Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

# Proteomic analysis of outer membrane proteins of *Edwardsiella tarda*

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**Keywords**
*Edwardsiella tarda,* matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry, outer membrane proteins.

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2009/1754: received 6 October 2009, revised 4 November 2009 and accepted 9 November 2009

doi:10.1111/j.1365-2672.2009.04627.x

## Abstract

**Aims:** The purpose of this study was to identify outer membrane proteins (OMPs) of *Edwardsiella tarda*.
**Methods and Results:** The OMPs from a virulent strain of *E. tarda* (ET-7) was extracted using lauroyl sarcosine method. The OMPs were analysed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), and protein spots were identified using matrix assisted laser desorption/ionization-time-of-flight mass spectrometry. A total of 21 proteins were identified from 24 protein spots observed on the 2D-PAGE gel. These proteins were identified as GroEL, antigenic proteins, ABC transporters, elongation factors, OmpA, PTSINtr with GAF domain, catalase C, glycolytic enzymes, DnaJ, transcriptional regulator, proteins mraZ and ccdA. Subcellular localizations, $\beta$-barrel OMPs and lipoproteins of identified proteins were predicted using PSORTb, PRED-TMBB and LipoP1.0 programme.
**Conclusions:** Identification, localization and possible functions of OMPs of *E. tarda* were studied.
**Significance and Impact of the Study:** These proteins could be used for development of novel drug targets, diagnostics or vaccine against edwardsiellosis.

## Introduction

*Edwardsiella tarda* is an important cause for haemorrhagic septicaemia in freshwater and marine fish species throughout the world. This organism is widely distributed in aquatic environments and has a broad host range, causing diseases in reptiles, amphibians, marine mammals and other warm-blooded animals including humans. In humans, it also causes gastro- and extra-intestinal infections (Austin and Austin 1999). Pathogenesis of *E. tarda* is multifactorial. Several potential virulence factors have been suggested to contribute to the pathogenesis of *E. tarda* (Srinivasa Rao *et al.* 2003).

The outer membrane proteins (OMPs) of bacteria have an important role in the interaction with hosts and in bacterial pathogenicity (Seltman and Holst 2002). In the present context, a complete genome sequence of *E. tarda* is not available; proteomic analysis of *E. tarda* OMPs may provide information necessary to understand the virulence factors and immunogenicity of the bacterium.

The most common approaches used in proteomic analysis are two-dimentional polyacrylamide gel electrophoresis (2D-PAGE) and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS). 2D-PAGE of proteins is currently the highest resolution analytical technique available for the study of protein profiles. 2D-PAGE along with mass spectrometry and bioinformatics is emerging as a powerful tool to study the proteins. MALDI-MS is an analytical technique that has already been proved to be a powerful method for the characterization and specific detection of various bacterial pathogen proteins (Jarman *et al.* 2000). Previously, MALDI-MS have been utilized for characterization of OMPs of fish bacterial pathogens, i.e. *Aeromonas salmonicida* (Ebanks *et al.* 2005); *Flavobacterium columnare* (Liu

*et al.* 2008) and *Edwardsiella ictaluri* (Dumpala *et al.* 2009).

Proteomics information on OMPs of *E. tarda* is very limited. Tan *et al.* (2002) have characterized extracellular proteins of *E. tarda* by 2D-PAGE and MALDI-TOF MS. A conserved 37 kDa OMP of Japanese isolates of *E. tarda* was characterized by 2D-PAGE and amino acid sequencing (Kawai *et al.* 2004). Verjan *et al.* (2005) identified seven antigenic proteins of *E. tarda*. Kumar *et al.* (2007) characterized the OMPs profiles of 16 isolates of *E. tarda*. Hou *et al.* (2009) have showed that EseD and Et18 antigen of *E. tarda* offer immunoprotection in Japanese flounder. Sakai *et al.* (2009) identified major antigenic proteins of *E. tarda* using hyper immune sera of Japanese flounder, and Jiao *et al.* (2009) identified potential antigens of *E. tarda* using *in vivo* induced antigen technology. The objective of this study was to identify OMPs of *E. tarda* by advance tools of proteomics.

## Materials and methods

### Bacterial strain

*Edwardsiella tarda* (ET-7) used in this study has been described in our previous research. Briefly, it was recovered from kidney of diseased snakehead (*Ophiocephalus punctatus*). It appeared as small, transparent colonies with minute black centre on Salmonella–Shigella agar medium. The isolate was identified by conventional biochemical tests, molecular techniques and 16S rRNA gene sequencing (Kumar *et al.* 2008). This strain is haemolysin positive and pathogenic for *Labeo rohita*. Further, OMPs profile of ET-7 has been characterized by SDS-PAGE and western blotting (Kumar *et al.* 2007), and it was also identified by using monoclonal antibodies (Kumar *et al.* 2009).

### Preparation of OMPs

The OMPs were obtained as per the method described by Bader *et al.* (2004). Briefly, *E. tarda* was cultured in brain heart infusion brain broth at 30°C. Bacterial cells were collected by centrifugation and then washed three times with chilled phosphate buffer saline. The pelleted bacteria were suspended in 0·01 mol $l^{-1}$ HEPES buffer with 1 mg DNase and RNase and then sonicated. Intact cells and large debris were removed by centrifugation at 6000 ***g*** for 15 min at 4°C. Supernatants were treated with lysozyme (0·1 mg $ml^{-1}$) and incubated at 37°C for 30 min. Supernatants were ultra-centrifuged at 41 000 ***g*** for 2 h at 4°C. The pellet was resuspended in 2·0% sodium lauroyl sarcosine and incubated at 25°C for 30 min to solubilize the inner membrane. The solution was then ultra-centrifuged at 41 000 ***g*** at 4°C for 2 h. The pellets containing OMPs were washed three times in cold sterile MQ distilled water and resuspended in rehydration buffer. Protein concentrations of OMPs were determined by the method of Bradford (1976).

### 2D-PAGE

Isoelectric focusing (IEF) was carried out using the method of 'in gel rehydration' with slight modifications (Gorg *et al.* 2000). An immobilized pH gradient (IPG) strip of pH 3–10 and length 17 cm (Bio-Rad, Hercules, CA, USA) was rehydrated overnight at 20°C with 500 $\mu$g proteins, which was mixed with 2D rehydration buffer. Strips were then focused on an IEF unit PROTEAN IEF Cell (Bio-Rad) at 20°C using the following four-step program: (i) 0–250 V for 2 h in linear mode; (ii) 250 V constant for 2 h in rapid mode; (iii) 250–5000 V for 4 h in linear mode; and (iv) 5000 V constant until 35 kVh is reached. The current limit was set at 50 $\mu$A per strip. After IEF, strip was equilibrated for 15 min in equilibration buffer I (6 mol $l^{-1}$ urea, 2% SDS (w/v), 0·375 mol $l^{-1}$ Tris; pH 8·8, 20% glycerol (v/v)) containing 130 m mol $l^{-1}$ DTT followed by equilibration buffer II containing 135 m mol $l^{-1}$ iodoacetamide instead of DTT for 15 min.

The OMPs were separated in second dimension on 12% SDS–PAGE gel (Laemmli 1970), which was made in casting cassette of 1-mm thickness. After 2 h of polymerization, the equilibrated IPG strip was placed on the dried surface of the gel and immobilized by adding 0·5% agarose solution containing bromophenol blue. Then the gels were placed in the vertical electrophoresis system (PROTEAN II XI; Bio-Rad). Running buffer was filled in upper and lower chambers, and electrophoresis was carried out at constant voltage of 200 V for 5–6 h. After the gel electrophoresis, gel was stained with coomassie brilliant blue R250 solution to visualize proteins and image of gel was acquired by Chemidoc (Model Universal Hood II; Bio-Rad, Segrate, Milan, Italy) using Quantity one software (Bio-Rad). The protein spots were also visualized using silver staining of the same gel.

### In-gel digestion with trypsin

The method described by Shevchenko *et al.* (1996) was followed. Major protein spots of interest were excised from the Coomassie Blue and silver stained 2D gel using spot picker (Model Investigator ProPic; Genomic Solutions Ltd, Coventry, UK) and collected in 96-well PCR plate. Digestion of proteins and spotting of peptides on MALDI target plate was carried out using protein digester (Model Investigator ProPrep, Genomic Solutions Ltd). Silver stained gel plugs were first treated with 50 $\mu$l of a freshly prepared 1 : 1 (v/v) mixture of the two

 

destaining reagents K3 [Fe(CN)6] and $Na_2S_2O_3$ dissolved in water for 30 min at room temperature. Then, all gel plugs (Coomassie Blue and silver stained) were treated two times (10 min each time) with 25 mmol $l^{-1}$ $NH_4HCO_3$–50% acetonitrile (ACN) (1 : 1). All destained gel plugs were then dehydrated by adding 100% ACN for 10 min and dried under vacuum. Then, the gel plugs were submerged with freshly prepared 10 mmol $l^{-1}$ DTT in 50 mmol $l^{-1}$ $NH_4HCO_3$ for 45 min at 56°C. After incubation, the excess liquid was removed and replaced quickly by the same volume of freshly prepared 55 mmol $l^{-1}$ iodoacetamide in 50 mmol $l^{-1}$ $NH_4HCO_3$ for 30 min and then dehydrated with 100% ACN. The dried gel pieces were rehydrated in 25 mmol $l^{-1}$ $NH_4HCO_3$ containing 0·02 $\mu$g $\mu l^{-1}$ of trypsin (Mass spectrometry grade; Promega) and incubated for 16 h at 37°C. The resulting peptides were extracted twice from the gel pieces using peptide extraction buffer (1 : 1 mixture of 70% ACN and 1% trifluoroacetic acid (TFA) and transferred to a new 96 well PCR plate.

### Mass spectrometric identification

Prior to mass spectrometric analysis, samples were desalted and concentrated on C-18 ZipTips (Millipore) using the manufacturer's protocol. ZipTips were eluted on MTP 384 target plate of MALDI-TOF with 2 $\mu$l of α-cyano-4-hydroxycinnamic acid (HCCA) saturated solution dissolved in 50% ACN, 0·2% TFA. Mass spectra of digested proteins were acquired on MALDI-TOF/TOF instrument (Model Autoflex II TOF/TOF50; Bruker Daltonik GmbH, Leipzig, Germany) in positive reflectron mode, in the detection range of 500–3000 *m/z*. External calibration to a spectrum, acquired for a mixture of peptides with masses ranging from 1046 to 2465 Da, was performed prior to acquisition. The proteolytic masses obtained were then processed through FLEX ANALYSIS ver. 2.4 program (Bruker Daltonik GmbH, Leipzig, Germany) for peak detection. Submission of peak lists to the database using the mascot search engine (http://www.matrixscience.com) to identify the proteins. Peptide mass tolerance was set to 50 with carbamidomethylcystein set as fixed modification, oxidation of methionine as variable modification and one missed cleavage site allowed. Most intense peaks in the each spectrum were selected for fragmentation by laser-induced dissociation. The MS/MS spectra were calibrated internally to the precursor ion mass and used for sequence specific search at mascot database (Matrix science). In addition, peptide mass fingerprint-based searches were carried out using only the set of peptide masses, in the same database without any constraints for isoelectric point (pI) and molecular mass. The whole procedure was repeated several times to ensure correct protein identification.

### Prediction of localization, β-barrel OMPs and lipoproteins

Subcellular localizations of identified proteins were predicted using PSORTb ver. 2.0.4 (http://psort.org). Transmembrane β-barrel OMPs in identified proteins were predicted using PRED-TMBB (http://biophycis.biol.uoa.gr/PRED-TMBB). Lipoproteins of identified proteins were predicted using LIPOP1.0 (http://cbs.dtu.dk/services/LipoP).

## Results

### 2D-PAGE

Ten protein spots were observed on coomassie blue stained 2D-PAGE gel and additional fourteen spots were observed in the same gel after silver staining. 2D-PAGE gel analysis was carried out within a pH range of 3–10, and we observed that most of the proteins were focused in the acidic pH range. The spot pattern of OMPs obtained after 2D-PAGE and Coomassie Blue and silver staining are depicted in Figs 1 and 2, respectively.

### MS or MS/MS identification

A total of 21 proteins were identified by MS analysis from 24 protein spots observed on 2D-PAGE gels. These

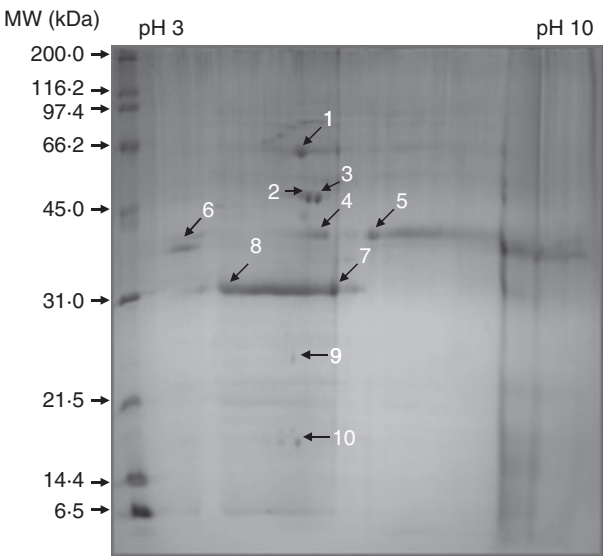


**Figure 1** 2D protein profiles of outer membrane proteins of *Edwardsiella tarda* on a Coomassie Blue stained gel.


 

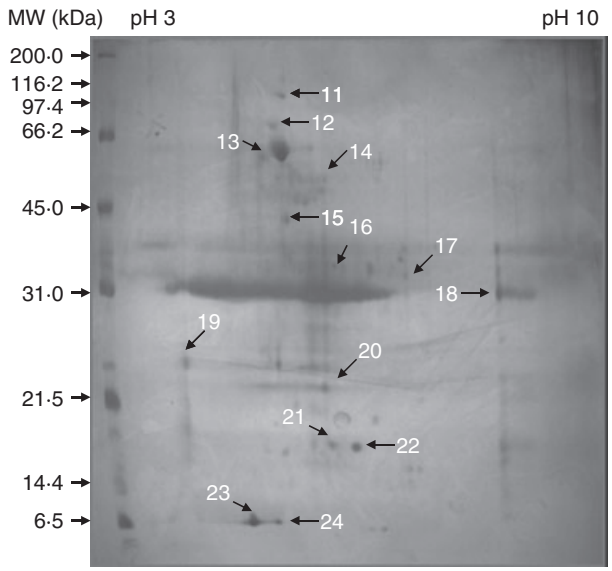


**Figure 2** 2D protein profiles of outer membrane proteins of *Edwardsiella tarda* on a silver stained gel.

proteins were identified as GroEL, antigenic proteins, ABC transporters, elongation factors, OmpA, PTSINtr with GAF domain, catalase C, glycolytic enzymes, DnaJ, transcriptional regulator, proteins mraZ and ccdA. One hypothetical protein (spot 7) was identified. Spots 19, 20 and 24 were unidentified. Details of identified proteins by MS analysis are given in Table 1. For further protein identification, we directly looked for sequence specific MS/MS-based database search whenever we were able to observe successful fragmentation of the most abundant peptide fragment in a MALDI-TOF MS, and the details of MS/MS analysis are given in Table 2.

### Prediction of localization, $\beta$-barrel OMPs and lipoproteins of identified proteins

Subcellular localization of 21 proteins was predicted by PSORTb. Out of 21 proteins, 1 protein (spot 10) was predicted as outer membrane, 11 proteins (spots 1, 5, 7, 8, 11, 13–16, 18 and 21) as cytoplasmic, 2 proteins (spots 6 and 9) as cytoplasmic membrane, spot 17 as periplasmic and localization of 6 proteins (spots 2, 3, 4, 12, 22 and 23) were unknown (Table 1). Ten proteins (spots 1–4, 6, 9–11, 17 and 23) were predicted as $\beta$-barrel OMPs by PRED-TMBB, and two proteins (spots 5 and 17) were predicted as lipoproteins by LipoP1.0.

## Discussion

Bacterial OMPs play a major role in suppressing the immune defence factors of the host and has a protective antigenicity (Lutwyche *et al.* 1995). 2D-PAGE and MALDI-TOF MS are important tools for protein identification of various bacterial pathogens. In the present study, OMPs of *E. tarda* were extracted, resolved and identified using proteomic tools. Spot 1 was identified as GroEL/Cpn60 protein, which prevents misfolding and promotes refolding of bacterial polypeptides under stress conditions. GroEL is also known as chaperone or heat shock protein (HSP). It may help in transporting proteins across bacterial cell membranes (Goulhen *et al.* 1998). Immunocytochemical experiments have also indicated that GroEL is involved in microbial pathogenicity and 66 kDa HSP of *Salmonella* enterica serotype *typhimurium*, GroEL of *Haemophilus ducreyi*, HSP60 of *Legionella pneumophila* and GroEL of *Clostridium difficile* are involved in interactions between the bacteria and host cells (Frisk *et al.* 1998). Cell surface localization of GroEL on OMPs of *A. salmonicida* has been also reported by Ebanks *et al.* (2005). GroEL of *E. tarda* has been identified as antigenic protein using hyper immune sera of Japanese flounder (Sakai *et al.* 2009). So, it is possible that GroEL of *E. tarda* is associated as a virulence factor during fish infection. Spot 14 was identified as HSP DnaJ, involved in protein folding and renaturation after stress. DnaJ chaperone machinery has been shown to be involved in bacterial invasion of epithelial cells and is essential for causing a systemic infection in the host (Takaya *et al.* 2004). It has been also identified on outer membrane of *Methylococcus capsulatus* by MS analysis (Berven *et al.* 2006). Therefore, it is not surprising to report the presence of DnaJ on the outer membrane of *E. tarda*.

Spots 2, 3 and 4 were identified as antigenic protein of *E. tarda*, also called as flagellin protein. All the three spots had a similar molecular weight (43·77 kDa), pI and amino acid sequences. In MS/MS analysis, most of the peaks in spots 2 and 3 were identified as antigenic proteins of *E. tarda*. However, spot 4 showed extra OmpC porin protein along with antigenic proteins. Our previous results by SDS–PAGE and western blotting indicate that this band (44 kDa protein) was common and predominant immunogenic band in all 16 isolates of *E. tarda* (Kumar *et al.* 2007). In a similar study, Kumar *et al.* (2009) have raised MAbs to 44 kDa OMP of *E. tarda*, and demonstrated their reactivity to whole bacterial cells. Both the studies clearly indicate that these proteins are located on outer membrane of *E. tarda*. Antigenic proteins of *E. tarda* have been reported as normal components of the bacterial cell wall and are involved in aromatic amino acid, sugar and probably nucleotide uptake, stress response and motility (Verjan *et al.* 2005). This protein of *E. tarda* has been also implicated in bacterial pathogenesis with regard to adhesion,

 

**Table 1** Outer membrane proteins of *Edwardsiella tarda* identified by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) analysis

| Spot No. | Protein identified | Accession number | Mascot Score | Nominal mass (Da) | pI | Sequence coverage (%) | Function | Localization |
|---|---|---|---|---|---|---|---|---|
| 1 | GroEL protein | O66198 | 84 | 56 421 | 4·84 | 28 | Protein folding | Cytoplasmic |
| 2 | Antigenic protein | Q3V737 | 99 | 43 774 | 5·20 | 34 | Pathogenesis | Unknown |
| 3 | Antigenic protein | Q3V737 | 80 | 43 774 | 5·20 | 32 | Pathogenesis | Unknown |
| 4 | Antigenic protein | Q3V737 | 56 | 43 774 | 5·20 | 34 | Pathogenesis | Unknown |
| 5 | Glyceraldehyde 3-phosphate dehydrogenase | AP_002398 | 65 | 39 162 | 6·01 | 27 | Glycolysis | Cytoplasmic |
| 6 | ABC transporter | Q81JQ2 | 54 | 34 491 | 5·76 | 28 | Export or import of substances | Cytoplasmic membrane |
| 7 | Hypothetical protein, oxidoreductase | POAG85 | 38 | 31 639 | 6·32 | 24 | Unknown | Cytoplasmic |
| 8 | Elongation factor Ts | Q831V0 | 36 | 32 113 | 4·87 | 20 | Translation | Cytoplasmic |
| 9 | ABC transporter | Q881A6 | 46 | 29 240 | 5·61 | 24 | Export or import of substances | Cytoplasmic protein |
| 10 | OmpA | Q4AT92 | 38 | 19 866 | 5·40 | 12 | Structural stability of outer membrane and conjugation | Outer membrane |
| 11 | PTSINtr with GAF domain | Q2J422 | 54 | 82 086 | 5·92 | 32 | Channel forming protein | Cytoplasmic |
| 12 | Catalase C | Q9X576 | 41 | 78 693 | 5·81 | 10 | Defense mechanism | Unknown |
| 13 | Glucose-6-phosphate isomerase | Q9KUY4 | 50 | 60 709 | 5·65 | 16 | Glycolysis | Cytoplasmic |
| 14 | Heat shock protein DnaJ | YP_839472 | 52 | 42 398 | 5·78 | 21 | Protein folding | Cytoplasmic |
| 15 | L-lactate dehydrogenase | Q87G18 | 36 | 41 487 | 6·04 | 16 | Glycolysis | Cytoplasmic |
| 16 | Elongation factor Ts | Q9X5Z9 | 40 | 29 989 | 5·50 | 19 | Translation | Cytoplasmic |
| 17 | Beta-lactamase CTX-M-3 | O33807 | 32 | 31 292 | 7·86 | 9 | Antibiotic resistance | Periplasmic |
| 18 | Formamidopyrimidine-DNA glycosylase | Q88CQ5 | 30 | 30 387 | 9·13 | 10 | DNA repair | Cytoplasmic |
| 21 | Transcriptional regulator slyA | P0A8W3 | 35 | 16 400 | 6·60 | 15 | Hemolysin activator | Cytoplasmic |
| 22 | Protein mraZ | Q4A666 | 26 | 17 080 | 7·82 | 10 | Cell wall biosynthesis | Unknown |
| 23 | Protein ccdA | P62553 | 47 | 8367 | 5·02 | 62 | Synthesis of cytochrome c | Unknown |

**Table 2** Outer membrane proteins of *Edwardsiella tarda* identified by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS)/MS analysis

| Spot No. | Peak mass (Da) | Protein identified | Mascot score | Nominal mass (Da) | pI | Sequence coverage for the PMF results |
|---|---|---|---|---|---|---|
| 1 | 1553·846 | 60 kDa chaperonin | 97 | 56 421 | 4·84 | AAVEEGVVAGGGVALVR |
| | 1845·910 | 60 kDa chaperonin | 119 | 56 421 | 4·84 | DTTTIIDGVGEEAAIQGR |
| | 2401·284 | 60 kDa chaperonin | 126 | 56 421 | 4·84 | ANDAAGDGTTTATVLAQAIVNEGLK |
| 2 and 3 | 1699·715 | Antigenic protein Et 46 | 78 | 43 774 | 5·20 | IQDADYATEVSNMSR |
| | 2065·009 | Antigenic protein Et 46 | 149 | 43 774 | 5·20 | IQDADYATEVSNMSR |
| | 2719·480 | Antigenic protein Et 46 | 216 | 43 774 | 5·20 | LTVQAQNGSNSSSDLQSIQDEITQR |
| | 2884·581 | Antigenic protein Et 46 | 170 | 43 774 | 5·20 | NANDGISLAQTTEGALNEVNDNLQNIR |
| 4 | 1263·513 | Outer membrane protein C, porin | 42 | 41 038 | 4·83 | FADYGSLDYGR |
| | 2065·009 | Antigenic protein Et 46 | 149 | 43 774 | 5·20 | IQDADYATEVSNMSR |
| | 2719·480 | Antigenic protein Et 46 | 216 | 43 774 | 5·20 | LTVQAQNGSNSSSDLQSIQDEITQR |
| 16 | 1534·674 | Elongation factor Ts | 45 | 31 732 | 5·18 | IAAEGVISGFVTADGK |
| | 1579·730 | Putative LysR-family transcriptional regulator | 35 | 31 750 | 5·57 | AVVALASADAGVALVPR |
| 23 | 1320·456 | Protein ccdA | 22 | 8367 | 5·02 | AENQEGMAEVAR |

motility and in phase variation (Tan *et al.* 2002). The 43·77 kDa OMP band could be further evaluated as candidate antigen for the development of a new vaccine or could be used as an antigen for detection of *E. tarda* infection in fish by immunoassays.

In MS, Spot 5 having a molecular weight of 39 kDa was identifed as GAPDH that catalyses the conversion of glyceraldehydes-3-phosphate into 1,3-bisphosphoglycerate. Previous study also showed that 37 kDa OMP of *E. tarda* is highly homologous to GAPDH (Kawai *et al.* 2004). This indicates that protein homologous to GAPDH is also present in *E. tarda* of Indian origin. This protein has been shown to be antigenic as well as protective in nature. Liu *et al.* (2005) reported that recombinant GAPDH protein offered protection to Japanese flounder against *E. tarda* infection. Sakai *et al.* (2009) also reported that GAPDH of *E. tarda* is antigenic protein for Japanese flounder. Therefore, it is suggested that GAPDH could be further evaluated as vaccine candidate against *E. tarda* in Indian major carps. Spots 13 and 15 were also identified as two other glycolytic enzymes, i.e. glucose-6-phosphate isomerase that catalyses the conversion of glucose-6-phosphate into fructose 6-phosphate and L-lactate dehydrogenase that catalyses the conversion of pyruvate into lactate. Outer surface localizations of glycolytic enzymes, i.e. GAPDH, phosphoglycerate kinase and enolase have been reported in OMPs of *E. tarda* and *A. salmonicida* (Kawai *et al.* 2004; Ebanks *et al.* 2005).

Bacterial outer membrane contain channel-forming proteins that involve in the export or import of a wide variety of substrates across extra and intracellular membranes, including metabolic products, lipids, sterols and waste products. Spots 6 and 9 were identified as ABC transporter proteins. Spot 10 was identified as OmpA, which is one of the major surface antigens of Gram-negative bacteria. It is required for bacterial conjugation and for maintaining the structural stability of the outer membrane (Ried and Henning 1987). Sakai *et al.* (2009) have reported that OmpA is also an antigenic protein of *E. tarda* and may play some role in pathogenesis.

One hypothetical protein (spot 7) was identified in the outer membrane but its function is not yet known. Spots 8 and 16 were identified as elongation factor Ts (EF-Tu), which is required for the chain elongation step in protein synthesis. Ebanks *et al.* (2005) have also demonstrated the surface localization of elongation factor Tu and Ts in the OMPs of *A. salmonicida* using MS analysis. Spot 12 was identified as catalase, which is an important enzyme required for overcoming the harmful effect of $H_2O_2$. Catalase has been predicted to be present on outer membrane surface of *Helicobacter pylori* and presented as an immunoreactive protein on the cell surface (Baik *et al.* 2004). Therefore, it may possible that catalase may play a role as a virulence factor in pathogenesis of *E. tarda*. Spot 17 was identified as beta-lactamase CTX-M-3 precursor, which is responsible for resistance to beta-lactam antibiotics like penicillins, cephalosporins, cephamycins and carbapenems and decreases the permeability of OMP. Studies have shown that expression of CTX-M-lactamase in *Escherichia coli* and *Klebsiella pneumoniae* results in decreased expression of OmpC and OmpK (Girlich *et al.* 2009). In our study, the presence of CTX-M-lactamase may apparently be the reason for absence or reduced expression of these proteins in the OMP profile of *E. tarda*. Liu *et al.* (2008) have also reported the presence of penicillin-binding protein in the outer membrane of *F. columnare*.

Spot 18 was identified as formamidopyrimidine DNA glycosylase, which is a bifunctional base excision repair enzyme that removes a wide range of oxidized purines from correspondingly damaged DNA (Serre *et al.* 2002). The localization of this enzyme is predicted to be cytoplasmic. However, Verjan *et al.* (2005) reported amino acid sequence similarity of antigenic protein 32 and antigenic protein 76 of *E. tarda* to glycosylase of *Yersinia pestis*. Therefore, it indicates that glycosylase may be antigenic or located on outer surface of *E. tarda*.

Spot 21 was identified as transcriptional regulator slyA, which is capable of activating the expression of the novel haemolysin. SlyA is directly or indirectly involved in the regulation of flagellin, iron and OMPs (Elliott *et al.* 1998). Haemolysin activator protein has been also reported on outer membrane surface of *M. capsulatus* (Berven *et al.* 2006). Further work can be undertaken to explore the role of SlyA gene in promoting haemolysin production in *E. tarda*.

Spot 22 was identified as MraZ protein, required for both cell wall biosynthesis and cell division. The MraZ family of proteins, also referred to as the UPF0040 family, is highly conserved in bacteria (Adams *et al.* 2005). Spot 23 was identified as ccdA protein involved in synthesis of cytochrome C. Synthesis of cytochrome C occurs on the outer surface of the cytoplasmic membrane in bacteria (Kranz *et al.* 1998).

Subcellular localizations, $\beta$-barrel OMPs and lipoproteins of identified proteins were predicted using PSORTb, PRED-TMBB and LipoP1.0 programme. Out of 21 identified proteins, ten proteins (spots 1–4, 6, 9–11, 17 and 23) were predicted as $\beta$-barrel OMPs and two proteins (GAPDH and beta-lactamase CTX-M-3) were predicted as lipoproteins. Out of them, one protein (beta-lactamase CTX-M-3) may be lipoprotein with integral $\beta$-barrel. The location of some of the proteins (antigenic proteins, catalase, mraZ and ccdA proteins) could not be predicted by PSORTb. These proteins may be localized on the outer membrane because of their functional characteristics.

 

Some of the identified proteins were predicted to be cytoplasmic (heat shock proteins, elongation factors, glycolytic enzymes, glycosylase and transcriptional regulator slyA), which could be because of cytoplasmic contamination during outer membrane preparation. It has been well documented that cytoplasmic, periplasmic and inner membrane proteins are present as contamination in OMPs preparation (Baik *et al.* 2004; Ebanks *et al.* 2005; Liu *et al.* 2008). The association of cytoplasmic proteins with outer membrane of *E. tarda* might provide new insights into the biological and pathogenic roles of these proteins in *E. tarda* infection.

In conclusion, identified proteins of OMPs could provide basic information for understanding the pathogenicity of *E. tarda* and would be useful for development of novel drug targets, diagnostics or vaccine against edwardsiellosis.

## Acknowledgement

The authors are thankful to Dr V.M. Katoch, Director General and Secreatry, Indian Council of Medical Research, New Delhi, India and Dr K. Venkatesan, Scientist 'F' for providing necessary facilities at National JALMA Institute for Leprosy & Other Mycobacterial Diseases, Agra, India. We also are thankful to Dr W.S. Lakra, Director, National Bureau of Fish Genetic Resources, Lucknow, India for their encouragement in the research work.

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