Immunochemical structure of the OmpD porin from *Salmonella typhimurium*

Shiva P. Singh,¹ Stephanie Miller,¹ Yvonne U. Williams,¹
Kenneth E. Rudd² and Hiroshi Nikaido³

Author for correspondence: Shiva P. Singh. Tel: +1 334 229 4301. Fax: +1 334 229 4288.
e-mail: SPSINGH@ASU.ALASU.EDU

¹ Biomedical Research and Training Programs, Alabama State University, Montgomery, AL 36101, USA

² National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD 20894, USA

³ Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA

The OmpD porin was isolated and purified from *Salmonella typhimurium* strain SH 7454 (*ompC*::Tn10), digested with cyanogen bromide (CNBr) and the peptide fragments were separated by SDS-PAGE. N-terminal sequencing identified a total of 96 residues from four distinct peptides. The sequence showed that OmpD is homologous to NmpC (75% identity), Lc (75%) and OmpC (70%) from *Escherichia coli*, and OmpC (68%) from *S. typhimurium*. The sequence was essentially identical to the translated sequence of an *nmpC*-like gene of *S. typhimurium*, currently placed at 38.6 centisomes of the chromosome. Our results and other data suggest, however, that this gene is actually the *ompD* gene, which is more correctly placed in the 34 centisome region of the chromosome. The CNBr-generated peptides were also screened with 16 anti-*S. typhimurium* OmpD monoclonal antibodies by Western blotting. These results, in conjunction with the prediction of the OmpD folding pattern based on the known three-dimensional structure of *E. coli* OmpF, showed a close immunological relationship among *S. typhimurium* OmpD and *E. coli* NmpC and Lc, and a strong conservation of sequences within the transmembrane β strands of these porins and *E. coli* OmpC, PhoE and OmpF, and *Salmonella typhi* OmpC.

Keywords: *Salmonella typhimurium*, OmpD, porins, amino acid sequence, monoclonal antibodies

INTRODUCTION

Porins are a family of pore-forming proteins commonly found in the outer membrane (OM) of Gram-negative bacteria (Lugtenberg & van Alphen, 1983; Nikaido & Vaara, 1985). They exist as homo- or heterotrimeric (Gehring & Nikaido, 1989) *in vivo* and function as water-filled channels allowing passive diffusion of nutrients across the OM (Nikaido & Vaara, 1985). Two general porins, OmpC and OmpF, are produced by *Escherichia coli* and *Salmonella typhimurium*, and a third porin, PhoE, with a preference for negatively charged solutes such as phosphate, is produced by both organisms when cells are cultured under special conditions (Lugtenberg & van Alphen, 1983; Nikaido & Vaara, 1985). The three-dimensional structures of OmpF and PhoE from *E. coli*

(Cowan *et al.*, 1992) and two porins from *Rhodobacter* species (Kreusch & Schulz, 1994; Weiss *et al.*, 1991) have been determined by X-ray crystallography. These proteins form hollow cylinders that consist of anti-parallel β strands; long hydrophilic loops of irregular length and short β -hairpin turns connect these strands on the external and periplasmic surfaces of the OM bilayer, respectively (Cowan & Rosenbusch, 1994; Cowan *et al.*, 1992; Jap *et al.*, 1991; Jeanteur *et al.*, 1994; Klebba *et al.*, 1990; Nikaido, 1992; Struyve *et al.*, 1993; Weiss *et al.*, 1991). The primary structure of porins varies significantly among Gram-negative bacteria (Gerbl-Rieger *et al.*, 1991), but amphiphilic β strands (7–14 residues each) in the porin barrel are structurally conserved (Gerbl-Rieger *et al.*, 1991; Jeanteur *et al.*, 1994). Generally, porins contain five or more surface epitopes, 6–25 residues in length (Klebba *et al.*, 1990; Puente *et al.*, 1989; Singh *et al.*, 1992, 1995, 1996; Tommassen *et al.*, 1993; van der Ley *et al.*, 1986b), which are partially obscured by the lipopolysaccharide (LPS) core and completely blocked by O-antigen sugars (Bentley & Klebba, 1988; Bowden *et al.*, 1995; van der

Abbreviation: OM, outer membrane.

The PIR accession number for the OmpD sequences reported in this paper is A57983.

Ley *et al.*, 1986a). These proteins also contain conserved buried epitopes that are localized on the transmembranous β strands (Jeanteur *et al.*, 1994; Klebba *et al.*, 1990; Puente *et al.*, 1989; Singh *et al.*, 1992, 1995, 1996; Tommassen *et al.*, 1993).

OmpD, a fourth general porin, is of special interest because it is regularly found in *S. typhimurium* but is absent from *E. coli* (Lee & Schnaitman, 1980; Lugtenberg & van Alphen, 1983; Nikaido & Vaara, 1985). This protein is regulated in a manner similar to *E. coli* porins Lc, encoded by bacteriophage PA-2, and NmpC, which also appears to be part of the integrated defective phage genome (Blasband *et al.*, 1986; Highton *et al.*, 1985). All three proteins are subject to catabolite repression (Lee & Schnaitman, 1980; Pugsley & Schnaitman, 1978) and their biosynthesis, unlike that of OmpC and OmpF, is not affected by the osmolarity of the growth medium (Pugsley & Schnaitman, 1978; Singh *et al.*, 1987). Recently, Hongo *et al.* (1994) sequenced a 849 bp segment of DNA located near the methyl-viologen-resistance-encoding gene on the *S. typhimurium* chromosome. The N-terminally truncated protein encoded by this segment was termed 'NmpC' of *S. typhimurium* because it is 83% identical to *E. coli* NmpC.

We have previously reported, based on the Western immunoblot reactivity of anti-OmpD mAbs, that *E. coli* NmpC, *S. typhimurium* OmpD and the Lc porins form a subfamily that is part of a larger family that includes OmpF, OmpC and PhoE (Singh *et al.*, 1992). The porins in this family show roughly 65% identity and 80% similarity (Blasband *et al.*, 1986; Mizuno *et al.*, 1983; Puente *et al.*, 1989). The amino acid sequences of NmpC and Lc from *E. coli* (Blasband *et al.*, 1986) and the NmpC-like protein from *S. typhimurium* (Hongo *et al.*, 1994) are known; however, the three-dimensional structure of none of these proteins is yet resolved, and the *S. typhimurium* OmpD has not been sequenced either. In this study, we identified 96 residues of *S. typhimurium* OmpD by N-terminal analysis of cyanogen bromide (CNBr)-generated peptides which show that OmpD is identical to the NmpC-like protein (Hongo *et al.*, 1994); the OmpD sequence also showed 68–75% identity with NmpC and Lc from *E. coli*, and OmpC from both *E. coli* and *S. typhimurium*. We also used a panel of 16 anti-OmpD mAbs to define several epitopes of *S. typhimurium* OmpD. The CNBr-cleaved peptides containing internal Met residues and antiporin mAbs, as employed in this study, have been used previously to study the immunochemical structure of other bacterial porins (Haase *et al.*, 1994; Klebba *et al.*, 1990; Murphy & Bartos, 1988; Mutharia & Hancock, 1985; Rawling *et al.*, 1995; Singh *et al.*, 1995). A folding pattern of OmpD is proposed; it predicts the same exposed loops, periplasmic turns and membrane-spanning β strands of this protein as seen in the known three-dimensional structure of *E. coli* OmpF (Cowan *et al.*, 1992).

METHODS

Bacterial strain and growth conditions. The culture media and growth conditions used in this study have been described previously (Singh *et al.*, 1992). *S. typhimurium* strains SH 7454,

SH 7455 and SH 7457 (*galE* rough mutants producing R_c chemotype LPS; provided by P. H. Mäkelä, National Public Health Institute, Helsinki, Finland) are *ompC::Tn10*, *ompD::Tn10 ompC::Tn10* and *ompD::Tn10* derivatives of SH 6749 (Sukupolvi *et al.*, 1984) and express the OmpD, OmpF and OmpC proteins, respectively, as their sole porin when grown under high osmolarity conditions (Singh *et al.*, 1987, 1992). *E. coli* strains JF 1054 (provided by J. Foulds, National Institute of Allergy and Infectious Diseases, MA, USA), PC 2086 (provided by J. Tommassen, State University of Utrecht, The Netherlands) and JF 703, JF 701 and JF 694 (provided by K. Gehring, University of California, Berkeley, USA) express NmpC, Lc, OmpC, OmpF and PhoE, respectively, as their sole porins (Gehring & Nikaido, 1989; Verhoef *et al.*, 1987).

Isolation and purification of OmpD. The native porin (trimer) was isolated and purified by solubilization of bacterial cell envelopes in 1% (w/v) SDS/0.5 M NaCl followed by size exclusion chromatography with Sephacryl S-200 (Gehring & Nikaido, 1989). Denatured monomeric porin was prepared by boiling the trimer at 100 °C for 5 min in 1% SDS (Klebba *et al.*, 1990).

The OM was isolated by suspending the cell pellet in 10 mM HEPES buffer, pH 7.4 and passing the suspension twice at 16000 p.s.i. (110400 kPa) through a French pressure cell (American Instrument). OM fragments were then purified by sucrose density gradient centrifugation (Smit *et al.*, 1975). LPS (R type) was isolated from *S. typhimurium* SH 7454 by the phenol/water extraction procedure described by Galanos *et al.* (1969).

Production of anti-OmpD mAbs. BALB/c mice were immunized with OmpD monomer and cell fusion was carried out as described previously (Singh *et al.*, 1992). Hybridomas were selected in hypoxanthine-aminopterin-thymidine medium (Kearney, 1984). Culture fluids from wells with colonies were assayed by ELISA against porin monomers, trimers, OM, LPS and whole cells. Hybridomas of interest were cloned by limiting dilution and injected into BALB/c mice for production of ascitic tumours (Kearney, 1984). The class and subclass of mAbs were determined by ELISA with goat antisera against mouse heavy and light chains μ , γ_1 , γ_2a , γ_2b , γ_3 , κ and λ (Fisher).

ELISA. ELISA was performed as described previously (Singh *et al.*, 1992).

CNBr digestion of OmpD. Protein concentrations were measured with the Micro BCA protein assay reagent (Pierce), with BSA as standard. Five milligrams of OmpD monomer was precipitated, washed with acetone and digested with 250 mg CNBr in 500 μ l 70% (v/v) trifluoroacetic acid (Garten *et al.*, 1975) by overnight incubation at room temperature. The digested sample was lyophilized twice, and then suspended in SDS-sample buffer (1 mg ml⁻¹) and stored at -80 °C for later use in gel electrophoresis.

Gel electrophoresis. SDS-PAGE was performed on 10–20% (w/v) polyacrylamide linear gradient gels as described previously (Singh *et al.*, 1992). The gel was stained with ammoniacal silver stain and the molecular masses of OmpD fragments were determined using a standard curve. For Western blots, proteins were electrophoretically transferred to nitrocellulose paper and immunoblots were performed as described previously (Pai *et al.*, 1992; Singh *et al.*, 1992).

Amino acid composition and N-terminal sequencing. Purified OmpD or its CNBr-generated peptides were separated by SDS-PAGE and transferred to Immobilon-P PVDF membranes (Millipore). The transfer was carried out in 10 mM CAPS according to the procedure of Matsudaira (1987). Protein bands

Table 1. Epitope specificity of anti-*S. typhimurium* OmpD mAbs

mAbs were raised to the purified OmpD monomer (DM). They were also characterized by ELISA as described previously (Singh *et al.*, 1992); all bound purified OmpD monomer but none reacted with OmpD trimer, OM, LPS or whole cells (data not shown).

mAb	Isotype	mAb reactivity in Western blots*				
		<i>E. coli</i> NmpC	<i>E. coli</i> Lc	<i>E. coli</i> OmpC	<i>S. typhi</i> OmpC	<i>E. coli</i> PhoE
DM51.1	IgG ₁	++	++	+	+	++
DM62.1	IgG ₁	++	++	++	+	++
DM9.2†	IgG ₂ b	++	++	++	++	++
DM20.2†	IgG ₂ b	+	++	++	++	+
DM22.2†	IgG ₁	++	++	++	++	++
DM49.2	IgG ₂ b	++	++	+	+	+
DM57.2	IgG ₂ b	+	+	++	++	+
DM2.1	IgG ₂ a	++	++	++	+	++
DM12.2	IgG ₂ b	++	++	++	+	+
DM11.2	IgG ₂ b	++	++	++	++	+
DM60.3	IgG ₂ b	++	++	+	+	+
DM26.1†	IgG ₂ b	++	++	—	+	+
DM46.2†	IgM	+	+	—	++	++
DM5.1	IgG ₁	++	++	—	—	—
DM19.1†	IgG ₂ a	++	++	—	—	—
DM24.4†	IgG1	++	++	—	—	—

* Cell envelopes from bacterial strains selectively expressing *E. coli* porins NmpC (JF 1054), Lc (PC 2086), OmpC (JF 703), OmpF (JF 701) and PhoE (JF 694), and *S. typhi* OmpC (Singh *et al.*, 1992) were separated by SDS-PAGE and the immunoblots were performed as described previously (Singh *et al.*, 1992). Immunoblot reactions of anti-porin mAbs were evaluated in comparison with the reactions of normal mouse serum and ascites from the cell fusion partner P3x63-Ag8.653, which is a nonsecretor of immunoglobulins (Kearney, 1984). Reactions were scored as negative (—), weakly positive (+) or strongly positive (++) in comparison with the intensity of reaction with the immunogen porin and negative controls. Immunoblot reactions of the mAbs with *E. coli* OmpF (data not shown) were generally similar to those indicated for *E. coli* OmpC.

† DM 9.2, 20.2, 22.2, 26.1, 46.2, 19.1 and 24.4 were designated mAbs 22, 18, 24, 29, 35, 27 and 25, respectively, in our previous report (Singh *et al.*, 1992).

on the PVDF membranes were localized by staining with 0.1% amido black. The sections of the membrane containing the desired bands were excised, hydrolysed with 6 M HCl and analysed in a Beckman model 6300 amino acid analyser. For N-terminal sequencing, CNBr-generated peptides on PVDF membranes were applied to a Beckman model LF3000G sequencer. The amino acid sequences of *S. typhimurium* OmpD were compared with those of NmpC (Blasband *et al.*, 1986), Lc (Blasband *et al.*, 1986) and OmpC (Mizuno *et al.*, 1983) from *E. coli*, and OmpC (Puentes *et al.*, 1989; Singh *et al.*, 1995) and the NmpC-like protein (Hongo *et al.*, 1994) from *S. typhimurium*, with the Best Fit program of the University of Wisconsin Genetics Computer Group, Madison, WI, USA.

PCR. A PCR amplification was performed using two primers that are homologous to nt 36–54 (5'-GACAAAGACAAA-ACCGTT-3') and 775–757 (5'-CGTCCAGCAGGTTGAT-TTT-3') on the *S. typhimurium* nmpC-like gene (GenBank accession number D26057; Hongo *et al.*, 1994). These primers were used to amplify genomic DNA from *S. typhimurium* strains SH 7454 (OmpD⁺), SH 7455 and SH 7457. The latter two strains contain a mutation made by a Tn10 insertion (*ompD159::Tn10*

Tc^S) and were subsequently selected for a tetracycline sensitive derivative that does not express OmpD. The reaction mixture (50 µl) contained 200 pmol of each primer, 200 µM of each deoxynucleoside triphosphate, 1.25 units *Taq* DNA polymerase and approximately 200 pmol of genomic DNA as template. Each amplification cycle included three reaction steps: 1 min at 94 °C for denaturation, 1 min at 55 °C for annealing and 2 min at 72 °C for extension. All together, 40 cycles were carried out by using a Programmable Thermocycler-100 (MJ Research). The products of amplification were visualized on an ethidium-bromide-stained agarose gel.

RESULTS AND DISCUSSION

mAb specificities

Nine previously unreported mAbs (DM 51.1, 62.1, 49.2, 57.2, 2.1, 12.2, 11.2, 60.3 and 5.1) were raised to *S. typhimurium* OmpD (Table 1). The test panel also included seven previously reported anti-OmpD mAbs (DM 9.2, 20.2, 22.2, 26.1, 46.2, 19.1 and 24.4; Table 1), all of which

Fig. 1. For legend see facing page.

recognized buried epitopes on the OmpD porin (Singh *et al.*, 1992, 1996). Specificities of the new mAbs were determined by their reactivity with purified porins, LPS, OM and intact whole cells in ELISA and their immunoblot reactivity with either denatured whole cell or cell envelope lysates. All the new mAbs bound purified OmpD monomer but did not react with porin trimer, OM, LPS or intact whole cells (data not shown); their epitope(s) are buried, either in the membrane bilayer or in the tertiary structure of OmpD.

Identity of CNBr-generated fragments

Since CNBr cleaves polypeptides at the C-terminal end of Met residues (Garten *et al.*, 1975), complete digestion of *E. coli* NmpC (and possibly *S. typhimurium* OmpD; see below) with CNBr would produce four peptides: aa 1–124, 125–241, 242–306 and 307–342 with calculated molecular masses of 13905, 12779, 7401 and 4010 Da, respectively. Likewise, *E. coli* Lc would produce four peptides that would be identical to NmpC in terms of their amino acid positions with only minor differences in calculated molecular masses. However, we detected at least five bands (fragments) with estimated molecular masses of 31.0, 24.5, 15.0, 13.2 and 10.5 kDa (f1–f5, respectively) in CNBr digests of *S. typhimurium* OmpD (data not shown). These fragments were identified from their N-terminal sequence(s) (see below), relative electrophoretic mobilities and/or by the process of elimination. Each fragment contained one to three distinct N-terminal sequences (in order of relative abundance): f1, aa 1–241 and 307–342; f2, 125–342; f3, 1–124, 242–342 and 125–241; f4, 125–241 and 1–124; and f5, 242–342 and 1–124 (Fig. 1). Thus, the main peptides in f1–f5 were considered to be aa 1–241, 125–342, 1–124, 125–241 and 242–342, respectively, based on the relative amounts of various peptides (as obtained by sequence analysis) in each band. The observed apparent molecular masses of OmpD fragments (see above) were generally comparable to the expected molecular masses of 26.7 (f1), 24.2 (f2), 13.9 (f3), 12.8 (f4) and 11.4 (f5) kDa as deduced from the NmpC sequence of *E. coli* (Blasband *et al.*, 1986). The association of a small peptide (307–342) with a much larger peptide (1–241) in fragment f1 was unexpected; however, such anomalous electrophoretic behaviour has been reported for other OM protein fragments (De Mot *et al.*, 1994). The fact that bands f3, f4 and f5 have fragments in common might be explained by insufficient separation/excision of these bands (10.5–15.0 kDa) prior to sequence analysis. The partial cleavage of proteins by CNBr resulting in two or more overlapping peptides, as observed in this study, has also been reported by several other investigators

(Garten *et al.*, 1975; Klebba *et al.*, 1990; Mutharia & Hancock, 1985; Rawling *et al.*, 1995; Singh *et al.*, 1995); it occurs due to the incomplete cleavage of Met–Ser or Met–Thr sequences which can form homoserine and are therefore not cleaved (Garten *et al.*, 1975; Mutharia & Hancock, 1985).

Similarity of N-terminal sequences with those of other enteric bacterial porins

Electrophoretically separated peptides of *S. typhimurium* OmpD were transferred onto PVDF membranes and the fragments f1–f5 were excised and sequenced. The sequences of the first 19–31 aa were identified, aligned and compared with the known sequences of NmpC from *E. coli* (Blasband *et al.*, 1986) and *S. typhimurium* (Hongo *et al.*, 1994) (Fig. 1). As mentioned earlier, the deduced sequence of the NmpC-like protein from *S. typhimurium* is partial (Hongo *et al.*, 1994) and lacks the residues corresponding to the first 58 aa in mature NmpC and Lc from *E. coli*. Identical and similar residues among these proteins, and aromatic residues which tend to flank the membrane-spanning β strands, were identified. Since the three-dimensional structure of NmpC is unknown at this time, the prediction of its exposed loops (L1–L8), periplasmic turns (T1–T8) and membrane-spanning β strands (S1–S16) was made (Fig. 1) by comparing the sequences of NmpC with *E. coli* OmpF (Cowan *et al.*, 1992). Three of these sequences (L4, L5 and L6) correspond to the regions with pronounced differences between the *E. coli* NmpC and the NmpC-like protein from *S. typhimurium* (Fig. 1).

The N-terminal sequence of OmpD which begins with AEVYN... (aa 1–32) aligned better with OmpC (81% identity) from *E. coli* and *S. typhimurium* than NmpC or Lc (74%) from *E. coli* (Table 2). Conversely, the C-terminal sequence (STFVD..., aa 307–326) was 90 to 95% identical with the NmpC-like protein from *S. typhimurium* and NmpC and Lc from *E. coli*, but only 80% identical with *E. coli* and *S. typhimurium* OmpC. The sequence TFGED... (aa 242–260) showed the lowest identity (37–47%) with OmpC from *E. coli* and *S. typhimurium*, and NmpC and Lc from *E. coli*, but 100% identity with the *S. typhimurium* NmpC-like protein. It is noteworthy that the TFGED... sequence corresponds to the externally-exposed loop L6. This is one of the three loops mentioned above that show the greatest divergence of sequences between *E. coli* NmpC and the *S. typhimurium* NmpC-like protein (Fig. 1). It is clear, based on the near identity (63 out of 65 residues) observed in the primary amino acid sequences, that *S. typhimurium* OmpD is

Fig. 1. Alignment of OmpD sequences with folding predictions. N-terminal sequences of the *S. typhimurium* OmpD (middle line) were compared with known amino acid sequences of *E. coli* NmpC (upper line) and the *S. typhimurium* NmpC-like protein ('NmpC') (bottom line). The residue numbers assigned to peptides refer to those of *E. coli* NmpC (Blasband *et al.*, 1986). Boldface dots indicating deletion of amino acid residues and extra sequences are included in the *E. coli* and *S. typhimurium* proteins to obtain the best-fit alignment (Best Fit algorithm; Genetics Computer Group, Madison, WI, USA). Identical (|) and similar (:) residues among the proteins are shown; aromatic residues are underlined. The externally exposed loops (L1–L8), periplasmic turns (T1–T8) and membrane-spanning β strands (S1–S16) were predicted by a comparison of the NmpC sequence with the three-dimensional structure of *E. coli* OmpF (Cowan & Rosenbusch, 1994; Jeanteur *et al.*, 1994).

Table 2. Comparison of amino acid sequences of OmpD with those of other bacterial porins

OmpD sequence*	Identity (%)†				
	<i>S. typhimurium</i> 'NmpC'	<i>E. coli</i> NmpC	<i>E. coli</i> Lc	<i>E. coli</i> OmpC	<i>S. typhimurium</i> OmpC
AEVYN... (31)	—	74	74	81	81
TGRTT... (26)	96	85	88	73	65
TFGED... (19)	100	47	42	37	37
STFVD... (20)	95	90	90	80	80
Overall (96)	97	75	75	70	68

* See Fig. 1. The numbers in parentheses indicate the total number of residues identified in the peptide.

† The N-terminal sequences of CNBr-generated fragments of *S. typhimurium* OmpD were aligned with known sequences using the Best Fit program of the University of Wisconsin Genetics Computer Group. The percentage identity was calculated on the basis of the number of residues identified in OmpD. The sequences of other bacterial porins were taken from the following references: *S. typhimurium* NmpC-like protein ('NmpC'), Hongo *et al.* (1994); *E. coli* NmpC, Blasband *et al.* (1986); *E. coli* Lc, Blasband *et al.* (1986); *E. coli* OmpC, Mizuno *et al.* (1983); *S. typhimurium* OmpC, Puente *et al.* (1989), Singh *et al.* (1995).

identical to the hypothetical translation product of what was named 'nmpC' of this organism. The two mismatches could be due to errors in either DNA or protein sequence determinations, or, alternatively, the sequence polymorphism due to *S. typhimurium* strain differences.

Overall, and as expected, the OmpD sequence also showed a high degree of identity with NmpC (75%), Lc (75%) and OmpC (70%) from *E. coli*, and OmpC (68%) from *S. typhimurium* (Table 2). Similarly, the amino acid compositions of *S. typhimurium* OmpD, despite some differences in the number of Asx, Glx, Gly and Lys residues, showed considerable similarity with NmpC from *E. coli* (data not shown). These results are consistent with our earlier report (Singh *et al.*, 1992) that OmpD is immunologically more related to NmpC and Lc porins of *E. coli* than OmpC from *E. coli* and *S. typhimurium*.

We do not know why *S. typhimurium* constitutively expresses OmpD in addition to the general porins OmpC and OmpF, or what role OmpD plays in the physiology or pathogenesis of this organism. The expression of NmpC in *E. coli* K-12 is prevented because of a IS5 insertion (Blasband *et al.*, 1986); possibly, in *S. typhimurium* the *ompD* gene is expressed because such an insertion sequence is absent in this organism.

mAb reactivity with CNBr-generated fragments

The OmpD protein from *S. typhimurium* was digested with CNBr, electrophoresed and transferred to nitrocellulose paper. The fragments were analysed for their immunoreactivity with a panel of 16 anti-OmpD mAbs. Each antibody gave a strong positive reaction with the undigested OmpD monomer in Western blots (data not shown), and the panel was classified into three distinct groups on the basis of its immunoreactivity with CNBr-generated peptides in Western blots (Table 3).

Group I contained two mAbs (DM 51.1 and 62.1) that reacted with band f1, f3, f4 and f5 (Table 3); failure of these antibodies to bind f2 indicates that the epitope(s) recognized by these mAbs is localized to transmembranous β strands S1–S3, between residues 1 and 124 (Fig. 1). Group II mAbs (DM 9.2, 20.2, 22.2, 49.2, 57.2 and 2.1) bound CNBr-generated fragments f1–f5 (Table 3), indicating that the epitope recognized by this panel of mAbs is likely to be localized at or near residue 124 (Fig. 1). This residue is within the eyelet-forming loop, L3 (Cowan *et al.*, 1992; Nikaido, 1992; Struyve *et al.*, 1993) which folds into the barrel leading to the inaccessibility of the epitope on the cell surface. Loop L3 contains one and a half turns of α -helix, contributes to the constriction of the pore and is responsible for ion selectivity (Cowan *et al.*, 1992). The sequence around residue 124 is well-conserved (Fig. 1; Jeanteur *et al.*, 1994; Nikaido, 1992) and, as predicted, all six mAbs in this group cross-react with *E. coli* NmpC, Lc, OmpC, PhoE, OmpF and *Salmonella typhi* OmpC (Table 1).

Group III mAbs (DM 12.2, 11.2, 60.3, 26.1, 46.2, 5.1, 19.1 and 24.4) reacted with all CNBr-generated fragments except f5 (Table 3), indicating that the epitope(s) recognized by these mAbs is likely to be localized on a buried region between residues 125 and 241 (Fig. 1). This region contains membrane-spanning β strands S6–S11 (Fig. 1). DM 12.2, 11.2 and 60.3 cross-react with all of the porins tested (Table 1); their epitope is likely to be present on S10, given the nearly total conservation of amino acids on this strand compared to other β strands (Jeanteur *et al.*, 1994). DM 26.1 and 46.2 also react with all of the porins except *E. coli* OmpC and OmpF (Table 1); their epitope is possibly localized on S8 which contains different residues at aa positions 172 and 176 in OmpC and OmpF compared to the rest of the porins tested (Jeanteur *et al.*, 1994). However, DM 5.1, 19.1 and 24.4 bound NmpC and Lc only (Table 1), their epitope is possibly localized on S9

Table 3. Reactivity of anti-OmpD mAbs with CNBr-generated peptides of *S. typhimurium* OmpD

Purified porin was digested with CNBr, separated by SDS-PAGE on 10–20% gradient gels and the peptides transferred to nitrocellulose. The paper was cut into strips which were incubated with individual mAbs, washed and amplified with goat anti-mouse Ig. The strips were then washed and developed with alkaline-phosphatase-labelled rabbit anti-goat Ig/nitro blue tetrazolium/bromochloroindolyl phosphate (Pai *et al.*, 1992). Reactions were scored as indicated in Table 1, footnote *.

mAb	Peptides					Probable epitope*
	f1	f2	f3	f4	f5	
DM51.1	++	—	+	+	+	TM (S1–S3; aa 1–124)
DM62.1	++	—	++	+	+	
DM9.2	++	+	++	+	++	
DM20.2	++	+	++	+	+	
DM22.2	++	++	+	+	+	L3 (adjacent to residue 124)
DM49.2	++	+	+	+	+	
DM57.2	+	+	+	+	+	
DM2.1	++	++	++	+	+	
DM12.2	++	++	+	+	—	TM (S6–S11; aa 125–241)
DM11.2	+	+	+	++	—	
DM60.3	++	+	+	+	—	
DM26.1	++	++	+	+	—	
DM46.2	++	++	+	+	—	
DM5.1	+	+	+	+	—	
DM19.1	++	+	++	++	—	
DM24.4	++	++	+	+	—	

* L1–L8, externally exposed loops; TM, transmembrane β strands S1–S16, as predicted in the OmpD model (Fig. 1).

since this strand in NmpC and Lc shows significant divergence of sequences (aa 187–191) from other porins.

We have thus identified several buried epitopes on the OmpD porin; these appear to be localized on β strands on peptides f3 (DM 51.1 and 62.1; aa 1–124; S1–S3) or f4 (DM 12.2, 11.2, 60.3, 26.1, 46.2, 5.1, 19.1 and 24.4; aa 125–241; S6–S11) or adjacent to residue 124 on loop L3 (DM 9.2, 20.2, 22.2, 49.2, 57.2 and 2.1) (Fig. 1). Although we were unable to identify any surface-exposed epitope, we can, nevertheless, draw significant conclusions from the mAb reactivity patterns observed in this study. First, *S. typhimurium* OmpD and *E. coli* NmpC and Lc are immunologically similar as evidenced by the strong cross-reactivity of all 16 mAbs in the panel with these porins (Table 1). This is consistent with our earlier report (Singh *et al.*, 1992) of a close immunological relationship among these proteins. Second, group II mAbs recognize an epitope(s) that is located at or near residue 124 within the eyelet-forming loop, L3 (Cowan *et al.*, 1992; Nikaido, 1992; Struyve *et al.*, 1993). However, this epitope is not accessible on the cell surface (Table 1), indicating that the three-dimensional structure of the porin is essential for an accurate interpretation of mAb reactivity. The hydropathy analysis can be misleading since we know that the transmembrane strands can be quite hydrophilic due to

the alternation of hydrophobic and hydrophilic residues (Cowan & Rosenbusch, 1994; Cowan *et al.*, 1992; Jeanteur *et al.*, 1994; Nikaido, 1992). Finally, a majority of the mAbs (13 out of 16) showed strong cross-reaction with *E. coli* OmpC, OmpF and PhoE, and *S. typhi* OmpC (Table 1) and, as stated above, all 16 mAbs reacted strongly with *E. coli* NmpC and Lc. These results suggest that the folding model of OmpD, based on the structure of *E. coli* OmpF (Cowan *et al.*, 1992), is likely to be essentially correct. They further confirm and strengthen the conclusions of several investigators (Cowan & Rosenbusch, 1994; Cowan *et al.*, 1992; Jeanteur *et al.*, 1994; Nikaido, 1992; Struyve *et al.*, 1993) that the β strands in enterobacterial porins are structurally conserved and that the evolutionary divergence of sequences in these proteins is confined to the externally exposed loops. The sequence data presented in this study should be useful in identification of the surface and other buried epitopes which may be important from standpoints of structure–function relationships, diagnostic assays and vaccine developments, including insertion and expression of foreign genes in *Salmonella* strains (Chatfield *et al.*, 1989; Hackett, 1993; Isibasi *et al.*, 1992; Klebba *et al.*, 1990; Kuusi *et al.*, 1981; Muthukumar & Muthukkaruppan, 1993; Puente *et al.*, 1995; Singh *et al.*, 1996; Tommassen *et al.*, 1993).

Revised map location of *ompD* DNA sequence

The *ompD* locus of *S. typhimurium* was mapped roughly in the 32–33 min region of the chromosome (Sanderson & Roth, 1988), and more recently at 33·7 min (now redefined as centisomes, 1 centisome = 1%) by physical methods using the *ompD159::Tn10* mutation (Liu *et al.*, 1993; Sanderson *et al.*, 1995). This *ompD/nmpC*-like locus (see below) of *S. typhimurium* is not equivalent to the *nmpC* of *E. coli*, which maps in the 12–13 min region of the chromosome (Pugsley & Schnaitman, 1978; Lee & Schnaitman, 1980).

As indicated earlier, OmpD is identical (63 out of 65 residues) to the hypothetical translation product of the *nmpC*-like gene from *S. typhimurium* (Hongo *et al.*, 1994). This sequence identity was further supported when we amplified an internal fragment of the *S. typhimurium nmpC*-like gene using PCR primers derived from bp 36–54 and 775–757 (Hongo *et al.*, 1994). We obtained an amplicon of the expected size (739 bp) using the genomic DNA prepared from the OmpD⁺ strain (SH 7454) but not the *ompD159::Tn10* strains (SH 7455 and SH 7457) (data not shown). This indicates that the mutation caused by the insertion and loss of tetracycline resistance of the *ompD159::Tn10* strain results in either loss of a primer site or rearrangement that does not allow amplification of the internal fragment specific for the *nmpC*-like gene from SH 7455 and SH 7457. These results further strengthen our view that the *nmpC*-like gene (Hongo *et al.*, 1994) is indeed *ompD* that encodes the OmpD protein.

Identification of the *nmpC*-like gene (Hongo *et al.*, 1994) as *ompD* now allows us to correct the placement of this DNA sequence (GenBank accession number D26057) on the *S. typhimurium* genetic map (Sanderson *et al.*, 1995). The previous placement was based solely on the identification of the *narK*-like gene within D26057 as the *narK* gene of *S. typhimurium* and an approximation of where the *narK* gene of *S. typhimurium* would map if it were in the analogous location as in *E. coli* (K. E. Rudd, unpublished results; Sanderson *et al.*, 1995). However, recently the *narK*-like gene *narU* of *E. coli* has been sequenced (GenBank accession number X94992; V. Bonnefoy, J. Ratouchniak, F. Blasco & M. Chippaux, unpublished results; Bonnefoy & DeMoss, 1994) and a comparison of the *narK*-like gene of *S. typhimurium* with both *narU* and *narK* of *E. coli* shows that the *narK*-like gene of *S. typhimurium* is more closely related to *narU* than to *narK*. Protein sequence comparisons indicate that the NarK-like *S. typhimurium* protein is 78·1% identical to NarK of *E. coli* (SWISS-PROT accession number P10903) and 85·5% identical to NarU of *E. coli* (GenBank accession number D26057). In addition, the map location of *ompD* in *S. typhimurium* is analogous to the map location of *narU* of *E. coli*, i.e. between *ogt* and *dcp* (Becker & Plapp, 1992; Berlyn *et al.*, 1996; Sanderson *et al.*, 1995), and is not consistent with the map location of the *E. coli narK* gene (Berlyn *et al.*, 1996). Therefore, the DNA sequence of the three *S. typhimurium* genes with GenBank accession number D26057 (Hongo *et al.*, 1994) should be placed at the *ompD* map location 33·7 Cs and the genes flanking the

S. typhimurium smvA gene in D26057 should be now designated *narU* and *ompD*, not *narK* and *nmpC*.

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