

Low molecular weight proteins of outer membrane of *Salmonella typhimurium* are immunogenic in *Salmonella* induced reactive arthritis revealed by proteomics

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Summary

In patients with reactive arthritis (ReA)/undifferentiated spondyloarthropathy (uSpA), synovial fluid mononuclear cells (SFMC) show proliferation to bacterial antigens that trigger ReA, i.e. *Chlamydia*, *Yersinia*, *Campylobacter*, *Shigella* and *Salmonella* species. We have shown previously that SFMC proliferate significantly to outer membrane proteins of *S typhimurium* in *Salmonella* induced ReA. In the present study we characterized the immunoreactive fractions of outer membrane protein (Omp) of *S typhimurium* in *Salmonella* induced ReA. Omp of *Salmonella* was isolated and fractionated by continuous elution sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using Prep-Cell into eight Omp fractions based on molecular weight. Twenty-three patients with ReA were screened for the bacterial trigger using the SFMC proliferative response to crude lysates of *Y enterocolitica*, *S flexneri*, *C jejuni* and *S typhimurium* using thymidine uptake assay. SFMC from patients with salmonella induced ReA were tested against eight fractions. Seven of 23 patients with ReA had *S typhimurium*-induced ReA. Of these seven patients, five patients SFMC had a significant stimulation index (SI) against < 22, 22–26, 25–35 and 28–40 kDa fractions of Omp. These fractions were analysed by SDS-PAGE and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, which revealed 10 proteins. These proteins were 37 kDa OmpA, 33 kDa TsX, 28 kDa putative Omp, 28 kDa Vac J, 39 kDa OmpD, 18 kDa OmpX, 23 kDa OmpW, 43 kDa OmpS1 and 19 kDa peptidoglycan-associated lipoprotein. In conclusion, for the first time we have identified some low molecular weight proteins in the Omps of *Salmonella* which are T cells immunoreactive in patients with salmonella induced ReA/uSpA.

Keywords: enteric bacteria, MALDI-TOF, seronegative spondyloarthropathy, synovial fluid analysis

Introduction

Reactive arthritis (ReA) and undifferentiated spondyloarthropathy (uSpA) are immune-mediated synovitis in response to distant gastrointestinal and genitourinary infections due to *Salmonella typhimurium*, *Shigella flexneri*, *Campylobacter jejuni* and *Yersinia enterocolitica* [1–3]. In sporadic ReA, we have reported previously that *S typhimurium* accounts for one-third of cases in our cohort of patients, in contrast to *Yersinia* and *Chlamydia* in western countries [4]. Immunoreactive antigenic fragments of *S typhimurium* [5,6] and T cell clones specific for *S typhimurium* have been derived from the synovial fluid (SF) of patients with ReA [7].

Some of the antigens recognized by T cells have been characterized in *Yersinia* and *Chlamydia* induced ReA. These include 19 kDa urease- β subunits, 23 kDa ribosomal protein, heat shock protein (hsp60) and outer membrane protein (Omp) in *Yersinia* ReA [8–10] and 60 kDa hsp and Omp of *Chlamydia* [11,12]. The antigenic targets in *Salmonella* induced ReA are less well characterized. Recently, we have shown that synovial fluid mononuclear cells (SFMC) respond more to crude Omp fraction than cytosolic fraction [13]. In typhoid fever and murine *S typhimurium* infection, the immune response is directed against Omp such as porins, OmpA and Omp-28 [14–17].

In this study, we fractionated outer membrane proteins by continuous elution sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then performed a lymphoproliferative assay using SFMC of patients with *Salmonella* induced ReA. The fractions which had a higher proliferative index were characterized further by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) to identify the protein profile.

Patients and methods

Selection of patients

Patients with ReA/uSpA who had active disease and consented to participate were included. ReA was defined as the presence of acute-onset asymmetrical oligoarthritis preceded by a history of diarrhoea within 4 weeks, and European Spondyloarthropathy Study Group criteria were used for classifying uSpA [18]. *Salmonella* induced ReA was defined if the SFMC showed a stimulation index (SI) of more than 2.5 to *S typhimurium* only. A stimulation index (SI) was calculated as mean counts per minute (cpm) with bacterial antigen/mean cpm with medium alone. If the SI has > 2.5 to more than one bacteria then *Salmonella* induced ReA was considered only if the SI against *S typhimurium* was 2.5 times higher than the next higher value.

Preparation and fractionation of outer membrane proteins (Omp) of *S. typhimurium*

S typhimurium (ATCC-13311) was grown in trypticase soy broth overnight at 37°C. *S typhimurium* cells were harvested by centrifugation (15 000 g, 15 min, 4°C) and washed three times with phosphate-buffered saline (PBS 0.15 M, pH 7.2). Ten g of bacterial pellets were suspended in 50 ml sonication buffer [50 mM Tris pH 7.5, containing 10 mM MgCl₂, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM ethyleneglycol tetraacetic acid (EGTA) and 1 mM phenylmethylsulphonyl fluoride (PMSF)] and then disrupted with an ultrasonicator (Sonics & Materials, Newtown, CT, USA). After centrifugation at 12 000 g for 20 min at 4°C the supernatant was collected, aliquoted and stored at -80°C. Omp was isolated from the crude lysate according to the method described by Osborn *et al.*, with minor modifications [19]. Briefly, the crude lysate of *S typhimurium* was centrifuged at 105 000 g for 1 h to obtain the cytoplasmic supernatant and membrane pellet. The membrane pellet was treated with 20 mM Tris-HCl (pH 7.5) containing 2.0% (wt/vol) sodium lauryl sarcosine (SLS) for 30 min at 37°C [20]. It was then centrifuged at 105 000 g to obtain the Omp pellet and washed three times with distilled water. The pellet was resuspended in distilled water, divided into aliquots and stored at -80°C until use. Lipopolysaccharide (LPS) contamination was detected using the *Limulus* amoebocyte lysate assay kit (Sigma, St Louis, MO, USA).

Omp was separated by continuous elution SDS-PAGE using Laemmli's buffer system [21] in a Prep-Cell (Bio-Rad, Hercules, CA, USA) using 10% resolving gel and 4% stacking gel in a 27-mm diameter tube [22]. The Omp pellet (20 mg) was solubilized in the sample buffer (0.06 M Tris-Cl pH 6.8; containing 2% SDS, 10% glycerol, 0.025% bromophenol blue and 5% 2-mercaptoethanol) by keeping in boiling water for 5 min before loading. Electrophoresis was conducted at 12 W for 8 h and, during this period, elution buffer (electrode buffer; 0.025 M Tris-glycine pH 8.3; containing 0.1% SDS) was pumped at a flow rate of 1 ml/min. Fractions (100 × 3 ml) were collected starting immediately after the dye front. Each fraction was analysed on mini-gel slabs and silver-stained [23] to visualize the eluted proteins. Tubes containing identical bands were pooled to provide 13 discrete fractions. Each fraction was concentrated on a Speed Vac and aliquoted (0.1 ml). SDS was removed using the method of Wessel and Flügge [24]. SDS-removed Omp fractions were autoclaved at 103.5 kPa pressure for 20 min and stored at -80°C. Removal of SDS was confirmed by the colorimetric estimation method [25]. Proteins quantification was done by Lowry's method and densitometry using bovine serum albumin (BSA) as a standard.

Lymphocyte proliferation assay

The *in vitro* SFMC proliferation assay against crude bacterial antigens (*S typhimurium*, *Escherichia coli*, *S flexneri*, *Y enterocolitica*, *C jejuni*) and fractions of Omp of *S typhimurium* were tested as described earlier [4]. These were tested at two different concentrations (5 and 10 µg/ml) and mononuclear cells were cultured for 5 days.

Human leucocyte antigen (HLA)-B27 typing

HLA typing was performed with nested polymerase chain reaction using B27-specific primers [26].

Proteomic analysis

Omp fractions which showed a significant SI were run on one-dimensional PAGE (15% resolving gel, 0.5 mm thick). Separated bands were digested in gel as per the protocol described earlier [27]. In brief, the Coomassie blue-stained bands were washed, in-gel reduced, S-alkylated and digested with trypsin (Promega, Madison, WI, USA) at 37°C overnight. Peptides were extracted, dried in a Speed Vac and resolubilized in 0.1% trifluoroacetic acid. Zip Tips (Millipore, Billerica, MA, USA) were used to desalt and concentrate the peptide mixture. Peptide mass fingerprinting was performed by spotting 0.3 µl of the extracted protein digest mixed with α-cyano-4-hydroxycinnamic acid (CHCA, Sigma) on a MALDI target plate. MS and MS/MS spectra were acquired in the positive ion mode on the Applied Biosystems 4700 Proteomics Analyser (Framingham, MA,

USA), which uses a 200-Hz diode-pumped Nd:YAG laser operating at 355 nm. The instrument was operated in the delayed extraction mode with a delay time of 200 ns. Spectra were obtained by accumulation of 1000–5000 consecutive laser shots. Close external calibration was performed with a mixture of six des-Arg1-bradykinin (904·4681), angiotensin I (1296·6853), Glu1-fibrinopeptide B (1570·6774), adrenocorticotrophic hormone (ACTH) (clip 1–17) (2093·0867), ACTH (clip 18–39) (2465·1989) and ACTH (clip 7–38) (3657·9294). Mass calibration for MS/MS spectra was performed by fragment masses of precursor Glu1-fibrinopeptide B (1570·6774). Peak harvesting was conducted using 4000 Series Explorer™ Software (Applied Biosystems).

Database searching using mass spectrometry data (MS or MS/MS) was performed using GPS version 3·5 software (Applied Biosystems) equipped with a MASCOT search engine. The database query was made for *Salmonella* species with a minimum number of matched masses set at 4. The observed monoisotopic masses were searched against the SWISS-PROT and NCBI nr databases. The maximum peptide precursor tolerance was set at 40 parts per million (p.p.m.) and MS/MS fragment tolerance was defined as 0·2 Da. At most one missed cleavage for tryptic peptides was allowed, and the modifications accepted were carbamidomethyl cysteines as fixed modification and oxidation of methionines as variable modification. Tandem MS was performed only in the cases where identification appeared ambiguous with MALDI-TOF MS data.

Statistical analysis

All data are means of results from triplicate wells. Statistical analyses were performed using SPSS version 9 software (SPSS, Chicago, IL, USA). Comparison between groups was performed by the Mann–Whitney two-tailed test; $P < 0·05$ was considered significant.

Results

Seven (ReA-3, uSpA-4) out of 23 patients with ReA/uSpA (ReA-7, uSpA-16) were categorised as *Salmonella* induced as their SFMC reacted specifically to crude lysate of *S typhimurium*. All were males with a median age of 23 years (17–53 y) and the median duration of current episode was 8 weeks (range 4 to 104 wks). The S.T. values against crude lysates of *S typhimurium* were 12, 4·7, 2·8, 8·3, 3·1, 14·1 and 3·7.

Fractionation profile of Omp

The yield of Omp was 1 mg/g of *S typhimurium* pellet. The Limulus test showed the presence of LPS at 6 ng/10 µg of Omp. Prep-Cell separation fractions with similar molecular weight were pooled into eight fractions. Each fraction had

more than one band, some distinctive and others overlapping with adjacent fractions (Fig. 1). The final SDS content was 5–10 µg/ml in the fractions except IV, V and VI, where it was below the detectable limit. These traces of SDS are below the reported toxic level for lymphoproliferation during culture studies [22].

Response of SFMCs to *S typhimurium* Omp and its fractions

Among the seven patients with *Salmonella* induced ReA, five patients showed significant cellular proliferation with Omp; two patients SFMCs did not show any response against Omp (Fig. 2). All five patients who had a proliferative response against Omp showed proliferative responses to one or more of fractions I–V (MW range of <22–40 kDa); all five patients showed responses to fraction I [median (range), 3·19 (1–34·5)], four to II [3·46 (1·43–54·6)], five to III [4·8 (1–40·7)], two to IV [2·23 (1·58–21·8)] and three to V [2·41 (1–6·59)]. Except for one patient, none of these individuals showed any response to fractions VI–VIII (MW > 40 kDa). There was no significant difference in the magnitude of responses to the fractions I, II, III, IV and V, while the responses to fractions I, II, III and IV were significantly ($P < 0·05$) higher than to fractions VI–VIII. Levels of response between fractions V and VI–VIII were not significantly different.

Identification of T cell immunodominant antigens

Because there was a good lymphoproliferative response to fractions I–IV of Omp, these fractions were analysed by MALDI-TOF MS. On 15% SDS-PAGE fractions I–III had

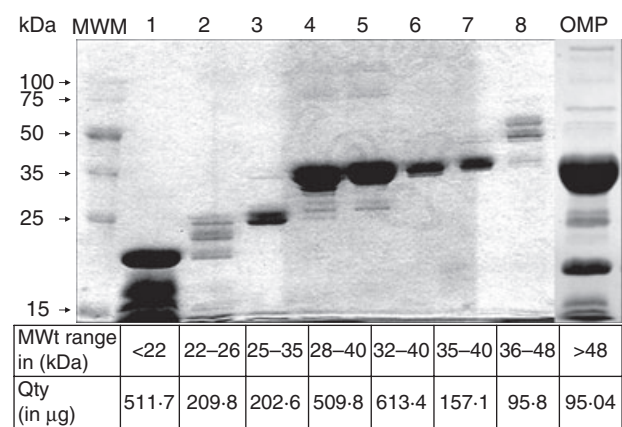


Fig. 1. Protein profile (sodium dodecyl sulphate-polyacrylamide gel electrophoresis, 10% gel, Coomassie stain) of the eight repooled Prep-Cell fractions (I–VIII) after SDS removal. MWM lane: molecular weight marker (in kDa); Omp lane: whole outer membrane proteins fraction. Molecular weight range and finally recovered amount of proteins of each fraction in the bottom of figure.

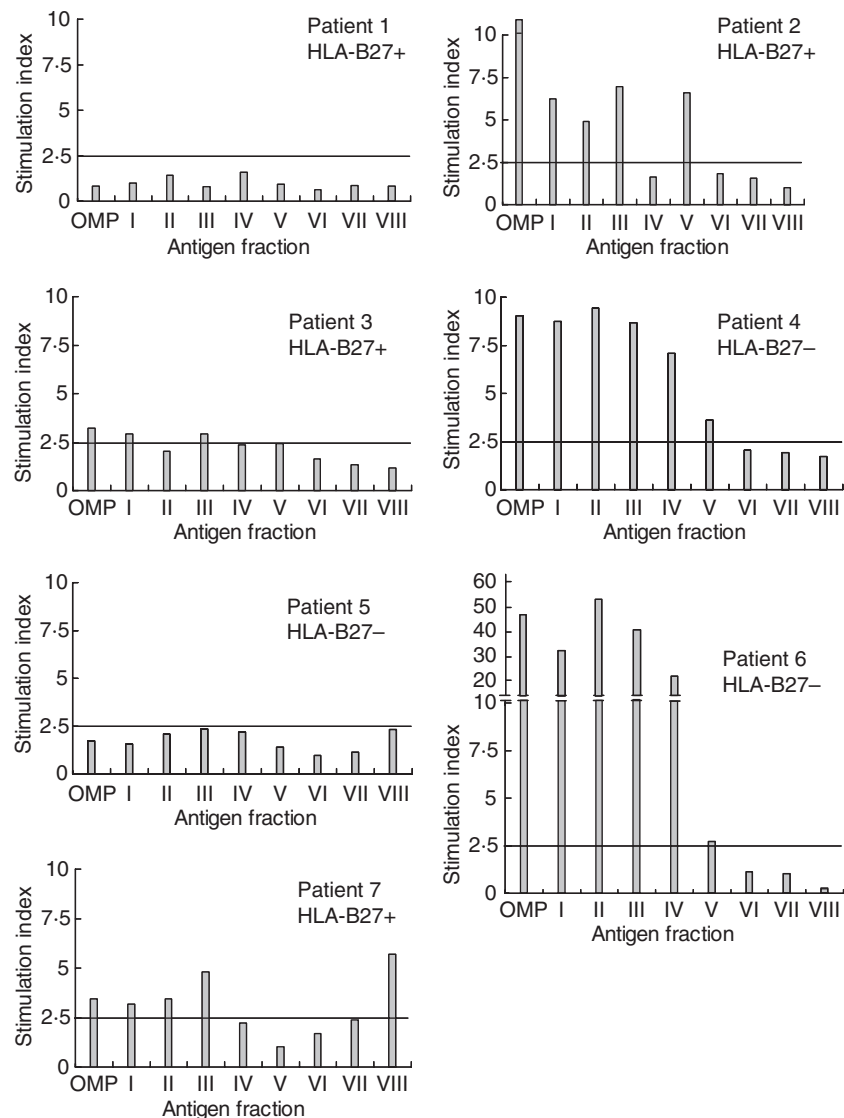


Fig. 2. Human synovial fluid mononuclear cells response to fractions of Omp. Synovial fluid mononuclear cells of *Salmonella* induced reactive arthritis patients ($n = 7$) were stimulated *in vitro* with I–VIII antigenic fractions of Omp of *S. typhimurium*. Each graph represents one individual. Stimulation index (SI) of ≥ 2.5 was taken as a positive response and is indicated by a horizontal dotted line.

three bands each, whereas fraction IV had four distinct protein bands (Fig. 3). MALDI-TOF MS analysis of these protein bands revealed 10 identifiable proteins from the database (Table 1). All fractions had OmpA, two fractions had OmpW and one fraction each had OmpX, peptidoglycan-associated lipoprotein precursor, putative Omp, nucleoside-specific channel-forming protein (Tsx), VacJ lipoprotein, OmpD and OmpS1.

Discussion

This work is in continuation with our previous work to identify the protein antigens that stimulate T cells in *Salmonella* induced ReA. We have shown previously that the Omp fraction contains immunodominant antigens. In this paper, we have shown for the first time that low molecular weight fractions of Omp (≤ 40 kDa) contain antigenic targets of synovial T cells in patients with *Salmonella* induced ReA/

uSpA. Further, using the proteomic approach, we identified 10 Omps in these immunodominant fractions.

The diagnosis of ReA is based on a typical clinical feature with a preceding history of infection. In the case of *Salmonella* induced ReA, documentation of preceding infection is difficult as IgG and IgA antibodies to *Salmonella* LPS do not distinguish between patients and control in an endemic country such as ours. We have already shown in our previous work that the SFMC response to crude bacterial antigens of *S. typhimurium* could be used to categorize these patients [4]. In the case of a cross-reactive response, we have defined the cut-off as used by Seiper *et al.* [28]. SFMC from patients with rheumatoid arthritis [4,29] and peripheral blood mononuclear cells (PBMC) from healthy controls [29], as well as patients with ReA/uSpA [4,29], do not show a proliferative response to crude bacterial lysates, hence in this study we have not included these controls. Furthermore, the aim of this study was to define the immunodominant fractions of Omp.

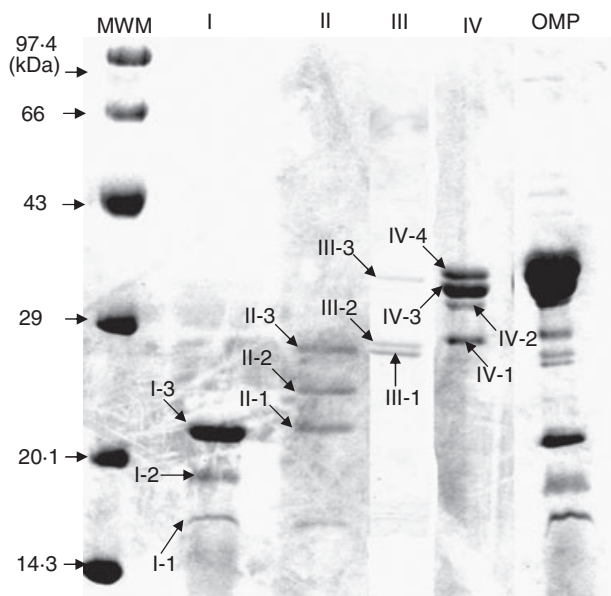


Fig. 3. Protein profile (sodium dodecyl sulphate-polyacrylamide gel electrophoresis, 15% gel, Coomassie stain) of the four immunodominant Omp fractions (I–IV). Each protein band (marked by numbered arrow) from every fraction was picked and processed for protein identification. MWM lane: molecular weight marker (in kDa), Omp lane: whole outer membrane proteins fraction.

The entire genome of *S typhimurium* has been sequenced and the Omp of *S typhimurium* contains 251 proteins [30]. Several approaches have been used to define T cell immunodominant antigens. Using the T cell immunoblotting method, antigens are separated by SDS-PAGE and blotted onto nitrocellulose membrane. The nitrocellulose membrane is then cut into strips based on molecular weight; the strips are then converted into particulate antigen which is used to stimulate lymphocytes [31]. Alternatively, two-dimensional gel is used to separate proteins which are transferred into soluble phase by electro-elution, and these protein fractions are then used for cellular proliferation [32]. The disadvantage of immunoblotting is the inability to quantify the proteins, whereas the second method yields fewer proteins. Thus, we have separated our Omp antigen by SDS-PAGE using the continuous electro-elution method [22] so that the proteins are available in soluble form and can be quantified. Furthermore, this method allows a higher amount of proteins to be separated. The only disadvantage of this method in comparison to two-dimensional gel is less resolution.

The main components of all Gram-negative bacterial Omps are LPS, lipoproteins and porins. LPS is capable of stimulating lymphocytes [33] and it can act as an adjuvant to Omp and enhance the immune response [34]. However, the LPS present in the crude Omp preparation was very small (6 ng/10 µg Omp), and unlikely to account for the proliferative response.

Our observation of low molecular weight fractions of *Salmonella* Omp to be stimulatory is similar to earlier reports in *Yersinia* and *Chlamydia* induced ReA, which have shown low molecular weight Omps to be immunogenic [8,12]. Ten Omps were identified in the immunodominant fractions of *Salmonella*. However, none of the identified Omps of *Salmonella* have significant homology to the *Yersinia* OmpH and *Chlamydia* Momp, the two well-known T cell immunodominant antigens in *Yersinia*-induced and *Chlamydia*-induced ReA, respectively [8,12]. This suggests the possibility that these Omps of *Salmonella* are specific for *Salmonella* induced ReA.

Of all the Omps, OmpA appears to be the major antigenic protein in *salmonella* induced ReA/uSpA as it is common to most of the stimulatory fractions. Since it is a heat modifiable protein, it was located at different molecular weight position in SDS PAGE fraction I to V of Omp due to variable changes in its mobility [35–36]. OmpA is a major antigenic outer membrane protein in *Salmonella* and other enteric bacteria [37,38]. No data are available on it being a T cell immunodominant antigen; however, it contains B cell immunodominant epitopes in patients with typhoid fever and murine *S typhimurium* infection [16].

We have found OmpD, which belongs to the porin family, in one of the stimulatory fraction. Porins are major outer membrane proteins, which contain N terminal conserved β -barrel regions [39]. Porins are well defined immunogens in various Gram-negative bacteria, including *Salmonella* infections. Immunodominant epitopes in porins are located on the outer surface, the periplasmic surface and on the β -barrel region [40,41]. In *Salmonella*, three porin species are OmpC, OmpF and OmpD [42,43]. OmpC and OmpD show a significant sequence similarity (roughly 62% identity and 80% similarity) and are known to be related immunologically. A large number of anti-OmpC as well as anti-OmpD monoclonal antibodies recognize the sequential antigenic determinants of the conserved β -barrel region, suggesting this region to be the major immunodominant site [44]. The porins from *Salmonella* also contain T cell immunodominant antigens and are involved in protective immunity in human typhoid fever as well as in murine salmonellosis [45,46]. In murine salmonellosis, OmpC was identified to contain T cell immunodominant epitopes [47]. The significant sequence similarity and immunological relatedness of OmpC with OmpD and OmpS1 (roughly 63% identity and 80% similarity) [48] indicate that these proteins may be T cell immunodominant antigens in *Salmonella* induced ReA/uSpA.

In conclusion, our study shows that low molecular weight fractions of Omp contain T cell immunodominant antigens. The T cell immunogenicity of the novel antigens identified in our study need to be confirmed through the use of T cell clones using recombinant purified proteins. This will help in identifying the T cell immunodominant antigens which can be used for vaccine or for developing a diagnostic test for *Salmonella* induced ReA.

Table 1. Protein identified in immunodominant fractions of the Omp.

Sample ID (fraction no. -band no.)	Annotation	Accession no.	Database searched	Mol. wt	pI	Peptide matched	Sequence covered (%)	Protein score	% CI	No. of peptide for MS/MS	Amino acid position of peptides for MS/MS	Total ion score	Total ion score CI%
I-1	Outer membrane protein X	gi56414068	NCBI-nr	18.5	5.74	7	9.04	114	100	2	83-95, 157-171	42.47	99.98
I-2	Peptidoglycan-associated lipoprotein precursor	gi56414134	NCBI-nr	18.9	6.29	8	12.5	275	100	7	104-113, 114-125, 138-151, 114-126, 152-166, 83-97, 61-82	48	99.9
I-2	Outer membrane protein A	P02936	Swiss-Prot	37.6	5.6	6	2.1	79.59	99.99	3	322-332, 256-267, 268-281	42.47	99.98
I-3	Outer membrane protein W	Q8ZP50	Swiss-Prot	22.9	5.57	5	8.1	204	100	4	141-150, 188-198, 151-167, 117-140	166.2	100
II-1	Outer membrane protein W	Q8ZP50	Swiss-Prot	22.9	5.57	3	3.7	113	100	3	188-198, 151-167, 117-140	92.58	100
II-2	Outer membrane protein A	P02936	Swiss-Prot	37.6	5.6	6	3.03	129	100	2	104-117, 195-217	90.2	100
II-3	Outer membrane protein A	P02936	Swiss-Prot	37.6	5.6	6	3.64	157	100	2	104-117, 139-163	120.8	100
III-1	Putative outer membrane protein	gi29141633	NCBI-nr	27.9	5.51	9	8.68	190	100	4	235-247, 235-248, 126-148, 149-179	96	100
III-1	Putative outer membrane protein	gi56413728	NCBI-nr	28	5.51	9	9.6	265	100	5	235-247, 235-248, 126-148, 149-179, 41-62	170	100
III-1	Outer membrane protein A	P02936	Swiss-Prot	37.6	5.6	7	10.47	250	100	4	223-231, 104-117, 195-217, 139-163	198	100
III-2	Nucleoside-specific channel- forming protein tsx	P0A261	Swiss-Prot	32.8	4.96	10	14.8	452	100	8	263-273, 112-123, 192-203, 53-66, 53-67, 91-106, 170-187, 204-224	365	100
III-2	VacJ lipoprotein	gi56412711	NCBI-nr	28.2	5.14	7	1.27	107	100	4	29-37, 185-193, 205-214, 194-204	55	99.99
III-3	Outer membrane protein A	gi7188820	NCBI-nr	26.77	5.7	4	7.92	148	100	2	104-117, 195-217	123	100
III-3	Outer membrane protein A	P02936	Swiss-Prot	37.6	5.6	19	25.3	210	100	n.d.	n.d.	n.d.	n.d.
IV-1	Outer membrane protein A	P02936	Swiss-Prot	37.6	5.6	13	19.75	128	100	n.d.	n.d.	n.d.	n.d.
IV-2	Outer membrane protein A	P02936	Swiss-Prot	37.6	5.6	13	17.07	128	100	n.d.	n.d.	n.d.	n.d.
IV-3	Outer membrane protein A	P02936	Swiss-Prot	37.6	5.6	19	37.36	226	100	n.d.	n.d.	n.d.	n.d.
IV-4	Outer membrane protein D	P37592	Swiss-Prot	39.6	4.66	9	21.91	367	100	5	149-156, 325-333, 334-344, 105-115, 38-58	306.5	100
IV-4	Outer membrane protein A	P02936	Swiss-Prot	37.6	5.6	6	2.13	107	100	5	322-332, 268-281, 104-117, 195-217, 139-163	70.8	100
IV-4	Outer membrane protein S1	Q56110	Swiss-Prot	43.2	4.46	1	18.36	82	99.99	1	104-114	82.1	100

n.d.: not done.

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