Lipopeptides: Status and Strategies to Control Fungal Infection

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Abstract

Global food security is become central focus, specifically because of threatening plant diseases caused by fungal phyto-pathogens and massive economic losses thereof. In the context of bio-control of fungal phyto-pathogens, lipopeptides produced by *Bacillus* sp. have been studied well. The three families of *Bacillus* lipopeptides are surfactin, iturins and fengycins, confirmed for their antagonistic activities against various fungal phyto-pathogens. In recent past lipopeptides produced by *Pseudomonas* sp. has also proven effective bio-control agents, specifically against fungal phyto-pathogens. On other hand echinocandins are novel class of anti-fungal lipopeptides produced by various *Aspergillus* sp., used successfully in treatment of serious fungal infections and currently in clinical trials. Here we summarized all available information and data of lipopeptides in focus of their use as bio-control agent for plant protection as well as in treatment of fungal diseases in human caused by different pathogenic fungi.

4.1 Introduction

Host defense peptides or antimicrobial peptides (AMPs) play an essential protective role in the innate immune system of all organisms. These antimicrobial peptides are multifunctional compounds with multiple utilities (Franco 2011). The increasing resistance of bacteria and fungi to multiple antibiotics is a major public health concern worldwide that leads to enormous efforts for developing new antibiotics with new modes of action. Two promising families of drugs that meet these criteria are antimicrobial peptides (AMPs) and lipopeptides (Boman 1998; Zasloff 2002). Lipopeptides differ from AMPs as the earlier are produced only in bacteria and fungi but later is produced by all forms of life. Lipopeptides are small molecules produced during cultivation on various carbon sources. They are formed as short linear peptides linked with a lipid tail or other lipophilic molecules to form cyclic structures (Arnusch et al. 2012; Raaijmakers et al. 2010; Mandal et al. 2013).

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Polymyxin A was the first antimicrobial lipopeptide described in detail. It was isolated in the year 1949 from the bacterium *Bacillus polymyxa* (Mandal et al. 2013) which is reclassified as *Paenibacillus polymyxa*. However, the effective lipopeptide production as a bio-surfactant was first reported from *Bacillus subtilis* IAM1213 (Jones 1949). Since then, various types of antimicrobial lipopeptides have been isolated from other *Bacillus* strains and their biosynthesis and antimicrobial activity studied in detail (Arima et al. 1968; Peypoux et al. 1984; Grangemard et al. 1999; Rivardo et al. 2009; Tareq et al. 2014). Other genera like *Pseudomonas*, *Streptomyces*, and *Aspergillus* representing Gram-negative bacteria, actinobacteria, and fungi are also reported for lipopeptide production (Cochrane and Vederas 2014; Morikawa et al. 2000; Higginbotham and Murphy 2010; Rao et al. 2013; Nielsen and Sørensen 2003). Recently, lipopeptides have received great attention for their cytotoxic, antitumor, immunosuppressant, and surfactant properties apart from antimicrobial property (Sørensen et al. 2001; Cameotra and Makkar 2004; Donadio et al. 2007; Song et al. 2011). Some of them like daptomycin (Song et al. 2014), caspofungin (Robbel and Marahiel 2010), micafungin (Ngai et al. 2011), and anidulafungin (Emiroglu 2011) have even reached the market with antibiotic status. Lipopeptides showed remarkable membrane-active and surface-interface properties resulting in a number of excellent biological activities, which are of great relevance in health care and biotechnology-based processes. These properties make lipopeptides as potent candidate drugs for the resolution of a number of global issues in medicine industry and environmental protection (George and Reboli 2012).

4.1.1 Origin and Structural Diversity of Lipopeptides

4.1.1.1 Lipopeptides Produced by Gram-Positive Bacteria (*Bacillus* sp.)

Broadly there are three types of lipopeptides, namely, surfactin, iturin, and fengycin, that are produced by various *Bacillus* species.

4.1.1.1.1 Iturin

Iturins usually have a molecular mass of ~1.1 kDa. They consist of two major parts: a peptide part composed of short amino acid sequence and carbon hydrophobic tail. This composition clearly indicates an amphiphilic character of these compounds and suggests cell membrane-based mechanism of action (Banat et al. 2010). The iturins are cyclic peptides containing seven amino acids (heptapeptide) linked to a fatty acid (β-amino) chain that varies between C14 and C17 carbon molecules. Bacillomycin D and bacillomycin L are important members of the iturin family and peptide-lipid antibiotics. Structural determination indicated that bacillomycins D and L consist of a heptapeptide chain linked to a liposoluble β-amino acid. Further aspartyl, glutamyl, asparaginyl, and glutarninyl amino acids were found as peptidic moieties (Arima et al. 1968; Stachelhaus et al. 1995; Moyne et al. 2001). Bacillomycin Lc, a new antifungal antibiotic of the iturin class, was isolated from a strain of *Bacillus subtilis*. The structure determined by chemical and spectrometric analyses showed differences from bacillomycin L. It also exhibited sequence changes from aspartate-1 to asparagine-1 and from glutamine-5 to glutamate-5 (Tenoux et al. 1991). Bacillomycin F, another variant, also displayed differences with other members of the iturin family. In fact, it is a mixture of homologous peptide-lipids, essentially containing $C_{51}H_{80}N_{12}0_{14}$ and $C_{52}N_{82}N_{12}0_{14}$. The lipid moiety consists of minor *iso*C_{I5}, *anteiso*C_{15} β-amino acids, and major *iso*C_{16}, *iso*C_{17} and *anteiso*C_{17} p-amino acids (Eshita et al. 1995). Mycosubtilin, a peptide-lipid antibiotic, was isolated from *Bacillus subtilis*. It was identified as a cyclopeptide composed of seven α-amino acids in an LDDLLDL sequence closed by a β-amino acid linkage similar to that found in other antibiotics of the iturin group (Peypoux et al. 1985). The unique biological and physicochemical properties make these molecules interesting for biocontrol, food, and pharmaceutical applications (Fig. 4.1).

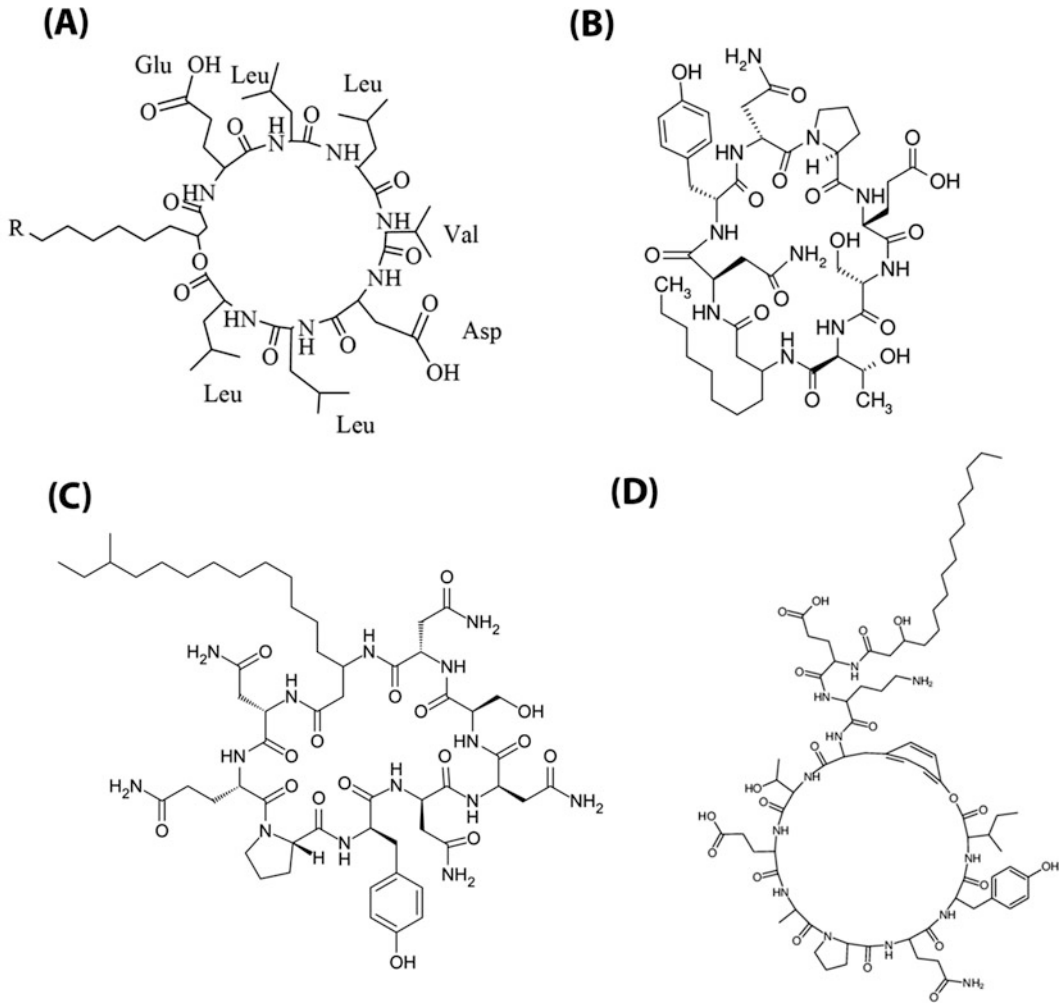

Fig. 4.1 Structure of different lipopeptides produced by *Bacillus* sp. (**a**) Surfactin (Peypoux et al. 1986). (**b**) Bacillomycin D (Liu et al. 2008). (**c**) Mycosubtilin (Peypoux et al. 1985). (**d**) Fengycin/plipastatin A (Peypoux et al. 1981)

4.1.1.1.2 Surfactin

Surfactin (~1.36 kDa) is an amphipathic cyclic heptapeptide composed of Glu-Leu-Leu-Val-Asp-Leu-Leu (ELLVDLL) with the chiral sequence LLDLLDL interlinked with β-hydroxy fatty acid with the chain length of 12–16 carbon atoms to form a cyclic lactone ring structure (Jacques 2011). The order of amino acids and the size of lipid moiety differ in a variety of surfactins (Tsan et al. 2007). In surfactin molecules while the hydrophobic amino acids are located at positions 2, 3, 4, 6, and 7, the Glu and Asp residues are located at positions 1 and 5, respectively. Usually, surfactin isoforms coexist in the cell as a mixture of several peptidic variants with different aliphatic chain lengths and differ in their biological properties accordingly (Roongsawang et al. 2002). However, the culture conditions decide the pattern of amino acids and β-hydroxy fatty acids in the surfactin molecules (Raaijmakers et al. 2010). The β-turn may be formed by an intramolecular hydrogen bond; thus, the β-sheet may depend on an intermolecular hydrogen bond (Fzb et al. 2004). The lipopeptide lichenysin produced by *B. licheniformis* structurally resembles surfactin

of *B. subtilis*, but the glutamic acid at position 1 in surfactins is replaced by glutaminyl residue in lichenysins. This local variation caused significant changes in various properties of the molecule compared to surfactin. Lichenysin exhibits superior surfactant power, low critical micellar concentration, and higher hemolytic activity. Lichenysin is also a better chelating agent because of increased association constants with Ca^{2+} and Mg^{2+} by a factor of 4 and 16, respectively (Peypoux et al. 1984; Romero et al. 2007a). Pumilacidins A, B, C, D, E, F, and G are cyclic acyl heptapeptide composed of a β-hydroxy fatty acid, 2 L-leucine, 2 D-leucine, L-glutamic acid, L-aspartic acid, and L-isoleucine (or L-valine) (Grangemard et al. 2001). They were first isolated from the culture broth of a strain of *B. pumilus*.

A surfactant named BL-86 is produced by *B. licheniformis* strain 86. It is a mixture of lipopeptides with the major component size ranging between 979 and 1,091 Da with variation in increments of 14 Da. The variation in molecular weight represents changes in the number of methylene groups of the lipid and/or peptide portion of the surfactant. It showed an ester carbonyl structure, which could be a part of a lactone ring connecting the β-position of the lipid to one of the carbonyl groups in the peptide (Naruse et al. 1990).

4.1.1.1.3 Fengycin

Fengycin represents the third family of lipopeptides after surfactin and iturin and is also called plipastatin (Akpa et al. 2001). These are bioactive lipopeptides produced by several strains of *B. subtilis* F-29-3. Fengycins effectively inhibit the growth of filamentous fungi but are ineffective against yeast and bacteria. The inhibition is antagonized by sterols, phospholipids, and oleic acid; however, addition of two other unsaturated fatty acids is found to increase the antifungal effect. Fengycins are broadly classified into two groups that differed by one amino acid. Fengycin A is composed of 1 D-Ala, 1 L-Ile, 1 L-Pro, 1 D-allo-Thr, 3 L-Glu, 1 D-Tyr, 1 L-Tyr, and 1 D-Orn, whereas fengycin B D-Val was replaced by D-Ala (Horowitz and Griffin 1991; Akpa et al. 2001). These bioactive molecules are lipodecapeptides containing a lactone ring with β-hydroxy fatty acid chain (either saturated or unsaturated). As mentioned the structure of fengycin contains a peptide chain of ten amino acids, cyclic decapeptides formed by lactonization (Il et al. 2010). The fatty acid moieties vary from C14 to C17 carbon atoms. The presence of different fatty acids yields different homologous compounds and isomers. Members of fengycin family also exhibit heterogeneity at the sixth position in peptide moiety. Based on variations in single amino acid (at the sixth position), fengycins are classified into two classes, namely, fengycin A and fengycin B. While fengycin A contains Ala at sixth position, it is replaced by valine in the case of fengycin B (Vanittanakom and Loeffler 1986; Bonmatin et al. 2003).

4.1.1.2 Lipopeptides Produced by Gram-Negative Bacteria (*Pseudomonas* sp.)

4.1.1.2.1 Viscosinamide

Viscosinamide produced by *Pseudomonas fluorescens* DR54 showed antagonistic properties against plant pathogenic fungi like *Pythium ultimum* and *Rhizoctonia solani*. However, bio-surfactant properties of viscosinamide differed from the known bio-surfactant viscosin that contained glutamine rather than glutamate at the second amino acid position. Isolation and determination of structure of this new compound with antibiotic and antifungal properties reveals promising perspectives for the application of *P. fluorescens* DR54 as a biological control agent. Tests performed in plants also showed that purified viscosinamide reduced the aerial mycelium development of both *P. ultimum* and *R. solani* (Lee et al. 2010; De Faria et al. 2011; Nielsen et al. 1999).

Since *P. ultimum* is a root pathogen, its growth inhibition using the viscosinamide has been studied on sugar beet seeds. Seeds infected with *P. ultimum* and *P. fluorescens* DR54 have improved plant emergence after 7 days compared to a control without the biocontrol strain. The

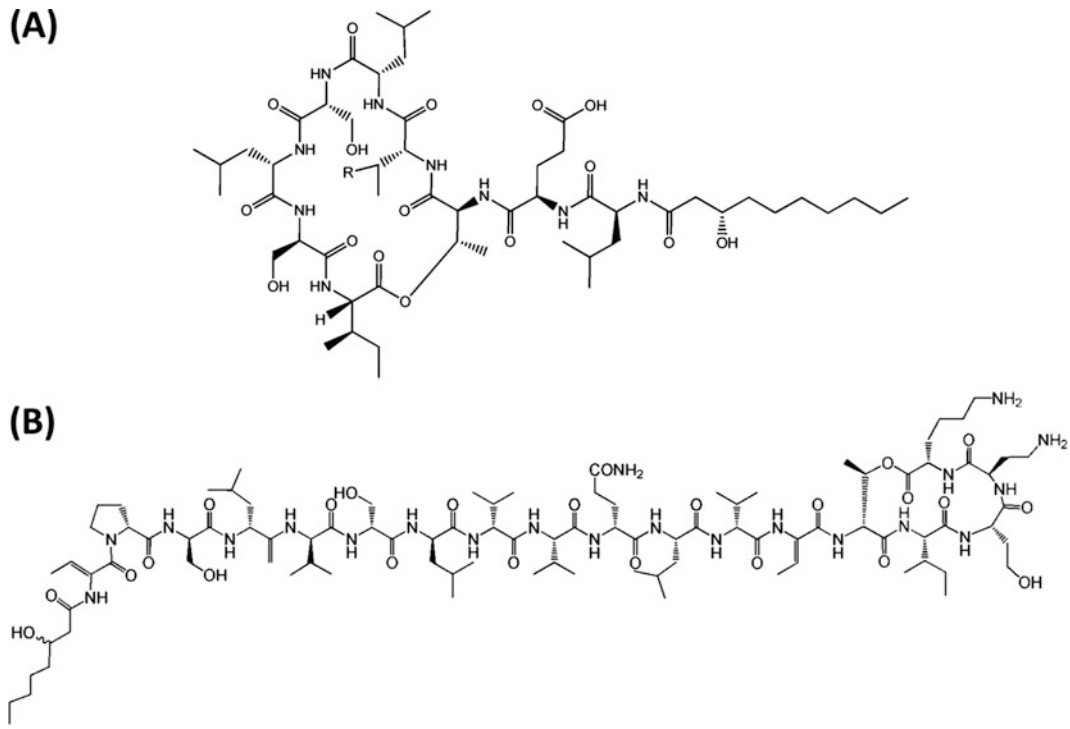

Fig. 4.2 Structure of lipopeptides produced by *Pseudomonas* sp. (**a**) Viscosin (R = H) or viscosinamide and massetolide (R = CH3) (Thrane et al. 2000). (**b**) Tolaasin (Hansen et al. 2000)

impact of *P. fluorescens* DR54 on the growth and activity of *P. ultimum* was also studied by direct microscopy after staining with the vital fluorescent dye like Calcofluor White and fluorescein diacetate. *P. fluorescens* DR54 caused reduction in *P. ultimum* mycelial density, oospore formation, and intracellular activity. Further, *P. ultimum* oospore formation was completely absent in the presence of *P. fluorescens* DR54. In vitro studies confirmed that purified viscosinamide induced encystment of *Pythium* zoospores (De Faria et al. 2011; Nielsen et al. 1999; Thrane et al. 1999) (Fig. 4.2).

4.1.1.2.2 Tolaasin

Tolaasin (tolaasin I, tolaasin II, and tolaasins A, B, C, D, and E are the available analogs) and few other metabolites are produced by *P. tolaasii*. All these analogs showed differences in their amino acid composition. All observed lipodepsipeptides of bacterial origin are maintained by the β-hydroxy octanoyl phi chain at the N-terminus, except tolaasin A, in which the acyl moiety is a gamma-carboxy butanoyl phi moiety (Mazzola et al. 2009; Andolfi et al. 2008). *P. tolaasii* is a causal organism of brown blotch disease to *Agaricus bisporus* and causes yellowing of *Pleurotus ostreatus* (Molinaro et al. 2003; Nutkins et al. 1991; Moquet et al. 1996; Cho et al. 2007).

Tolaasin is a lipodepsipeptide, found to be produced by a pathogen of mushroom species. During development of lysis bioassay for tolaasin, it was also found to cause hemolysis. Hemolysis by tolaasin involves a dose-dependent manner that is maximal between pH 6.0 and 7.0 and increased with increase in temperature. Tolaasin can also be produced in a growth-dependent manner, and its production always initiates during exponential growth of *P. tolaasii* and continued till the stationary phase. Ultrastructural studies performed to identify the mechanism of action showed disruption of the *A. bisporus* plasma membrane and vacuole

membranes by tolaasin. Multilamellar liposomes inoculated with tolaasin demonstrated loss of lytic activity and prevented tolaasin-induced hemolysis, suggesting tolaasin induces partitions and forms pores in erythrocyte membranes that cause lysis by a colloid osmotic mechanism. Tolaasin is phytotoxic when infiltrated into leaves of *Nicotiana tabacum* and was shown to be active against a range of basidiomycetes and Gram-positive bacteria (Soler-Rivas and Arpin 1999; Bassarello et al. 2004; Coraiola et al. 2006).

4.1.1.2.3 Tensin

Tensin is an antifungal cyclic lipopeptide produced by *P. fluorescens* strain 96.578. The molecular formula is $C_{67}H_{116}N_{12}O_{20}$, representing an exact molecular weight of 1408.84 Da. The NMR data obtained for tensin predicted it as a cyclic lipopeptide composed of a 3-hydroxydecanoyl residue in combination with 11 amino acid residues that include 5 leucine (Leu), 1 isoleucine (Ile), 1 aspartic acid (Asp), 1 glutamine (Gln), 1 glutamic acid (Glu), 1 threonine (Thr), and 1 serine (Ser) amino acids (Rainey et al. 1991). *P. fluorescens* is already used as a biocontrol agent against fungi such as *R. solani*. Various studies suggested that tensin is effective against radial growth of *R. solani* (Cho et al. 2010).

4.1.1.2.4 Syringotoxin and Syringopeptin

Syringotoxin is produced by *P. syringae* pv. *syringae* which is a known pathogen of various species of citrus trees. Peptide moiety of bioactive molecule contains amino acids in order of Ser-Dab-Gly-Hse-Orn-aThr-Dhb-(3-OH)Asp-(4-Cl)Thr with the terminal carboxy group closing a macrocyclic ring on the OH group of the N-terminal Ser (Nielsen et al. 2000). Syringopeptin, another major phytotoxic antibiotic produced by *P. syringae* pv. *syringae*, is also a lipodepsipeptide. The amino acid sequence is Ser-Ser-Dab-Dab-Arg-Phe-Dhb-4(Cl)Thr-3(OH)Asp with the beta-carboxy group of the C-terminal residue closing a macrocyclic ring on the OH group of the N-terminal Ser (Couillerot et al. 2009; Ballio et al. 1990) (Fig. 4.3).

4.1.1.2.5 Pseudophomins

The pseudophomins are also cyclic lipodepsipeptides grouped into pseudophomins A and B. They are isolated from *P. fluorescens* strain BRG100, a bacterium with potential application as a biocontrol agent for plant pathogens and agricultural weeds. Pseudophomin B showed higher antifungal activity against the phytopathogens such as *Phoma lingam*/*Leptosphaeria maculans* and *Sclerotinia sclerotiorum*. In contrast, pseudophomin A showed stronger inhibition toward green foxtail (*Setaria viridis*) and induced root germination than pseudophomin B (Segre et al. 1989; Galonić et al. 2007).

4.1.1.2.6 Pseudomycins

Pseudomycins are peptide antimycotics that are isolated from *P. syringae*, another plant-associated bacterium. Pseudomycins contain three analogs classified as A–C that contain amino acids hydroxyl aspartic acid, aspartic acid, serine, arginine, lysine, and diaminobutyric acid. The molecular mass of pseudomycins A–C is 1,224, 1,208, and 1,252 Da, respectively, as determined by plasma desorption mass spectrometry. Pseudomycin D, on the other hand, has a molecular mass of 2,401 Da and is more complex than pseudomycins A–C (Wilson Quail et al. 2002; Pedras et al. 2003). Pseudomycin A is the predominant peptide of the pseudomycin family that possesses selective phytotoxicity and is effective against human pathogen *Candida albicans* (Harrison et al. 1991).

4.1.1.2.7 Massetolide A

Massetolide A is isolated from *P. fluorescens* SS101, identified as a biocontrol agent. *P. fluorescens* SS101 was effective in preventing infection of tomato (*Lycopersicon esculentum*) leaves by *P. infestans* and significantly reduced the expansion of existing late blight lesions. Purified massetolide A displayed significant control of *P. infestans* both locally and systemically via induced resistance. This study suggests that

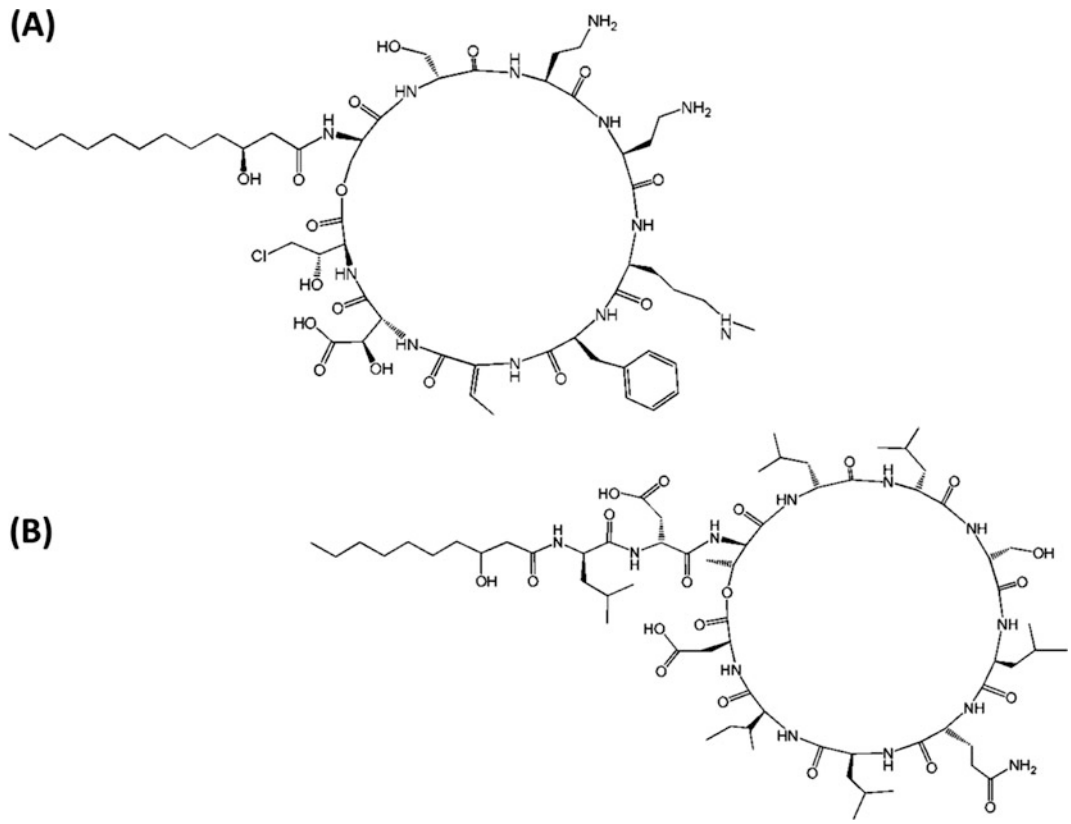

Fig. 4.3 Structure of lipopeptides produced by *Pseudomonas* sp. (**a**) Syringomycin (Mátyus et al. 2008). (**b**) Amphisin (Nielsen and Sørensen 2003)

the cyclic lipopeptide massetolide A is a metabolite with versatile functions in the ecology of *P. fluorescens* SS101 and in interactions with tomato plants and the late blight pathogen *P. infestans* (Ballio et al. 1994; Sun et al. 2001). Thus, the number of *Pseudomonas* strains has shown promising results in biological control of late blight caused by *P. infestans*.

4.1.1.3 Fungal Lipopeptides

4.1.1.3.1 Echinocandins

The echinocandins produced by fungi comprise a new class of antifungal lipopeptide agents. Echinocandins isolated for the first time from *Aspergillus rugulosus* and *A. nidulans* exhibited antifungal and anti-yeast activities (Tran et al. 2007; De Bruijn et al. 2008). Compounds under this group are amphiphilic, cyclic hexapeptides with an N-linked acyl lipid side chain. The molecular weight of these compounds is around 1,200 Da (Tóth et al. 2012). So far, three echinocandin compounds have been determined for structural elucidation. While the echinocandin B (ECB) structure was determined by chemical degradation studies in combination with X-ray crystallographic analysis, structures of echinocandins C and D were elucidated by their conversion into a common intermediate isoform derived from the echinocandin B (Benz et al. 1974; Denning 2003). They are all acylated by a linoleic acid molecule at the N-terminus. The synthesis of echinocandins C and D was carried out using the stereocontrolled synthesis of all constituents such as (2S,3S,4S)-3-hydroxy-4-methylproline, (2S,3R)-3-hydroxyhomotyrosine, and γ,δ-dihydroxyornithine (Benz et al. 1974; Messik and Oberthür 2013). The

common feature to these lipopeptides is a cyclic peptide and an N-terminal fatty acid group; however, the fatty acid group is usually either branched or unbranched with a chain length of 14–18 carbon atoms. Both the cyclic structure (Shamala et al. 1976; Kurokawa and Ohfune 1993) and an acyl side chain are essentially required for the biological activity of the compound. This emphasizes the importance of the acyl group for the mechanism of antimicrobial action (Journet et al. 1999; Rodriguez et al. 1999; Debono et al. 1989).

Deacylation of ECB has been accomplished using an *Actinoplanes utahensis* culture to design new derivatives with enhanced efficiency (Debono et al. 1989). The biological activity was restored with the reintroduction of the N-acyl group that might play certain structural requirements. Indeed, small groups such as acetyl, benzoyl, or cyclohexanoyl were effective in restoring activity, and also increase in the chain length from C12 up to C17–C18 was proportional to the increase in activity (Debono et al. 1995).

The echinocandin drugs available in the market consist of three agents, caspofungin (Cancidas; Merck and Co., Whitehouse Station, New Jersey, USA), micafungin (Mycamine; Astellas Pharmaceuticals, Grand Island, New York, USA), and anidulafungin (Eraxis; Pfizer Pharmaceuticals, New York, USA). Echinocandins exhibit antimicrobial activity by blocking the synthesis of 1-3-β-D-glucan, a critical component of the fungal cell wall, through noncompetitive inhibition of the enzyme 1-3-β-D-glucan synthase. Osmotic disruption after loss of cell wall integrity is mainly responsible for fungicidal activity (Boeck et al. 1989; Hobbs et al. 1988). The echinocandin antifungal spectrum is restricted with few exceptions to *Candida* spp. and *Aspergillus* spp. Echinocandin antifungal agents have been studied in various clinical settings ranging from invasive, oral, and esophageal candidiasis and for treatment of invasive aspergillosis. At present echinocandin drugs are available only as intravenous injections in the market (Fig. 4.4).

(a) *Caspofungin*

Caspofungin (caspofungin acetate) is a semisynthetic water-soluble lipopeptide produced as a fermentation product of the fungus *Glarea lozoyensis*. This also belongs to the echinocandin family and is a derivative of pneumocandin B_o. Studies demonstrated that caspofungin has antifungal activity against yeasts of the genus *Candida* (including isolates resistant to azoles and amphotericin B). This also includes species of the genus *Candida* that are resistant (*Candida krusei*) or less susceptible to azoles (*Candida dubliniensis*, *Candida glabrata*) or resistant to amphotericin B (Diekema et al. 2005; Douglas 2006; Barchiesi et al. 1999). Several species of *Aspergillus* and certain dimorphic fungi, such as *Histoplasma*, *Blastomyces*, and *Coccidioides*, were also inhibited by caspofungin (Vazquez et al. 1997; Bachmann et al. 2002; Kurtz et al. 1994). In vitro and in vivo experiments confirmed the additive or synergic activity of caspofungin with amphotericin B and triazoles against different fungi. It also possesses activity against *Pneumocystis carinii* (Marco et al. 1998).

The β(1,3)-D-Glucan is found to be an essential component of the cell walls of numerous fungal species as the solid three-dimensional matrix formed by chains of β(1,3)-D-glucan gives shape and mechanical strength to the cell wall. Caspofungin blocks the synthesis of β(1,3)-D-glucan by noncompetitive inhibition of the enzyme β(1,3)-D-glucan synthase and showed potential antifungal effects (Pfaller et al. 1998; Zhang et al. 2006; Georgopapadakou 1997). Clinical trials proved caspofungin to be well tolerated and effective for invasive aspergillosis patients, oropharyngeal candidiasis, esophageal candidiasis, and invasive candidiasis. It showed an efficacy that is equivalent to amphotericin B (Robbel and Marahiel 2010; Douglas 2001; Georgopapadakou 2001; Groetzner et al. 2008; Groll and Walsh 2001; Herbrecht et al. 2010; Mora-Duarte et al. 2002). Experimental studies carried out in several models showed correlation with the in vitro susceptibility data of caspofungin.

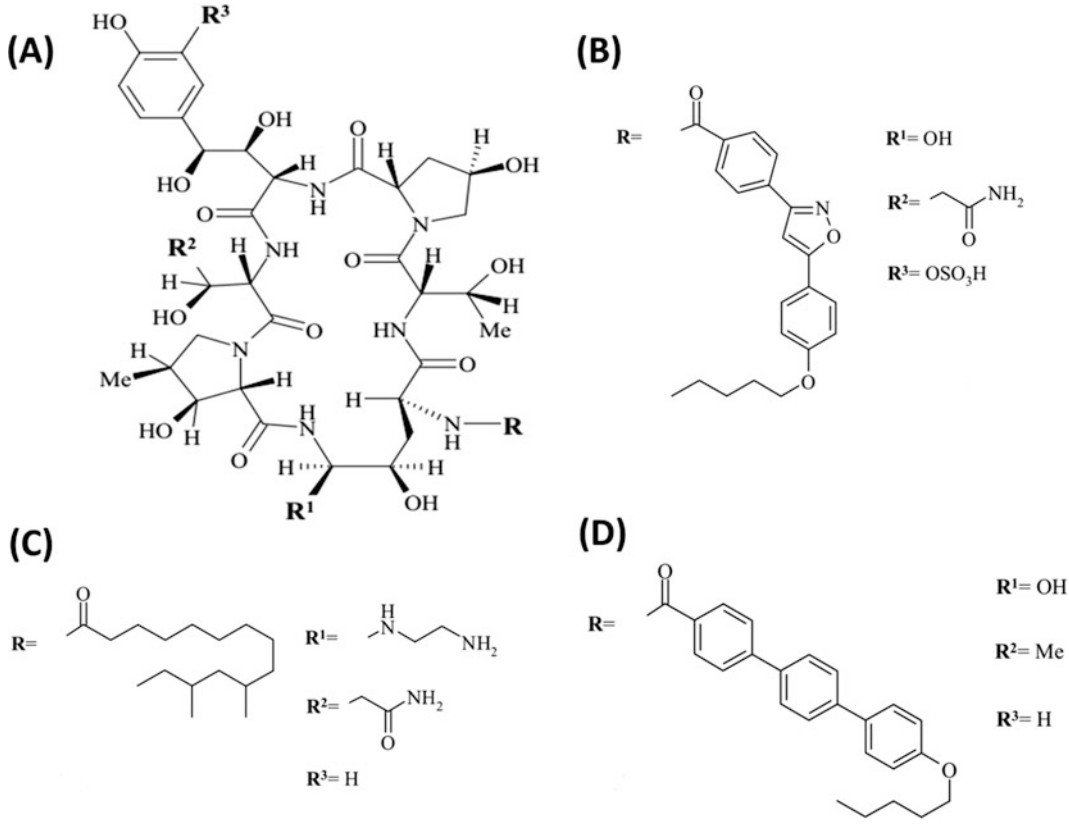

Fig. 4.4 Structure of some fungal lipopeptides (**a**) Echinocandin B. (**b**) Micafungin. (**c**) Caspofungin. (**d**) Anidulafungin

Several studies have also demonstrated that caspofungin is effective against disseminated candidiasis in immune-competent or immunosuppressed mice (Kartsonis et al. 2005a; Keating and Figgitt 2003; Abruzzo et al. 1997). Experimental data confirmed its efficacy in immunosuppressed mice infected with in vitro fluconazole-resistant or dose-dependent susceptible isolates of *C. krusei*, *C. glabrata*, and *C. albicans* (Graybill et al. 1997a, b). Caspofungin administered (0.05–5 mg/kg/day) by the intravenous or intraperitoneal route into the infected animals significantly prolonged the survival and reduced the renal fungal load.

Several experimental studies of invasive aspergillosis in immunocompromised rodents have demonstrated the efficacy of caspofungin (Abruzzo et al. 2000; Sionov et al. 2006; Chabrol et al. 2010; Kartsonis et al. 2005b). However, caspofungin did not protect mice against lethal infection with *C. neoformans* even at high doses of 40 mg/kg/day. These experimental data confirm the resistance of *C. neoformans* observed in vitro (Kartsonis et al. 2005a).

Caspofungin was compared with the reference treatment trimethoprim plus sulfamethoxazole in mice pretreated with steroids and infected with *P. carinii* (Maertens et al. 2004) by administering through subcutaneous or oral route. Results suggested 90 % reduction in number of pulmonary cysts in subcutaneous administration. Though similar results were observed for the reference treatment, the effect was more rapid with caspofungin (4 days) when compared to reference treatment (14 days). However, the oral route of administration was always found to be less effective for caspofungin. Moreover,

a prophylactic effect was observed in the same models upon daily subcutaneous administration of caspofungin (Madureira et al. 2007; Powles et al. 1998).

(b) *Micafungin*

Micafungin is used worldwide in chemotherapy of life-threatening fungal infections. It is the second approved antifungal agent in the echinocandin series. FR901379, which is known as a seed compound of micafungin, was first discovered at Fujisawa Pharmaceutical Co., Ltd, Japan, in the year 1989 (Fortún et al. 2009). Micafungin is a water-soluble antifungal agent with molecular weight of 1292.26 Da that was derived from *Coleophoma empetri* (Spreghini et al. 2012; Hashimoto 2009; Mikamo et al. 2000). The water solubility of micafungin is attributed to a sulfate moiety in the molecule. Micafungin is a potent inhibitor of 1,3-β-D-glucan synthase, an enzyme involved in cell wall biosynthesis in several pathogenic fungal species. Micafungin acts in a concentration-dependent manner as a noncompetitive inhibitor in the formation of the enzyme 1,3-β-D-glucan synthase. This enzyme is necessary for synthesis of 1,3-β-D-glucan, a glucose polymer crucial to the structure and integrity of the cell wall of several fungal pathogens (Tóth et al. 2012; Barrett 2002; Vicente et al. 2003; Onishi et al. 2000; Tawara et al. 2000). Fungal cells that are unable to synthesize this polysaccharide cannot maintain their shape and lack adequate rigidity to resist osmotic pressure, which results in fungal cell lysis. This mechanism is found to be unique to the echinocandin class of antifungal agents. It contains the potential in additive or synergistic activity with other antifungals like polyenes and azoles. Apart from cell wall structure, glucan is also involved in cell growth and division (Nishiyama et al. 2002). Micafungin demonstrates a prolonged concentration-dependent post-antifungal effect. Fungal cell walls are usually made up of chitin as a major component along with mannoproteins. The selective antifungal effect of echinocandin activity is because of varied quantities of fungal cell wall components among different fungal species (Wiederhold et al. 2008; Groll et al. 2001). Of significance, the cell walls of zygomycetes and cryptococci lack 1,3-β-D-glucan, which explains the poor activity of echinocandins, including micafungin, against selective fungal pathogens (Vicente et al. 2003; Andes et al. 2008).

Micafungin is the second antimicrobial agent in the echinocandins class that is approved for use in clinical practice. Though it is a potent antifungal, it showed slow fungicidal activity against clinical isolates like *Candida albicans*, *C. dubliniensis*, *C. tropicalis*, *C. glabrata*, and *C. krusei*. The activity was somewhat higher (MIC90s) for *C. parapsilosis*, *C. lusitaniae*, and *C. guilliermondii* (Vicente et al. 2003; Mariné et al. 2007; Nakai et al. 2002; Laverdiere et al. 2002). Micafungin did not show activity against basidiomycetous yeasts, *Cryptococcus neoformans* or *Trichosporon* species in vitro, but it displayed inhibitory activity against *Aspergillus* species at lower concentrations than amphotericin B and itraconazole (Vicente et al. 2003; Andes et al. 2008; Laverdiere et al. 2002; Ostrosky-Zeichner et al. 2003).

Although clinical data are still lacking, the role of micafungin appears to be similar to that of caspofungin. The initial approval has been granted for treatment of esophageal candidiasis and prophylaxis in subjects with neutropenia. Pharmacokinetic and pharmacodynamic studies revealed no adverse effects, and safety is reported similar to those of other agents in the echinocandin class.

4.1.1.4 Clinical Efficacy

Micafungin is found to be clinically effective in treatment of *Candida* and *Aspergillus* infections as most trials that were conducted in Japan, South Africa, the United States, Latin America, and Germany produced positive results. The US FDA has approved micafungin as a therapeutic agent for the treatment of esophageal candidiasis. However, the FDA did not approve micafungin to use for children as safety and efficacy studies in this population have not been established.

Candida Infections The safety and efficacy of micafungin in the treatment of esophageal candidiasis in AIDS patients was evaluated (Uchida et al. 2000; Fujie et al. 2001), and doses of micafungin were determined as 12.5, 50, 75, and 100 mg/day through intravenous injection. Clinical improvement was noted for patients who received 75 or 100 mg/day showing favorable clinical and endoscopic outcomes with rapid response within a duration of 3–5 days (Fujie et al. 2001; Pettengell et al. 2004). In a multinational, double-blind, non-inferiority study, a single high dose of micafungin (150 mg/day) with fluconazole (200 mg/day) (Fujie et al. 2001) also showed similar results. In another open-label, non-comparative study of micafungin for the treatment of candidemia patients, the overall success was noted in 83.2 % of patients (De Wet et al. 2004). Similarly, results of several smaller trials also supported the efficacy of micafungin in the management of candidemia (Kohno et al. 2013; Denning et al. 2006). An interesting report noted that a successful outcome with topical application of micafungin is in the treatment of refractory yeast-related corneal ulcers (Hachem et al. 2008).

Mold Infections Although data is available on using micafungin for the treatment of refractory aspergillosis, it is from open trials and not examined in a randomized, controlled manner. In a phase 2 multinational study, efficacy of micafungin as primary or salvage therapy for invasive aspergillosis was assessed (Kohno et al. 2004). Another study involving stem cell transplant (SCT) recipients evaluated the safety and efficacy of micafungin in combination with other antifungal drugs for the treatment of refractory aspergillosis (De Wet et al. 2004; Matsumoto et al. 2005). Overall, significant *Aspergillus*-infected patients had a satisfactory response. In addition, several case reports have described success with micafungin in severely compromised hosts with refractory aspergillosis (Moretti et al. 2014; Kontoyiannis et al. 2009; Ota et al. 2004). Considering the poor prognosis in patients with refractory aspergillosis, the aforementioned data from these unpublished trials and case reports are encouraging. Most available data were in the setting of refractory aspergillosis with micafungin used as salvage therapy in combination with other drugs. Data for its use as monotherapy, particularly for the initial treatment of invasive aspergillosis, are lacking.

A phase 3, large, randomized, double-blind, multi-institutional comparative study was conducted in the United States and Canada that evaluated the efficacy of micafungin as prophylaxis during the pre-engraftment period of neutropenia (Yokote et al. 2004; Chandrasekar et al. 2004). However, whether micafungin should be preferred to fluconazole as prophylaxis during the pre-engraftment period in SCT recipients and whether the improved long-term survival seen with fluconazole will also be seen with micafungin are important questions that need to be addressed (Van Burik et al. 2004; Hiramatsu et al. 2009).

Urinary Sepsis *Candida glabrata* is frequently resistant to fluconazole, and in advanced renal failure, the safe use of this and other recommended drugs is limited. Patients suffering from renal and severe urinary sepsis by *C. glabrata* are successfully treated with micafungin. In fact, amphotericin B, fluconazole, and flucytosine are recommended for effective treatment of symptomatic candiduria (Marr et al. 2000; De Pauw and Donnelly 2007). However, in renal failure these agents are contradicted, and due to significant toxicity risk, their utilities are substantially limited. Fluconazole is extensively excreted by the kidneys reaching high urinary concentrations. Non-*C. albicans* species, notably *C. glabrata*, is nowadays frequently resistant to fluconazole therapy. Echinocandins are potent antifungal agents used to treat these strains as they exert activity by inhibiting β-D-glucan synthase (Franco 2011; Zasloff 2002), a major component of the fungal cell wall (Ullmann et al. 2012). Micafungin, like the entire class of echinocandin drugs, has a broad spectrum of activity on

Candida species, and it is now considered a first-line therapy in candidiasis. In general, micafungin is well tolerated and has a low potential of drug interaction, its clearance is independent from glomerular filtration, and there are no contradictions for dose adjustment even in severe renal failures. All echinocandins are highly protein bound (99 %) and share the major pharmacokinetic disadvantage of poor glomerular filtration and tubular secretion resulting in very low urinary concentrations (Jones 1949; Arima et al. 1968). For that reason, even if they have a very favorable profile in terms of efficacy and safety, their use seems precluded in fungal urinary tract infections. However, despite micafungin being minimally excreted in urine, its wide distribution in many organs and tissues, including the kidneys, liver, and spleen, has been shown in animal models (Nishiyama et al. 2002; Mermel et al. 2009; Sucher et al. 2009). The observed tissue concentrations were severalfold in excess of the MIC against clinical isolates of *Candida* spp. and *Aspergillus* spp. Few reports in the literature are available on the safe and effective use of echinocandins (i.e., caspofungin and micafungin) in candiduria (Petraitis et al. 2002; Niwa et al. 2004; Lagrotteria et al. 2007; Kauffman 2005). Of these, six were of candiduria successfully treated with caspofungin (collected by Merck Research Laboratories retrospectively from a phase 2–3 clinical study). To our knowledge, only three cases of candiduria treated with micafungin are reported in the literature. There are some features in treatment where firstly the patient was treated with a dose of 200 mg daily of micafungin (a dose higher than the standard recommended dose) without adverse effects. The choice of using a dosage greater than usual was empirical and was motivated by the possibility of achieving higher renal tissue concentration with such dosage. In conclusion the present case shows that micafungin can achieve clinically relevant fungal sterilization even in urine and there is room to consider micafungin in the armamentarium against *C. glabrata* urinary infections, notably in the context of renal failure where other antifungal effective drugs are unsafe or contradicted.

(c) *Anidulafungin*

Anidulafungin is a semisynthetic product of echinocandin B which is a fermentation product of the mold *A. nidulans*. It was developed by Eli Lily, underwent preclinical and clinical studies at Vicuron Pharmaceuticals, and was sold to Pfizer and marketed under the name Eraxis™. Anidulafungin (Eraxis; Pfizer) is the newest echinocandin antifungal approved by the US Food and Drug Administration recently and currently used for the treatment of esophageal candidiasis, candidemia, and deep-tissue candidiasis.

Anidulafungin is a novel echinocandin and has several advantages over existing antifungals. The unique features of anidulafungin are its slow degradation in humans where it undergoes biotransformation rather than being metabolized. It has potent in vitro activity against *Aspergillus* and *Candida* species, including the strains that resist fluconazole or amphotericin B. Results of various clinical trials indicate anidulafungin as effective in treatment of esophageal candidiasis, including azole-refractory disease. The results of a recent study comparing fluconazole versus anidulafungin demonstrated the superiority of anidulafungin in the treatment of candidemia and invasive candidiasis (IC). Studies evaluating the concomitant use of anidulafungin, amphotericin B, voriconazole, or cyclosporine did not show significant drug-drug interactions or any adverse effects. To date, anidulafungin appears to have an excellent safety profile. Based on the early clinical experience, it appears that anidulafungin will be a valuable asset in the management of serious and difficult-to-treat fungal infections.

Anidulafungin has potent in vitro fungicidal activity against a broad range of *Candida* species, including *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. famata*, *C. rugosa*, and *C. stellatoidea* (Table 4.1) (Cochrane and Vederas 2014; Morikawa et al. 2000; Higginbotham and Murphy 2010). Anidulafungin is also effective against species of *Candida* that are intrinsically resistant to azoles (*C. krusei*), amphotericin B (*C. lusitaniae*), or other echinocandins (*C. parapsilosis*) (Rao

Table 4.1 Selected antifungal lipopeptide antibiotics currently in preclinical and clinical development

Compound name (commercial name)	Therapeutic area	Method of manufacture	Lead compound and producing organism	Company	Phase
Caspofungin (Cancidas)	Antifungal	Semisynthetic	Echinocandins and *Glarea lozoyensis*	Merck and Co.	Market
Micafungin (Mycamine)	Antifungal	Semisynthetic	Echinocandins and *Coleophoma empetri*	Astellas Pharmaceuticals	Market
Anidulafungin (Eraxis)	Antifungal	Semisynthetic	Echinocandins and *Aspergillus nidulans*	Pfizer Pharmaceuticals	Market

Adopted from Giovanna Pirri et al. (2009)

Table 4.2 Lipopeptides as biocontrol of fungal phytopathogens

Plant disease	Phytopathogen	Lipopeptide-producing microorganism	Lipopeptide inhibiting the phytopathogen	Ref.
Damping-off bean	*Pythium ultimum*	*Bacillus subtilis* M4	Iturin/fengycin	Ongena et al. (2005)
Gray mold disease of apple	*Botrytis cinerea*	*Bacillus subtilis* M4	Fengycin	Ongena et al. (2005)
Powdery mildew of cucurbits	*Podosphaera fusca*	*Bacillus subtilis*	Iturin/fengycin	Romero et al. (2007b)
Fusarium head blight (FHB) in wheat and barley and ear rot in corn	*Gibberella zeae* (anamorph of *Fusarium graminearum*)	*Bacillus subtilis* JA; JA026	Fengycin	Liu et al. (2005)
Sugar beet seed infection	*Rhizoctonia solani*	*Pseudomonas fluorescens* strain 96.578	Tensin	Nielsen et al. (2000)
Sclerotinia stem rot disease	*Sclerotinia sclerotiorum*	*Bacillus amyloliquefaciens*	Surfactin/fengycin	Alvarez et al. (2012a)
Rice blast	*Magnaporthe grisea*	*Chromobacterium* sp. C61	Chromobactomycin	Kim et al. (2014)

et al. 2013; Nielsen and Sørensen 2003). Anidulafungin has also demonstrated excellent in vitro activity against several species of *Aspergillus* (Table 4.2) (Nielsen and Sørensen 2003; Sørensen et al. 2001; Cameotra and Makkar 2004). Anidulafungin also demonstrates additive effects in vitro in combination with amphotericin B against species of the genera *Aspergillus* and *Fusarium* by synergistic activity when combined with itraconazole or voriconazole against *Aspergillus* species (Donadio et al. 2007; Song et al. 2011).

Mechanism of Action Anidulafungin is a semisynthetic lipopeptide synthesized from fermentation products of *A. nidulans*. The compound is a noncompetitive inhibitor of 1,3-β-D-glucan synthase, which results in the selective inhibition of the synthesis of glucan, a major structural component of the cell wall of many pathogenic fungi that is absent in mammalian cells. A difference in glucan content determines the excellent activity of anidulafungin in fungi and the paucity of adverse effects in humans (Rivardo et al. 2009).

4.1.2 Lipopeptides Against Fungal Phytopathogens

Some bacterial species live in association with plant roots and other parts. The natural antagonistic property showed by these microorganisms (notably belonging to the *Bacillus* and closely related *Paenibacillus* genera and *Pseudomonas*

sp.) has emerged as promising alternatives to reduce the use of chemical pesticides and other toxic substances in agriculture (Sobel et al. 2000; McSpadden Gardener 2004; Cawoy et al. 2011; Jacobsen et al. 2004). The disease protection properties of these microorganisms rely on three main traits.

The *first* and foremost among these is a high ecological fitness to efficiently colonize in the roots, which is a prerequisite to proficiently compete for space and nutrients in the microenvironment of the rhizosphere. *Second* is their capacity to secrete highly active antimicrobial substances with strong antagonistic activity toward various plant pathogens. The *third* is their ability to trigger plant immune response in tissues which imparts resistance state that makes the host less susceptible to subsequent infection (Pérez-García et al. 2011; Lugtenberg and Kamilova 2009; Chen et al. 2000). The efficient antimicrobial substance production which leads to direct antagonism of phytopathogens is a key biocontrol mechanism (Berendsen et al. 2012; Chen et al. 2009). Antimicrobial production efficiency and a high rhizosphere fitness possibly explain the strong biocontrol potential of *Bacillus* both in vitro and under field conditions for their successful marketing commercially (Rückert et al. 2011; Pal and Mc Spadden Gardener 2006; Janisiewicz and Korsten 2002; Larkin and Tavantzis 2013; Spadaro and Gullino 2005; Király et al. 2008). Among the *Bacillus* antimicrobial products, cyclic lipopeptides (LPs) such as surfactin, iturin, and fengycin families are of high interest not only due to their high production rate in bioreactors by *B. subtilis* or *B. amyloliquefaciens* but also because of the secretion of these compounds in relevant amounts under natural conditions in the rhizosphere environment (Yang et al. 2013; Kinsella et al. 2009; Nihorimbere et al. 2009, 2012; Dietel et al. 2013; Yaryura et al. 2008) (Fig. 4.5).

One of the *B. subtilis* strain designated as JA was found to antagonize the growth of *Gibberella zeae*. The electrospray ionization mass spectrometry (ESI/MS) analysis of the product revealed the antifungal lipopeptide production by this strain. A mutant of this strain exhibited favorable properties including the high yield of antifungal lipopeptide production with relatively faster growth over the parent strain, which suggested that this strain would be a promising biocontrol candidate in agriculture (Makovitzki et al. 2007).

The potential of *B. subtilis* strain M4 at protecting plants against fungal diseases was demonstrated in different pathosystems, and fengycin-like lipopeptide production was reported by this strain. The protective effect against damping-off of bean seedlings which is caused by *Pythium ultimum* (gray mold of apple and in other postharvest diseases) was demonstrated. The protection ability was also established by the strong biocontrol activity of lipopeptide-enriched extracts applied onto infected tissues of plants. Apart from this, root pre-inoculation with strain M4 enabled the host plant to respond more efficiently to subsequent pathogen infection on leaves and other parts of the plant. The mechanisms by which *Bacillus* spp. suppress disease in infected plants are yet to be discovered. These antifungal properties of stain M4 emphasize the interest of *B. subtilis* as a pathogen antagonist and plant defense-inducing agent. The production of cyclic fengycin-type lipopeptides may be closely related to the expression of these two biocontrol qualities (Liu et al. 2005).

Apart from members of *Bacillus* genus, a *Chromobacterium* sp. strain C61 that displayed antifungal activities was also used successfully as a biocontrol agent for various plant diseases under field conditions. The analysis of C61 culture filtrates exposed an antifungal cyclic lipopeptide, *chromobactomycin*, that contained a unique nonameric peptide ring. The chromobactomycin inhibited the growth of several phytopathogenic fungi in vitro as well as plant applications. It significantly reduced disease severity caused by several pathogens and inhibited the mycelial growth of *R. solani*, *Pyricularia grisea*, *B. cinerea*, *Alternaria longipes*, and *C. gloeosporioides* (Ongena et al. 2005) (Fig. 4.6).

Isolation and characterization of bacterial antagonist for use as biological control of phytopathogenic fungi like rice blast fungus has been studied. The antimicrobial substance was further

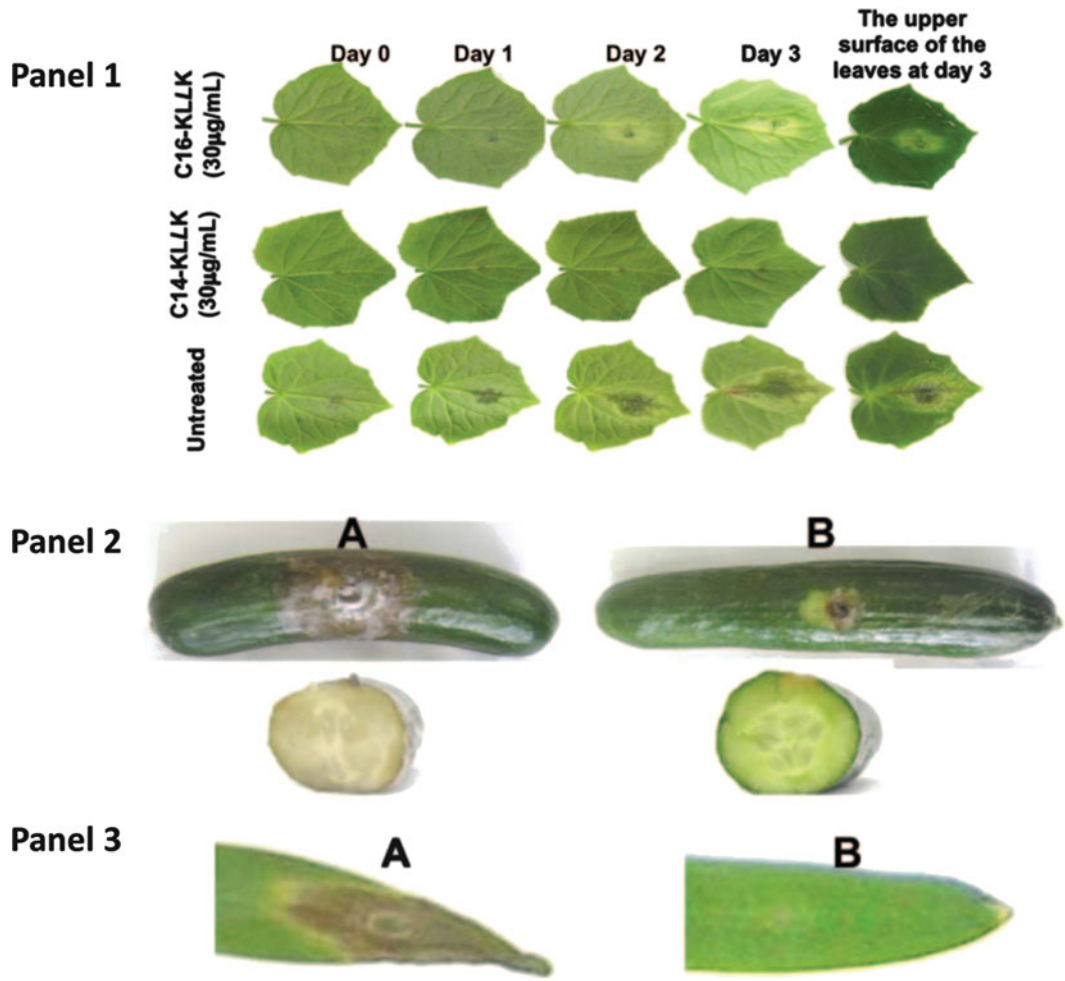

Fig. 4.5 Panel 1: Fungicidal activities of the lipopeptides (C14-KLLK and C16-KLLK) upon artificial infection of gray mold (*Botrytis cinerea*) on cucumber leaves for 4 days after infection and 3 days after treatment. Panel 2: Cucumber fruits infected with *B. cinerea* conidia 3 days after the last treatment with double-distilled water (*A*) or 30 mg/l C14-KLLK (*B*). Panel 3: Corn leaves infected with *Cochliobolus heterostrophus*, untreated (*A*) or treated with 30 mg/l C14-KLLK (Debois et al. 2014)

purified and characterized the antifungal molecule produced by the antagonist. The strain was isolated from soil and identified as *B. licheniformis* BC98 that showed high antagonist antifungal activity against the rice blast fungus *Magnaporthe grisea*. This bacterial strain also inhibited the growth of other phytopathogens such as *Curvularia lunata* and *Rhizoctonia bataticola*. Biochemical and mass studies of biologically active fractions revealed it as a lipopeptide with molecular mass of 1,035 Da. However, it was identified as surfactin after NMR analysis. Microscopic analysis of the effect of the antagonist on *M. grisea* revealed bulbous hyphae showing patchy and vacuolated cytoplasm under the electron microscope. The antagonist inhibited germination of *M. grisea*, and therefore it is considered as a potential candidate for control of rice blast disease (Romero et al. 2007b).

Podosphaera fusca is the main causal agent of cucurbit *powdery mildew*, and four *Bacillus subtilis* strains, UMAF6614, UMAF6619, UMAF6639, and UMAF8561, were found to inhibit the growth of *P. fusca*. Therefore, they were studied for their ability to suppress the

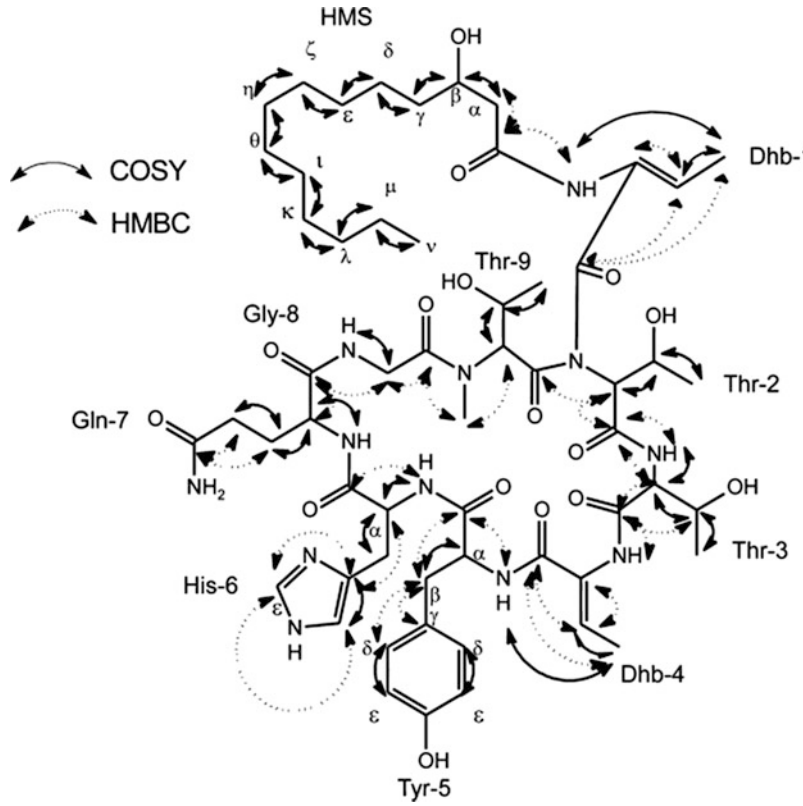

Fig. 4.6 Chemical structure of chromobactomycin. *Dhb* dihydroxybutyric acid, *Gln* glutamine, *Gly* glycine, *His* histidine, *HMS* β-hydroxymyristate, *Thr* threonine,*Tyr* tyrosine (Ongena et al. 2005)

disease on melon. Experiments were performed using detached leafs and seedling assays. They were also further subjected to elucidate the mode of action involved in their biocontrol performance. Three lipopeptide antibiotics, i.e., surfactin, fengycin, and iturin A or bacillomycin, were identified in butanolic extracts of *B. subtilis* culture filtrate. The purified lipopeptide fractions (bacillomycin, fengycin, and iturin A) have shown strong inhibitory effects on *P. fusca* conidia germination and provided interesting evidence of their presumed involvement in the antagonistic activity. This also suggested that the iturin and fengycin families of lipopeptides have a major role in the antagonism of *B. subtilis* toward *P. fusca* (Alvarez et al. 2012a) (Fig. 4.7).

The antifungal compounds identified as iturin, surfactin, and fengycin isoforms produced by two previously isolated *Bacillus* sp. strains, ARP23 and MEP218, were found to be effective against *Sclerotinia sclerotiorum*. These strains were further assessed for their ability to control *sclerotinia stem rot* in soybean. The field trials showed effective results. While the surfactin C15 and fengycin A (C16–C17) and B (C16) isoforms were produced by strain ARP23, the major lipopeptide produced by strain MEP218 was iturin A C15. Mycelial growth, morphology, and sclerotial germination were altered in the presence of lipopeptides. Foliar application of *Bacillus amyloliquefaciens* strains on soybean plants prior to *S. sclerotiorum* infection also revealed significant protection against sclerotinia stem rot. Strains ARP23 and MEP218 were renamed or identified as strains belonging to the species *B. amyloliquefaciens* and were concluded to produce antifungal compounds belonging to the cyclic lipopeptide family. As sclerotinia stem rot was considered as one of the most severe soybean diseases worldwide, these results proposed the potential of *B. amyloliquefaciens* strains ARP23 and MEP218 to control plant diseases caused by *S. sclerotiorum* as biocontrol agent (Kim et al. 2014) (Fig. 4.8).

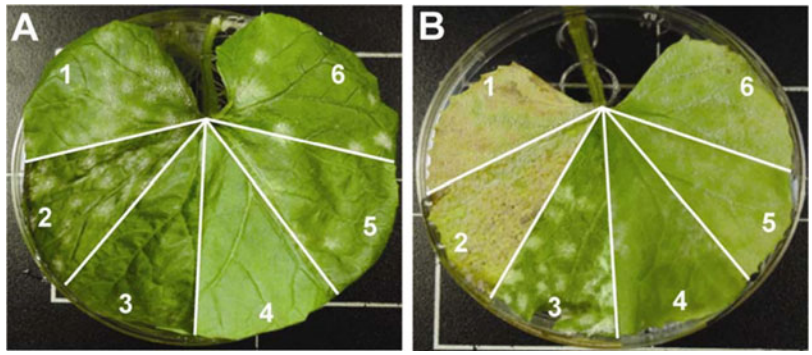

Fig. 4.7 Powdery mildew symptoms on melon leaves following treatments with butanolic extracts of cell-free filtrates of the antagonistic strain *Bacillus subtilis* UMAF6639. Treatments are *1* untreated control, *2* nutrient broth control, *3* washed cells from stationary phase, *4* cell-free filtrate nondiluted, and *5* and *6* cell-free filtrates 1:4- and 1:16-fold diluted, respectively. Panels are compositions of photographs of leaves inoculated with conidia of *Podosphaera fusca* and treated as described, taken 7 (**a**) and 16 (**b**) days after treatments (Fzb et al. 2004)

Fig. 4.8 Systemic protection against *Botrytis cinerea* B05 infection by synthetic ultrashort lipopeptides in cucumber and *Arabidopsis* seedlings. (**a**) The first leaf of cucumber plants (*white arrows*) was infiltrated with 100 µl of water (MOCK) or 100 µl of a 12.5 M solution of P1 24 h before inoculation of the second and third leaves with *B. cinerea* mycelium. Symptoms were assessed 3–4 days after infection. (**b**) *Arabidopsis rosette* leaves 3–4 days after infection with *B. cinerea* spores. Four leaves from each plant rosette were treated with 100 µl of water (MOCK) or 100 µl of 12.5 M peptides. Pathogen inoculation was done on non-treated leaves. Three to four days later, infected leaves were detached and analyzed (Tendulkar et al. 2007)

4.2 Conclusion

Lipopeptides are amphiphilic molecules with diverse physiochemical properties. Because of their broad-spectrum antimicrobial activities, they are used as various biocontrol agents. Moreover, presence-specific amino acid residues import certain functions to the lipopeptides that are important for biological activity. Though the molecular pattern is not conserved in all lipopeptides, structural homologues are interestingly more active in comparison to others. In fact, this is the reason why some *Bacillus* strains are more efficient than others against pathogenic strains. Therefore, the identification and understanding of putative receptors and their molecular mechanism is essential. However, efficiency

of biological control properties of lipopeptides can be improved by using different combinations of production mechanism or by improving the production strains or in combination. Large-scale production of these lipopeptides is essential for biotechnological studies including therapeutic applications.

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