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

Sandy Ladeuze,1 Nathalie Lentz,1 Laurence Delbrassinne,2 Xiaomin Hu,3 and Jacques Mahillon1*

Laboratory of Food and Environmental Microbiology, Université Catholique de Louvain, B-1348 Louvain-la-Neuve, Belgium1; Scientific Institute of Public Health, 14 Rue Juliette Wytman, B-1050 Brussels, Belgium2; and State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan 430071, China3

Received 25 October 2010/Accepted 22 January 2011

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-Leu-L-Ala-L-Val-L-Val). 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-Leu, D-Ala (cesA), L-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), Al-tayar 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 (cesAΔK) 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. Non-diluted, 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. Rajkov, 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,
Verticillium dahliae
Trichoderma viride
Stemphylium vesicarium
Sclerotinia minor
Sclerotinia cinerea
Rhizoctonia solani
Penicillium chrysogenum
Mortierella isabellina
Monographella nivalis
Magnaporthe grisea
Macrophomina phaseolina
Cladosporium cucumerinum
Botrytis aclada
Aspergillus niger
Alternaria alternata

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 lated at the center of a petri dish and incubated at 25°C to

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 an arc of 90° 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.

As shown in Table 2, six species were 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 cesAKO 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 cesAKO 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-
The parasitic strategy (biotrophic, hemibiotrophic, necrotrophic) was high in sensitivity and was inhibited by 1 ng of cereulide (from strain F4810/72); however, the chemical structures and major properties of valinomycin are convenient, but results should be interpreted with care. Althoung 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 its fungistatic effect. This issue, however, first requires further experimental investigations.

We thank the personnel of the Food and Environmental Microbiology Laboratory for time spent in discussions and helpful comments. This work was supported by research grants from the National Fund for Scientific Research (FNRS), by the Université Catholique de Louvain (UCL), and by the Federal Public Service, Belgian Science Policy.

REFERENCES

1. Altay, M., and A. D. Sutherland. 2006. Bacillus cereus is common in the environment but emetic toxin producing isolates are rare. J. Appl. Microbiol. 100:7–14.
2. Andersson, M. A., et al. 2007. Toxicological profile of cereulide, the Bacillus cereus toxin, in functional assays with human, animal and bacterial cells. Toxicon 49:351–367.
3. Andersson, M. A., et al. 2004. Sperm bioassay for rapid detection of cereulide-producing Bacillus cereus in food and related environments. Int. J. Food Microbiol. 94:175–183.
4. Andersson, M. A., R. Mikkola, J. Helin, M. C. Andersson, and M. Salkinoja-Salonen. 1998. A novel sensitive bioassay for detection of Bacillus cereus emetic toxin and related depsipeptide ionophores. Appl. Environ. Microbiol. 64:1338–1343.
5. Blackwell, M., D. S. Hibbett, J. W. Taylor, and J. W. Spatafora. 2000. Research Coordination Networks: a phylogeny for kingdom Fungi (Deep Hypha). Mycologia 92:829–837.
6. Dierick, K., et al. 2005. Fatal family outbreak of Bacillus cereus-associated food poisoning. J. Clin. Microbiol. 43:4277–4279.
7. Dommel, M. K., et al. 2010. Identification of the main promoter directing cereulide biosynthesis in emetic Bacillus cereus and its application for real-time monitoring of cec gene expression in foods. Appl. Environ. Microbiol. 76:1232–1240.
8. Ehling-Schulz, M., et al. 2006. Cereulide synthetase gene cluster from emetic Bacillus cereus: structure and location on a mega virulence plasmid related to Bacillus anthracis toxin plasmid pX01. BMC Microbiol. 6:20–20.
9. Hoton, F. M., L. Andrup, I. Svæciecka, and J. Mahillon. 2005. The cereulide genetic determinants of emetic Bacillus cereus are plasmid-borne. Microbiology 151:2121–2124.
10. Hoton, F., et al. 2009. Family portrait of Bacillus cereus and Bacillus weihenstephanensis cereulide-producing strains. Environ. Microbiol. Rep. 1:177–183.
11. Kokkonen, M. L., Ojala, P. Parikka, and M. Jestoǐ. 2010. Mycotoxin production of selected Fusarium species at different culture conditions. Int. J. Food Microbiol. 143:27–25.
12. Lahatli, R., and M. Hiji. 2010. Screening, identification and evaluation of potential biocontrol fungal endophytes against Rhizoctonia solani AG3 on potato plants. FEMS Microbiol. Lett. 311:152–159.
13. Reference deleted.
14. Lücking, G., M. K. Dommel, S. Scherer, A. Fouet, and M. Ehling-Schulz. 2009. Cereulide synthesis in emetic Bacillus cereus is controlled by the transcription state regulator AprB, but not by the virulence regulator PlcR. Microbiology 155:925–931.
15. Mahler, H., et al. 1997. Fulminating liver failure in association with the emetic toxin of Bacillus cereus. N. Engl. J. Med. 336:1142–1148.
16. Matter, A. M., S. B. Hoot, P. D. Anderson, S. S. Neves, Y.-Q. Cheng. 2009. Valinomycin biosynthetic gene cluster in Streptomyces: conservation, ecology and evolution. PLoS One 4:1–10.
17. Mikkola, R., N.-E. L. Saris, P. A. Grigoriev, M. A. Andersson, and M. Salkinoja-Salonen. 1999. Isoprenoid properties and mitochondrial effects of cereulide. Eur. J. Biochem. 265:112–117.
18. Park, C. N., J. M. Lee, D. Lee, and B. S. Kim. 2008. Antifungal activity of cereulide, a peptide antibiotic produced by Streptomyces sp. strain M10 antagonistic to Botrytis cinerea. J. Microbiol. Biotechnol. 18:880–884.
19. Patterson, E. L. and D. P. Wright, Jr. 1970. Process for controlling insects, nematodes and mites using valinomycin. U.S. patent 3520973.
20. Pettit, G. R., et al. 1999. Antineoplastic agents. Part 409. Isolation and

| Fungus                  | F4810/72 | F4810/72 cesA4KO | IS075 | IS075 cesA4KO |
|-------------------------|---------|-----------------|-------|--------------|
| Alternaria alternata    | +       | +               |       | +            |
| Botrytis cinerea        | +       | +               |       | +            |
| Cladosporium cucumerinum| +       | +               |       | +            |
| Magnaporthe grisea      | +       | +               |       | +            |
| Mortierella isabellina  | +       | +               |       | +            |
| Monographella nivalis   | +       | +               |       | +            |
| Sclerotinia minor       | -       | -               |       | -            |
| Verticillium dahliae    | -       | -               |       | -            |

* 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 cesA4KO mutants. The growth medium used was PDA-0.2. The + and – symbols refer to the presence and absence of growth inhibition, respectively.
structure of montanastatin from a terrestrial actinomycete. Bioorg. Med. Chem. 7:895–899.

21. Rajkovic, A., et al. 2008. Heat resistance of Bacillus cereus emetic toxin, cereulide. Lett. Appl. Microbiol. 46:536–541.

22. Rajkovic, A., M. Uyttendaele, and J. Debevere. 2007. Computer aided boar semen motility analysis for cereulide detection in different food matrices. Int. J. Food Microbiol. 114(1):92–99.

23. Rasko, D. A., et al. 2007. Complete sequence analysis of novel plasmids from emetic and periodontal Bacillus cereus isolates reveals a common evolutionary history among the B. cereus-group plasmids, including Bacillus anthracis pXO1. J. Bacteriol. 189:52–64.

23a. Saris, N.-E. L., et al. 2009. Microbial toxin’s effect on mitochondrial survival by increasing K⁺ uptake. Toxicol. Ind. Health 25:441–446.

24. Shinagawa, K., H. Konuma, H. Sekita, and S. Sugii. 1995. Emesis of rhesus monkeys induced by intragastric administration with the Hep-2 vacuolation factor (cereulide) produced by B. cereus. FEMS Microbiol. Lett. 130:87–90.

25. Shiota, M., et al. 2010. Rapid detoxification of cereulide in Bacillus cereus food poisoning. Pediatrics 125:e951–e955.

26. Teplova, V. V., R. Mikkola, A. A. Tonshin, N. E. Saris, and M. S. Salkinoja-Salonen. 2006. The higher toxicity of cereulide relative to valinomycin is due to its higher affinity for potassium at physiological plasma concentration. Toxicol. Appl. Pharmacol. 216:39–46.

27. Turnbull, P. C. B., J. M. Kramer, K. Jorgensen, R. J. Gilbert, and J. Melling. 1979. Properties and production characteristics of vomiting, diarrheal, and necrotizing toxins of Bacillus cereus. Am. J. Clin. Nutr. 32:219–228.