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Detection and phylogenic analysis of one anthrax virulence plasmid pXO1 conservative open reading frame ubiquitous presented within *Bacillus cereus* group strains

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Abstract

The presence of one of the anthrax virulence plasmid pXO1 conserved fragments was analyzed in 24 *Bacillus cereus* and *B. thuringiensis* strains, including 6 *B. thuringiensis* subspecies, by polymerase chain reactions. Twelve out of 24 strains showed PCR-positive for an ORF101 homologous sequence. Two pXO1-ORF101-like fragments from a *B. cereus* B-4ac and a commercial *B. thuringiensis kurstaki* HD1 were cloned, sequenced and expressed in *Escherichia coli*. Toxicity assays revealed that the product encoded by the pXO1-ORF101-like fragment had no impact on either Vero cells or Chinese Hamster Ovary cells, suggesting that this fragment probably not contribute to enterotoxic activity. Sequence alignment of the pXO1-ORF101 from three *Bacillus anthracis* and ORF101-like fragments from other 12 *B. cereus* group isolates indicated high identity (more than 90%) and the presence of subgroup- and strain-specific SNPs among these fragments.

Keywords: pXO1; ORF101; *Bacillus cereus*, *Bacillus thuringiensis*; SNP

Bacillus anthracis is a member of *Bacillus cereus* group [1,2]. It harbors the plasmid pXO1, which is essential to express anthrax toxin and finally cause acute fatal disease anthrax and contains as many as 143 open reading frames (ORFs) [3–5]. The known toxin genes (*cya*, *lef*, and *pagA*) and toxin regulatory elements (*atxA* and *pagR*) are located in the 44.8-kb putative pathogenicity island (PAI) of *B. anthracis* [5,6]. Many of the pXO1 ORFs have been detected in closely related *Bacillus* species especially within the *B. cereus* group [6]. Besides, some reports revealed that three IS*231*-like transposase genes presented in pXO1 and other *Bacillus* species [7], and that a substantial conservation between pXO1 sequences and DNA from other *Bacillus* species was noticed [6]. The presence of pXO1-like fragment in PAI of *B. anthracis* in other *B. cereus* group strains raises questions of conservation of functions involved in pathogenesis and evolution.

The ORF101 included in the putative PAI is one of the anthrax virulence plasmid pXO1 conservative fragments within *B. cereus* group and nearly identical to part of the enterotoxin gene *bceT* [6,8]. The *bceT* gene from *B. cereus* B-4ac was initially reported to encode toxic product [9]. Restriction enzyme analysis and similarity comparison intimated that the published *bceT* gene sequence consisted of a putative promoter for a putative sodium-dependent proline transporter, a fragment with homology to DNA translocation genes, and a fragment in the complementary strand having 93% similarity to pXO1-ORF101 of *B. anthracis* (AF065404, 121815–122327) as Pannucci and the colleagues described [6], suggesting that the reported enterotoxic activity of *bceT* encoding protein could be endowed partially from the fragment with high similarity to pXO1-ORF101 [8].

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This initiated our interest for a more thorough investigation of the pXO1-ORF101 homology in *B. cereus* group bacteria in order to determine whether pXO1-ORF101 is likely involved in food poisoning and the phylogenetic relatedness involved in pathogenesis among *B. anthracis* and related *B. cereus* group.

Materials and methods

Bacterial strains and growth conditions. *Bacillus cereus* group strains used in this study are listed in Table 1. *Escherichia coli* M15 [pREP4] (Qiagen, Germany) was used as the host strain to express the His-tagged recombinant protein. Bacteria were cultivated in Luria–Bertani (LB) medium [10] at 30 °C (for *B. cereus* group strains) or 37 °C (for *E. coli*). Antibiotics in agar plates and broth media were in the following concentrations: 100 μg/ml ampicillin and 50 μg/ml kanamycin.

DNA manipulations. DNA extraction and PCR were performed as described [11]. A primer pair Bam-orf101-F (5′-ATCCGAGGATCCAACTATTTATCTTTAGAAGAGGCA-3′) and Pst-orf101-R (5′-ATCCGACTGCAGTATTCTTCTATGTCTTCTTTTACT-3′), which introduced *Bam*H I and *Pst*I restriction site, respectively (underlined), was used for sequencing and cloning of pXO1-ORF101-like fragments. Enzyme digestion, ligation, transformation, and agarose gel electrophoresis were conducted by standard procedures, as described by the supplier.

Cloning of ORF101-like fragment. Two pXO1-ORF101-like fragments from *Bacillus thuringiensis* subsp. *kurstaki* HD1 and *B. cereus* B-4ac were amplified by PCR using the high fidelity expand polymerase (Roche Diagnostics), respectively. The amplified fragments were digested by *Bam*H I and *Pst*I, and ligated into the His-tagged expression vector pQE30 (Qiagen, Germany), resulting in the plasmids pAT101-1 and pAT101-2, respectively. The recombinant plasmids pAT101-1 and pAT101-2 were transferred into *E. coli* M15 (pREP4), while plasmids pQE30 and pQE40 were introduced into M15 (pREP4) as controls, resulting in recombinant *E. coli* strains M15 (pAT101-1), M15 (pAT101-2), M15 (pQE30), and M15 (pQE40), respectively.

Overproduction and purification of the fusion protein. For overproduction of the fusion proteins of a 6× His-tag and the pXO1-ORF101-like fragment encoding peptide, 400 μl of an overnight culture of the transformed *E. coli* strain was used to inoculate 20 ml of fresh LB medium with ampicillin (100 μg/ml) and kanamycin (50 μg/ml), and the resulting culture was grown at 37 °C to an optical density of 0.6 at 600 nm. The culture was induced by adding isopropyl-D-thiogalactopyranoside (IPTG) to a

Table 1
Genotypic and phenotypic characterization of bacterial strains used in this study

Strain	Source and characterization	Accession No. of pXO1-ORF101 like sequence	Reference
B. cereus			
B-4ac	The source of the *bceT* clone	DQ449036	[9]
F4433/73	Enterotoxic strain		[17]
F3502/73	Emetic		[17]
CIP 5832	Probiotic strain		[17]
DBt248	Danish soil isolate, originally produced spherical crystal, non-toxic to Vero cell and negative in enterotoxin Western analysis. Unknown serotype		[17]
MADM 1279	Isolated from sugar candy		[17]
MS 10362	Isolated from ice creme, produce brown pigment	DQ449037	[17]
AND1309	Isolated from curry powder, *hblA* negative		[17]
AND1313	Isolated from curry powder, *hblA* negative		[17]
421-3	Negative by PCR for hblA, and negative by Oxoid test for Hbl-L2, but positive with the Tecra assay and by PCR for the nheA	DQ449038	[17]
421-4	Negative by PCR for hblA, and negative by Oxoid test for Hbl-L2, but positive with the Tecra assay and by PCR for the nheA	DQ449039	[17]
ATCC 33018		DQ449040	ATCC
B. thuringiensis subsp. *kurstaki*			
AW05	HD73, toxic to Lepidoptera larvae		[20]
HD1	Used for control of Lepidoptera larvae	DQ449045	[8]
DMU67	Indistinguishable from strain HD1 by RAPD fingerprinting	DQ449044	[21]
KTo	Flagella serotype H3, toxic to Lepidoptera larvae		[22]
Bt657	Isolated from sliced sausage	DQ449046	
B. thuringiensis subsp. *israelensis*			
HD567	Used for control of Dipteran larvae		[8]
4Q2-72			BGSC
Other subspecies			
alesti HD4			[8]
indiana HD521		DQ449042	[8]
dakota HD932		DQ449043	[8]
tenebrionis DSM5526		DQ449041	[8]
B. mycoides DSMZ 2048	Type strain	DQ449035	[17]