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Food and Indoor Air Isolated *Bacillus* Non-Protein Toxins: Structures, Physico-Chemical Properties and Mechanisms of Effects on Eukaryotic Cells

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Abbreviations

AFC	atom/fragment contribution
ATP	adenosine 5'-triphosphate
BCF	bioconcentration factor
BLM	black-lipid membrane
CCCP	carbonyl cyanide m-chlorophenylhydrazone
CFU	colony-forming unit
CMC	critical micelle concentration
COSY	correlation spectroscopy
Da	dalton
DDT	1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane
DMPC	dimyristoylphosphatidylcholine
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
EEC	European Economic Community
ERETIC	electronic reference to access in vivo concentrations
ESI	electrospray ionization
EU	European Union
FCCP	carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
GC	gas chromatography
GRAS	generally regarded as safe
IT-MS	iontrap mass spectrometry
HSQC	heteronuclear multiple quantum correlation
m/z	mass-to-charge ratio
MS	mass spectrometry
MALDI-TOF	matrix-assisted laser desorption/ionization time-of- flight
MS/MS	tandem mass spectrometry
NAD, NAD^+ and NADH	nicotinamide-adenine dinucleotide and its oxidized and reduced forms
NK	natural killer
NMR	nuclear magnetic resonance
PCB	polychlorinated biphenyls
PN	pyridine nucleotides
PTP	permeability transition pore
QSAR	quantitative structure-activity relationship
TQ-MS	triple quadrupole mass spectrometry
RLM	rat liver mitocondria
ROS	reactive oxygen species
RP-HPLC	reversed-phase high-performance liquid chromatography
SMILES	simplified molecular input line entry system
SPE	solid-phase extraction
TOCSY	total correlation spectroscopy
UV	ultraviolet

Abstract

We report here the structures and properties of heat-stable, non-protein, and mammalian cell-toxic compounds produced by spore-forming bacilli isolated from indoor air of buildings and from food. Little information is available on the effects and occurrence of heat-stable non-protein toxins produced by bacilli in moisture-damaged buildings. Bacilli emit spores that move in the air and can serve as the carriers of toxins, in a manner similar to that of the spores of toxic fungi found in contaminated indoor air. *Bacillus* spores in food cause problems because they tolerate the temperatures applied in food manufacture and the spores later initiate growth when food storage conditions are more favorable.

Detection of the toxic compounds in *Bacillus* is based on using the change in mobility of boar spermatozoa as an indicator of toxic exposure. GC, LC, MS, and nuclear magnetic resonance NMR spectroscopy were used for purification, detection, quantitation, and analysis of the properties and structures of the compounds. Toxicity and the mechanisms of toxicity of the compounds were studied using boar spermatozoa, feline lung cells, human neural cells, and mitochondria isolated from rat liver. The ionophoric properties were studied using the BLM (black-lipid membrane) method.

One novel toxin, forming ion channels permeant to $K^+ > Na^+ > Ca^{2+}$, was found and named amylosin. It is produced by *B. amyloliquefaciens* isolated from indoor air of moisture-damaged buildings. Amylosin was purified with a RP-HPLC and a monoisotopic mass of 1197 Da was determined with ESI-IT-MS. Furthermore, acid hydrolysis of amylosin followed by analysis of the amino acids with the GS-MS showed that it was a peptide. The presence of a chromophoric polyene group was found using a NMR spectroscopy. The quantification method developed for amylosin based on RP-HPLC-UV, using the macrolactone polyene, amphotericin B (MW 924), as a reference compound.

The *B. licheniformis* strains isolated from a food poisoning case produced a lipopeptide, lichenysin A, that ruptured mammalian cell membranes and was purified with a LC. Lichenysin A was identified by its protonated molecules and sodium- and potassium-cationized molecules with MALDI-TOF-MS. Its protonated forms were observed at *m/z* 1007, 1021 and 1035. The amino acids of lichenysin A were analyzed with ESI-TQ-MS/MS and, after acid hydrolysis, the stereoisomeric forms of the amino acids with RP-HPLC.

The indoor air isolates of the strain of *B. amyloliquefaciens* produced not only amylosin but also lipopeptides: the cell membrane-damaging surfactin and the fungicidal fengycin. They were identified with ESI-IT-MS observing their protonated molecules, the sodium- and potassium-cationized molecules and analysing the MS/MS spectra. The protonated molecules of surfactin and fengycin showed *m/z* values of 1009, 1023, and 1037 and 1450, 1463, 1493, and 1506, respectively.

Cereulide (MW 1152) was purified with RP-HPLC from a food poisoning strain of *B. cereus*. Cereulide was identified with ESI-TQ-MS according to the protonated molecule observed at *m/z* 1154 and the ammonium-, sodium- and potassium-cationized molecules observed at *m/z* 1171, 1176, and 1192, respectively. The fragment ions of the MS/MS spectrum obtained from the protonated molecule of cereulide at *m/z* 1154 were also interpreted. We developed a quantification method for cereulide, using RP-HPLC-UV and valinomycin (MW 1110, which structurally resembles cereulide) as the reference compound. Furthermore, we showed empirically, using the BLM method, that the emetic toxin cereulide is a specific and effective potassium ionophore of whose toxicity target is especially the mitochondria.

1. Introduction

The many species of the genus *Bacillus* produce valuable metabolites with the potential for technical and scientific applications. On the other hand, several strains of *Bacillus* species also produce compounds toxic to mammalian cells. It is worth noting that some *Bacillus* species with a GRAS (generally regarded as safe) status are able to produce toxins. *Bacillus* species are able to produce physically and chemically resistant endospores if the conditions needed for the vegetative bacteria to survive are limited. These spores can survive extreme environmental conditions such as dryness, heat, radiations and chemical treatments. For instance, it was shown that *B. pumilus* spores were resistant to the ultraviolet (UV) radiation conditions occurring on Mars (Newcombe et al. 2005). Such resistant endospores can remain dormant for long periods and are one reason why *Bacillus* species are ubiquitous in diverse environments. The ubiquitous nature of *Bacillus* species (McKillip 2000) suggests that certain toxic strains of the *Bacillus* group are likely to be common in water-damaged indoor environments and in food. The toxins produced by *Bacillus* can be divided into the heat-labile, such as proteins, and heat-stable such as peptides. Information on the chemical and biological properties and evaluation of the toxicity, frequency and dose of exposure form the basis on which estimations of hazardous effects can be made.The heat stability and resistance of the toxins to enzymes indicate persistence in environment, whereas high levels of hydrophobicity suggest that the substances may permeate the cell membrane and accumulate in the fatty tissues. The present study is focused on those indoor air and food poisoning *Bacillus* species that produce environmentally resistant heat-stable non-protein compounds toxic to mammalian cells.

1.1. Microbes and toxic metabolites contaminating food

In most food poisoning cases the source of intoxication remains unknown (McCabe-Sellers & Beattie 2004). On the other hand, when bacteria are responsible for poisonings, serious foodborne illnesses may occur. *Bacillus* species are capable of forming spores and growing under both aerobic and anaerobic conditions, indicating that it is almost impossible to avoid *Bacillus* contamination in food. Other foodborne hazards are caused by the spore-forming bacteria *Clostridium perfringens* and *C. botulinum*, which grow under anaerobic conditions. Most *Bacillus* strains are believed to be harmless if found in low concentration such as < 1000 colony forming units (CFU)/g. However, at suitable growing temperatures (20-50 °C) an organism such as *B. cereus* may multiply in 20 min (Maier et al. 1999). This means that starting from < 1000 CFU/g it requires less than 1.5 hours to reach >10 000 CFU/g, which is considered as a food spoilage amount of *B. cereus* (Notermans and Batt. 1998, Gilbert et al. 2000). Furthermore, certain *Bacillus* strains are pathogenic and form hazardous toxic metabolites. Therefore, serious bacterial food poisonings are usually ill-fated combinations of improper food handling and accidents.

It was estimated that in the USA food poisoning cases annually cost 2-3 billion dollars (McCabe-Sellers & Beattie 2004) and that every year there are 27 000 cases in which *B. cereus* is involved (Mead et al. 1999). *Bacillus cereus* caused 104 documented food poisoning outbreaks in Taiwan and 50% of the ready-to-eat food items contained *B. cereus* (Fang et al. 2003). As many as 5% of foodborne outbreaks have been connected with *B. cereus* in the Netherlands, England, France, and the USA (Rosenquist et al. 2005). Food intoxications in Japan caused by *B. cereus* during 1982-2001 were especially of the emetic types (Kawamura-Sato et al. 2005).

The strains of *B. cereus* produce food poisoning protein and peptide toxins: as many as 10 different heat-labile protein enterotoxins causing abdominal cramps, diarrhea, and nausea and the heat-stable peptide toxin cereulide which causes vomiting (Kotiranta et al. 2000). Some of these enterotoxins are formed in the gut and cause problems when food containing live toxin-producing bacteria is ingested (Le Loir et al. 2003). Heating of food at high temperature may prevent this intoxication by killing the vegetative bacteria and destroying the heat-labile enterotoxins. The situation is a different with the heat-stable toxins, because they may pass through many food-processing steps before causing intoxication.

Foodborne bacteria such as *Staphylococcus aureus, Escherichia coli,* and *C. perfringens* also produce several heat-stable protein enterotoxins that cause intoxication at low nanogram concentrations (Bennett 2005, Balaban and Rasooly 2000, Schmitt et al. 1999, Le Loir et al. 2003**,** Lund et al. 2000, Shatursky et al. 2000). *Escherichia coli, B. cereus, C. botulinum* and *C. perfringens* produce heat-labile protein enterotoxins. The *E. coli* enterohemorrhagic heat-labile protein toxin causes bloody diarrhea and *C. botulinum* heat-labile botulin causes botulism. The enterotoxins are either a single protein such as the *B. cereus* enterotoxins cytotoxic CytK (34 kDa, Lund et al. 2000), hemolysin I, 55 (kDa), hemolysin II (30 kDa), mouse lethal toxin (MLT, 33-34 kDa, Shinagawa et al. 1991) and enterotoxin FM (45 kDa, Asano et al. 1997) or multi component proteins as hemolytic three-unit hemolysin BL (HBL) which consists of 36-, 35-, and 45- kDa proteins (Beecher et al. 1995a) or nonhemolytic enterotoxin (NHE), a three-component toxin consisting of 39-, 45-, and 105-kDa proteins (Lund and Granum 1996). Most of the enterotoxins act as porins in cells of the small intestinal epithelium causing diarrhea, abdominal cramps, and pain (Laohachai et al. 2003, Le Loir et al. 2003). Some are both hemolytic and cytotoxic (Schmitt et al. 1999, Lund et al. 2000).

Cereulide is a heat-stable cyclic dodecadepsipeptide (Agata et al. 1994), tolerating autoclaving at 121 °C for 15 min (Shinagawa et al. 1995), pH 2 and 11, and proteolytic enzymes. Thus it appears impossible to inactivate it during normal food-processing procedures. In fatal food poisoning cases caused by *B. cereus* (Mahler et al. 1997, Dierick et al. 2005) the fulminant liver failures of patients were connected with cereulide intoxication. The concentrations provoking the acute toxicity effect of cereulide in humans and monkeys were estimated as 8-10 µg /kg of the body weight (Agata et al.1995, Shinagawa et al. 1995, Jääskeläinen et al. 2003a).

The protein enterotoxins produced by *C. perfringens* can cause problems by contaminating various meat products, whereas *C. botulinum* and the protein toxin botulin are found in contaminated smoked fish, honey, and canned products. The *B. cereus* spores were hydrophobic and accumulated in the fat droplets of milk, e.g. when *B. cereus* was found in 56% of pasteurized milk samples (Larsen and Jorgensen 1997). When 325 food samples were examined, one third of the total and half of the cream cheese and spreading butter samples contained *B. cereus* (Schlegelova et al. 2003). Of 48 901 ready-to-eat food samples examined 98.7% contained *B. cereus*-like organisms at concentrations of < 1000 CFU/g, 0.7% at 1000-10 000 CFU/g, and 0.5 % at >10 000 CFU/g. The samples of cucumber, tomato (2.6%), rice (1.3%), dessert with milk and flour (1.0%), dessert with milk and rice (3.1%), and cake custard (1.2%) contained >10 000 CFU/g of *B. cereus* (Rosenquist et al. 2005). Studies of *B. cereus* growing in food products show that in meat, rice, and milk products the *B. cereus* doubling times vary from 20 min to 3 h at 30 °C. The lowest doubling times were in milk (20-36 min), cooked rice (26-31 min), and infant formulas (56 min) (Sutherland et al. 1996). Thus, in the milk and cooked rice *B. cereus* is able to multiply from 1000 CFU/g to 10 000 CFU/g in 1-2 h and infant formula in 3 h.

Recently, it was reported that cereulide-producing *B. cereus* were isolated from commercial infant foods (Shaheen et al. 2006). Spores of all cereulide-producing *B. cereus* strains were more heat-resistant and the average amount of germinating spores were higher for cereulide-nonproducing *B. cereus* strain spores at a temperature range of 4-48 °C (Carlin et al. 2006). In contrast, most psychrotrophic isolates of *B. cereus* are mainly unable to produce cereulide; the first was reported only recently (Altayar and Sutherland. 2006). Food poisoning isolates of psychrotrophic *B. cereus* strains were connected with the production of enterotoxins (Dufrenne et al. 1995).

Cereulide production in *B. cereus* strains is dependent on growing conditions: pH, temperature, oxygen, amino acids, growth media, or food. The growing conditions of those *B. cereus* strains capable of producing cereulide vary (Rajkovic, et al. 2006). The *B. cereus* strains 5964a and NS117 produced more cereulide when growing in nonshaken vs. shaken milk, while the aeration of cultures negatively affected cereulide production (Rajkovic et al. 2006). The *B. cereus* strain F4810/72, growing in baby food, also grewn better under stationary conditions (Shaheen et al. 2006), while the *B. cereus* strains NC7401 and F4810/72 produced less cereulide in static incubations compared with incubation on a rotary shaker (the cultures were grown in trypticase soy broth) (Häggblom et al. 2002). The cereulide production in *B. cereus* strains B203, B116 and F-4810/72 was increased in the presence of oxygen (Jääskeläinen et al. 2004). Cereulide production was reduced when *B. cereus* strain NC7401 grew in food at low pH (Agata et al. 2002). The amino acids *L*-Leu, *L*-Val, and *L*-Thr were essential to cereulide production in *B. cereus* strain NC7401 (Agata et al. 1999, Kuse et al. 2000). Addition of the amino acids *L*-Leu and *L*-Val increased cereulide production in *B. cereus* strains B203, B116, and F-4810/72 (Jääskeläinen et al. 2004).

Bacillus species, other than *B. cereus*, e.g. *B. subtilis, B. licheniformis*, and *B. amyloliquefaciens* were also associated with food poisoning cases (Kramer and Gilbert

1989, Beattie and Williams 1999, Salkinoja-Salonen et al. 1999a). Putative emetic toxins were produced by strains of *B. subtilis*, *B. mojaviense,* and *B. pumilus* isolated from tap water, foods, and spices (From et al. 2005). Infant cereals and dried formulas causing vomiting were associated with *B. subtilis* and *B. cereus* contamination (Duc et al. 2005). Several species of *Bacillus* are capable of producing heat-stable non-protein compounds toxic to eukaryotic cells, hence are potentially able to intoxicate food products.

Various non-protein heat-stable mycotoxins are produced by molds such as *Aspergillus, Fusarium*, and *Penicillium* growing in wet crop fields or when cereals, grain, corn and peanuts are stored under moist conditions. These mycotoxins include aflatoxins (Frisvad et al. 2005, McKean et al. 2006), fumonisins (McKean et al. 2006), zearalenone (Alm et al. 2006), ochratoxin (Ringot et al. 2006), T-2, diacetoxyscirpenol, deoxynivalenol (vomitoxin), and nivalenol from the trichothecene group (Schollenberger et al. 2006), all of which are acutely toxic and have caused human illness. Trichothecenes induced apoptosis and caused vomiting (Rocha et al. 2005). The toxicity of deoxynivalenol, zearalenone and fumonisin B1 targetted the mitochondria in a human intestinal cell line Caco-2 (Kouadio et al. 2005). Deoxynivalenol, T-2 toxin, and zearalenone were toxic to three human epithelial cell lines at exposure concentrations from 100 ng/ml to 1 µg/ml after 2-4-d exposures (Calvert et al. 2005). Ochratoxin A inhibited mitochondrial respiration and disturbed cellular signaling and regulation (Ringot et al. 2006). Moniliformin from *Fusarium* is cardiotoxic and also affects smooth muscle cells (Kamyar et al. 2006). The cation-selective channel-forming mycotoxins beauvericin and enniatin are produced by *Fusarium* frequently found in corn, cereals, and grain (Logrieco et al. 2002, Jestoi et al. 2004, Kamyar et al. 2004, Kouri et al. 2005).

1.2. Microbes and toxic metabolites in moisture-damaged buildings

There is indication that over half of the buildings in Finland have moisture problems (Koskinen et al. 1999, Lappalainen et al. 2001). Mold problems have been reported in as many as 27-56% of buildings in North America, 15-48% in Europe and 15% in Finland (Górny et al. 2002). These data suggest that moisture damage and mold problems affect an enormous number of people in Finland. In 2004 there were 1.4 million buildings in Finland (Tilastokeskus (Statistics, Finland) 2005), suggesting that 700 000 moisture-damaged and 200 000 moldy buildings exist in Finland. This is due mainly to our climate and also the energy crisis during the 1970s, which affected building technology in Scandinavia (Bornehag et al. 2005). Building materials such as gypsum liners, insulation materials, and plastic flooring together with the moisture problem, offer suitable environments for microbes.

Molds, fungi, bacteria, and their metabolites cause health problems in water-damaged buildings (Husman 1996, Andersson et al. 1997, 2005, Peltola et al. 1999, 2001, Salkinoja-Salonen et al. 1999b, Suominen et al. 2001, Nevalainen and Seuri 2005). Fungi such as *Fusarium*, *Aspergillus, Penicillium*, and *Stachybotrys* have been associated with

asthma, allergy, and rhinitis in indoor air (Nevalainen et al. 1992, Andersson et al. 1997) (see also Chapter 1.1). *Trichoderma harzianum* isolated from moisture-damaged buildings produces peptaibols toxic to mammalian cells (Peltola et al. 2004).

Most indoor air studies have been focused on the problems caused by molds and fungi whereas the role of bacteria and their toxins has remained more or less unclear. The spores of *Streptomyces californicus* and *B. cereus* isolated from indoor air induced inflammatory responses in murine macrophages and reduced cell viability more efficiently than did the spores of *Penicillium, Aspergillus* and *Stachybotrys* (Hirvonen et al. 2005). Bacteria grow more rapidly than fungi and may be the first microbes to cause problems in moisture-damaged buildings. Bacteria such as *Streptomyces griseus*, which produce valinomycin, were reported from moisture-damaged buildings (Andersson et al. 1998), as was *B. cereus*, which produces cereulide, from construction materials (Mikkola et al. 1999) and from settled dust and building materials (Andersson et al. 2002). The occurrence of *S. griseus* was connected with moisture damage in houses (Rintala et al. 2004). Inhalation of *Bacillus* spores can serve as routes for toxin exposure, as can inhalation of fungal spores and their toxins (Kuhn et al. 2005, Black et al. 2006).

Persistent and hydrophobic non-protein toxins such as valinomycin or cereulide may sorb into hydrophobic indoor materials such as plastic products. Degradation of these substances by mammalian or microbial enzymes has not been reported. Therefore, such substances may remain active long after being produced. In contrast, protein toxins are sensitive to microbial enzymes and can be inactivated by environmental conditions such as heat, pH changes, or draft. However, protein toxins may also cause damage because the bacteria or their toxins can be transported as aerosols or dust into contact with the skin or respiratory tracts of occupants. Indeed, *B. cereus* secretes protein toxins such as pore-forming HBL, NHE and phospholipases that are associated with the incidence of a serious eye disease, endophthalmitis (Beecher et al 1995b, 2000). One of the well-known hazardous bacteria, *B. anthracis*, is a close relative of *B. cereus* and produces the protein anthrax toxins. Examples of other protein toxins produced by *Bacillus* are the fungicidal entomocin 9 produced by *B. thuringiensis* (Cherif et al. 2003), as well as hemolytic cereolysin, and necrotic and hemolytic CytK produced by *B. cereus* (Shinagawa 1991, Lund et al. 2000). The ribonuclease called barnase is produced by *B. amyloliquefaciens*, provokes failures in perfused rat kidney of (Ilinskaya and Vamvakas 1997), and is cytotoxic to human cells (Prior et al. 1996).

High levels of *Bacillus* species in the indoor air generally indicate previous water damage and lack of adequate maintenance of the building or house. Infants, the elderly, and other individuals with immune system deficiencies are sensitive to microbial contaminations of indoor air. Occupants of moisture-damaged buildings suffer from irritations of the eyes, illnesses of the upper and lower respiratory systems, tiredness, faintness, allergies, and neurological problems (Peltola et al. 2001).

1.3. Overview of the industrial applications of *Bacillus* species

Bacteria of the *Bacillus* group are widely used for the manufacture of important industrial enzymes, food products, pesticides, and insecticides (Fogarty and Kelly 1990, Beynon and Beaumont 1998, Outtrup and Jörgensen 2002). Bacilli secrete exocellular enzymes (Priest 1977), e.g. α-amylases and proteases, producing over one-half of the total commercial enzyme volume (Crueger and Crueger 1982, Fogarty and Kelly 1990). The proportions of enzymes of *Bacillus* origin used in the technical, food, and feed industries constitute 56%, 29%, and 15%, respectively of the total enzyme market in 1999 (Outtrup and Jorgensen 2002). One example is serine proteinase subtilisin (stable under alkaline conditions), important to detergent industry, which is produced by *B. licheniformis* and *B. amyloliquefaciens*. Alkaline proteases are used in many protein hydrolysates as infant food formulations (Gupta et al. 2002). *Bacillus subtilis* (natto) is used for the fermentation of soy.

Certain strains of *Bacillus* have GRAS status and are used in industry and agriculture (Schallmey et al. 2004). *Bacillus thuringiensis*, which closely related to *B. cereus*, emits effective CryT proteins against insect larvae that are used as an insecticidal (Schallmey et al. 2004). Due to their ability to form spores, *Bacillus* species can be easily stored for later applications, such as pesticide use.

Bacillus amyloliquefaciens strain RC-2 containing iturins (Yoshida et al. 2001, Hiradate et al. 2002), has been proposed as an agent to protect mulberry leaves against *Colletotrichum dematium. Bacillus amyloliquefaciens* strain FZB24 is a commercial biopesticide producing the auxin indole-3-acetic acid, and the enzyme phytase (Kilian et al. 2000, Idriss et al. 2002). *Bacillus* species are also used as feed additives; e.g *B. subtilis*, *B. licheniformis*, and *B. pumilus* are used in fermentation processes in foods such as ugba, ogiri, ogiri-saro, and dawadawa in Africa and natto, thua-nao, and kinema in Asia (Beaumont 2002, Amoa-Awua et al. 2006). Certain *Bacillus* species produce nature's most powerful biosurfactants such as surfactin from *B. subtilis* or lichenysin A from *B. licheniformis*, which has been considered for use in improving oil recovery (Schaller et al. 2004). Such biosurfactants have also been considered for medical use (Singh and Cameotra 2004). Many strains of *Bacillus* produce several bacteriocin-like substances, e.g. some strains of *B. cereus* produce the bacteriocin cerein 7, a pore-forming peptide of 3940 Da (Oscariz et al. 1999, Oscariz and Pisabarro 2000). The lantiobiotics are lanthionine-containing peptide antibiotics, such as mersacidin (1825 Da) from *Bacillus* strains HIL-Y85, 54728, which is active against methicillin-resistant *Staphylococcus aureus* MRSA strains (Chatterjee et al. 2005). The bacteriocin subtilin, produced by *B. subtilis* ATCC 6633, structurally resembles nisin and inhibits the germination of spores of *Clostridium* and *B. cereus* (Chatterjee et al. 2005).

The usefulness and application of many *Bacillus* species and, on the other hand, their ability to produce toxic compounds call for careful monitoring of their environmental occurrence. Recently, one commercial enzyme producer published data on the non-toxicity of their enzyme-producing *Bacillus* strains (Pedersen et al. 2002).

1.4. Properties and biological effects of non-protein toxins from the genus *Bacillus*

Many microbial peptides are synthesized non-ribosomally by peptide synthetases which are multienzyme complexes (Hancock and Chapple 1999). *Bacillus subtilis* and *B. amyloliquefaciens* use 4-7% of their genomes for producing bioactive compounds (Stein 2005). Cereulide from *B. cereus* is also produced non-ribosomally (Horwood et al. 2004, Ehling-Schulz et al. 2005a) and recently it was shown that cereulide production is plasmid-mediated (Hoton et al. 2005). *Bacillus subtilis* strain A1/3 is exceptionally rich in the genes coding for synthetases of polyketides and non-ribosomal peptides and produces nine different bioactive compounds (Hofemeister et al. 2004).

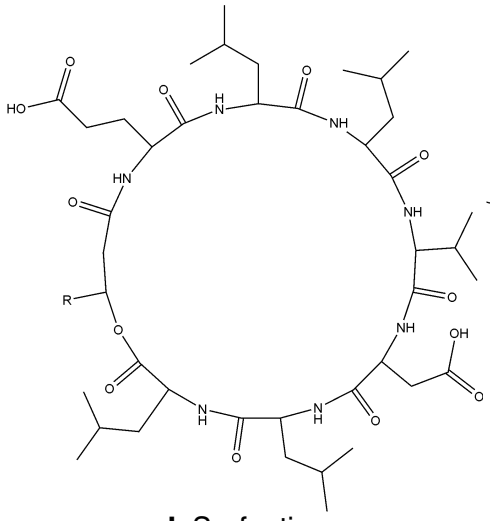
Surfactin, lichenysin A, fengycin, tyrocidine, bacitracin and mycosubtilin are produced by non-ribosomal peptide synthesis (Konz et al. 1997, Mootz and Marahiel 1997, Yakimov et al. 1998, Duitman et al. 1999, Koumoutsi et al. 2004). Approximately 300 precursors for non-protein compounds have been found, whereas peptide production is limited to a range of 2-48 residues, in contrast to ribosomal synthesis in which 21 different amino acids are used for peptide or protein production (Marahiel et al. 1997). The *Bacillus* group produces a diverse collection of antibacterial, antiviral or antitumoral compounds, and some are toxic to eukaryotic cells. The toxins belong to various chemical groups, as listed in Table 1 and Figure 1. The structures include cyclic lipopeptides and cyclic peptides, phospholipid oligopeptides, a small-molecular-weight compound with functional amine groups NH_2 as the side chain, and cationic sugar derivatives. The peptides have molecular masses in the range from 500 to 4000 Da and those of non-peptides from 129 to 555 Da, averaging 1000 Da and 300 Da, respectively.

Most of the toxic non-protein substances from *Bacillus*, such as those listed in Table 1, are well known but their role as contaminants in food or moisture-damaged buildings is incompletely understood. The reasons are historical, since most of these substances were found mainly before 1980, whereas the toxicity or poisoning related to such substances was established much later. For instance, the microbial role in indoor air problems has been studied for the past 20 years and only in the recent years has the role played by bacterial metabolites received justified attention. Most of the studies have been focused on those *Bacillus* species with capable of producing antimicrobially active substances for medical purposes or for protecting agricultural crops. However, many *Bacillus*-derived antibiotics, e.g. tyrocidine, gramicidin S, and polymyxin B, are too toxic for use as drugs administered orally. Several compounds produced by *Bacillus* and related genera are fungicidal. The fungal cell resembles the mammalian cell in having similar organelles and phospholipid membranes, except that fungi contain ergosterol instead of cholesterol. It is a difficult pharmaceutical task to develop agents toxic to fungi but non-toxic toward mammalian cells.

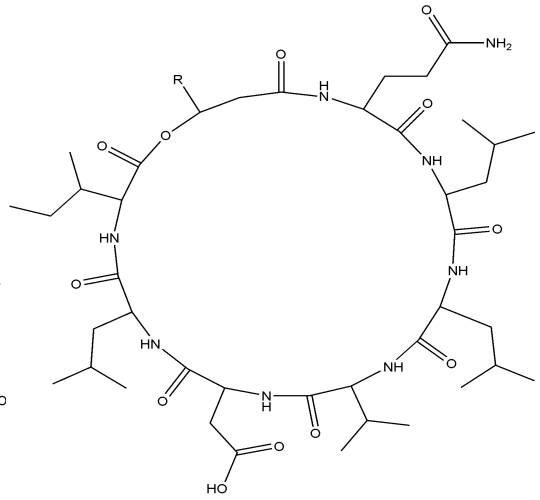
Table 1. *Non-protein fungi and/or mammalian cells toxic compounds from Bacillus and related genera.*

Bacteria	Compound	Structure class	Molecular mass (Da)	Biological effects on eukaryotic cells	Reference
B. cereus	Azoxybacilin	An unusual amino acid containing an azoxy group	161	Fungicidal	Aoki et al. 1996
B. subtilis	Bacillomycins	Cyclic lipoheptapeptide	1030 1044	Fungicidal, Hemolytic	Besson et al. 1984, Quentin et al. 1982, Volpon et al. 1999
B. subtilis	Bacillopeptin	Cyclic lipoheptapeptide	1030 1044	Antifungal hemolytic	Kajimura et al. 1995, Quentin et al. 1982
B. subtilis	Bacilysocin	Phospholipid	470	Fungicidal	Tamehiro et al. 2002
B. subtilis *B. licheniformis*	Bacitracin A (B, C)	Cyclic heptapeptide	1423	Nephrotoxic, allergen	Butaye et al. 2003
B. circulans	Butirosin A	Aminoglycoside	555	Nephrotoxic	Cox and Serpersu 1997, Beck et al. 1980,Takeda et al. 1978
B. cereus	Cereulide	Cyclic dodecadepsipeptide	1152	Inhibition of NK cells, sperm toxic, cytotoxic	Paananen et al. 2002, Wang et al. 1995
B. cereus	Cispentacin	2-aminocyclo- pentane-1-carboxylic acid	129	Fungicidal	Jetnwaney et al. 1997
B. subtilis *B. amyloliquefaciens,* *B. thuringiensis*	Fengycins	Cyclic lipodecapeptide	1449-1505	Antifungal	Kim et al. 2004 Koumoutsi et al. 2004
Paenibacillus polymyxa	Fusaricidin A-D	Cyclic hexadepsipeptide	886-960	Antifungal	Beatty and Jensen. 2002, Kajimura and Kaneda 1997
Bacillus sp. sun hua	Macrolactin A	Macrolactone	402	Cytotoxic	Gustafson et al. 1989, Han et al. 2005
Brevibacillus brevis	Gramicidin (Dubois)	Linear pentadecapeptide group of A, B, C, D	1930 (A)	Cytotoxic	Doebler 1999
Brevibacillus brevis	Gramicidin S	Cyclic decapeptide	1141	Toxic to human red cells	Lee and Hodges 2003
Brevibacillus brevis	Tyrocidine	Cyclic decapeptide	1309 1271	Hemolytic	Qin et al. 2003
B. licheniformis	Halobacillin	Cyclic lipoheptapeptide	1035	Cytotoxic	Trischmann et al. 1994

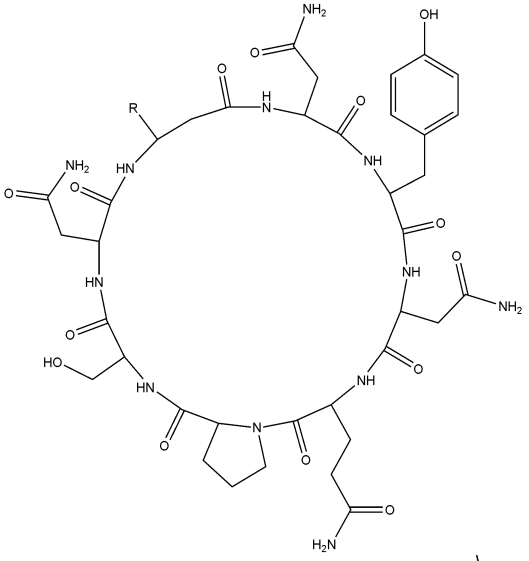
Bacteria	Compound	Structure class	Molecular mass (Da)	Biological effects on eukaryotic cells	Reference
B. cereus	Homocereulide	Cyclic dodecadepsipeptide	1166	Cytotoxic	Wang et al. 1995
B. licheniformis	Isohalobacillin B	Cyclic lipoheptapeptide	1035	ACAT inhibitor	Hasumi et al. 1995
B. amyloliquefaciens *B. subtilis* *Paenibacillus koreensis*	Iturin A	Cyclic lipoheptapeptide	1030 1044	Fungicidic Hemolytic	Besson et al.1984, Quentin et al. 1982, Chung et al. 2000
B. cereus	Kanosamine	3-amino-3-deoxy-D-glucose	179	Fungicidal	Milner et al. 1996
B. thuringiensis	Kurstakins	Cyclic lipoheptapeptide	878-906	Fungicidal	Hathout Y et al. 2000
B. licheniformis	Lichenysin A	Cyclic lipoheptapeptide	992-1034	Hemolytic	Grangemard et al. 2001
Paenibacillus polymyxa	LI-F	Cyclic depsihexapeptide containing 15-guanidino-group	947	Fungicidal	Kuroda et al. 2000, Kurusu et al. 1987
B. subtilis	Mycobacillin	Cyclic tridecapeptide	1528	Fungicidal ATP release starvation Hemolytic activity	Chowdhury et al. 1998, Banerjee 1977, Mannanov and Sattarova 2001
B. cereus	Mycocerein	Octapeptide	1000	Fungicidal	Wakayama et al. 1984
B. subtilis	Mycosubtilin	Cyclic lipooctapeptide	1030 1044	Fungicidal Hemolytic	Besson et al. 1979, Besson et al. 1989
B. licheniformis	Amoebiens, fungicin M4	Peptides Cyclic peptide	770- 3400	Inhibit amoebas Fungicidal, cytotoxic	Galvez et al. 1994, Lebbadi et al.1994a
B. cereus *B. subtilis*	Plipastatins	Cyclic lipodecapeptide	1449-1527	Inhibitors of phospholipase A2	Volpon et al. 2000, Nishikiori et al. 1986
Paenibacillus polymyxa	Polymyxin B	Cyclic decapeptide	1203	Nephrotoxic	Ouderkirk et al. 2003
Paenibacillus kobensis	Polymyxin M (Mattacin)	Cyclic decapeptide	1157	Fungicidal	Martin et al. 2003
B. pumilus	Pumilacidin	Cyclic lipoheptapeptide	1035-1077	Antiulcer activity in rat	Naruse et al. 1990
B. subtilis	Rhizocticin A	Phosphono-oligopeptide		Antifungal	Kugler et al. 1990
B. amyloliquefaciens, *B. subtilis*	Surfactin	Cyclic lipoheptapeptide	993-1035	Hemolytic Cytotoxic	Vollenbroich et al. 1997, Dufour et al. 2005
B. cereus	Zwittermicin A	Aminopolyol	396	Fungicidal	Silo-Suh et al.1998



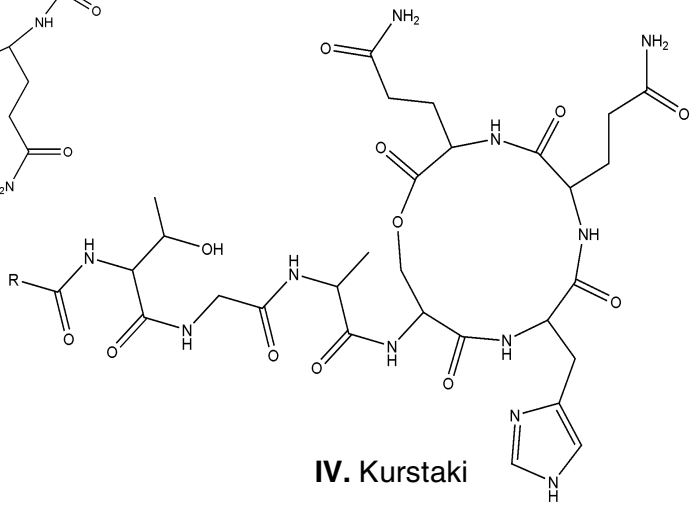
I. Surfactin



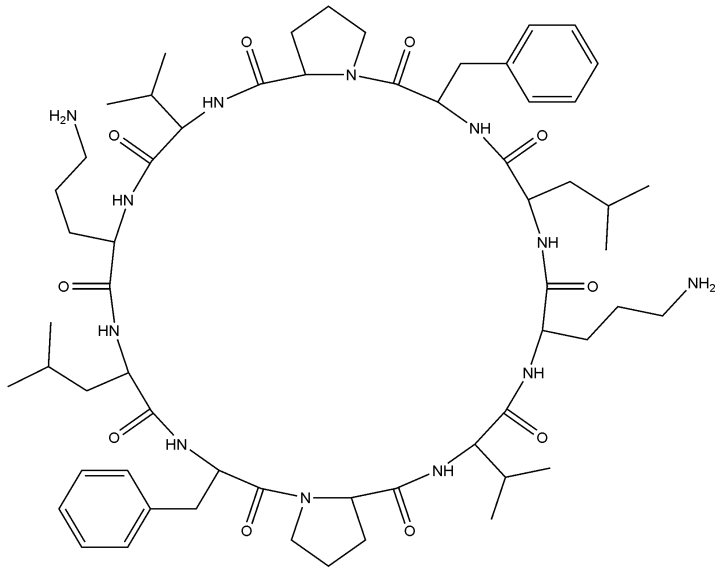
II. Lichenysin A



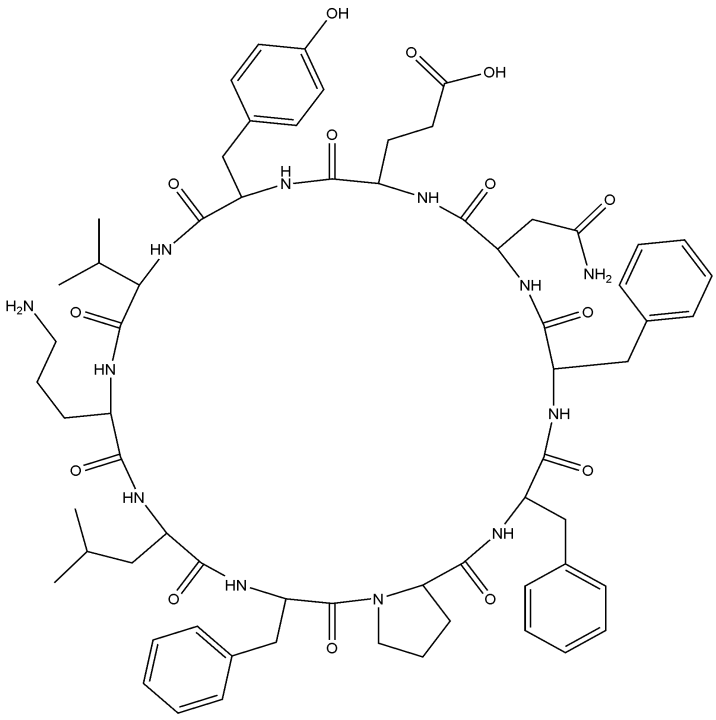
III. Mycosubtilin



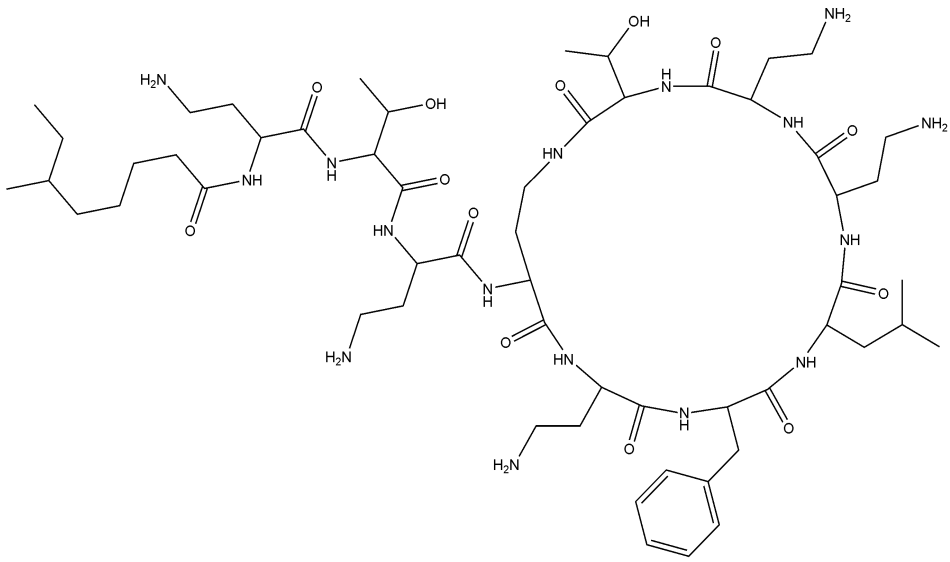
IV. Kurstaki



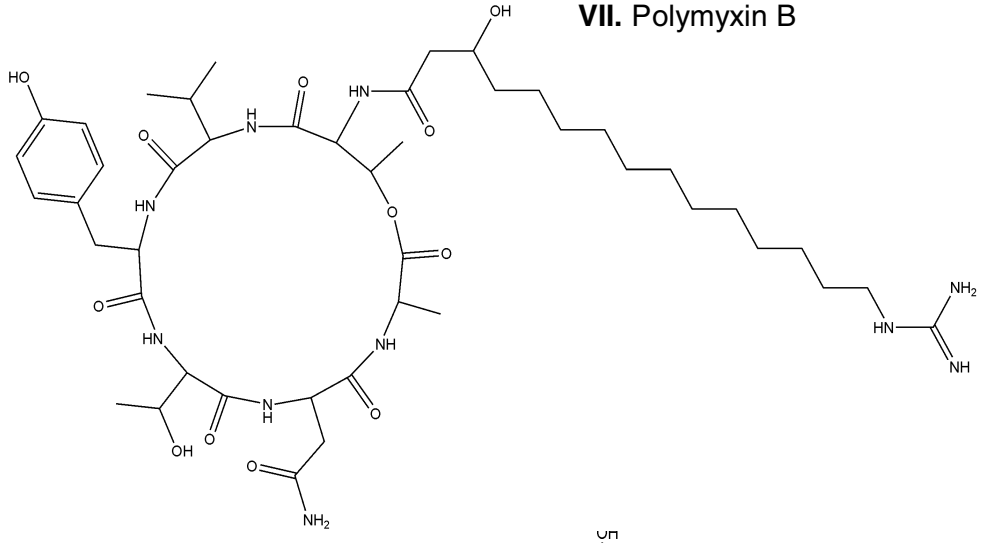
V. Gramicidin S



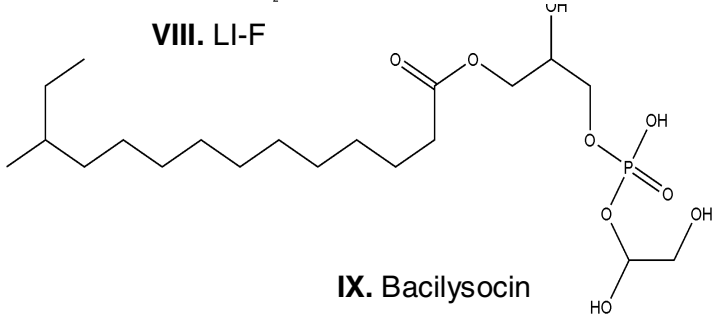
VI. Tyrocidine A



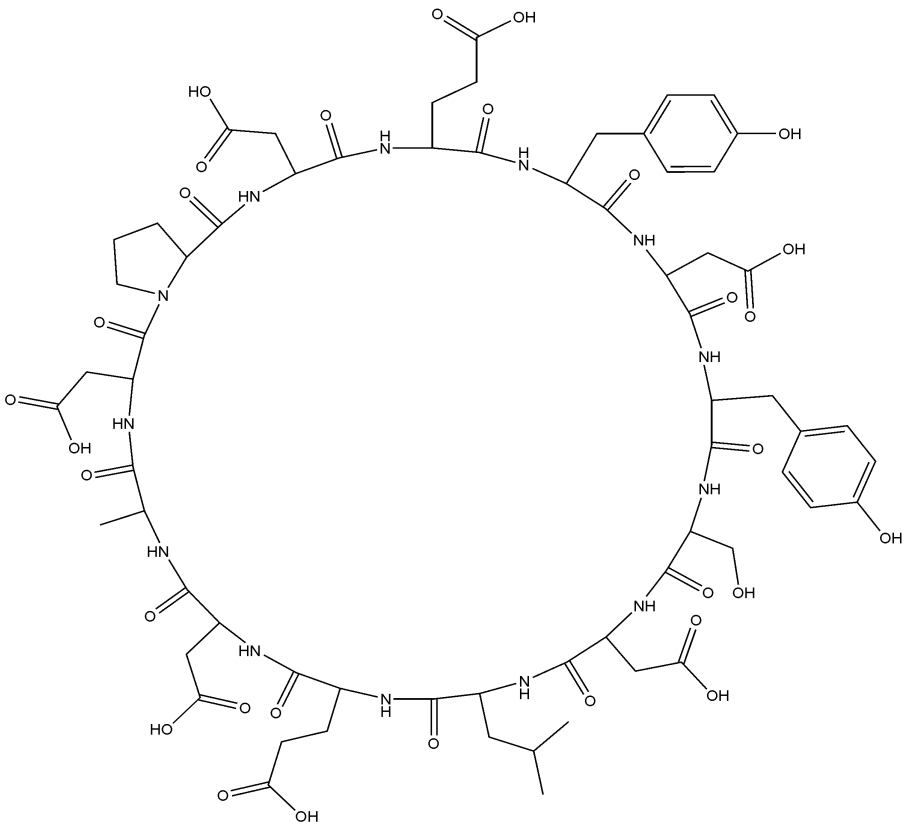
VII. Polymyxin B



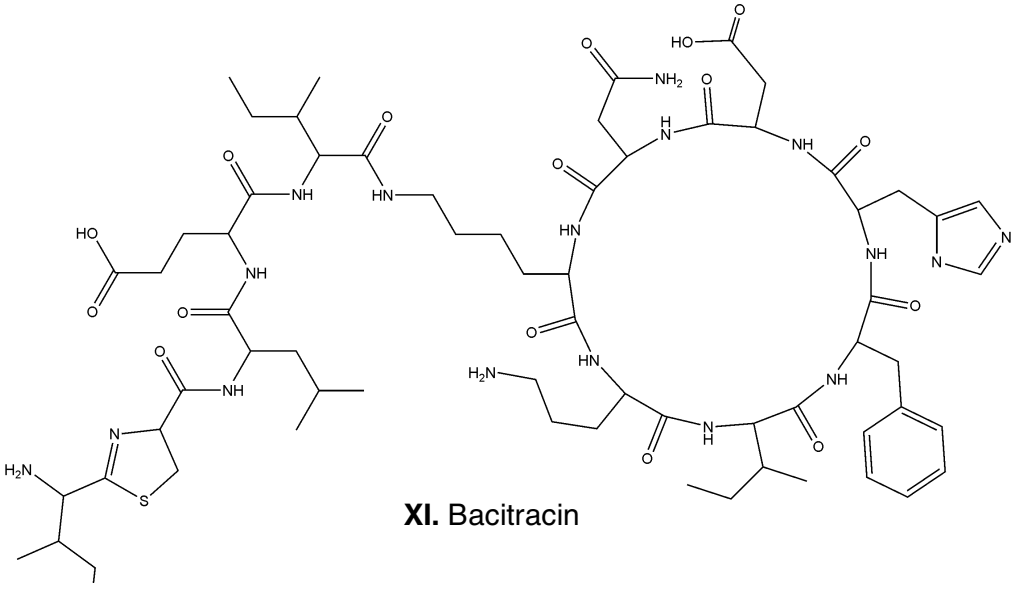
VIII. LI-F



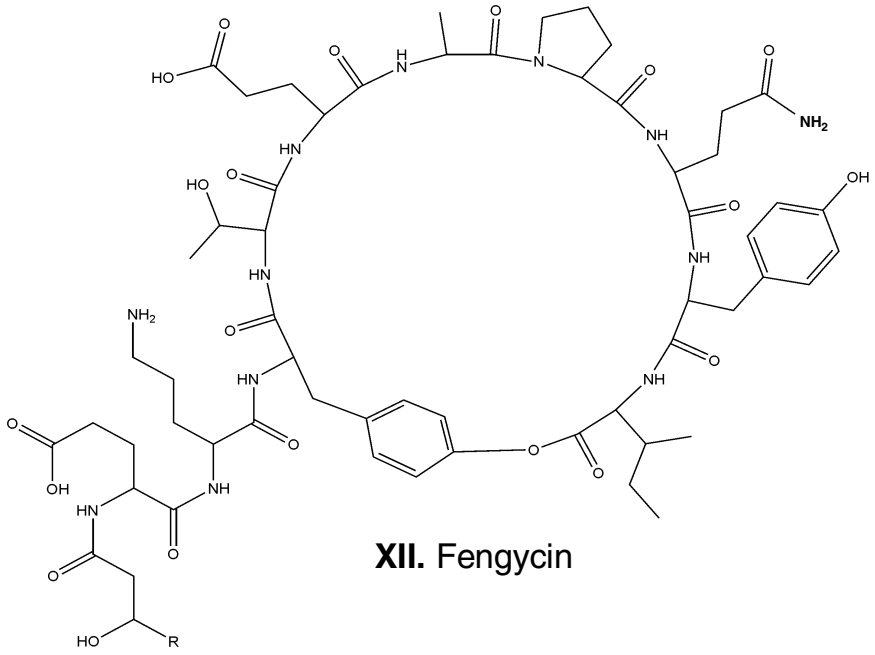
IX. Bacilysocin



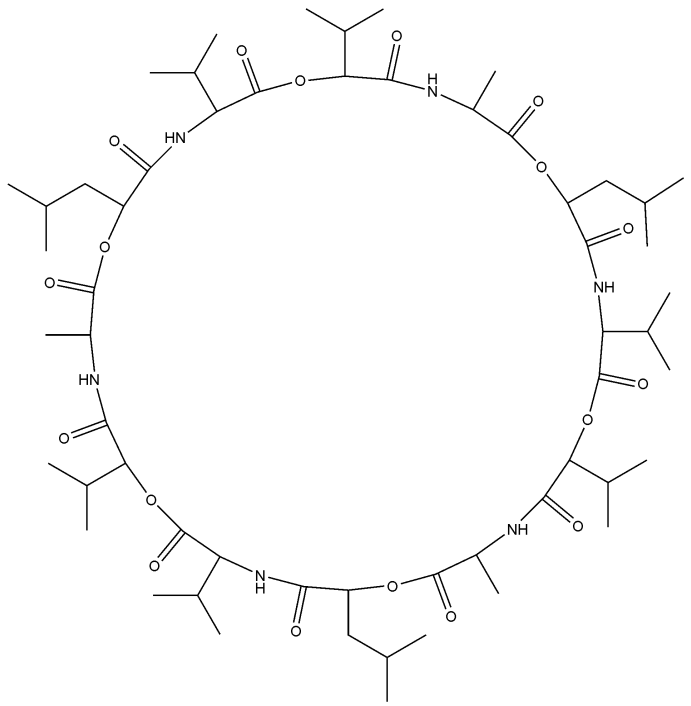
X. Mycobacillin



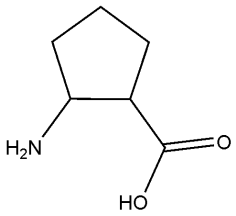
XI. Bacitracin



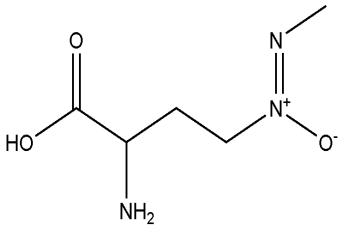
XII. Fengycin



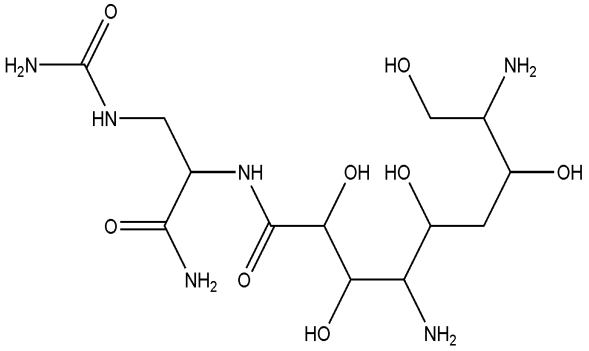
XIII. Cereulide



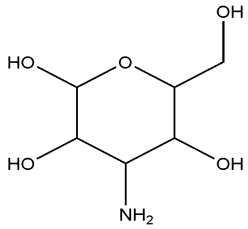
XIV. Cispentacin



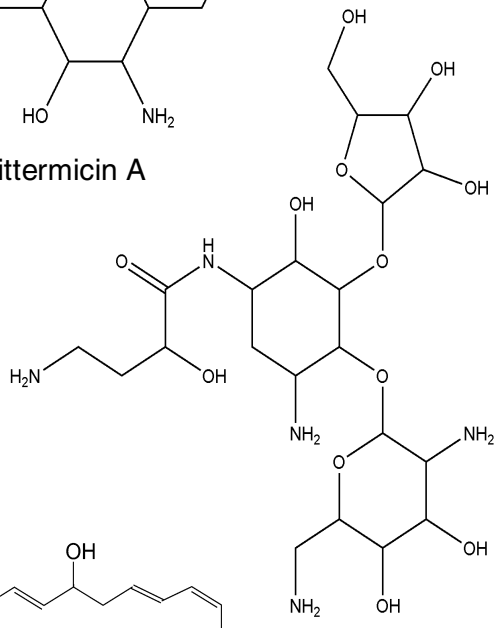
XV. Azoxybacilin



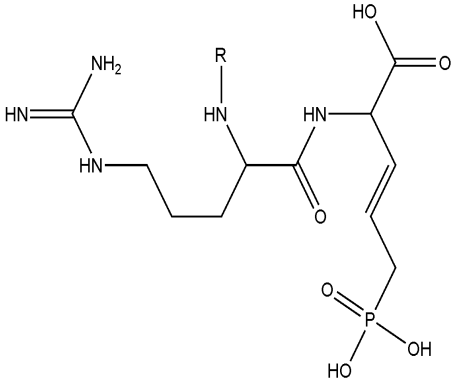
XVI. Zittermicin A



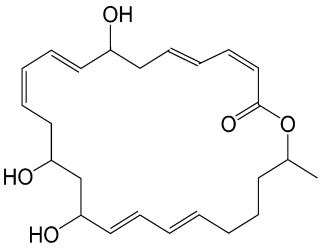
XVII. Kanosamine



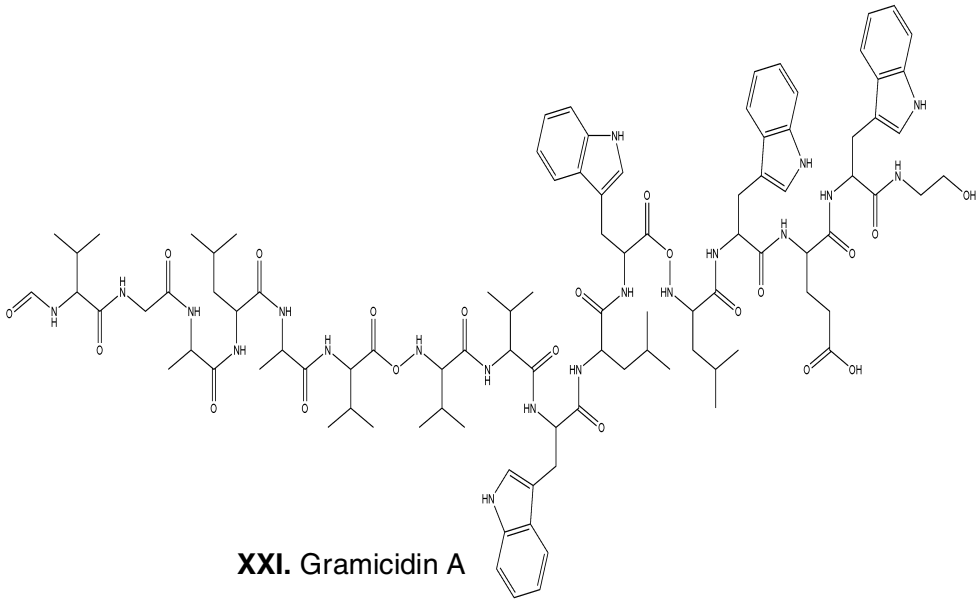
XVIII. Butirosin



XIX. Rhizoticin



XX. Macrolactin A



XXI. Gramicidin A

Figure 1. *Structures of compounds toxic toward eukaryotic cells produced by strains of Bacillus and species of related genera. R in the structures stands for n- , iso,- and anteiso- branching hydrocarbons with different lengths (C10-C20), depending on the compounds. In compound XIX, R = H or Val.*

Bacillus strains produce compounds (Table 1 and Fig. 1) that alter the functions of biological membranes in different ways. Substances affecting the plasma membrane integrity (such as I, II in Fig. 1) may cause leaking of the cell contents, initiating necrosis. Ionophoric substances (such as V, XII, XXI in Fig. 1) form ion channels or operate as ion carriers. Such substances alter the permeability of plasma and/or organelle membranes. Maintenance of the transmembrane electrical potential as well as the proper ionic concentrations of cells or organelles is critical to cellular functions. Many biochemical functions are regulated by ion fluxes across membranes: signals, transporting properties, adenosine 5'-triphosphate (ATP) production, maintenance of the cell size, and change in the conformation of integral membrane proteins, disturbing immunological recognition. Membrane potential differences alter the conformation of membrane proteins such as Na^+/K^+ channels and may initiate apoptosis (Bacso et al. 1996).

Thus, there are good reasons for investigating indoor air and food environmental *Bacillus* strains for their abilty to produce toxic compounds. The experimental work and the literature review in this thesis evaluate the potential hazards of non-protein heat-stable compounds produced by strains of *Bacillus* and related genera.

1.4.1. Lipopeptides

Several species of the genus *Bacillus* produce various lipopeptides (Table 1 and Fig. 1). *Bacillus* lipopeptides consist of a peptide part containing 7-11 amino acids, either cyclic or linear or a combination of these. β-hydroxy or β-amino fatty acids form the lipid part connected to the peptide backbone. Lipopeptides are thus amphiphilic, in which acid or basic amino acids serve as polar groups and fatty acids partly as neutral hydrophobic moieties, most are active biosurfactants. The hydrocarbon length of the fatty acids and amino acid composition may vary, depending on the nutrition of the bacteria, and affect the properties of the lipopeptides. The cyclic structure of the peptide part protects the lipopeptide from enzymatic cleavage and maintains its general stability. Many lipopeptides show antimicrobial or antiviral activity and affect mammalian cell functions. Both the phospholipids of the biomembranes and the lipopeptides have amphilic structures. Thus, lipopeptides are capable of penetrating into cells, with the lipophilic hydrocarbon chain interacting with the plasma membrane lipid moiety while the polar amino acids in the peptide part interact with the polar phosphatidyl moieties. Whether lipopeptides are able to damage the integrity of the plasma membrane or create ion-selective pores depends on the nature of the lipopeptides and on the phospholipids of the membranes.

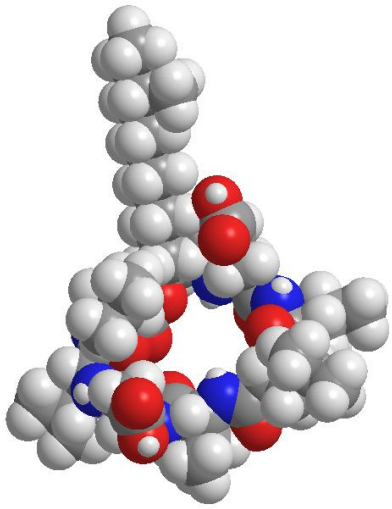
1.4.1.1. Lipopeptides of the surfactin family

Lipopeptides of the surfactin family (surfactin, lichenysins, and pumilacidins) contain a cyclic heptapeptide acylated with β-hydroxy fatty acids. The length of the carbon chain of β-hydroxy fatty acids ranges from C13 to C18 with *n*, *iso*, or *anteiso* branchings. These peptides are powerful biosurfactants, produced by strains of several *Bacillus* species. Since surfactin (I in Fig. 1 and Fig. 2) was discovered (Arima et al. 1968) and synthesized (Nagai et al. 1996), its properties have been studied widely and interesting applications from industry to medical use were proposed (Singh and Cameotra 2004). Surfactin (I in Fig. 1 and Fig. 2) contains a heptapeptide (Glu1-Leu2-Leu3-Val4-Asp5-Leu6-Leu7). The natural analogue as lichenysin A (II in Fig. 1 and Fig. 2) has Gln in position 1 and Ileu at position 7 and pumilacidin has Leu at position 4, whereas other variations are located at position 7. Lichenysin A is produced by *B. licheniformis* during anaerobic and aerobic growth. It was first isolated from oil wells and its structure was elucidated by Yakimov et al. (1995, 1999).

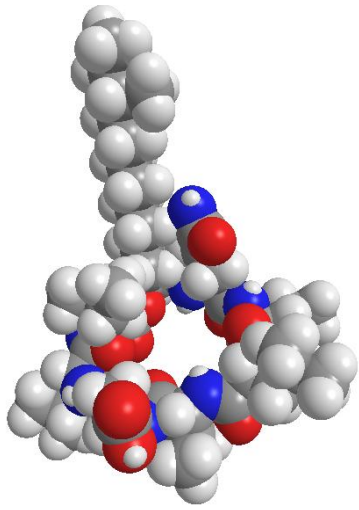
Surfactin in concentrations of 30-64 μM was cytotoxic to several human and animal cell lines (Vollenbroich et al. 1997) and provoked hemolysis (Dufour et al. 2005). Lichenysin A had a stronger surfactant effect and was hemolytic at 10-times lower concentration (15 μM) than surfactin (Grangemard et al. 2001). Surfactin was reported to lyse protozoan membranes (Gould et al. 1971) and to inhibit starfish oocyte maturation at a concentration of 3 μM (Toraya et al. 1995). Surfactin showed insecticidal activity against the fruit fly *Drosophila melanogaster* (Assie et al. 2002). Inactivation of enveloped viruses such as vesicular stomatitis virus (VSV), simian foamy virus (SFV), and suid herpesvirus 1 (SHV-

1) by surfactin depended on its hydrophobicity: the C14 and C15 isoforms were more antiviral than C13 (Kracht et al. 1999). The biological effects of surfactin and lichenysin A are probably due to their strong surfactant properties. However, surfactin also forms cation-selective $K^+ > Na^+$ channels in BLM (Sheppard et al. 1991).

Surfactin-producing *B. subtilis* strains have high swarming motility and biofilm formation, whereas surfactin-nonproducing strains did not swarm or form biofilm (Connelly et al. 2004). Surfactin promotes bacterial cell motion by lowering the surface tension (Kinsinger et al. 2003, Hofemeister et al. 2004, Mukherjee and Das 2005).



Surfactin



Lichenysin A

Figure 2. *Structures of surfactin and lichenysin A. Atom colors: blue is nitrogen, red is oxygen, gray is carbon, and white is hydrogen.*

The amphiphilic structures of the lipopeptides surfactin (I in Fig. 1) and lichenysin A (II in Fig. 1) explain their ability to form micelles and to penetrate the plasma membrane. Their basic structures are closely similar (Fig. 2), containing a cyclic peptide with seven amino acid residues associated with the β-hydroxy fatty acid. The main difference is shown in Fig. 2 near a hydrocarbon of β-hydroxy fatty acids, since surfactin has Glu (oxygen [red] with an OH group) in contrast to lichenysin A Gln (nitrogen [blue] with an NH group).

1.4.1.2. Lipopeptides of the iturin family

Lipopeptides of the iturin family, such as iturins, bacillomycins (Table 1), and mycosubtilin (III in Fig. 1) contain a cyclic heptapeptide acylated with β-amino fatty acids with a chain length of C_{14} to C_{16} (Peypoux et al. 1978, Hourdou et al. 1989). Iturin A contains the heptapeptide Asn1-Tyr2-Asn3-Gln4-Pro5-Asn6-Ser7, whereas in the other members the amino acid residues in the heptapeptides vary slightly; e.g. mycosubtilin (III in Fig. 1) has Asn1-Tyr2-Asn3-Gln4-Pro5-Ser6-Asn7.

Iturin A retained 100% biological activity after heating for 30 min at 100 °C and for 60% after autoclaving for 20 min at 120 °C, as measured with the fungal inhibition test (Yu et al. 2002). Another antifungal substance from *B. subtilis* YM was highly similar to iturin A, and heat-resistant at 100 °C for 1 h, and also resistant towards proteolytic enzymes (Chitarra et al. 2003).

Biological membranes consist of various lipids, sterols, and proteins all of which can affect the activity of channel-forming substances. Biological effects of the iturin family peptides are due to their capability of forming ion-conducting pores (Maget-Dana and Peypoux 1994). Iturin A and bacillomycin L (Table 1) provoked hemolysis and released potassium from erythrocytes (Latoud et al. 1986, Aranda et al. 2005). Iturin A induced morphological changes in human erythrocytes (Thimon et al. 1994). Iturin A, bacillomycins, and mycosubtilin (III in Fig. 1) formed channels in BLM (Maget-Dana et al. 1985a,b, Maget-Dana and Ptak 1990). Mycosubtilin altered the permeability of the plasma membrane, releasing nucleotides, proteins, and lipids from yeast cells (Besson and Michel 1989) and lysing erythrocytes (Besson et al. 1989).

The lipopeptides of the iturin family are more active in membranes containing cholesterol, such as mammalian cells, than in the ergosterol-containing fungal cells. Iturin A released K^+ ions more actively from the cells of *Saccharomyces cerevisiae* mutants containing cholesterol in the membrane instead of ergosterol (Latoud et al.1990). Nucleotides, proteins, polysaccharides, and lipids leaked from *S. cerevisiae* cells exposed to iturin A (Latoud et al. 1987) while the lethal concentration in *S. cerevisiae* was 10-60 μg/ml (Besson et al. 1984). Iturin and bacillomycin L form ion pores by aggregation in the membranes and interacting with sterols (Quentin et al. 1982, Maget-Dana and Peypox 1994, Volpon et al. 1999). Mycosubtilin formed pores in dimyristoylphosphatidylcholine (DMPC) membranes by interacting with the phospholipids, forming a (1:2) complex with cholesterol, thus stabilizing the ion pore (Maget-Dana and Ptak 1990).

The Tyr residue in the peptide backbone of the iturin family peptides plays a role in interacting with sterols and forming pores. Bacillomycin L forms pores in membranes by interacting with sterols due to its Tyr residue which forms a hydrogen bond with the hydroxyl group of sterols (Volpon et al. 1999). The Tyr residue enhanced the membrane activity of iturin A (Harnois et al. 1989). The cyclic tridecapeptide mycobacillin (X in Fig. 1) lacks the hydrocarbon tail and was proposed to bind cholesterol by its Tyr residue (Mukherjee and Bose 1978).

Bacillus strains that produce iturins are applicable as fungicides. Iturin A was antagonistic against *Fusarium oxysporum*, which cause potato diseases (Han et al. 2005). *Bacillus amyloliquefaciens* strain B94 produces iturins that inhibit fungal plant pathogens (Yu et al. 2002). *Bacillus subtilis* strain FZB24 is used in commercial biocontrol products against fungal plant pathogens; it produces iturin resembling compounds (Kilian et al. 2000). Surfactin- and iturin-containing *Bacillus* strains have been used to promote plant growth.

The lipopeptides iturin A (Table 1) and surfactin (Fig. 2) together play a role in adhesion of *B. subtilis* on solid surfaces by changing the hydrophobicity of the cells (Ahimou et al. 2000), similar to that by surfactin (1.4.1.1). The utilization of substrate is promoted in *Bacillus* by secreting of surface active substances like iturin or surfactin (Mukherjee and Das 2005).

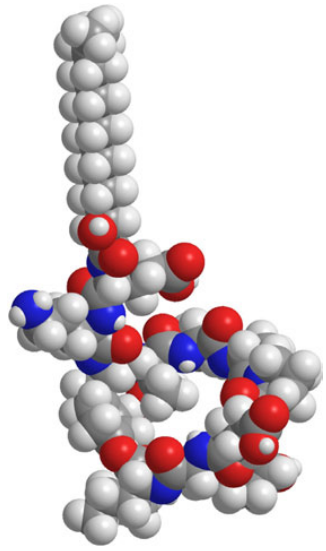


Figure 3. *Structure of fengycin. Atom colors are as in Fig. 2.*

1.4.1.3. Fengycins and various lipopeptides

Fengycins (III in Fig. 1 and Fig. 3) and the structurally similar plipastatins (Table 1) are distinguished from the other lipopeptides by the way in which the β-hydroxy fatty acid is linked with the polar dipeptide which is associated with the cyclic octapeptide (Fig. 3). The cyclic structure of fengycin *cyclo*(*D*-*allo*-Thr1-*L*-Glu2-*D*-Ala3/*D*-Val3-*L*-Pro4-*L*-Gln5-*L*-Tyr6-*L*-Ile7-*D*-Tyr8)*D*-Orn9-*L*-Glu10-β-OH-FA is formed when *D*-Tyr8 is connected with amide bonds to *D*-*allo*-Thr1 and *D*-Orn9 and forms a lactone bond with *L*-Ile7 (XII in Fig. 1 and Fig. 3). The structure of fengycin A contains *D*-Ala3 instead of the *D*-Val3 of fengycin B. Fengycins have stereoisomeric composition different from those of plipastatin. Fengycins contain *D*-Tyr8 instead of the *L*-Tyr8 of plipastatins and *L*-Tyr6 instead of the *D*-Tyr of plipastatins (Volpon et al. 2000).

Hathout et al. (2000) reported from *B. thuringiensis kurstaki* HD-1 an antifungal compound structurally resembling plipastatin and fengycins. Fengycins and plipastatins inhibit phospholipase A_2, an enzyme affecting inflammation, acute hypertensions, and blood platelet aggregation (Volpon et al. 2000). *Bacillus thuringiensis* strain CMB26 produced an analogue of fengycin with a double bond in the fatty acid. It was fungicidal, bactericidal, and insecticidal, and more effective against fungi than was iturin or surfactin (Kim et al. 2004). In low molar ratios from 0.1 to 0.5 of fengycin/dipalmitoyl-phosphatidylcholine (DPPC) membrane fengycin forms pores and at a ratio of > 0.66 it acts as a detergent that solubilities membrane (Deleu et al. 2005). Analogues of iturin and fengycin were reported with a double bond in the fatty acid part. The fatty acids in iturins were predominantly C_{16} and C_{17} (Vater et al. 2002, Deleu et al. 2005). Recently *B. subtilis* strain GA1 was described as producing three lipopeptides: surfactin, fengycin, and iturins. It was effective against gray mold which causes disease in apples (Toure et al. 2004).

Another group of cyclic hexapeptide lipopeptides contains six amino acids acylated with 15-guanidino-n-hydroxypentadecanoic acid (bacillopeptins, fusaridins (Table 1) and LI-F (VIII in Fig. 1)). The fusaricidins (Kajimura and Kaneda 1997, Beatty and Jensen 2002) and LI-F (Kurusu et al. 1987, Kuroda et al. 2000) from *Paenibacillus polymyxa* were fungicidal and antibacterial. LI-F compounds containing an azole group were toxic to ddY mice (Kuroda et al. 2001). The target of the azole group containing fungicidals target is cytochrome P450 which is also present in mammalian cells (Yoshida 1988), while azole antibiotic drugs are toxic when administered orally (Mclean et al. 2002) and impair mitochondrial functions (Rodriquez and Acosta 1996).

Interestingly, the first cyclic lipopeptide daptomycin (1620 Da) from *Streptomyces roseosporus* has been approved (USA) for use as an antibacterial drug against gram-positive bacterial infections as MRSA. It acts by forming ion channels in membranes and high doses may cause renal dysfunction (Silverman et al. 2003).

1.4.2. Miscellaneous *Bacillus* toxins

Rhizocticins (XIX in Fig. 1) produced by *B. subtilis* are oligopeptides with a phosphono group. Rhizocticins mislead the fungal peptide transport system with an oligopeptide part into transporting it inside the cell where the toxic phosphono group is realized (Kugler et al. 1990). The cyclic tridecapeptide mycobacillin (X in Fig. 1) is also produced by *B. subtilis.* Mycobacillin liberates ATP from *Aspergillus niger*, causing depletion of energy (Chowdhury et al. 1998), and is hemolytic at an exposure concentration of 25 µg/ml (Banerjee 1977). *Bacillus cereus* secretes the fungicidal octapeptide mycocerein of whose amino acid composition is similar to that of the iturin group (Wakayama et al. 1984). *Bacillus licheniformis* F2.2 isolated from a fermented food produced plipastatin, surfactin, and the new 1193-Da non-peptide surfactant BL1193 (Thaniyavarn et al. 2003).

The cationic surfactant polymyxin B (VII in Fig. 1) is a cyclic decapeptide from *Paenibacillus polymyxa* that is effective against gram-negative bacteria. It contains 2, 4 di-aminobutyric acid (Rosenberg and Ron 1999). At least 15 different polymyxins have been found, e.g. mattacin 1157 Da (polymyxin M) produced by *Paenibacillus kobensis* M. It is structurally related to polymyxin B. Both bind to lipopolysaccharide (LPS), which is thought to be reason for their effectivity against gram-negative bacteria (Martin et al. 2003). Polymyxin B caused renal dysfunction in 14% of patients treated with it against a multiresistant bacterial infection (Ouderkirk et al. 2003). The fungicidal 770-Da peptide A12-C was isolated from *B. licheniformis* 12 which contained Glu, Arg, Ala, Pro, Tyr and Orn (Galvez et al. 1993a). The strain *B. licheniformis* M-4 produces a 3400-Da fungicidal peptide fungicin M4 containing Glu, Arg, Pro, Tyr, Val, Met and Orn (Lebbadi et al. 1994a).

Bacillus licheniformis strains also produce peptides called amoebicins (Table 1) because they inhibit the amoeba *Naegleria fowleri. Bacillus licheniformis* A12 produced two amoebicins, the peptides A12-A and A12-B, which had the same mass of 1430 Da and contained Asp, Glu, Ser, Pro, and Tyr (Galvez et al. 1993b). They were active against yeast, fungi, and bacteria, and ruptured membrane of the amoeba *Naegleria fowleri*. *Bacillus licheniformis* D-13 produced three amoebicin peptides with the same masses (1870 Da) and contained Asp, Glu, Val, and Leu (Galvez et al. 1994). These amoebicins inhibited several bacteria and were cytotoxic to murine cells (Galvez et al. 1994). A12 and D13 tolerated heating at 100 °C for 30 min, pH 2.5, and activity of proteolytic enzymes. Peptide A12 lost 75% of its activity at pH 9.5 but D13 was active (Galvez et al. 1993b, 1994). The *B. licheniformis* strain M-4 produced three antifungal amoebicins: 3000 - 3200-Da peptides containing Asp, Glu, Ser, Thr, Pro, and Tyr (Lebbadi et al. 1994b). It was shown that amoebicin m4-A decreased the membrane potential of *B. megaterium* in a manner similar to that of gramicidin D, formed pores in liposomes, and finally lysed both of these (Lebbadi et al. 1994c). Bacitracin (XI in Fig. 1) produced by strains of *B. licheniformis* and *B. subtilis* is known as an antibacterial substance but is also nephrotoxic (Butaye et al. 2003) and possesses fungicidal activity against *Botrytis cinerea* (Gebhardt et al. 2002).

The peptide gramicidins and tyrocidine are produced by *Brevibacillus brevis*. Gramicidin S (V in Fig. 1) and tyrocidine are cyclic decapeptides. Tyrocidine is a group of compounds tyrocidine A (VI in Fig. 1), B, and C. Tyrocidine and gramicidin S cause hemolysis (Prenner et al. 1999, Qin et al. 2003). Both substances are toxic during internal use or when inhaled but are topically used to treat bacterial infections of the skin. Gramicidin S and the polymyxins share two unique features with antimicrobial peptides from animals, plants and insects: they are polycationic and amphipathic (Zhang et al. 2000). Linear gramicidin (gramicidin D, Dubos and Hotchkiss 1941) pentadecapeptide is a group of compounds A (90% of the total mixture), B, C, and D. The structure of gramicidin A (XXI in Fig. 1) is HCO-*L*-Val-Gly-*L*-Ala-*D*-Leu-*L*-Ala-*D*-Val-*L*-Val-*D*-Val-*L*-Trp-*D*-Leu-*L*-Trp-*D*-Leu-*L*-Trp-*D*-Leu-*L*-Trp-$NHCH_2CH_2OH$. The amino acid residues of gramicidin A have hydrophobic side chains, *D* and *L* forms of amino acid residues alternate and both ends of the peptide are modified (blocked). Gramicidin A contains valine at the position 1 and form head-to-head dimers (the β-helix coils) that act as H^+-, K^+-, Na^+-selective channels (Busath 1993). The K^+ and Na^+ channels of gramicidin A have selectivity ratio K:Na of 1:3 (Wallace 2000).

Bacillus sp. sunhua produces the 402-Da macrolactin A (XX in Fig. 1) which is a cytotoxic macrolide. A total of 45% of the macrolactin was stable after heating at 100 °C for 30 min and 60-100% at pH 5-13, but it was unstable at less than pH 3 (Han et al. 2005). Macrolactin A which inhibited *B. subtilis* (5 μg per agar plate) and *Staphylococcus aureus* (20 μg per agar plate), was antiviral and cytotoxic to cancer cells in concentrations from 3 to 8 μg/ml (Gustafsson et al. 1989). Plans have been formed to use *Bacillus* sp. sunhua as a biocontrol agent in potato planting (Han et al. 2005).

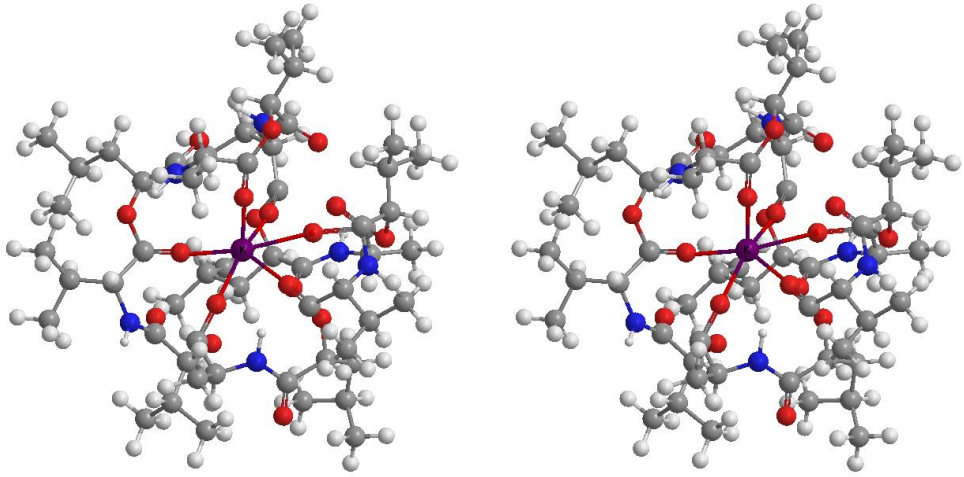
Butirosin (XVIII in Fig. 1) is an aminoglycoside toxic to the kidney and is produced by *B. circulans* (Cox and Serpersu 1997). Bacilysocin is a 470-Da non-peptide produced by *B. subtilis* 168. It is a new fungicide with a phosholipid structure (Tamehiro et al. 2002). A non-protein acetone-soluble substance from *Bacillus atrophaeus* inhibited growth of several fungi. It also affected plasma membranes and membrane organelles of the A*scosphaera apis* pathogen of honeybee larvae (Youssef and Knobletta 1998).

Bacillus pumilus is known to produde the antibiotic pumilacidins (Table 1). In addition some *B. pumilus* strains also produce compounds toxic to mammalian cells. The strains of *B. pumilus* originating from different environmental sources produced heat-stable and proteolysis-resistant toxins (Suominen et al. 2001). Strains of *B. pumilus* and *B. simplex* producing compounds toxic to boar sperm were isolated from indoor air (Peltola et al. 2001). Toxic strains of *B. pumilus* were also reported from the air of cotton mills (Hoult and Tuxford 1991). Munimbazi and Bullerman (1998) isolated from dried Ndagala fish a strain of *B. pumilus* that produced a heat-stable (121 °C, 15 min), pH (2-10)-stable and proteolytic enzyme-resistant antifungal peptide that was soluble in polar organic solvents. Recently, an antifungal and antibacterial substance of < 3 kDa and with an unknown structure was reported from *B. pumilus*; the substance was resistant to pronase and had a stable shelf life for of at least 8 d in agar (Bottone and Peluso 2003).

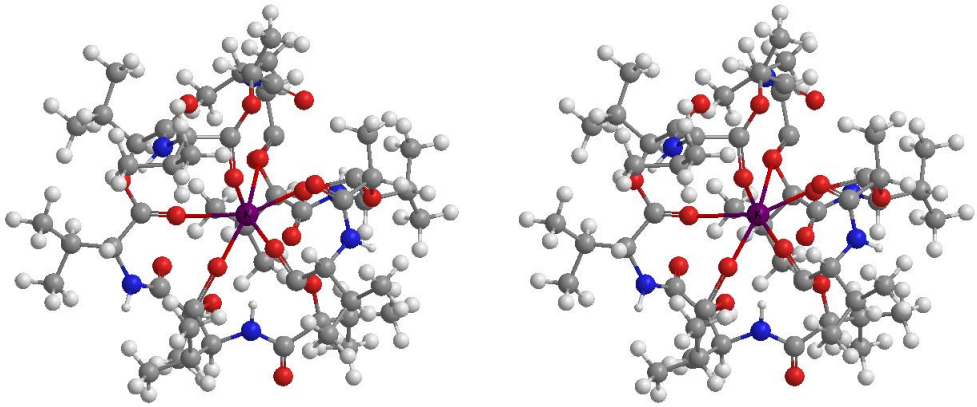
1.4.3. Cyclic depsipeptides

In cyclic depsipeptides the peptide backbones consist of hydroxy acids together with amino acids, forming a cyclic structure. Cyclic depsipeptides are able to bind alkali metal ions to the cavity of the molecule, which makes them act as ionophores (Ballard et al. 2002). A similar ion cavity is found in cyclic ethers such as 18-crown-6, which also binds potassium with six single-ion pairs of electrons of the oxygen atoms in the ether bonds. The ion-binding properties of the ionophoretic cyclic depsipeptides are depentent on the size of the ring and the complex-forming groups determining the cavity in which the ions fit. The conformations of cyclic depsipeptides are depentent on chemical environment, e.g. solvents or membrane lipids, which makes it possible to bind ions and to release the ions.

Cereulide *cyclo*(*D*-Ala-*D*-*O*-Leu-*L*-Val-*L*-*O*-Val)$_3$ (Fig. 4 and XIII in Fig. 1), whose structure was determined by Agata et al. 1994 and homocereulide *cyclo*(*D*-*O*-Leu *D*-Ala *L*-*O*-Val-*L*-Val)$_2$*D*-*O*-Leu-*D*-Ala-*L*-*allo*-*O*-Ileu-*L*-Val (Wang et al.1995) are potent ionophores from *B. cereus* that structurally resemble valinomycin *cyclo*(*D*-Val-*L*-*O*-Ala-*L*-Val-*D*-*O*-Val)$_3$ (Fig. 4) from *Streptomyces* strains. These substances are cyclic dodecadepsipeptides and transport potassium ions across lipid membranes. Their structures take shape where single pairs of electrons of the oxygen atoms from the six ester bonds form complexes with potassium ion. Cereulide and valinomycin are carriers (not channel formers) for potassium and have the stereoisomer *cyclo*(*DDLL*)$_n$ configuration. Interestingly, the some compounds that have the stereoisomeric *cyclo*(*DLDL*)$_n$ configuration are able to orientate in membranes so that they form tubular ion channels (Hartgerink et al. 1998).



Cereulide



Valinomycin

Figure 4. *Stereo views of cereulide and valinomycin with the same atom colors in Fig. 2, in which violet is potassium ion.*

The stereo view in Fig. 4 shows how the outer layer forms a hydrophobic belt around the structurally similar valinomycin and cereulide, making them permeable through to the cell membrane. The inward-oriented oxygen atoms from six ester bonds can form with unpaired electron complexes with positively charged alkali metals and ammonium ions. The structures of cereulide and valinomycin make them hosts to ions of alkali metals such as Li^+, Na^+, K^+, and Rb^+. The size of the molecules cavity of is favorable for binding with potassium and also with the same-sized ammonium ion.

Cereulide (Fig. 4) is the only potassium ionophore carrier found in *Bacillus* species and it was synthesized by Isobe et al. (1995). Cereulide causes emetic symptoms (Agata et al. 1995) and may cause fatal liver failure (Mahler et al. 1997). It was shown in many studies to be highly toxic to human cells such as colon, neural, airway epithelial, and HeLa cells (Jääskeläinen et al. 2003a), neural cells (Teplova et al. 2004), and triggers apoptosis in natural killer (NK) cells (Paananen et al. 2002). Cereulide was also found in construction materials (Mikkola et al. 1999) and indoor environments (Andersson et al. 2002). However, the mode of action and specific mitochondrial toxicity were not clear before 1999, after which its ionophoretic properties were studied experimentally using BLM in this thesis. Valinomycin (Fig. 4) is regarded as extremely toxic (Sigma-Aldrich, material safety data sheets). Several valinomycin-producing *Streptomyces griseus* strains was found in air of elementary schools and settled dust of children day-care centers (Andersson et al. 1998). *Streptomyces griseus* strains were are commonly found in moisture-damaged buildings (Rintala et al. 2004). It was reported that *B. amyloliquefaciens* (three strains out of the 18 studied), *B. subtilis* (one strain out of the 19 studied) and *B. pumilus* (12 strains out of the 14 studied) also produced valinomycin (Wulff et al. 2002). Since Wulff et al. identified valinomycin using retention data of

HPLC without MS data, their results are uncertain. Cereulide could be considered as an analogue of valinomycin. Subtle changes in the outer layer hydrocarbons of the analogues can affect the stability constant of the valinomycin K^+ complex, as shown in the work of Ovchinnikov (1974) and Teplova et al. (2006). Thus, valinomycin analogues such as cereulide will have different ionophoretic, properties depending on the K^+ concentration in the environment. *In vivo* cereulide induced emetic symptoms in the Asian house shrew (*Suncus murinus*) at 13 μg/kg effective $dose_{50}$ (ED_{50} value, oral administration) at doses 60 times lower than valinomycin (Agata et al. 1995). The small structural difference between cereulide versus valinomycin changes the biological properties. The physical and chemical properties of cereulide and valinomycin are very similar, whereas the biological effects are different: cereulide is an emetic substance at low doses and the physiogical K^+ concentration in (blood) cereulide is more active (Teplova et al. 2006).

Bacillus cereus strains also produce homocereulide (Table 1) which resembles cereulide (see p. 27). Cereulide and homocereulide were cytotoxic toward murine leukemia and colon cancer cells at 18 pM (0.021 ng/ml) and 16 pM (0.018 ng/ml), respectively (Wang et al. 1995). Valinomycin possesses toxic activity toward VSV and some gram-positive, but not gram-negative bacteria (Pettit et al. 1999). Valinomycin provoked toxic effects similar to those of cereulide, inhibiting boar sperm cell motility and swelling mitochondria (Andersson et al. 1998) and causing apoptosis in NK cells (Paananen et al. 2000). Valinomycin caused apoptosis and intracellular acidification in murine hematopoietic cells (Furlong et al. 1998). Valinomycin was 500-10 000 times more cytotoxic than montanastatin, an analogue of valinomycin with a cyclic structure *cyclo*(*D*-Val-*L*-*O*-Ala-*L*-Val-*D*-*O*-Val)$_2$. Both compounds were coproduced by *Streptomyces anulatus* (Pettit et al. 1999). Valinomycin inhibited several gram-positive bacteria towards which montanastatin was inactive (Pettit et al. 1999). The ionophoric properties of montanastatin are unknown.

1.5. Ionophores impair mitochondrial functions

Mitochondria regulate cells in life and death, finally transmitting the apoptosis (programmed death) signal to the cell. In recent years impaired mitochondria were suspected of playing a role in the etiology of several chronic diseases (DiMauro and Davidzon 2005).

1.5.1. Mitochondrial inner membrane functions

Mitochondria are the site of ATP production associated with respiration of cells. The electron transfer chain (inner membrane, cristae Fig. 5 and Fig. 6), where complex II led by the citric acid cycle is located in the mitochondrial matrix. Sugars and fatty acids are used as fuels to build up an electric transmembrane potential (100-150 mV) in the mitochondria (Fig. 6). This potential energy is converted to chemical energy in the form of

ATP (V in Fig. 6,). As many as 90% of the reactive oxygen species (ROS) are generated by aerobic metabolism at steps I - IV of the respiratory chains in the mitochondrial inner membrane (Fig. 6).

Figure 5. *The mitochondria possess two membranes (orange) separated by intermembrane space (white), the permeable outer membrane around the mitochondria and the impermeable inner membrane which forms folds called cristae, as well as the matrix (gray).*

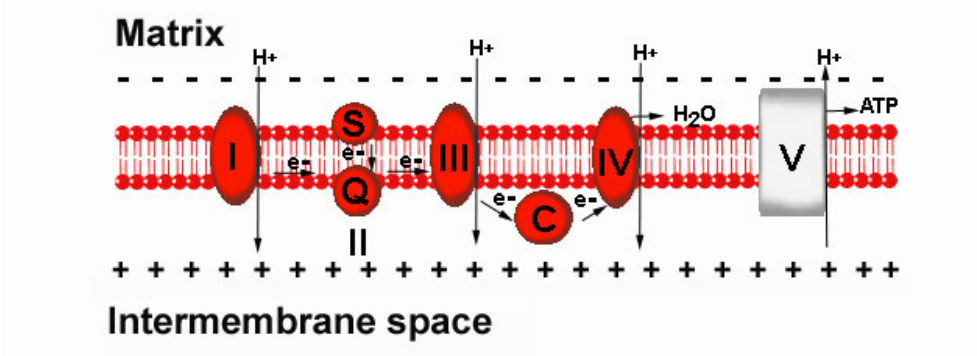


Figure 6. *Functions of the mitochondrial inner membrane: I, NADH dehydrogenase (proton pump); II, The succinate dehydrogenase (S) which is part of the tricarboxylic acid cycle, coenzyme Q (Q); III, cytochrome c reductase (proton pump), cytochrome c (C); IV, cytochrome c oxidase (proton pump, conversion of oxygen to water); V, ATP synthase.*

1.5.2. Ionophores have different effect on mitochondria, impairing their functions

An intact inner membrane is essential for maintaining electrical potential in the mitochondria. Ionophores decrease inner membrane potential and increase oxidative stress by forcing mitochondria to maintain membrane potential, causing higher oxygen

consumption and increase in ROS. High contents of ROS can damage the unshielded mitochondrial DNA. Mutations in circular mitochondrial DNA were associated with several diseases (DiMauro and Davidzon 2005). Ionophoretic toxins destroy the osmotic balance of cell and interfere with ATP production.

A decreased mitochondrial inner membrane potential triggers apoptotic cell death. Toxins can damage mitochondria so that they become inefficient, similar to the situation noted in studies on aging. It was observed that old mice had more lymphocytes with lower mitochondrial membrane potentials than young mice (Mather and Rottenberg 2002). It was also shown that aged neurons have significantly reduced mitochondrial membrane potential (Murchison el al. 2004). Continued exposure to potassium ionophores may induce osmotic swelling and lysis of mitochondria.

There are other ionophores known as food contaminants that may cause problems similar to those caused by cereulide. Enniatins A, B are cyclohexadepsipeptide from the fungus *Fusarium* and form $K^+ > Ca^{2+} > Na^+$ channels (Ovchinnikov 1974, Kamyar et al. 2004). Beauvericin from the enniatin group is also a cyclohexadepsipeptide and forms cation channels (Kouri et al. 2003) and induce changes in homeostasis, inducing Ca^{2+} influx in mitochondria from pig myocytes as well as decreasing the intracellular ratio of K^+ to Na^+ (Kouri et al. 2005). The enniatin group of cyclohexadepsipeptides show the stereoisomeric *cyclo*(*DLDLDL*) configuration (see also Chapter 1.4.3). Thus, forming tubular ion channels may explain observed channel properties of enniatins.

Some ionophoric growth promotors and anticoccidal substances are commonly used as feed additives. Carboxylic Ca^{2+}, Mg^{2+}, K^+, and Na^+ ionophores such as monensin, lasalocid, salinomycin, narasin, and laidlomycin produced by *Streptomyces* species, maduramycin produced by *Actinomadura yumaensis*, and semduramicin from *Actinomadura roseorufa* are used as growth promotors or to prevent coccidiosis caused by *Eimeria* in animals (horse, cattle, sheep, and poultry) (Butaye et al. 2003). However, the use of ionophores as feed additives has caused several accidental cases of poisoning among pets and domestic animals (Van der Linde-Sipman et al. 1999). Narasin from eggs is shown heat-stable (Rokka et al. 2005). Recently, it was shown that ionophoric animal food additives may act as mitochondriotoxins (Hoornstra et al. 2003). Lasalocid transported biogenic amines across the lipid bilayer membrane when studied using the BLM technique (Kinsel et al. 1982a,b). The indoor air-isolated *Trichoderma harzanium* produced the peptaibol peptide, which formed potassium channels, inhibited motility, and depolarized mitochondria of sperm cells (Peltola et al. 2004). Mitochondrial dysfunctions affect especially those organs rich in mitochondria, such as the central nervous system, liver, lung, kidney, and the heart.

1.6. Synergistic effects of *Bacillus* toxins

Several *Bacillus* strains produce toxic compounds that together, in some cases, act more efficiently than when alone. Coproduction of iturin and/or surfactin and/or fengycin in the same *Bacillus* strains is common. Surfactins are found as coproducts with iturin or plipastatins from *B. subtilis* (Sandrin et al. 1990, Roongsawang et al. 2002). The non-peptides zwittermicin A (XVI in Fig. 1) and kanosamine (XVII in Fig. 1) purified from *B. cereus* were active against several gram-positive and gram-negative bacteria, were fungicidal, and together showed synergistic effects on microbes (Milner et al. 1996, Silo-Suh et al. 1998). Combination of the antifungal agents LI-F (VIII in Fig. 1) and azole acted synergistically against fungi (Kuroda et al. 2000). Sensitivity to the ionophores valinomycin (Fig. 4) and A23187 of *Escherichia coli* was increased 50 - 100 times by the presence of polymyxin B (VII in Fig. 1) (Alatossava et al. 1985). Bacillomycin D (Table 1) and fengycin (Fig. 3) act in a synergistic manner against fungi (Koumoutsi et al. 2004). Surfactin (Fig. 2) also acts synergistically with iturin A (Table 1) against fungi (Maget-Dana et al. 1992, Thimon et al. 1992). Iturin and surfactin interact and increase strongly in hemolytic activity compared with their individual effects (Maget-Dana et al. 1992, Thimon et al. 1992). Iturin A and surfactin synergy was explained as resulting from a mixed micelle formation (Thimon et al. 1992). The synergistic effects of toxins from *Bacillus* species (from coproduction in one strain or in separate strains) should be considered when estimating the biotoxin hazards in food or indoor environments.

2. Aims of the present study

This study focused on the non-protein heat-stable toxins produced by bacilli isolated from food, food poisoning cases and moisture-damaged buildings in which the inhabitants suffered from ill health associated with the indoor environment.

What are the physical, chemical, and biological properties of such *Bacillus* toxins?
To address this topic the following tasks were executed:

- Purification and characterization of toxins,
- Investigation of toxicity to different mammalian cells and cell lines,
- Explaining the biological effects of the toxins,
- Developing detection and quantitation methods for the toxins.

3. Material and methods

3.1. Origins of the toxic *Bacillus* strains

We examined the toxic *B. cereus* strains F4810/72, NC7401, and F5881 (I-II) and the *B. licheniformis* strains 553/1, 553/2, and 575U/5 associated with food poisoning cases (III). Five fungicidal and boar sperm-toxic *B. amyloliquefaciens* strains (1/117, 2/117, 5/117, 2.14, 19b) were isolated from water-damaged building material and indoor dust (IV, V). The reference strains used were from similar sources or from different culture collections (I-V).

3.2. Evaluation of the toxicity of the *Bacillus* strains using eukaryotic cells

The tools used for the detection of toxic *Bacillus* strains included inhibition of boar sperm cell motility and/or antagonism toward fungal growth on agar plates. The boar sperm cells consist of a head, midpiece, and flagellum. The midpiece is crowded with mitochondria that produce ATP to move the sperm cell by its propelling flagellum. Toxicity to exposed boar sperm cells can be observed as a loss of motility. This is done with the light microscope by estimating the exposure dose (ED_{50}) that converts 50% of the cells to a non-motile state.

The changes in membrane potentials of the cell and mitochondrial membranes were studied using functional fluorochrome stains. Damage of to the plasma membrane was recorded using the vital stains calcein acetoxymethyl (AM) ester and propidium iodide (III), rhodamine 123 and ethidium bromide (III). Decreases of mitochondrial membrane potential were assessed using JC-1 stain (IV, V).

3.4. Purification of toxins

Methanol was selected as the extractant for active compounds from the biomasses obtained from plate-cultivated *Bacillus*. Further purification of toxins from the methanol extract was obtained using solid-phase Sep-Pak C18 cartridges and RP-HPLC. The RP-HPLC fractions were evaporated and redissolved in methanol before performing the boar sperm motility inhibition test.

The RP-HPLC was used as the main tool for purification, amino acid analysis, and the MS studies of the toxins. Prior to the RP-HPLC purifications the bacterial methanol extracts were fractioned with solid-phase Sep-Pak C18 cartridges to pre-purify or to concentrate the toxin. The columns used for RP-HPLC were C8 and C18 phases with eluent compositions

of methanol/water with 0.1% formic acid and acetonitrile (0.1% trifluoroacetic acid)/water (0.075% trifluoroacetic acid).

3.4. BLM

In BLM method artificial membranes from phospholipids or purified cell membranes are formed to simulate biological membranes. With BLM it is possible to measure changes in lipid film conductance using electrodes. For this bilayer membranes are created in the pinhole of the BLM cuvette containing the electrolytic solution and then the test substances are added to the cuvette (II). BLM is used to resolve the ionophoretic properties of compounds: the selectivity for different anions or cations and to establish whether it forms a channel or acts as a carrier.

Diagram 1 shows a route of the study and the methods. References to the methods used are shown in Table 2.

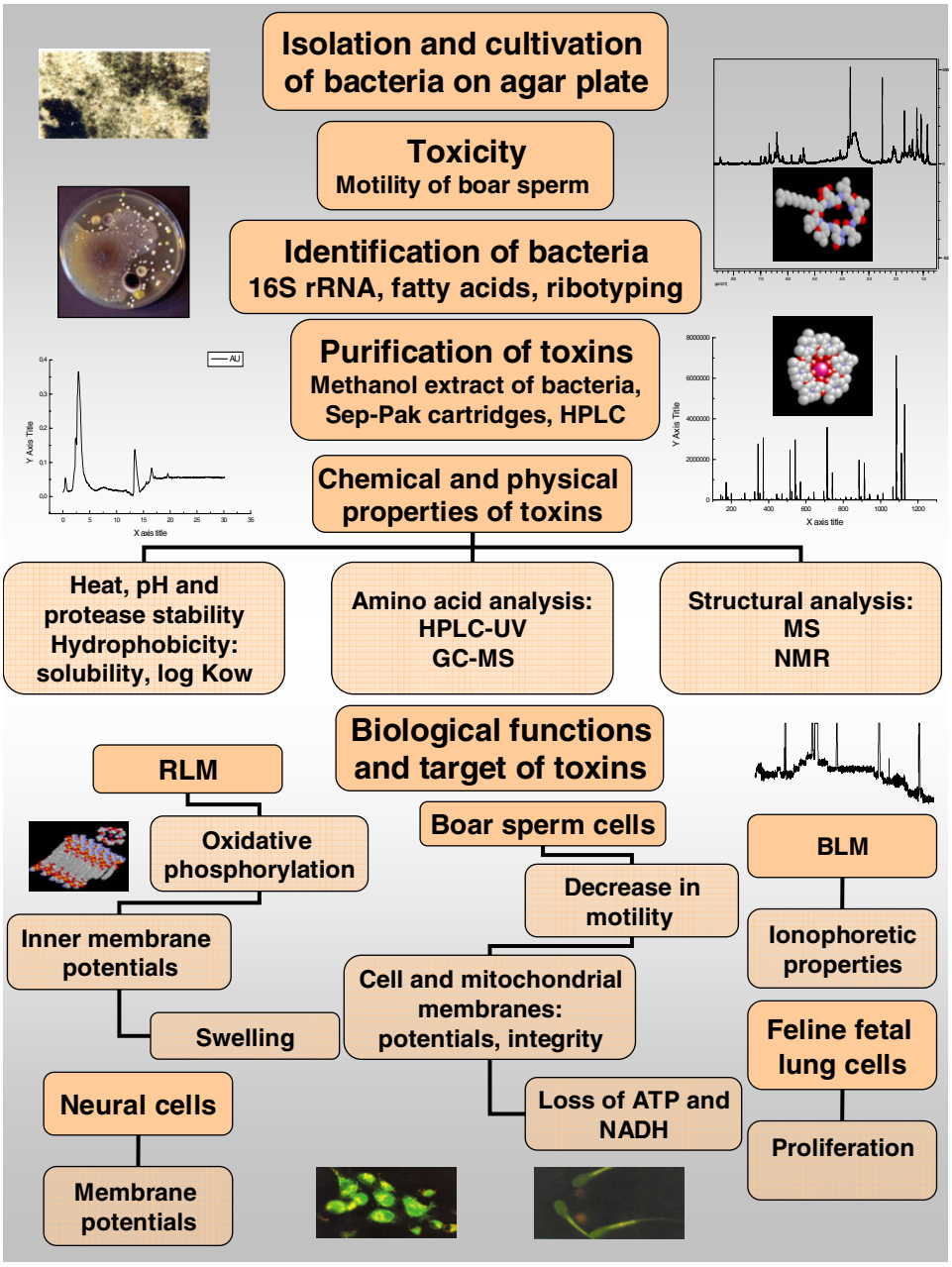


Diagram 1. *Basic procedures used in this study to investigate the Bacillus toxins.*

Table 2. *List of methods used in this study.*

Method	References
Identification of the bacteria	
Whole fatty acid analysis	I-V
Partial 16S rRNA sequence	I, III, IV
Automated ribotyping	IV
Microscopy	
Photo (epifluorescence)	IV, V
Confocal (scanning)	III
Electron (transmission)	I,II
Mass and structural analysis	
ESI-IT-MS	IV,V
MALDI-TOF-MS	II
ESI-TQ-MS	I, III
NMR	V
Amino acid analysis	
RP-LC	III
GC-MS	IV
Purification and quantification	
RP-LC	I-V
Ion conductivity	
Black-lipid membrane (BLM)	II,V
Biological effect	
Boar sperm cells	I-V
Rat liver mitochondria (RLM)	II, V
Feline fetal lung cells	IV
Human neural cells	IV
Hydrophobicity	
LogKow Program	II, this thesis

4. Results and discussion

This study focused on characterizing the non-protein compounds isolated from *Bacillus* species implicated in food poisoning and from moisture damaged-buildings in which the inhabitants suffered from ill health associated with the indoor environment. Toxic *Bacillus* strains were traced, using boar sperm cells as indicators of toxicity. *Bacillus* extracts toxic to boar sperm were fractioned with the RP-HPLC and the toxic fractions were examined using MS, NMR and BLM methods and mammalian cells as well as isolated RLM as indicators of toxicity.

Purification, identification, and quantification of the emetic toxin cereulide produced by *B. cereus* strains was shown here in a novel bioassay (I), as were the mitochondrial toxicity and ionophoretic properties of cereulide (II). *Bacillus licheniformis* strains isolated from food poisoning cases were analyzed for the presence of compounds toxic toward boar sperm cells. The biosurfactant lichenysin A was isolated and its biological target in the sperm cells was identified (III). A novel toxin named amylosin and the biosurfactant surfactin were isolated from *B. amyloliquefaciens* strains and their properties characterized (IV, V).

The *Bacillus* toxins studied can be categorized into three groups according their toxicities:

A. Compounds affecting the mammalian plasma membrane and causing leakage of cell contents.
B. Compounds generating ion-selective channels both through the plasma membrane and the mitochondria and also causing depletion of cellular ATP and NADH.
C. Compounds that permeate the plasma membrane without damaging it and affect organelles inside the cells such as the mitochondria.

4.1. Tolerance towards heat, varied pH, and protease of the toxins

The enzymes and protein toxins in the *Bacillus* extracts were deactivated by heating in a water bath at 100 °C for 20 min. Boar sperm cells were used as the indicator to detect the presence of toxic substances. The toxicity response detected in the extract was scored towards treatment with protease, acid (pH 2), and alkali (pH 12). The *B. cereus* emetic toxin cereulide was resistant to trypsin, pepsin, and pronase (mixture of carboxypeptidase, zinc endopeptidases, aminopeptidases, and serine protease), heating at 121 °C for 15 min, pH 2 and pH 12 (I), similar to the result described by Shinagawa et al. (1995). The lichenysin A, surfactin, and amylosin were also acid/base-resistant and heat-stable. Cyclic compounds are more inert to chemical and physical treatments than related linear compounds. Such stable compounds can be expected to be persistant and to remain in the environment long after the death of the viable cell that produced them.

4.2. Purification of the toxins

4.2.1. Development of the extraction method for the heat-stable *Bacillus* toxins

This thesis was the first description of the methods used to purify cereulide from plate-cultivated cells of *B. cereus*, followed by extraction with methanol (I). The methods included ammonium acetate precipitation, followed by ethanol and chloroform extraction of the precipitate and Sep-Pak C18 cartridge fractionation to purify cereulide from the *B. cereus* extract (I). Cereulide purification was improved by leaving out the ammonium sulfate precipitation and pre-purifying the methanol extract of plate-cultivated *B. cereus* cells directly with Sep-Pak C18 (II). We used methanol as solvent because it possesses good penetration properties and dissolves several classes of hydrophilic and hydrophobic compounds. Methanol precipitates proteins, which reduces contamination in subsequent purification steps.

In previously publications on purification of cereulide (Agata et al. 1994, Wang et al. 1995) and homocereulide (Wang et al.1995) time-consuming methods based on liquid culture of *B. cereus* were employed. Agata et al. (1994) precipitated liquid culture with ammonium sulfate, extracted precipitate with ethanol, and used various solvent partitions, followed by silica gel thin-layer chromatography (TLC) purification and a C18 cartridge. Wang (1995) extracted cells of *B. cereus* with chloroform/methanol, followed by silica gel TLC purification.

We found that heat-stable boar sperm-toxic compounds other than cereulide were also soluble in 50-100% methanol/water. Solid-phase extraction (SPE) cartridges based on RP material such as C8 or C18 were convenient for concentrating and pre-purifying the moderate to highly hydrophobic toxins cereulide, surfactin, lichenysin A, and amylosin from the *Bacillus* methanol/water extracts (I-V). Therefore we used the method of plate cultivation of *Bacillus* followed by methanol extraction and Sep-Pak cartridge pre-purification in all subsequent studies.

Cereulide is soluble in pentane (Häggblom et al. 2002, Jääskeläinen et al. 2003b, Shaheen et al. 2006) but the *B. amyloliquefaciens* toxin amylosin did not dissolve in pentane, chloroform, or 100% acetonitrile (IV). Instead of using methanol as we did to extract lichenysin A (III) and surfactin (IV) from plate-cultivated *Bacillus* cells, these compounds were purified by acid precipitation (pH 2) from liquid cultures of *Bacillus*, followed by extraction with tetrahydrofuran (Yakimov et al. 1995).

Previously foam fractionation followed by acid precipitation from *B. subtilis* cultures was used to concentrate *Bacillus* surfactants such as surfactin (Cooper et al. 1981). An automated accelerated solvent-extraction (ASE) method, benefitting from pressure, heat, and properties of the solvent mixture, may be used for fast-extraction procedures to mine toxins from complex matrices such as foods (Jääskeläinen et al. 2003b). Due to the closed

system as an ASE method, personal exposure to the toxins is reduced compared with manual extraction. One interesting extraction technique yet to be applied to bacterial toxins would be supercritical fluids such as carbon dioxide or pressurized hot water, which are non-toxic solvents.

4.2.2. Purifications and quantitation of toxins by RP-HPLC

Since the hydrophobicity of the toxins studied here (see 4.3) varied from moderate to high, the RP-HPLC method was suitable for the purification of these toxins. The RP-HPLC-UV methods for purification and quantification of cereulide were developed by us using valinomycin (Sigma-Aldrich) as the standard compound (I, II). To date, cereulide is not available commercially. The same methods were subsequently used by us to purify and quantify valinomycin produced by *Streptomyces griseus* strains isolated from indoor air (Andersson et al. 1998). In these methods acetonitrile/water with trifluoroacetic acid was used as the eluent in RP-HPLC and C8 and C18 columns. Later we purified and quantified cereulide using methanol/water with formic acid, which is less hazardous than acetonitrile/water with trifluoroacetic acid, but comparably efficient (Teplova et al. 2006). Both cereulide and valinomycin exhibit activity in RP-HPLC in similar way and valinomycin was an excellent reference compound for cereulide analysis. Agata et al. (1994) used a C18 column and 95% methanol with trifluoroacetic acid as eluent and Wang et al. (1995) used C18 and methanol as eluent in cereulide analysis. However, in these studies valinomycin was not used to quantify cereulide.

The limit for reliable detection of cereulide and valinomycin in the RP-HPLC-UV method was rather high, 5-10 µg per injection, because of the UV absorbances (215 nm) of these compounds are low. For this reason, a step for the concentration and pre-purifications by Sep-Pak Cartridges of the methanol extracts obtained from the bacteria was needed. Currently, valinomycin is offered commercially (Sigma-Aldrich, product numbers V0627, 94675 and V3639) and the company referees to our work (Andersson et al. 1998).

In the present thesis lichenysin A was purified with RP-HPLC from methanol extracts of *B. licheniformis*, using acetonitrile/water with trifluoroacetic acid and a C8 column. Commercial surfactin, structurally similar to lichenysin A, was used as a standard compound to quantify lichenysin A from the methanol extracts of *B. licheniformis* (III). Developing the HPLC method using methanol/water with formic acid and an analytical and semi-preparative C2/C18 column (C18 in V) for toxins from *B. amyloliquefaciens* strains resulted protocols in which two toxins, amylosin and surfactin, could be purified within a single run (IV, V). The exact concentration of amylosin in methanol solution was determined using the ERETIC NMR method (V). Amylosin and the macrolactone polyene amphotericin B (924 Da) showed similar UV absorption at 382 nm in concentrations of 1 µg per injection. This made it possible to develop an analytical RP-HPLC-UV method to quantify the novel toxin, amylosin (V).

Analytical methods resembling those used by us have been used for the detection and purification of other ionophores and metabolites from *Bacillus* species. The RP-HPLC-MS method for quantifying cereulide was developed by Häggblom et al. (2002) using valinomycin as standard and RP-HPLC conditions similar to those used by us (II). Cyclic fusaricins were isolated from cultures of *Paenibacillus polymyxa,* extracted with methanol, and purified with size-exclusion chromatography, a Sep-Pak C18 cartridge, and HPLC (Beatty and Jensen 2002). Liquid chromatography-MS detection and fluoroimmunoassay were used to analyze the residues of carboxylic ionophores from eggs and animal tissues intended for human consumption (Crooks et al. 1998, Kennedy et al. 1998, Matabudul et al. 2000, 2002, Turnipseed et al. 2001). The LC-MS/MS methods were validated to analyze residues of coccidiostats such as lasalocid, monensin, salinomycin, and narasin from eggs and broilers (Rokka and Peltonen 2006). Following C18 cartridge purification heat-stable toxins resembling cereulide in hydrophobicity were found in *B. firmus*, *B. megaterium*, *B. simplex*, *and B. licheniformis* (Taylor et al. 2005). In their study toxicity was detected using MTT method and Hep-2 cell vacuolation. The toxins from *B. megaterium* and *B. licheniformis* did not form vacuoles in Hep-2 cells as does cereulide (Taylor et al. 2005).

4.3. Hydrophobicity of the heat-stable toxins isolated from *Bacillus*

4.3.1. Significance of hydrophobicity

Passive penetration and the potential for bioaccumulation in tissue of any substance through the plasma membrane is dependent on the hydrophobicity of the substance. It is well known that hydrophobic xenobiotic compounds such as DDT (Table 3) and PCB can bioaccumulate in the fatty tissue of animals and humans. Thus, exposure to persistent, toxic hydrophobic compounds at concentrations that are too low to induce acute intoxication may much later leave the tissue under certain conditions. Metabolites of toxins are usually more polar (and less hydrophobic) than the original toxin and may lose the ability to accumulate in animal cells and tissues. The logarithm of the octanol/water partition coefficient (log K_{ow}) is one of the parameters used in the quantitative structure-activity relationship (QSAR) method developed for pharmaceutical, biochemical, and toxicological purposes (Meylan and Howard 2000).

Hydrophobicity indices such as log K_{ow} values can be used to estimate the potential for membrane penetration and bioaccumulation. A bioaccumulating compound fits the following criteria: $5.0 < \log K_{ow} < 7.5$, which corresponds to a bioconcentration factor (BCF) > 5000 (EEC, 1993).

4.3.2. Methods used for assessment of log K_{ow}

Log K_{ow} values can be estimated by analyzing the octanol/water solubility ratio of the compound using RP-HPLC with reference compounds of known log K_{ow} values or by calculating from the structure of the compound with computer programs such as LogKow (Meylan and Howard 2000). The LogKow program uses the atom/fragment contribution (AFC) method in which the log K_{ow} of fragments and the correction factor coefficients were derived by multiple regression from a large database of compounds with experimentally determined log K_{ow} values (Meylan and Howard 2000). Estimation of 10 589 compounds with the LogKow program resulted in a high correlation coefficient (r^2 = 0.943) compared with the experimental log K_{ow} values (Meylan and Howard 2000). The structure of the compound is introduced into the LogKow program in the form of SMILES (simplified molecular input line entry system) notation. After calculation of the log K_{ow} fragments of the compound, the results are tabulated and the log K_{ow} of the compound is estimated. The LogKow program is allowed for use of log K_{ow} estimation of lubricants for the purpose of award eco-label (EU, 2005).

RP-HPLC can be used to determine the log K_{ow}, using methanol/water (or other solvent miscible with water) as the eluent and hydrophobic alkyl chains (C2-C18) bonded to silica columns as the stationary phase, and using reference compounds for calibration (ASTM 1992, Saski et al. 1997, Kolari et al. 1998,).

4.3.3. Log K_{ow} values of the toxins

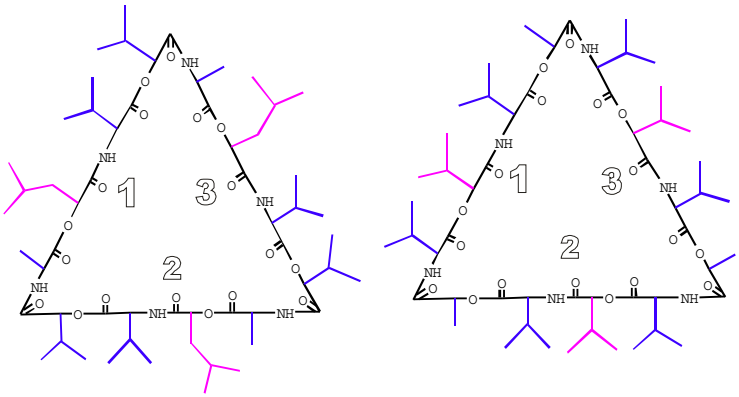


Figure 7. *The structures of cereulide (L-O-Val-L-Val-D-O-Leu-D-Ala)$_3$ (1) and valinomycin (L-O-Ala-L-Val-D-O-Val-D-Val)$_3$ (2). The hydrophobic side chains of the amino and hydroxy acids form the lipophilic outer layers of cereulide and valinomycin, respectively. O-Leu is 2-hydroxyisocaproic acid, O-Val is 2-hydroxyisovaleric acid, and O-Ala is lactic acid.*

Cereulide and valinomycin consist of three repeating units that can be arranged as shown by 1, 2, and 3 in Fig. 7. Cereulide and valinomycin have identical backbones and the 42-Da difference in the molecular masses is due to the side chains of the outer layers, in which cereulide has three *D*-*O*-Leu (magenta) instead of three *D*-*O*-Val (magenta) in valinomycin. Thus, cereulide (log K_{ow} 7.49, Table 3) has three times more CH_2 units than valinomycin in its outer layer. Translated into log K_{ow} values, this means 1.5 (one methylene CH_2 corresponds to 0.5 log K_{ow} units in the LogKow program) and valinomycin log K_{ow} is 5.99 (Table 3). The LogKow program version 1.57 calculated for cereulide and valinomycin a correction factor for the three fragments (-C(=O)-O-C-C(=O)-N-) whose coefficient is 0.5 (total 1.5); this calculation was missing in program version 1.66. Despite the different numerical values obtained for cereulide and valinomycin, the difference in log K_{ow} values between valinomycin and cereulide was in both cases 1.5 log units (corresponding to a difference of the 31 times of the K_{ow} values) in both LogKow program versions.

Table 3. *Log K_{ow} values of the toxins studied and of selected reference compounds.*

Substance	Log K_{ow}	References
Amylosin	3-4	This thesis, HPLC estimation
Surfactin	C15: 5.55, C14: 5.05, C13: 4.55, C12: 4.05	This thesis, calculated
Lichenysin A	C15: 4.89, C14: 4.39, C13: 3.89, C12: 3.39	This thesis, calculated
Cereulide	7.46 (5.96)	II, (Teplova et al. 2006), calculated
Valinomycin	5.99 (4.49)	II, (Teplova et al. 2006), calculated
Amphotericin B	2.46	Zhao et al. 2001, calculated
DTT 1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane	6.3	Lintelmann et al. 2003
Benzo(a)pyrene	6.13	Lintelmann et al. 2003

Values in parentheses are calculated using LogKow program version 1.66 (Teplova et al. 2006).

Despite the similarity in chemical structures of valinomycin and cereulide, cereulide provoked emesis in model animals (*Suncus murinus*) 60 times more effectively than did valinomycin (Agata et al. 1995). Cereulide is more potent as a K^+ ionophore at physiological K^+ concentrations (Teplova et al. 2006). The cereulide toxicity threshold (0.5 ng/ml) toward boar sperm cells was four times lower than that of valinomycin (2 ng/ml) (I). These differences between the biological effects cereulide and of valinomycin may be due to the fact that cereulide has a 31-times higher K_{ow} value than valinomycin: the calculated log K_{ow} of cereulide is 7.46 and that of valinomycin 5.99 (II, LogKow version 1.57).

Due the varying hydrocarbon length and branching of the fatty acid chain of lichenysin A and surfactin molecules (from C12 to C17) the log K_{ow} values of these compounds are also different. In Table 3 the log K_{ow} values are calculated from *iso*-C12 to *iso*-C15 fatty

acid-containing lichenysin A and surfactin (*iso*-C15 fatty acid-containing surfactin and lichenysin A are the main parts of these compounds).

The log K_{ow} of amylosin (Table 3) was estimated with RP-HPLC (using a C18 column and methanol/water with formic acid as eluent) and including surfactin and amphotericin B (4.2.2.2) as references. Since the amylosin retention time was higher than that of amphotericin B (log K_{ow} 2.46) and lower than that of surfactin-containing *iso*-C12 fatty acid (log K_{ow} 4.05), the amylosin log K_{ow} was estimated to be between 3 and 4.

4.4. Amino acid analysis of the toxins

The amino acid analysis was carried out with RP-HPLC-UV or alternatively with GC-MS from hydrolysates of purified peptides. Using Marfey reagent the stereoisomeric forms of the amino acid residues *L*-Gln, *L*-Leu, *D*-Leu, *L*-Val, *L*-Asp, *D*-Leu and *L*-Ile of lichenysin A were analyzed with RP-HPLC as diastereomeric 2,4-dinitrophenyl-5-*L*-alaninamide derivatives (III).

The usefulness of the GC-MS method in amino acid residue analysis was noted when the *B. amyloliquefaciens* toxins surfactin and amylosin amino acid residues were analyzed as *tert*-butyl dimethylsilyl derivatives (IV). The amino acid residue composition of amylosin (Leu, Pro, Ser, Asp/Asn, Glu/Gln, and Tyr) resembles those found in lipopeptides belonging to the ituric group, such as iturins, bacillomycins, as well as fengycin (see pp. 22-24). However, none of these had a chromophoric group such as that found in amylosin.

The advantage of using GC-MS compared with HPLC-UV is that the HPLC-UV method relies only on the retention time, whereas in the GC-MS method verification of the amino acids is possible using both the retention time and the mass spectra of the derivatized amino acids. The GC-MS method using a chiral column would also give stereoisomeric information on the amino acids (Zampolli et al. 2006). LC-MS/MS methods have been developed to analyze underivatized amino acids (Qu et al. 2002, Piraud et al. 2005) and the micro-LC-MS method for stereoisomers analysis of underivatized amino acids (Zeleke et al. 2006).

4.5. Mass spectrometry of the toxins

ESI-TQ, ESI-IT, and MALDI-TOF MSs were used to characterize and identify the toxins.

4.5.1. Selective ion complexes of ionophoric toxins in MS and MS/MS analysis

We showed that cereulide forms cationized molecules not only with K^+ and Na^+ but also with $NH4^+$ (I, II). This was done using ESI-TQ and MALDI-TOF MS. The interpreted ESI-TQ-MS/MS spectrum of cereulide was first published in 1998 (I, Fig. 2B, and Table 1) using the protonated molecule $[M+H]^+$ of cereulide with a *m/z* of 1154 as precursor ion. In these studies valinomycin was used as the reference compound. Cereulide (C) and valinomycin (V) formed protonated molecules $[C+H]^+$ *m/z* 1154, $[V+H]^+$ *m/z* 1112, cationized molecules $[C+NH4]^+$ *m/z* 1171, $[V+NH4]^+$ *m/z* 1129, $[C+Na]^+$ *m/z* 1176, $[V+Na]^+$ *m/z* 1134, $[C+K]^+$ *m/z* 1192, and $[V+K]^+$ *m/z* 1150 in the ESI-MS experiments (I, II, and Andersson et al. 1998, Fig. 2A). We showed that cereulide forms a 1:1 complex with K^+ using BLM data (II).

Suwan et al. (1995) were the first to show that cereulide forms a complex with the alkali metals base in the NMR data. However, these authors concluded incorrectly that cereulide forms an ion-dipole with the carbonyl oxygen of amino acids residues *D*-Ala and *L*-Val. This was unfortunate since their NMR studies and comparison of their results with many NMR studies of valinomycin such as that of Haynes et al. (1969) showed that ion-dipole bonds are formed between cations as K^+ and the six carbonyl oxygens of the oxy acids. The hexagonal cylinder-like structure of cereulide is similar to that of valinomycin. NMR studies of cereulide have confirmed its similarity to valinomycin in the mode of binding cations (Pitchayawasin et al. 2003). However in that study there was no direct mention of ion-dipole interaction between the cation and the carbonyl oxygen of *D*-*O*-Leu and *L*-*O*-Val. Thus, it would be more correct to state that K^+ is hosted inside cereulide by the ion-dipole interactions of three carbonyl oxygens of the *D*-*O*-Leu residues on one side of the plane and three carbonyl oxygens of the *L*-*O*-Val residues on the other side of the plane.

Later the complexation of cereulide with alkali metal and ammonium ions was confirmed by the MS and NMR studies of Pitchayawasin et al. (2003). In ^{1}H-NMR using $CDCl_3$, studies of valinomycin and cereulide showed similar tendencies in the proton shifts of NH and alpha protons complexing with K^+, compared with the ^{1}H-NMR shift of the uncomplexed state or the proton complex (Haynes et al. 1969, Fig. 1, Pitchayawasin et al. 2003, Fig. 4). Thus, formations of these complexes showed equal molecular conformational changes in valinomycin and in cereulide as observed in NMR.

The ionic radii of ammonium and potassium are similar; thus cereulide and valinomycin complex these ions similarly. Adding volatile ammonium salt (which is more suitable than potassium salt for the mass spectrometry instrument) prior to the ESI-TQ-MS analysis (valinomycin was dissolved in 50% methanol containing 5 mM ammonium acetate) increased the intensity of the ammonium-cationized molecule of valinomycin at *m/z* 1129 (Andersson et al. 1998, Fig. 2).

Similar enchancement of cereulide intensity with ESI-TQ-MS analysis was shown for the ammonium-cationized molecule of cereulide at *m/z* 1170 (Paananen et al. 2002, Fig. 1).

The abundant ammonium-cationized molecule of cereulide at *m/z* 1171 was also noted when cereulide was purified using the method of protein precipitation with ammonium acetate before the MS analysis (I, Fig. 1A). When cereulide was analyzed using MALDI-TOF-MS (Fig. 2 in II) without adding excess ammonium, the relative abundance of the ammonium-cationized molecule of cereulide at *m/z* 1171 was smaller than the sodium- or potassium-cationized molecules of cereulide at *m/z* 1176 and 1192, respectively. For identification of a compound it is necessary to perform MS/MS analysis because different compounds can have the same mass ions when analyzed in low-resolution MS but rarely the same MS/MS spectrum. The ammonium-cationized molecules of cereulide and valinomycin are better precursor ions for MS/MS analysis than the corresponding sodium- or potassium-cationized molecules. Using ammonium-cationized molecules as precursor ions, fragmentation is achieved at a lower energy input and thus results in a clearer MS/MS spectrum. The sensitivity of ESI-MS analysis may be increased by adding ammonium to the solution, as in valinomycin (Andersson et al. 1998).

With HPLC-MS analysis the fmol level of cereulide can be detected (Häggblom et al. 2002, Pitchayawasin et al. 2003). The protonated and cationized molecules of cereulide and valinomycin originate from the sample or from the solvent used in the analysis. The selectivity of cereulide and valinomycin affinities for the ESI-MS for the ions was: potassium > ammonium > sodium ions> proton. If the ionic concentration varies from sample to samples, it may cause problems with calculations of the results of the quantitative analysis since both the protonated and cationized molecule peak areas should be integrated. We conclude that adding an excess amount of ammonium acetate to the standards and to the samples or eluent is an advantage in LC-MS analysis, beause it would set the ionic conditions in the standards and the samples at equal levels. Detecting very low concentrations of cereulide from cells or tissues may be important for biochemical or medical purposes. Since the peak concentration in HPLC is inversely proportional to the square of the column radius, micro- and nano-LC columns can also be used to increase sensitivity (Kostiainen et al. 2003).

Two homologs, 1138 Da and 1166 Da, of cereulide were reported by Pitchayawasin et al. (2004) and 1166-Da homocereulide (see also pp. 32, 34) was also reported by Wang et al. (1995). Since the differences are ±14 Da and cereulide (1152 Da) consists of a three-repeating unit, this is no more the case with homologs that have two repeating units and one modified unit. Pitchayawasin et al. (2004) proposed that in the 1166-Da homolog of cereulide (1152 Da) *O*-Val was changed to *O*-Leu and in the 1138-Da homolog *O*-Leu to *O*-Val. Since the amounts of these homologs were 10% of the total cereulide (1152 Da) produced, this may not affect the toxicity. However, some strains or growth conditions of *B. cereus* could produce a higher amount (over 10% of the total cereulide) of such homologs, which should be considered in the RP-HPLC-MS analysis. The homologs may possess affinity for K^+ different from that of cereulide, due to their minor structural difference. It was shown that cereulide toxicity is based on its extremely high affinity for K^+ (Teplova et al. 2006).

4.5.2. Mass spectrometric characteristics of the lipopeptide toxins and amylosin

Membrane-destroying substances from *B. licheniformis* and *B. amyloliquefaciens* were identified as lichenysin A and surfactin, respectively, with MS and MS/MS analysis using MALDI-TOF, ESI-TQ and ESI-IT MS (III, IV). Identification of lipopeptides having a 1-Da difference in their masses (or even having an equal mass number) with MS analysis is not specific enough, considering inaccuracy in mass detection (typically up to 0.50 Da in low-resolution mass spectrometers). Such lipopeptide identification requires MS/MS analysis and/or use of reference compounds. The amino acids sequence of lichenysin A was interpreted from ESI-TQ MS/MS data (III), while that of surfactin (from *B. amyloliquefaciens* 19b) was identified by comparing its ESI-IT-MS/MS spectrum with the MS/MS spectra of commercial surfactin (Sigma-Aldrich). Surfactin and lichenysin A formed three peaks each in UV absorption at 215 nm in the HPLC elution profiles (III, Fig. 1 and IV, Fig. 2) and the MS total ion chromatograms (IV, Fig. 2). The different peaks were due to the carbon chain lengths of tridecanoic, tetradecanoic and pentadecanoic 3-hydroxylated fatty acids of these lipopeptides. These lipopeptides, containing 3-hydroxylated fatty acids, formed protonated and potassium- and sodium-cationized molecules as shown in the mass spectra of lichenysin A, using MALDI-TOF-MS (III, Fig. 1).

The protonated molecules at *m/z* 995 and 1009 and the corresponding sodium-cationized molecules at *m/z* 1017 and 1031 of surfactin were used as precursor ions in ESI-IT-MS/MS analysis (Fig. 8 A and B). The distinctive high abundance of fragment ions at *m/z* 686 and 708 were observed, using protonated and sodium-cationized molecules of surfactin as precursor, respectively. Fragmentation patterns similar to those in Fig. 8 A and B were found in all studied MS/MS spectra (containing different carbon chain lengths) obtained from protonated and sodium-cationized molecules of surfactin and lichenysin A. The abundant protonated molecule at *m/z* 685 was obtained by Yakimov et al. (1999) from an alkaline-hydrolyzed product of lichenysin A and also from the MS/MS spectra of native surfactin and lichenysin A when these lipopeptides were analyzed using fast-atom bombardment MS. The origin of the MS/MS fragment ions at *m/z* 686 and 708 is the opening of molecules between the hydroxy acid Ile in lichenysin A and Leu in surfactin and fragmentation between Leu and Gln (lichenysin A) and Glu (surfactin) (see also I, II in Fig. 1). The fragment ions at *m/z* 686 and 708 represent $[HN\text{-}LeuLeuValAspLeuIle\text{-}COOH+H]^+$ and $[HN\text{-}LeuLeuValAspLeuIle\text{-}COOH+Na]^+$ of lichenysin A (see also II in Fig. 1) and $[HN\text{-}LeuLeuValAspLeuLeu\text{-}COOH+H]^+$ and $[HN\text{-}LeuLeuValAspLeuLeu\text{-}COOH+Na]^+$ of surfactin (see also I in Fig. 1), respectively.

Thus observation of fragment ions *m/z* 686 and 708 in the MS/MS spectra of lichenysin A and surfactin can be used for identification of these lipopeptides The mass spectrum of surfactin from *B. amyloliquefaciens* 19b (Fig. 8C) observed in the negative mode shows a monoisotopic distribution of deprotonated molecules at *m/z* 1007, 1021, and 1035. The protonated molecules of lichenysin A at *m/z* 1007, 1021, and 1035 and the corresponding sodium-cationized molecules at *m/z* 1029, 1043, and 1057 and the potassium-cationized

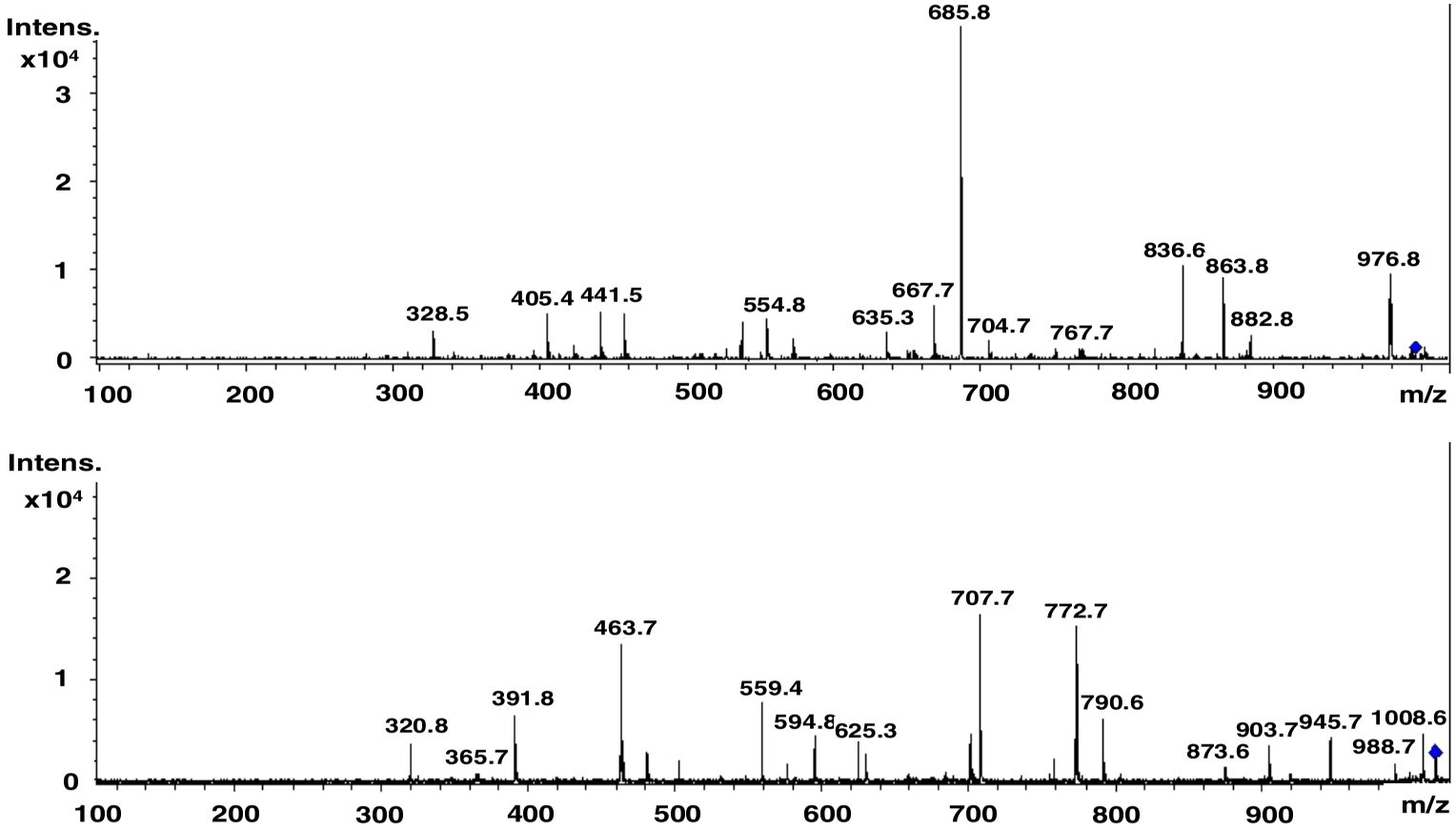
molecules at *m/z* 1045, 1059, and 1073 are shown in the MALDI-TOF-MS spectra of lichenysin A (Fig. 1 in III). The MS and MS/MS spectral data as described above and the use of negative-mode MS are tools that can be used for identifying lichenysin A, surfactin, and other similar cyclic lipopeptides.

The MS and MS/MS data were used for identification of fengycin A and B (see 1.4.1.3.) produced by *B. amyloliquefaciens* 19B. The protonated molecules of fengycin A β-OH-FA(C15) at *m/z* 1450, β-OH-FA(C16) at *m/z* 1463, and β-OH-FA(C18) at *m/z* 1493 and of fengycin B and β-OH-FA(C17) at *m/z* 1506 and the sodium-cationized molecules of fengycin A β-OH-FA(C16) at *m/z* 1484 and β-OH-FA(C17) at *m/z* 1500 and of fengycin B β-OH-FA(C17) at *m/z* 1528 were observed from *B. amyloliquefaciens* 19B using LC-ESI-IT-MS (Fig. 9A). These observed mass ions corresponded to published MS data on fengycin A and B (Vater et al. 2002, Madonna et al. 2003, Hofemeister et al. 2004, Kim et al. 2004, Koumoutsi et al. 2004, Wang et al. 2004).

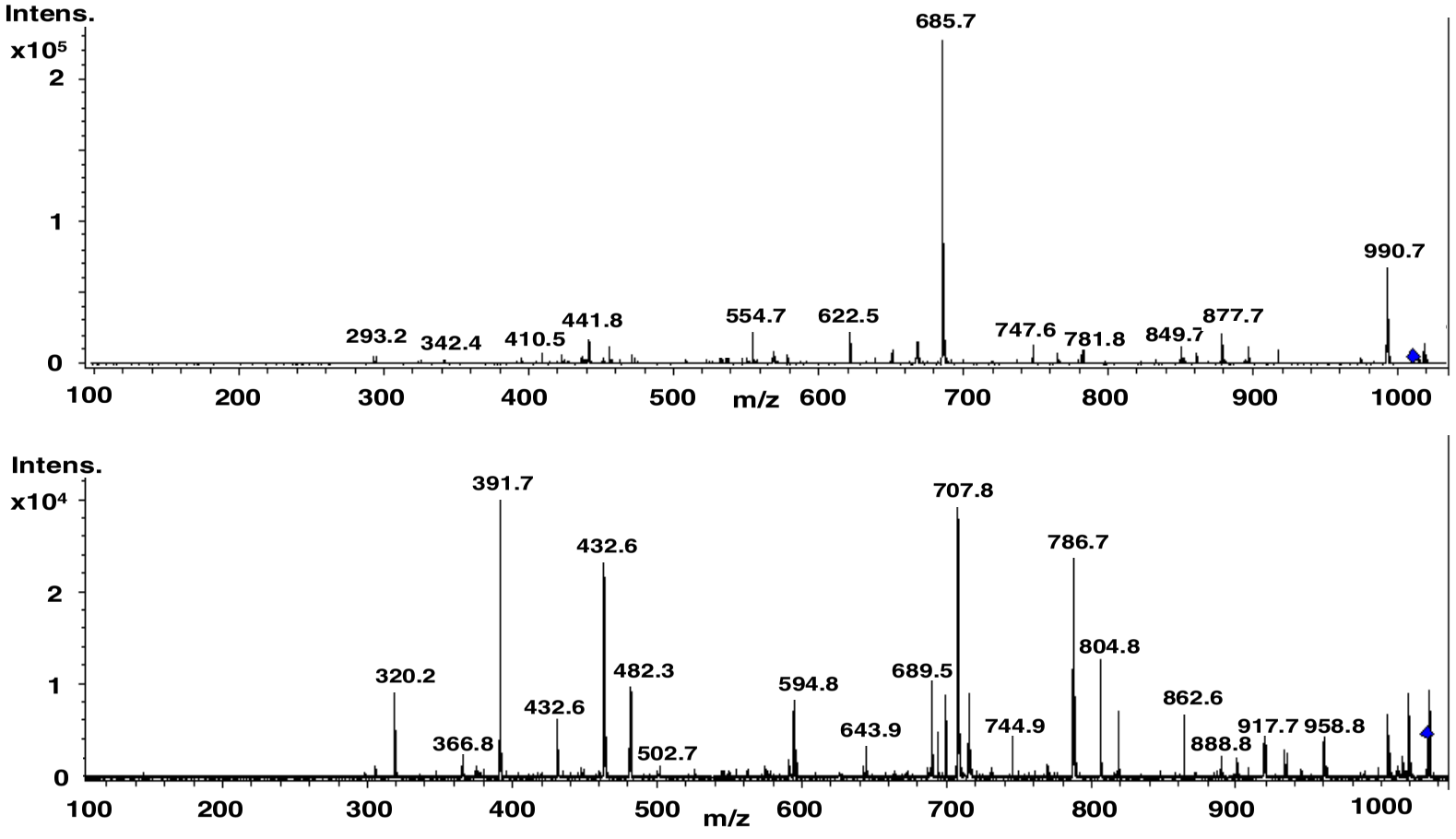
The MS/MS fragmentation of the protonated molecule at *m/z* 1492 (Fig. 9B) provided the fragment ions at *m/z* 967 and 1080, while the protonated molecule of *m/z* 1506 (Fig. 9C) provided the fragment ions at *m/z* 995 and 1108. The origin of the MS/MS fragment ions at *m/z* 967 and 1080 (fengycin A) and *m/z* 995 and 1108 (fengycin B) is fragmentation of the fengycin A and B protonated molecules between Orn and Tyr (product ions *m/z* 967 and 995) or Glu and Orn (product ions *m/z* 1080 and 1108) (see XII in Fig. 1). Thus, the fragment ions represent [*cyclo*(Ile-Tyr-Gln-Pro-Ala/Val-Glu-Thr-Tyr)-Orn+H]$^+$ and [*cyclo*(Ile-Tyr-Gln-Pro-Ala/Val-Glu-Thr-Tyr)+H]$^+$ of fengycin A and B. The calculated monoisotopic masses of the fragment ions were *m/z* 1080.5 (Ala, fengycin A), *m/z* 1108.6 (Val, fengycin B), *m/z* 966.5 (Ala, fengycin A), and *m/z* 994.4 (Val, fengycin B), respectively. These MS/MS results are consistent with previously published MS/MS data on fengycin (Madonna et al. 2003, Wang et al. 2004). The MS and MS/MS analyses suggest that *B. amyloliquefaciens* 19B produced lipopeptides fengycin A and B.

When the sperm-toxic fraction of *B. amyloliquefaciens* 19B methanol extract was analyzed with HPLC-ESI-IT-MS (Fig. 2B, C in IV) or using a syringe pump to inject the sperm-toxic fraction into the ESI-IT-MS (Fig. 1C, V), the mass ion at *m/z* 1220 was observed in the positive-ionization mode. The MS technique can be used to determine compound monoisotopic molecular masses during positive- and negative-ionization experiments. In the MS analysis of amylosin the deprotonated molecule at *m/z* 1196 (IV and Fig. 1 D in V) was obtained in the negative-ionization mode and therefore the mass ion at *m/z* 1220 was assumed to represent the sodium-cationized molecule of amylosin. Thus, the monoisotopic molecular mass of the toxic compound was set at 1197 Da. Therefore, *B. amyloliquefaciens* strain 19B produced at least the lipopeptides surfactin and fengycin A and B and the novel toxin amylosin.

A.


Intens.
x10⁴
3
2
1
0
685.8
836.6
863.8
976.8
667.7
328.5
405.4
441.5
554.8
635.3
704.7
767.7
882.8
100
200
300
400
500
600
700
800
900
m/z
Intens.
x10⁴
2
1
0
707.7
772.7
463.7
559.4
391.8
790.6
320.8
594.8
625.3
903.7
945.7
1008.6
365.7
873.6
988.7
100
200
300
400
500
600
700
800
900
m/z

B.


Intens.
x10⁵
2
1
0
685.7
990.7
293.2
342.4
410.5
441.8
554.7
622.5
747.6
781.8
849.7
877.7
100
200
300
400
500
m/z
600
700
800
900
1000
Intens.
x10⁴
2
1
0
391.7
707.8
432.6
786.7
804.8
320.2
482.3
689.5
594.8
862.6
432.6
643.9
744.9
917.7
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400
500
m/z
600
700
800
900
1000

C

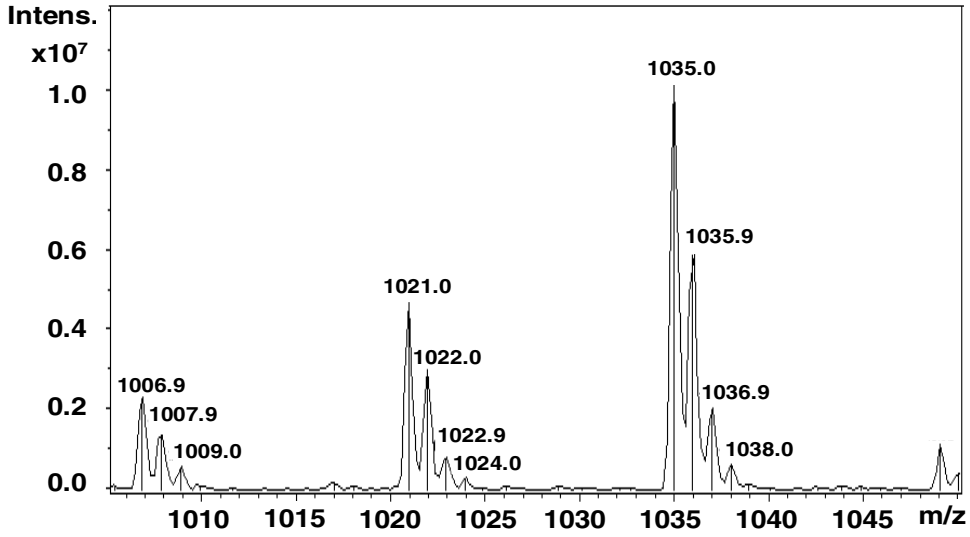
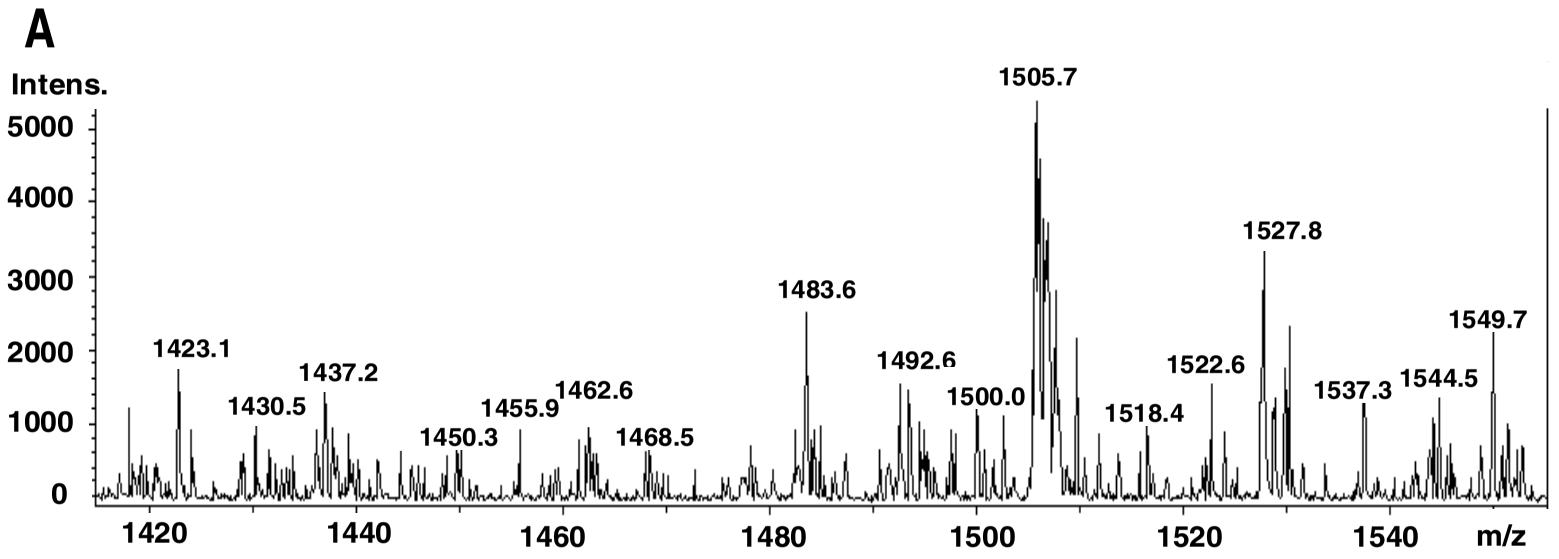


Figure 8. *ESI-IT-MS and MS/MS spectra of surfactin isolated from B. amyloliquefaciens strain 19B. (A) MS/MS using the protonated molecule at m/z 995 (upper panel) and sodium-cationized molecule at m/z 1017 (lower panel) as precursor ions (B) MS/MS using the protonated molecule at m/z 1009 (upper panel) and sodium-cationized molecule at m/z 1031 (lower panel) as precursor ions (C) Deprotonated molecules of surfactin obtained in the negative-ion mode MS.*

The toxins of *B. cereus*, *B. licheniformis*, and *B. amyloliquefaciens* investigated here had masses of from 1000 to 1200 Da. This range is similar to that of most toxic substances from the *Bacillus* species represented in Table 1. This size of toxic cyclic peptides may possess enough functional groups to initiate bioactivity as well as having hydrophobicity high enough to passively permeate the cell membranes.

MALDI-TOF-MS can be used for analyzing products of microbial metabolism and for direct detection of compounds of known toxicity as well as for solving taxonomic problems. Easy sample preparation and recent developments in small benchtop MALDI-TOF-MS instruments have made this an attractive technique for microbial toxin analysis.
The MS data on heat-stable *Bacillus* toxins and valinomycin from *Streptomyces griseus,* obtained using MS methods, and on the observed mass ions are compiled in Table 4.


A
Intens.
5000
4000
3000
2000
1000
0
1423.1
1430.5
1437.2
1450.3
1455.9
1462.6
1468.5
1483.6
1492.6
1500.0
1505.7
1518.4
1522.6
1527.8
1537.3
1544.5
1549.7
1420
1440
1460
1480
1500
1520
1540
m/z

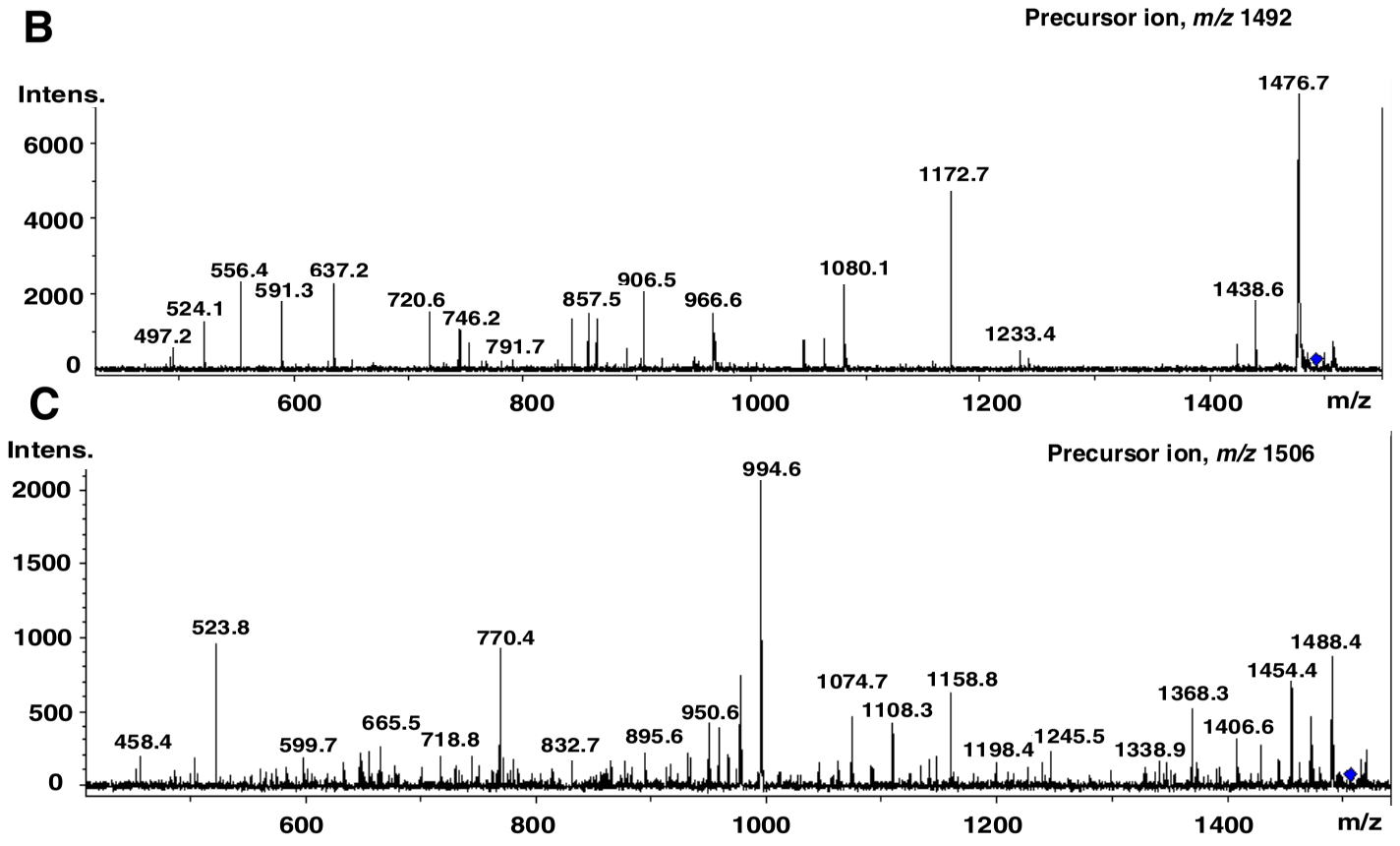


Figure 9.*. The ESI-IT-MS spectrum of fengycin homologs produced by B. amyloliquefaciens 19B (A) and the ESI-IT-MS/MS spectra obtained from precursor ions of m/z 1492 (B) and 1506 (C).*

Table 4. *Molecular ions in MS analysis, precursor ions in MS/MS analysis, and corresponding fragment ions and the MS method used for analyzing Bacillus toxins and valinomycin from Streptomyces griseus.*

Analytes	Cereulide			Valinomycin	Lichenysin A	Surfactin		Amylosin		Fengycin	
MS method	ESI-TQ-MS[I]	MALDI-TOF-MS[II]	ESI-IT-MS[1]	ESI-TQ-MS[2]	MALDI-TOF-MS[III]	ESI-IT-MS[IV]		ESI-IT-MS[IV]	MALDI-TOF-MS[IV]	ESI-IT-MS[3]	
	m/z										
$[M+H]^+$	1154	-	-	1112	1007, 1021, 1035	995,1009, 1023, 1037				1450,1463, 1484,1493, 1500,1506, 1528*	
$[M+NH_4]^+$	1171	1170	1171	1129	-						
$[M+Na]^+$	1176	1176	1176	1134	1029, 1043, 1057	1017,1031, 1045, 1059		1220	1220		
$[M+K]^+$	1192	1192	1192	1150	1045, 1059, 1073	1047, 1061 1075,					
$[M-H]^-$						1007, 1021, 1034		1196			
MS/MS method	TQ-MS			TQ-MS	TQ-MS	IT-MS				ESI-IT-MS	
Precursor ions	1154			1129	1022	1009	1031			1492	1506
The abundant fragments ions	1126 1083 1055 941 769 741 698 556 499 385 357 314			1112 1084 913 885 741 714 543 515 371 343 172	909 796 686 681 581 468 355 227	991 878 850 782 748 686 622 557 441	805 787 708 690 595 482 432 392 320			1477 1439 1173 1080 967 907 858 721 637 591 556 524	1488 1454 1368 1246 1159 1108 1075 995 951 897 770 524

Superscripts I-V refer to the original articles listed on p. 6, [1]Teplova et al. 2006, [2]Andersson et al. 1998, and [3]this thesis.

4.6. NMR analysis of amylosin

The structural properties of amylosin were determined using NMR (V). The HPLC-SPE-NMR technique (the single peaks of amylosin from several HPLC runs were pooled in the SPE cartridge and later released to the NMR tube) with a 500-MHz NMR spectrometer equipped with a 5-mm probe (Bruker Biospin, Rheinstetten, Germany) was used to obtain the ^{1}H-NMR spectrum of amylosin. This ^{1}H-NMR spectrum of the HPLC-purified amylosin was used as a reference in the NMR experiments performed with a 600-MHz NMR spectrometer equipped with a TXI 1-mm MicroProbe (Bruker Biospin, Fällanden, Switzerland). With a 1-mm probe it was possible to carry out several NMR experiments at a concentration of 66 mM using small amount (400 µg) of the amylosin (V).

The data of the NMR experiments, using ^{1}H-NMR, HSQS, and COSY, and the simulated ^{1}H-NMR spectrum using the Perch program (PERCH Solutions Ltd, Kuopio, Finland) showed that amylosin contained a polyene structure with a conjugated double-bond system of hexaene. The conjugated chromophore system of amylosin resembles that found in polyene macrolides (Fig. 1 in V). The TOCSY and HSQC data showed that amylosin contained the amino acids: Glu/Gln, Asp/Asn, Leu/Ile, Tyr, and Pro (V, Fig. 2) and supported the results obtained with GC-MS amino acids analysis (IV, p. 319) expect that Ser was not found in NMR (see 4.4). The ERETIC NMR method was used in quantitation of amylosin (see 4.2.2). This NMR methodology has been used in pharmaceutical applications (Pan et al. 2006).

The polyene macrolides are fungicidal substances that intercorporate with ergosterol to form channels or pores in the cell membrane of fungi. Medical use of polyene macrolides is limited because they are also active in cholesterol-containing membranes i.e. they are potentially toxic to animal and human cells. Amphotericin B is a fungicidal cyclic compound produced by *Streptomyces*. It has an amphoteric structure with hydrophilic hydroxyl groups on one side of the molecule and a hydrophobic polyene structure on the other side. It binds to the fungal ergosterols and distrupts the fungal cell membrane, causing leakage of cell components, and is also nephrotoxic (Goldman and Koren 2004). Compounds having polyene structure are known to be produced by *Bacillus* species. Cytotoxic macrolactin A (XX in Fig. 1), isolated from *Bacillus* species (Gustafsson et al. 1989), has 12 olefinic methine carbons. It disrupted the mycelia of *Streptomyces scabies* by a mechanism resembling that of polyene macrolides (Han et al. 2005). Thus, macrolactin A may have potential pore-forming properties. Antibacterial difficidin and oxydifficidin, which contain diene and triene structures, and 7-*O*-macrolactin which has polyene structures, are cyclic macrolides produced by *B. subtilis* (Hofemeister et al. 2004). It is interesting that the bacteriostatic compound bacillaene (580 Da) isolated from *B. subtilis* (bacillaene structure was partially characterized) contained a hexaene structure whose ^{1}H-NMR spectrum in the polyene range resembles that of amylosin (Patel et al. 1995).

Pigments protect some *Bacillus* strains from UV radiation as in *B. atrophaeus* DSM 675, whose spores contain a red pigment resembling carotene (Moeller et al. 2005). Therefore, amylosin may also play a role in protecting *B. amyloliquefaciens* against UV radiation.

4.7. Detection methods for cereulide and cereulide-producing strains of *B. cereus*

Several methods have been described in the literature to detect cereulide and cereulide-producing strains of *B. cereus*. Dilution methods such as Hep-2 cell vacuolation titer are suitable for semiquantitatively comparing toxicity but too imprecise for detecting the exact concentration of cereulide. Tests in which the detection of toxicity is based on light microscopy for observing changes in cells exposed to toxin may also suffer from person-related factors. The mitochondrial uncoupling properties of cereulide have been used to develop a quantitative method for detecting cereulide using RLM (Kawamura-Sato et al. 2005); however such a method can also detect other ionophoric toxins in food, such as coccidiostats (see p. 32).

The fastest and most specific method for detecting cereulide in food is the RP-HPLC-MS method in which the retention time, MS spectra, and MS/MS spectra can be used to identify and quantify cereulide. MALDI-TOF-MS can also be used to identify cereulide from bacterial extracts (II, Fig. 2). The RP-HPLC-UV method is suitable for detecting cereulide in cell extracts of *B. cereus* strains.

Basically, the same toxicity detection methods can be used for species of *Bacillus* other than those producing cereulide (Beattie and Williams 1999, Pedersen et al.2002, From et al. 2005, Hsieh et al. 2004). Using small-acid soluble proteins of *Bacillus* spores as biomarkers and analyzing the cell extracts with MALDI-TOF-MS may support identification of *Bacillus* species and toxins (Hathout et al. 2003).

In comparing the toxicity of various strains affecting target cells in the same manner, two situations related to toxicity must be considered: either the toxins are the same or they are different. In the first case different strains can produce the same toxin in varying amounts under equal conditions. On the other hand, one toxin can be more powerful but produced in smaller amounts than the other.

Detecting the existence (past or present) of strains potentially able to produce cereulide can be done with polymerase chain reaction methods, but genetic methods tell nothing about the cereulide contents of samples.

Table 5. *Compilation of methods used to detect cereulide and cereulide producing strains of B. cereus.*

Cereulide	
Method	**Reference**
Oxygen consumption by rat liver mitochondria	Kawamura-Sato et al. 2005
Cytotoxicity assay using Chinese hamster ovary cells	Beattie and Williams 1999, Pedersen et al. 2002
RP-HPLC-ESI-MS	Häggblom et al. 2002
RP-HPLC-UV, MALDI-TOF-MS	I,II Teplova et al. 2006
Hep-2 cell culture using basic 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as indicator (MTT assay)	Finlay et al. 1999, Toh et al. 2004, Taylor et al. 2005
Hep-2 cell vacuolation, titer method	Hughes et al. 1988, Agata et al 1994
Boar sperm motility test	I, II, Andersson et al. 2004
Detection of cereulide-producing strains of *B. cereus*	
Method	**Reference**
Fourier transform infrared spectroscopy	Ehling-Schulz et al. 2005b
Polymerase chain reaction methods	Ehling-Schulz et al. 2004, Horwood et al. 2004, Nakano et al. 2004,Toh et al. 2004

4.8. Biological properties of the toxins

4.8.1. Mammalian cells

Using specific fluorochromic stains it is possible to differentiate between several modes of toxicity: live and dead stainings calcein AM ester and ethidium homodimer-1 (I), ethidium bromide and rhodamine 123 (III), and PI (IV, V) or the height of transmembrane electric potential monitoring stains JC-1 (IV, V) and rhodamine 123 (II). In this way it is possible to obtain more specific information on toxin targets inside the cells.

4.8.1.1. Cereulide

Cereulide (0.5 ng/ml) inhibited motility and depolarized the mitochondrial membrane in the absence of damage to the plasma membrane in boar sperm cells in a manner similar to that of valinomycin (2 ng/ml). Cereulide (I, Fig. 1; II, Fig. 1) and valinomycin (Andersson et al. 1998, Fig. 1) also swelled the mitochondria of the boar sperm cells. Cereulide was a more potent toxin in the boar sperm cells than valinomycin (I, Table 1). The same was observed with isolated RLM with these toxins in a medium whose ionic composition matched that of blood plasma (Teplova et. al. 2006). Hyperpolarization of the cell

membrane by exposure to valinomycin or cereulide was reported in boar sperm cells (Hoornstra et al. 2003). Gramicicin D depolarized and valinomycin hyperpolarized human B lymphoblastoid JY cell plasma membranes and endorsed killer activity of T cells (Bacso et al. 1996). Exposure of rat hepatocytes to the protonophore gramicidin A, the potassium carrier valinomycin, and the calcium carrier ionomycin first increased the sodium contents in the cell together with loss of potassium, increased the water content, and finally altered the K:Na ratio from 10:1 to 1:1 and affecting the cell morphology (Bolkent and Zierold 2002). Initiating the apoptosis cascade has also been connected to the depolarization of mitochondria.

4.8.1.2. Amylosin

Amylosin hyperpolarized the plasma membrane and depolarized the mitochondrial membrane of boar sperm cells at the same concentrations (30-60 ng/ml) without permeabilizing the plasma membrane to propidium iodide (V). The toxic effects of amylosin increased with longer exposure times, as shown by the lower hydrophobicity (Table 3) of amylosin compared with the other toxins studied.

4.8.1.3. Surfactin and lichenysin A

Surfactin from *B. amyloliquefaciens* and lichenysin A from *B. licheniformis* were toxic to boar sperm cells at concentrations of 4-8 µM. These substances decreased sperm motility, disrupted the cell membrane, and provoked an acrosome reaction (III, Fig. 2). The critical micelle concentration (CMC) of surfactin is dependent on the ionic strength. The CMC of surfactin was 7.5 µM in 100 mM NaCl (Heerklotz and Seelig 2001). The boar sperm MR-A extender contains 126 mM Na^+ (Na^+ is a main ion of the extender; IV), which indicates that surfactin destabilized the cell membrane of boar sperm at its CMC. This shows that the micelles of surfactin and lichenysin A disrupted the plasma membrane; however, it is not known whether surfactin or lichenysin A forms micelle aggregates in the medium or after embedding into the membrane. With lichenysin A 100% hemolysis of blood was observed at 15 µM instead of the 200 µM required for hemolysis by surfactin (Grangemard et al. 2001). Surfactin is more anionic at pH 7 (see also Chapter 1.4.1.1, Fig. 2) because it contains Asp and Glu in contrast to lichenysin A, which contains Asn and Glu. This may lead to differential activity in membranes.

Lipopeptides such as lichenysin A and surfactin are biosurfactants; thus their effect on cell membranes as detergents is clear but they also have other biological functions. Surfactin and lichenysin belong structurally to lactones known as bioactive compounds. Lactones and cyclic peptides blocked the active sites of protease enzymes (Gaczynska and Osmulski 2005). Indeed, lichenysin A and the structurally similar isohalobacillin inhibited cholesterol acyltransferase (Hasumi et al. 1995) as did also the cation channel-forming beauvericin (Tomoda et al. 1992). When the effects of surfactin on vesicles made of

palmitoyloleylphoshatidylcholine (POPC) was studied, it were found that surfactin released the content of vesicles below its CMC (Carrillo et al. 2003).

The toxicity to boar sperm of methanol extracts of *B. subtilis* FZB42-containing surfactin, bacillomycin, and fengycin was low compared with the high toxicity of similarly prepared extracts of *B. amyloliquefaciens* 19B-containing amylosin, surfactin and fengycin. The *B. subtilis* FZB42 and *B. amyloliquefaciens* 19B extracts contained similar amounts of lipopeptides. This also indicates that the effects of amylosin are different from those of the lipopeptides. Amylosin-producing strains of *Bacillus* have high mammalian toxicity. Iturin and bacillomycin, which belong to the iturinic group (Vater et al. 2002), have low toxicity to boar sperm cells. The cholesterol content of boar sperm cells is low (cholesterol/ phospholipid molar ratio 0.26, Parks and Lynch 1992) and that in mitochondria virtually zero (Colbeau et al. 1971); thus compounds needing cholesterol to form pores, e.g fengycin and iturin (see also iturins, Chapter 1.4.1.2), are less active toward boar sperm cells than other mammalian cells.

4.8.1.2. Cultured cells

Exposure of human neural cells (Paju) to amylosin rendered them permeable to PI, whereas in boar sperm cells this effect was not detected after similar exposure (IV). Feline lung cells died rapidly (IV, V) when exposed to amylosin, possibly due to loss of selective membrane permeability to sodium and/or potassium ions and by depletion of cellular ATP (one toxicity parameter used when boar sperm cells were exposed to amylosin). The depletion of cellular ATP was proposed to be one reason for the irreversible cell injury shown when the effects of cyanide, oligomycin, and CCCP in rat hepatocytes were studied (Nieminen et al. 1994).

4.8.2. *Bacillus* toxins that altered functions of the mitochondria

4.8.2.1. Cereulide

Potassium carriers such as cereulide were proposed to be specific mitochondriotoxins (II). Cereulide toxicity to RLM was studied in detail (II). Cereulide induced mitochondrial dysfunctions at low exposure concentrations (0.5 ng/ml) in the presence of potassium ions by lowering the mitochondrial membrane potential (I, II). Changes in the membrane potential in isolated mitochondria exposed to the toxins were studied, using rhodamine 123, which is a cationic, water-soluble, lipophilic chloride salt that is taken up by mitochondria with membrane potential (negative inside) and monitored as a quenching of fluorescence in buffer solution. When the mitochondrial membrane potential decreases, rhodamine returns to the buffer and an increase in fluorescence is seen. The potassium concentration in the cytosol is higher than that of sodium, while in the blood plasma the concentration of sodium is higher than that of potassium. Thus, using an external medium

low in potassium is a physiologically relevant condition. In a sodium medium cereulide had only minor effects on the mitochondria (II). Addition of cereulide first increased the membrane potential of the mitochondria in a sodium medium, and then decreased it, and addition of potassium in this state accelerated decay. Swelling of the mitochondria in response to cereulide exposure was shown both in isolated RLM (II, Fig. 5) and in boar sperm cells (II, Fig. 1). The mitochondrial inner membrane is folded, containing a high surface area (see also Chapter 1.5.1., Fig. 5). Exposure to a potassium ionophore mediates potassium intake followed by water diffusion into the matrix and subsequent swelling of the mitochondria (II, Fig. 1). Thus, cereulide disrupt the osmotic balance of the mitochondria and provokes dysfunctions, e.g. in ATP production. The effect of cereulide on mitochondria was similar to that of its structural relative valinomycin. We concluded that cereulide is mitochondriotoxic due to its ionophoretic properties (II).

4.8.2.2. Amylosin

Exposure to purified amylosin uncoupled isolated RLM, decreased the mitochondrial potential, and caused oxidation of the PN and diminished production of ATP (V). These mitochondrial dysfunctions were concentration-dependent and potassium- and sodium-selective. Using potassium-selective electrodes it was shown that amylosin forms potassium-permeant channels in the mitochondria (Fig. 10). Amylosin induced swelling of mitochondria in manner similar to that of valinomycin and cereulide. The differencein effects between amylosin and cereulide or valinomycin was that the latter were not active in sodium media whereas amylosin affected mitochondria in sodium media with half of the effectivity seen in potassium media. It was shown that the effects of amylosin in mitochondria were not due to the mitochondrial permeability transition pore (PTP) opening, but instead were mediated by formation of independent channels.

Amylosin formed individual potassium (sodium, calcium) channels. The mitochondrial inner membrane lacks sterols (Colbeau et al. 1971), showing that amylosin is able to form channels without interacting with sterols, thus differing from the polyene macrolides. Iturin A was also unable (unpublished results) to release potassium from the mitochondria, as was expected since it needs sterol to form channels.

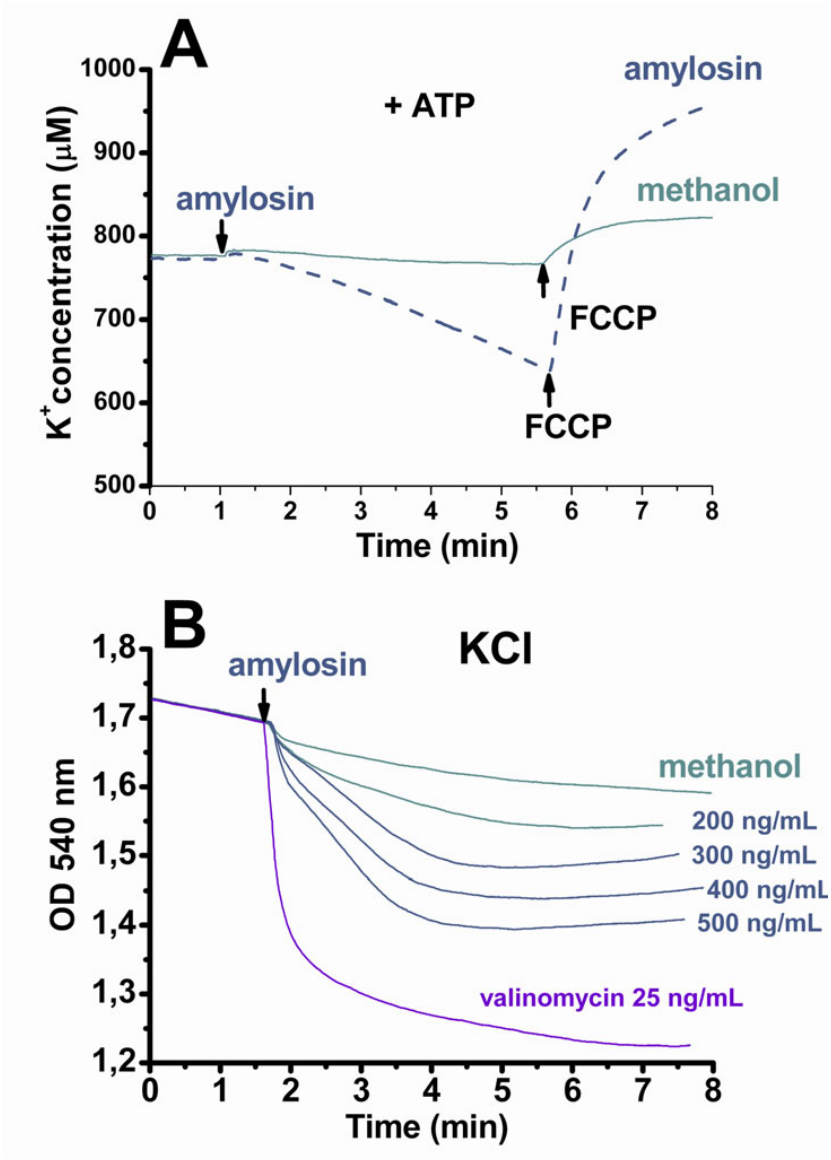


Figure 10. *Isolated rat liver mitochondria were used to study how amylosin affected mitochondrial functions. Amylosin induced channels (A) and swelling in mitochondria (B). Figures are modified using Figs. 6B and 7A (V). Potassium chloride (KCl) 0.7 mM (A) and 120 mM (B), +ATP, adenosine 5'-triphosphate is added, 1 mM (A).*

Isolated RLM were used as tools to study the effects of amylosin in membranes. A potassium-selective electrode was used to monitor the K^+ concentration in the external medium when RLM were exposed to amylosin (Fig. 10A). Amylosin forms channels in the mitochondrial inner membrane and the positive potassium ions are exported through the channels due to electrostatic attraction (see also Chapter 1.5.1, Fig. 6). When the

mitochondrial membrane was depolarized with the protonophore uncoupler FCCP the flow of potassium ions was reversed (Fig. 10). Amylosin did not affect the natural ATP-dependent mitochondrial potassium channels and the ion channels formed by amylosin were ATP-independent (V, Fig. 6).

The optical density of the mitochondria suspension of measured at 540 nm was decreased when the mitochondria were exposed to amylosin, indicating swelling of the mitochondria (Fig. 10, B), which was concentration-dependent. Amylosin also swelled the heads of boar sperm cells, showing formation of similar channels in the plasma membrane (V, Fig. 5D). The potassium carrier valinomycin was used as a reference. The experiment in Fig. 10A was carried out at an amylosin concentration of 200 mM in K^+ medium. Amylosin also inhibited oxidative phosphorylation both in potassium and in sodium media (V, Fig. 8).

4.8.3. Ionophoretic properties of toxins analyzed with the black-lipid membrane (BLM) technique

For the BLM studies soybean L-α-phosphatidylcholine (Asolectin, type II-S) was used. Its approximate composition was: phosphatidylcholine (24%), phosphatidylethanolamine (20%), inositol phosphatides (14%), the remainder being other phospholipids, lipids, and carbohydrates (Ardhammar et al. 2002).

Agata et al. (1994) proposed that cereulide is a K^+ ionophore and Suwan et al. (1995) showed in NMR that cereulide forms alkali metal complexes and is a potential potassium ionophore. We first suggested that cereulide forms channels (I); however, analyzing it with BLM and also isolated RLM showed that cereulide acts as a potassium carrier causing swelling and stimulating respiration in the presence of potassium (II).

The ionophoretic properties of cereulide were experimentally determined for the first time using BLM (II). Cereulide iniated increase in conductance of the BLM at a concentration as low as < 0.2 ng/ml in 100 mM KCl media (II, Fig. 3). We found that the conductance provoked by cereulide was cation-selective in a carrier manner and increased stoichiometrically with the concentration of cereulide. Cereulide was highly selective for potassium ion: it carried potassium three orders of magnitude more effectively than sodium ions or protons and two orders of magnitude more effectively than calcium ions. The ionophoretic selectivity of cereulide resembles that of valinomycin in BLM. Lichenysin A and surfactin were not active in BLM; however, surfactin formed channels when glycerol monooleate lipids were used to generate the BLM, but was not active when phosphatidyl choline was used (Sheppard et al. 1991). Surfactin formed a cation-selective $K^+ > Na^+$ channel in glycerol monooleate lipid membrane at a 1M salt concentration. The pore-forming properties of surfactin may be due to its ability to form dimers in the membranes (Sheppard et al.1991, Carrillo et al. 2003). The activities of iturin and surfactin in artificial membranes of glycerol monooleate differ from those observed in phosphatidylcholine. Iturin is active in both membranes, whereas surfactin only forms

pores in glycerol monooleate. Surfactin also needs to form dimers with salts before it enters the membrane and forms pores (Sheppard et al. 1991).

Interestingly, methanol extracts of *B. licheniformis* strains 553/1 and BAS50 which produce lichenysin A and type strain DSM13 formed K^+ channels in BLM. Lichenysin A-producing strains 553/1 and BAS50 were manyfold more active than *B. licheniformis* type strain DSM13 (Grigoriev et al. unpublished data). Channels generated in the BLM by the extract obtained from strain 553/1 were open for a longer time and were more cation-selective (anion/cation selectivity 28) than the channels observed using the extract from strain BAS50 (anion/cation selectivity 3.8). Purified lichenysin A was not active in the BLM. When the *B. licheniformis* extracts were fractioned with HPLC, the channel-active compound(s) was located in the fractions containing polar compounds. In addition, this channel-forming substance(s) was non-toxic to boar sperm cells. Further purification and properties of this channel-forming substance(s) are still under investigation. Recently it was shown that the genome of strain DSM13 contains genes with the potential for biotechnological applications (Veith et al. 2004).

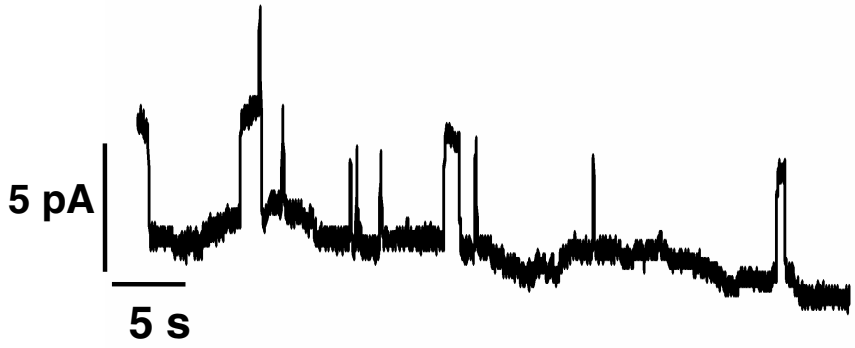


Figure 11. *Typical recording for a channel-forming substance observed when amylosin was analyzed with BLM. Opening (the conductivity increased for a short time) and closing (conductivity returned to the baseline) of single potassium channels are seen here. The amylosin concentration was < 20 nM in 1 M KCl medium and the applied voltage was 100 mV.*

Carriers act differently from channel formers in the BLM. The conductivity increases when the concentration of carriers increases, whereas channel formers induce jumps in conductivity in single-channel experiments (open and closed state of channels). Amylosin is two times more effective in forming potassium channels than sodium channels. Dependence of conductance for potassium on the concentration of amylosin in BLM is seen in Fig. 11. The conductivity for K^+ began to increase at a concentration 30 ng/ml (V, Fig. 4B), which is close to the concentration at which amylosin-exposed boar sperm cell membranes were depolarized.

We concluded that the toxicity of amylosin is due its channel-forming properties. Amylosin forms cation-selective channels in the order $K^+ > Na^+ > Ca^{2+}$ of 26 > 15 >3.5; thus, amylosin was two times more selectively for potassium than for sodium. Gramicidin A has a similar selectivity ratio $K^+ > Na^+$ of 20.6 > 14.6 in DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) membrane (Pfeifer et al. 2006), but also formed H^+ channels whereas amylosin did not. When exposed to 1 µg/ml gramicidin D, NG108-15 neuroblastoma X glioma hybrid cells lost their capacity to generate action potentials (Doebler 1999). Since the balance in Na^+ and K^+ plays a role in formation of the action potential, a channel-forming compound such as amylosin may disturb it.

Potassium and sodium channel-forming amylosin affects mammalian cells in a mode that is different from that of the potassium carriers cereulide and valinomycin, which are clearly mitochondrial toxins. The capacity of valinomycin to transport and release potassium was estimated as 10^4 potassium ions per second, whereas the quantity of potassium that can pass through channel-forming compounds such as gramicidin A is 1000 times larger. Amylosin as a channel-forming substance is thus an effective potassium and sodium transporter. The properties of heat-stable *Bacillus* toxins and valinomycin from *Streptomyces griseus,* including their origin, chemical properties, toxicity toward mammalian cells, as well as mitochondrial and ionophoretic properties are compiled in Table 6.

Table 6. *Site of the toxinogenic bacteria and toxins found: their effects on mammalian cells and ionophoric properties as revealed by BLM.*

Bacteria and site	Name and class of toxin	Ref	MW	Boar sperm cells	Isolated rat liver mitochondria	Black-lipid membrane	Human NK cells	Human neural cells	Fetal feline lung cells
B. cereus Food *S. griseus* Water-damaged buildings	Cereulide, Dodecadepsi-peptide Valinomycin[1], Dodecadepsi-peptide	I, II	1152 1110	Motility decreased Depolarized mitochondrial membrane Swelling of the mitochondria	Depolarized mitochondrial membrane Swelling of the mitochondria	Potassium ionophore	Swelling[2,3] of mitochondria Apoptosis [2,3]	-[4]	-
B. licheni-formis Food	Lichenysin A, Lipopeptide	III	1006 1020 1034	Motility decreased Damage to cell membrane Acrosome reaction Loss of intracellular ATP and NADH	-	No function	-	-	-
B. amylo-liquefaciens Water-damaged buildings	Amylosin Peptide, containing polyene chromophore	IV, V	1197	Motility decreased Depolarized mitochondrial membrane and cell membrane Loss of intracellular ATP and NADH	Depolarized mitochondrial membrane Oxidation of pyridine nucleotides Swelling of the mitochondria Uncoupling of oxidative phosphorylation	Potassium, sodium, and calcium channels	-	Depolarized mitochondrial membrane and plasma membrane	Cytotoxic
B. amylo-liquefaciens Water-damaged buildings	Surfactin Lipopeptide	IV	1007 1021 1035	Motility decrease Damage to cell membrane Loss of intracellular ATP and NADH	No function	No function	-	-	-

[1]Andersson et al. 1998, [2]Paananen et al. 2000, [3]Paananen et al. 2002, [4] - Not analyzed

5. Conclusions and remarks

The results of this study and added with those in the literature showed that *Bacillus* species common in food and in the environment, growing under anaerobic and aerobic conditions over a broad temperature range produced thermally stabile non-protein compounds toxic to mammalian cells. In the present study toxins were found belonging to the cyclic lipopeptide, peptide and cyclic depsipeptide chemical groups with MW of 1000-1500. They were ionophoretic, causing imbalance in the homeotasis of ions in cells, and damaging the plasma membrane, causing leakage of the cell contents. Here we showed that the ionophoric *Bacillus* toxins investigated altered the mitochondrial functions of mammalian cells. The properties of compounds indicate their environmental toxicity: i.e. their persistence, hydrophobicity and toxicity, to mammalian cells.

5.1. Conclusions

I. Cereulide, a cyclic depsipeptide (1152 Da), is a food poisoning toxin produced by certain strains of *B. cereus.* Cereulide was purified from *B. cereus* extracts using RP-HPLC. Identification of cereulide was performed using MS spectra and we represent the first time the interpreted MS/MS spectrum of cereulide (I). The RP-HPLC-UV method was developed, in which valinomycin was selected as the standard compound for quantifying cereulide.

II. The potassium carrier properties of cereulide were discovered by black lipid membrane (BLM) method and were published for first time. The log K_{ow} values of cereulide (7.46) and valinomycin (5.99) were calculated. Studies of the effects of cereulide on mitochondria and on BLM showed that cereulide is a more potent potassium carrier ionophore than valinomycin, the most potent natural potassium ionophore previously known. We established that the toxicity of cereulide is due to its potassium carrier properties.

III. A toxin was found, using boar sperm cells as the indicator, from *B. licheniformis* associated with a case of food poisoning. The substance was identified as the lipopeptide lichenysin A, using MS and MS/MS data and by stereoisomeric *D* and *L* amino acid RP-HPLC-UV analysis. The toxicity target of lichenysin A is the cell membrane. This toxin damaged the cell membrane of boar sperm and provoked the acrosome reaction.

IV. Fungicidal and boar sperm-toxic strains of *B. amyloliquefaciens* were isolated from a moisture-damaged building in which the occupants suffered from respiratory symptoms and nervous illness. The toxic substance of these *B. amyloliquefaciens* strains was purified with RP-HPLC. The monoisotopic molecular mass of the toxic substance was 1197 Da, analyzed by RP-HPLC-MS. Amino acids were found after acid hydrolysis of the 1197-Da substance, using GC-MS analysis. The 1197-Da substance was toxic to fungi, boar sperm cells, feline fetal lung cells, and human neural cells. BLM analysis showed that the

substance formed channels conductive for potassium, sodium, and calcium ions. The same strains of *B. amyloliquefaciens* also produced another toxic substance. It damaged the boar sperm cell membrane and was identified as the lipopeptide surfactin, using commercial surfactin as a reference and the data of the RP-HPLC-MS/MS spectrum. The amino acids of surfactin were analyzed, as mentioned above. The structure and toxic effects of surfactin resemble those of lichenysin A (III). *Bacillus amyloliquefaciens* strain 19B also produced the fungicidal fengycin A and B, identified by MS and MS/MS analysis.

V. The novel toxic 1197-Da substance from *B. amyloliquefaciens* was named amylosin. An RP-HPLC-UV quantitation method for amylosin was developed, using commercially available amphotericin B as the reference compound. The method is based on the precisely determined concentration of purified amylosin by ^{1}H-NMR, using the ERETIC method. The amylosin chromophore possesses a polyene structure, as determined with NMR. Amylosin generates Na^{+}- and K^{+}-permeant channels as shown with BLM. A detailed study of the eukaryotic membrane-affecting properties of amylosin was executed, using isolated RLM. We proposed that the cytotoxicity of amylosin to sperm cells and feline fetal lung cells is due to its channel forming properties. This conclusion was supported by the results of its effects on the plasma transmembrane electric potential effect of mitochondria and plasma membranes of boar sperm cells.

The *Bacillus* toxins and their main toxicity properties studied (I-V) are shown in Table 7.

Table 7. *Mode of actions and toxicity thresholds of Bacillus toxins studied (I-V).*

Compound	Mol. mass Da	Mode of action	Toxicity threshold to mammalian cells
Cereulide	1152	Ionophore carrier for K^{+}	0.5 nM
Amylosin	1197	Ionophore channels for K^{+}, Na^{+}, and Ca^{2+}	30 nM
Surfactin	1035 (major)	Damaged cell membrane	4-8 µM
Lichenysin A	1034 (major)	Damaged cell membrane	4-8 µM

5.2. Remarks

Our results showed that *Bacillus* strains isolated from indoor air and food produced four different thermally stable peptides toxic to mammalian cells. One is a potassium ionophore carrier (cereulide), the second a novel potassium channel-forming peptide (amylosin) which was purified and its structure partially characterized, while the third (surfactin) and the fourth (lichenysin A) are membrane-disrupting and potential channel-forming lipopeptides. The antifungal lipopeptide (fengycin) was also found. Frequent

exposure to or ingestion of such compounds produced by *Bacillus* may represent a serious risk to health. Some strains producing lipopeptides are halotolerant up to a concentration of 16% NaCl and produce the lipopeptides in environments with up to 8% salt concentration (Roongsawang et al. 2002), indicating that such strains have the ability to grow and form toxins in salt-preserved food.

Cereulide is not commercially available. Therefore, in the present study we quantified cereulide using valinomycin (commercially available), which highly resembles cereulide, as the standard. Cereulide and valinomycin have identical UV-absorbing chromophores, ester, and amide bonds (see Fig. 7) in the UV range 200-220 nm. However, we cannot exclude the possibility that the molar extinction coefficient values of valinomycin and cereulide may differ, causing small errors in quantitation of cereulide in RP-HPLC-UV.

Valinomycin and cereulide can also act differently in the ESI technique used in RP-HPLC-ESI-MS analysis to quantify cereulide. In the MS the possible dissimilar properties of valinomycin and cereulide of complexing cations may cause inaccuracy in quantification of cereulide. The toxicity studies indicated that the potassium complex of cereulide was more stable that of valinomycin (Teplova et al. 2006).

In general surfactin is produced at high frequence by *Bacillus* species. The strains of *B. amyloliquefaciens* studied here produced surfactin in high amounts (6% of dry weight) and coproduced a new fungicidal substance (amylosin) in amounts constituting 1% of dry weight (IV). Lichenysin A production by *B. licheniformis* was also relatively high. Thus, high concentrations of surfactin or lichenysin A could be attained in indoor materials contaminated with *B. amyloliquefaciens* strains producing surfactin or in food by lichenysin A-producing *B. licheniformis* strains. *Bacillus amyloliquefaciens* was previously reported to produce surfactin and to possess lytic effects on protozoa. Surfactin is a persistent, hydrophobic molecule and can contaminate lungs and other tissues when dust or aerosols are inhaled. Surfactin may also damage the eyes when exposed to dust containing it. The eyes are protected against mechanical injury by a film consisting of oil, water, and mucus; the biosurfactant surfactin can destroy this film.

Surfactin and lichenysin A are able to bind the divalent ions of calcium and magnesium to form a more hydrophobic complex. Lichenysin A makes a 2:1 and surfactin a 1:1 complex with calcium; this may indicate that surfactin and lichenysin A can bind cadmium as well. Cadmium is toxic to mitochondria (Pathak and Khandelwal 2006) and surfactin or lichenysin A are potentially able to form hydrophobic complexes with cadmium and increase cadmium concentration in cells.

The toxicity threshold of amylosin for inhibiting boar sperm motility was 30 nM (V), thus 15 and 60 times higher than that observed for valinomycin (2 nM) and for cereulide (0.5 nM) (I), respectively. Amylosin depolarized the plasma membrane of boar sperm cells (IV, V), whereas cereulide and valinomycin had no effect (Hoornstra et al. 2003). Exposure to amylosin caused dysfunction in all studied cells: boar sperm, neural, and feline fetal lung cells. The toxicity of amylosin was explained by its channel-forming

properties found with BLM. Isolated RLM were used to show how amylosin forms independent potassium channels with no association with the mitochondrial ATP-regulated K^+ channel. One possible target of amylosin may be the central nervous system. Amylosin has the potential to disturb the signal system of neurons by forming sodium channels, as shown in BLM. Amylosin also forms calcium channels and can disturb calcium transport in cells. Calcium modulates many processes such as neurotransmission, enzyme, and hormone secretion, as well as many biological processes, e.g. cell cycle regulation and programmed cell death (Belkacemi et al. 2005)

Bacillus amyloliquefaciens strain 19B, found in indoor air, also produced fengycin in addition to amylosin and surfactin. There is an interesting relationship between fengycin and the pulmonary surfactant protein that aids the lung in breathing and contains the lipid dipalmitoylphosphatidylcholin (DPPC) (Yu and Possmayer 2003). Fengycin disturbs the organization of the DPPC monolayers in a concentration-dependent manner (Deleu et al. 2005). The pulmonary surfactant protein contains DPPC and forms surface-active films on the alveolar surfaces. Fengycin may thus impair the function of the pulmonary surfactant essential for breathing.

Similar toxins were found from *Bacillus* strains isolated from moisture-damaged buildings and cases of food poisoning. Such occurrence is also common for toxin-producing fungi (see Chapters 1.1. and 1.2.). The structurally highly similar lichenysin A and surfactin were produced by *Bacillus* isolated from food poisoning and indoor environments, respectively. Cereulide was produced by *B. cereus* strains isolated from food poisoning (I). Cereulide-producing *B. cereus* strains were also isolated from the construction materials of buildings (Mikkola et al. 1999), filler material from hospitals, and filler material and dust from private dwellings (Andersson et al. 2002).

The *Streptomyces* species, such as *S. griseus*, which produce valinomycin (Andersson et al. 1998) are not isolated from food; however, there is one report of valinomycin produced by *Bacillus* species (Wulff et al. 2002). Valinomycin is a heat-stable substance possessing toxicity toward mammalian cells similar to the toxicity shown by cereulide (I, II, Andersson et al. 1998, Paananen et al. 2000, Teplova et al. 2006) but provokes an emetic symptom in *Suncus murinus* at 60 times higher concentration than cereulide (Agata et al. 1995). Shinagawa et al. (1995) estimated that oral administration of 70 µg of cereulide could provoke an emetic reaction in rhesus monkey. Jääskeläinen et al. (2003a) suggested that rhesus monkeys and humans are equally sensitive to cereulide and the amount of cereulide needed to provoke the emetic symptoms in humans by ingestion (60 kg) is 400-500 µg. Assuming that valinomycin causes the emesis in rhesus monkeys and humans similar to that observed in *Suncus murinus,* 4.2 mg and 24-30 mg of valinomycin are required for emetic reaction in rhesus monkeys and humans, respectively. Thus, if heat-stable valinomycin produced by *Bacillus* strains contaminates food, a high amount (20-30 mg) of highly toxic valinomycin must be ingested before the body is alarmed by vomiting, compared with a dose 60 times smaller in cereulide. On the other hand, vomiting could be considered as a first toxic reaction (in which the body is alarmed) and effects on the organs as a secondary (serious) toxic reaction to cereulide (or valinomycin). Most

probably, valinomycin-producing *Bacillus* species could not produce enough valinomycin in contaminated food to provoke vomiting. However, secondary serious intoxication could be caused by valinomycin before the body is alarmed by the vomiting. In the food poisoning cases, involving the *B. cereus* strains that produce cereulide, fatal dysfunctions of the liver were reported (Mahler et al. 1997, Dierick et al. 2005) and valinomycin definitely has the potential to provoke toxic effects similar to those of cereulide. Therefore, the potential of *Bacillus* species to produce valinomycin (or valinomycin analogues) should be monitored and studied in more detail.

Since some *B. cereus* strains that produce enterotoxins or cereulide can grow even at refrigerated temperatures, it is advisable to store leftover portions of cooked rice, pasta, or milk-containing foods in the freezer. In addition, the labels of many milk products, at least in Finland recommend storage temperatures of 0-8 °C. Many studies have shown that *B. cereus* spores can survive pasteurization and 10% of the non-emetic or non-diarrheal strains of *B. cereus* have a minimum growing temperature of 4 °C, whereas for 50% of the diarrheal strains it is 7 °C (Pielaat et al. 2005, Carlin et al. 2006). These facts suggest that to prevent *B. cereus* from growing, the recommended storage temperature of industrial milk products and convenience foods should be 0-4 °C instead of the more commonly used 0-8 °C to improve food safety.

Due to the value of *Bacillus* species in industrial fermentations on one hand and their ability to form toxic compounds on the other, it is necessary to explore their non-toxicity before use as production organisms for large-scale biotechnical applications. This especially concerns genetically modified strains. Our results illustrate and reinforce the need to detect and identify toxic *Bacillus* non-protein metabolites. We therefore suggest that appropriate analytical HPLC-ESI-MS methods be developed and that an ESI-MS library of the known *Bacillus* toxins be created to accomplish this task.

Tiivistelmä

Tämän väitöskirjan aiheena on sisäilmasta ja ruoka-aineista eristettyjen itiöitä muodostavien Basillusten tuottamien lämmönkestävien toksisten ei-proteiini yhdisteiden rakenne ja toksisuus nisäkäs soluille Basillusten erittämien toksisten ei-proteiini yhdisteiden esiintymisestä, vaikutuksista ja kulkeutumistavoista kosteusvaurioisten rakennusten sisäilmassa ei ole paljon tutkimustietoa. Basillukset muodostavat ilmassa liikkuvia itiöitä, jotka voivat toimia toksiinien kantajina, kuten sisäilmasta eristetyt toksisten sienien itiöt. Ruoka-aineissa Basillusten itiöt ovat ongelmana, koska ne kestävät ruuanvalmistuksessa käytettäviä lämpötiloja ja ovat valmiita lisääntymään ruoka-aineissa suotuisissa olosuhteissa.

Basillusten toksiset yhdisteet löydettiin käyttäen toksisuus indikaattorina sian siittiöiden liikkuvuuden muutosta toksisen altistuksen johdosta. Yhdisteiden puhdistuksessa, detektoinnissa ja rakenteen analysoimisessa käytettiin kaasu- ja nestekromatografiaa, massaspektrometriaa ja ydinmagneettista resonanssispektroskopiaa. Yhdisteiden toksisuutta ja vaikutustapoja tutkittiin käyttäen sian siittiöitä, kissan keuhkosoluja, ihmisen hermosoluja ja rotan maksasta eristettyjä mitokondrioita sekä BLM:ää (Black lipid membrane) ioniforistен ominaisuuksien selvittämiseen.

Tämän väitöskirjatyön tuloksena löydettiin uusi $K^+ > Na^+ > Ca^{2+}$ ionikanavan muodostava toksiini joka sai nimen amylosiini. Amylosiinia tuottivat kosteusvaurioisen rakennuksen sisäilmasta eristetyt *B. amyloliquefaciens* kannat. Amylosiini puhdistettiin nestekromatografilla ja monoisotooppinen atomipaino 1197 Da selvitettiin sähkösumutusionisaatio ioniloukku massaspektrillä. Lisäksi sen todettiin olevan peptidi happohydrolyysin ja aminohappojen kaasukromatografisen massaspektrometri analyysin avulla. Sen rakenteen todettiin sisältävän kromoforisen polyeeni ryhmän käyttämällä ydinmagneettista resonanssispektroskopiaa. Amylosiinille kehitettiin nestekromatografinen kvantitointimenetelmä käyttäen makrolaktoni polyeenia amphoterisin B:tä (moolimassa 924 g/mol) referenssiaineena.

Ruoka-aine myrkytyksestä eristetty *B. licheniformis* kanta tuotti solukalvoa rikkovaa lipopeptidiä, likenysiini A:ta, joka puhdistettiin nestekromatografilla. Likenysiini A identifioitiin matriisiavusteisella laserionisaatio lentoaika-massaspektrometrillä sen tuottamien protonoituneiden sekä kationioituneiden ammonium, natrium, kalium molekyylien avulla. Sen tuottamat protonoidut molekyylit olivat *m/z* 1007, 1021 ja 1035. Likenysiini A:n aminohapot analysoitiin sähkösumutus-ionisaatio kolmoiskvadrupolin massaspektrometrin tuottaman tandem-massaspektrin sekä lipopeptidin happohydrolyysin ja aminohappojen nestekromatografisen analyysin avulla. Sisäilmasta eristetyn *B. amyloliquefaciens* kannan havaittiin tuottavan amylosiinin lisäksi lipopeptideja: sienille toksista fengysiinia ja solukalvoa hajottavaa surfaktiinia. Ne identifioitiin sähkösumutusionisaatio ioniloukku massaspektrillä niiden tuottamien protonoituneiden ja kationisoituneiden ammonium, natrium, kalium molekyylien sekä analysoimalla niiden

tuottamat tandem massaspektrit. Surfaktiinin tuottamat protonoidut molekyylit olivat *m/z* 1009, 1023 ja 1037 ja fengysiinin *m/z* 1450, 1463, 1493 ja 1506.

Kereulidi (moolimassa 1152 g/mol) puhdistettiin nestekromatografisesti ruokamyrkytys tapaukseen liittyvästä *B. cereus* kannasta. Kereulidi identifioitiin sen sähkösumutusionisaatio kolmoiskvadrupoli massaspektrometrin tuottamien protonoidun molekyylin *m/z* 1154, ammonium *m/z* 1171, natrium *m/z* 1176 ja kalium *m/z* 1192 kationisoituneiden molekyylien avulla sekä tulkitsemalla kereulidin protonoidun molekyylin *m/z* 1154 tandem-massaspektrin tuote-ionit. Kereulidille kehitettiin nestekromatografinen kvantitointimenetelmä käyttäen sitä muistuttavaa valinomysiinia (moolimassa 1110 g/mol) referenssiaineena. Lisäksi osoitettiin empiirisesti, käyttämällä BLM:ää, että *B. cereuksen* tuottama emeettinen toksiini, kereulidi on tehokas ja spesifinen kalium ionifori, jonka toksinen vaikutus kohdistuu erityisesti mitokondrioiden toimintaan.

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References

Agata N, Mori M, Ohta M, Suwan S, Ohtani I, Isobe M. 1994 A novel dodecadepsipeptide, cereulide, isolated from *Bacillus cereus* causes vacuole formation in HEp-2 cells. FEMS Microbiol Lett. 121:31-34.

Agata N, Ohta M, Mori M, Isobe M. 1995 A novel dodecadepsipeptide, cereulide, is an emetic toxin of *Bacillus cereus*. FEMS Microbiol Lett. 129:17-20.

Agata N, Ohta M, Mori M, Shibayama K. 1999 Growth conditions of and emetic toxin production by *Bacillus cereus* in a defined medium with amino acids. Microbiol Immunol. 43:15-18.

Agata N, Ohta M, Yokoyama K. 2002 Production of *Bacillus cereus* emetic toxin (cereulide) in various foods. Int J Food Microbiol. 73:23-27.

Ahimou F, Jacques P, Deleu M. 2000 Surfactin and iturin A effects on *Bacillus subtilis* surface hydrophobicity. Enzyme Microb Technol. 10:749-754.

Alatossava T, Jutte H, Kuhn A, Kellenberger E. 1985 Manipulation of intracellular magnesium content in polymyxin B nonapeptide-sensitized *Escherichia coli* by ionophore A23187. J Bacteriol. 162:413-419.

Alm H, Brussow KP, Torner H, Vanselow J, Tomek W, Danicke S, Tiemann U. 2006 Influence of Fusarium-toxin contaminated feed on initial quality and meiotic competence of gilt oocytes. Reprod Toxicol. 22:44-50.

Altayar M, Sutherland AD. 2006 *Bacillus cereus* is common in the environment but emetic toxin producing isolates are rare. J Appl Microbiol. 100:7-14.

Amoa-Awua WK, Terlabie NN, Sakyi-Dawson E. 2006 Screening of 42 *Bacillus* isolates for ability to ferment soybeans into dawadawa Int J Food Microbiol. 106:343-347

Andersson MA, Jääskeläinen E, Mikkola R, Teplova V, Veijalainen P, Apetroaie C, Hoornstra D, Kroppenstedt R, Salkinoja-Salonen MS. 2005 Indoor bacilli and streptomycetes produce substances toxic to mammalian cells. In Bioaerosols, Fungi, Bacteria, Mycotoxins and Human Health: Patho-physiology, Clinical Effects, Exposure Assessment, Prevention and Control in Indoor Environments and Work. Eckardt Johanning, MD, MSc, Editor. Boyd Printing Company, Albany, New York 12210 pp. 292-299.

Andersson MA, Jääskeläinen EL, Shaheen R, Pirhonen T, Wijnands LM, Salkinoja-Salonen MS. 2004 Sperm bioassay for rapid detection of cereulide producing *Bacillus cereus* in food and related environments. Int J Food Microbiol. 94**:** 1453–1457.

Andersson MA, Nikulin M, Koljalg U, Andersson MC, Rainey F, Reijula K, Hintikka EL, Salkinoja-Salonen M. 1997 Bacteria, molds, and toxins in water-damaged building materials. Appl Environ Microbiol. 63:387-393.

Andersson MA, Mikkola R, Kroppenstedt RM, Rainey F, Peltola J, Helin J, Sivonen K, Salkinoja-Salonen MS. 1998 The mitochondrial toxin produced by *Streptomyces griseus* strains isolated from an indoor environment is valinomycin. Appl Environ Microbiol. 64:4767-4773.

Andersson M, Mikkola R, Apetroaie C, Hoornstra D, Nieminen T, Salkinoja-Salonen MS. Fungicidic and mitochondriotoxic Bacilli frequent in water damaged buildings. In Indoor air 2002: Proceeding of the 9th Conference on Indoor Air Quality and Climate, Vol 1, H. Levin, ed., Indoor Air 2002, Santa Cruz, California, pp. 34-39.

Aoki Y, Yamamoto M, Hosseini-Mazinani SM, Koshikawa N, Sugimoto K, Arisawa M. 1996 Antifungal azoxybacilin exhibits activity by inhibiting gene expression of sulfite reductase. Antimicrob Agents Chemother. 40:127-1232.

Aranda FJ, Teruel JA, Ortiz A. 2005 Further aspects on the hemolytic activity of the antibiotic lipopeptide iturin A. Biochim Biophys Acta. 1713:51-56.

Ardhammar M, Lincoln P, Norden B. 2002 Invisible liposomes: refractive index matching with sucrose enables flow dichroism assessment of peptide orientation in lipid vesicle membrane. Proc Natl Acad Sci U S A. 99:15313-15317.

Arima K, Kakinuma A, Tamura G. 1968 Surfactin, a crystalline peptide lipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. Biochem Biophys Res Commun. 31:488–494.

Asano SI, Nukumizu Y, Bando H, Iizuka T, Yamamoto T. 1997 Cloning of novel enterotoxin genes from *Bacillus cereus* and *Bacillus thuringiensis*. Appl Environ Microbiol. 63:1054-1057.

Assie LK, Deleu M, Arnaud L, Paquot M, Thonart P, Gaspar CH, Haubruge E. 2002 Insecticide activity of surfactins and iturins from a biopesticide *Bacillus subtilis* Cohn (S499 strain). Biol Wet. 67:647-655.

ASTM (American Society for Testing and Materials) E 1147-92. Standard test method for partition coefficient (n-octanol/water) estimation by liquid chromatography. Annual Book of ASTM Standards, PA, USA. Vol.14.01, pp 712–715.

Bacso Z, Matko J, Szollosi J, Gaspar R Jr, Damjanovich S. 1996 Changes in membrane potential of target cells promotes cytotoxic activity of effector T lymphocytes. Immunol Lett. 51:175-180.

Balaban N, Rasooly A. 2000 Staphylococcal enterotoxins. Int J Food Microbiol. 1:1-10.

Ballard CE, Yu H, Wang B. 2002 Recent developments in depsipeptide research. Curr Med Chem 9:471-498.

Banerjee P. C. 1977 Lytic effect of mycobacillin and its derivatives on erythrocytes Antimicrob Agents Chemother. 12: 124-125.

Beattie SH, Williams AG. 1999 Detection of toxigenic strains of *Bacillus cereus* and other *Bacillus* spp. with an improved cytotoxicity assay. Lett Appl Microbiol. 28:221-225.

Beatty PH, Jensen SE. 2002 *Paenibacillus polymyxa* produces fusaricidin-type antifungal antibiotics active against *Leptosphaeria maculans*, the causative agent of blackleg disease of canola. Can J Microbiol. 48:159-169.

Beaumont M. 2002 Flavouring composition prepared by fermentation with *Bacillus* spp. Int J Food Microbiol. 75:189-196.

Beck H, Eikenberg P, Sack K. 1980 Experimental study on renal tolerability of aminoglycosides butirosin and bekanamycin. Arzneimittelforschung. 30:288-294.

Beecher DJ, Olsen TW, Somers EB, Wong AC. 2000 Evidence for contribution of tripartite hemolysin BL, phosphatidylcholine-preferring phospholipase C, and collagenase to virulence of *Bacillus cereus* endophthalmitis. Infect Immun. 68:5269-5276.

Beecher DJ, Schoeni JL, Wong AC. 1995a Enterotoxic activity of hemolysin BL from *Bacillus cereus*. Infect Immun. 63:4423–4428.

Beecher DJ, Pulido JS, Barney NP, Wong AC. 1995b Extracellular virulence factors in *Bacillus cereus* endophthalmitis: methods and implication of involvement of hemolysin BL. Infect Immun. 63:632-639.

Belkacemi L, Bedard I, Simoneau L, Lafond J. 2005 Calcium channels, transporters and exchangers in placenta: a review. Cell Calcium. 37:1-8.

Bennett RW. 2005 Staphylococcal enterotoxin and its rapid identification in foods by enzyme-linked immunosorbent assay-based methodology. J Food Prot. 68:1264-1270.

Besson F, Michel G. 1989 Action of mycosubtilin, an antifungal antibiotic of *Bacillus subtilis*, on the cell membrane of *Saccharomyces cerevisiae*. Microbios 59:113-121.

Besson F, Peypoux F, Michel G. 1979 Interactions between bacterial membranes and peptidolipids: lysis of *Micrococcus luteus* protoplasts by derivatives of peptidolipidic antibiotics from *Bacillus subtilis*. Biochim Biophys Acta. 552:558-562.

Besson F, Quentin MJ, Michel G. 1989 Action of mycosubtilin on erythrocytes and artificial membranes. Microbios. 59:137-143.

Besson F, Peypoux F, Quentin MJ, Michel G. 1984 Action of antifungal peptidolipids from *Bacillus subtilis* on the cell membrane of *Saccharomyces cerevisiae*. J Antibiot (Tokyo) 37:172-177.

Beynon RJ, Beaumont A. 1998 Bacillolysin. In: Barrett AJ, Rawlings ND, Woessner JF (eds) Handbook of proteolytic enzymes. Academic, London, pp. 1047-1050.

Black JA, Foarde KK, Menetrez MY. 2006 Solvent comparison in the isolation, solubilization, and toxicity of *Stachybotrys chartarum* spore trichothecene mycotoxins in an established in vitro luminescence protein translation inhibition assay. J Microbiol Methods. 66:354-361.

Bolkent S, Zierold K. 2002 Effects of the ionophores valinomycin, ionomycin and gramicidin A on the element compartmentation in cultured rat hepatocytes. Toxicol In Vitro. 16:159-165.

Bornehag CG, Sundell J, Hagerhed-Engman L, Sigsgaard T. 2005 Association between ventilation rates in 390 Swedish homes and allergic symptoms in children. Indoor Air. 15:275-280.

Bottone EJ, Peluso RW. 2003 Production by *Bacillus pumilus* (MSH) of an antifungal compound that is active against *Mucoraceae* and *Aspergillus* species: preliminary report. J Med Microbiol. 52:69-74.

Busath DD. 1993 The use of physical methods in determining gramicidin channel structure and function. Annu Rev Physiol. 55:473-501.

Butaye P, Devriese LA, Haesebrouck F. 2003 Antimicrobial growth promoters used in animal feed: effects of less well known antibiotics on gram-positive bacteria. Clin Microbiol Rev. 16:175-88.

Calvert TW, Aidoo KE, Candlish AG, Fuat AR. 2005 Comparison of in vitro cytotoxicity of Fusarium mycotoxins,deoxynivalenol, T-2 toxin and zearalenone on selected human epithelial cell lines. Mycopathologia. 159:413-419.

Carrillo C, Teruel JA, Aranda FJ, Ortiz A. Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. Biochim Biophys Acta. 2003 1611:91-97.

Carlin F, Fricker M, Pielaat A, Heisterkamp S, Shaheen R, Salonen MS, Svensson B, Nguyen-the C, Ehling-Schulz M. 2006 Emetic toxin-producing strains of *Bacillus cereus* show distinct characteristics within the *Bacillus cereus* group. Int J Food Microbiol. 109:132-138.

Chatterjee C, Paul M, Xie L, van der Donk WA. 2005 Biosynthesis and mode of action of lantibiotics. Chem Rev 105:633-684.

Cherif A, Chehimi S, Limem F, Hansen BM, Hendriksen NB, Daffonchio D, Boudabous A. 2003 Detection and characterization of the novel bacteriocin entomocin 9, and safety evaluation of its producer, *Bacillus thuringiensis* ssp. entomocidus HD9. J Appl Microbiol. 95:990-1000.

Chitarra GS, Breeuwer P, Nout MJ, van Aelst AC, Rombouts FM, Abee T. 2003 An antifungal compound produced by *Bacillus subtilis* YM 10-20 inhibits germination of *Penicillium roqueforti* conidiospores. J Appl Microbiol. 94:159-166.

Chowdhury B, Das SK, Bose SK. 1998 Use of resistant mutants to characterize the target of mycobacillin in *Aspergillus niger* membranes.Microbiology. 144:1123-1130.

Chung YR, Kim CH, Hwang I, Chun J. 2000 *Paenibacillus koreensis* sp. nov., a new species that produces an iturin-like antifungal compound. Int J Syst Evol Microbiol. 50:1495-1500.

Colbeau A, Nachbaur J, Vignais PM. 1971 Enzymic characterization and lipid composition of rat liver subcellular membranes. Biochim Biophys Acta. 249:462-492.

Connelly MB, Young GM, Sloma A. 2004 Extracellular proteolytic activity plays a central role in swarming motility in *Bacillus subtilis*. J Bacteriol. 186:4159-4167.

Cooper DG, Macdonald CR, Duff SJ, Kosaric N 1981 Enhanced Production of Surfactin from *Bacillus subtilis* by Continuous Product Removal and Metal Cation Additions.Appl Environ Microbiol. 42:408-412.

Cox JR, Serpersu EH. 1997 Biologically important conformations of aminoglycoside antibiotics bound to an aminoglycoside 3'-phosphotransferase as determined by transferred nuclear Overhauser effect spectroscopy. Biochemistry. 36:2353-2359.

Crooks SR, Fodey TL, Gilmore GR, Elliott CT. 1998 Rapid screening for monensin residues in poultry plasma by a dry reagent dissociation enhanced lanthanide fluoroimmunoassay. Analyst. 123:2493-2496.

Crueger W, Crueger A. 1982 Enzymes. In Biotechnology: a textbook of industrial microbiology (eds.) Crueger W, Crueger A., Science Tech. Inc., Madison, Wis. USA pp. 161-174.

Deleu M, Paquot M, Nylander T. 2005 Fengycin interaction with lipid monolayers at the air-aqueous interface-implications for the effect of fengycin on biological membranes. J Colloid Interface Sci. 283:358-365.

Dierick K, Van Coillie E, Swiecicka I, Meyfroidt G, Devlieger H, Meulemans A, Hoedemaekers G, Fourie L, Heyndrickx M, Mahillon J. 2005 Fatal family outbreak of *Bacillus cereus*-associated food poisoning. J Clin Microbiol. 43:4277-4279.

DiMauro S, Davidzon G. 2005 Mitochondrial DNA and disease. Ann Med. 37:222-232.

Doebler JA. 1999 Gramicidin toxicity in NG108-15 cells: protective effects of acetamidine and guanidine. Cell Biol Toxicol. 15:279-289.

Dubos RJ, Hotchkiss RD. 1941 Production of bactericidal substances by aerobic sporulating bacilli. J Exp Med 17:405–422.

Duc Le H, Dong TC, Logan NA, Sutherland AD, Taylor J, Cutting SM. 2005 Cases of emesis associated with bacterial contamination of an infant breakfast cereal product. Int J Food Microbiol. 102:245-251.

Dufrenne J, Bijwaard M, Giffel M, Beumer R, Notermans S. 1995 Characteristics of some psychrotrophic *Bacillus cereus* isolates. Int J Food Microbiol. 27:175-183.

Dufour S, Deleu M, Nott K, Wathelet B, Thonart P, Paquot M. 2005 Hemolytic activity of new linear surfactin analogs in relation to their physico-chemical properties. Biochim Biophys Acta 1726:87-95.

Duitman EH, Hamoen LW, Rembold M, Venema G, Seitz H, Saenger W, Bernhard F, Reinhardt R, Schmidt M, Ullrich C, Stein T, Leenders F, Vater J. 1999 The mycosubtilin synthetase of *Bacillus subtilis* ATCC6633: a multifunctional hybrid between a peptide synthetase, an amino transferase, and a fatty acid synthase. Proc Natl Acad Sci U S A. 96:13294-13299.

EEC. 1993 Technical guidance documents in support of the risk assessment directive (93/67/EEC) for substances notified in accordance with the requirements of Council Directive 67/548/EEC. Brussels, Belgium.

Ehling-Schulz M, Vukov N, Schulz A, Shaheen R, Andersson M, Märtlbauer E, Scherer S. 2005a Identification and Partial Characterization of the Nonribosomal Peptide Synthetase Gene Responsible for Cereulide Production in Emetic *Bacillus cereus* Appl Environ Microbiol. 71:105-113.

Ehling-Schulz M, Svensson B, Guinebretiere MH, Lindback T, Andersson M, Schulz A, Fricker M, Christiansson A, Granum PE, Martlbauer E, Nguyen-The C, Salkinoja-Salonen M, Scherer S. 2005b Emetic toxin formation of *Bacillus cereus* is restricted to a single evolutionary lineage of closely related strains. Microbiology. 151:183-197.

Ehling-Schulz M, Fricker M, Scherer S. 2004 Identification of emetic toxin producing *Bacillus cereus* strains by a novel molecular assay. FEMS Microbiol Lett. 232:189-195.

EU, 2005/360/EC: Commission Decision of 26 April 2005 Establishing ecological criteria and the related assessment and verification requirements for the award of the Community eco-label to lubricants.

Fang TJ, Wei QK, Liao CW, Hung MJ, Wang TH. 2003 Microbiological quality of 18 °C ready-to-eat food products sold in Taiwan. Int J Food Microbiol. 80:241-250.

Finlay WJJ, Logan NA, Sutherland AD. 1999 Semiautomated metabolic staining assay for *Bacillus cereus* emetic toxin. Appl Environ Microbiol. 65:1811-1812.

Fogarty WM, Kelly CT. 1990 Recent advances in microbial amylases. In: Fogarty WM, Kelly CT (eds) Microbial enzymes and biotechnology, 2nd edn. Elsevier Applied Science, London, pp. 71-132

Frisvad JC, Skouboe P, Samson RA. 2005 Taxonomic comparison of three different groups of aflatoxin producers and a new efficient producer of aflatoxin B1, sterigmatocystin and 3-O-methylsterigmatocystin, *Aspergillus rambellii* sp. nov. Syst Appl Microbiol. 28:442-453.

From C, Pukall R., Schumann P, Hormazabal V, Granum PE 2005 Toxin-producing ability among *Bacillus* spp. outside the *Bacillus cereus* group. Appl Environ Microbiol. 71:1178-1183.

Furlong IJ, Mediavilla CL, Ascaso R, Rivas AL, Collins MKL. 1998 Induction of apoptosis by valinomycin: mitochondrial permeability transition causes intracellular acidification. Cell Death Differ. 5:214-221.

Gaczynska M, Osmulski PA. 2005 Small-molecule inhibitors of proteasome activity. Methods Mol Biol. 301:3-22.

Galvez A, Maqueda M, Martinez-Bueno M, Lebbadi M, Valdivia E. 1993a Isolation and physico-chemical characterization of an antifungal and antibacterial peptide produced by *Bacillus licheniformis* A12. Appl Microbiol Biotechnol. 39:438-442.

Galvez A, Valdivia E, Gonzalez-Segura A, Lebbadi M, Martinez-Bueno M, Maqueda M. 1993b Purification, characterization, and lytic activity against *Naegleria fowleri* of two amoebicins produced by *Bacillus licheniformis* A12. Appl Environ Microbiol. 59:1480-1486.

Galvez A, Maqueda M, Cordovilla P, Martinez-Bueno M, Lebbadi M, Valdivia E. 1994 Characterization and biological activity against *Naegleria fowleri* of amoebicins produced by *Bacillus licheniformis* D-13. Antimicrob Agents Chemother. 38:1314-1319.

Gebhardt K, Schimana J, Muller J, Fiedler HP, Kallenborn HG, Holzenkampfer M, Krastel P, Zeeck A, Vater J, Holtzel A, Schmid DG, Rheinheimer J, Dettner K. 2002 Screening for biologically active metabolites with endosymbiotic bacilli isolated from arthropods. FEMS Microbiol Lett. 217:199-205.

Gilbert RJ, de Louvais J, Donovan T, Little C, Nye K, Ribeiro CD, Richards J, Roberts D, Bolton FJ. 2000 Guidelines for the microbiological quality of some ready-to-eat foods sampled at the point of sale. Commun Dis Public Health. 3:163-167.

Goldman RD, Koren G. 2004 Amphotericin B nephrotoxicity in children. J Pediatr Hematol Oncol. 26:421-426.

Gould AR, May BK, Elliott WH. 1971 Studies on the protoplast-bursting factor from *Bacillus amyloliquefaciens*. FEBS lett. 14:320-322

Gorny RL, Reponen T, Willeke K, Schmechel D, Robine E, Boissier M, Grinshpun SA. 2002 Fungal fragments as indoor air biocontaminants. Appl Environ Microbiol. 68:3522-3531.

Grangemard I, Wallach J, Maget-Dana R, Peypoux F. 2001 Lichenysin: a more efficient cation chelator than surfactin. Appl Biochem Biotechnol. 90:199-210.

Gupta R, Beg QK, Lorenz P. 2002 Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol. 59:15-32.

Gustafsson K, Roman M, Fenical W. 1989 The macrolactins, a novel class of antiviral and cytotoxic macrolides from a deep-sea marine bacterium. J Am Chem Soc 111:7519–7524.

Han JS, Cheng JH, Yoon TM, Song J, Rajkarnikar A, Kim WG, Yoo ID, Yang YY, Suh JW. 2005 Biological control agent of common scab disease by antagonistic strain *Bacillus* sp. sunhua. J Appl Microbiol. 99:213-221.

Hancock RE, Chapple DS. 1999 Peptide antibiotics. Antimicrob Agents Chemother. 43:1317-1323.

Harnois I, Maget-Dana R, Ptak M. 1989 Methylation of the antifungal lipopeptide iturin A modifies its interaction with lipids. Biochimie. 71:111-116.

Hartgerink JD, Clark TD, Ghadiri MR. 1998. Peptide nanotubes and beyond. Chem Euro J. 4:1367-1372.

Hasumi K, Takizawa K, Takahashi F, Park JK, Endo A. 1995 Inhibition Acyl-CoA: Cholestrol acyltransferase by isohalobacillin, a complex of novel cyclic acylpeptides produced by *Bacillus* sp. A1238. J. Antibiotics. 48:1419-1424.

Hathout Y, Ho YP, Ryzhov V, Demirev P, Fenselau C. 2000 Kurstakins: a new class of lipopeptides isolated from *Bacillus thuringiensis*. J Nat Prod. 63:1492-1496.

Hathout Y, Setlow B, Cabrera-Martinez RM, Fenselau C, and Setlow P. 2003 Small, acid-soluble proteins as biomarkers in mass spectrometry analysis of *Bacillus* spores Appl and Environ Microbiol. 69:1100-1107.

Haynes DH, Kowalsky A, Pressman BC. 1969 Application of nuclear magnetic resonance to the conformational changes in valinomycin during complexation. J Biol Chem. 244:502-505.

Heerklotz H, Seelig J. 2001 Detergent-like action of the antibiotic peptide surfactin on lipid membranes. Biophys J. 81:1547-1554.

Hiradate S, Yoshida S, Sugie H, Yada H, Fujii Y. 2002 Mulberry anthracnose antagonists (iturins) produced by *Bacillus amyloliquefaciens* RC-2. Phytochemistry 6:693-698.

Hirvonen MR, Huttunen K, Roponen M. 2005 Bacterial strains from moldy buildings are highly potent inducers of inflammatory and cytotoxic effects. Indoor Air. 15:65-70.

Hofemeister J, Conrad B, Adler B, Hofemeister B, Feesche J, Kucheryava N, Steinborn G, Franke P, Grammel N, Zwintscher A, Leenders F, Hitzeroth G, Vater J. 2004 Genetic analysis of the biosynthesis of non-ribosomal peptide- and polyketide-like antibiotics, iron uptake and biofilm formation by *Bacillus subtilis* A1/3. Mol Genet Genomics. 272:363-378.

Hoornstra D, Andersson MA, Mikkola R, Salkinoja-Salonen MS. 2003 A new method for in vitro detection of microbially produced mitochondrial toxins. Toxicol In Vitro. 17:745-751.

Horwood PF, Burgess GW, Oakey HJ. 2004 Evidence for non-ribosomal peptide synthetase production of cereulide (the emetic toxin) in *Bacillus cereus*. FEMS Microbiol Lett. 236:319-24.

Hoton FM, Andrup L, Swiecicka I, Mahillon J. 2005 The cereulide genetic determinants of emetic *Bacillus cereus* are plasmid-borne. Microbiology. 151:2121-2124.

Hourdou ML, Besson F, Tenoux I, Michel G. 1989 Fatty acids and β-amino acid syntheses in strains of *Bacillus subtilis* producing iturinic antibiotics. Lipids. 24:940-944.

Hoult B, Tuxford AF. 1991 Toxin production by *Bacillus pumilus* J Clin Pathol. 44:455-458.

Hsieh FC, Li MC, Lin TC, Kao SS. 2004 Rapid detection and characterization of surfactin-producing *Bacillus subtilis* and closely related species based on PCR. Curr Microbiol. 49:186-191.

Hughes S, Bartholomew B, Hardy JC, Kramer JM. 1998 Potential application of a Hep-2 cell assay in the investigation of *Bacillus cereus* emetic-syndrome food poisoning. FEMS Microbiol Lett. 52:7-12.

Husman T. 1996 Health effects of indoor-air microorganisms. Scand J Work Environ Health. 22:5-13.

Häggblom MM, Apetroaie C, Andersson MA, Salkinoja-Salonen M. 2002 Quantitative analysis of cereulide, the emetic toxin of *Bacillus cereus*, produced under different conditions. Appl Environ Microbiol. 68:2479–2483.

Idriss EE, Makarewicz O, Farouk A, Rosner K, Greiner R, Bochow H, Richter T, Borriss R. 2002 Extracellular phytase activity of *Bacillus amyloliquefaciens* FZB45 contributes to its plant-growth-promoting effect. Microbiology. 148:2097-2109.

Ilinskaya ON, Vamvakas S. 1997 Nephrotoxic effects of bacterial ribonucleases in the isolated perfused rat kidney.Toxicology 120:55-63.

Isobe M, Ishikawa T, Suwan S, Agata N, Ohta M. 1995 Synthesis and activity of cereulide, a cyclic dodecadepsipeptide ionophore as emetic toxin from *Bacillus cereus*. Bioorg. Med. Chem. Lett. 5:2855-2858.

Jestoi M, Rokka M, Yli-Mattila T, Parikka P, Rizzo A, Peltonen K. 2004 Presence and concentrations of the Fusarium-related mycotoxins beauvericin, enniatins and moniliformin in finnish grain samples. Food Addit Contam 21:794-802.

Jethwaney D, Hofer M, Khaware RK, Prasad R. 1997 Functional reconstitution of a purified proline permease from Candida albicans: interaction with the antifungal cispentacin. Microbiology. 143:397-404.

Jääskeläinen EL, Teplova V, Andersson MA, Andersson LC, Tammela P, Andersson MC, Pirhonen TI, Saris NE, Vuorela P, Salkinoja-Salonen MS. 2003a In vitro assay for human toxicity of cereulide the emetic toxin produced by food poisoning *Bacillus cereus.* Toxicol In Vitro 17:737-744.

Jääskeläinen EL, Häggblom MM, Andersson MA, Vanne L, Salkinoja-Salonen MS. 2003b Potential of *Bacillus cereus* for producing an emetic toxin, cereulide, in bakery products: quantitative analysis by chemical and biological methods. J Food Prot 66:1047-1054.

Jääskeläinen EL, Häggblom MM, Andersson MA, Salkinoja-Salonen MS. 2004 Atmospheric oxygen and other conditions affecting the production of cereulide by *Bacillus cereus* in food. Int J Food Microbiol. 96:75-83.

Kajimura Y, Kaneda M. 1997 Fusaricidins B, C and D, new depsipeptide antibiotics produced by *Bacillus polymyxa* KT-8: isolation, structure elucidation and biological activity. J. Antibiot 50:220-228.

Kajimura Y, Sugiyama M, Kaneda M. 1995 Bacillopeptins, new cyclic lipopeptide antibiotics from *Bacillus subtilis* FR-2. J Antibiot (Tokyo). 48:1095-1103.

Kamyar M, Rawnduzi P, Studenik CR, Kouri K, Lemmens-Gruber R. 2004 Investigation of the electrophysiological properties of enniatins. Arch Biochem Biophys. 429:215-223.

Kamyar MR, Kouri K, Rawnduzi P, Studenik C, Lemmens-Gruber R. 2006 Effects of moniliformin in presence of cyclohexadepsipeptides on isolated mammalian tissue and cells. Toxicol In Vitro. doi:10.1016/j.tiv.2006.03.001.

Kawamura-Sato K, Hirama Y, Agata N, Ito H, Torii K, Takeno A, Hasegawa T, Shimomura Y, Ohta M. 2005 Quantitative analysis of cereulide, an emetic toxin of *Bacillus cereus*, by using rat liver mitochondria. Microbiol Immunol. 49:25-30.

Kennedy DG, Hughes PJ, Blanchflower WJ. 1998 Ionophore residues in eggs in Northern Ireland: incidence and cause. Food Addit Contam. 15:535-541.

Kilian M, Steiner U, Krebs B, Junge H, Schmiedeknecht G, Hain R. 2000 FZB24® *Bacillus subtilis* - mode of action of a microbial agent enhancing plant vitality Pflanzenschutz-Nachrichten 1:72-93.

Kim PI, Bai H, Bai D, Chae H, Chung S, Kim Y, Park R, Chi YT. 2004 Purification and characterization of a lipopeptide produced by *Bacillus thuringiensis* CMB26. J Appl Microbiol. 97:942-949.

Kinsel JF, Melnik EI, Lindenbaum S, Sternson LA, Ovchinnikov YuA. 1982a The effect of amine structure on complexation with lasalocid in model membrane systems. I. Identification of charged complexes in lipid bilayer membranes. Biochim Biophys Acta. 684:233-240.

Kinsel JF, Melnik EI, Sternson LA, Lindenbaum S, Ovchinnikov YuA. 1982b The effect of amine structure on complexation with lasalocid in model membrane systems. II. Ionophore selectivity for amines in lipid bilayers and at oil/water interfaces. Biochim Biophys Acta. 692:377-383.

Kinsinger RF, Shirk MC, Fall R. 2003 Rapid surface motility in *Bacillus subtilis* is dependent on extracellular surfactin and potassium ion. J Bacteriol. 185:5627-5631.

Kolari M, Mattila K, Mikkola R, Salkinoja-Salonen MS. 1998 Community structure of biofilms on ennobled stainless steel in Baltic Sea water. J Ind Microbiol Biotechnol 21:261-274.

Konz D, Klens A, Schorgendorfer K, Marahiel MA. 1997 The bacitracin biosynthesis operon of *Bacillus licheniformis* ATCC 10716: molecular characterization of three multi-modular peptide synthetases. Chem Biol. 4:927-937.

Koskinen OM, Husman TM, Meklin TM, Nevalainen AI. 1999 The relationship between moisture or mould observations in houses and the state of health of their occupants. Eur Respir J. 14:1363-1367.

Kostiainen R, Kotiaho T, Kuuranne T, Auriola S. 2003 Liquid chromatography /atmospheric pressure ionization-mass spectrometry in drug metabolism studies. J Mass Spectrom. 38:357-372.

Kotiranta A, Lounatmaa K, Haapasalo M. 2000 Epidemiology and pathogenesis of *Bacillus cereus* infections. Microbes Infect. 2:189-198.

Kouadio JH, Mobio TA, Baudrimont I, Moukha S, Dano SD, Creppy EE. 2005 Comparative study of cytotoxicity and oxidative stress induced by deoxynivalenol, zearalenone or fumonisin B1 in human intestinal cell line Caco-2. Toxicology. 213:56-65.

Koumoutsi A, Chen XH, Henne A, Liesegang H, Hitzeroth G, Franke P, Vater J, Borriss R. 2004 Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. J Bacteriol. 186:1084-1096.

Kouri K, Duchen MR, Lemmens-Gruber R. 2005 Effects of beauvericin on the metabolic state and ionic homeostasis of ventricular myocytes of the guinea pig. Chem Res Toxicol. 18:1661-1668.

Kouri K, Lemmens M, Lemmens-Gruber R. 2003 Beauvericin-induced channels in ventricular myocytes and liposomes.Biochim Biophys Acta. 1609:203-210.

Kracht M, Rokos H, Ozel M, Kowall M, Pauli G, Vater J. 1999 Antiviral and hemolytic activities of surfactin isoforms and their methyl ester derivatives J. Antibiot. 52**:** 613-619.

Kramer JM, Gilbert RJ. 1989 *Bacillus cereus* and other *Bacillus* species. In:. Doyle MP, Editor, *Foodborne Bacterial Pathogens*, Marcel Dekker, New York, pp. 21-70.

Kuhn RC, Trimble MW, Hofer V, Lee M, Nassof RS. 2005 Prevalence and airborne spore levels of *Stachybotrys* spp. in 200 houses with water incursions in Houston, Texas Can J Microbiol. 51:25-28.

Kugler M, Loeffler W, Rapp C, Kern A, Jung G. 1990 Rhizocticin A, an antifungal phosphono-oligopeptide of *Bacillus subtilis* ATCC 6633: biological properties. Arch Microbiol. 153:276-281.

Kuroda J, Fukai T, Nomura T. 2001 Collision-induced dissociation of ring-opened cyclic depsipeptides with a guanidino group by electrospray ionization/ion trap mass spectrometry.J Mass Spectrom. 36:30-37.

Kuroda J, Fukai T, Konishi M, Uno J, Kurusu K, Nomura T. 2000 LI-F antibiotics, a family of antifungal cyclic depsipeptides produced by *Bacillus polymyxa* L-1129. Heterocycles 53:1533-1549.

Kurusu K, Ohba K, Arai T, Fukushima K. New peptide antibiotics 1987 LI-F03, F04, F05, F07, and F08, produced by *Bacillus polymyxa*. I. Isolation and characterization. J. Antibiot 40:1506-1514.

Kuse M, Franz T, Koga K, Suwan S, Isobe M, Agata N, Ohta M. 2000 High incorporation of L-amino acids to cereulide, an emetic toxin from *Bacillus cereus*. Bioorg Med Chem Lett. 10:735-773.

Laohachai KN, Bahadi R, Hardo MB, Hardo PG, Kourie JI. 2003 The role of bacterial and non-bacterial toxins in the induction of changes in membrane transport: implications for diarrhea. Toxicon. 42:687-707.

Lappalainen S, Kähkönen E, Loikkanen P, Palomäki E, Lindroos O, Reijula K. 2001 Evaluation of priorities for repairing in moisture-damaged school buildings in Finland. Building and Environment. 36:981-986.

Larsen HD, Jorgensen K. 1997 The occurrence of *Bacillus cereus* in Danish pasteurized milk. Int. J. Food Microbiol. 34:179-186.

Latoud C, Peypoux F, Michel G, Genet R, Morgat JL. 1986 Interactions of antibiotics of the iturin group with human erythrocytes. Biochim Biophys Acta. 856:526-535.

Latoud C, Peypoux F, Michel G. 1987 Action of iturin A, an antifungal antibiotic from *Bacillus subtilis*, on the yeast *Saccharomyces cerevisiae*: modifications of membrane permeability and lipid composition. J Antibiot. 40:1588-1595.

Latoud C, Peypoux F, Michel G. 1990 Interaction of iturin A, a lipopeptide antibiotic, with *Saccharomyces cerevisiae* cells: influence of the sterol membrane composition. Can J Microbiol. 36:384-389.

Le Loir Y, Baron F, Gautier M. 2003 *Staphylococcus aureus* and food poisoning Genet Mol Res. 2:63-76.

Lebbadi M, Galvez A, Maqueda M, Martinez-Bueno M, Valdivia E. 1994a Fungicin M4: a narrow spectrum peptide antibiotic from *Bacillus licheniformis* M-4. J Appl Bacteriol. 77:49-53.

Lebbadi M, Galvez A, Valdivia E, Martinez-Bueno M, Maqueda M. 1994b Purification of amoebolytic substances from *Bacillus licheniformis* M-4. Arch Microbiol. 162:98-102.

Lebbadi M, Galvez A, Valdivia E, Martinez-Bueno M, Maqueda M. 1994c Biological activity of amoebicin m4-A from *Bacillus licheniformis* M-4. Antimicrob Agents Chemother. 38:1820-1823.

Lee DL, Hodges RS. 2003 Structure-activity relationships of de novo designed cyclic antimicrobial peptides based on gramicidin S. Biopolymers. 71:28-48.

Lintelmann J, Katayama A, Kurihara N, Shore L, Wenzel A. 2003 Endocrine disruptors in the environment (IUPAC Technical Report). Pure Appl Chem. 75: 631-681.

Logrieco A, Rizzo A, Ferracane R, Ritieni A. 2002 Occurrence of beauvericin and enniatins in wheat affected by *Fusarium avenaceum* head blight. Appl Environ Microbiol. 68:82-85.

Lund T, De Buyser ML, Granum PE. 2000 A new cytotoxin from *Bacillus cereus* that may cause necrotic enteritis. Mol Microbiol. 38:254-261.

Lund T, Granum PE. 1996 Characterization of a non-haemolytic enterotoxin complex from *Bacillus cereus* isolated after a foodborne outbreak. FEMS Microbiol Lett. 141:151-156.

Madonna AJ, Voorhees KJ, Taranenko NI, Laiko VV, Doroshenko VM. 2003 Detection of cyclic lipopeptide biomarkers from *Bacillus* species using atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry. Anal Chem. 75:1628-1637.

Maget-Dana R, Heitz F, Ptak M, Peypoux F, Guinand M. 1985a Bacterial lipopeptides induce ion-conducting pores in planar bilayers. Biochem Biophys Res Commun. 129:965-971.

Maget-Dana R, Peypoux F. 1994 Iturins, a special class of pore-forming lipopeptides: biological and physicochemical properties.Toxicology. 87:151-174.

Maget-Dana R, Ptak M, Peypoux F, Michel G. 1985b Pore-forming properties of iturin A, a lipopeptide antibiotic. Biochim Biophys Acta. 815:405-409.

Maget-Dana R, Ptak M. 1990 Iturin lipopeptides: interactions of mycosubtilin with lipids in planar membranes and mixed monolayers. Biochim Biophys Acta. 1023:34-40.

Maget-Dana R, Thimon L, Peypoux F, Ptak M. 1992 Surfactin/iturin A interactions may explain the synergistic effect of surfactin on the biological properties of iturin A. Biochimie. 74:1047-1051.

Mahler H, Pasi A, Kramer JM, Schulte P, Scoging AC, Bar W, Krahenbuhl S. 1997 Fulminant liver failure in association with the emetic toxin of *Bacillus cereus*. N Engl J Med. 336:1142-1148.

Maier SK, Scherer S, Loessner MJ. 1999 Long-chain polyphosphate causes cell lysis and inhibits *Bacillus cereus* septum formation, which is dependent on divalent cations. Appl Environ Microbiol. 65:3942-3949.

Mannanov RN, Sattarova RK. 2001 Antibiotics Produced by *Bacillus* Bacteria. Chem Nat Comp. 37:117-123.

Marahiel MA, Stachelhaus T, Mootz HD. 1997 Modular peptide synthetases involved in nonribosomal peptide synthesis. Chem Rev. 97:2651-2674.

Martin NI, Hu H, Moake MM, Churey JJ, Whittal R, Worobo RW, Vederas JC. 2003 Isolation, structural characterization, and properties of mattacin (polymyxin M), a cyclic peptide antibiotic produced by *Paenibacillus kobensis* M. J Biol Chem. 278:13124-13132.

Matabudul DK, Conway B, Lumley ID. 2000 A rapid method for the determination of lasalocid in animal tissues and eggs by high performance liquid chromatography with fluorescence detection and confirmation by LC-MS-MS. Analyst. 125:2196-2200.

Matabudul DK, Lumley ID, Points JS. 2002 The determination of 5 anticoccidial drugs (nicarbazin, lasalocid, monensin, salinomycin and narasin) in animal livers and eggs by liquid chromatography linked with tandem mass spectrometry (LC-MS-MS). Analyst. 127:760-768.

Mather MW, Rottenberg H. 2002 The inhibition of calcium signaling in T lymphocytes from old mice results from enhanced activation of the mitochondrial permeability transition pore. Mech Ageing Dev. 123:707-724.

McCabe-Sellers BJ, Beattie SE. 2004 Food safety: emerging trends in foodborne illness surveillance and prevention.J Am Diet Assoc. 104:1708-1717.

McKean C, Tang L, Tang M, Billam M, Wang Z, Theodorakis CW, Kendall RJ, Wang JS. 2006 Comparative acute and combinative toxicity of aflatoxin B(1) and fumonisin B(1) in animals and human cells. Food Chem Toxicol. 44:868-876.

McKillip JL. 2000 Prevalence and expression of enterotoxins in *Bacillus cereus* and other *Bacillus* spp., a literature review. Antonie Van Leeuwenhoek. 77:393-399.

McLean KJ, Marshall KR, Richmond A, Hunter IS, Fowler K, Kieser T, Gurcha SS, Besra GS, Munro AW. 2002 Azole antifungals are potent inhibitors of cytochrome P450 mono-oxygenases and bacterial growth in mycobacteria and streptomycetes. Microbiology. 148:2937-2949.

Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, and Tauxe RV. 1999 Food-related illness and death in the United States. Emerg Infect Dis 5:607–625.

Meylan WM, Howard PH. 2000 Estimating Log P with Atom/fragments and Water Solubility with Log P. Drug Des Discov. 19:67-84.

Mikkola, R, Andersson MA, Peltola J, Saris NE, Grigoriev P, and Salkinoja-Salonen MS. 1999 Purification and properties of toxins isolated from *Streptomyces griseus* and *Bacillus cereus* found in construction materials of building. Proceeding of the 8th Conference on Indoor Air Quality and Climate, Indoor air 1999, Vol 4, Edinburg, UK, pp. 1110-1111.

Milner JL, Silo-Suh L, Lee JC, He H, Clardy J, Handelsman J**.** 1996 Production of kanosamine by *Bacillus cereus* UW85. Appl Environ Microbiol. 62:3061-3065.

Moeller R, Horneck G, Facius R, Stackebrandt E. 2005 Role of pigmentation in protecting *Bacillus* sp. endospores against environmental UV radiation. FEMS Microbiol Ecol. 51:231-236.

Mootz HD, Marahiel MA. 1997 The tyrocidine biosynthesis operon of *Bacillus brevis*: complete nucleotide sequence and biochemical characterization of functional internal adenylation domains. J Bacteriol. 179:6843-6850.

Mukherjee AK, Das K. 2005 Correlation between diverse cyclic lipopeptides production and regulation of growth and substrate utilization by *Bacillus subtilis* strains in a particular habitat. FEMS Microbiol 54:479-489.

Mukherjee S, Bose SK. 1978 Some observations on the nature of reactive groups involved in reversal of mycobacillin inhibition by sterols and lipids. J Antibiot 31:147-149.

Munimbazi C, Bullerman LB. 1998 Isolation and partial characterization of antifungal metabolites of *Bacillus pumilus*. J Appl Microbiol. 84:959-968.

Murchison D, Zawieja DC, Griffith WH. 2004 Reduced mitochondrial buffering of voltage-gated calcium influx in aged rat basal forebrain neurons. Cell Calcium. 36:61-75.

Nagai S, Okimura K, Kaizawa N, Ohki K, Kanatomo S. 1996. Study on surfactin, a cyclic depsipeptide. II. Synthesis of surfactin B2 produced by *Bacillus* natto KMD 2311. Chem Pharm Bull. 44:5–10.

Nakano S, Maeshima H, Matsumura A, Ohno K, Ueda S, Kuwabara Y, Yamada TA. 2004 PCR assay based on a sequence-characterized amplified region marker for detection of emetic *Bacillus cereus*. J Food Prot. 67:1694-1701.

Naruse N, Tenmyo O, Kobaru S, Kamei H, Miyaki T, Konishi M, Oki T. 1990 Pumilacidin, a complex of new antiviral antibiotics. Production, isolation, chemical properties, structure and biological activity. J Antibiot (Tokyo). 43:267-280.

Nevalainen A, Seuri M. 2005 Of microbes and men. Indoor Air. 15:58-64.

Newcombe DA, Schuerger AC, Benardini JN, Dickinson D, Tanner R, Venkateswaran K. 2005 Survival of spacecraft-associated microorganisms under simulated martian UV irradiation. Appl Environ Microbiol. 71:8147-8156.

Nieminen AL, Saylor AK, Herman B, Lemasters JJ. 1994 ATP depletion rather than mitochondrial depolarization mediates hepatocyte killing after metabolic inhibition. Am J Physiol Cell Physiol. 267:C67-C74.

Nishikiori T, Naganawa H, Muraoka Y, Aoyagi T, Umezawa H. 1986 Plipastatins: new inhibitors of phospholipase A2, produced by *Bacillus cereus* BMG302-fF67. III. Structural elucidation of plipastatins. J Antibiot (Tokyo). 39:755-761.

Notermans S, Batt CA. 1998 A risk assessment approach for food-borne *Bacillus cereus*and its toxins. Journal of Applied Microbiology Symposium Supplement. 84: 51S-61S.

Oscariz JC, Lasa I, Pisabarro AG. 1999 Detection and characterization of cerein 7, a new bacteriocin produced by *Bacillus cereus* with a broad spectrum of activity. FEMS Microbiol Lett. 178:337-341.

Oscariz JC, Pisabarro AG. 2000 Characterization and mechanism of action of cerein 7, a bacteriocin produced by *Bacillus cereus* Bc7. J Appl Microbiol. 89:361-369.

Ouderkirk JP, Nord JA, Turett GS, Kislak JW. 2003 Polymyxin B nephrotoxicity and efficacy against nosocomial infections caused by multiresistant gram-negative bacteria. Antimicrob Agents Chemother. 47:2659-2662.

Outtrup H, Jorgensen ST. 2002 The importance of *Bacillus* species in the production of industrial enzymes. In: Berkeley R, Heyndrickx M, Logan N, De Vos P (eds), Applications and systematics of *Bacillus* and relatives. Blackwell publishing, UK, pp 206–218.

Ovchinnikov YA. 1974 Membrane active complexones. Chemistry and biological functions. FEBS Lett. 44:1-21.

Paananen A, Mikkola R, Sareneva T, Matikainen S, Andersson MA, Julkunen I, Salkinoja-Salonen MS, Timonen T. 2000 Inhibition of human natural killer (NK) cell function by valinomycin, a toxin from *Streptomyces griseus* in indoor air. Infect Immun. 68:165-169.

Paananen A, Mikkola R, Sareneva T, Matikainen S, Michael H, Andersson MA, Julkunen I, Salkinoja-Salonen MS, Timonen T. 2002 Inhibition of human natural killer cell activity by cereulide , a toxin from *Bacillus cereus*. Clin Exp Immunol. 129:420-428.

Pan C, Liu F, Ji Q, Wang W, Drinkwater D, Vivilecchia R. 2006 The use of LC/MS, GC/MS, and LC/NMR hyphenated techniques to identify a drug degradation product in pharmaceutical development. J Pharm Biomed Anal. 40:581-590.

Parks JE, Lynch DV. 1992 Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. Cryobiology. 29:255-266.

Patel PS, Huang S, Fisher S, Pirnik D, Aklonis C, Dean L, Meyers E, Fernandes P, Mayerl F. 1995 Bacillaene, a novel inhibitor of procaryotic protein synthesis produced by *Bacillus subtilis*: production, taxonomy, isolation, physico-chemical characterization and biological activity. J Antibiot. 48:997-1003.

Pathak N, Khandelwal S. 2006 Influence of cadmium on murine thymocytes: potentiation of apoptosis and oxidative stress. Toxicol Lett. 165:121-132.

Pedersen PB, Bjørnvad ME, Rasmussen MD, Petersen JN. 2002 Cytotoxic Potential of Industrial Strains of *Bacillus* sp. Regul Toxicol Pharmacol. 36:155-161.

Peltola J, Ritieni A, Mikkola R, Grigoriev P, Pocsfalvi G, Andersson M, Salkinoja-Salonen MS. 2004 The Biological effects of peptaibols from *Trichoderma harzianum* on mammalian cells. Appl Environ Microbiol.70:4996-5004.

Peltola J, Andersson MA, Haahtela T, Mussalo-Rauhamaa H, Rainey FA, Kroppenstedt R M, Samson RA, Salkinoja-Salonen MS. 2001 Toxic-Metabolite-Producing Bacteria and Fungus in an Indoor Environment. Appl Environ Microbiol. 67:3269-3274.

Peltola J, Andersson, MA, Mikkola R, Mussalo-Rauhamaa H, Salkinoja-Salonen MS**.** 1999 Membrane toxic substance from water damaged construction materials and fungal pure cultures. In E. Johanning (Ed). Bioaerosols, Fungi and Mycotoxins: Healt effects, Assessment, Prevention and Control. Eastern New York Occupational and Enrironmental Health Center, Albany New York, USA, pp. 432-443.

Pettit GR, Tan R, Melody N, Kielty JM, Pettit RK, Herald DL, Tucker BE, Mallavia LP, Doubek DL, Schmidt JM. 1999 Antineoplastic agents. Part 409: Isolation and structure of montanastatin from a terrestrial *actinomycete*. Bioorg Med Chem. 75:895-899.

Peypoux F, Guinand M, Michel G, Delcambe L, Das BC, Lederer E. 1978 Structure of iturin A, a peptidolipid antibiotic from *Bacillus subtilis*. Biochemistry 17:3992-3996.

Pfeifer JR, Reiss P, Koert U. 2006 Crown ether-gramicidin hybrid ion channels: dehydration-assisted ion selectivity. Angew Chem Int Ed Engl. 45:501-504.

Pielaat A, Fricker M, Nauta MJ, van Leusden FM. Biodiversity in *Bacillus cereus* RIVM report 250912004/2005, National Institute for Public Health and the Environment, 3720 Bilthoven, the Netherlands pp. 1-84.

Piraud M, Vianey-Saban C, Bourdin C, Acquaviva-Bourdain C, Boyer S, Elfakir C, Bouchu D. 2005 A new reversed-phase liquid chromatographic/tandem mass spectrometric method for analysis of underivatised amino acids: evaluation for the diagnosis and the management of inherited disorders of amino acid metabolism. Rapid Commun Mass Spectrom. 19:3287-3297.

Pitchayawasin S, Kuse M, Koga K, Isobe M, Agata N, Ohta M. 2003 Complexation of Cyclic Dodecadepsipeptide, Cereulide with Ammonium Salts. Bioorg Med Chem Lett. 13:3507-3512.

Pitchayawasin S, Isobe M, Kuse M, Franz T, Agata N, Ohta, M. 2004 Molecular diversity of cereulide detected by means of nano-HPLC-ESI-Q-TOF-MS. Intern J Mass Spec. 235:123-129.

Prenner EJ, Lewis RN, McElhaney RN. 1999 The interaction of the antimicrobial peptide gramicidin S with lipid bilayer model and biological membranes. Biochim Biophys Acta. 1462:201-221.

Priest FG. 1977 Extracellular enzyme synthesis in the genus *Bacillus*. Microbiol Mol Biol Rev. 41:711-753.

Prior TI, Kunwar S, Pastan I. 1996 Studies on the activity of barnase toxins in vitro and in vivo. Bioconjug Chem. 7:23-29.

Qin C, Zhong X, Bu X, Ng NL, Guo Z. 2003 Dissociation of antibacterial and hemolytic activities of an amphipathic peptide antibiotic. J Med Chem. 46:4830-4833.

Qu J, Chen W, Luo G, Wang Y, Xiao S, Ling Z, Chen G. 2002 Rapid determination of underivatized pyroglutamic acid, glutamic acid, glutamine and other relevant amino acids in fermentation media by LC-MS-MS. Analyst. 127:66-69.

Quentin MJ, Besson F, Peypoux F, Michel G. 1982 Action of peptidolipidic antibiotics of the iturin group on erythrocytes. Effect of some lipids on hemolysis. Biochim Biophys Acta. 684:207-211.

Rajkovic A, Uyttendaele M, Ombregt SA, Jääskeläinen E, Salkinoja-Salonen M, Debevere J. 2006 Influence of type of food on the kinetics and overall production of *Bacillus cereus* emetic toxin. J Food Prot.69:847-852.

Ringot D, Chango A, Schneider YJ, Larondelle Y. 2006 Toxicokinetics and toxico-dynamics of ochratoxin A, an update. Chem Biol Interact. 159:18-46.

Rintala H, Hyvärinen A, Paulin L, Nevalainen A. 2004 Detection of *Streptomycetes* in house dust - comparison of culture and PCR methods. Indoor Air. 14:112-119.

Rocha O, Ansari K, Doohan FM. 2005 Effects of trichothecene mycotoxins on eukaryotic cells: a review. Food Addit Contam. 22:369-378.

Rodriquez RJ, Acosta D Jr. 1996 Inhibition of mitochondrial function in isolated rate liver mitochondria by azole antifungals. J Biochem Toxicol. 11:127-131.

Rokka M, Eerola S, Perttilä U, Rossow L, Venäläinen E, Valkonen E, Valaja J, Peltonen K. 2005 The residue levels of narasin in eggs of laying hens fed with unmedicated and medicated feed. Mol Nutr Food Res. 49:38-42.

Rokka M, Peltonen K. 2006 Simultaneous determination of four coccidiostats in eggs and broiler meat: validation of an LC-MS/MS method. Food Addit Contam. 23:470-478.

Roongsawang N, Thaniyavarn J, Thaniyavarn S, Kameyama T, Haruki M, Imanaka T, Morikawa M, Kanaya S. 2002 Isolation and characterization of a halotolerant *Bacillus subtilis* BBK-1 which produces three kinds of lipopeptides: bacillomycin L, plipastatin, and surfactin. Extremophiles 6:499-506.

Rosenberg E, Ron EZ. 1999 High- and low-molecular-mass microbial surfactants Appl Microbiol Biotechnol. 52:154-162.

Rosenquist H, Smidt L, Andersen SR, Jensen GB, Wilcks A. 2005 Occurrence and significance of *Bacillus cereus* and *Bacillus thuringiensis* in ready-to-eat food. FEMS Microbiol Lett. 250:129-136.

Salkinoja-Salonen MS, Vuorio R, Andersson MA, Kampfer P, Andersson MC, Honkanen-Buzalski T, Scoging AC. 1999a Toxigenic strains of *Bacillus licheniformis* related to food poisoning. Appl Environ Microbiol. 65:4637-4645.

Salkinoja-Salonen MS, Andersson MA, Mikkola R, Paananen A, Peltola J, Mussalo-Rauhanmaa H, Vuorio R, Saris NE, Grigorjev P, Helin J, Koljalg U, Timonen T. 1999b Toxigenic microbes in indoor environment: identification, structure and biological effects of the aerosolizing toxins. In: Johanning E (ed.) Bioaerosols, fungi and mycotoxins: health effects, assessment, prevention and control. Eastern New York Occupational and Environmental Health Center, Albany, New York, pp 359–374.

Sandrin C, Peypoux F, Michel G. 1990 Coproduction of surfactin and iturin A, lipopeptides with surfactant and antifungal properties, by *Bacillus subtilis*. Biotechnol Appl Biochem. 12:370-375.

Saski EK, Mikkola R, Kukkonen JVK, Salkinoja-Salonen MS. 1997 Bleached kraft pulp mill discharged organic matter in recipient lake sediment:Environmental and molecular properties. Environ Sci Pollut Res. 4:194-202.

Schaller KD, Fox SL, Bruhn DF, Noah KS, Bala GA. 2004 Characterization of surfactin from *Bacillus subtilis* for application as an agent for enhanced oil recovery. Appl Biochem Biotechnol. 115:827-836.

Schallmey M, Singh A, Ward OP**.** 2004 Developments in the use of *Bacillus* species for industrial production. Can J Microbiol. 50:1-17.

Schlegelova J, Brychta J, Klimova E, Napravnikova E, Babak V. 2003 The prevalence of and resistance to antimicrobial agents of *Bacillus cereus* isolates from foodstuffs. Vet Med. 11:331-338.

Schmitt CK, Meysick KC, O'Brien AD. 1999 Bacterial toxins: friends or foes? Emerg Infect Dis. 2:224-234.

Schollenberger M, Muller HM, Rufle M, Suchy S, Plank S, Drochner W. 2006 Natural occurrence of 16 fusarium toxins in grains and feedstuffs of plant origin from Germany. Mycopathologia. 161:43-52.

Shaheen R, Andersson MA, Apetroaie C, Schulz A, Ehling-Schulz M, Ollilainen VM, Salkinoja-Salonen MS. 2006 Potential of selected infant food formulas for production of *Bacillus cereus* emetic toxin, cereulide. Int J Food Microbiol. 107:287-294.

Shatursky O, Bayles R, Rogers M, Jost BH, Songer JG, Tweten RK. 2000 *Clostridium perfringens* beta-toxin forms potential-dependent, cation-selective channels in lipid bilayers. Infect Immun. 68:5546-5551.

Sheppard JD, Jumarie C, Cooper DG, Laprade R. 1991 Ionic channels induced by surfactin in planar lipid bilayer membranes. Biochim Biophys Acta. 1064:13-23.

Shinagawa K, Ichikawa K, Matsusaka N, Sugii S. 1991 Purification and some properties of a *Bacillus cereus* mouse lethal toxin. J Vet Med Sci. 53:469-474.

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

Silo-Suh LA, Stabb EV, Raffel SJ, Handelsman J. 1998 Target range of zwittermicin A, an aminopolyol antibiotic from *Bacillus cereus*. Curr Microbiol. 37:6-11.

Silverman JA, Perlmutter NG, Shapiro HM. 2003 Correlation of daptomycin bactericidal activity and membrane depolarization in *Staphylococcus aureus*. Antimicrob Agents Chemother. 47:2538-2544.

Singh P, Cameotra SS. 2004 Potential applications of microbial surfactants in biomedical sciences. Trends Biotechnol. 22:142-146.

Stein T. 2005 *Bacillus subtilis* antibiotics: structures, syntheses and specific functions.Mol Microbiol. 56:845-857.

Suominen I, Andersson MA, Andersson MC, Hallaksela AM, Kämpfer P, Rainey FA, Salkinoja-Salonen M. 2001 Toxic *Bacillus pumilus* from indoor air, recycled paper pulp, Norway spruce, food poisoning outbreaks and clinical samples. Syst Appl Microbiol. 24:267-276.

Sutherland JP, Aherne A, Beaumont AL. 1996 Preparation and validation of a growth model for *Bacillus cereus*: the effects of temperature, pH, sodium chloride and carbon dioxide. Int J Food Microbiol. 30:359-372.

Suwan S, Isobe M, Ohtani I, Agata N, Mori M, Ohta M. 1995 Structure of cereulide, a cyclic dodecadepsipeptide toxin from *Bacillus cereus* and studies on NMR characteristics of its alkali metal complexes including a conformational structure of the K^+ complex. J Chem Soc Perkin Trans. I :765-775.

Takeda K, Kinumaki A, Hayasaka H, Yamaguchi T, Ito Y. 1978 Mutational biosynthesis of butirosin analogs. II. 3', 4'-Dideoxy-6'-N-methylbutirosins, new semisynthetic aminoglycosides. J Antibiot. 31:1031-1038.

Tamehiro N, Okamoto-Hosoya Y, Okamoto S, Ubukata M, Hamada M, Naganawa H, Ochi K. 2002 Bacilysocin, a novel phospholipid antibiotic produced by *Bacillus subtilis* 168. Antimicrob Agents Chemother. 46:315-320.

Taylor JM, Sutherland AD, Aidoo KE, Logan NA. 2005 Heat-stable toxin production by strains of *Bacillus cereus, Bacillus firmus, Bacillus megaterium, Bacillus simplex* and *Bacillus licheniformis*. FEMS Microbiol Lett. 242:313-317.

Teplova V, Jääskeläinen E, Salkinoja-Salonen M, Saris NE, Serlachius M, Li FY, Andersson LC. 2004 Differentiated Paju cells have increased resistance to toxic effects of potassium ionophores. Acta Biochim Pol. 51:539-544.

Teplova VV, Mikkola R, Tonshin AA, Saris NE, Salkinoja-Salonen MS. 2006 The higher toxicity of cereulide relative to valinomycin is due to its higher affinity for potassium at physiological plasma concentration. Toxicol Appl Pharmacol. 210:39-46.

Thaniyavarn J, Roongsawang N, Kameyama T, Haruki M, Imanaka T, Morikawa M, Kanaya S. 2003 Production and characterization of biosurfactants from *Bacillus licheniformis* F2.2. Biosci Biotechnol Biochem. 67:1239-1244.

Thimon L, Peypoux F, Exbrayat JM, Michel G. 1994 Effect of iturin A, a lipopeptide from *Bacillus subtilis* on morphology and ultrastructure of human erythrocytes. Cytobios.79:69-83.

Thimon L, Peypoux F, Maget-Dana R, Roux B, Michel G. 1992 Interactions of bioactive lipopeptides, iturin A and surfactin from *Bacillus subtilis*. Biotechnol Appl Biochem. 16:144-151.

Tilastokeskus (Statistics Finland) 2005 Asuntokanta 2004.

Toh M, Moffitt MC, Henrichsen L, Raftery M, Barrow K, Cox JM, Marquis CP, Neilan BA. 2004 Cereulide, the emetic toxin of *Bacillus cereus*, is putatively a product of nonribosomal peptide synthesis. J Appl Microbiol. 97:992-1000.

Tomoda H, Huang XH, Nishida H, Nagao R, Okuda S, Tanaka H, Omura S, Arai H, Inoue K. 1992 Inhibition of acyl-CoA:cholesterol acyltransferase activity by cyclodepsi-peptide antibiotics. J Antibiot. 45:1626-1632.

Toraya T, Maoka T, Tsuji H, Kobayashi M. 1995 Purification and structural determination of an inhibitor of starfish oocyte maturation from a *Bacillus* species. Appl Environ Microbiol. 61:1799-804.

Toure Y, Ongena M, Jacques P, Guiro A, Thonart P. 2004 Role of lipopeptides produced by *Bacillus subtilis* GA1 in the reduction of grey mould disease caused by *Botrytis cinerea* on apple. J Appl Microbiol. 96:1151-1160.

Trischmann JA, Jensen PR, Fenical W. 1994 Halobacillin: a cytotoxic cyclic acylpeptide of the iturin class produced by a marine *Bacillus*. Tetrahedron Lett. 35:5571-5574.

Turnipseed SB, Roybal JE, Pfenning AP, Gonzales SA, Hurlbut JA, Madson MR. 2001 LC/MS confirmation of ionophores in animal feeds. J AOAC Int. 84:640-647.

Van der Linde-Sipman JS, Van den Ingh TS, Van Nes JJ, Verhagen H, Kersten JG, Beynen AC, Plekkringa R. 1999 Salinomycin-induced polyneuropathy in cats: morphologic and epidemiologic data. Vet Pathol. 36:152-156.

Vater J, Kablitz B, Wilde C, Franke P, Mehta N, Cameotra SS. 2002 Matrix-assisted laser desorption ionization-time of flight mass spectrometry of lipopeptide biosurfactants in whole cells and culture filtrates of *Bacillus subtilis* C-1 isolated from petroleum sludge. Appl Environ Microbiol. 68:6210-6219.

Veith B, Herzberg C, Steckel S, Feesche J, Maurer KH, Ehrenreich P, Baumer S, Henne A, Liesegang H, Merkl R, Ehrenreich A, Gottschalk G. 2004 The complete genome sequence of *Bacillus licheniformis* DSM13, an organism with great industrial potential. J Mol Microbiol Biotechnol. 7:204-211.

Vollenbroich D, Pauli G, Ozel M, Vater J. 1997 Antimycoplasma properties and application in cell culture of surfactin, a lipopeptide antibiotic from *Bacillus subtilis*. Appl Environ Microbiol. 63:44-49.

Volpon L, Besson F, Lancelin JM. 1999 NMR structure of active and inactive forms of the sterol-dependent antifungal antibiotic bacillomycin L. Eur J Biochem. 264:200-210.

Volpon L, Besson F, Lancelin JM. 2000 NMR structure of antibiotics plipastatins A and B from *Bacillus subtilis* inhibitors of phospholipase A(2). FEBS lett. 485:76-80.

Wakayama S, Ishikawa F, Oishi K. 1984 Mycocerein, a novel antifungal peptide antibiotic produced by *Bacillus cereus*. Antimicrob Agents Chemother. 26: 939-940.

Wallace BA. 2000 Common structural features in gramicidin and other ion channels. Bioessays. 22:227-234.

Wang J, Liu J, Wang X, Yao J, Yu Z. 2004 Application of electrospray ionization mass spectrometry in rapid typing of fengycin homologues produced by *Bacillus subtilis*. Lett Appl Microbiol. 39:98-102.

Wang GYS, Kuramoto M, Yamada K, Yazawa K, Uemura D. 1995 Homocereulide, an extremely potent cytotoxic depsipeptide from the marine bacterium *Bacillus cereus.* Chem Lett. 9:791-792.

Wulff EG, Mguni CM, Mansfeld-Giese K, Fels J, Lübeck M, Hockenhull J. 2002 Biochemical and molecular characterization of *Bacillus amyloliquefaciens, B. subtilis* and *B. pumilus* isolates with distinct antagonistic potential against *Xanthomonas campestris* pv. *campestris* Plant Pathol. 51:574-584.

Yakimov MM, Timmis KN, Wray V, Fredrickson HL. 1995 Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniformis* BAS50. Appl Environ Microbiol. 61:1706-1713.

Yakimov MM, Kroger A, Slepak TN, Giuliano L, Timmis KN, Golyshin PN. 1998 A putative lichenysin A synthetase operon in *Bacillus licheniformis*: initial characterization. Biochim Biophys Acta. 1399:141-153.

Yakimov MM, Abraham WR, Meyer H, Laura Giuliano, Golyshin PN. 1999 Structural characterization of lichenysin A components by fast atom bombardment tandem mass spectrometry. Biochim Biophys Acta. 1438:273-280.

Yoshida S, Hiradate S, Tsukamoto T, Hatakeda K, Shirata A. 2001 Antimicrobial activity of culture filtrate of *Bacillus amyloliquefaciens* RC-2 isolated from mulberry leaves. Phytopathology. 91:181-187.

Yoshida Y. 1988 Cytochrome P450 of fungi: primary target for azole antifungal agents. Curr Top Med Mycol. 388-418.

Youssef NN, Knobletta J. 1998 Culture filtrate of *Bacillus atrophaeus* induced abnormalities in *Ascosphaera apis*. Mycologia. 90:937-946.

Yu GY, Sinclair JB, Hartman GL, Bertagnolli BL. 2002 Production of iturin A by *Bacillus amyloliquefaciens* suppressing Rhizoctonia solani. Soil Biol Biochem 7:955-963.

Yu SH, Possmayer F. 2003 Lipid compositional analysis of pulmonary surfactant monolayers and monolayer-associated reservoirs. J Lipid Res. 44:621-619.

Zampolli M, Meunier D, Sternberg R, Raulin F, Szopa C, Pietrogrande MC, Dondi F. 2006 GC-MS analysis of amino acid enantiomers as their N(O,S)-perfluoroacyl perfluoroalkyl esters: application to space analysis. Chirality. 18:279-295.

Zeleke JM, Smith GB, Hofstetter H, Hofstetter O. 2006 Enantiomer separation of amino acids in immunoaffinity micro LC-MS. Chirality. 18:544-550.

Zhang L, Dhillon P, Yan H, Farmer S, Hancock RE. 2000 Interactions of bacterial cationic peptide antibiotics with outer and cytoplasmic membranes of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother. 44:3317-3321.

Zhao YH, Le J, Abraham MH, Hersey A, Eddershaw PJ, Luscombe CN, Butina D, Beck G, Sherborne B, Cooper I, Platts JA. 2001 Evaluation of human intestinal absorption data and subsequent derivation of a quantitative structure-activity relationship (QSAR) with the Abraham descriptors. J Pharm Sci. 90:749-784.