The cereulide genetic determinants of emetic
*Bacillus cereus* are plasmid-borne

*Bacillus cereus* is a spore-forming ubiquitous bacterium recognized as a food-spoilage hazard. Indeed, many isolates have been shown to be responsible for clinical infections and food-poisoning outbreaks (Ghelardi et al., 2002; Chan et al., 2003; Musa et al., 1998). Their incidence is probably much higher than is generally reported owing to the differences that exist between the reporting procedures of different countries and the symptomatic similarities with other pathogenic bacteria such as *Staphylococcus aureus* or *Clostridium perfringens*. Two types of food poisoning are caused by *B. cereus*, namely diarrhoeal and emetic types. The diarrhoeal syndrome seems to be induced by the simultaneous action of several factors, mainly the haemolytic (Hbl) and non-haemolytic (Nhe) toxins, and is characterized by abdominal pain and diarrhoea (Beecher *et al*., 1995). The emetic type, characterized by nausea and vomiting, involves a small cyclic dodecadepsipeptide \([\text{(D-O-Leu-D-Ala-1-O-Val-1-Val)}]\) named cereulide (Agata *et al*., 1995). Due to its chemical structure, cereulide was thought to be synthesized non-ribosomally and, recently, partial identification and characterization of the...
non-ribosomal peptide synthetase gene have been carried out (Toh et al., 2004; Horwood et al., 2004; Ehling-Schulz et al., 2005). Cereulide acts as an ionophore through mitochondrial membranes and interferes with oxidative phosphorylation. Preferentially produced in starchy food, mainly boiled rice, cereulide induces the emetic syndrome between 1 and 5 h after consumption of the contaminated food. This suggests that the toxin is pre-formed in the food before bacterial sporulation.

Cereulide cannot be inactivated by standard heat treatment or by digestion on account of its acid- and protease-resistant properties (Melling et al., 1976; Shinagawa et al., 1995).

Generally, food-borne illnesses caused by B. cereus are relatively mild and do not last more than 24 h. However, the emetic syndrome can have a fatal and fulminant outcome (Mahler et al., 1997). This was the case in Kinrooi (Belgium) in 2003, when a 7-year-old girl died after consuming pasta contaminated by B. cereus. Cereulide is thought to have been responsible for the child’s death (Dierick et al., 2005).

To date, the location of the cereulide genetic determinants has remained unknown. A potential extrachromosomal location was thus investigated by plasmid profiling and curing experiments in strains collected from emetic isolates from food-poisoning outbreaks, including Kinrooi 5975c isolated from the Belgian lethal case in 2003. This study shows that the genomic location of genetic determinants involved in cereulide production is extrachromosomal and is found on a plasmid named pCERE01.

Plasmid content profiling of several strains, including Kinrooi 5975c, was performed according to the method developed by Jensen et al. (1995) (Fig. 1a). The initial plasmid isolation revealed important genomic similarities between the five emetic-positive strains (lanes 3–7). This observation confirmed the genomic similarity among cereulide-producing strains that we had already observed by PFGE (Dierick et al., 2005). Moreover, a large number of plasmids, with sizes ranging from around 2 to about 350 kb, was identified in all the strains tested. The five emetic strains (lanes 3–7) showed particularly high numbers of plasmid bands, reaching 10 for B. cereus strain Kinrooi 5975c. Interestingly, it was noted that one similar large plasmid was present in all five emetic isolates as well as in the non-emetic strain B. cereus 17532 (Fig. 1a).

Hybridization (Sambrook et al., 1989) was performed using the PCR product amplified by emetic B. cereus-specific primers as probe (Ehling-Schulz et al., 2004). As shown in Fig. 1(b), the probe specifically hybridized to a large plasmid in all five emetic strains studied, including the emetic reference strain F4810/72. The name pCERE01 was proposed for the cereulide plasmid present in strain Kinrooi 5975c. Its size was estimated to about 200 kb. The probe did not hybridize with the cereulide non-producing strains.

To check whether the presence of the pCERE01 plasmid was indeed correlated with cereulide production, B. cereus strain Kinrooi 5975c was chosen to perform several independent curing experiments. Six different cured derivatives were selected and plasmid profiling was performed.

![Fig. 1. Plasmid profiling and hybridization. (a) Plasmid gel electrophoresis of the strains under study. Lanes: C, strain AND508 (Andrup et al., 1993) used as reference for large plasmids and containing the 128 kb completely sequenced plasmid pBT0xis (Berry et al., 2002); 1–8, B. cereus isolates 16198, 17544, F4810/72 (emetic reference strain), 10329, 17533, Kinrooi 5965b, Kinrooi 5975c and 17532, respectively; 9, B. thuringiensis GBJ038 serovar kurstaki HD73 harbouring pHT73 and pAW63 (72 kb) (Wilcks et al., 1998); 10 and 11, B. cereus AND2023 and 17534, respectively. (b) Southern hybridization of the plasmids isolated by gel electrophoresis [shown in (a)] with a probe specific for emetic B. cereus strains. Arrows indicate the five hybridizing plasmids [also shown in (a)]. (c) Plasmid profiling of derivatives of B. cereus Kinrooi 5975c. Lanes: C, strain AND508 used as reference for large plasmids; 1–7, Kinrooi 5975c, KC1, KC2, KC3, KC4, KC5 and KC6, respectively. * indicates that the strains are positive in cereulide-specific PCR and in the sperm toxicity assay (Table 1). Arrows indicate pCERE01.](image-url)
Table 1. Characterization of the *B. cereus* strains evaluated in the study

<table>
<thead>
<tr>
<th><em>B. cereus</em> strain</th>
<th>Origin</th>
<th>Relevant characteristics</th>
<th>Southern hybridization</th>
<th>Cereulide-specific PCR</th>
<th>Sperm toxicity*</th>
<th>Source or reference†</th>
</tr>
</thead>
<tbody>
<tr>
<td>F4810/72</td>
<td>Vomit</td>
<td>Reference strain for cereulide production</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Andersson et al. (1998)</td>
</tr>
<tr>
<td>ATCC 14579</td>
<td>Vomit</td>
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<td>–</td>
<td>ND</td>
<td>–</td>
<td>Ivanova et al. (2003)</td>
</tr>
<tr>
<td>AND2023</td>
<td>Food isolate</td>
<td>Reference strain</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>L. Andrup</td>
</tr>
<tr>
<td>16198</td>
<td>Danish parsley</td>
<td>Reference strain</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>A. Wilcks</td>
</tr>
<tr>
<td>17544</td>
<td>French apples</td>
<td>Reference strain</td>
<td>–</td>
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<td>A. Wilcks</td>
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<tr>
<td>10329</td>
<td>Pasta</td>
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<td>+</td>
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<td>+</td>
<td>A. Wilcks</td>
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<tr>
<td>17533</td>
<td>Danish blackcurrant</td>
<td>Reference strain</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>A. Wilcks</td>
</tr>
<tr>
<td>17532</td>
<td>Danish redcurrant</td>
<td>Reference strain</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>A. Wilcks</td>
</tr>
<tr>
<td>5965b</td>
<td>Pasta</td>
<td>Kinrooi outbreak</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Dierick et al. (2005)</td>
</tr>
<tr>
<td>5975c</td>
<td>Vomit</td>
<td>Kinrooi outbreak</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Dierick et al. (2005)</td>
</tr>
<tr>
<td>KC1</td>
<td>5975c derivative</td>
<td>pCERE01−</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>This study</td>
</tr>
<tr>
<td>KC2</td>
<td>5975c derivative</td>
<td>pCERE01+</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td>This study</td>
</tr>
<tr>
<td>KC3</td>
<td>5975c derivative</td>
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<td>–</td>
<td>–</td>
<td>This study</td>
</tr>
<tr>
<td>KC4</td>
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<tr>
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<td>This study</td>
</tr>
<tr>
<td>KC6</td>
<td>5975c derivative</td>
<td>pCERE01−</td>
<td>ND</td>
<td>–</td>
<td>–</td>
<td>This study</td>
</tr>
</tbody>
</table>

ND, Not determined
*Toxicity was observed as inhibition of sperm motility. +, Toxic (<10% of the spermatozoids were motile); –, non-toxic (>60% of the spermatozoids were motile).
†A. Wilcks, Department of Microbiological Food Safety, Danish Institute for Food and Veterinary Research.

(Fig. 1c). Plasmid isolation gel electrophoresis revealed that, in all the strains, one or several plasmids were missing in comparison with the original strain. Interestingly, the four isolates cured of the pCERE01 large plasmid (KC1, KC3, KC5 and KC6) were negative in the emetic-toxin-specific PCR assay, while the cured derivatives still harbouring pCERE01 (KC2 and KC4) amplified the cereulide-producing *B. cereus* specific fragment (Ehling-Schulz et al., 2004) (Table 1). A sperm bioassay (Andersson et al., 2004) was then carried out to distinguish between cereulide-producing and non-producing strains. The four derivatives cured for pCERE01 (KC1, KC3, KC5 and KC6) showed no apparent cereulide production in contrast to the other cured derivatives (KC2 and KC4) that still produced cereulide (Table 1). This result confirmed that the loss of pCERE01 from the original strain is associated with the loss of cereulide production.

Finally, preliminary conjugation experiments using cereulide-producing strain Kinrooi 5975c indicated that it can act both as donor and recipient cell for the transfer of the conjugative plasmid pXO16 (Jensen et al., 1995). However, pCERE01 conjugation or mobilization could not be observed by PCR screening of transconjuguants, meaning that pCERE01 transfer occurs at a frequency lower than $10^{-2}$ transconjuguants per recipient cell (data not shown), if at all. Transfer of pCERE01 to other members of the *B. cereus* group would be very interesting to investigate in light of the discussion of the evolution and genetic relationship among members of the *B. cereus* group. To monitor this transfer, a transposon insertion with a resistance marker in the plasmid is in progress.

To conclude, the fatal outbreak that occurred in Belgium 2 years ago underlines the potentially deadly character of the cereulide-producing *B. cereus* strains. Given the possible severity of the emetic syndrome, identification and characterization of the genetic determinants involved in cereulide synthesis is of prime importance. Hence, increased interest in this subject has been registered recently with the publication of several studies highlighting a non-ribosomal synthesis of the emetic toxin and a partial identification and characterization of the synthetase gene responsible for its production (Toh et al., 2004; Horwood et al., 2004; Ehling-Schulz et al., 2005). *B. cereus* is very closely related to two other members of the *B. cereus* group, the causative agent of anthrax, *Bacillus anthracis*, and the insect pathogen *Bacillus thuringiensis*. These bacteria have been suggested to belong to the same species (Helgason et al., 2000). In *B. anthracis* and *B. thuringiensis*, major virulence factors are located extrachromosomally on large plasmids and now we have reported the equivalent genetic organization in the emetic type of *B. cereus*, with the genetic basis of cereulide being encoded on the large plasmid pCERE01.

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