Short communication

*Bacillus cereus* produces several nonproteinaceous insecticidal exotoxins

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Abstract

*Bacillus cereus* is mainly known as a human food-borne opportunistic pathogen. Here, we used biological assays and HPLC to investigate the ability of *B. cereus* to produce insecticidal exotoxins during the stationary growth phase. None of the 575 *B. cereus* strains screened produced detectable levels of β-exotoxin I, a small, heat-stable insecticidal nucleotide analogue. However, six out of a subset of 270 *B. cereus* strains produced several small, nonproteinaceous insecticidal exotoxins different from β-exotoxin I. Thus, *B. cereus* can secrete a large array of proteinaceous and nonproteinaceous toxins acting on insects and mammals.

Keywords: *Bacillus cereus*; Toxin; Insecticide

1. Introduction

*Bacillus cereus* is genetically very similar to *Bacillus thuringiensis* (Helgason et al., 2000). *B. cereus* is considered to be a human opportunistic pathogen (Drobniewski, 1993), while *B. thuringiensis* is an entomopathogen widely used as a biological insecticide. During the sporulation phase, *B. thuringiensis* produces endotoxins—the Cry proteins—which are highly specific against several insect pests (Schnepf et al., 1998). Most of the Cry proteins are encoded by genes located on plasmids. In addition to the endotoxins, *B. thuringiensis* also produces nonproteinaceous insecticidal exotoxins. The best known of these exotoxins is β-exotoxin I, a heat-stable, water-soluble, and low molecular weight compound (701 Da). About 50% of the *B. thuringiensis* strains secrete detectable amounts of this toxin during the stationary growth phase (Espinasse et al., 2002). β-Exotoxin I is highly toxic by oral route to a wide range of insect species. It is a phosphorylated adenine nucleotide analogue that inhibits DNA-dependent rRNA polymerase in vitro (Sebesta et al., 1981).

The absence of Cry plasmids in *B. cereus* appears to be the main difference between this species and *B. thuringiensis*. Despite the absence of Cry plasmids in *B. cereus*, some strains of this species were shown to secrete a heat-stable, insecticidal exotoxin (Krieg and Lysenko, 1979; Ohba et al., 1981). However, this exotoxin was not characterised and it is not clear whether it is β-exotoxin I. At least one nonproteinaceous exotoxin secreted by *B. cereus* has been characterised: this exotoxin is the cereulide, a cyclic dodecadepsipeptide that induces vomiting in some cases of gastroenteritis (Agata et al., 1995).

In this study, we used insect bioassays to determine the ability of a large number of *B. cereus* strains to produce insecticidal exotoxins in standard culture conditions. We found that a few strains of this species produce such toxins, that appear to be of nonproteinaceous nature. We have subsequently determined by HPLC analysis that none of these exotoxins were β-exotoxin I.
2. Materials and methods

2.1. Bacterial strains and supernatant preparations

The *B. cereus* strains tested for insecticidal exotoxin production were from the La Minière INRA collection, with the exception of 10 strains that were from the Anne-Brit Kolstø collection (School of Pharmacy, University of Oslo, Norway). These strains were isolated as described by Ohba and Aizawa (Ohba and Aizawai, 1986) from soil or from insects samples collected throughout the world. Luria–Bertani broth (100 ml in a 1-L flask) was inoculated with single colonies picked from agar plates, and grown at 30°C and 175 rpm. At mid-sporulation stage, the cultures were centrifuged and the supernatants were filtered through a membrane (pore size = 0.2 μm, Nalgene Sterilization Unit, Nalge Nunc International, Rochester, NY, USA) and stored at −20°C until use. *B. thuringiensis* 407 Cry- is a serovar H1 strain that has been cured of plasmids bearing the δ-endotoxin genes by successive cultures at 40°C (Lereclus et al., 1989).

2.2. Insect bioassays

We used a free ingestion method to determine the toxicity of supernatants towards laboratory colonies of *Anthonomus grandis* (cotton boll weevil), *Spodoptera littoralis* (cotton leafworm), and *Aphis fabae* (black bean aphid). For *A. grandis* and *S. littoralis*, the bioassays were carried out as described earlier (Espinasse et al., 2002). For *A. fabae*, 10 young adults were placed in a glass cylinder closed on one side with Saran film and on the other side by two Parafilm membranes. We added 25 μl of test sample to 225 μl of artificial diet (mixture of amino acids, vitamins, sucrose, ovalbumin, and Wesson salts), and inserted the mixture between the two Parafilm membranes. Three cylinders were used for each sample. Larval population size was assessed 5 days after infestation. Growth inhibition rates were calculated using the formula [(control−treated)/control].

The concentration of β-exotoxin I or the amount of culture supernatant inducing 50% mortality (lethal concentration 50, LC₅₀) was calculated using a log-probit method (Finney, 1971).

2.3. Detection and quantification of β-exotoxin I

β-Exotoxin I in the culture supernatant was determined by an HPLC method as previously described (Gohar and Perchat, 2001). The detection limit of this method was 2 μg/ml of β-exotoxin I.

2.4. Partial characterisation of exotoxins

The culture supernatant was incubated with pronase (4 U/mg, Sigma, Ref. P-6911) at 37°C for 1 h. The insecticidal activity of the pronase-treated sample was compared to that of a control sample submitted to the same procedure, except that pronase was omitted. The approximate molecular sizes of the exotoxins were determined by assaying the insecticidal activity of the culture supernatants after ultrafiltration on a 1-kDa pore size membrane (Microsep Filtron, Filtron Technology, Northborough, MA, USA). For ammonium sulphate precipitation, the culture supernatants were incubated for 1 h on ice with ammonium sulphate at 85% saturation. The insecticidal activity of the pellet was then assayed. Heat stability was assessed by performing the toxicity assay after incubating the culture supernatants for 20 min either at 90°C (treated) or on ice (control).

3. Results and discussion

Of the three insect species used in the toxicity assays, *A. grandis* was the most sensitive to β-exotoxin I with an LD₅₀ of 5.8 μg/ml (95% confidence limits: 3.9–7.8 μg/ml). Therefore, we tested the toxicity of the culture supernatants of 575 *B. cereus* strains from the La Minière INRA collection against this coleopteran species. Only four of these supernatants were toxic to *A. grandis*. The toxicity profile of these four strains against *A. grandis*, *S. littoralis*, and *A. fabae* is reported in Table 1: as shown in the table, this profile is different from the β-exotoxin I profile. We then used HPLC to detect β-exotoxin I in the culture supernatant of a subset of 55 strains, including the four toxic strains. None of the *B. cereus* strains tested produced detectable amounts of β-exotoxin I. Therefore, the four entomopathogenic strains produced another exotoxin active against *A. grandis*.

Whereas, *A. grandis* was shown to be the most sensitive to β-exotoxin I of the insect species used in this study, this may not be true to other exotoxins. Therefore, we tested the toxicity of culture supernatants from 207 *B. cereus* strains—including the four strains toxic to *A. grandis*—towards *S. littoralis* and *A. fabae*. Five of these supernatants (2.4%) were active against *A. fabae* and none against *S. littoralis* (Table 1). HPLC showed that β-exotoxin I was not produced by these five strains. We partially characterised the toxic compounds secreted by these strains, and by the strain Be 352-1 selected on *A. grandis*. In all cases, the active compound was resistant to proteolysis and was not

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Supernatant toxicity against <em>A. grandis</em>, <em>S. littoralis</em> and <em>A. fabae</em> for the active strains of <em>B. cereus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td><em>A. grandis</em></td>
</tr>
<tr>
<td>Be 137-1</td>
<td>85 (3)</td>
</tr>
<tr>
<td>Be 352-1</td>
<td>100 (2)</td>
</tr>
<tr>
<td>Be 622-1</td>
<td>98 (3)</td>
</tr>
<tr>
<td>AH 682</td>
<td>98 (2)</td>
</tr>
<tr>
<td>Bc 544-1</td>
<td>8 (1)</td>
</tr>
<tr>
<td>Bc 634-1</td>
<td>11 (1)</td>
</tr>
<tr>
<td>β-Exo</td>
<td>100</td>
</tr>
</tbody>
</table>

Values refer to the average percentage of nymphs mortality for *A. grandis* and *S. littoralis*, and to the average percentage of reduction in nymph population for *A. fabae*. Numbers in brackets refer to the number of independent replicates. Strain AH 682 comes from the Anne-Brit Kolstø collection. β-Exo: β-exotoxin at 80 μg/ml.
precipitated by ammonium sulphate. Thus, these exotoxins were not proteinaceous. Furthermore, differences in the heat stability, filtration behaviour through a 1-kDa pore size membrane and insecticidal spectrum of these exotoxins suggested that the different entomopathogenic strains did not produce the same compound (Tables 1 and 2). For example, strains Bc 544-1 and Bc 634-1 were the sole strains active only on *A. fabae,* the toxic compound was retained on a 1-kDa membrane only for strain 544-1, and no other strain than AH 682 produced a heat-stable toxin.

Some strains of *B. thuringiensis* also produce small non-proteinaceous insecticidal toxins different from β-exotoxin I. For example, an exotoxin similar to β-exotoxin I was described by Levinson et al. (Levinson, 1990) and named β-exotoxin II. A sigma exotoxin (Argauer et al., 1991) and an M-exotoxin (Horak et al., 1996) have also been briefly described in this species. Hence, *B. thuringiensis* and *B. cereus* both produce a set of small nonproteinaceous insecticidal exotoxins that are only produced by a small proportion of strains, in contrast to the widely distributed β-exotoxin I. *B. cereus* has also been shown to secrete during the vegetative phase a family of insecticidal proteins named Vip (Warren et al., 1996). In addition, strains of *B. cereus* were found to be pathogens of Lepidoptera (To et al., 1975) and Diptera (Kaaya and Darji, 1989). While *B. cereus* is considered mainly as a human opportunistic pathogen producing haemolysins and enterotoxins, these data show that it can also be a pathogen of insects.

Acknowledgment

We thank Anne-Brit Kolsto for providing us with *B. cereus* strains.

### Table 2

Partial characterisation of the compounds contained in the culture supernatant of six strains of *B. cereus*

<table>
<thead>
<tr>
<th>Strain</th>
<th>MW (kDa)</th>
<th>Pronase resist.</th>
<th>Heat stability</th>
<th>AS precipitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bc 137-1</td>
<td>&lt;1</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bc 352-1</td>
<td>&lt;1</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bc 622-1</td>
<td>&lt;1</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AH 682</td>
<td>&lt;1</td>
<td>+</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>Bc 544-1</td>
<td>&gt;1</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bc 634-1</td>
<td>&lt;1</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

MW, approximate molecular weight as determined by ultrafiltration on a 1 kDa membrane; <1 kDa, not retained on the membrane; and >1 kDa, retained on the membrane. Pronase resist.: +, resistance to proteolysis by pronase. Heat stability: 0, not stable; +, stable. AS precipitation: precipitation by ammonium sulphate; 0, no precipitate. Strain AH 682 comes from the Anne-Brit Kolsto collection.

### References


