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3 # Lipoproteins from *Clostridium perfringens* and their protective efficacy
4 in mouse model

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ABSTRACT

Clostridium perfringens is an obligately anaerobic rod-shaped bacterium and etiological agent for several diseases in humans and animals. The pathogen has been listed as Validated Biological Agent and warrants development of medical countermeasures. The homologs of some of the lipoproteins identified from various fractions of *C. perfringens* in our previous studies were observed to be virulence determinants in other pathogenic bacteria. Three putative virulence associated lipoproteins; polysaccharide deacetylase family protein, probable ion-uptake ABC transporter, and a putative lipoprotein of no known function are reported here with respect to their immuno-protective potentials. The three proteins were over expressed and purified to near homogeneity. The lipoproteins were shown to be exposed on the *C. perfringens* surface and, hence, accessible to antibodies and potentially visible to the host immune system. Immunization of mice with purified recombinant proteins elicited protective immunity against challenge with *C. perfringens* in mouse gas gangrene model. Distribution and relationship of orthologous proteins across other bacterial select agents especially among the members of *Firmicutes*, was carried out to look for conserved antigenic determinants. 27–39

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44 ## 1. Introduction

45 *Clostridium perfringens* is an obligately anaerobic rod-shaped bacterium and the five toxinotypes of the pathogen are etiological agent for several diseases in humans and animals. Besides its medical and veterinary significance, *C. perfringens* has been listed as Validated Biological Agent (Ecker et al., 2005). The organism produces several toxins for disease manifestation and on the basis of their differential production, the species has been divided into five toxinotypes (A–E) (Petit et al., 1999). *C. perfringens* Type A strains cause gas gangrene and gastrointestinal diseases in humans (food poisoning, antibiotic-associated diarrhea, sporadic diarrhea, sudden infant death syndrome), apart from those responsible for diarrhea in animals (Titball and Rood, 2001). Gas gangrene is the most destructive of all clostridial diseases and is characterized by rapid destruction of tissue with production of gas (Stevens et al., 1997). The disease resulted in death of hundreds of thousands of soldiers as a result of battlefield injuries during the two World Wars (approximately 1% of wounded personnel during World War II and 10% of wounded personnel during World War I) and *C. perfringens* was recognized as the most important causal organism (MacLennan, 1943). 45–64

It is extremely difficult to treat gas gangrene as the infection advances rapidly and becomes established in tissues that are deprived of blood. During the two World Wars, antisera raised against toxoids of all five species of clostridia associated with gas gangrene were shown to be of some therapeutic value if administered soon after trauma. Vaccine development against gas gangrene has received little attention, however, active immunization using inactive toxin was demonstrated to yield positive results in animal model (Titball et al., 1998; Stevens et al., 2004). Role of non-toxin protein antigens for bacterial disease presentation, especially at the stage of colonization has been recently recognized (Drudy et al., 2004; Sengupta et al., 2010). 65–76

Much attention has recently focused on the development of protein-based vaccines with the potential advantages of being antigenically conserved across strains, ability to induce long-lasting memory responses, and relatively inexpensive to produce by recombinant DNA techniques. Surface outer membrane proteins including lipoproteins have been of interest for understanding the colonization of infectious diseases and vaccine development. Some of these surface proteins play vital role in early stages of attachment and host colonization and facilitate the progression of host–pathogen interactions (Gardiner et al., 2014). 77–86

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Bacterial lipoproteins are known to be involved in iron uptake (Reidl and Mekalanos, 1996), adhesion (Odenbreit et al., 1999), cytokine stimulation in host (Ma and Weis, 1993), and induction of proliferation and immunoglobulin production in mouse B cells (Honarvar et al., 1994). Moreover, lipoproteins serve as a pathogen-associated molecular patterns (PAMPs) for pathogenic bacteria and initiate an immune response via TLR2 (Thakran et al., 2008). The conserved structure of PAMPs activate macrophages, monocytes, and neutrophils to induce production of cytokines and low molecular mass mediators, resulting in initiation of the innate immune response (O' Brien et al., 2005). Several lipoproteins have been reported as virulence determinant and/or vaccine target candidates. Lipoproteins are often shown to be protective in vaccinated animals against diseases caused by several bacterial pathogens including *Leptospira* sp. (Gardiner et al., 2014), *Streptococcus pneumoniae* (Whalan et al., 2006), *Streptococcus suis* (Aranda et al., 2012), *Francisella tularensis* (Sjöstedt et al., 1992), *Helicobacter pylori* (Keenan et al., 2000), *Pseudomonas aeruginosa* (Finke et al., 1990), *Borrelia burgdorferi* (Steere et al., 1998), *Staphylococcus aureus* (Mishra et al., 2012), *Neisseria meningitides* (Masignani et al., 2003).

In our previous proteomic elucidation of extracellular and surface protein fractions of *C. perfringens*, several proteins were found to be lipoprotein either by annotation in the database or by *in silico* prediction (Alam et al., 2009; Sengupta et al., 2010). For instance, polysaccharide deacetylase identified in our study has been shown to be a virulence factor in *S. pneumoniae* and *Bacillus anthracis* (Vollmer and Tomasz, 2002; Balomenou et al., 2013). Similarly, the ATP-binding cassette (ABC) transporters play a key role in maintenance of bacterial fitness and are important for bacterial pathogenesis (Hammerschmidt, 2009). The importers of gram-positive bacteria are part of multi-domain structure of ABC transporters and are synonymous to periplasmic solute binding protein (SBP) of gram-negative bacteria. They are anchored to the cell exterior via lipid groups conferring affinity, specificity, and directionality to the importers. In Gram-positive bacteria SBPs are processed as extracellular surface lipoproteins (Mariotti et al., 2013) and function by binding a wide range of solutes, including oxyanions, amino acids, peptides, carbohydrates and siderophores. ABC transporters associated with the uptake of metal ions have been shown to play key roles in bacterial virulence including attachment of some pathogenic bacteria to host cells (Garmory and Titball, 2004).

The homologs of some of the lipoproteins identified from various fractions of *C. perfringens* in our previous studies (Alam et al., 2009; Sengupta et al., 2010) were observed to be virulence determinants in other pathogenic bacteria. Three putative virulence associated lipoproteins; polysaccharide deacetylase family protein, probable ion-uptake ABC transporter, and a putative lipoprotein of no known function are reported here with respect to their immuno-protective potentials. Immunization of mice with purified recombinant proteins showed that they are capable of eliciting protective immunity against challenge with *C. perfringens* in mouse model. Given that these proteins are important for pathogenesis, we also sought to determine the distribution and relationship of orthologous proteins across other bacterial select agents especially among the members of *Firmicutes*.

2. Materials and methods

2.1. Bacterial strain and growth conditions

C. perfringens ATCC 13124 was obtained from Becton Dickinson India Pvt. Ltd., India. Cells were anaerobically grown in thioglycolate broth (TGB) containing 5 g of yeast extract, 15 g of tryptone, 5.5 g of glucose, 0.5 g of sodium thioglycolate, 2.5 g of sodium chloride, 0.5 g of L-cystine, 0.001 g of resazurin, 0.75 g of agar, and 1000 ml of distilled water. On some occasion, cells were also grown in TPYG broth containing 30 g of pancreatic digest of casein, 5 g of yeast extract, 20 g of glucose, 1 g of sodium thioglycollate, 250 mg of cycloserine, 76 mg of sulfamethoxazole, 4 mg of trimethoprim, and 1000 ml of distilled water. The culture was maintained at 4 °C in cooked meat medium (CMM) containing 454 g of beef heart infusion, 20 g of proteose peptone, 2 g of dextrose, 5 g of sodium chloride, and 1000 ml of distilled water. The media were procured either from Oxoid Ltd., England, or from Difco Laboratories, France, and were prepared anaerobically by standard methods using a gassing manifold and serum vials as described earlier (Sengupta et al., 2010). For preparation of the exoproteome or total cellular protein, *C. perfringens* cells were also grown in RPMI 1640 broth medium at 37 °C under anaerobic conditions as described previously (Shimizu et al., 2002). For the above purpose, cells were also grown in reinforced clostridial medium (RCM) containing 3 g of yeast extract, 10 g of beef extract, 10 g of peptone, 5 g of dextrose, 1 g of soluble starch, 5 g of NaCl, 3 g of sodium acetate, 5 g of cystein HCl, and 1000 ml of distilled water. The three lipoproteins were cloned in *Escherichia coli* JM107 cells and further sub-cloned in *E. coli* strain BL21-DE3 (Novagen, Germany). Single bacterial colony of *E. coli* strains were taken from the culture grown on Luria Bertani (LB) agar and grown in LB broth using ampicillin (final concentration of 100 μg/ml) for vector pTZ57R/T (Fermentas) and *E. coli* JM107 cells and kanamycin (final concentration of 25 μg/ml) for pET 28 'a' vector and *E. coli* BL21-DE3 strain.

2.2. Amplification, cloning, expression and purification of recombinant lipoproteins

Total DNA was extracted from *C. perfringens* ATCC 13124 according to the procedure of Marmur (29). Based on the published genome sequence, oligonucleotide primers were designed to amplify open reading frames (ORFs) of polysaccharide deacetylase (CPF_0823), ABC transporter (CPF_0440), and putative lipoprotein (CPF_1780). The details regarding primers, PCR conditions and target genes are described in Supplementary Table 1. Purified amplicons were first cloned into a TA cloning vector pTZ57R/T (Fermentas) according to the manufacturer's instructions. The ligated products were transformed into *E. coli* JM107 cells. Plasmids were isolated from 10 randomly selected clones and were tested for the presence of the insert by size determination on an agarose gel (1.5%) and PCR amplification of the target gene.

Recombinant plasmids were double digested with corresponding restriction enzymes to obtain insert and the genes were sub-cloned into expression vector pET 28 'a' (Novagen, Germany). Two clones positive for the insert were subjected to double-pass sequencing to check for possible mismatches using an in house automated sequencer (ABI Prism, model 3730, USA). The expressed recombinant proteins were induced for 4 h at 37 °C in 250-ml LB medium cultures containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The over-expressed recombinant proteins were purified to near-homogeneity by using Ni–NTA Sepharose resin (Qiagen, Germany) according to the manufacturer's instructions. The eluted proteins were dialysed against 50 mM Tris–HCl (pH 8.0) buffer, quantified, and stored in aliquots at −70 °C till further use. The quantitation of protein was carried out according to the method of Bradford (1976) by using the Quick Start Bradford protein assay kit (Bio-Rad) according to the manufacturer's instructions. The protein concentration was calculated using bovine serum albumin (BSA) as the standard. The purified recombinant proteins were further confirmed by MS/MS analysis of tryptic digests using in house MALDI-TOF-TOF instrument (AB Sciex, USA) as described earlier (Kumar et al., 2013).

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