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Antifungal Activity Displayed by Cereulide, the Emetic Toxin Produced by *Bacillus cereus*▽

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In this study, the fungistatic activity of *Bacillus cereus* cereulide-producing strains was demonstrated against nine fungal species. The role of cereulide was confirmed using plasmid-cured derivatives and *ces* knockout mutants. The fungistatic spectra of cereulide and valinomycin, a chemically related cyclododecadepsipeptide, were also compared and found to be similar but distinct.

Food poisoning by emetic *Bacillus cereus* strains has been shown to cause fatal liver failure (6, 15, 25). Cereulide, the emetic toxin of *B. cereus*, is a cyclic dodecadepsipeptide composed of three repetitions of two amino acids and two hydroxy acids (D-*O*-Leu-D-Ala-L-*O*-Val-L-Val)$_3$. Its chemical structure is closely related to that of valinomycin, which is produced by various *Streptomyces* spp. (16). Cereulide is a heat-stable toxin (4, 21, 24, 27) and is synthesized enzymatically by a nonribosomal peptide synthesis (NRPS) whose genetic determinants are located on the pCERE01 plasmid of the *B. cereus* emetic strain Kinrooi 5975c (9), the pBC270 element from *B. cereus* strain AH187 (23), and the pBCE4810 plasmid from *B. cereus* reference strain F4810/72 (8). These elements contain a 23-kb gene cluster (*ces*) involved in the cereulide synthesis. The two largest genes, named *cesA* (~10 kb) and *cesB* (~8 kb), lead to the incorporation and modification of D-*O*-Leu, D-Ala (*cesA*), L-*O*-Val, and D-Val (*cesB*), which compose the basic tetradepsipeptide motif of cereulide (7, 14).

Cereulide can cause stomach pain and vomiting, respiratory distress, and occasional loss of consciousness, possibly leading to coma and ultimately death of the individual (6). This toxin is also an ionophoric molecule with a high degree of affinity for K^+ ions (26). It is lipophilic and specifically targets mitochondrial membranes (4), where it leads to cellular dysfunctions, such as swelling mitochondria and blocking oxidative phosphorylation (2, 17).

It has been shown that valinomycin and cereulide display similar activities, since they block the motility of boar spermatozoa through the dissipation of mitochondrial internal membrane potential, a property often used for the detection of cereulide (3, 4, 22, 23a). Given these similarities, it was interesting to consider the known activities of valinomycin to hypothesize on the actual "raison d'être" of cereulide. Valinomycin is toxic for some insects, nematodes, and Gram-positive bacteria at very low concentrations (18, 19, 20). Although the antifungal activity of valinomycin has been reported (18), Altayar and Sutherland (1) showed that cereulide does not have any antifungal activity on rich medium. Since fungi are ubiquitous organisms living in the same environment as *B. cereus* (11, 12), a possible competition between these organisms could, however, be expected. The purpose of this study was to assess the potential antifungal activity of three emetic *B. cereus* strains and to highlight the specific contribution of cereulide using isogenic cereulide-producing and cereulide-minus (cured or mutated) strains. Comparison with the antifungal activity spectrum of valinomycin was also performed.

Six strains of *B. cereus* were used in this study. The emetic strain Kinrooi 5975c came from a fatal case of food intoxication and is considered a high producer of cereulide (6). Strain KC1 (Kinrooi Cured 1) was obtained by curing Kinrooi 5975c from its pCERE01 plasmid that carries the cereulide genetic determinants (9). The reference emetic strain, F4810/72, was isolated from a victim of food poisoning caused by cereulide (23), and strain IS075 was isolated from a vole (10). The *cesA* knockout derivatives ($cesA^{KO}$) of strains F4810/72 and IS075 were also constructed. The *cesA* synthetase genes were inactivated via homologous recombination by an inactivated copy bearing an internal deletion and the insertion of a spectinomycin resistance gene marker. The final construction was checked by PCR, which could discriminate between the wild-type and tagged versions.

A total of 27 fungi comprising different families were tested for their sensitivity to cereulide: 19 *Ascomycota* (10 different orders), 2 *Zygomycota*, 1 *Basidiomycota*, and 5 *Oomycota*. Nondiluted, 5-times-diluted (PDA-0.2), and 10-times-diluted (PDA-0.1) potato dextrose agar (PDA) (Oxoid) were used for fungal culture. Growth of *Oomycota* was obtained in V8 medium, which contains (per liter) 100 ml of V8 juice.

BHI (brain heart infusion) medium, solidified with 2% agar, was used for the biomass production of the six *B. cereus* strains. Cereulide was extracted from the bacterial cultures using a protocol adapted from Andersson et al. (3), and extract concentrations were determined by liquid chromatography-mass spectrometry (LC-MS), using commercial valinomycin as an external standard (L. Delbrassinne, M. Andjelkovic, A. Rajkovic, N. Bottledoorn, J. Mahillon, and J. Van Loco, submitted for publication). Samples of 7,281 ng/ml and 3,987 ng/ml were obtained for the Kinrooi 5975c and F4810/72 emetic strains,

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TABLE 1. Sensitivity of fungi to cereulide and valinomycin

Fungus	Sensitivity[a]								
	PDA			PDA-0.2			PDA-0.1		
	Kinrooi	KC1	Val	Kinrooi	KC1	Val	Kinrooi	KC1	Val
Alternaria alternata	−	−	+	+	−	+	+	−	+
Aspergillus niger	−	−	+	−	−	+	−	−	+
Botrytis aclada	−	−	−	−	−	−	−	−	−
Botrytis allii	−	−	−	−	−	−	−	−	−
Botrytis cinerea	+	−	+	+	−	+	+	−	+
Cladosporium cucumerinum	+	−	+	+	−	+	+	−	+
Colletotrichum gloeosporioides	−	−	+	/	/	/	/	/	/
Didymella lycopersici	−	−	−	−	−	−	−	−	−
Fusarium graminearum	−	−	+	−	−	+	−	−	+
Macrophomina phaseolina	−	−	−	−	−	+	−	−	+
Magnaporthe grisea	+	−	+	+	−	+	+	−	+
Monographella nivalis	+	−	+	+	−	+	+	−	+
Mortierella isabellina	+	−	+	+	−	+	+	−	+
Nectria radicicola	−	−	−	−	−	−	−	−	−
Penicillium chrysogenum	−	−	−	−	−	−	−	−	−
Rhizoctonia solani	−	−	−	−	−	−	−	−	−
Sclerotinia cinerea	−	−	−	−	−	−	−	−	−
Sclerotinia minor	+	−	+	+	−	+	+	−	+
Stemphylium vesicarium	−	−	−	−	−	+	+	−	+
Trichoderma viride	−	−	−	−	−	−	−	−	−
Verticillium dahliae	−	−	−	+	−	−	+	−	−
Zygorhynchus moelleri	−	−	−	−	−	−	−	−	−

[a] Sensitivity of 22 fungi to cereulide extract from *B. cereus* Kinrooi 5975c and its cured derivative KC1 and to valinomycin (Val) on rich (PDA) and diluted (PDA-0.2 and PDA-0.1) media. For Kinrooi 5975c extract and valinomycin, 20 μl corresponding to 146 ng of cereulide was dropped on the edge of every tested fungus. The + and − symbols refer to the presence and absence of growth inhibition, respectively. A slash indicates the absence of fungal growth.

respectively, in contrast to the 52 ng/ml of isolate IS075. For KC1, a Kinrooi 5975c derivative cured of its "cereulide" plasmid (9), and for both *cesA*^{KO} mutants, results of LC-MS were below the limit of detection (LOD; 1 ng/ml). The cereulide activity of each extract was also assessed by boar semen bioassay (3).

Antifungal activity was tested as follows. Fungi were inoculated at the center of a petri dish and incubated at 25°C to reach a diameter of ca. 3 cm. The contour of the strain was marked, and 20-μl volumes of extracts were spotted as a 90° arc along the edge of the colony. For each colony, two facing 90° arcs (including a negative control) were deposited at the edge. After a second incubation period, their growth was recorded. In the case of inhibition, the fungus stopped growing where the toxin was placed but displayed a continuous growth on the negative control. All tests were performed in triplicate, and no difference was observed among them.

The potential antifungal activities of the Kinrooi 5975c extract and its cured derivative KC1 were first evaluated in three growth conditions of the fungi: PDA, PDA-0.2, and PDA-0.1. For each fungus, 20 μl of bacterial extract was spotted along the edge of the colonies and their potential effect was recorded as a growth inhibition of the fungus. As shown in Table 1, *Botrytis cinerea*, *Cladosporium cucumerinum*, *Magnaporthe grisea*, *Monographella nivalis*, *Mortierella isabellina*, and *Sclerotinia minor* displayed inhibition by the methanol-water extract from the Kinrooi 5975c strain on all media. *Alternaria alternata* and *Verticillium dahliae* were sensitive to the Kinrooi 5975c extract on both PDA-0.2 and PDA-0.1, while *Stemphylium vesicarum* was inhibited by the extract only on the most diluted medium, PDA-0.1. Interestingly, none of the fungal strains had their growth inhibited by the extract of strain KC1, cured of the pCERE01 plasmid.

In order to further demonstrate that the toxic activity displayed in Table 1 was due to cereulide and not to another toxic component carried by the "cereulide" plasmid, all of the 22 fungal species were tested against methanol-water extracts from the *B. cereus* emetic strains F4810/72 (ca. 80 ng) and IS075 (ca. 1 ng) and their *cesA*^{KO} derivatives. Only those species that were sensitive to Kinrooi 5975c are reported; none of the others were sensitive to *B. cereus* F4810/72 or IS075 (data not shown).

As shown in Table 2, six species were inhibited by the extract of F4810/72, which contained approximately 50% less cereulide than the Kinrooi 5975c extract. Two species were also sensitive to the much-less-concentrated cereulide extract from strain IS075. The data also clearly indicated that no inhibition was observed with both *cesA*^{KO} mutants, confirming that the observed fungal growth inhibition was indeed due to the presence of the cereulide.

It was also interesting to compare these inhibition activities observed with the cereulide with those reported for valinomycin. As shown in Table 1, using the same amount of both toxins (146 ng), the effects were similar, but slightly different. In all growth conditions, the six species sensitive to Kinrooi 5975c on all media were also inhibited by valinomycin. The *A. alternata* and *Stemphylium vesicarium* species, sensitive to Kinrooi only under PDA-0.2 and/or PDA-0.1 growth conditions, were also sensitive to valinomycin but under different growth conditions (Table 1). Interestingly, the cereulide and valinomycin toxins clearly differentiated for five species: while *V. dahliae* was inhibited only by cereulide (on PDA-0.2 and PDA-0.1), *Asper-*

TABLE 2. Fungal sensitivity to cereulide extracts from two emetic strains and their *cesA*^{KO} derivatives

Fungus	Sensitivity[a]			
	F4810/72	F4810/72 *cesA*^{KO}	IS075	IS075 *cesA*^{KO}
Alternaria alternata	+	−	−	−
Botrytis cinerea	+	−	−	−
Cladosporium cucumerinum	+	−	−	−
Magnaporthe grisea	+	−	+	−
Mortierella isabellina	+	−	+	−
Monographella nivalis	+	−	−	−
Sclerotinia minor	−	−	−	−
Verticillium dahliae	−	−	−	−

[a] Sensitivity of the eight fungi (which were previously shown to be sensitive to the cereulide extract from *B. cereus* Kinrooi 5975c [Table 1]) to extracts from the F4810/72 and IS075 emetic strains (containing 80 and ca. 1 ng, respectively, in the 20 μl used) and their corresponding *cesA*^{KO} mutants. The growth medium used was PDA-0.2. The + and − symbols refer to the presence and absence of growth inhibition, respectively.

gillus niger, *Fusarium graminearum*, and *Colletotrichum gloeosporioides* (in all growth conditions tested) and *Macrophomina phaseolina* (in diluted medium) turned out to be sensitive to valinomycin only.

Five *Oomycota* (*Pythium ultimum* var. *sporangiiferum*, *P. ultimum* var. *splendens*, *Phytophthora cactorum*, *Phytophthora drechsleri*, and *Phytophthora citrophthora*) were also tested against valinomycin and cereulide extracts. However, under the conditions used, no growth inhibition could be detected (data not shown).

Using the cured, cereulide-minus derivative of an emetic strain first, and subsequently the *cesA*^{KO} mutants of two cereulide-producing *B. cereus* strains as negative controls, it was possible to demonstrate that the growth inhibition of several fungal species was specifically due to the presence of cereulide. The 22 fungal species used in this work have been placed in the phylogeny tree for kingdom Fungi from Blackwell et al. (5). The parasitic strategy (biotrophic, hemibiotrophic, necrotrophic, or saprophytic) of each species has also been examined, but no correlation could be drawn between these two classifications and the cereulide sensitivity of fungi.

Another interesting observation of the present work is the differential sensitivity of the fungal species to both cereulide and valinomycin. For instance, *Mortierella isabellina* and *Magnaporthe grisea* were highly sensitive and were inhibited by 1 ng of cereulide (extract from IS075 strain); *A. alternata*, *Botrytis cinerea*, *Cladosporium cucumerinum*, and *Monographella nivalis* were inhibited by 80 ng of cereulide (from strain F4810/72); while *Sclerotinia minor* and *V. dahliae*, less sensitive to cereulide, were blocked only by the Kinrooi 5975c extract (ca. 146 ng of cereulide). Similar observations can be made for valinomycin.

Using commercial valinomycin as a reference molecule is convenient, but results should be interpreted with care. Although the chemical structures and major properties of valinomycin and cereulide are related, their activity spectra on fungi differed. Teplova et al. (26) showed that cereulide affinity for the K^+ ion is higher than that of valinomycin. At the same concentration, cereulide should thus show a higher toxicity than valinomycin; however, *A. niger*, *Colletotrichum gloeosporioides*, *F. graminearum*, and *Macrophomina phaseolina* were inhibited by valinomycin (Table 1) but not by cereulide at the same concentration. On the other hand, *V. dahliae* seemed to be insensitive to the action of valinomycin, whereas it was sensitive to the Kinrooi 5975c extract on diluted media.

The fungistatic activity of cereulide is an important piece of information that can help us to suggest a hypothesis about its biological "raisons d'être." In the environment, or in starchy food products, *B. cereus* shares its niches with various types of fungi. It is reasonable to assume that cereulide could help the emetic strains to settle more efficiently in these environments thanks to their fungistatic effect. This issue, however, first requires further experimental investigations.

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