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Multilocus sequence typing reveals that *Bacillus cereus* strains isolated from clinical infections have distinct phylogenetic origins

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Abstract

Eight strains of *Bacillus cereus* isolated from bacteremia and soft tissue infections were assigned to seven sequence types (STs) by multilocus sequence typing (MLST). Two strains from different locations had identical STs. The concatenated sequences of the seven STs were aligned with 65 concatenated sequences from reference STs and a neighbor-joining tree was constructed. Two strains were distantly related to all reference STs. Three strains were recovered in a clade that included *Bacillus anthracis*, *B. cereus* and rare *Bacillus thuringiensis* strains while the other three strains were assigned to two STs that were more closely affiliated to most of the *B. thuringiensis* STs. We conclude that invasive *B. cereus* strains do not form a single clone or clonal complex of highly virulent strains.

Keywords: *Bacillus anthracis*; *Bacillus cereus*; *Bacillus thuringiensis*; MLST; Clone; Phylogeny

1. Introduction

Bacillus cereus is commonly found in the soil and associated with plants [1]. It is best known for causing two forms of mild food poisoning characterized by predominantly diarrhoea or vomiting [2,3]. Occasionally *B. cereus* is implicated in more serious invasive infections. For example, it is responsible for a particularly severe form of endophthalmitis which may result in loss of functional vision or even blindness [4], and bacteremia in the immunocompromised host [5] as well as in preterm neonates [6]. Meningitis, pneumonia, urinary tract infections and fatal fulminant liver failure have also been attributed to *B. cereus* infections (reviewed in [3]). *B. cereus* strains secrete a battery of extracellular enzymes and toxins including phospholipases C, sphingomyelinase and various hemolysins that contribute to their virulence [7,8].

B. cereus is the parent species of a wider group of bacteria encompassing several additional species, *Bacillus anthracis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, *Bacillus thuringiensis* and *Bacillus weihenstephanensis* that are so closely related to *B. cereus* that they are generally considered a single taxospecies [1,9]. Plasmid encoded virulence factors distinguish *B. anthracis*, the causative agent of anthrax [10], and *B. thuringiensis*, a common insect pathogen that is used as a commercial bioinsecticide [11]. *B. mycoides* and *B. pseudomycoides* strains are differentiated from *B. cereus* by their distinctive rhizoid colony morphology [12] and *B. weihenstephanensis* strains are relatively psychrotolerant [13]. Accordingly, the *B. cereus* group can be treated as a

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single species for population analysis and has been subject to both multilocus enzyme electrophoresis (MEE) [14,15] and multilocus sequence typing (MLST) [16,17] analyses that have indicated a reasonably strong clonal structure to the organisms with evidence for specific clones associated with the emetic form of food poisoning [18], periodontal disease [14] and some serovars of *B. thuringiensis* with particular entomopathogenic traits [19].

In this study we have examined eight strains of *B. cereus* isolated from cases of bacteremia and from soft tissue infections by MLST to assess the phylogenetic origins of the strains. We show that two of the strains were identical for the seven partial gene sequences that we analyzed, while the other strains were phylogenetically distinct. We conclude that strains of *B. cereus* involved in opportunistic infections do not belong to a single clonal complex.

2. Materials and methods

2.1. Strains and molecular methods

Five strains of *B. cereus* were received from J. McLauchlin (Food Safety Microbiology Laboratory, Health Protection Agency, London) that had been associated with bacteremia (Table 1). Three strains were isolated from soft tissue infections and identified as *B. cereus* following growth on *B. cereus* selective agar (Oxoid) and phenotypic inspection (Table 1). Methods for strain preservation, bacterial growth, isolation of DNA have been described previously [17]. We amplified by PCR fragments of seven housekeeping genes (*glpF*, *gmk*, *ilvD*, *pta*, *pur*, *pycA* and *tpi*) as described on the website for MLST of *B. cereus* (www.pubmlst.org/bcereus) using the standard primers with primer option 1 for the *ilvD* gene. PCR fragments were sequenced in both directions using the amplification primers to provide unambiguous sequence data.

2.2. Data analysis

The allele sequences were compared with existing allele sequences using the *B. cereus* MLST website and given new allele numbers if they differed from known alleles. The seven allele numbers define a sequence type (ST) a number for which was assigned to each new allele combination. The concatenated sequences for the seven gene fragments for all sequence types were constructed and downloaded using the *B. cereus* MLST website. Multiple alignment of all the concatenated sequences was carried out using CLUSTAL W [20]. Neighbor-joining trees were derived from the alignments using the tree building facility in CLUSTAL W and visualized using TreeView (www.taxonomy.zoology.gla.ac.uk/rod/treeview.html). Clonal groups or lineages were further analyzed using SplitsTree [21]. The position and frequency of polymorphisms in the allele nucleotide sequences were detected using the START program [22].

3. Results

3.1. Phylogenetic origins of the invasive strains

All eight clinical strains of *B. cereus* were isolated in 2003. Five strains were derived from blood of patients of varying age and health and the remaining three from tissue infections (Table 1). The last three cases responded successfully to ciprofloxacin treatment. Most of the clinical isolates had unique combinations of alleles providing STs that were new to the database, the exception was strain 172560W which shared the same ST as a strain of *B. thuringiensis* serovar pakistani isolated in Chile. Strains R2955/03 and R3149/03 had identical STs despite their origins from different hospitals (Table 1).

We constructed a neighbor-joining tree from the concatenated sequences of the seven alleles for the clinical isolates together with concatenated sequence from selected STs from the database (Fig. 1). Two of the isolates, R3238/03 (ST-72) and R3098/03 (ST-74), were distantly related to all other STs and formed independent lines of descent. Indeed ST-72 and ST-74 each had unique alleles which did not occur in any other ST in the database (the allele numbers and sequences for all STs are available from www.pubmlst.org/bcereus).

Table 1
Origins of *Bacillus cereus* isolates used in this study

Strain	Source	Patient/age	Predisposing factors	Location in UK	Sequence type (ST)
R2955/03	Blood	Male, 50	Unknown	Bristol	73
R3039/03	Blood	Male, 70	Unknown	Leicester	75
R3098/03	Blood	Male, 90	Post blood transfusion	Hull	74
R3149/03	Blood	Female, 86	Myeloma	Manchester	73
R3238/03	Blood	Female, 65	Unknown	Oxford	72
168287M	Hematoma	Male, 66	Post-operative infection following prosthetic elbow joint surgery	Glasgow	77
191560K	Calf tissue	Female, 58	Insect bite	Glasgow	76
172560W	Burn wound	Female, 49	20% full thickness burns to legs	Glasgow	18