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Antibiotic Compounds from *Bacillus*: Why are they so Amazing?

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Abstract: The constant increase of multi-drug resistant pathogens stimulates research, more than ever, to identify and develop new antibacterial compounds. The recent advances in genome sequencing have highlighted the genus *Bacillus* as an unexpected source of antibiotic-like compounds. This review focus on the different class of antimicrobial molecules produce by *Bacillus* genus such as polyketides, nonribosomal peptide, bacteriocins as well as other unusual peptides.

Key words: *Bacillus*, polyketides, nonribosomal peptides, bacteriocins, lantibiotics

INTRODUCTION

The discovery of penicillin by A. Fleming more than 90 years ago has revolutionized the medicine and allowed to treat millions of people from bacterial infections. Unfortunately, the intensive use and misuse of antibiotics have generated a strong selective pressure for the emergence of resistant strains. Actually, the constant increase of multi-drug resistant pathogenic microorganisms stim ulates more than ever effort to identify and develop new antibacterial compounds. In that field, the recent advances in genome sequencing have highlighted the genus *Bacillus* as an unexpected source of antibiotic-like compounds. Indeed, for some of them, such as *Bacillus subtilis*, more than 4% of the genome has been found potentially devoted to the synthesis of Polyketides (PKs), Nonribosomal Peptide (NRPs), bacteriocins as well as other unusual antibiotics (Kunst *et al*., 1997; Chen *et al*., 2009a; Arguelles-Arias *et al*., 2009). In the past, these compounds had to be identified by intensive screening for antimicrobial activity against appropriate targets and subsequently purified using fastidious methods prior to assess their potential utilization as antibacterial or antifungal compound. However, since the advent of the genomic era, available bacterial genomes are screened for bacteriocin, Polyketide Synthetase (PKS) and Nonribosomal Peptide-Synthetase (NRPS) gene clusters using appropriate bioinformatics tools, rendering the identification of new compounds more easy-going (Tapi *et al*., 2010; Caboche *et al*., 2008).

Polyketides and non-ribosomal peptide: Polyketides (PKs) and Nonribosomal Peptides (NRPs) are synthesized by large multimodular synthetase by elongation of activated monomers of amino and hydroxyl acid building blocks. The genes coding for these synthetases are clustered in operons than can span over 150 kb. NRPSs are organized in modules responsible for the incorporation of a specific amino acid. The modules consist of three main core domains that catalyze a specific reaction for the incorporation of a monomer. Firstly, the adenylation domain (A) selects and activates the cognate amino acid as an amino acyl-adenylate. Then after, Thiolation domain (T) covalently binds the activated monomer to the synthetase through a phosphopantetheinyl arm. Finaly, the condensation domain (C) catalyses the formation of the peptide linkage between the activated amino acids from two adjacent T modules. Two additional specific modules are also requested for NRPs synthesis. These are the loading module, composed only by one A and T domain, involved in the activation and incorporation of the first amino-acid of the NRP and a termination module containing a Thioesterase (TE) domain, which releases the newly synthetized peptide from the synthetase. Beside these three main domains, many NRPSs feature more specialized domains within modules that allow residue modifications, namely Epimerisation (E), Methylation (M), Oxidation (Ox), Reduction (R), Formylation (F) and Heterocyclisation (Cy). These specialized domains enable NRPSs to synthesize an impressive number of diversified structures with broad range of biological activities that could not be obtained by the ribosomal machinery (Felnagle *et al*., 2008). This diversity of structure is responsible for their board-range of biological activities, targets, mechanisms of action, allowing their exploitation by the biopharmaceutical industry. Bacitracin, for instance, used in human medicine, inhibit the biosynthesis of the bacterial cell wall by interacting with undecaprenyl pyrophosphate, involved in peptidoglycan synthesis (Stone and Strominger, 1971). Since its US FDA approval in 2010, bacitracin is used for the treatment of infants with *staphylococcal* pneumonia and empyema. Mechanistically, NRPS could function based on two distinct schemes. Linear NRPSs proceed synthesis starting with the loading module, following by addition of specific amino acid according the ordered fashion of the different modules

in the synthetase and ending with the cleavage of the peptide from the synthetase by the TE domain. Iterative NRPSs use successively, one, several or the entire set of modules constituting the synthetases (Mootz *et al*., 2002). Since the discovery of gramicidin from *B. brevis*, the first NRP compound characterized in the early '70, many bioactive NRPs have been isolated and their biosynthetic clusters characterized. Recently, the Norine database containing hundreds of NRPS molecules has been created, providing users with an interesting computational tool for systematic study of these molecules (Caboche *et al*., 2008). With emergence of powerful bioinformatics and molecular tools, identifying new metabolites by genome mining has become a reality (Ansari *et al*., 2004; Lanen and Shen, 2006). For instance, a bioinformatics analysis of the genome of *B. thuringiensis* led to the prediction of an NRPS constituted of seven modules that could be involved in the synthesis of a heptalipopeptide similar to kurstakin (Abderrahmani *et al*., 2011). The discovery of new natural products by genome mining is an encouraging sign, suggesting that this methodology could lead to the isolation of novel molecules of pharmacological interest.

PKSs assemble the core structure of polyketides from acyl-Coenzyme A (acyl-CoA) monomers in a head-to-tail fashion (Jenke-Kodama and Dittmann, 2009; Hertweck *et al*., 2007). Type I PKSs are similar to NRPSs in that the different catalytic domains are found in a single polypeptide and are further subdivided into iterative and modular synthases. An iterative type I PKS is a monomodular synthase in which a single set of catalytic domains is used repeatedly in a highly programmed fashion. By contrast, modular PKSs feature several separate modules that do not repeat. Type II PKSs possess a biosynthetic mechanism analogous to iterative type I PKSs but harbour their catalytic domains on mono-or bi-functional proteins (Hertweck *et al*., 2007). Several bacteria, including *B. subtilis* and fungi, but mostly plants, possess type III PKSs (Nakano *et al*., 2009; Resmi and Soniya, 2012; Li *et al*., 2011). They consist of a single multimodular protein synthesizing molecules devoid of any antibimicrobial activities (Felnagle *et al*., 2008). Each PKS module is composed by at least three core domains: an Acyltransferase Domain (AT) which selects the appropriate monomers, an acyl Carrier Protein Domain (ACP) that carry the activated monomer through the formation of a thioester linkage and a Ketosynthase (KS) domain responsible for the condensation between the activated monomer and the polyketide intermediate present on two adjacent ACP domains. Additional secondary domains such as Ketoreductase (KR), Oxidation (Ox), Dehydratase (DH), Methyltransferase (MT), Enoylreductase (ER) and Methylation (M) domains are involved in the chemical modification of the growing polyketide. Type II PKSs often feature a Cyclase (Cy) domain leading to the formation of aromatic structures. Similarly to NRPS, the last module contains a Thioesterase (TE) domain that catalyzes the release of the final product from the enzyme (Meurer *et al*., 1997). Due to their versatile assemblage mechanism, polyketides exhibit remarkable diversity both in terms of structure and biological activities. For instance, three functional gene clusters directing the synthesis of difficidin, macrolactin and bacillaene were identified in *B. amyloliquefaciens* (Arguelles-Arias *et al*., 2009; Chen *et al*., 2007). Difficidin is an unsaturated 22-membered macrocylic polyene lactone phosphate ester with broad-spectrum antibacterial activity (Wilson *et al*., 1987; Zimmerman *et al*., 1987). It inhibits protein biosynthesis and was recently shown promising in its suppressive action against *Erwinia amylovara*, a devasting plant pathogen causing necrotrophic fire blight disease of apple, pear and other rosaceous plants (Zweerink and Edison, 1987; Chen *et al*., 2009b). By contrast, macrolactin and bacillaene have not yet been demonstrated to be directly related to biocontrol, although they are both antimicrobial agents that could be potentially useful in human medicine. Macrolactin, which consists of a 24-membered ring lactone, had the ability to inhibit murine melanoma cancer cells as well as mammalian herpes simplex viruses. It was also shown effective in protecting lymphoblast cells from HIV (Gustafson *et al*., 1989). Similarly to difficidin, bacillaene is an inhibitor of prokaryotic protein synthesis constituted by an open-chain enamine acid with an extended polyene system. This compound displays antimicrobial activity toward human pathogens such as *Serratia marcescens*, *Klebsiella pneumoniae* and *Staphylococcus aureus* (Patel *et al*., 1995).

NRPS/PKS hybrid synthetases: Several compounds isolated from bacteria are synthesized by NRPS/PKS hybrid synthetases. These metabolites are composed of a polyketide backbone featuring incorporated amino acids in the case of a PKS-NRPS hybrid or a peptidyl chain harbouring ketone group characteristic of an NRPS-PKS hybrid. Among them are the three iturin lipopeptides, namely mycosubtilin (Duitman *et al*., 1999), iturin A (Tsuge *et al*., 2001) and bacillomycin D (Hofemeister *et al*., 2004), produced by different strain of *Bacillus*. Iturins are amphiphilic cyclic peptides composed of seven α-amino acids linked to a single β-amino fatty acid. The length of the fatty acid moiety may vary from C14 to C17 and different homologous compounds with a linear or branched fatty acid moiety are usually co-produced (Hofemeister *et al*., 2004). This amphiphilic structure confers interesting biological properties to these secondary metabolites. For instance, mycosubtilin, produced by *B. subtlis* ATCC6633, was found very effective against the human pathogenic

yeast *Candida albicans* (Fickers *et al*., 2008; 2009) and as biocontrol agent to prevent *Pythium* infections of tomato seedlings (Leclere *et al*., 2005). The gene clusters involved in mycosubtilin is composed of one operon that span over 38 kb. It consists of four ORFs, designated *fenF*, *mycA*, *mycB* and *mycC*. The mycosubtilin synthetase reveals features unique for peptide synthetases as well as for fatty acid synthases. MycA subunit combines functional domains derived from peptide synthetases, amino transferases and fatty acid synthases. The growing understanding of PKS and NRPS mechanisms has led to the development of genetic engineering technique yielding to the synthesis of "synthetic" compounds. These methods consist of adding, subtracting or permuting modules or catalytic domains of a known natural synthase to obtain NRPs and PKs presenting modified structures and functions (Cane *et al*., 1998).

Bacteriocin: Beside NRP and PK molecules, *Bacillus* genus is also able to produce interesting ribosomally synthetised peptides, the so-called bacteriocin (Lee and Kim, 2011; Abriouel *et al*., 2011). Based on their structure and biological activities, bacteriocins could be divided into three main groups. Class I bacteriocins, the so-called lantibiotics, are characterized by their unusual amino acids such as Lanthionine (Lan), Methyllanthionine (MeLAn) and dehydrated residues. Lan and MeLan are enzymatically synthesized by the cyclisation of a free cysteine and a dehydrated residue, namely Dehydroalanine (Dha) and didehydrobutyrine (Dhb), respectively. DhA and Dhb are obtained by the dehydratation of a serine or a threonine (McAuliffe *et al*., 2001). Lantibiotics can be further subdivided into two types based on their structure and mode of posttranslational modifications. Type A lantibiotics exhibit linear secondary structures and are positively charged. Two distinct LanB and LanC enzymes are involved in their post-translational modification and they are processed by a LanP protease. By contrast, Type B lantibiotics, exhibit a globular structure and are non-charged. They are modified by a single LanM enzyme and processed by a LanT transporter with N-terminal-associated protease activity (Willey and Donk, 2007). Beside this, two-component lantibiotics, consisting of a two peptides system that acts synergistically were also reported. Since a single LanM enzyme modifies these peptides, they were classified as type B lantibiotics (Asaduzzaman and Sonomoto, 2009). Compared to lantibiotics, class II bacteriocins are non-modified peptides that are synthesized ribosomally. They are characterized by a molecular weigh below 5 kDa. They could be further subdivided into three subclasses (IIa, IIb, IIc) based on structural properties, activity and mode of action (Nes and Tagg, 1996; Klaenhammer, 1993). Class IIa bacteriocins are characterized by an hydrophobic N-termini containing the YGNGV consensus sequence and a disulfide bridge. Class IIb feature two-component non-modified bacteriocin whereas class IIc regroups all the other molecules that do not correspond to class IIa and IIb. Class III bacteriocins are heat sensitive molecules with a molecular weight higher than 30 kDa. Its only representative among the *Bacillus* genera is the megacin A-216 produced by *B. megaterium* that only exhibits a phospholipase A2 activity (Tersch and Carlton, 1984). The type IA bacteriocin subtilin, produced by *B. subtilis* ATCC6633, is the model lantibiotic produced by members of the *Bacillus* genus. Active subtilin contains eight post-transcriptionally modified residues: four methyllanthionines (Abu-S-Ala), one didehydrobutyrine, one dehydroalanine and one lanthionine (Ala-S-Ala) and one D-alanine (Banerjee and Hansen, 1988). Despite their relative variability of structure, lantibiotics share in common some characteristic features concerning their mode of synthesis. The gene cluster involved in their synthesis, which is approximately 10-15 kb, is composed of a structural gene as well as other genes necessary for the modification, transport, regulation and immunity of the producer strain. The subtilin biosyntheric gene cluster is composed of 10 ORFs, namely *spaBTCSIFEGRK*, forming four distinct operons (*spaBTC*, *spaS*, *spaIFEG*, spa*RK*). *SpaS* encodes a premature and inactive peptide of 56 residues with a 24 residues signal sequence. SpaB and SpaC are involved in the dehydratation of serine and threonine, which are required for the formation of Dha and Dhb, respectively, as well as that of lanthionine, which is the result of the cyclisation of Dha with thiol group of a free cysteine. Presubtilin is exported by the ABC type transporter protein SpaT and is then processed by the serine protease subtilisin to form a bioactive subtilin. Self-protection of the producer strain from the synthesized lantibiotics is ensured by different immunity mechanisms. The protein SpaI, a membrane-bound protein, interacts specifically with subtilin causing its inactivation. Beside this, the immunity complex formed by SpaF, SpaE and SpaG proteins pumps subtilin out of the producer strain. The production of lantibiotic is regulated at the transcriptional level in a cell-density-dependent manner. It was evidenced that some lantibiotic, such as subtilin, can act as auto-inducing peptides (Kleerebezem *et al*., 2004). Two proteins, spaR and spaK, corresponding to a response regulator protein and a sensor kinase, respectively, form the regulatory system. After subtilin concentration in the culture medium reaches a certain threshold, it activates the membrane-bound SpaK protein which then autophosphorylates. This leads to the phosphorylation of SpaR, which in phosphorylated form can recognize the binding domain on three promoters upstream of

spaS, *spaBTC* and *spaIFEG*, resulting in subtilin production. In addition, *SpaRK* expression is controlled by the sporulation transcription factor sigma H (Stein *et al*., 2002). All of these highlight that lantibiotics synthesis is correlated to both to cell density and to sporulation mechanism. The biological activity of lantibiotics is centered on Gram-positive bacteria and consists of pores formation in target cell membranes and/or inhibition of peptidoglycane synthesis by interacting with undecaprenylpyrophosphoryl-MurNAc-(pentapeptide)-GlcNAc, the so-called lipid II (McAuliffe *et al*., 2001; Bierbaum and Sahl, 2009). For some lantibiotics, such as epidermin, lipid II serve as a docking molecule that facilitate the pore formation (Parisot *et al*., 2008; Brotz and Sahl, 2000).

Unusual peptide: *Bacillus* sp were also found able to synthetize others unusual antibiotic peptides. Among them, rhizocticins are phosphonate oligopeptide antibiotics containing the C-terminal nonproteinogenic amino acid (Z)-l-2-Amino-5-Phosphono-3-Pentenoic Acid (APPA) (Borisova *et al*., 2010; Kino, 2010; Kino *et al*., 2009). The molecules are synthesized by the so-called L-amino acid ligase. These enzymes are able to catalyze the formation of an alpha-peptide bond from L-amino acids in an ATP-dependent manner. For instance, YwfE from *B. subtilis* 168, was the first reported L-amino acid ligase able to synthesizes various dipeptides (Tabata *et al*., 2005). Rhizocticin A, is known to inhibit the growth of yeast and filamentous fungi but is not active against bacteria. Rhizocticins enters the target cell via the oligopeptide transport system and must be cleaved by a peptidase in order to release the active APPA, which inhibit threonine synthase, an enzyme that catalyse the Pyridoxal 5'-Phosphate (PLP)-dependent conversion of phosphohomoserine to L-threonine (Kino *et al*., 2009; Laber *et al*., 1994; Diddens *et al*., 1979). Hence, APPA interferes with threonine synthesis ultimately affecting protein synthesis and leading to growth inhibition (Borisova *et al*., 2010). Beside APPA based antibacterial compounds, some strains of *B. subtilis* produce the dipeptide bacilysin composed of L-alanine and the unusual amino acid L-anticapsin (Walker and Abraham, 1970). Anticapsin is able to inhibit glucosamine synthetize and thus causes a failure in the bacterial wall synthesis and finaly the growth of bacteria (Kenig *et al*., 1976).

CONCLUSION

The amazing biological properties of de different type of Bacillus's compounds described in this review could be one answer to the multi-drug resistant concerns. To date, it is estimated that only a small fraction of the antimicrobial molecules potentially produced by Gram-positive bacteria has been identified. Research to discover these compounds is sure to be ongoing for many more years.

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