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MINIREVIEW

Cereulide and Valinomycin, Two Important Natural Dodecadepsipeptides with Ionophoretic Activities

MAGDALENA ANNA KROTEŃ, MAREK BARTOSZEWICZ* and IZABELA ŚWIĘCICKA

Department of Microbiology, Institute of Biology, University of Białystok,
Świerkowa 20B, 15-950 Białystok, Poland

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Abstract

Cereulide produced by *Bacillus cereus sensu stricto* and valinomycin synthesized mainly by *Streptomyces* spp. are natural dodecadepsipeptide ionophores that act as potassium transporters. Moreover, they comprise three repetitions of similar tetrapeptide motifs synthesized by non-ribosomal peptide synthesis complexes. Resemblances in their structure find their reflections in the same way of action. The toxicity of valinomycin and cereulide is an effect of the disturbance of ionic equilibrium and transmembrane potential that may influence the whole organism and then cause fatal consequences. The *vlm* and *ces* operons encoding valinomycin and cereulide are both composed of two large, similar synthetase genes, one thioestrase gene and four other ORFs with unknown activities. In spite of the characterization of valinomycin and cereulide, genetic determinants encoding their biosynthesis have not yet been clarified.

Key words: *Bacillus cereus*, cereulide, valinomycin, ionophore

Introduction

The life and growth of both prokaryotic and eukaryotic cells is an incessant exchange of molecules with the extracellular environment. Depending on the kind of transported molecules and necessary energy expenditures three distinct ways of this process can be distinguished: (i) simple diffusion, (ii) passive transport which does not require energy, and finally (iii) active transport supported by energy accumulated in ATP for carrying molecules unlike their gradient of concentrations. Especially ions and large hydrophobic particles penetrate into cells using specific carriers. Thus ionophores, lipid-soluble molecules transporting ions across the lipid bilayers are essential (Booth, 1988; Bethal, 2006). One of the best characterized ionophores is valinomycin, but much attention is also paid to cereulide because of mutual resemblances with valinomycin and its pathological impact. Valinomycin is produced mainly by members of the genus *Streptomyces* (Actinobacteria), non-motile, filamentous and specific spore forming Gram-positive with strong similarities to fungi in their colony morphology (Goodfellow *et al*., 1992; Cheng, 2006). Additionally, these bacteria are able to produce several extracellular hydrolytic enzymes and at least two third of known antibiotics (Omura *et al.*, 2001). While cereulide, also regarded as emetic toxin, is synthesized by *Bacillus cereus sensu stricto* (Agata *et al*., 1995; Granum and Lund, 1997; Hoton *et al*., 2005), a Gram-positive spore former predominantly found in soil, but also in a wide range of different foodstuffs like fried and boiled rice, milk, eggs, noodles, pasta, potatoes, bread, meat, vegetables, bakery and starchy products, including ready-to-eat ones (Finlay *et al*., 2002; Jääskeläinen *et al*., 2003a; Jensen *et al*., 2003; Guinebretiere *et al*., 2006; Bartoszewicz *et al*., 2008). Moreover, *Bacillus cereus* and its relatives such as *Bacillus thuringiensis* can also be found in arthropod guts (Święcicka and Mahillon, 2006) and mammalian gastrointestinal tracts (Święcicka *et al*., 2006; Wilcks *et al*., 2006). The significance of these bacilli is much higher as they may cause two different kinds of food poisonings: (i) emetic syndrome characterized by vomiting and related to cereulide, and (ii) diarrhoeal syndrome caused by several enterotoxins such as hemolysin BL, non-hemolytic enterotoxin and cytotoxin K (Granum and Lund, 1997; Hansen and Hendriksen, 2001; Bartoszewicz *et al*., 2006; Michelet *et al*., 2006). *B. cereus s.s.* could also be the causative agent of

* Corresponding author: M. Bartoszewicz, Department of Microbiology, Institute of Biology, University of Białystok, Świerkowa 20B, 15-950 Białystok, Poland; phone (+48) 857457365; fax (+48) 857457302; **e-mail: mbartosz@uwb.edu.pl**

periodontitis, osteomyelitis, ocular and wounds infections (Drobniewski, 1993; Helgason *et al*., 2000).

Valinomycin with its chemical formula cyclo(L-Val-D-α-hydroxyisovaleric acid-D-Val-L-Lac-L-Val-)$_3$ is an antibiotic substance with wide range of activity against different bacteria such as *Mycobacterium* spp., *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Micrococcus luteus*, fungies like *Candida albicans* and *Cryptococcus neoformans* (Pettit *et al*., 1999; Park *et al*., 2008), and virus-infected cells (Cheng, 2006). Recently, several authors also pointed out its role against SARS disease and tumor cells (Cheng, 2006). Moreover, Pettit *et al*. (1999) provided proof that a 10 μM valinomycin solution reduces vesicular stomatitis virus in Vero cells due to the lower level of virion production. Unfortunately, because of its toxicity to eukaryotic cells, valinomycin cannot be used in human therapy. Cereulide, the valinomycin-related dodecadepsipeptide described by Agata *et al.* (1994, 1995), is linked to emetic syndrome of human food poisoning, usually mild illness with occasionally fatal consequences (Mahler *et al*., 1997; Dierick *et al*., 2005). In spite of several resemblances in chemical composition, the similarity between cereulide and valinomycin remains largely unknown. In this paper we present current knowledge about those two natural ionophores, their positive and negative impact on human activity and their potential application in medical treatment.

Genesis and structure of natural ionophores

Transmembrane ion carriers are divided into two classes. The first class includes transporters that combine ions outside the cell, then move across the membrane and release ions in the inner cellular environment after conformational changes. The second class is composed of channel formers, hydrophilic pores in the membrane allowing ions to pass through, simultaneously preventing their contact with the hydrophobic membrane interior. So far a few different channel formers, like natural gramicidin or alamethicin and several natural mobile carriers such as nonactin (ammonium), nigericin (K^+, Rb^+), salinomycin (K^+) and narasin (K^+, Na^+, Rb^+) have been characterized (Radko *et al*., 2006). Most of them are regarded as antibiotics produced mostly by *Streptomyces* spp. but nowadays also synthetic ionophores are constructed and synthesized. Their size usually varies from 200 to 2000 Da, depending on the number of similar and repeatable subunits in their structure. Their main function is highly specific regulation of basic cations amounts: Na^+, K^+, Mg^{2+}, Ca^{2+}.

According to the International Union of Pure and Applied Chemistry (IUPAC) (http://www.iupac.org) depsipeptides are defined as peptides with at least one peptide bond replaced by an ester bond like in montanastatin (produces by *Streptomyces anulatus*), enniatin (from *Fusarium scirpi*), salinomycin (synthetised by *Streptomyces albus*) and monensin (from *Streptomyces cinnamonensis*). Recently valinomycin synthesized mainly by *Streptomyces tsusimaensis*, *S. griseus* and *B. subtilis* (Wulff *et al*., 2002; Cheng, 2006), and cereulide from *B. cereus* undergo intensive studies (Dierick *et al*., 2005; Hoton *et al*., 2005). The chemical structures of both ionophores as well as their repeatable motifs are presented in Figure 1.

As shown, cereulide consists of four repetitions of [D-α-hydroxy-4-methylpentanoic acid, D-Alanine, L-α-hydroxyisovaleric acid, and L-Valine] fragment respectively (Agata *et al*., 1994), whereas in valinomycin D- and L-*O*-valine are found by turns with lactic acid and hydroxyvalerate acid (Brockmann and Schmidt-Kastner, 1955). From *in silica* analyses it was also suggested that D-α-hydroxyisovaleric acid (D-Hiv), L-lactic acid (L-Lac) and L-valine (L-Val) may be biosynthetic precursors in the valinomycin biosynthesis pathway (Cheng, 2006), but this needs to be clarified by performing ATP exchange assays.

The action of cereulide and valinomycin is linked to their hydrophilic interior which allows K^+ ions to pass through. From the outside the ionophores are lipophilic and exactly fit the membrane. Moreover, the structure of the cavity inside the cyclic molecule supplies the ability of differ amino- and 2-hydroxyacids to hold K^+ there (Duax *et al*., 1996). Thus potassium may move through lipid membranes in accordance with its electrochemical gradient.

The chemical composition, cyclic structure and presence of D-amino acids in both cereulide and valinomycin and genetic data are all strong evidences indicating that these ionophores are biosynthesized by a nonribosomal peptide synthetase system (Marahiel *et al*., 1997; Siebier and Marahiel, 2003; Ehling-Schulz *et al*., 2006; Cheng, 2006). Still, there is a deficiency of data concerning cereulide synthesis, though on the basis of structural similarities we could expect a mechanism related to that proposed for valinomycin. That process requires four modules grouped in two synthetases and encoded by two large genes in the valinomycin synthetase operon, which are responsible for appropriate transformations (*i.e.* epimerization) and incorporation of precursors. The activity of synthetases results in the formation of a tetradepsipeptide structure. Probably after next two rounds of this synthetase complex, a linear 12-element particle is formed. It undergoes cyclisation and then achieves the final form of a cyclic dodecadepsipeptide (Cheng, 2006). Also, the machinery for cereulide production exploits two synthetases that form a tetrapepside structure. These fragments are joined into a linear particle. Subsequent cyclization leads to mature cereulide molecule.

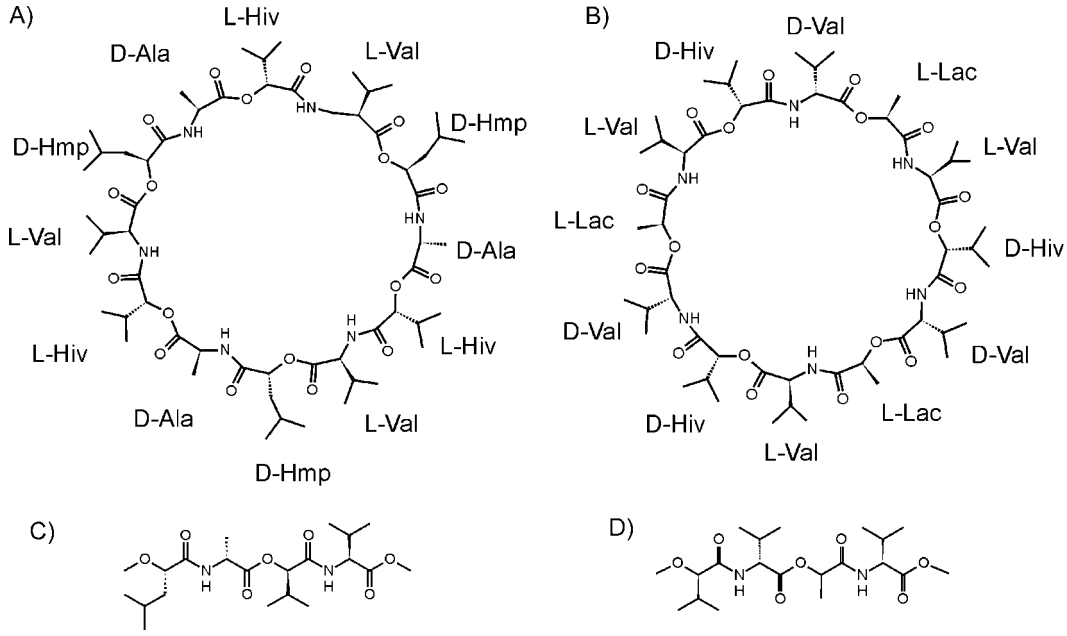

Fig. 1. Chemical structure of two related cyclic dodecadepsipeptides, (A) cereulide from *Bacillus cereus* and (B) valinomycin from *Streptomyces tsusimaensis*, and (C, D) their tetradepsipeptide constant motifs. Val-valine, Hmp-D-α-hydroxy-4-ethylpentanoic acid, Ala-alanine, Hiv-hydroxyisovaleric acid. Letters L and D indicate enantiomers.

Although conditions required for valinomycin synthesis are poorly described and they may depend on bacterial species, cereulide is most effectively formed at 10–15°C in the presence of oxygen. Temperatures above 37°C or the lack of oxygen do not permit cereulide synthesis (Finlay *et al*., 2000; Jääskeläinen *et al*., 2004). This emetic toxin is usually detectable at the end of *B. cereus* log-phase growth (Häggblom *et al*., 2002).

Genetic basis of cereulide and valinomycin synthesis and inheritance

Comparison of operons encoding cereulide and valinomycin. It has been previously suggested that depsipeptides are synthesized by way of non-ribosomal peptide synthesis (NRPS) by specific enzymatic complexes, each encoded by several genes (Marahiel *et al*., 1997; Horwood *et al*., 2004; Cheng, 2006). However, until now only a few such NRPS complexes have been well characterized, especially these responsible for cereulide and valinomycin production (Ehling-Schulz *et al*., 2006; Cheng, 2006).

Cereulide synthesis is encoded by a plasmid-located *ces* operon that can easily be detected by PCR and RT-PCR (Ehling Schulz *et al*., 2004b; Hoton *et al*., 2005; Ehling-Schulz *et al*., 2006; Fricker *et al*., 2007). The emetic 270 kb plasmid pBc270 from reference *B. cereus* strain F4810/72 is related to a single evolutionary lineage of emetic *B. cereus sensu stricto* isolates (Ehling-Schulz *et al*., 2005; Rasko *et al*., 2007) but recently Thorsen *et al*. (2006) also described *B. weihenstephanensis* isolates producing cereulide. Nevertheless, it still appears that an emetic characteristic is restricted to closely related bacilli.

The 23 kb *ces* operon from *B. cereus* F4810/72 was sequenced and described, including large flanking regions (Ehling-Schulz *et al*., 2006). Its organization is presented in Figure 2. As shown, it comprises seven genes with different activities responsible for activation, epimerization and cyclisation processes. The special interest focuses especially on two largest genes named *cesA* (~10kb) and *cesB* (~8kb) because their expression leads to the incorporation and modification of D-*O*-Leu, D-Ala (*cesA*), L-*O*-Val, and D-Val (*cesB*) which compose the tetradepsipeptide repetitive motif in the cereulide ring structure. Moreover, the *ces* operon contains epimerase and thioestrase domains that may act in the cleavage and cyclisation of the tetradepsipeptide motif, as has been suggested elsewhere for valinomycin (Cheng, 2006). Additionally in the *ces* operon five more genes, *cesH*, *cesP*, *cesT*, *cesC* and *cesD* have been described. The *cesH* gene shows high similarity with hydrolases from *B. cereus s.l.*, while the *cesP* gene appears to be similar to phosphopantetheinyl transferase gene involved in surfactin and gramicidin synthesis. The *cesT* gene shows homology with thioestrase gene connected with NRPS. Finally *cesC* and *cesD* genes encode a putative ABC transporter proposed to be the whole synthetase transporter (Ehling-Schulz *et al*., 2006).

Recently Lucking *et al*. (2009) demonstrated that cereulide synthesis is independent of the *plcR* gene,

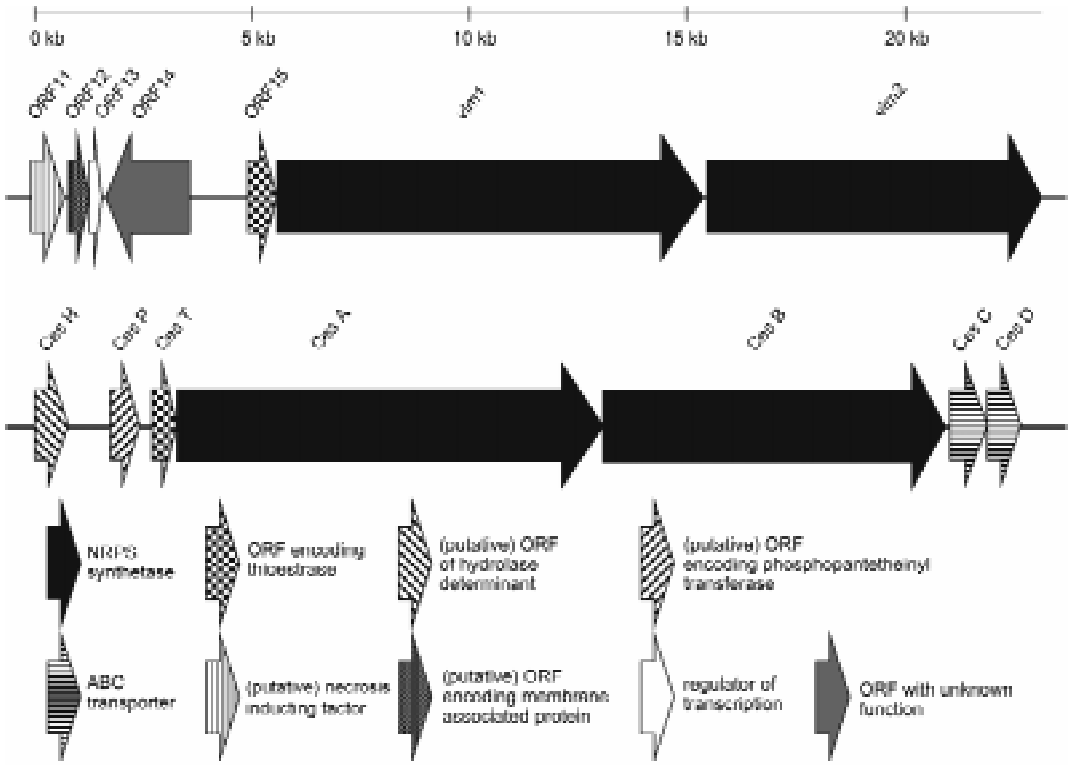

Fig. 2. Organisation of operons encoding (A) valinomycin and (B) cereulide based on Cheng (2006) and Ehling-Schulz *et al.* (2006), respectively.

NRPS synthetases are of critical importance in valinomycin and cereulide biosynthesis, other genes play a supporting role. Arrows below indicate putative functions of ORFs in both operons.

B. cereus specific virulence regulator but appears to be controlled by AbrB, a transition state regulator. Overexpression of this factor leads to non toxic phenotype of emetic strains.

In the 23-kb *vlm* operon encoding valinomycin there are also seven identified ORFs (Cheng, 2006). Sequencing analyses and subsequent comparison with other known NRPS operons revealed that two largest ORFs named *vlm1* (~10 kb) and *vlm2* (~8 kb) are synthetase genes directly involved in incorporation of valinomycin precursors and dodecadepsipeptide synthesis. Moreover, it was suggested that *vlm1* and *vlm2* correspond to *cesA* and *cesB*, respectively. The remaining genes are supposed to be necrosis inducing factor (OFR11), membrane associated protein (ORF12), regulator of transcription (ORF13), and, similarly to *ces* operon, thioestrase (ORF15). ORF14 is still a mystery, its function is unknown and in contrast to all remaining ORF's it is orientated in the opposite direction (Cheng, 2006).

Background of valinomycin and cereulide inheritance. The location of the *ces* operon on large plasmid potentially enables horizontal gene transfer with emetic isolates as donors and non-emetic strains as receptors (Ehling-Schulz *et al*., 2006; Hoton *et al*., 2005). Since Van der Auwera *et al*. (2007) have shown that conjugation takes place in different matrices including foodstuffs and that plasmids could initiate their own transfer, the problem of genetic exchange elevates its significance (Van der Auwera *et al*., 2008). If the cereulide plasmid is conjugative we should expect *ces*-positive isolates to be genetically diverse in opposition to observed facts indicating the clonal structure of emetic strains (Ehling-Schulz *et al*., 2005). Thus, conjugation embracing transfer of emetic plasmid is extremely rare or even impossible.

Several plasmids similar to emetic pBc270 have also been described. For example, pBc10987 from pathogenic *B. cereus* ATCC 10987 is about 208 kb but it shows high homology with the *ces* operon flanking regions (Ehling-Schulz *et al*., 2006). Moreover, pXO1 from *B. anthracis* shows almost the same fragments as the plasmid present in *B. cereus* F4810/72 indicating common ancestry of all these strains (Rasko *et al*., 2004; Ehling-Schulz *et al*., 2005; Ehling-Schulz *et al*., 2006). So far little has been established regarding the evolution of large plasmids in the *B. cereus* group, but this problem is of extreme importance because plasmid-borne features are species-typical (for *B. thuringiensis* – plasmids with *cry* genes; for *B. anthracis* pXO1 and pXO2; for emetic *B. cereus* – pCER270). Moreover, they are responsible for the specific pathogenicity of these bacteria (Van der Auwera *et al*., 2005; Święcicka *et al*., 2008).

Loci of the *vlm* operon have not been established but its presence was demonstrated in *Streptomyces* as well as in *Bacillaceae*, two groups of just slightly related bacteria (*Streptomyces* belong to *Actinobacteria* while *Bacillaceae* are members of *Firmicutes*). Still, there is no data from, for example, hybridization studies indicating chromosomal or plasmid origin of the *vlm* operon, but its wide distribution indirectly supports the expectation of plasmid origin. Moreover *vlm* operon contains 70.7% CG, being characteristic for *Streptomyces* sp. bacteria (Cheng, 2006) whereas *Bacillus* sp. strains in general contain much less G+C (Priest, 1993).

Mode of action of valinomycin and cereulide

Potential risks and influence of cereulide and valinomycin on organisms. Cereulide, a heat-stable toxin withstanding autoclaving at 121°C for 15 min (Shinagawa *et al*., 1995) or even 90 min heating at 126°C (Turnbull *et al*., 1979; Rajkovic *et al*., 2008), tolerates also pH 2 and 11, as well as proteolytic enzymes. Thus, it seems it is rather impossible to inactivate cereulide during normal, daily food-processing procedures, so it has a huge significance for health hazard and food poisoning epidemiology as shown by Mahler *et al*. (1997) and Dierick *et al*. (2005). Moreover cereulide-related diseases are often mistaken with those caused by staphylococci because symptoms of both intoxications show high resemblances (Granum and Lund, 1997; Bartoszewicz *et al*., 2006). First signals of acute emetic poisoning appear 1–6 h after intoxication and they consist of abdominal pain, vomiting and respiratory distress (Granum and Lund, 1997; Salkinoja-Salonen *et al*., 1998). A girl who died in 2003 in Belgium suffered from severe pulmonary hemorrhage, coma, diffuse bleeding, muscle cramps and metabolic acidosis (Dierick *et al*., 2005). In this case a postmortem liver biopsy showed microvascular and extensive coagulation necrosis, the presence of emetic *B. cereus* was confirmed in the liver and in the spleen, probably as a result of posthumous transfer. Interestingly, Briley *et al*. (2001) described non-typical outbreaks in which skin contact with *B. cereus* contaminated foodstuff resulted also in emesis suggesting that cereulide could be absorbed by the skin, epidermis and mucous membranes.

Valinomycin poisonings have not been widely reported. However, the antibiotic may react as an irritant in the case of the skin or eye contact. As pointed out in the Material Safety Data Scheet for valinomycin supplied by the manufacturers, inhalation of this substance can lead to breathing disturbances while ingestion can cause loss of conscious. Moreover, severe valinomycin over-exposure may result in death. Lethal doses (LD_{50}) of valinomycin for mouse were established to be at the level of 2.5 mg kg^{-1} of body mass, whereas LD_{50} in acute dermal toxicity for rabbit equals 5 mg kg^{-1} of body mass. Valinomycin may also provoke several chronic effects, like damage of the central and peripheral nervous system, eyes, lens and cornea. It is also suspected to cause tremors, convulsions, aggressive behavior, and often affects heart and kidneys, but no clinical evidence has been presented. Valinomycin also demonstrates positive antifungal, insecticidal, nematocidal, antibacterial and antitumor activities (Perkins *et al*., 1990; Pettit *et al*., 1999; Paananen *et al*., 2005; Cheng, 2006; Park *et al*., 2008), while cereulide is being known only with toxicity (Agata *et al*., 1995; Dierick *et al*., 2005).

Mode of action of valinomycin and cereulide on cells. The symptoms of cereulide and valinomycin intoxication are results of organism hemostasis disturbance and previous cells dysfunctions. In every cell, due to ion carriers plasma membranes guarantee keeping of stable inner environment and electric membrane potential between opposite sides of the membrane. Among ionophores, the essential function, required especially for receiving signals from the surroundings, is removing Na^+ from the cell, and uptaking K^+ performed by potassium-sodium ion pump. The disturbance caused by cereulide depends on transmembrane potential. Sufficient electrochemical gradient causes K^+ migration towards membrane side with negative charge. The lack of electric potential leads to leak out of potassium ions down the gradient of concentration (Andersson *et al*., 2007). The loss of K^+ gradient takes effect in severe dysfunctions, like swelling mitochondria in cells affected by cereulide. Mikkola *et al*. (1999) suggested that mitochondria shape changes as a result of phospholipase A_2 activation and permeability modification stimulated by Ca^{2+}. Modification of shape probably also accompanies the blockade of oxidative phosporylation and finally causes reduction of spermatozoa motility, as has been proven by several authors (Häggblom *et al*., 2002; Ehling-Schulz *et al*., 2004a; Hoton *et al*., 2005; Rajkovic *et al*., 2006). Moreover, these suppositions are supported by mitochondrial membrane depolarization caused by cereulide, simultaneously depriving synthetase ATP driving force (Andersson *et al*., 2007).

Modification in membrane permeability and reduction of energy production lead to reduction of immunological activity of natural killer cells (NK) (Paananen *et al*., 2000). Cereulide and valinomycin affect susceptible cells leading to formation of large vacuoles in the cytoplasm, moreover they are responsible for changes in the nucleus, mild chromatin condensation and distortion of mitochondrial cristae. Then, both decadepsipeptides diminish cytokine production, which could depress the immunological potential of

Table I
Comparison of cereulide and valinomycin action on cells and organisms.

Action	Valinomycin	Cereulide	References
Respiratory distress	+	+	Granum and Lund, 1997
Abdominal pain, vomiting	N.D.	+	Granum and Lund, 1997, Dierick *et al*., 2005
Loss of consciousness, coma	+	+	Dierick *et al*., 2005
Metabolic acidosis	N.D.	+	Dierick *et al*., 2005, Mahler *et al*., 1997
Mortality	+	+	Dierick *et al*., 2005
Pulmonary hemorrhage, diffuse bleeding, muscle cramps	N.D.	+	Dierick *et al*., 2005
Damage of central and peripheral nervous system	+	N.D.	Briley *et al*., 2001
Effect on skin, eyes	+	+	Mikkola *et al*., 2004, Rajkovic *et al*., 2006
Swelling mitochondria, blockade of oxidative phosphorylation, reduction of spermatoza motility	N.D.	+	Jääskeläinen *et al*., 2003b, Ehling-Schulz *et al*., 2004a
Toxicity to NK	+	+	Paananen *et al*., 2002
Diminished cytokine production	+	+	Paananen *et al*., 2002
Increased apoptosis	+	+	Paananen *et al*., 2000, Paananen *et al*., 2005

intoxicated organisms. On the other hand they increase apoptosis (Paananen *et al*., 2002; Pannanen *et al*., 2005). This process starts when a specific signal is received through the aid of apoptosis receptors like Fas, TNF-α or DR located on the surface of the cell. Transduction of this signal leads to the initiation of a cascade of further processes. There are two main types of apoptosis, caspase-3 dependent and caspase-3 independent (Maianski *et al*., 2003). Interestingly valinomycin and cereulide may induce both ways of apoptosis, but there is evidence that valinomycin-induced apoptosis is predominantly caspase-independent (Paananen *et al*., 2005).

Finally, the reaction to cereulide and valinomycin depends on the target cells. HeLa and T cells are much less sensitive to intoxication than NK while monocytes demonstrate no changes. The motility of boar spermatozoa is drastically reduced while bovine spermatozoa do not react at all. In general, all sensitive cells react rapidly to cereulide and valinomycin exposition, probably due to lipophilicity which enables simple migration of toxins through tissues in the same way as di(para-chloro-phenyl)-trichloroethane (DDT), the chemical pesticide widely used in the middle of the XXth century (Beart, 2006). Mode of action of cereulide and valinomycin on cells and organisms are compared in Table I.

Conclusion. Several authors have mentioned the huge resemblance of cereulide and valinomycin. Moreover, both toxins are being intensively examined and numerous functional similarities, approximate toxicity and mechanism of action have been described. However, biochemical resemblances have no counterpart in genetics because the *ces* and *vlm* operons demonstrate significant differences. Interestingly, the presence of *ces* operon is restricted to a single evolutionary line of emetic *B. cereus* isolates, whereas *vlm* operon is widely distributed. Thus it may provide helpful data concerning bacterial evolution and horizontal gene transfer.

Actually, the interest in cereulide and valinomycin concentrates especially on their toxicity and practical application. Valinomycin has strong anti-tumor, antibacterial and even anti-viral activity, but its negative influence on the human body restricts its use in therapy. On the other hand, cereulide is known only because of its poisonous potential. Accurate analyses of the chemical structure and mode of action of both substances should allow the future construction of their derivatives with modified properties: reduced toxicity and improved clinical activity.

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