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.

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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 pre-term 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...
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.zool-ogy.gla.ac.uk/rod/treerview.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>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Patient/age</th>
<th>Predisposing factors</th>
<th>Location in UK</th>
<th>Sequence type (ST)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R2955/03</td>
<td>Blood</td>
<td>Male, 50</td>
<td>Unknown</td>
<td>Bristol</td>
<td>73</td>
</tr>
<tr>
<td>R3059/03</td>
<td>Blood</td>
<td>Male, 70</td>
<td>Unknown</td>
<td>Leicester</td>
<td>75</td>
</tr>
<tr>
<td>R3098/03</td>
<td>Blood</td>
<td>Male, 90</td>
<td>Post blood transfusion</td>
<td>Hull</td>
<td>74</td>
</tr>
<tr>
<td>R3149/03</td>
<td>Blood</td>
<td>Female, 86</td>
<td>Myeloma</td>
<td>Manchester</td>
<td>73</td>
</tr>
<tr>
<td>R3238/03</td>
<td>Blood</td>
<td>Female, 65</td>
<td>Unknown</td>
<td>Oxford</td>
<td>72</td>
</tr>
<tr>
<td>168287M</td>
<td>Hematoma</td>
<td>Male, 66</td>
<td>Post-operative infection following prosthetic elbow joint surgery</td>
<td>Glasgow</td>
<td>77</td>
</tr>
<tr>
<td>191560K</td>
<td>Calf tissue</td>
<td>Female, 58</td>
<td>Insect bite</td>
<td>Glasgow</td>
<td>76</td>
</tr>
<tr>
<td>172560W</td>
<td>Burn wound</td>
<td>Female, 49</td>
<td>20% full thickness burns to legs</td>
<td>Glasgow</td>
<td>18</td>
</tr>
</tbody>
</table>
The *B. anthracis–B. cereus–B. thuringiensis* population comprises two major clades: clade 1 which encompasses *B. anthracis*, numerous *B. cereus* and rare *B. thuringiensis* strains, and clade 2 which comprises *B. cereus* and *B. thuringiensis* strains (Fig. 1). A third clade of mixed organisms has been labeled as “others” in Fig. 1. Within these clades various lineages have been defined [21] and most of the clinical *B. cereus* isolates were associated with these lineages.

One strain from septicemia (R 3039/03) and two soft tissue isolates (191560K and 168297M) were recovered in lineage Cereus III, a member of clade 1, as STs 75, 76 and 77, respectively (Table 1). A splitsgraph of this cluster revealed the close phylogenetic origins of STs 57, 60, and 76 and the more loosely affiliated STs 27, 60 and 77 (Fig. 2A). There was considerable variation among alleles in this lineage but it was notable that strain R3039/03, shared the *gmk1* and *pta1* alleles with *B. anthracis* strains.

Of the remaining three strains, two identical isolates from blood (strains R2955/03 and R3149/03) and one from a soft tissue infection (172560M) were assigned...
to separate lineages in clade 2 which comprises a mixed population of B. cereus and B. thuringiensis strains (Fig. 1). Strain 172560M was recovered in lineage Kurstaki. Split decomposition of the STs in this lineage indicated three principal groups or sub-lineages, designated (a), (b) and (c) in Fig. 2B with the pathogen assigned to sub-lineage (b). This sub-lineage was defined by alleles gmk9 and tpi7 with variation in the other alleles.

Two identical isolates from separate cases of bacteremia were assigned to ST-73 in lineage Tolworthi. Split decomposition of this lineage indicated two groups of STs that we have labeled (a) and (b) in Fig. 2C with the clinical isolates located in sub-lineage (a). To determine further the structure and origins of these sub-lineages we undertook an evaluation of the roles of recombination versus mutation in their evolution.

### 3.2. Mutation versus recombination in the evolutionary descent of B. cereus strains from clinical sources

If we assume an ancestral ST for a group or sub-lineage based on the maximum number of shared alleles within the group, it is possible to compare variant (derived) STs with the assumed ancestor [23]. Single base differences between two alleles are likely to be due to mutation, double changes are less likely to be due to mutation, and when alleles differ at multiple sites it is more likely that the observed variation is due to lateral gene transfer, especially if the variant allele occurs in a non-related ST elsewhere in the dataset [23]. We have used these criteria to indicate the relative roles of recombination and mutation in the B. cereus group (Fig. 2).

In lineage Cereus III, ST-57 was designated the ancestor and while single nucleotide changes are extant,
there is strong evidence for recombinational exchange especially in ilvD, pta and tpi. In sub-lineage Kurstaki (b), many variant alleles differed by a single nucleotide change (Fig. 2B). The replacement of purl2 in ST-18 by pur28 in ST-51, on the other hand, involved nine changes to an allele which suggests recombinational exchange between members of the sub-lineage. Nevertheless, the evolutionary trend in sub-lineage Kurstaki (b) appeared to be biased towards the mutational. Finally, two blood isolates were assigned to sub-lineage Tolworthi (a). The clone ST-24 is the likely ancestor of this group as indicated by split decomposition and shared alleles (Fig. 2C). The star-like nature of the splits graph, and preponderance of single and double mutational changes suggest predominance of mutation over recombination in this group.

4. Discussion

Opportunistic infections typically occur when an otherwise harmless organism is introduced into a body tissue in sufficient numbers that the organisms can proliferate. B. cereus has an array of toxins which enable it to colonize the host and it was possible that clones or clonal complexes with a particular array of virulence factors may be responsible for invasive infections. For example, there is evidence that isolates of B. cereus causing periodontal disease are associated with a particular clonal group [14] and the ST-26 strains which cause the emetic form of food poisoning are strongly clonal [18]. However, the results from this study suggest that the ability of B. cereus strains to cause invasive disease is not restricted to a particular clonal group of strains, but is a feature of a genomically diverse group of organisms. This lack of correlation between virulence potential and MLST genotype has also been noted in Staphylococcus aureus [23] and contrasts with community acquired pathogens such as Neisseria meningitidis [24] and Streptococcus pneumoniae [25] in which certain clones are particularly invasive. However, we emphasize that this conclusion is based on a small sampling of strains from clinical sources and, it is possible that when more strains have been analyzed, virulent clones or clonal complexes will emerge such as ST-73 in lineage Tolworthi.

The B. anthracis–B. cereus–B. thuringiensis population is evolving in two major clades: clade 1 comprising B. anthracis, B. cereus and rare B. thuringiensis strains, and clade 2 encompassing a mixed population of B. cereus and B. thuringiensis strains [17]. The recovery of three of the clinical B. cereus isolates in clade 1, closely related to B. anthracis and, in the case of strain R3039/03, sharing gmk1 and pta1 alleles with B. anthracis strains, is consistent with this part of the population encompassing virulent strains. B. cereus G9241 which was associated with an illness resembling inhalation anthrax and contains the anthrax toxin genes but lacks the polyglutamate capsule [26] is also loosely related to clade 1 (Fig. 1). It was therefore a little surprising to find clinical isolates also in clade 2 which is generally associated with B. thuringiensis strains which, with rare exceptions are considered harmless [27]. B. cereus 172560W from a burns patient was identical to a strain of B. thuringiensis serovar pakistani. We have examined strain 172560W microscopically and it does not make a visibly detectable crystal protein, therefore by definition is not a strain of B. thuringiensis.

Evidence for lateral gene transfer among B. cereus and B. thuringiensis populations has been provided by MEE studies [15]. Here we indicated the relative contributions of mutation and recombination in the evolution of these bacteria by scrutinizing the distribution of nucleotide changes in alleles among closely related strains. Our data suggest that recombination and mutation both contribute to the evolution of the population with predominance of mutation in sub-lineages Kurstaki (b) and Tolworthi (a). Similar analyses indicate that recombinational exchange is also less common than mutation in the related, low G+C organism, S. aureus [23].

B. cereus has a history of association with invasive infection [28]. These examples of strains isolated in 2003 indicate that it continues to pose a threat to both healthy and immunocompromised individuals. Here we have established that the bacteria responsible for these infections are not restricted to a single clonal group or lineage within B. cereus but are genomically diverse.

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References


