2. Review of Literature
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2.1. Introduction

Mosquitoes are found throughout the world except in places that are permanently frozen. There are about 3,500 species, of which nearly three-quarters are native to the humid tropics and subtropics (Reiter, 2001). Mosquito-borne diseases are among the world's leading causes of illness and death. Despite great strides over the last 50 years, the World Health Organization estimates that more than 300 million clinical cases of mosquito-borne illnesses occur each year (Kalluri et al., 2007).

2.2. Mosquitoborne diseases

Malaria (Italian mala aria, bad air) is a protozoan (genus Plasmodium) infection transmitted by mosquitoes of the genus Anopheles. The four species of Plasmodium that infect humans appear to have evolved from a common ancestor during the early Tertiary period, some 60 million years ago (Garnham, 1963). Malaria is the world's most important parasitic infection, even with years of continual efforts, malaria is still one of the major causes of morbidity and mortality affecting third-world countries and still a threat to over 2 billion people, representing approximately 40% of the world's population in about 100 countries. Best estimates currently describe the annual global burden of malaria as 300-500 million cases and 1-2 million deaths (Na-Bangchang & Congpuong, 2007).

Lymphatic filariasis, a dreadful disease in humans and which afflicts more than 120 million people worldwide, is caused by the parasitic nematodes Wuchereria bancrofti, Brugia malayi and Brugia timori. The Indian subcontinent that comprises Bangladesh, India, Maldives, Nepal and Sri Lanka harbors 50 per cent of the world's lymphatic filarial disease burden. As per recent epidemiologic estimates on prevalence of W. bancrofti and B. malayi, about 428 million people are at risk, with 28 million microfilaria carriers and 21 million clinical cases spread out in 13 States and 5 Union Territories of India (Rao, 2005).
Dengue is an expanding health problem. About two-fifths of the world population is at risk of acquiring dengue with 50-100 million cases of acute febrile illness yearly including about 500,000 cases of DHF/DSS (Simasathien & Watanaveeradej, 2005). An outbreak of chikungunya virus is currently ongoing in many countries in Indian Ocean since January 2005. The current outbreak appears to be the most severe and one of the biggest outbreaks caused by this virus. India, where this virus was last reported in 1973, is also amongst the affected countries (Lahariya & Pradhan, 2006). Japanese encephalitis (JE)-epidemics has been reported in many parts of the country. The incidence has been reported to be high among pediatric group with high mortality (Kabilan et al., 2004).

2.3. Control of Mosquito borne diseases

Apart from chemotherapy, the mosquito borne diseases can be controlled by implementing vector control measures by means of applying insecticides thereby reducing the breeding potential of the mosquitoes. There was success in vector control between 1950 and 1970 but worldwide resistance followed it to organosynthetic insecticides wherever they were used intensively. Insect resistance to one or more categories of insecticides has limited the effectiveness of these compounds, and their non-selective mode of action adversely affects non-target organisms (Regis et al., 2001). For instance, global DDT spraying to control mosquito populations succeeded for only 8 years, as mosquito resistance appeared thereafter (Baird, 2000). As a result, synthetic chemical insecticides are being phased out in many countries and furthermore, many governments restrict chemical insecticide use owing to concerns over their environmental effects on non-target beneficial insects and especially on vertebrates through contamination of food and water supplies (Federici et al., 2003).

As a result, the World Health Organization facilitated the replacement of these chemicals with bacterial insecticides through the development of standards for their registration and use. The current interest in the development of biological agents for the control of vectors, especially mosquitoes is an indication of concern and sheer helplessness faced by the scientific community in the recrudescence of mosquito borne diseases like malaria, and dengue in epidemic proportions which was under control.
during fifties (Rajagopalan, 1981). The simplest definition for biological control is, "direct and indirect manipulation of natural enemies (pathogens, parasites and predators) to increase the incidence of mortality in the population under attack" (Laird, 1963). The different biological control agents being studied in different parts of the world for the control of vector mosquitoes includes, many naturally occurring predators, parasites, and pathogens of vector insects including fungi, and bacteria.

2.3.1. Bacterial Larvicidal agents

*Bacillus thuringiensis*

*Bacillus thuringiensis* (Bt) is a gram-positive bacterium that produces insecticidal crystal protein toxins during sporulation. *B. thuringiensis* was first discovered in diseased silkworms in 1901 (Ishiwata, 1901). In 1977, the first Bt strain, ONR-60A demonstrating a high level of larvicidal activity, was isolated from a mosquito-breeding pond in the Negev Desert of Israel and was found to be highly active towards dipteran larvae (Goldberg & Margalit, 1977). This strain was later identified as a new H antigenic type H-14 of *B. thuringiensis* and assigned the name subspecies *israelensis* (de Barjac, 1978). This ubiquitous spore forming bacterium was an eye-opener for the biologists to explore the potential of these crystalliferous bacteria in biological control of vector mosquitoes. Ever since, the discovery of the first Bt strain capable of killing mosquito larvae, several subspecies of Bt have been isolated from a range of environments, including insects, soil, dust from stored grain, and leaves of coniferous and deciduous trees (Martin & Travers, 1989; Smith & Couche, 1991; Meadows et al., 1992; Bernhard et al., 1997). There are several reports from different parts of the world about the occurrence of mosquitocidal strains belonging to different subspecies/serotypes. Thus, so far around 36 subspecies/serotypes of *B. thuringiensis* toxic to different species of mosquitoes were reported from different parts of the world (Balaraman, 2005). It contains four major proteins - Cyt1A (27.3 kDa), Cry4A (128 kDa), Cry4B (134 kDa) and Cry11A (72 kDa) - in three different inclusion types assembled into a spherical parasporal body held together by lamellar envelope (Ibarra and Federici, 1986).
**Bacillus sphaericus**

*Bacillus sphaericus* (Bs) is widespread in soil and aquatic environments (Manonmani et al., 1990). The mosquitocidal strain studied extensively belongs to serotype H5a5b (e.g., strains 1593 and 2362), which is highly toxic. In general, members of highly toxic groups have certain positive characteristics which are relevant to their use as microbial insecticides: their toxic crystals are protected within the exosporium, they are stable over a range of temperatures, and they may remain insecticidal in polluted water (Baumann et al., 1991; Berry et al., 1993). The major components of the crystal are two proteins i.e. the binary or Bin toxin of 51 and 42 kDa (Baumann et al., 1985). Neither protein alone is toxic to larvae nor both are required for toxicity (Baumann et al., 1987; Broadwell et al., 1990). In addition to the binary toxin, many strains of Bs produce other mosquitocidal toxins during vegetative growth that are referred to as Mtx toxins. But these are not as toxic as the Bin toxin (Delécluse et al., 2000). The target spectrum of Bs is restricted to mosquitoes, and its highest activity is against *Culex* and certain *Anopheles* species (Delécluse et al., 2000). Resistance to Bs has already been reported in field populations of *Culex* mosquitoes (Rao et al., 1995; Yuan et al., 2000; Nielsen-Leroux et al., 2002; Olivera et al., 2004).

**Bacillus alvei & Bacillus brevis**

From rice fields of Pondicherry, India two indigenous bacterial pathogens of mosquitoes viz. *Bacillus alvei* and *Bacillus brevis* were isolated from dead culicine larvae. The cell mass of these bacteria was highly effective against the larvae of *Culex fatigans*, *Anopheles stephensi* and *Aedes aegypti* (Balaraman et al., 1979). Recently Khyami Horani et al. (1999) isolated *B. brevis* toxic to *Culiseta longiareolata* (Diptera: Culicidae) from soil and water samples collected from Jordan.

**Bacillus circulans**

A new strain of *Bacillus circulans* isolated from a larva of *Cx. quinquefasciatus* showed larvicidal activity on 3 mosquitoes of medical importance. Compared to *B. sphaericus* strain 2362, this *B. circulans* isolate proved less toxic to *Cx. quinquefasciatus* and *Anopheles gambiae* but was 107 times more toxic to *Ae. aegypti*. The tests have
showed that the toxicity of the bacterial culture of *B. circulans* resulted from its spores and not from the insecticidal effect of chitinases or exotoxins (Darriet & Hugard, 2002)

**Brevibacillus laterosporus**

*Brevibacillus laterosporus* comb. nov. (Shida et al., 1996), previously classified as *Bacillus laterosporus* (Laubach & Rice, 1916), is an aerobic spore-forming bacterium that is characterized by its ability to produce a canoe-shaped lamellar parasporal inclusion adjacent to the spore. Some strains produce crystalline inclusions of various shapes and sizes, which are released separately from spores during lysis of the sporangium. *B. laterosporus* has the potential to be used as a biological control agent which, in comparison with strains of *B. thuringiensis* and *B. sphaericus*, demonstrates a very wide spectrum of biological activities. Toxicity towards larvae of the mosquitoes *Cx. quinquefasciatus* and *Ae. aegypti* has been reported (Rivers et al., 1991).

Despite showing such wide-ranging biological activities, *B. laterosporus* has not been seriously considered for use in biological control, most probably because the observed mosquitocidal activity is generally much weaker than that of *B. thuringiensis* ssp. *israelensis*. Yet Orlova et al. (1998) demonstrated that crystalliferous strains of *B. laterosporus* presented LC_{50} values similar to those attained with *B. thuringiensis* ssp. *israelensis* in bioassays employing larvae of three species of mosquitoes, with the larvicidal activity of *B. laterosporus* being associated with spores and crystalline inclusions.

**Bacillus subtilis**

*Bacillus subtilis* is a ubiquitous bacterium commonly recovered from water, soil, air and decomposing plant residue. The bacterium produces an endospore that allows it to endure extreme conditions of heat and dessication in the environment. *B. subtilis* produces a variety of proteases and other enzymes that enable it to degrade a variety of natural substrates and contribute to nutrient cycling. However, under most conditions the organism is not biologically active but exists in the spore form (Alexander, 1977).
As early as in 1989, Gupta and Vyas reported a strain of *B. subtilis* capable of infecting and causing mortality of larvae of *Anopheles culicifacies*, the primary vector of malaria in central India. Recently, Das and Mukherjee (2006) have reported the mosquito larvicidal activity of *B. subtilis* (DM-03 & DM-04) strains. The mosquito larvicidal activity is by the cyclic lipopeptides (CLPs) secreted by *B. subtilis* strains. The LC₅₀ of the crude CLPs secreted by *B. subtilis* DM-03 and DM-04 strains against third instar larvae of *Cx. quinquefasciatus* was 120.0±5.0 and 300.0±8.0 mg/l respectively post 24 h of treatment. Also, physico-chemical factors such as pH of water, incubation, temperature, heating and exposure to sunlight hardly influenced the larvicidal potency of these CLPs and were safe to Indian major carp *Labeo rohita*, a non-target aquatic organism.

*Clostridium bifermantans*

A nationwide screening program in Malaysia for microbial control agents of mosquitoes resulted in the isolation of *Clostridium bifermantans*, an anaerobe, by Lee & Seleena (1990). This new strain of *C. bifermantans*, individualized as serovar *malaysia* (C.b.m.) according to its specific H antigen is toxic to mosquito and blackfly larvae when given orally. The toxicity occurs in sporulated cells, which contain, in addition to spores, heat labile proteinaceous parasporal inclusion bodies and feather-like appendages. The amino acid content of the inclusion bodies is similar to that of *B. thuringiensis* ssp. *israelensis* and *B. sphaericus* crystals (de Barjac, 1990). Cells of *C. bifermantans* ssp. *malaysia* are safe for laboratory mammals and goldfish (Thiery et al., 1992).

Another strain of *C. bifermantans* toxic to mosquito larvae on ingestion was isolated from a soil sample collected from secondary forest floor (Seleena et al., 1997). This strain was designated as serovar *paraiba* (*C. b. paraiba*) according to its specific H antigen. *C. bifermantans paraiba* is most toxic to *Anopheles maculatus* Theobald larvae and its activity is as high as that of *B. thuringiensis* subsp. *israelensis*. 
2.3.2. **Bacterial Pupicidal agent**

**Pseudomonas fluorescens**

Since 1977, biological control of mosquito was carried out by biolarvicides and till 2002 none of the biological control agents were reported to be mosquito pupicidal i.e. the ability to kill the pupal stages of mosquitoes. The first bacterium known to exhibit mosquito pupicidal activity is a gram-negative bacterium *Pseudomonas fluorescens*. The metabolites were toxic to larvae and pupae of mosquitoes (Prabakaran et al., 2003).

A formulation was developed from the metabolite(s) of *P. fluorescens* Migula strain (VCRC B426) and tested against 4th-instar larvae and pupae of three species of vector mosquitoes, *An. stephensi* Liston, *Cx. quinquefasciatus* Say and *Ae. aegypti* (L). The larvae and pupae of *An. stephensi* were the most susceptible followed by those of *Cx. quinquefasciatus* and *Ae. aegypti*, and the dosage requirement for pupal mortality was less than that required for larval mortality. The LC50 dosage requirements for larvae of these mosquito species were, respectively, 70.4, 511.5 and 757.3 μg protein ml\(^{-1}\), whereas for pupae they were, respectively, 2.0, 9.4 and 19.2 μg protein ml\(^{-1}\). The lethal fraction was purified from the culture broth and its molecular mass, as determined by high performance liquid chromatography (HPLC), was 44kDa (Prabakaran et al., 2003). Further, an emulsifiable concentrate (EC) formulation developed from a metabolite of *P. fluorescens* was tested for efficacy against *Cx. quinquefasciatus* larvae and pupae under field conditions. At application rates of 100, 200, 300 ml/m\(^2\), the formulation caused 100% elimination of larvae and pupae at day 1 after treatments and >80% reduction in pupal density for periods of 7, 12 and 11 days in cesspits and 5, 9 and 10 days in U-shaped drains (Sadanandane et al., 2003).

2.4. **Bacterial diversity of mangrove swamps and sediments**

Estuarine, mangrove and coral reef environs in Gulf of Mannar was studied by Kannapiran et al. (1999) for the isolation of magnetotactic bacteria. Totally 37 strains were isolated with predominance of *Bacillus* spp. followed by *Pseudomonas* spp. *Spirillum* spp., and *Vibrio* spp. Based on the fatty acid profile, a few of the strains were
identified as *Pseudomonas mesophilico, Pseudomonas caryophylli* and *Bacillus cereus*. The sediments of the mangrove swamps of Cochin showed the presence of *Aeromonas* sp., *Alcaligenes* sp., *Bacillus* sp., *Flavobacterium* sp. *Micrococcus* sp., *Pseudomonas* sp. and *Vibrio* sp (Joseph & Chandrika, 2000).

### 2.5. Bacterial diversity of mangroves of Andaman & Nicobar Islands

The mangroves of Andaman & Nicobar Islands constitute 9.4% of land area and ~10.95% of the forest cover of these islands (Bandyopadhyay, 1991). The fauna and flora of these islands was studied by Sinha (1994).

Shome et al. (1995) investigated the bacterial flora of mangrove litter fall and underneath sediments from South Andaman. Thirty-eight bacterial isolates were obtained from *Rhizophora, Avicenia* and *Nypa* species inhabited areas. The cultural, morphological and biochemical features revealed that most of the isolates belong to *Bacillus* spp (50%). In addition *Aeromonas, Vibrio, Escherichia, Enterobacter, Corynebacterium, Kurthia, Staphylococcus, Micrococcus* and *Listeria* were also present. Most of the isolates were gram positive (76.3%), motile (87%) and fermentative bacteria ranging from 6.9% for dulcitol to 82.1% for dextrose.

Serpentine soils collected from Saddle Hills, Chidyatapu and Rutland of Andaman Islands, India were analyzed for physico-chemical and microbiological characteristics and compared with those from adjacent non-serpentine localities. The serpentine soils contained high levels of nickel (1740.0 - 8033.4 mg/kg dry soil), cobalt (93.2 - 533.4 mg/kg dry soil) and chromium (302.9 - 4437.0 mg/kg dry soil), in addition to 62-152 g of iron and 37-60 g of magnesium per kg dry soil. Characteristically the serpentine soils showed low microbial density (6.2-11.3 × 10^6 colony forming unit/g soil) and activity (1.7-3.5 μg fluorescein/g dry soil/h) than non-serpentine outcrops. Serpentine microbial population was dominated by bacteria, which represented 5.12 to 9.5 × 10^6 cfu/g of soil, while the fungal population ranged from 0.17 to 3.21 × 10^6 cfu/g of soil (Pal et al., 2005).
2.6. Mosquitocidal bacteria from mangrove

*C. bifermentans* and *B. thuringiensis* subsp. *israelensis/tochigiensis* were isolated from mangrove swamps and mangrove sediments of Malaysia and Japan (de Barjac et al., 1990; Maeda et al., 2001). In the light of this, the mangrove forests of Andaman-Nicobar Islands were explored for the presence of mosquitocidal bacteria. Though there are few reports on the microbial diversity of these islands, there are no studies on the mosquitocidal bacterial flora of these islands.

The present investigation relates to the isolation, identification of *B. subtilis* from mangrove forests and the characterization of its mosquitocidal toxins.

2.7. Biology of *B. subtilis*

2.7.1. Taxonomy and Characterization

The genus *Bacillus* is a large and heterogeneous collection of aerobic or facultatively anaerobic, rod shaped, endospore-forming bacteria that are widely distributed in the environment. Many kinds of species belong to this genus. They are known to have acidophilic, alkalophilic, thermophilic or other properties (Goto et al., 2000). The genus *Bacillus* encompasses 203 validly described species (http://www.bacterio.cict.fr/b/bacillus.html) exhibiting a wide range of nutritional requirements, physiological and metabolic diversity and DNA base composition (Claus & Berkeley, 1986).

*B. subtilis* is a ubiquitous soil microorganism that contributes to nutrient cycling due to the various enzymes produced by members of the species. Although the actual numbers in existence in the environment has not been determined. This bacterium occurs at population levels of $10^6$ to $10^7$ per gram of soil (Alexander, 1977). However, unless a soil has been recently amended with organic matter providing readily utilizable nutrients, the bacterium exist in the endospore stage. About 60 to 100% of soil bacilli populations exist in the inactive spore state (Alexander, 1977).
Historically, prior to the monographs of Smith in 1946 and 1952, *B. subtilis* was a term given to all aerobic endospore-forming bacilli (Logan, 1988). The *Bacillus* species *subtilis, licheniformis* and *pumilus* are closely related and there has been difficulty in distinguishing among the three species that historically were grouped together as the subtilis-group or subtilis-spectrum (Gordon, 1973). These three species clustered together (78%) in the "*subtilis*" group in a numerical classification based on 118 unit characteristics of 368 strains of *Bacillus* (Priest et al., 1988). The reclassification of genus *Bacillus*, which began in 1991, revealed at least eight genera: *Alicyclobacillus* (Wisotzkey et al., 1992), *Aneurinibacillus* (Shida et al., 1996), *Bacillus, Brevibacillus* (Shida et al., 1996), *Gracilibacillus* (Waino et al., 1999), *Paenibacillus* (Ash et al., 1991, 1993), *Salibacillus* (Waino et al., 1999) and *Virgibacillus* (Heyndrickx et al., 1999). Since these eight genera consist of more than 100 species that have similar characteristics, identifying them is difficult. The identification of *Bacillus* species has been performed mainly with morphological and physiological criteria, and this method is widely employed in various fields. However, the process used requires skillful techniques and is very complex and time-consuming. Hence, the reliance on only biochemical-based identification could lead to inaccurate identification of the genus *Bacillus*.

2.7.2. Molecular Taxonomy

With the advance of genetic engineering, the randomly amplified polymorphic DNA (RAPD) method (Zahner et al., 1999), the hybridization method (te Giffel et al., 1997) and restriction mapping (Henderson et al., 1995) were adapted for the identification of *Bacillus* species. Recently MALDI-TOF-MS and oligonucleotide microarrays are also being employed for the identification (Wang et al., 2002; Bavykin et al., 2007).

The utility of the rRNA sequence as a taxonomic tool has been amply demonstrated in bacteria, where 16S rRNA sequence analyses have completely redefined phylogenetic relationships previously too dependent on cellular metabolism (Fox et al., 1980; Lane et al., 1985; Woese, 1987; Woese & Fox, 1977). In addition to highly
conserved areas that have been used to study the relationships among distant taxa, the 16S sequence contains more variable regions that have been useful in the differentiation of genera and species (Goebel 1987). This differentiation has been accomplished through the use of probes which have been generated by using conserved 16S sequences as universal primers for polymerase chain reaction (PCR) amplification (Saiki et al., 1988) of certain variable 16S regions (Barry et al., 1990).

16S rDNA probes have been used for the identification of campylobacters (Gorkiewicz et al., 2003), gram positive cocci (Song et al., 2003), Klebsiella (Li et al., 2004), Nocardia (Roth et al., 2003), Leuconostoc (Kim et al., 2003), Streptomyces (Park et al., 2003), Clostridia (Song et al., 2002), Vibrio (Spragano et al., 2002) and also for Bacillus sps. (Goto et al., 2000, Wattiau et al., 2001, Nair et al., 2002; Blackwood et al., 2004).

DNA base (GC) composition of species within a genus should not differ by more than 10 to 12 % mol GC. Nonetheless, values within the Bacillus genus ranged from 33 to 65 % mol GC in 1993, although many of the species did cluster at 40 to 50 % mol G_C (Priest, 1993). Subsequently, recent phylogenetic analyses have reclassified some of the Bacillus species into new genera, including Paenibacillus, Geobacillus, and Brevibacillus (Ash et al., 1993; Xu & Cote, 2003). It has been reported that there are two subspecies within B. subtilis viz. B. subtilis subsp. subtilis and B. subtilis subsp. spizizenii, which share phenotypic profiles but are segregated based on DNA reassociation values of 58 to 69%, in addition to minor polymorphisms in the 16S rRNA gene between the type strains (Nakamura et al., 1999). Due to these recent advances, it has become increasingly difficult to classify species within the Bacillus genus, as many share similar physiology, metabolism, and morphology as well as highly conserved 16S rRNA genes.

The use of a conserved, housekeeping gene necessary for the survival of the organism was reported as the desirable alternative in molecular taxonomy. Protein coding genes exhibit much higher genetic variation than 16S rRNA gene and can be used for classification and identification of closely related taxa (Mollet et al., 1997; Kim et al., 1999; Yamamoto & Harayama, 1995). Chun and Bae (2000) demonstrated the use of
**gyrA** sequences (coding for DNA gyrase subunit A) for accurate classification of *Bacillus subtilis* and related taxa, including *Bacillus amyloliquefaciens, Bacillus vallismortis, Bacillus mojavensis, Bacillus atrophaeus* and *Bacillus licheniformis*. Apart from *gyrA* gene, *rpoB* gene, is described as possessing the same key attributes as 16S rDNA, in that it is common to all bacteria and is a mosaic of conserved as well as variable sequence domains (Dahllof et al., 2000). Most importantly, the *rpoB* gene exists as a single copy in bacterial genomes (Mollet et al., 1997). The RpoB protein has been used to infer relationships between archaeal orders (Matte-Tailliez et al., 2002) and Gram-positive and Gram negative bacteria (Morse et al., 2002).

### 2.7.3. Bioactive metabolites of B. subtilis

The rhizobacterium *B. subtilis* has been used for genetic and biochemical studies for several decades and is regarded as paradigm of Gram-positive endospore-forming bacteria (Moszer et al., 2002). The potential of *B. subtilis* to produce antibiotics has been recognized for 50 years. Several hundred wild-type *B. subtilis* strains have been collected, with the potential to produce more than two dozen antibiotics with an amazing variety of structures. If all pathways are considered, their production requires more than 350 kb (NRPSs, 200 kb; PKSs, 76 kb; lantibiotics, 50 kb; others > 20 kb), corresponding to a remarkable 10% of the annotated ORFs. It should be emphasized that all investigated *B. subtilis* strains produce individual antibiotic cocktails encompassing only a portion of the compounds depicted above; the average of a *B. subtilis* genome that is devoted to antibiotic production is about 4–5% of 4.2- Mbp (Kunst et al., 1997; Stein, 2005). Peptide antibiotics represent the predominant class. They exhibit highly rigid, hydrophobic and/or cyclic structures with unusual constituents like D-amino acids and are generally resistant to hydrolysis by peptidases and proteases (Katz and Demain, 1977).

#### 2.7.3.1. Lipopeptides

*B. subtilis* produces various lipopeptides (Table. 1). Lipopeptides consist of a peptide part containing 7-11 amino acids, either cyclic or linear or a combination of both. Beta hydroxy or beta amino fatty acids form the lipid part connected to the peptide backbone. Thus lipopeptides are amphphilic, in which acid or basic amino acids serve as
### Table 1. Lipopeptides produced by *Bacillus subtilis*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Class (based on structure)</th>
<th>Biological activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillomycins</td>
<td>Cyclic lipoheptapeptide</td>
<td>Fungicidal &amp; Hemolytic</td>
<td>Besson et al., 1977; Besson &amp; Michel, 1988</td>
</tr>
<tr>
<td>Bacillopeptin</td>
<td>Cyclic lipoheptapeptide</td>
<td>Antifungal &amp; hemolytic</td>
<td>Keneda &amp; Kajimura 2002 Kajimura et al., 1995</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>Cyclic heptapeptide</td>
<td>Antibacterial</td>
<td>Azevedo et al., 1993</td>
</tr>
<tr>
<td>Bacilysocin</td>
<td>Phospholipid</td>
<td>Fungicidal</td>
<td>Tamehiro et al., 2002</td>
</tr>
<tr>
<td>Fengycin</td>
<td>Cyclic lipodecapeptide</td>
<td>Antifungal</td>
<td>Vanittanakom et al., 1986; Koumoutsi et al., 2004</td>
</tr>
<tr>
<td>Iturin A</td>
<td>Cyclic lipoheptapeptide</td>
<td>Antibacterial, Fungicidal &amp; hemolytic</td>
<td>Besson et al., 1976 ; 1978</td>
</tr>
<tr>
<td>Mycobacillin</td>
<td>Cyclic tridecapeptide</td>
<td>Fungicidal &amp; hemolytic</td>
<td>Majumdar &amp; Bose, 1958</td>
</tr>
<tr>
<td>Mycosubtilin</td>
<td>Cyclic lipooctapeptide</td>
<td>Fungicidal &amp; hemolytic</td>
<td>Peypoux et al., 1976; Besson &amp; Michel, 1989</td>
</tr>
<tr>
<td>Plipastatin</td>
<td>Cyclic lipodecapeptide</td>
<td>Antifungal</td>
<td>Tsuge et al., 2007</td>
</tr>
<tr>
<td>Rhizoctin-A</td>
<td>Phosphono-oligopeptide</td>
<td>Antifungal</td>
<td>Kugler et al., 1990</td>
</tr>
<tr>
<td>Surfactin</td>
<td>Cyclic lipoheptapeptide</td>
<td>Antibacterial, biosurfactant</td>
<td>Arima et al., 1968; Vater et al., 2002</td>
</tr>
</tbody>
</table>
polar groups and fatty acids partly as neutral hydrophobic moieties. The hydrocarbon length of the fatty acids and amino acid composition may vary, depending on the nutrition of the bacteria and this may 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 activities. Both the phospholipids of the biomembranes and the lipopeptides have amphiphilic 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 ions elective pores depends on the nature of the lipopeptides and on the phospholipids of the membranes.

2.7.3.2. Lipopeptides of the surfactin family

Lipopeptides of the surfactin family includes surfactin, lichenysins and pumilacidins. These peptides are produced by strains of several Bacillus species and are powerful biosurfactants. Since the discovery of surfactin in sixties (Arima et al., 1968) and its synthesis in nineties (Nagal et al., 1996), its properties have been studied widely and interesting applications in industry and medical field have been proposed (Singh and Cameotra, 2004). Surfactin contains a heptapeptide (Glu1-Leu2-Leu3-Val4-Asp5-Leu6-Leu7) (Fig. 1).

2.7.3.3. Surfactin

The history of surfactin dates back to 1968, when Arima et al. reported the presence of a new biologically active compound in the culture broth of a B. subtilis strain. It was named surfactin because of its exceptional surfactant activity and its structure was elucidated as that of a macrolide lipopeptide (Kakinuma et al., 1969). Although other lipopeptides have been discovered since then, surfactin remains the main representative of the family. It is only from 1980 surfactin has drawn the attention of several researchers, as an attractive alternative or supplement to chemical surfactants that have a detrimental effect on the environment.
Figure 1. Structure of Surfactin

\[ \text{C}_{100}\text{H}_{134}\text{N}_{12}\text{O}_{29} \cdot 4\text{H}_2\text{O} \]

(I)

3\((R)\)-OH-Dec-\(d\)-Leu-\(d\)-Asp-\(d\)-allo-Thr-\(d\)-Leu-\(d\)-Leu-\(d\)-Ser-\(L\)-Leu-\(L\)-Gln-\(L\)-Leu-\(L\)-Ile-\(L\)-Asp
2.7.3.4. Structure of surfactin

This compound has been characterized as a cyclic lipopeptide containing a carboxylic acid (3-hydroxyl-13-methyl tetradecanoic acid) and seven amino acids (Kakinuma et al., 1969a, 1969b). This peptide is glutamic acid (N-bounded to the carboxylate of the fatty acid) L-leucine-D-leucine-L-valine-L-aspartic acid-D-leucine-L-leucine (bounded to the 3 hydroxy function) (Cooper et al., 1981). A lactone bridge between the beta-hydroxyl function of the acid and the carboxy-terminus function of the peptide confers a cyclic structure to this molecule (Fig. 1). Natural surfactin is a mixture of isoforms with differing molecular weight (Vater et al., 2002) and the commercial surfactin supplied by Sigma, (St Louis, USA) has the molecular weight of 1,036 Daltons.

2.7.3.5. Isoforms of surfactin

The isoforms of surfactin differ in their physicochemical properties due to variation in the chain length and branching of its hydroxyl fatty acid component (Hosano & Suzuki, 1983; Peypoux et al., 1994) as well as substitutions of the amino acid components of the peptide ring (Bonmatin et al., 1994; Itokawa et al., 1994). These variations rather than being genetically determined, depend on the specific B. subtilis strain and the nutritional and environmental conditions (Peypoux and Michel, 1992). When B. subtilis S 499 was grown on a culture medium containing L-alanine as nitrogen source, a mixture of surfactins was obtained. It contained a peptide sequence, which differs from that of standard surfactin by the replacement of the L-valine residue by L-alanine residue in position 4 (Peypoux et al., 1994). Kowall et al. (1998) separated isoforms of surfactin from B. subtilis OKB 105 by reverse phase HPLC and characterized them by NMR and MS methods. Gao et al., (2003), studied B. subtilis B2 strain and the results indicated that the surfactin isoforms of B2 strain was cyclic lipodecapeptide L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu containing fatty acid with a side chain length of 13, 14 and 15 carbon atoms. Recently, Bacillus coagulans has been found to produce several surfactins. Four main components with molecular weights 1007, 1021 and 1035 Da were separated. Their structures have been confirmed by spectrometric and spectroscopic studies and by acid hydrolysis. The compounds were found to represent two pairs of surfactin isoforms in which beta-hydroxy-iso-C14 or anteiso-C15 fatty acids
are linked to the [Leu7] or [Val7] heptapeptide moiety by both amide group and a lactone bond (Huszcza & Burczyk, 2006).

2.7.3.6. **Biosynthesis of surfactin**

Two mechanisms for the biosynthesis of peptides are known to exist in bacteria and fungi,

(i) the ribosomal synthesis of linear precursor peptides that are subjected to post-translational modification and proteolytic processing

(ii) the non-ribosomal synthesis of peptides by large megaenzymes, the non-ribosomal peptide synthetases (NRPSs)

A number of peptides with highly variable structures and a broad range of biological activities are produced in bacteria and fungi as secondary metabolites via non-ribosomal mechanism (Guenzi et al., 1998). The utilization of the thiotemplate mechanism for non-ribosomal synthesis of peptides was first described by Lipmann (1980) and has recently been reviewed by several authors (Sieber & Marahiel, 2003; Finking & Marahiel, 2004; Walsh, 2004). The synthesis involves large multienzymes, the Non Ribosomal Peptide Synthetases (NRPSs) that are composed of modularly arranged catalytic domains, which catalyse all necessary steps in peptide biosynthesis.

Genetic studies were undertaken to identify the genes required for the production of surfactin (Nakano et al., 1988; Nakano & Zuber, 1989; Nakano et al., 1991; Tsuge et al., 1996; Quadri et al., 1998). Surfactin biosynthesis is catalysed non-ribosomally by the action of a large multienzyme complex consisting of four modular blocks, called surfactin synthetases (Fig. 2) (Bruner et al., 2002). The surfactin synthetases, SrfA-A, SrfA-B and SrfA-C, are encoded by the first three genes of the 25 kb srfA operon (Schneider & Marahiel, 1998). The unimodular enzyme SrfA-C, bears a thioesterase domain, SrfA-C-TE, located at its C-terminal end (Hsieh et al., 2004). The mechanism of surfactin excretion is not known, as an active transporter has not been found, implying passive diffusion across the cytoplasmic membrane (Stein, 2005)
Figure 2. Surfactin biosynthesis by the modular peptide synthetase

(A) The srf operon (top) with the three genes srfA-A, srfA-B, and srfA-C coding for the surfactin synthetase subunits shown below the genes. Bars indicate the positions of modules within the protein, whereas the individual domains are shown as colored balls: A, adenylation domain; PCP, peptidyl carrier protein domain; C, condensation domain; E, epimerization domain; TE, thioesterase domain. The 4-phosphopantetheinyl cofactors with their active thiol groups are shown with the corresponding peptides attached at their current synthesis states. The growing peptide chain is passed from left to right, until the linear product at the last PCP domain is cyclized to the lipopeptide by the TE domain.

(B) The SNAC (S-N-acetyl cysteamine) acyl peptide mimic can be cyclized by the genetically excised SrfTE domain. The native peptide (R DLeu) and the soluble substrate (R DOrn) are illustrated. The -hydroxy fatty acid (FA) is likely to be attached by the N-terminal C domain.
2.7.3.7. **Biological properties of surfactin**

Surfactin, produced by *B. subtilis* was first identified as a potent inhibitor of fibrin clotting (Arima et al., 1968) and later found to lyse erythrocytes as well as spheroplasts and protoplasts of some bacterial species (Bernheimer & Avigad, 1970). Surfactin is antibacterial, antitumoral and hypocholesterolemic agent (Tsukagoshi et al., 1970; Kameda et al., 1972; Imai et al. 1971). It is the most powerful biosurfactant known lowering the surface tension of water from 72 to 27 mN/m\(^2\) (Cooper et al., 1981). Biosurfactants provide an advantage over synthetic surfactants because most are biodegradable and, hence, less toxic (Nakano et al., 1988). Since the biosurfactants are natural compounds they have industrial importance. They are distinguished by excellent surface- and membrane-active properties along with superior emulsifying and foaming properties that can be utilized in food biotechnology and in the agricultural sector.

Diverse activities have been demonstrated by surfactin, including emulsification, foaming (Razafindralambo et al., 1993), inhibition of starfish oocyte maturation (Toraya et al. 1995), antiviral and antimycoplastic activities (Vollenbroich et al., 1997). Viruses, which are covered by a lipoprotein membrane, such as herpes- and retroviruses, are efficiently inactivated by this biosurfactant. Surfactin showed insecticidal activity against the fruit fly *Drosophila melanogaster* (Assie et al., 2002).

Platelet aggregation was inhibited and the density of platelet-rich plasma (PRP) clots was decreased by the preincubation of PRP with surfactins. The findings suggest that surfactins are able to prevent a platelet aggregation leading to an inhibition of additional fibrin clot formation, and to enhance fibrinolysis with facilitated diffusion of fibrinolytic agents (Lim et al., 2005).

On top of such properties, surfactin can differentiate viral cells from *Mycoplasma* cells and proposals have been made to employ it to ensure the safety of pharmaceutical products (Nissen et al., 1997; Vollenbroich et al., 1997). Kumar et al. (2007) reported that using surfactin, mycoplasma were eliminated from an extensively infected irreplaceable hybridoma cell line. There were apparent indications of limited elimination, suggesting
the possible usefulness of surfactin in achieving decontamination. The effect of surfactin on the proliferation of LoVo cells, a human colon carcinoma cell line, was examined by Kim et al. (2007). Surfactin strongly blocked the proliferation of LoVo cells by inducing pro-apoptotic activity and arresting the cell cycle. The results suggested that surfactin might have anti-cancer properties as a result of its ability to down-regulate the cell cycle and suppress its survival. Whang et al. (2007) investigated potential application of two biosurfactants, surfactin (SF) and rhamnolipid (RL), for enhanced biodegradation of diesel-contaminated water and soil. The results confirmed their diesel biodegradation efficiency in diesel/soil systems.