Comparative Analysis of Antimicrobial Activities of Valinomycin and Cereulide, the *Bacillus cereus* Emetic Toxin\(^\dagger\)

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Cereulide and valinomycin are highly similar cyclic dodecadepsipeptides with potassium ionophoric properties. Cereulide, produced by members of the *Bacillus cereus* group, is known mostly as emetic toxin, and no ecological function has been assigned. A comparative analysis of the antimicrobial activity of valinomycin produced by *Streptomyces* spp. and cereulide was performed at a pH range of pH 5.5 to pH 9.5, under anaerobic and aerobic conditions. Both compounds display pH-dependent activity against selected Gram-positive bacteria, including *Staphylococcus aureus, Listeria innocua, Listeria monocytogenes, Bacillus subtilis, and Bacillus cereus* ATCC 10987. Notably, *B. cereus* strain ATCC 14579 and the emetic *B. cereus* strains F4810/72 and A529 showed reduced sensitivity to both compounds, with the latter two strains displaying full resistance to cereulide. Both compounds showed no activity against the selected Gram-negative bacteria. Antimicrobial activity against Gram-positive bacteria was highest at alkaline pH values, where the membrane potential (\(\Delta \Psi\)) is the main component of the proton motive force (PMF). Furthermore, inhibition of growth was observed in both aerobic and anaerobic conditions. Determination of the \(\Delta \Psi\), using the membrane potential probe DiOC\(_2\)(3) (in the presence of 50 mM KCl) in combination with flow cytometry, demonstrated for the first time the ability of cereulide to dissipate the \(\Delta \Psi\) in sensitive Gram-positive bacteria. The putative role of cereulide production in the ecology of emetic *B. cereus* is discussed.

Members of the *Bacillus cereus* group, also known as *Bacillus cereus* sensu lato, are Gram-positive, rod-shaped, facultative anaerobic, spore-forming bacteria (30, 31). This group consists of the species *B. cereus*, *B. weihenstephanensis*, *B. mycoides*, *B. pseudomycoides*, *B. anthracis*, and *B. thuringiensis* (16, 30, 31, 70). *B. cereus* is found predominantly in the soil (3, 30, 70, 75) and has been isolated from the rhizosphere (3, 5), the air (70), the gut of invertebrates (30, 39), and a wide range of foods (16, 70). It is a notorious food spoilage organism (4, 70) and the causative agent of two types of food-borne disease (16, 22, 70), the emetic and diarrheal syndromes. The emetic toxin cereulide is produced by emetic *B. cereus* (1, 2, 22, 70) and some psychrotolerant *B. weihenstephanensis* strains (72, 73). This food intoxication, expressed by vomiting, is usually mild but occasionally results in fatalities (12, 42, 70). The toxin was found to be proteolytic resistant and highly heat and pH stable (57, 67, 68, 74). Cereulide is a so-called cyclic dodecadepsipeptide containing a three-repeat sequence [cycl(o-(O-Val-d-Lac–O-Leu–d-Ala–)], and its \(K^+\) ionophoric properties have been established in black-lipid membranes and by assessing its impact on mitochondrial swelling and function (1, 32, 45, 68). Recent research focused mainly on the genetic factors involved in the production of cereulide, the identification of transcriptional regulators, detection methods, and the impact of environmental conditions on cereulide levels reached, including a variety of (model) foods (13, 15, 17, 38, 69, 71, 78). Yet, besides its role in food poisoning and its effect on mammalian cells, the biological relevance of cereulide production in the environment, including its impact on other bacteria, has not been addressed. Production of ionophores has been described for a range of microbial species, including *Streptomyces*, *Streptoverticillum*, *Nocardiopsis*, *Nocardia*, *Actinomadura*, *Bacillus*, and several fungi (10, 21, 32, 52). These compounds can be divided into three major classes on the basis of their mode of transport: the neutral ionophores, carboxylic ionophores, and the channel-forming quasi-ionophores (10, 53). Notably, some information is available on the antimicrobial activity of the neutral ionophore valinomycin, a well-known peptide antibiotic that is produced by members of the soil-dwelling Gram-positive genus *Streptomyces*. Valinomycin shows high similarity with cereulide, being a cyclic dodecadepsipeptide also but containing the three-repeat sequence cycl(o-(d-Val-d-HyIva–d-Lac–)], where HyIva is \(\alpha\)-hydroxyisovaleric acid and Lac is lactic acid (8, 41, 54). This peptide, like cereulide, has a central hydrophilic cavity in which a \(K^+\) ion can be accommodated (18, 55, 56). Hydrophobic side chains of valine and hydroxyisovaleric acid allow for its diffusion through the hydrophobic interior of cell membranes, a process driven by the existing \(K^+\) and/or charge gradients (33). A wide range of biological activities has been reported for valinomycin, including insecticidal, nematicidal, antiviral, and apoptosis-inducing effects in human cells (11, 62). Valinomycin has become a useful tool in the study of ion transport in biological systems, and its inhibitory effect on intact cells and the growth of bacteria and yeast has been studied, although to a limited extent (25, 26, 61, 64). The function of cereulide in *B. cereus* growth and ecology is unknown. Optimal conditions (20) and oxygen dependency (29) for cereulide synthesis suggest that it may be produced in a range of environments outside the human host, where it may play a role in competition of emetic *B. cereus* with other microorganisms for nutrients. To maximize chances of...
survival, microorganisms have developed strategies to outgrow or eliminate competition, and one of these strategies embraces the production of antimicrobial compounds. Antibiotics produced by bacteria can be specific, effective only against closely related species, or broad spectrum, depending on their mode of action (59). These compounds are usually synthesized during post-logarithmic growth, when nutrients and space for microbial multiplication become limited (43), which is accordingly observed for the synthesis of cereulide (20, 23, 72).

Therefore, this study was designed to assess the antimicrobial activity of cereulide and valinomycin using a selection of Gram-negative and Gram-positive bacteria at a range of pH 5.5 to pH 9.5 and under anaerobic and aerobic conditions. Moreover, combining fluorescent probes with flow cytometry allowed for the determination of the impact of these ionophores on the membrane potential (ΔΨ) in selected target organisms and in the pH range indicated. The impact of our findings on emetic B. cereus ecology is discussed.

MATERIALS AND METHODS

Bacterial strains and culture conditions. A selection of facultative anaerobic bacteria was used, including Gram-negative Escherichia coli K-12 and Salmonella enterica subsp. enterica serovar Typhimuiron II 505, and Gram-positive Staphylococcus aureus ATCC 29213, Listeria innocua, Listeria monocytogenes EGD-e, Bacillus subtilis 168, enterotoxigenic Bacillus cereus ATCC 10987 and ATCC 14579, and emetic Bacillus cereus F48107 and AS29. All strains were grown in brain heart infusion (BHI) broth (Becton Dickinson, France) incubated at 30°C with shaking for 200 rpm (Innova 4335, New Brunswick Scientific, Netherlands) for 17 h unless stated otherwise. Cell cultures were maintained at −80°C in 20% glycerol as a cryoprotectant. Absorbance at 600 nm was measured using a Novaspec II spectrophotometer (Pharmacia Biotech, United Kingdom).

Chemicals. A valinomycin ready-made solution (1 mg/ml dimethyl sulfoxide (DMSO)) and nigericin were obtained from Sigma-Aldrich. Cereulide was synthesized as described by Biesta-Peters et al. (6). The final concentration of valinomycin and cereulide in the exposure experiments was 9 μM in the presence of 1% (vol/vol) DMSO. At this concentration, DMSO neither affected the growth nor the survival of the selected strains in this study. The concentrations of valinomycin and cereulide used in this study are within the range of values reported in the literature, including environmental and (model) food samples (23, 29, 64, 65).

Drop dilution plate assay. To determine the effect of cereulide and valinomycin on the growth of the different test strains, BHI pour plates (0.5% [wt/vol] agar) adjusted to the appropriate pH, containing approximately 1 × 10^8 CFU/ml (per 1 ml) prepared. After solidification, 5 μl of valinomycin (179.97 μM), cereulide (167.84 μM), both containing 4% DMSO, was spotted directly on the agar surface. As a control, 5 μl of 4% DMSO was spotted. After the drops dried, plates were incubated overnight at 30°C, after which the inhibition zones were measured. All experiments have been performed using two independent biological duplicates.

Microtiter plate assay. To determine the effect of valinomycin and cereulide on the growth performance of the selected strains, spectrophotometric growth curves were obtained using a 96-well microtiter plate reader (SpectraMax Plus384; Molecular Devices, United Kingdom). The optical density at 600 nm (OD_600) of each well was automatically measured every 5 min for 24 h, with shaking for 30 s before each read. Inocula were prepared by resuspending overnight culture cells in 0.01 M phosphate buffer (PPi) to an OD_600 of 2, reaching a final start OD_600 of 0.1 in a 96-well microtiter plate (Greiner Bio-One, Germany). Plates were prepared by dispensing 180 μl of BHI broth, using the appropriate pH, into each well, supplemented with 10 μl of the proper inoculum, which was finalized by the addition of the control or test compound. Growth performance was tested at a range of pH values, namely, pH 5.5, 6.5, 7.5, 8.5, and 9.5, in aerobic and anaerobic conditions. Anaerobic conditions were created by covering the 96-well plate with optical adhesive film (MicroAmp). Each condition was tested using biological triplicates. The generated data were corrected for growth medium background signals. Log_10-transformed growth curves were fitted according to the Zwietering growth model (79) using the solver from Microsoft Office Excel 2003. For each growth curve generated, the specific growth rate (μ) and duration of lag phase (λ) were determined and used to calculate the average μ and λ for each condition, as well as their standard errors. Statistical significance between two conditions was established by performing Student’s t tests (two sided) and was considered significant when P values were <0.05.

Survival and recovery assay. To study the effects of exposure to valinomycin and cereulide on survival and growth, pH-adjusted BHI broth (50 ml in 250 ml Erlenmeyer flasks) containing approximately 1 × 10^4 CFU ml^−1 (absorbance at 600 nm, 0.1) was supplemented with cereulide or valinomycin to a final concentration of 9 μM and 1% (vol/vol) DMSO. As a control, cultures were exposed to 1% (vol/vol) DMSO. Cultures were grown aerobically at 30°C and 200 rpm. At regular intervals, appropriately diluted 50-μl aliquots of cells were surface plated in duplicate on BHI agar plates (Eddy Jet; IUL Instruments, Spain). Plates were incubated for 24 h at 30°C, and cell counts were expressed in log_10 CFU ml^−1. Each experiment was repeated twice, using two independent biological duplicates.

Detection and measurement of the membrane potential. The ΔΨ was determined using the BacLight bacterial membrane potential kit (Invitrogen) in combination with flow cytometry. Briefly, cells were diluted to approximately 1 × 10^8 CFU ml^−1 in phosphate-buffered saline (pH 5.5, 7, or 8.5) containing 50 mM KCl and 100 mM NaCl. Cells were complemented and incubated for 5 min with 10 μl (per ml cells) 100% DMSO, 500 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP) in DMSO, 1 mg/ml valinomycin in DMSO, 1 mg/ml cereulide in DMSO, or 100 μM nigericin in DMSO. Cell suspensions (1 ml) were stained with 10 μl of 3 mM DiOC_6(3) for 30 min at 30°C. The stained cells were analyzed by flow cytometry using a FACSCaliber (BD), with 488-nm excitation and detection through a 525- to 530-nm and 610-nm band-pass (−20-nm bandwidth) filter. A ratiometric method was used to obtain cell size-independent values as previously described by Novo et al. (49). Each experiment was performed using three independent biological duplicates.

RESULTS

Antibacterial activity of valinomycin and its dependency on pH. First, the impact of pH on the activity of valinomycin was assessed. Notably, the two components contributing to the proton motive force (PMF), namely, the membrane potential (ΔΨ) and the transmembrane pH gradient (ΔpH; signifying the difference between the intracellular and extracellular pH values), are closely interlinked, influencing the ability of active and passive membrane transport and ATP synthesis (7, 46–48). At acidic pH values, the ΔpH is the main component of the PMF, whereas at alkaline pH, the ΔΨ is the main component. This interlinkage suggests that the antibacterial activity of K^+ ionophores could also depend on the pH of the medium and the ability of a microorganism to compensate and maintain its ion gradients and PMF. In this experiment, we investigated the effect of exposure to valinomycin on the growth of a selection of Gram-negative and Gram-positive bacteria at a broad pH range. Growth (OD_600) in BHI was monitored for 24 h in a 96-well-microtiter-plate format during aerobic conditions. Figure 1 illustrates the effect of increasing the extracellular pH and the addition of valinomycin on aerobic growth of B. subtilis 168. These results were representative for all Gram-positive strains tested in this study, albeit the degree of inhibition varied. Although lag times and growth rates changed as a result of divergence from the optimal pH range for growth, the presence of valinomycin clearly decreased growth speed and/or caused increasing bacteriostatic activity as pH values became more alkaline. The left panel of Fig. 2 depicts the relative lag time and relative growth rate of a selection of Gram-positive bacteria in aerobic conditions. The growth rates of L. monocytogenes, B. cereus ATCC 10987 (Fig. 2), L. innocua, and S. aureus (data not shown) when exposed to valinomycin decreased with increasing pH values, whereas growth rates of B. subtilis, B. cereus ATCC 14579, and B. cereus F4810 remained largely unaffected up to pH 7.5 for B. subtilis and pH 8.5 for the...
latter two strains. In all cases, the lag phase appeared to be affected significantly, showing the largest effects for *L. monocytogenes*, *B. subtilis*, and *B. cereus* ATCC 10987 at alkaline pH. Notably, *B. cereus* ATCC 14579 and both emetic *Bacillus* strains were less sensitive to valinomycin, exhibiting outgrowth in 24 h at pH values up to pH 8.5 (Fig. 2 and data not shown). Gram-negative strains tested were found to be insensitive under all conditions tested, which confirms and expands upon previous reports (9, 64, 66).

**Sensitivity to valinomycin is reduced under anaerobic conditions.** To determine the effect of oxygen on the antibacterial activity of valinomycin, cells were grown in BHI at different pH values in the presence and absence of oxygen with or without 9 μM valinomycin (Fig. 2). Valinomycin extended the lag phase in close correlation with increasing pH values during aerobic and anaerobic conditions. Yet the lengths of the respective valinomycin-induced lag phases were generally shorter in the absence of oxygen. Although the sensitivities of the individual strains varied, this tendency was found for all tested Gram-positive strains. Valinomycin also reduced the relative growth rates of selected *L. monocytogenes*, *B. subtilis*, and *B. cereus* ATCC 10987 strains, with the largest effect again observed at alkaline pH (Fig. 2). Similar to aerobic conditions, the addition of valinomycin did not affect the growth of Gram-negative bacteria in anaerobic conditions (data not shown).

**Cereulide affects growth kinetics of Gram-positive bacteria.** The impact of pH on the antibacterial properties of synthetic cereulide (6) was assessed using a drop dilution plate assay. In

![FIG. 1. Effect of pH and valinomycin on growth performance of *Bacillus subtilis*. Cells were grown in BHI at 30°C without (left) or with (right) 9 μM valinomycin at different pH values: pH 5.5 (open triangles), pH 6.5 (closed squares), pH 7.5 (closed triangles), pH 8.5 (closed circles), and pH 9.5 (crosses).](http://aem.asm.org/)

![FIG. 2. Impact of oxygen and pH on relative lag time and bacterial growth rate upon exposure to valinomycin. *Listeria monocytogenes* (*Lm*), *Bacillus subtilis* 168 (*Bs*), *Bacillus cereus* ATCC 10987, *Bacillus cereus* ATCC 14579, and the emetic toxin-producing strain *Bacillus cereus* F4810/72 were grown in BHI in the presence and absence of 9 μM valinomycin at different pH values: pH 5.5 (white), pH 6.5 (light gray), pH 7.5 (dark gray), pH 8.5 (striped), and pH 9.5 (black). Left and right panels show growth in the presence and absence of oxygen, respectively. Due to the variations in lag phases and growth rates in control experiments with the different bacteria and pH values tested, relative lag times (top) and relative growth rates (bottom) have been obtained by the deviation of valinomycin over control results. Lag times acquired from valinomycin-exposed samples equaling the experimental upper limit of 24 h are marked with an “a.” When significantly different (*P* < 0.05), bars are marked with an asterisk. A relative growth rate of 1 (line) depicts no difference in growth rates between the absence (control) and presence of valinomycin.)
congruence with the results with valinomycin, Gram-positive bacteria appeared to be most susceptible at high pH (data not shown), and pH 8.5 was selected for subsequent experiments. Selected strains were incubated in BHI (pH 8.5, 30°C) in the absence and presence of 9 μM cereulide, and growth (OD600) was followed for 24 h in a 96-well spectrophotometer. All tested Gram-positive strains proved sensitive to the presence of cereulide as reflected in increased lag times, with the exception of the two emetic B. cereus strains used in this study (data not shown). Figure 3 illustrates the growth inhibitory effect of 9 μM cereulide on B. subtilis 168 and B. cereus ATCC 10987, with the emetic B. cereus strains (Fig. 3 and data not shown) showing resistance to cereulide. Interestingly, B. cereus ATCC 14579 revealed to be less susceptible to both valinomycin and cereulide but did not exhibit full resistance to cereulide, in contrast to the tested emetic B. cereus strains. Similar to data obtained in aerobic cultures, no growth effects were noticed for Gram-negative E. coli and S. Typhimurium exposed to cereulide (data not shown).

**Bactericidal and bacteriostatic activity.** Exposure to valinomycin or cereulide induced bacteriostatic conditions in selected Gram-positive bacteria. Close examination of the different growth curves revealed small drops in OD600 during the lag phase. To investigate if this phenomenon is related to changes in cell viability, 1 x 10⁷ CFU ml⁻¹ of selected strains were exposed to 9 μM valinomycin or 9 μM cereulide or 1% DMSO as a control. Exposures took place in BHI at pH 8.5, and samples were collected at regular time intervals. Next to measuring the OD at 600 nm, samples were plated on BHI to monitor viable counts. B. cereus ATCC 10987 (Fig. 4) and B. subtilis (data not shown) appeared to be highly sensitive to valinomycin as reflected in the 3-log reduction of the viable count in the first hours of exposure. B. cereus ATCC 14579 (data not shown) and the emetic strain B. cereus F4810/72 (Fig. 4) were revealed to be less susceptible to the bactericidal properties of valinomycin, showing a 2-log reduction and less than 1-log reduction in viable counts, respectively. Noteworthy, increasing the pH to 9.5 enhances the bactericidal effects of valinomycin, resulting in a 4.5-log reduction for B. cereus ATCC 10987, 4-log reduction for B. cereus ATCC 14579, and 3.5-log reduction for B. cereus F4810/72. Exposure of B. cereus ATCC 10987 cells to cereulide at pH 8.5 resulted in a 1.5-log reduction

![Figure 3](image-url)

**FIG. 3.** Impact of cereulide on growth of selected *Bacillus* strains at pH 8.5. *Bacillus subtilis* 168 (A), *Bacillus cereus* ATCC 10987 (B), and emetic *Bacillus cereus* F4810/72 (C) were grown for 24 h in BHI at 30°C in the presence (triangles) and absence (crosses) of 9 μM cereulide.

![Figure 4](image-url)

**FIG. 4.** Bactericidal or bacteriostatic properties of valinomycin and cereulide at pH 8.5. OD₆₀₀ values (open symbols) and viable counts (closed symbols) of *Bacillus cereus* ATCC 10987 and *Bacillus cereus* F4810/72 cultures exposed to 9 μM valinomycin (squares) or 9 μM cereulide (circles) in BHI. The nonexposed control cultures are depicted with triangles.
reduction, confirming its bactericidal properties (Fig. 4). Interestingly, the emetic strain B. cereus F4810/72 proved to be, although to a lesser extent compared to other nonemetic Bacillus strains, sensitive to valinomycin (Fig. 4). Yet, growth and survival seemed unaffected when exposed to cereulide (Fig. 3 and 4), indicating innate immunity of the producer cells to cereulide.

The addition of valinomycin and cereulide results in loss of membrane potential. The K⁺ ionophore valinomycin is widely used to study membrane energetics and causes dissipation of the membrane potential (ΔΨ). The impact of cereulide on bacterial energetics has not been reported up to now, although activity was tested against eukaryotic cells and isolated mitochondria. The bacteriostatic and bactericidal activity of cereulide and valinomycin at high pH may be attributed to the fact that, e.g., ΔΨ is the main or sole component of the PMF under these conditions (7). The effect of valinomycin and cereulide on membrane (de)polarization of Gram-positive bacteria was investigated by exposing the cells to these compounds in the presence of 50 mM KCl and 100 mM NaCl at pH 5.5, 7, or 8.5 and subsequent staining with the ΔΨ probe DiOC₂(3) and quantification with flow cytometry. Figure 5 shows a fluorescence histogram revealing the detrimental effect on ΔΨ in B. subtilis cells exposed to either valinomycin or cereulide, as indicated by the downshift in red emission of the fluorescent dye, signifying extensive depolarization of the cells. Using the proton ionophore CCCP, complete dissipation of ΔΨ was achieved, whereas exposure to 1% DMSO as a control had no effect. In the presence of the K⁺/H⁺ exchanger nigericin that dissipates the ΔpH (7), the addition of valinomycin or cereulide resulted in complete dissipation of the ΔΨ at all pH values tested. Similar results were obtained with cells from B. cereus ATCC 10987, L. innocua, and L. monocytogenes (data not shown). In Fig. 6, the responses of two nonemetic and one emetic B. cereus strain at different pH values are shown. Although growth effects are predominately linked to higher pH values, cells were readily (partially) depolarized by valinomycin and cereulide at all pH values, although the extent varied per strain. B. cereus ATCC 10987 demonstrated a dramatic loss of ΔΨ, whereas B. cereus ATCC 14579 and F4810/72 displayed residual polarization at higher pH values, signifying maintenance of a ΔΨ. The latter data are in line with the observed resistance to valinomycin and cereulide in the growth experiments.

**DISCUSSION**

This study showed that the B. cereus emetic toxin cereulide, like its structural homologue valinomycin, produced by soil-dwelling Streptomyces spp., dissipates the membrane potential in Gram-positive bacteria at a broad pH range. Notably, significant inhibition of growth and cellular inactivation are observed only at alkaline pH values, a condition where the membrane potential is the main component of the proton motive force, whereas at neutral pH values, and even more pronounced at acid pH values, the pH gradient (pH inside – pH outside) is the dominant factor (7, 46, 47). In this way, bacteria maintain a similar magnitude of proton motive force at a broad pH range with various contributions of the membrane potential and pH gradient, allowing generation of an increasing pH gradient at acidic conditions, thereby maintaining the intracellular pH at neutral to alkaline pH values (7). Under aerobic and anaerobic conditions, the PMF is generated by H⁺-pumping electron transfer chain activity and H⁺ ATPase, respec-
tively (25). In aerobic respiratory conditions, ATP is generated mainly via H⁻ influx via H⁻-ATPase, whereas in anaerobic conditions, ATP generated via substrate-level phosphorylation is used to extrude protons via this H⁻-ATPase. Despite basic differences in energy generation in anaerobic and aerobic conditions, growth of sensitive facultative anaerobic bacteria is affected in both conditions.

All tested Gram-positive bacteria, including *Bacillus* spp., *Listeria* spp., and *Staphylococcus aureus*, show inhibition of growth, although to different extents, whereas none of the tested Gram-negative bacteria are affected. This is in line with previous data obtained with valinomycin and other ionophores, which showed that Gram-negative bacteria are protected by the presence of an outer membrane that prevents access of these compounds to the inner membrane, acting as a selective permeability barrier between the cytoplasm and the outside environment (25, 61). Notably, enterotoxigenic *B. cereus* strain ATCC 14579 showed increased resistance to both valinomycin and cereulide. One could speculate that resistance to these compounds may involve activation of, e.g., antibiotic and/or multidrug resistance mechanisms in this strain. Obviously, more detailed experiments are required to elucidate the underlying mechanisms. Notably, emetic *B. cereus* displayed reduced sensitivity to valinomycin and appeared to be resistant to cereulide. This is in line with expectations where antimicrobial peptide- and ionophore-producing microorganisms are protected by so-called innate immunity (43, 59). An obvious candidate for such a role is the putative cereulide secretion machinery encoded by genes located on the *cereulide* plasmid (15, 58). Further experiments including targeted deletion mutants will allow for the identification and characterization of genes conferring (innate) immunity of producer cells.

Production of cereulide could provide producing strains with a competitive advantage toward other Gram-positive bacteria in alkaline environments. An interesting question thus arises if and where environments exist with alkaline pH and sufficient potassium levels and whether emetic *B. cereus* strains have been isolated from these. Recent papers and reviews highlighted the unique features of emetic *B. cereus* and discussed their presence in a wide range of environments, including soil and plant rhizosphere and specifically paddy rice fields (3, 5, 28, 30, 50, 70). Potassium levels in soil are generally low and in the range of 0.1 to 4 mM (35, 40, 60, 63, 77). However, potassium levels in (organic) manure, plants, and the guts of insects can reach values up to 200 mM (27, 34, 76, 77). The potassium concentration and pH in the soil could transiently increase in decaying plant material which involves microbial metabolism of proteinaceous compounds, resulting in production of ammonia and a concomitant increase of pH. Environments known to reach high pH values are the mid- and hindgut of insects (14, 19, 30). Here, pH values >8 have been determined. However, up to now emetic *B. cereus* strains are not regarded as regular inhabitants of insects. Whether they can occupy this niche in certain conditions remains to be clarified. The fact that cereulide can inhibit Gram-positive bacteria that may occupy similar niches as emetic *B. cereus* may offer an explanation for maintenance of the plasmids carrying the genetic information for production of this dodecadepsipeptide. The so-called pXO1-like virulence megaplasmids have been sequenced and characterized, revealing the presence of a 24-kb *ces* gene cluster coding for the enzyme machinery mediating synthesis, modification, and secretion of cereulide (15, 58). The valinomycin-encoding gene cluster *vlm* from *Streptomyces* spp. (51) was shown to be structurally highly similar to the *ces* gene cluster (32, 44), but comparative sequence analysis revealed them to be highly divergent from each other at the DNA level. It was suggested that the *vlm* and *ces* gene clusters may share a relatively distant common ancestor, but these two gene clusters have since evolved independently (44). The capacity to produce valinomycin may provide competitive advantage to *Streptomyces* spp. in a range of environments, and a similar situation may occur in cereulide-producing *emetic* *B. cereus*. This could offer an explanation for maintenance of *B. cereus cer* genes in a range of environments outside the human host and would fit with the optimum temperature found for cereulide production, i.e., at a range from 5 to 20°C (20, 23, 65, 72, 73). The fact that cereulide production is activated at high cell densities, i.e., in the transition phase (38), may point to an additional role in intercellular signaling and/or cannibalism when nonproducing cells are targeted cells and killed, finally leading to a release of amino acids that subsequently serve as nutrients for the surviving population as described for stationary-phase cultures of *B. subtilis* (37). Alternatively, cereulide could play a role in intercellular signaling, thereby affecting cellular differentiation in the targeted subpopulation, resulting in phenotypic differentiation within emetic *B. cereus* populations. Lopez et al. (36) recently showed that exposure of *B. subtilis* cells to nystatin and surfactin resulted in loss of intracellular K⁺ with concomitant activation of KinC, a membrane protein kinase. This kinase controls activation of a set of genes involved in the production of extracellular polysaccharides that constitute the matrix of the biofilm, thus stimulating biofilm formation in *B. subtilis*. Since cereulide, like valinomycin, acts as a K⁺ ionophore, efflux of K⁺ from the cells down a concentration gradient will result in lowering of intracellular K⁺ levels, which could act as a signal for sensor kinases present in *B. cereus* group members, conceivably affecting cell physiology, including activation of pathways associated with cellular differentiation, such as production of matrix components and/or sporulation. Recently, evidence has been provided that transition state regulator AbrB, which is linked to biofilm formation in *B. subtilis* (24), controls production of cereulide in *B. cereus* F4810/72 (38). The role of cereulide production in biofilm formation of emetic *B. cereus* remains to be elucidated and is therefore currently studied in our laboratory.

In conclusion, our data show that cereulide, the toxin produced by emetic *B. cereus*, dissipates the membrane potential in sensitive Gram-positive bacteria, thereby providing a possible biological function of this K⁺ ionophore in the environment, i.e., outside the human host. Whether cereulide, besides its putative role in microbial warfare, has a role in intercellular signaling, cellular differentiation, and biofilm formation in emetic *B. cereus* remains to be elucidated.

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