isogenic wild-type parent. However, Meyer *et al.* (1998) found that there was no statistically significant difference between the LD₅₀ 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.

ACKNOWLEDGEMENTS

We are indebted to Philip Youderian and Alexis Kalergis for encouragement and critical reading of this manuscript. We thank Stanley Maloy and Ana María Cordano for generous gifts of bacterial strains. This work was supported by grants 1960255 and 1990153 from FONDECYT. C.A.S. was supported by a fellowship from CONICYT.

REFERENCES

- Anderson, R. P. & Roth, J. R. (1981). Spontaneous tandem genetic duplications in *Salmonella typhimurium* arise by unequal recombination between ribosomal RNA (*rnn*) cistrons. *Proc Natl Acad Sci USA* **78**, 3113–3117.
- Barrett, E. L. & Riggs, D. L. (1982). Evidence for a second nitrate reductase activity that is distinct from the respiratory enzyme in *Salmonella typhimurium*. *J Bacteriol* **150**, 563–571.
- Bauer, F. J., Rudel, T., Stein, M. & Meyer, T. F. (1999). Mutagenesis of the *Neisseria gonorrhoeae* porin reduces invasion in epithelial cells and enhances phagocyte responsiveness. *Mol Microbiol* **31**, 903–913.
- Bäumler, A. J., Tsois, R. M., Ficht, T. A. & Adams, L. G. (1998). Evolution of host adaptation in *Salmonella enterica*. *Infect Immun* **66**, 4579–4587.
- Bernardini, M. L., Sanna, M. G., Fontaine, A. & Sansonetti, P. J. (1993). OmpC is involved in invasion of epithelial cells by *Shigella flexneri*. *Infect Immun* **61**, 3625–3635.
- Blanco, L. P., Toro, C. S., Romero, J. M., Santiviago, C. A. & Mora, G. C. (1997). *Salmonella typhi* Ty2 OmpC porin induces bactericidal activity on U937 monocytes. *Microbiol Immunol* **40**, 999–1004.
- Buchmeier, N., Bossie, S., Chen, C. Y., Fang, F. C., Guiney, D. G. & Libby, S. J. (1997). Sly, a transcriptional regulator of *Salmonella typhimurium*, is required for resistance to oxidative stress and is expressed in the intracellular environment of macrophages. *Infect Immun* **65**, 3725–3730.
- Calderón, I., Lobos, S. R. & Mora, G. C. (1984). The hemolytic effect of *Salmonella typhi* Ty2 porins. *Eur J Biochem* **141**, 579–583.
- Casse, F., Chippaux, M. & Pascal, M. C. (1973). Isolation from *Salmonella typhimurium* LT2 of mutants lacking specifically nitrate reductase activity and mapping of the *chlc* gene. *Mol Gen Genet* **124**, 247–251.
- Davies, R. L., Arkinsaw, S. & Selander, R. K. (1997). Evolutionary genetics of *Pasteurella haemolytica* isolates recovered from cattle and sheep. *Infect Immun* **65**, 3585–3593.
- Dorman, C. J., Chatfield, S., Higgins, C. F., Hayward, C. & Dougan, G. (1989). Characterization of porin and *ompR* mutants of a virulent strain of *Salmonella typhimurium*: *ompR* mutants are attenuated *in vivo*. *Infect Immun* **57**, 2136–2140.
- Echeita, M. A. & Usera, M. A. (1998). Chromosomal rearrangements in *Salmonella enterica* serotype Typhi affecting molecular typing in outbreak investigations. *J Clin Microbiol* **36**, 2123–2126.
- Farr, S. B. & Kogoma, T. (1991). Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. *Microbiol Rev* **55**, 561–585.
- Gennis, R. B. & Stewart, V. (1996). Respiration. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, vol. 1, pp. 217–261. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- George, J. T., Wallace, J. G., Morrison, H. R. & Harbourne, J. F. (1972). Paratyphoid in man and cattle. *Br Med J* **3**, 208–211.
- Hongo, E., Morimyo, M., Mita, K., Machida, I., Hama-Inaba, H., Tsuji, H., Ichimura, S. & Noda, Y. (1994). The methyl viologen-resistance-encoding gene *smvA* of *Salmonella typhimurium*. *Gene* **148**, 173–174.
- Kleckner, N., Bender, J. & Gottesman, S. (1991). Uses of transposons with emphasis on Tn10. *Methods Enzymol* **204**, 139–180.
- Krawiec, S. & Riley, M. (1990). Organization of the bacterial chromosome. *Microbiol Rev* **54**, 502–539.
- Lan, R. T. & Reeves, P. R. (1996). Gene transfer is a major factor in bacterial evolution. *Mol Biol Evol* **13**, 47–55.
- Lee, D. & Schnaitman, C. (1980). Comparison of outer membrane proteins produced by *Escherichia coli* and *Salmonella typhimurium*. *J Bacteriol* **142**, 1019–1022.
- Liu, S.-L. & Sanderson, K. E. (1995a). Genomic cleavage map of *Salmonella typhi* Ty2. *J Bacteriol* **177**, 5099–5107.
- Liu, S.-L. & Sanderson, K. E. (1995b). Rearrangements in the genome of the bacterium *Salmonella typhi*. *Proc Natl Acad Sci USA* **92**, 1018–1022.
- Liu, S.-L. & Sanderson, K. E. (1996). Highly plastic chromosomal organization in *Salmonella typhi*. *Proc Natl Acad Sci USA* **93**, 10303–10308.
- Lobos, S. R. & Mora, G. C. (1991). Alterations in the electrophoretic mobility of OmpC due to variations in the am-

- monium persulfate concentration in sodium dodecylsulfate-polyacrylamide gel electrophoresis. *Electrophoresis* **12**, 448–450.
- McClelland, M. & Wilson, R. K. (1998).** Comparison of sample sequences of the *Salmonella typhi* genome to the sequence of the complete *Escherichia coli* K-12 genome. *Infect Immun* **66**, 4305–4312.
- Maloy, S. R. (1990).** *Experimental Techniques in Bacterial Genetics*. Boston, MA: Jones & Bartlett.
- Maloy, S. R. & Nunn, W. (1981).** Selection for loss of tetracycline resistance by *Escherichia coli*. *J Bacteriol* **145**, 1110–1112.
- Meyer, P. N., Wilmes-Riesenberg, M. R., Stathopoulos, C. & Curtiss, R. (1998).** Virulence of a *Salmonella typhimurium* OmpD mutant. *Infect Immun* **66**, 387–390.
- Morymio, M. (1988).** Isolation and characterization of methyl viologen-sensitive mutants of *Escherichia coli* K-12. *J Bacteriol* **170**, 2136–2142.
- Morymio, M., Hongo, E., Hama-Inaba, H. & Machida, I. (1992).** Cloning and characterization of the *mvrC* gene of *Escherichia coli* K-12 which confers resistance against methyl viologen toxicity. *Nucleic Acids Res* **20**, 3159–3165.
- Negm, R. S. & Pistole, T. G. (1998).** Macrophages recognize and adhere to an OmpD-like protein of *Salmonella typhimurium*. *FEMS Immunol Med Microbiol* **20**, 191–199.
- Ng, I., Liu, S.-L. & Sanderson, K. E. (1999).** Role of genomic rearrangement in producing new ribotypes of *Salmonella typhi*. *J Bacteriol* **181**, 3536–3541.
- Nikaido, H. (1996).** Outer membrane. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, vol. 1, pp. 29–47. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- Nikaido, H. & Vaara, M. (1985).** Molecular basis of bacterial outer membrane permeability. *Microbiol Rev* **49**, 1–32.
- Ojeniyi, A. (1984).** ‘*Salmonella hirschfeldii*’ in poultry and man in Ibadan, Nigeria. *Bull World Health Organ* **62**, 773–775.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanderson, K. E., Hessel, A. & Rudd, K. (1995).** Genetic map of *Salmonella typhimurium*, Edition VIII. *Microbiol Rev* **59**, 241–303.
- Schnaitman, C. A. (1971).** Solubilization of the cytoplasmic membrane of *Escherichia coli* by Triton X-100. *J Bacteriol* **108**, 545–552.
- Selander, R., Li, J. & Nelson, K. (1996).** Evolutionary genetics of *Salmonella enterica*. In *Escherichia coli and Salmonella: Cellular and Molecular biology*, 2nd edn, vol. 2, pp. 2691–2707. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- Singh, S. P., Miller, S., Williams, Y. U., Rudd, K. E. & Nikaido, H. (1996).** Immunochemical structure of the OmpD porin for *Salmonella typhimurium*. *Microbiology* **142**, 3201–3210.
- Spector, M. P., Garcia del Portillo, F., Bearson, S. M. D., Mahmud, A., Magut, M., Finlay, B. B., Dougan, G., Foster, J. W. & Pallen, M. J. (1999).** The *rpoS*-dependent starvation-stress response locus *stiA* encodes a nitrate reductase (*narZYWV*) required for carbon-starvation-inducible thermotolerance and acid tolerance in *Salmonella typhimurium*. *Microbiology* **145**, 3035–3045.
- Thomas, G. W. (1978).** *Salmonella paratyphi* B in cattle. *Vet Rec* **103**, 512.
- Tufano, M. A., Rossano, F., Catalanotti, P., Liguori, G., Capasso, C., Ceccarelli, M. T. & Marinelli, P. (1994).** Immunobiological activities of *Helicobacter pylori* porins. *Infect Immun* **62**, 1392–1399.
- Zahrt, T. C. (1998).** *A genetic analysis of the Salmonella typhi host range*. PhD thesis, University of Illinois.
- Zahrt, T. C. & Maloy, S. (1997).** Barriers to recombination between closely related bacteria: MutS and RecBCD inhibit recombination between *Salmonella typhimurium* and *Salmonella typhi*. *Proc Natl Acad Sci USA* **94**, 9786–9791.
- Zahrt, T. C., Mora, G. C. & Maloy, S. (1994).** Inactivation of mismatch repair overcomes the barrier to transduction between *Salmonella typhimurium* and *Salmonella typhi*. *J Bacteriol* **176**, 1527–1529.

Received 27 February 2001; accepted 26 March 2001.