Classification of Bacillus Beneficial Substances Related to Plants, Humans and Animals

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Genus Bacillus is a spore-forming bacterium that has unique properties in cell differentiation, allowing the forming of spores in stress conditions and activated in the vegetative cell, with suitable environments occurring during the life cycle acting as a trigger. Their habitat is mainly in soil; thus, many species of Bacillus are associated with plants as well as rhizosphere bacteria and endophytic bacteria. Signal transduction is the principal mechanism of interactions, both within the cell community and with the external environment, which provides the subsequent functions or properties for the cell. The antimicrobial compounds of Bacillus sp. are potentially useful products, which have been used in agriculture for the inhibition of phytopathogens, for the stimulation of plant growth, and in the food industry as probiotics. There are two systems for the synthesis of these substances: nonribosomal synthesis of cyclic lipopeptides (NRPS) and polyketides (PKS). For each group, the structures, properties, and genes of the main products are described. The different compounds described and the way in which they co-exist exhibit the relationship of Bacillus substances to plants, humans, and animals.

Keywords: Quorum sensing, quorum quenching, cyclic lipopeptides, polyketides, bacteriocins

Spore-forming Bacillus species have been well known for many years as plant biocontrol producers (fungicides, bactericides, and fertilizers), probiotics, and pathogens. Their properties are diverse depending on the species or subspecies. The Bacillus sp. is used as a genetic model of Gram-positive bacteria that show a large number of strains in the database for the whole genome. They are also closely related to many species in the group Bacillaceae such as B. subtilis, B. amyloquefaciens, and B. licheniformis; the distance of the number of nucleotide substitutions between species is less than 0.31 [62]. According to the genetics, gene transfer occurs spontaneously among these strains. The gene evolution is also caused by horizontal gene transfer; for example, gene transfer from Archaea to Bacillus species is about 1.7% [24]. B. subtilis has been observed in the upper layers (1–3 cm) of a variety of soils. Through sporulation, B. subtilis adapts to unfavorable conditions with a highly resistant dormant endospore, surviving for years before revitalization via spore germination and outgrowth. Endospore formation takes the unusual form of asymmetric cell division, followed by the engulfment of the forespore by the mother cell. Dormant spores show properties that differ from those of growing cells, with an increased resistance to the effects of chemicals, heat, mechanical disruption, UV irradiation, and enzymes [56]. Each spore comprises a thick, proteinaceous shell – known as the coat – and internally a cortex, inner membrane, and core. Analysis of these components explains the high resistance of the dormant spore, and further shows their changing roles when spore germination is triggered by an external catalyst. A germinant penetrates the coat and cortex to reach a germination receptor in the inner membrane. Depending on the particular germinant and specific receptor of the spore, a range of gene products and their coding genes (ger genes) are exhibited and identified by mutations at various stages of spore germination [9, 15, 22, 39, 47]. The various mutants show differing responses to germinants as a result of gene and operon duplication and divergence. In addition to nutrients, spores are germinated by a variety of non-nutrient stimuli [16, 55], which allows the non-nutrient chemical-, enzyme-, or pressure-based germination of spores in many species of endospore-forming bacteria.

Why have these spores remained of interest to many fields in current study? Because they contain endospores that can release to be free in environments. The spores...
have cortex peptidoglycan and a coat structure, which combines obvious different structures and protects the spore cell from stress conditions [36, 40, 41]. The spores can become animated when they are triggered by some nutrients or chemicals, and then go through outgrowth, increasing the amount of vegetative cell. It is difficult to kill the Bacillus strains; this is an advantage for beneficial applications as they have a long life cycle, but is a disadvantage for controlling spore-forming diseases. Bacillus anthracis, animal and human pathogens known as anthrax, is a biological weapon worldwide and remains a serious problem for the US army [23] as it cannot be killed completely from the spores. Knowledge of the sporulation [20] and germination processes [37] is the major key to understanding and controlling this organism. To scientific advantage, Bacillus sp. is the friendliest species for the human, animal, plant, and environment. It also produces different kinds of enzymes used in industry, such as amylase, lipase, protease, and laccase [50]. The Bacillus species is found mainly in soil and in the gastrointestinal tract in both animals and humans. As such, it has been reported to be an antimicrobial producers and probiotic bacteria.

In this article, the antimicrobial compounds produced from Bacillus sp. are the effective products for beneficial uses. There are two systems involved in antimicrobial compounds synthesis: nonribosomal peptide synthetases (NRPS) and polyketide synthases (PKS). The bacterial community is also involved in the regulation of synthesized metabolites and the efficiency of compound activities in environments.

**Interaction and Community of Bacillus sp. in Habitats**

The fundamental function of active compounds from Bacillus species is the inhibition of plant pathogens. Many species are plant-associated bacteria, such as B. subtilis, B. amyloliquefaciens, B. licheniformis, B. pasteurii, B. cereus, B. pumilus, B. mycoides, and B. sphaericus, which have been reported to be elicitors of induced systemic resistance (ISR) [29]. The bacteria associate with plant-form sessile biofilm (exopolysaccharide/ lipopolysaccharide) to attach and colonize on the plant surface. The genes involved in biofilm and fruiting body formation are epsA–O, which is an operon consisting of 15 gene exopolysaccharides in B. subtilis [25]. The swarming motility controlled by gene swrA is also involved in the colonization of surfaces. Furthermore, the genes RBAM00750, RBAM00751, and RBAM00754 of B. amyloliquefaciens are involved in surface adhesion or biofilm formation [6]. Endophytic bacterium B. subtilis was isolated from plant stem and also showed strong inhibition of phytopathogens [31]. The mechanism of entry to the plant cell is not demonstrated; colonization of bacteria in the cell may involve the biofilm and fruiting body formation as described above. The rhizobacteria can use root exudates as signal molecules to penetrate into the root of a plant. The bacterial populations behave as biofilm communities for attachment and aggregation with plants. Then, bacterial colonies move into the root system through the swarming process. It has been reported that a Bacillus cyclic lipopptide, surfactin, has the role of stable biofilm formation and is involved in surface motility [4, 26]. Cell–cell communication is an important mechanism for a microbial community in habitats, known as quorum sensing. The quorum-sensing system has a function to coordinate gene expression and regulate virulence production [14]. There are two types of signal communication; intraspecies communication and interspecies communication. The interspecies signaling, such as antibiotics molecules, can transfer the signal to other bacteria; thereby the bacteria can also “eavesdrop” and lead to alteration in factors contributing to the virulence or persistence of bacterial pathogens as well as influencing the development of beneficial microbial communities. The interspecies signaling is involved in virulence factors, biofilm, and acts as an autoinducer [53]. Therefore, if the quorum sensing is interrupted, it will decrease the virulence of pathogens. Gram-negative bacteria produce N-acyl homoserine lactones (AHLs) as a signal molecule showing virulence cell of pathogens, whereas Gram-positive bacteria have modified peptides to signal virulence regulation, called autoinducing peptides (AIP). The AIP of Staphylococcus aureus are peptides consisting of 7–9 amino acids that form a thioacetamide at the C-terminal (Fig. 1). The ComX and CSF (Competence and Sporulation Factor, also PhrC) peptides are extracellular signaling of B. subtilis; these affect its differentiation. The signal peptides are generated by cleavage from larger precursor peptide, and subsequent modifications by substitution with isoprenyl groups, resulting in the formation of lactone and thiolactone rings and lanthionines [3, 35]; for example, the c-butyrolactones of Streptomyces spp. and lantibiotic (lanthionine-containing antibiotic) mersacidin of Bacillus sp. [53, 57].

In contrast, there are 3 types of quorum quenching, which is enzymatic degradation of AHLs; known as AHL acylases (or AHL amidases), AHL lactonases, and AHL oxidoreductase. The AHL acylases cleave the molecule (quorum sensing) into a free homoserine lactone and a fatty acid, whereas the AHL lactonases hydrolyze the lactone ring, yielding a homoserine [2, 11]. The third enzyme also cleaves the molecule to yield homoserine lactone [59]. Quorum-quenching bacteria in environments have been reported upon; each sample obtained at least one quorum-quenching bacterium [8]. For instance, B. thuringiensis is able to break down the signal of quenching through
degradation with AHL lactonases (AiiA), resulting in the silencing of virulence in pathogens without changing the number or composition of cells [45, 63]. The AiiA, quorum-quenching activity, was found in Bacillus spp. and also found in Gram-negative bacteria, such as Agrobacterium tumefaciens, Pseudomonas aeruginosa, Arthrobacter sp., Rhodococcus sp., Variovorax paradoxus, and Acinetobacter [46, 51]. The AiiA showed another role in the rhizosphere competence of B. thuringiensis on the plant root system, suggesting that AiiA is involved in the cell metabolism or survival mechanism during cell growth [45]. Therefore, the viability of B. thuringiensis is protected from AiiA to form root colonization.

**Groups of Bacillus Substances and Their Applications**

Bioactive compounds of Bacillus sp. are divided into two systems; (i) nonribosomal synthesis of cyclic lipopeptides (NRPS) and (ii) polyketides (PKS), which are controlled by many genes (Table 1). Most of the genes are clustered

Table 1. Major compounds from nonribosomal peptide synthetases and polyketide synthases.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Gene cluster involved</th>
<th>Bacillus species</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iturin</td>
<td>itu, lpa</td>
<td>B. subtilis, B. amyloliquefaciens</td>
<td>Antifungal, hemolytic activities</td>
</tr>
<tr>
<td>Fengycin</td>
<td>fen, Pps</td>
<td>B. subtilis, B. amyloliquefaciens</td>
<td>Anti-filamentous fungi</td>
</tr>
<tr>
<td>Surfactin</td>
<td>srf, ycx, aat, sfp</td>
<td>B. subtilis, B. amyloliquefaciens</td>
<td>Antiviral, antimycoplasm activities (Vollenbroich et al. [61])</td>
</tr>
<tr>
<td>BacillomycinD</td>
<td>bmy</td>
<td>B. amyloliquefaciens</td>
<td>Antifungal</td>
</tr>
<tr>
<td>Bacillibactin</td>
<td>dhb</td>
<td>B. subtilis, B. amyloliquefaciens</td>
<td>Iron transport system; siderophores</td>
</tr>
<tr>
<td>Putative peptide</td>
<td>nrs</td>
<td>B. amyloliquefaciens</td>
<td>Siderophores (Herzner et al. [17])</td>
</tr>
<tr>
<td>Bacylins/anticapsin</td>
<td>bac, ywf</td>
<td>B. subtilis, B. amyloliquefaciens</td>
<td>Antimicrobial activity</td>
</tr>
<tr>
<td>ZwittermicinA</td>
<td>zwit</td>
<td>B. cereus</td>
<td>Broad spectrum of antibacterial (He et al. [19])</td>
</tr>
</tbody>
</table>

**Nonribosomal peptide synthetases (NRPS)**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Gene cluster involved</th>
<th>Bacillus species</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subtilin</td>
<td>spa</td>
<td>B. subtilis</td>
<td>Antimicrobial activity (lantibiotic)</td>
</tr>
<tr>
<td>SubtilosinA</td>
<td>sho, alb</td>
<td>B. subtilis, B. amyloliquefaciens</td>
<td>Antibacterial activity</td>
</tr>
<tr>
<td>TasA</td>
<td>tas</td>
<td>B. subtilis</td>
<td>Antibacterial activity</td>
</tr>
<tr>
<td>Sublancin</td>
<td>sun, bdb</td>
<td>B. subtilis</td>
<td>Antimicrobial activity (not lantibiotic)</td>
</tr>
<tr>
<td>Macrolactin</td>
<td>mln</td>
<td>B. amyloliquefaciens</td>
<td>Anti-Gram-positive bacteria</td>
</tr>
<tr>
<td>Bacillaene</td>
<td>bae, pksX</td>
<td>B. subtilis, B. amyloliquefaciens</td>
<td>Antibacterial activity</td>
</tr>
<tr>
<td>Difficidin</td>
<td>dif</td>
<td>B. amyloliquefaciens</td>
<td>Antibacterial activity</td>
</tr>
<tr>
<td>Mersacidin</td>
<td>mrs</td>
<td>B. amyloliquefaciens</td>
<td>Inhibit cell wall biosynthesis, anti-Gram-positive bacteria (Brotz et al. [5])</td>
</tr>
</tbody>
</table>

*Refers to compounds produced from both NRPS and PKS.*
Different species of *Bacillus* offer different advantages in biotechnology niches, due to divergent characteristics. Lipopeptides (LPs) are active compounds showing antimicrobial activity and act as immune stimulators by reinforcing host resistance in terms of root colonization. The cyclic LPs consist of surfactin, fengycin or plipastatin, and iturin families; these are major groups containing base structures related to other NRPS products. In the iturin family, the main structure is heptapeptides linked to a $\beta$-amino fatty acid chain with a length of 14 to 17 carbons (Fig. 2). The differences in heptapeptides show derivative compounds of iturin (*e.g.*, bacillomycin, mycosubtilin). The members of surfactin are composed of a $\beta$-hydroxy fatty acid with 7 amino acids, whereas 10 amino acids linked to a $\beta$-hydroxy fatty acid chain show in fengycin groups. The LPs have two roles in direct antagonism; through the interruption of membrane permeabilization properties of pathogens, and through root colonization in the rhizosphere competence to induce host plant immunization. However, the LPs cannot act with only one function to cope with pathogenic organisms. They act in a synergistic manner and also work with other rhizosphere microbial populations. The control of plant diseases has been reported with surfactin and iturin [34], surfactin and fengycin [44], and iturin and fengycin [52]. Moreover, the effect of these compounds on plant cells is specific to the kind of plant because of differing composition in phytosterols, mainly composed of sitosterol, stigmasterol, campesterol, and in some species cholesterol [43]. These affect the penetration of compounds or microbes in order to protect host plants. The transcriptional mechanisms are also important keys to synthesis compounds; these are controlled by both nutritional conditions (carbon, nitrogen, iron) and physicochemical conditions (temperature, pH, oxygen). The expression of surfactin is pH dependent, whereas that of mycosubtilin is oxygen dependent [17]. Both temperature and pH influenced iturin D and subtilosin A expressions [60]. Effective plant protection, it is proposed, is to form a consortium that produces various compounds in synchronicity, and independent to environments. The consortium should be developed in signal cell communication, transcriptional expression, and the secretion system towards widespread use in host plants.

**Fig. 2.** The structures of iturin, surfactin, and fengycin (KEGG structure). The cyclic lipopeptides contain fatty acid chain linked with amino acids (see text). The derivatives of compounds in each group come from different amino acid components.
Polyketides are active compounds that exhibit major antibacterial, immunosuppressive, or antitumor activities. Their syntheses have ribosomal mechanisms using the polyketide synthase gene cluster (PKS multienzyme system). There are 3 operons in the PKS (pks1, pks2 and pks3), which are identified in B. amyloliquefaciens [7]. The pks1, pks2, and pks3 genes use the same biosynthesis pathway, type I PKS containing β-ketoacyl synthase (KS), trans-acyltransferase (AT), and acyl carrier proteins (ACP) for basic domain, and subsequently the intermediate compounds might combine with different groups in the elongation step to generate individual compounds or a novel bioactive compound. Bacillaene, encoded by the bae (pks1) gene, contains a linear structure conjugated to hexaene (Fig. 3). The macroactins consist of 24-membered ring lactones with modifications, such as the attachment of glucose β-pyranoside, or they occur as linear analogs [54]. This is controlled by the mln (pks2) gene. The dif (pks3) genes produce difficidin/oxydifficidin that is a highly unsaturated 22-member macrolide with a rare phosphate group. The modular PKS system starts at the C3 precursor and terminates at module 11 for macrolactin [54] and difficidin [7], whereas the bacillaene synthesis terminates at modules 16 and 17 [38]. Similar to B. subtilis, the PKS system produces polyketide-like compounds having antibacterial activity, which are encoded by the pksX operon. The bae gene cluster showed similarity to the pksX sequence region, and they contain two hybrid NRPS-PKS at some parts of genes. The pks2 and pks3 genes may be transferred from other soil bacteria or evolved from the ancestral pks operon by several gene duplications [7]. However, these occurrences are not involved in the modular PKS system. B. subtilis lacks the sfp gene, which has the function of 4'-phosphopantetheine transferase; this results in an inability to synthesize bacillaene, macrolactin, and difficidin. However, B. subtilis can produce difficidin because of Sfp-type PPTases (pptS gene), which is a cognate 4'-phosphopantetheine transferase (PPTase) for the postranslational modification of fatty acid synthases (FAS). The B. subtilis A1/3 contains pksM and pksR genes for difficidin and bacillaenes biosynthesis [21]. Apart from the PKS system, lantibiotics are ribosomally synthesized peptides having antimicrobial activity that are found in Bacillus species; for example, subtilin and nisin are from B. subtilis, and mersacidin is from B. amyloliquefaciens [19]. These peptides are classified in bacteriocins, which are antimicrobial peptides produced by ribosomal synthesis.

Bacteriocins of genus Bacillus are divided into 3 classes that are independent from lactic acid bacteria.
bacteriocins. Class I contains posttranslationally modified peptides, such as subtilin, mesarcidin, lichenidcin, and subtilosin A; this class is similar to LAB bacteriocins called lantibiotics. Class II is non-modified peptides (e.g., coagulin, thurincin, thuricin, lichenin), and class III features large proteins (e.g., megacin) [1]. The lantibiotics are well known, the most characterized, and used in the food, agricultural, and pharmaceutical industries. They are small molecules (3–10 kDa), containing precursors of unusual amino acids (lantionine and methylthionine), which are modified by the dehydration of serine and threonine and the addition of cysteine residues. There are 2 groups of lantibiotics; linearly shaped lantibiotics (e.g., subtilin, nisin, epidermin) and globularly shaped lantibiotics (e.g., mesarcidin, subtilosin A, sublancin) (Fig. 4). Subtilin has bactericidal activity synthesized by the spa operon [30]; it is produced at high levels in starvation conditions. It is likely that subtilin is released to inhibit other bacterial growth, allowing Bacillus to then uptake greater nutrient supply. Entianin is a novel compound, a subtilin-like lantibiotic, of Bacillus subtilis subsp. spizizenii DSM15029; it has autoinduction and antibiotic activities as subtilin [13]. The cyclic, anionic peptide subtilosin A contains head-to-tail amino acids with three disulfide bonds and a linkage of a thiol to α-carbon of amino acids. It showed bactericidal activity against human pathogens and hemolytic activity. Sublancin is an S-linked glycopeptide containing two disulfide bridges; it is not a lantibiotic, as shown in a revised structure consisting of a sugar linked to cysteine-22 residues (Fig. 4). It is a stable peptide and tolerant to both low and high pH; the loss of antimicrobial activity results from a mistake of glycosylation with the correct disulfide connectivity [42]. Quorum sensing or cell–cell communication is associated with the induction of antimicrobial activity and other peptides as signal transducers. The bacteriocins have a role as autoinducers in the activation of gene clusters [27, 28]. Moreover, bacteriocin-like inhibitory substances (BLIS) have been reported in various Bacillus species. It is interesting, as it exhibits a broad spectrum of antimicrobial activity, and it is also stable at a wide range of temperature and pH. The bacteriocins or BLIS have the potential ability to prevent or control both spoilage and pathogenic microorganisms. They are applied in probiotics for human use (as a dietary supplement), as animal feed, and are found in the food supply as preservatives.

Within the three groups of antimicrobial compounds, there are specific applications relating to the broad functions of antimicrobial activity (Table 1). The cyclic lipopeptides are used in crops, to protect plants from phytopathogens. The compounds are tolerant to enzymes (pronase, proteinase K) and organic solvents (butanol), and are stable in low pH and high temperature [52]. These properties render lipopeptides suitable for usage in agriculture as a biocontrol and biofertilizing agent. Additionally, the surfactins are important metabolites involved in quorum sensing of cell–cell interactions in Bacillus sp. It has a PPTase-dependent gene involved in NRPS for surfactin and biofilm formation. Another lipopeptides producer, Pseudomonas sp., exhibited different systems of pathway synthesis and possessed good potential compounds for biocontrol [49]. The polyketide and bacteriosin from PKS systems are commonly used in pharmaceutical industries. Moreover, the polyketides are also used as biocontrol agents and the bacteriocins are commonly used in the food industry as probiotics and preservatives. The Bacillus species are classified to be generally recognized as safe (GRAS) strains, and as such their usage is allowed in the food industry. There are many commercial products available as dietary supplements for human use (e.g., B. cereus, B. clausii, B. pumilus [12]), and for veterinary use (e.g., B. licheniformis, B. coagulans, B. clausii, B. cereus [10]). In these applications, the vegetative cell and spore of Bacillus can stimulate immune systems in the gastrointestinal tract. The secretion of compounds, such as coagulin, amicoumacin, and subtilisin, effects the suppression of pathogens, a characteristic that provides high potential as a property of probiotics. Therefore, there are three mechanism steps of Bacillus interaction with hosts; (1) stimulation of immune systems, (2) suppression of gastrointestinal pathogens, and (3) secretion of antimicrobial compounds. Moreover, it has been reported that more than 10% of inoculated Bacillus spore germination showed growth and reforming sporulation [58]. However, recognition of GRAS status, as safe for use in the food industry, is strain specific; for example, B. subtilis var. Natto is a safe strain for the production of proteolytic enzyme; B. subtilis bacterium is not identified as a safe strain. This genus produces the Bacillus enterotoxins, Nhe and Hbl, which are unsafe for humans. There are many applications of Bacillus substances apart from those previously mentioned, including use as biosurfactant, biofuel, and with various enzymes in various industries. Therefore, it is necessary to closely consider the specific purposes in the analysis of synthesis pathway and gene regulation of various metabolites, and then to match the unique properties to specialized roles. There is also a need for further study of cell differentiation in spore-forming bacteria, with greater knowledge enabling the identification and development of the products of genus Bacillus towards wide-ranging commercial applications.

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