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Exoproteome analysis of a novel strain of *Bacillus cereus* implicated in disease resembling cutaneous anthrax

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ABSTRACT

Bacillus cereus belongs to *B. cereus sensu lato* group, shared by six other related species including *Bacillus anthracis*. *B. anthracis* is the causative agent for serious illness affecting a wide range of animals as well as humans and is a category A Biological and Toxin Warfare (BTW) agent. Recent studies indicate that a *Bacillus* species other than *B. anthracis* can cause anthrax-like disease and role of anthrax virulence plasmids (pXO1 and pXO2) on the pathogenicity of *B. cereus* has been documented. *B. cereus* strain TF5 was isolated from the tissue fluid of cutaneous anthrax-like skin lesions of a human patient from an anthrax endemic area in India. The strain harboured a PA gene, however, presence of pXO1 or pXO2-like plasmids could not be ascertained using reported primers. Abundant exoproteome of the strain in the early stationary phase was elucidated using a 2-DE MS approach and compared with that from a reference *B. cereus* strain. Analysis of proteins showing qualitative and quantitative differences between the two strains indicated an altered regulatory mechanism and putative role of S-layer protein and sphingomyelinase in the pathogenesis of strain TF5. Phylogenetic analysis of the S-layer protein indicated close affiliation of the strain with anthracis-like *B. cereus* strains such as *B. cereus* var. anthracis strain CI; whereas sphingomyelinase exhibited specific relationship with all the strains of *B. anthracis* apart from that with anthracis-like *B. cereus* strains.

1. Introduction

Anthrax is caused by the Gram-positive spore-forming bacterium *Bacillus anthracis* and is a serious illness affecting a wide range of domestic and wild animals, as well as humans. Apart from other criteria, the stability of spores makes *B. anthracis* a category A Biological and Toxin Warfare (BTW) agent and concern for the disease caused by this organism has grown in the shadows of bioterrorist attacks such as those in Japan in 1993 and the United States in 2001. Cutaneous anthrax normally occurs through skin contact with infected animals or animal products while the disease manifests in different forms through ingestion or inhalation route of exposure, the latter being envisaged mode of infection in a bioterrorism or warfare scenario. In cutaneous form, infection results in lesions at the points of contact and oedema, often followed by meningitis or sepsis. The septic phase progresses rapidly and results in death within a few hours (Turnbull, 2008). The pathogenicity of the species *B. anthracis* is largely governed by the two plasmids pXO1 and pXO2, which encode the tripartite toxin and the poly-c-D-glutamic acid capsule, respectively. In *B. anthracis*, chromosomally encoded and plasmid-encoded genes are linked by regulatory mechanisms; some chromosomal genes have been shown to be regulated by the plasmid-encoded regulator AtxA (Mignot et al., 2003).

B. anthracis belongs to *Bacillus cereus sensu lato* group, shared by six other species including *B. cereus*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis*, and *Bacillus cytotoxicus* (Soufiane and Cote, 2009). The *B. cereus* group includes several closely related species that are divergent phenotypically and pathogenically and in some cases these differences are attributed to the presence of virulence plasmids rather than chromosomal differences (Salter, 2011). The species specific idiosyncrasies of *B. anthracis*, like the toxins and capsule are plasmid-encoded; however, other virulence related features like hemolysis, motility, and resistance to antibiotics are encoded on the chromosome (Rasko et al., 2005). Further, strains of *B. anthracis* are typically non-motile, characterized by the presence of four prophages, and a nonsense mutation in the plcR regulator gene that regulates the expression of at least 45 genes including many virulence determinants.

Recent studies indicate that a member of *B. cereus* group other than *B. anthracis* can cause anthrax-like disease and the effect on

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the pathogenicity of *B. cereus* due to acquisition of anthrax virulence plasmids (pXO1 and pXO2) by transformation, has been documented (Han et al., 2006; Hernandez et al., 1998, 1999; Hoffmaster et al., 2004, 2006; Klee et al., 2006, 2010; Leendertz et al., 2004, 2006). For instance, *B. thuringiensis* ser konkukian was shown to cause tissue necrosis and *B. cereus* strain E33L was the causative agent of anthrax-like disease in zebras; though the two strains did not possess any of the virulence plasmids (pXO1 or pXO2) of *B. anthracis* (Hernandez et al., 1998, 1999; Han et al., 2006). Other examples of atypical virulent strains causing a disease similar to inhalation anthrax-like disease with plasmid-encoded virulence factors include *B. cereus* G9241 that harbors a pXO1-like plasmid (pBCXO1) and a second plasmid (pBC210) encoding a polysaccharide capsule (Hoffmaster et al., 2004). The strain was identified as the causative agent of a fatal disease similar to inhalation anthrax (severe pneumonia) in a Louisiana welder in 1994. Another strain *B. cereus* 03BB102 that possesses a plasmid (p03BB102_179) containing both the anthrax toxin and capsule biosynthesis genes, was reported by the same group a few years later (Hoffmaster et al., 2006).

Klee et al. (2010) reported the complete genome sequence of a *Bacillus* strain isolated from a chimpanzee that had died with clinical symptoms of anthrax. The strain was designated *B. cereus* variety (var.) anthracis strain CI and differed from typical *B. anthracis* strains in that it was motile and lacked the four prohages and the nonsense mutation. It exhibited a mosaic genomic structure and a close genetic affiliation to *B. thuringiensis* ser konkukian strain 97–27 and *B. cereus* E33L than to *B. anthracis* strains; plasmids observed were identical to the anthrax virulence plasmids pXO1 and pXO2. Twelve genomic islands were identified in *B. cereus* var. anthracis strain CI which encoded genes absent in some or all of the compared strains; six of these regions were co-localized with genes correlated to mobile genomic elements i.e. integrases, recombinases, and transposases. Three of the islands were unique to *B. cereus* var. anthracis strain CI and were possibly strain specific. The authors suggested that the "bacterium has evolved from a *B. cereus* strain independently from the classic *B. anthracis* strains and established a *B. anthracis* lifestyle".

With the prevalence of virulent anthrax-like strains possibly arising from multiple nonpathogenic ancestors, it is tempting to believe that origins of pathogenicity in *Bacillus* group is primarily due to the events of horizontal gene transfer (HGT) of toxin containing plasmids. However, another hypothesis proposes that "multiple historical origins of pathogenic strains have occurred because of existing pre-adaptations or newly arising adaptive changes in the genomes of nonpathogenic ancestors" (Zwick et al., 2012). It is postulated that "the most evolutionarily flexible portions of the bacterial genome are regulatory sequences and transcriptional networks" (Scott and Dyer, 2012). Emerging and atypical strains in *B. cereus* group, causing anthrax-like disease in humans and animals detected in different regions of globe, are matter of deep concern from both public health and bioterrorism point of view, as the disease they cause are eventually misdiagnosed using standard microbiological methods and the strains are likely to evade detection methodologies using classical targets. Here we report proteomic characterization of a novel *B. cereus* strain TF5 isolated from a patient showing anthrax-like symptoms. Strain TF5 was identified as *B. cereus* using 16S rRNA gene sequencing and other diagnostic features; however, using primers pXO1-110 (Hoffmaster et al., 2006), PA gene could be amplified from the total DNA of the strain. The exoproteome of this novel and atypical strain of *B. cereus* was compared with that of a reference *B. cereus* strain (MTCC430) in order to elucidate putative extracellular effectors responsible for the disease manifestation.

2. Materials and methods

2.1. Ethical and safety considerations

Animal experiments were approved by the institutional Animal Ethical Committee at DRDE, Gwalior and all efforts were made to minimize suffering of mice. All the experimentations with virulent strains were carried out in a high containment facility (biosafety level 3) under strict safety guidelines and the work was approved by institutional biosafety committee at Defence Research and Development Establishment (DRDE), Gwalior, India.

2.2. Bacterial strain and growth conditions

B. cereus strain TF5 was isolated from tissue fluid of a patient showing cutaneous lesions that resembled cutaneous anthrax. Isolation was carried out during a surveillance study for cutaneous anthrax in the anthrax endemic tribal area of Paderu, Vishakhapatnam, India. *B. cereus* MTCC430, a reference strain of *B. cereus*, was obtained from Institute of Microbial Technology, Chandigarh, India. Non-pathogenic *B. anthracis* Sterne strain was procured from Institute of Veterinary and Preventive Medicine, Ranipet, Vellore, India. The bacterial strains were routinely grown at 37 °C on Brain Heart Infusion (BHI) agar containing calf brain infusion, 12.5 g; beef heart, 5 g; peptone, 10 g; NaCl, 5 g; D-glucose, 29 g; Na_2HPO_4, 2.5 g; agar, 15 g; and distilled water 1 L.

2.3. Preparation of extracellular protein fraction

Extracellular protein was prepared from *B. cereus* strain TF5 and *B. cereus* MTCC430 after growing the cells at 37 °C in BHI broth medium dialysed against distilled water prior to autoclaving in order to remove larger media proteins and other macromolecules. After growth till early stationary phase, cells were harvested by centrifugation at 10,000*g* for 10 min at 4 °C. The extracellular proteins in the supernatant were concentrated and purified as described earlier (Sengupta et al., 2010). Briefly, protein was precipitated with 10% (wt/vol) trichloroacetic acid overnight at 4 °C and centrifuged at 10,000*g* for 10 min at 4 °C. The resulting protein precipitate was washed twice with cold acetone containing 0.07% β-mercaptoethanol, air-dried, and stored at −80 °C until use. Total protein concentration was determined according to the method of Bradford (1976) using Quick Start Bradford Protein Assay kit (Bio-Rad, USA) as per manufacturer's instructions. The protein concentration was calculated using bovine serum albumin (BSA) as standard.

2.4. Two dimensional gel electrophoresis (2-DE)

2-DE was performed as described earlier (Sengupta et al., 2010). The protein pellet was resuspended in sample rehydration buffer (8 M urea, 2% w/v CHAPS, 15 mM DTT and 0.5% v/v IPG buffer pH 3–10). The isoelectric focusing was performed using immobilized pH gradient (IPG) strips (Bio-Rad, USA) of 7 cm size with a pH range from 4 to 7. For the first dimension 500 μg of protein samples in 150 μl of rehydration solution was used to passively rehydrate IPG strips overnight. The proteins were then focused for 10,000 VHr at 20 °C under mineral oil on a Protean IEF Cell (Bio-Rad, USA). After focusing, the strips were incubated for 10 min, in 2 ml of equilibrium buffer I (6 M urea, 30% w/v glycerol, 2% w/v SDS and 1% w/v DTT in 50 mM Tris/HCl buffer, pH 8.8) followed by equilibrium buffer II (6 M urea, 30% w/v glycerol, 2% w/v SDS and 4% w/v iodoacetamide in 375 mM Tris/HCl buffer, pH 8.8). After the equilibration steps the strips were transferred to 12%