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COMPARATIVE CHARACTERIZATION OF PORINS FROM *SALMONELLA TYPHI* 0-901 AND *SALMONELLA TYPHIMURIUM* RA-30

BAHMAN TABARAIE, Ph.D., BAL K. SHARMA,* M.D., F.A.M.S., MEHDI NEJATI, M.Sc.P.H., HOJAT AHMADI, Ph.D., PARVEEN SHARMA,* Ph.D., AND NIRMAL K. GANGULY,* M.D., F.A.M.S.

*From the Department of Bacterial Vaccines & Antigens, Production & Research Complex, Pasteur Institute of Iran, Tehran, I.R. Iran, and the *Departments of Internal Medicine and Experimental Medicine & Biotechnology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India.*

ABSTRACT

Porins from *Salmonella typhi* 0-901 and *Salmonella typhimurium* Ra-30 were characterized and compared. The elution profile of porins from these salmonella species on Sepharose-48 and HPLC appear to be very similar. The findings were confirmed by the electrophoretic pattern which showed three types of porins, i.e. OmpC, OmpD and OmpF in both species. These porins appear to be similar, if not identical, as the LPS-absorbed antiporin antibodies reacted with homologous as well as heterologous antigens. The results of this study favour the use of porins as a common immunogen to control salmonellosis since porin patterns were found to be quite similar among the serotypes of salmonellae, unlike other enterobacterial species.

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INTRODUCTION

The outer surface of Gram-negative bacteria is comprised of 40% lipopolysaccharides and 60% proteins.[1] Though the protein composition is relatively simple, the electrophoretic profile shows diversity among many species such as *Escherichia coli*, *Haemophilus influenzae*, *Vibrio cholerae*, etc.[2-4] In contrast, outer membrane proteins of salmonella species are very homologous.[5] Porins, the hydrophilic non-specific pore-forming channels[6] constituting major OMP, exist in much higher copies per cell [7] which are organized as trimers of three identical subunits .[8,9] *E. coli* B/r produces only one porin (OmpF) under normal growth conditions,[8] while *E. coli* K-12 [9] produces two, OmpF and OmpC, and *S. typhimurium* LT2 produces three porins, OmpF, OmpC and OmpD corresponding to the molecular weight of 36 kDa, 35 kDa and 34 kDa respectively.[10]

Since the reports regarding the different types of porins among salmonella species are lacking, and porins of *S. typhi* and *S. typhimurium* have been shown to be good immunogens,[11-13] we have made an attempt to characterize and compare the porins of two different serogroups of salmonellae, i.e. *S. typhi* 0-901 and Ra-chemotype of rough *S. typhimurium*-30 in order to assess their utility as a common immunogen to control salmonella infection.

Address for correspondence:
Dr. Bahman Tabaraie, Department of Bacterial Vaccines & Antigens, Production & Research Complex, Pasteur Institute of Iran, 25 km. Tehran-Karaj Highway, Tehran, I.R. Iran.
Tel: 548846 - 548341, Fax: 548246

MATERIAL AND METHODS

Bacterial strains

A rough strain of *S. typhimurium* 386 (SF1591) Ra-30 was procured from Max Planck Institute for Immunology, Frieburg, Germany and the non-motile strain of *S. typhi* O-901 (B-34-2) was supplied by the Central Research Institute, Kasauli, India. These strains were used for porin preparation.

Preparation of porins

Prior to porin extraction, flagellae were removed from the motile strain by shearing the thick bacterial suspension (grown in L-broth containing 0.5% dextrose on shaker at 37°C), in 10 mM Tris buffer (pH 7.8) containing 3 mM Na_3N, in Sorval OmniMixer for 1 min at 4°C. The cells were washed twice with the same buffer.

Semi-purified porins were prepared as described by Nurminen,[14] with slight modifications. Trition X-100 lysozyme-EDTA-treated cell envelopes were dialyzed extensively at room temperature against distilled water containing 32 mM Na_3N. Then the pH of the dialysate was raised to 8.0 by adding Tris (pH 8.8). The contents were centrifuged (3900 g for 15 min) and the sediment discarded. The supernatant was treated overnight with trypsin (0.5 mg/mL) with gentle shaking at 37°C. The next day the solution was again centrifuged and concentrated. 15 mg of this semi-purified preparation was dissolved in 3 mL of 0.02% Triton X-100 (W/V) and then subjected to double chromatographic perfusion through a sepharose-48 column (2.5 × 79.5 cm) pre-equilibrated with 0.01 M Tris buffer (pH 8.0) containing 0.02% Triton X-100 and 3 mM Na_3N. The sample was eluted with the same buffer. Optical density of each fraction was measured at 280 nm and the porin-containing fractions were pooled, dispensed and lyophilized. The column was calibrated with standard high molecular weight protein markers to assess the apparent molecular weights of the porin aggregates and the gel filtration properties were expressed in terms of distribution coefficient (Kav) values and Tunable Absorbance Detector: U.V. Waters - 486.

High pressure liquid chromatography (HPLC)

10 µg of porin sample was then applied onto HPLC (Waters 991 system) in reduced and native state using 2 PAK™ 300-SW column (7.5 mm × 30 cm) connected in series with a flow rate of 1 mL/min.

Preparation of LPS

Crude LPS was prepared by the conventional hot phenol-water extraction procedure of Westphal and Jann[15] after enzymatic cell digestion.[16] The crude preparation was then eluted through a Sepharose-4B column. 20 µL from each fraction was assayed with thiobarbituric acid for LPS and the absorbance at 260 nm determined to detect nucleic acids. LPS-containing peaks were pooled and lyophilized after dialysing against pyrogen-free distilled water. LPS from Ra chemotype of *S. typhimurium* was purchased from Sigma Chemicals.

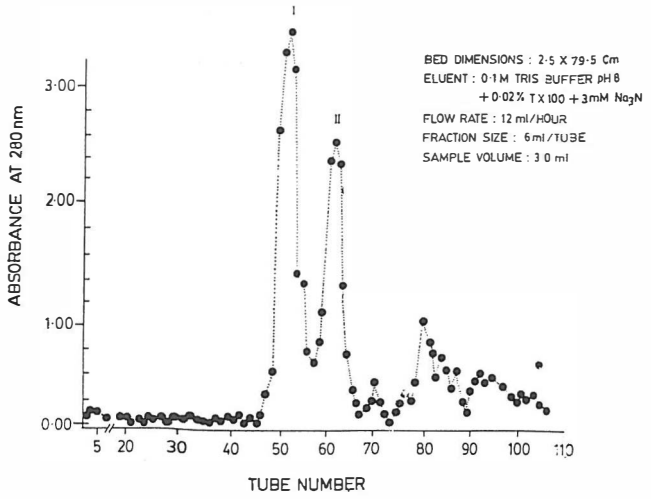


Fig. 1a. Elution profile for *Salmonella typhimurium* Ra-30 semi-purified porins on Sepharose - 4B column chromatography. Illustrating two major protein peaks (I & II) and a few spaced minor peaks.

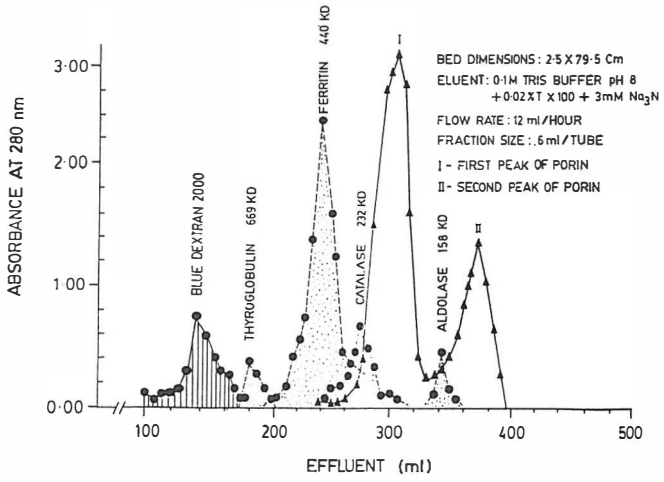


Fig. 1b. Elution profile for *Salmonella typhi* 0-901 porins and standard molecular weight protein markers (Pharmacia) on Sepharose - 4B column to calculate the Kav value of the peaks.

Preparation of antisera

White New Zealand rabbits (2.0 -2.5 kg) were selected. Prior to immunization serum samples from each experimental animal was screened against Widal antigens particularly for groups D and B by slide and tube agglutination test. Rabbits showing serum titer 80 or more during screening were rejected for immunization.

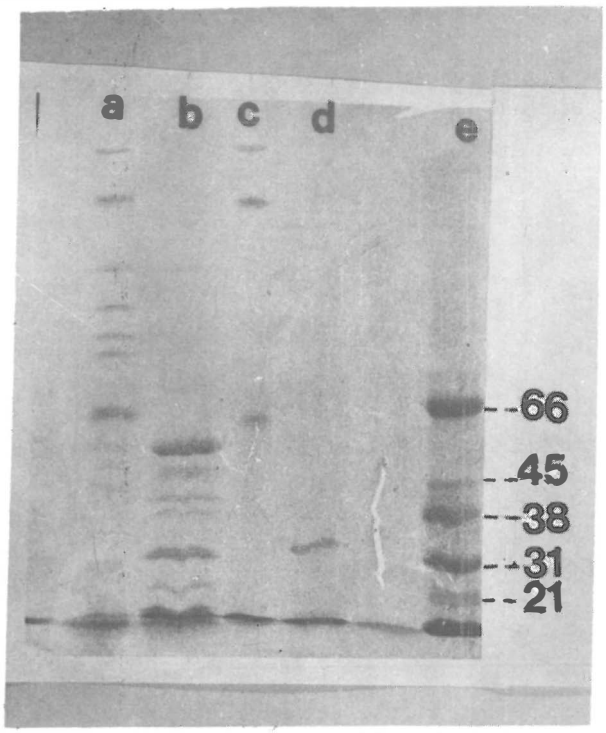


Fig. 2. Protein patterns of porins from *Salmonella typhi* 0-901 on SDS - PAGE (10% acrylamide). TX-100 treated lysozyme - EDTA cell
b) in reduced state, semipurified porins; (Lane c) non-reduced conditions; (Lane d) in reduced state; (Lane e) molecular weight markers.

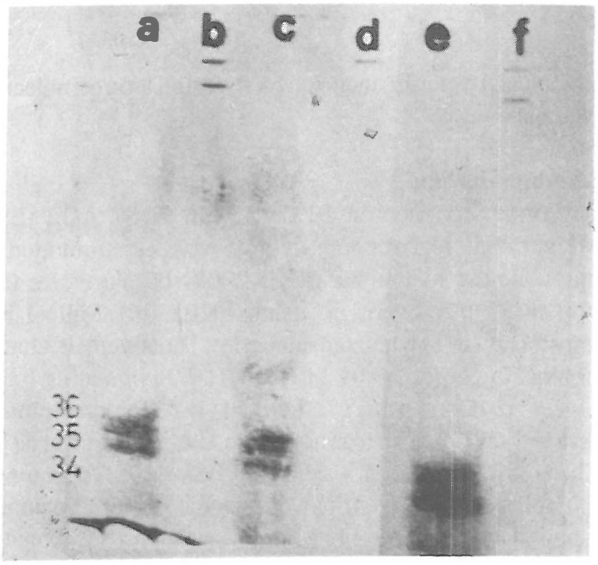


Fig. 3. Electrophoretic patterns of porins on SDS - PAGE (12% acrylamide) containing 4 M urea. Pooled peak I and II of *Salmonella typhi* 0-901 porins; (Lane a) reduced condition; (Lane b) non-reduced. Peak I; (Lane c) reduced; (Lane d) non-reduced. Pooled peak I and II of *Salmonella typhimurium* Ra-30 porins (Lane e) reduced; (Lane f) non-reduced.

Antiporin antiserum

Antiporin antibodies against *S. typhi* 0-901 were raised in a group of four rabbits by giving 100 µg of porins subsequently on days 0,14, 28 and 42 subcutaneously. The immune sera collected 10 days after the last injection were pooled. The pooled serum samples were inactivated at 56°C for 30 min. Mertiolate was then added to a final concentration of 1:10,000 and the serum stored at -20°C.

Anti-LPS antibodies absorption

Hyperimmune serum was absorbed 3 times each by incubating 1 mL of serum with 2 mg of smooth *S. typhi* O-901 LPS for 6 hours at 37°C with gentle shaking. The absorbed antibodies were removed by centrifugation at 105,000 g. Complete removal of anti-LPS antibody was confirmed with ELISA.

Anti-LPS antiserum

Anti-LPS antibodies were also raised in rabbits by giving 25, 50, 100 and 200 µg of LPS intravenously at 3-day intervals, followed by 250 µg and 300 µg at 7- day intervals. Finally 4 boosters of 300 µg each were given fortnightly. Hyperimmune sera collected 10 days after the last injection were pooled. The pooled serum was inactivated and stored as described above.

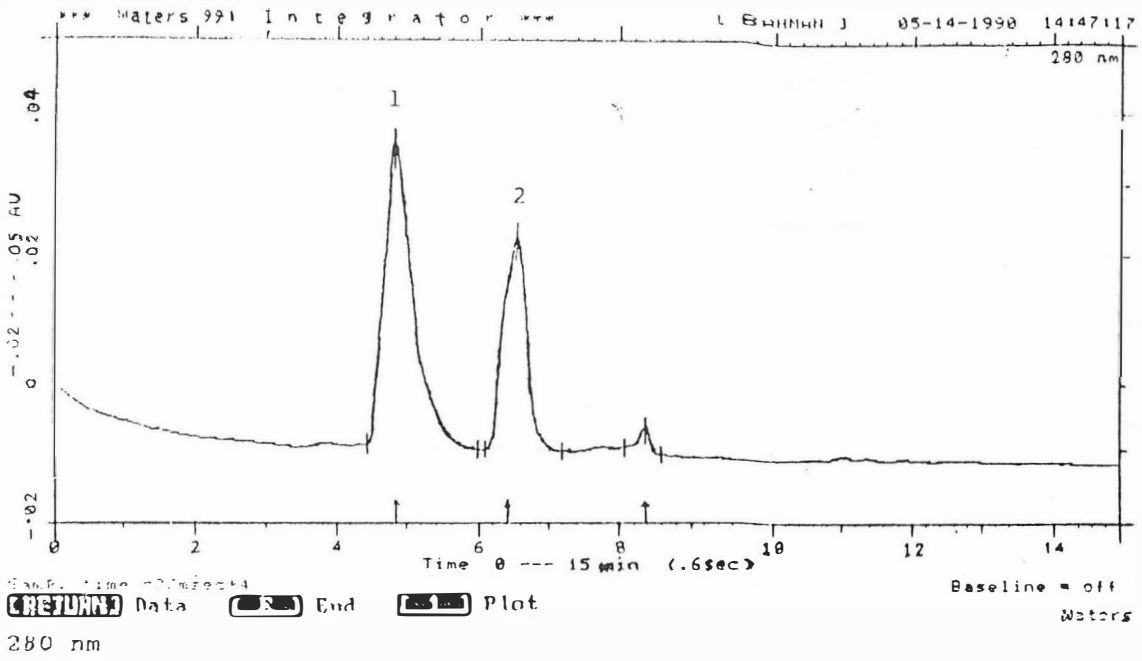
Quantitative analysis

Protein concentrations were measured by modified Lowry's method of Wessel and Flugge[17] in which measurement was done at 215 nm using BSA as standard.

Semi-quantitative estimation of LPS contaminant in porin preparations was performed by Limulus amebocyte lysate assay using Single E-toxoid Test Kit (Sigma Technical Bulletin No. 210, 1988).

Enzyme linked immunosorbent assay (ELISA)

Antibodies to either porins or LPS were detected by ELISA as described by Kuusi et al.[18] and Colwell et al.[19] with slight modifications. Flat bottom microtiter plates (Nune, Denmark) were coated with 200 µL of antigens diluted in buffer. The optimum antigen concentration used for coating the microtitration wells were 2 µg/mL in 0.1 M Tris buffer (pH 8.5) for *S. typhi* 0-901 porins and 4 µg/mL of LPS in 0.1 M carbonate buffer (pH 9.6). The plates were kept overnight at room temperature in a humid chamber. The next day the plates were washed three times with phosphate buffer saline (pH 7.4) containing 0.05% (V/V) Tween-20 (PBS-T). 250 µL of PBS-T containing 3% BSA was then added to each well and plates were kept at 37°C for 1 h. Thereafter the plates were washed three times with PBS-T. 200 µL of two-fold serially diluted immune serum was then added to each well and plates were incubated at 37°C for 2 h. The plates were again washed and 200 µL of optimally diluted (1: 2000) peroxidase-labelled swine anti-rabbit



No.	Retention time	Height [AU]	Left time	Right time	Area [AU×min]	Area [x]	Mark
1	4.84	0.0204	4.44	6.01	0.012074	79.211	I
2	6.42	0.0151	6.10	7.19	0.002613	17.145	I
3	8.39	0.0033	8.09	8.59	0.000555	3.644	I

Fig. 4. Elution profile for *Salmonella typhi* 0-901 porins (chromatographed) on HPLC system showing two peaks of high molecular weight protein aggregates with minor protein contamination.

immunoglobulin (Dakopatts, Denmark) in PBS-T was added to each well. After 1 h of incubation at 37°C the plates were washed again and 200 µL of freshly prepared substrate solution was added to each well and the plates kept in the dark for 10 to 30 min at room temperature. Absorbance was measured at 492 nm in a microplate reader MPR-A4 (Euro-Genetic, Belgium). The titer was taken as reciprocal log dilution of the serum giving an absorbance value at 492 nm.

SDS-polyacrylamide gel electrophoresis

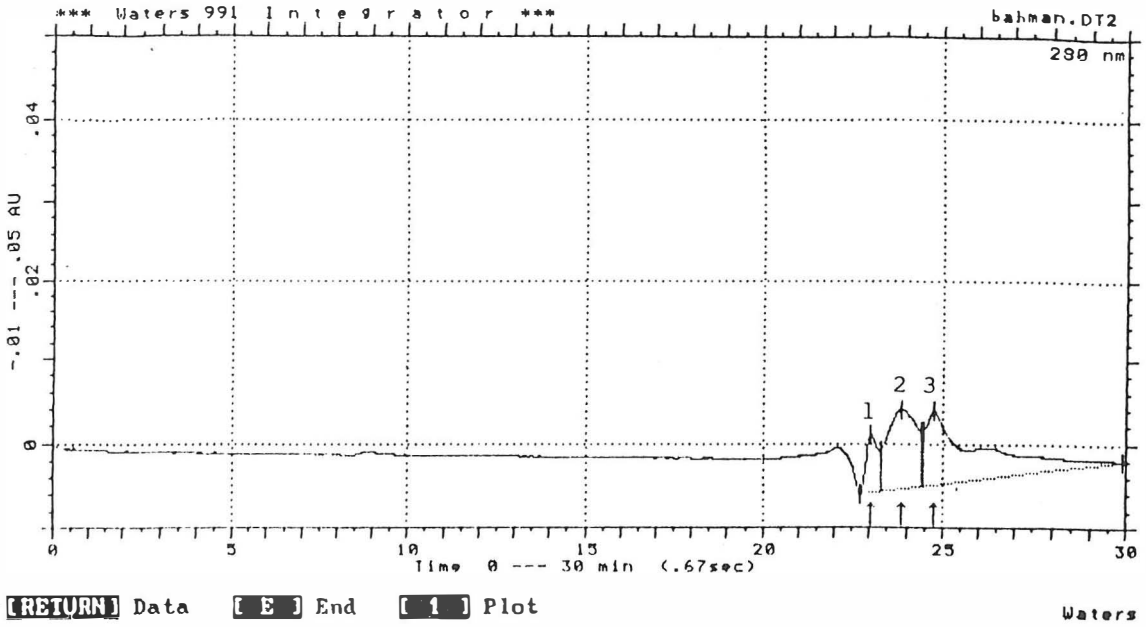
Slab gel electrophoresis was performed in discontinuous buffer system by the method of Laemmli [20] in a LKB 2001-001 electrophoretic unit. Porins were subjected to the gel in the order of their purification processes. Porins (75µg) were applied onto the gel either under non-reduced conditions or under reduced conditions to maintain the native trimeric aggregate or monomeric forms of porins. The gel were run at 60 mA for 4 h in 10% resolving gel and 4.5% of stacking gel. To enhance the resolution of different porin monomeric bands, 12% acrylamide gel containing 4 M urea was also used. Standard low molecular weight protein markers (Sigma Chemicals) were also included for the estimation of molecular weights.

Immunoblotting

Porins separated on 10% gel with SDS-PAGE (LKB Midgel 2050 electrophoretic unit) were electroblotted on nitrocellulose membrane (LKB 2005-105, pore size 0.45 µm) at 35 mA overnight using LKB 2051 Midgel blot transfer unit in Towbin transfer buffer. Blots were incubated in blocking buffer (0.01 M PBS pH 7.2 containing 0.3% Tween - 20) for 2 h at 37°C. Diluted (1: 200) hyperimmune sera was absorbed on to nitrocellulose strips for 1 h at 37°C. These strips were then washed in PBS (pH 7.2) containing 0.05% Tween-20. The strips were incubated with peroxidase-labelled swine antirabbit immunoglobulin (Dekopatts, Denmark) for 2 h at 37°C, then washed and developed in freshly prepared DAB substrate solution.

RESULTS

The elution profile of semi-purified porins from *S. typhi*



[280 nm

No.	Retention time	Height [AU]	Left time	Right time	Area [AU×min]	Area [x]	Mark
1	23.05	0.0068	22.74	23.31	0.002564	9.927	
2	23.88	0.0094	23.31	24.43	0.008546	33.088	V
3	24.77	0.0086	24.43	24.46	0.000214	0.0829	V

Fig. 5. Elution profile for *Salmonella typhi* 0-901 (rechromatographed) under reducing conditions on HPLC system showing three closely spaced peaks corresponding to OmpC, OmpD and OmpF of *Salmonella typhimurium*.

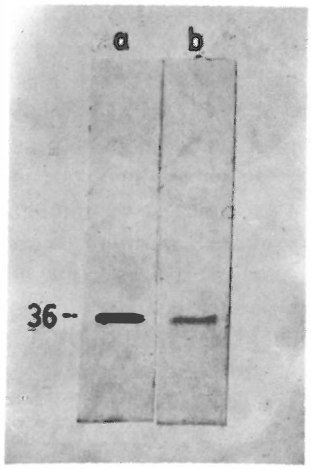


Fig. 6. Western blot analysis of rabbit anti-porin antibodies of *Salmonella typhi* 0-901 (LPS-adsorbed) when subjected to transblotted - porin strips of *Salmonella typhi* 0-901 (lane a) and *Salmonella typhimurium* Ra - 30 (lane b).

0.901 through Sepharose-48 column (Fig. 1a) shows that the porins were eluted in two major symmetric peaks at Kav values of 0.78 and 1.02 with apparent molecular weights of 221 kDa and 134 kDa, respectively. An exactly similar elution profile was observed when porins from *S. typhimurium* Ra-30 were eluted under similar conditions (Fig. 1b). The extent of purity of the porin preparation at different purification steps was cross-checked with SDS-PAGE using 10% gel. The porins were resolved onto this gel under reduced and non-reduced conditions (Fig. 2). Unheated Trition X-100 treated lysozyme-EDTA cell envelope showed many protein bands (Fig. 2, lane a). After trypsinization the number of such bands was reduced to two major bands corresponding to 221 kDa and 134 kDa besides several closely spaced bands probably of the OmpA of the cell envelope with a molecular weight of 65 kDa (Fig. 2, lane c). On the other hand, concerning the heated sample (at 100°C), when resolved on SDS PAGE gel, the two porin aggregates collapsed into a single band of 36 kDa (Fig. 2, lanes b&d). In order to resolve the trimeric aggregates of *S. typhi* 0-901 porins into their native monomers and compare these with *S. typhimurium* Ra-30 porins, samples from rechromatographed pooled fractions were resolved on to a 12% gel containing 4 M urea, under reduced conditions. As expected, the two trimeric aggregates of both the porins

Table I. ELISA titer of *S. typhi* 0-901 antiporin antibodies (adsorbed and non-adsorbed) and *S. typhi* 0-901 anti-LPS antibodies in immune rabbit sera.

Type of antiserum	Immunoglobulin titer* to		
	***S. typhi* 0 - 901 Porin**	***S. typhimurium* Ra - 30 Porin**	***S. typhi* 0 - 901 LPS**
S. typhi 0-901 anti-porin (non- adsorbed)	4.3	3.6	2.5
S. typhi 0-902 anti-porin (LPS-adsorbed)	3.4	3.1	< 2.0
Pre-immune	< 2.0	< 2.0	< 2.0

ELISA titer of *S. typhimurium* Ra - 30 anti-porin against homologous porin was 3.7 and no detectable titer against *S. typhimurium* Ra-30 LPS was observed in homologous or heterologous antisera.

* ELISA titer is expressed as reciprocal $\log_{10}$ dilution of serum giving an absorbance value at 492 nm.

(Fig. 3, lanes b&f) resolved into three bands with molecular weights of 36 kDa, 35 kDa and 34 kDa corresponding to three monomers of *S. typhimurium* Ra-30 (Fig. 3, lanes a&e), i.e. OmpC, OmpD and OmpF, respectively. A similar electrophoretic profile was observed when single trimeric aggregates were run under the same conditions (Fig. 3, lanes d&c).

To analyse the protein more objectively, 10 µg (per 10 µL) of chromatographed *S. typhi* 0-901 porin aggregates was injected to HPLC. The chromatographic profile showed two major peaks at retention times of 4.84 and 6.42 min along with a minor protein contaminant with a retention time of 8.39 min (Fig. 4). This contamination was removable as this could not be observed when rechromatographed samples were introduced onto HPLC in reduced form (Fig. 5). Two major peaks were resolved into three closely spaced peaks at retention times of 23.05, 23.88 and 24.77 min (Fig. 5), thus simulating the electrophoretic pattern of *S. typhimurium* Ra-30 porins (Fig. 3). The contamination of LPS from 2.5% can possibly be decreased to 0.1% if double chromatographic perfusion is applied.

To confirm immunological identity between the porins from two species, ELISA and immunoblotting techniques were performed. Table I shows that when *S. typhi* 0-901 antiporin antibodies were titered against purified *S. typhi* 0-901 and *S. typhimurium* Ra-30 porins, the respective titers were 4.3 and 3.6, whereas it was 3.4 and 3.1 when LPS-absorbed antiporin antibodies were used. We could not detect either the LPS or the anti-LPS antibodies from the rechromatographed porins or the antiporin antibodies respectively with ELISA.

Homology of similar extent was observed with 36 kDa bands on nitrocellulose strips when transblotted porins of *S. typhi* 0-901 and *S. typhimurium* Ra - 30 were subjected to LPS-adsorbed *S. typhi* anti-porin antibodies (Fig. 6). Moreover no band could be visualized when the strips were treated with anti-LPS antibodies.

DISCUSSION

The human antibody response to natural salmonella infection has been primarily attributed to homologous strain specific epitopes in the O-side chains of LPS [21, 22] rather than the broadly shared core or lipid-A moiety.[23] The strong immunoprotective activities observed against lethal doses of homologous or heterologous strains of salmonella pathogens when the mice were immunized with Re-chemotype of rough S. *minnesota*[24] are contrary to the above hypothesis.[21-23] These observations and the antigenic cross reactivities of outer membrane proteins observed in different salmonella species,[25] enabled us to characterize porins, the major constituent of OMPs which may be common in different salmonella serotypes.

In order to minimize configurational changes during the isolation process a mild enzymatic extraction technique using non-ionic detergent (0.2% Triton X-100) was adopted to obtain semi-purified porins. These were further purified by chromatographic perfusion. It was found that the molecular parameters of the two major protein peaks observed in the elution profile of *S. typhi* 0-901 porins were identical to those obtained with *S. typhimurium* Ra - 30 porins.

Similar patterns were reflected on 10% SDS gel electrophoresis. Porin aggregates of both *S. typhi* 0-901 and *S. typhimurium* Ra-30 constituting two different peaks on 12% SDS-gel containing 4 M urea (under reduced condition), resolved into three monomers of 36 kDa, 35 kDa and 34 kDa corresponding to OmpC, OmpD and OmpF porins respectively. Similarly, similar types of profiles from the rechromatographed aggregates of porins–when subjected to HPLC under reducing conditions–resolved into three fractions. Thus in these two salmonella strains no ambiguity in porins as is prevalent in *E. coli* strains could be observed.[8,9] Although it is difficult to obtain porins completely free from LPS contamination, the tendency of LPS to form aggegates of higher molecular weight as compared to that of porins during extraction with two major peaks at Kav values of 0.218 and 0.574 can be exploited advantageously to reduce LPS contamination from 2.8% in semi-purified preparations to 0.5% by single chromatographic perfusion and to 0.1% when rechromatographed, while the preparation has not been subjected to any harsh treatment during isolation techniques.

The similar type of homology was evident even when the LPS-adsorbed antiporin antibodies were allowed to react against homologous or heterologous antigens with ELISA and immunoblotting technique. These parameters have also been confirmed from the sera obtained from typhoidal patients (under publication). Thus it is apparent from the present investigation that under normal growth conditions, these two salmonella species produce similar porins (OmpC, OmpD and OmpF) with at least some common immunogenic domains, and LPS contamination does not appear to play any significant role in antigenicity or cross-reactivities of porins. Hence, it is possible that porins from *S. typhi* or other medically important salmonellae may be a putative common immunogen which may find application in the prevention or diagnosis of salmonellosis in man and animals.

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