Genetics and Biochemistry of Sporulation in Endospore-Forming Bacteria (*Bacillus*): A Prime Example of Developmental Biology

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1 Introduction

Endospore-forming bacilli constitute a prominent group of bacteria, not only for the pathogenic species it includes (i.e., *Clostridium botulinum*, *Clostridium difficile*, *Bacillus anthracis*), but also for its saprophytic (i.e., *Bacillus subtilis*) and industrially important microorganisms (i.e., *C. acetobutylicum*). Additional significant spore-forming species include the genera *Desulfotomaculum*, *Paenibacillus*, and *Alicyclobacillus*. Other recently described Gram-positive bacteria such as *Caldalkalibacillus thermarum* TA2.A1 (Peddie et al. 1999; Xue et al. 2006), which is a member of alkaliphilic bacteria but otherwise related to the *Bacillales* order, has been recently shown to contain at least three annotated operons involved in spore germination (de Jong et al. 2020), including the genes gerABC and yndE. As this alkalophilic bacterium is old in terms of evolution, it has to be assumed that the ability of endospore-forming emerged soon in the evolution of Gram-positive bacteria.

The bacteria exhibiting this exclusive ability, when encounter unappropriate physicochemical conditions initiate the formation of important small molecules, that are collectively known as "alarmones" which are part of the heat shock response

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in *B. subtilis* (Schäfer et al. 2020); two of them classic and well known such as pppGpp, ppGpp, and the newest one pGpp, with at last recognized effect as alarmone (Yang et al. 2020), all of them involved in a classical bacterial response known as "stringent response" and recently also found in metazoan (Ito et al. 2020). These elements (highly conserved in Nature and known for more than five decades; Cashel and Gallant 1969) show a variety of pleiotropic effects and are involved in a number of metabolic pathways in bacteria, including the development of endospores. Therefore the alarmones represent a new way for bacterial survival (Fernández-Coll and Cashel 2020). In addition, it has been shown recently that the ComX q*uorum sensing* peptide of *B. subtilis* positively affects the sporulation process (Špacapan et al. 2020). Differentiation processes in *B. subtilis,* such as endospore formation, involve multiple paralog Rap-Phr systems that are highly redundant, and that according to Gastélum and colleagues in 2020, interconnect this first-order morphogenetic event with others such as the development of competence.

B. subtilis is, therefore, and without a doubt, the best-known Gram-positive bacterial rod, and contains three subspecies [i.e., *subtilis* (Nakamura et al. 1999), *spizizenii* (Nakamura et al. 1999), and *inaquosorum* (Rooney et al. 2009)]. These three subspecies are so similar (they share *ca* 3300 ORFs) that they can only be differentiated by phylogenetic analysis of multiple proteins, as their 16S rRNAs exhibit an extremely high sequence identity (for a genomic insight into the taxonomic status of the three *B. subtilis* subspecies, see Yi et al. 2014).

Although endospore-forming bacteria can exhibit different metabolic and genetic abilities, they all belong to the phylum Firmicutes and share the capacity to survive harsh environmental conditions via the production of highly resistant endospores; this is a superior biological development, normally subjected to catabolic regulation (Schaeffer et al. 1965). These highly resistant structures have been recently reviewed from the point of view of the different technologies usable today that cause endospore death (Cho and Chung 2020). Espores from *B. subtilis* have been used recently in chickens with positive results as adjuvants in vaccines against the avian influenza H9N2 orthomyxovirus (Lee et al. 2020)

Endospore formation follows the same genetic program in all bacteria, with little variation from species to species; this fact led Hutchison and coworkers to suggest in 2014 that "*a robust and sophisticated developmental framework was already in place in the last common ancestor of all extant Firmicutes*." Nearly 90 different bacterial genera can form endospores and, although Gram-positive microorganisms are predominant among them, this endospore-forming group also includes many Gram-negative species.

This survival structure was originally described by Ferdinand Julius Cohn, in the nineteenth century (1875). The author, although a botanist, became one of the founding fathers of modern bacteriology and microbiology by demonstrating the ability of *Bacillus* to form endospores and describing the basic steps in spore formation (Drews 2000). Cohn, due to his background in algal taxonomy, also made a significant contribution to bacterial taxonomy, although his bacterial classification was not accepted by many of his colleagues, who still believed that bacteria

could spontaneously arise from decaying biological matter (Cohn 1875; Drews 2000).

The number of endospores produced by bacteria can vary from one (monosporic species), two (bisporic), or many (polysporic), and they always are genetically identical copies of the vegetative cells. The morphogenetic process resulting in endospore production is usually initiated by a lack of nutrients essential for vegetative growth (mainly nitrogen source depletion). This process is tightly regulated by SPO genes and different σ factors, that define the sporulation stages, and terminates with the formation of a multilayer, refractive, highly resistant structure that can withstand the challenges posed by factors such as extreme temperatures and DNA-damaging agents (Errington 2003). This survival structure is what microbiologists call "endospore," characterized by its metabolically inactive "dormant state." In some bacterial groups, however, the sporulation process gives rise to multiple intracellular offsprings, some of which do not undergo a dormancy period; many of these spore-forming bacteria, although hard to grow axenically, were identified as Clostridia, one of the endospore-forming bacterial group (Hutchison et al. 2014).

Unraveling the mechanism of endospore formation, triggered by starvation, resulted not only in the understanding of this basic bacterial morphogenetic process and in obtaining a variety of mutants with different metabolic and genetic abilities, but also in the discovery of novel non-Firmicutes and remote bacterial strains displaying certain characteristics of the Firmicutes. One of these traits is the resistance to soil-dwelling predatory microorganisms, such as the delta proteobacterium *Myxococcus xanthus*. It is well known that nondomesticated strains of *B. subtilis*, capable of producing bacillaene (a polyene antibiotic), can resist the attack of the predatory bacteria, eventually forming spores and hence becoming fully resistant to the predator. On the other hand, laboratory strains of *B. subtilis*, usually unable to produce the antibiotic, are easily predated by *M. xanthus* (Müller et al. 2014).

Starvation-induced sporulation is the last survival resort for some bacteria. The sporulation process involves a cellular decision-making stage (commitment point), that can last several hours depending on the bacterium, accompanied by the development of actinomycin resistance (Sterlini and Mandelstam 1969). During this time, the bacterium explores other possibilities of survival, such as the secretion of enzymes to use alternative food sources, production of antibiotics to eliminate competing microorganisms, and the induction of cell competence to uptake exogenous DNA. Sporulation is suppressed until all other possibilities are shown inviable and, once the commitment point is reached, sporulation is irreversible. The sporulation process is spatially and temporally orchestrated and represents one of the most thoroughly investigated cellular processes. Some of the genes involved were mapped on the *B. subtilis* chromosome (Piggot and Coote 1976; Piggot and Hoch 1985) by means of either transformation or transduction. Sporulation studies in *Bacillus* and *Clostridium* determined that, although the process is continuous, it can be structured into several stages. Sporulation starts with Stage 0: the decision to sporulate and ends with Stage VI/VII: spore release (Fig. 1), as proposed by Ryter in 1965. Already in 1974, Hranueli et al. proposed that spore formation in *Bacillus* involves at least 37 operons. For a recent review, see Setlow and Johnson (2019).

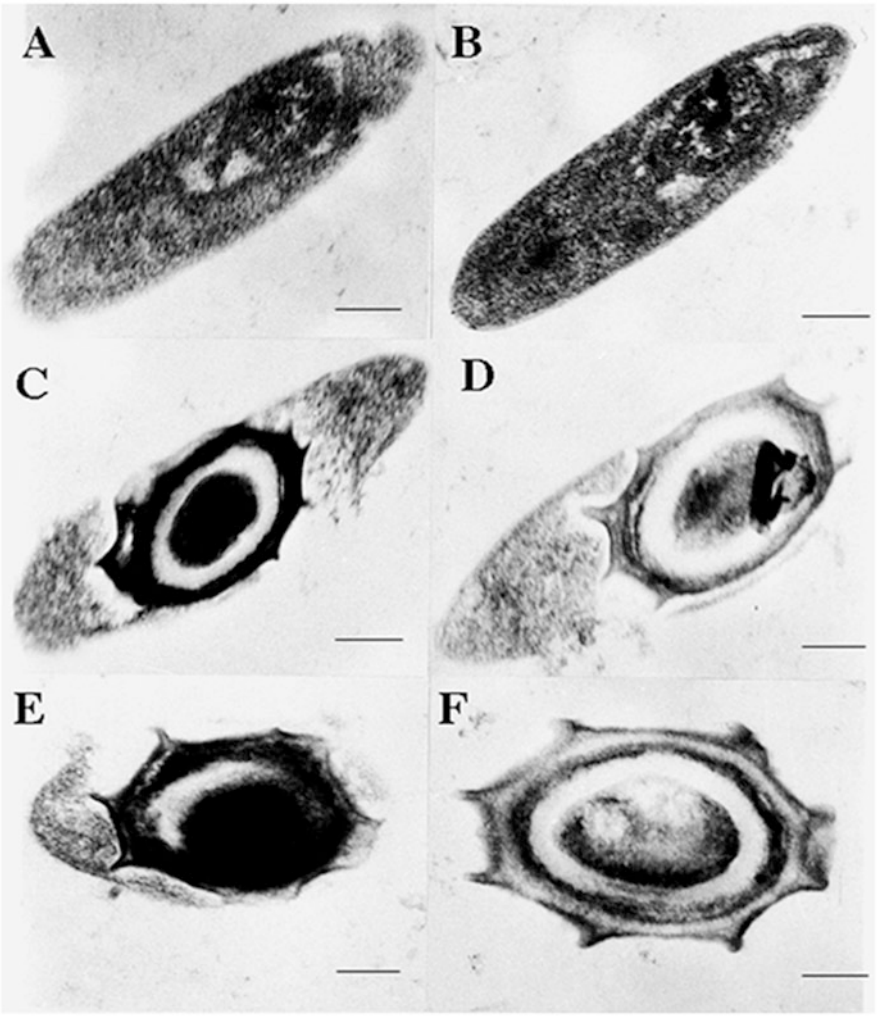

Fig. 1 Sporulation stages in the Gram-positive bacteria *Paenibacillus favisporus*. (**a**, **b**) Fore-spore formation; (**c**) spore maturation, displaying the typical surface of the spores from this species; **d**, **e,** and **f**) lysis of sporangium and spore release (modified from Velázquez et al. 2004). Scale bar is 0.7 mm (**a**, **b**, **c**, and **d**) or 0.2 mm (**e** and **f**)

The years 1996 and 1997 saw the publication, in Microbiology and Nature respectively, of first the computerized genetic map of *B. subtilis* (Biaudet et al. 1996) and then the complete sequence of *B. subtilis* genome (Kunst et al. 1997). *B. subtilis* genome spans 4,214,810 base pairs, encompassing 4100 protein-coding genes, as well as at least ten prophages or their remnants and a large number of genes for using a variety of nutrients, many of plant origin. More recently, the publication of complete genome sequences, such as that of *Clostridium perfringens* (Shimizu

et al. 2002), has permitted to carry out comparative genomics with other important Gram-positive anaerobic sporulating rods.

Unraveling this complex genetic and biochemical pathway not only contributes to a better knowledge of the biology of sporulating Gram-positive bacteria, but could also result in the discovery of novel antibiotics, or even contribute to the knowledge of associated flavors in certain beverages, such as the Chinese Maotai Liquor (Wang et al. 2018).

Endospore formation is a major morphological feature used in bacterial taxonomy and the characteristics of the spore, such as location within the sporangium (mother cell), sporangium distension and number of spore per sporangium, are also important for the classification of both aerobic and anaerobic spore-forming Gram-positive bacilli.

Starvation is not the only trigger for sporulation, in fact, siderophore production is another factor affecting endospore formation. Grandchamp and coworkers demonstrated in 2017 that the production of bacillibactin facilitates sporulation, and even enterobactin (a siderophore from *E. coli*) induces *B. subtilis* sporulation. However, while the uptake of either siderophore involves binding to just one protein (FeuA), the onset of sporulation in the presence of the siderophores requires a different protein for each siderophore, such as the esterase BesA for bacillibactin and the esterase YbbA for enterobactin (Grandchamp et al. 2017).

B. subtilis spores have recently found quite unusual applications (Sun et al. 2020). The authors used spore coat proteins CotB and CotC as anchors for the heterogenous antigen in a system grass carp reovirus combined with the genes cwlJ and sleB able to control the pore germination. Heterologous antigens using this method were able to elicit a strong humoral as well as cellular response in *Ctenopharyngodon idella.*

One tends to consider the SPO proteins (all those so far related to the sporulation process) as exclusive of those bacteria able to carry on with the formation of endospores, but the truth of the matter is that there is a large variety of bacterial species (including *Escherichia coli*) that contain sporulation-related repeated domains, known to bind peptidoglycan and also to enhance the activity of the penicillin-binding proteins and hence of the transpeptidase activity (Pazos et al. 2020).

The study of endospore formation in *B. subtilis* has been an important increase of our knowledge in terms of genetics, biochemistry, and developmental biology, but indeed it has resulted in practical applications. One of these has been the development of a new strain of *B. subtilis* that harboring the β-lactam-induced regulatory system BlaR1/BlaI from *Staphylococcus aureus*, which can be used as an efficient biosensor to evaluate the presence of β-lactams in solution (Lautenschläger et al. 2020). Another interesting application involving the spores of *Bacillus subtilis* is related to the use of these spores to prepare vaccines against *B. anthracis*. So, Oh, and colleagues reported in 2020 the obtention of a new *B. subtilis* strain that originates spores with the anthrax protective antigen on the surface. All in all, and as Errington and van der Aart have recently proposed *B. subtilis* has been and still is a workhorse as a model for studying cellular development, including the generation

of asymmetry, cell fate, and prokaryotic morphogenesis in general (Errington and van der Aart 2020).

2 Genes and Factors Affecting Endospore Formation

The initiation of sporulation is a prime example of developmental biology in Gram-positive bacteria that strongly involves biochemical and genetic factors. It occurs in Nature constantly in this group of bacteria, when encountering inappropriate physicochemical conditions, and the picture of the whole process may be altogether blurred by the continuous growing of *B. subtilis* under laboratory conditions in what has been denoted as "loss of social traits during domestication process of *Bacillus subtilis*" (Barreto et al. 2020).

Endospore formation depends on a major signal transduction system known as "the sporulation phosphorelay" that controls phosphorylation of the key Spo0A transcription factor (Burbulys et al. 1991; Ohlsen et al. 1994; Wang et al. 2001), as well as the synthesis of sporulation-specific sigma factors (Fimlaid et al. 2015) involved in the subsequent sporulation stages. Most of the biochemical changes during sporulation appear to occur during the first two "sporulation stages" mentioned above (0 and II); during this period, a new cell differentiates within the mother cell and isolates itself, although it maintains a specialized connection system to the mother cell, to receive from her a variety of nurturing compounds, such as activators and sigma factors.

Initiation of sporulation in *B. subtilis* stops normal growth (stage 0; Fig. 2), this is followed by the synthesis of a septum (stage II, see below). The Spo0A protein is activated through phosphorylation (Sonenshein 2000) in stage 0 and is responsible for the regulation, either directly or indirectly, of more than 500 genes (Fawcett et al. 2000). When studying the σ factors involved in the sporulation process, it soon became clear that a single vegetative σ factor could not be responsible for the RNA transcription carried out from a variety of promoters which, in addition, are different from those responsible for vegetative growth and primary metabolism. Further proof of this was provided by Linn and coworkers that, already in 1973, demonstrated that the activity of the vegetative sigma subunit of *B. subtilis* RNA polymerase dramatically decreases once sporulation starts, and its levels remained low throughout the sporulation process. These findings were confirmed by Brevet the following year (Brevet 1974).

An example of activation of sporulation-specific genes/regulons is the cascade reaction initiated by the arbB gene, which encodes a protein (ArbB) that acts as a repressor of spo0H expression. The gene spo0H encodes the σH protein (Weir et al. 1991), which regulates the expression of σF protein, responsible for entering sporulation stage II (Wu et al. 1992; Sonoda et al. 2015). Figure 3 summarizes the different σ factors involved in the main sporulation stages.

Briefly, the spore formation pathway mainly depends on two pivotal kinases integrated into the phosphorelay process of sporulation. The main activators and

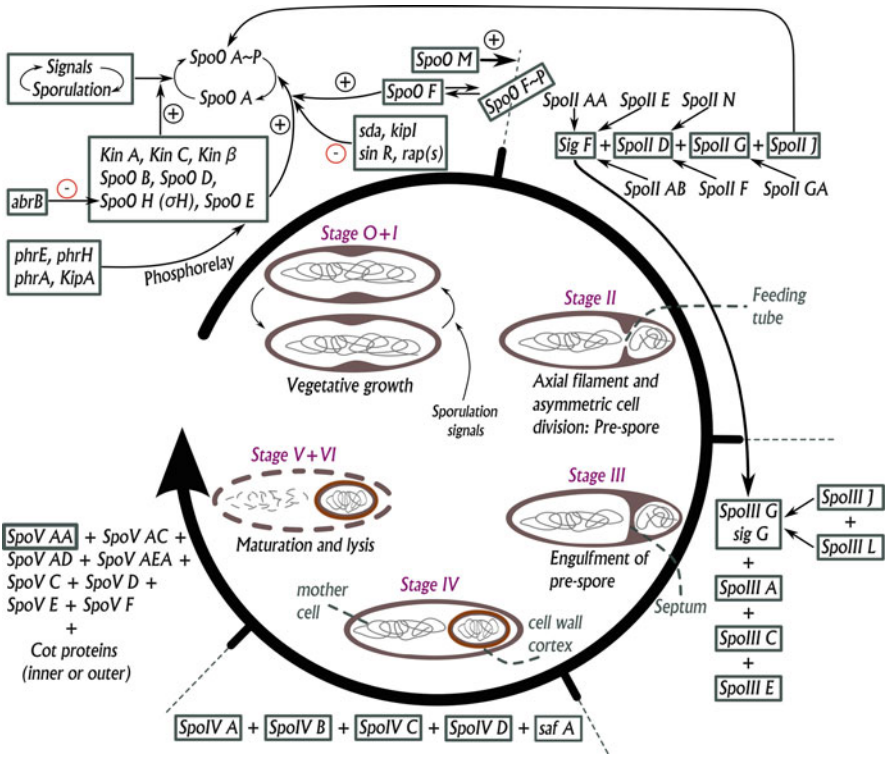

Fig. 2 Key stages of the sporulation cycle in *Bacillus subtilis*. Sporulation phases 0 to VI are indicated in the diagram, and the main genes involved in the process are summarized. Stage VI represents the final events leading to spore maturation inside the mother cell, while stage VII requires mother cell lysis for the spore to be released

repressors systems required for sporulation initiation are depicted in Tables 1, 2, 3, 4, and 5.

Bacteria rely on histidine kinases to react to a variety of external signals, and this also applies to sporulation. KinA is perhaps the main histidine kinase involved in the initiation of endospore formation in the family *Bacillaceae*. Winnen and collaborators described in 2013 that this kinase had an N-terminal region (residues 1–382) spanning three tandem Per-ARNT-Sim (PAS) domains, believed to constitute the sensor sporulation module. Upon nutrient starvation in endospore-forming bacteria, KinA inhibits the antikinase activity of KipI (gene homologues of kipI are found almost throughout all bacterial kingdom; Jacques et al. 2011a), hence allowing sporulation. KipI and KipA are the fourth and fifth genes, respectively, of a seven-cistron operon that is upregulated by high glucose concentrations and down-regulated in the presence of nitrogen. The combined actions of KinA and Kipl trigger the regulatory pathway known as the sporulation phosphorelay, which in turn activates Spo0A (the main component of the sporulation cascade). The protein Sda (Fig. 4) is also involved in KinA phosphorylation, as well as in replication and

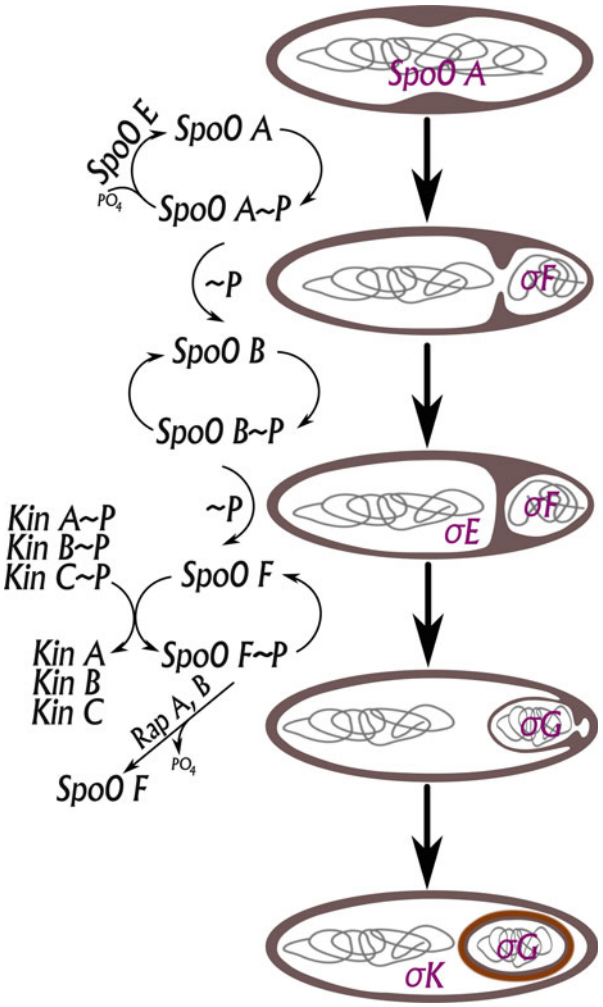

Fig. 3 Summary of sigma factors involved in *Bacillus subtilis* sporulation. SpoIIT is involved in the activation of σE in the mother cell, whereas SpoIIIL is required for σG activity in the forespore (Meeske et al. 2016)

sporulation coordination (Veening et al. 2009). The gene products involved in stage 0 are depicted in Tables 1 and 2.

Sporulating stages II and III involve a differentiation program that lasts 5 hours, and, according to Eichenberger and coworkers in 2004, it involves 383 genes epistatically controlled by transcription factors σ^E, σ^F, σ^G (they activate 81 genes), and σ^K. This stage is characterized by an asymmetric division that gives rise to a sporangium, formed by the mother cell and separated from the future forespore by a closing Z-ring that leaves a narrow tunnel, also known as the "feeding tube" (Mastny et al. 2013) that links both compartments. The tunnel also contains a DNA filament that extends from the mother cell. As it is, and before the beginning of the asymmetric division to form the prospore, an axial DNA filament is formed containing two chromosomes along the longest axis of the cell, and firmly attached to each pole

Table 1 Main activator proteins involved in *Bacillus* (mainly *subtilis*) initiation of sporulation

Locus/gene/ protein activators	Effect/regulation	Map position Degrees/ coordinates[e]	Reference
kinA (synonym spo IIJ, spoIIF, gsiC, scoD)[d] Encodes a 68.99 kDa protein	Transfers phosphate to Spo0F and SpoA transcription factors Autophosphorylates	118°	Wang et al. (2001) Perego et al. (1989) Tojo et al. (2013)
kinB Encodes a 47.7 kDa protein	Transfers phosphate to the Spo0F transcription factor. Expressed and activated before KinA	280°	Dartois et al. (1996) Tojo et al. (2013)
kinC (synonym ssb) Encodes a 48.68 kDa protein	Two-component sensor kinase, phosphorylates Spo0F and Spo0A, part of the phosphorelay	124°	LeDeaux and Grossman (1995) Kobayashi et al. (1995) Jiang et al. (2000a)
spo0A (synonyms spo0C, spo0G, spoIIL, sof-1) Encodes a 29.5 kDa protein	Activates sporulation-specific genes and non-specific (>500) Phosphorelay regulator coordinates DNA replication and initiation of sporulation by binding to sites close to the oriC	217°	Kudoh et al. (1984, 1985) Ferrari et al. (1985) Molle et al. (2003)
spo0B (synonym spo0D) Encodes a 22.40 kDa Protein	Phosphotransferase initiation	240°	Ferrari et al. (1982) Bouvier et al. (1984)
spo0G (synonym spoA)[f]	Not involved in competence development	217°	Ionesco et al. (1970) Sadaie and Kada (1983)
spo0F Encodes a 14.09 kDa protein	Phosphotransferase initiation	323°	Shimotsu et al. (1983) Trach et al. (1985)
spo0D BSU_17920	Phosphatase	240°	Ionesco et al. (1970)
spo0E Encodes a 9.79 kDa Protein	Spo0A-P phosphatase	115°	Perego and Hoch (1987)
spo0H (σH) Encodes a 25.3 kDa protein Expression requires spoA expression Regulated by external pH changes	Activates phrE gene[a] (Phosphatase RapE inhibitor) Transcribes early stationary phase genes, also involved in competence	11°	Weir et al. (1984) Dubnau et al. (1987, 1988) Cosby and Zuber (1997)

(continued)

Table 1 (continued)

Locus/gene/ protein activators	Effect/regulation	Map position Degrees/ coordinates[e]	Reference
spo0J (synonyms ParB, spo0JB) Encodes a 32.06 kDa protein	Involved in catabolite repression of sporulation and chromosome segregation Not involved in competence development	359°	Hranueli et al. (1974) Sadaie and Kada (1983) Mysliwiec et al. (1991) Ireton et al. (1994)
spo0K 5 genes operon	Oligopeptide transport system Involved also in competence development	104°	Rudner et al. (1991)
Spo0L	Spore cortex lytic enzyme	115°	Kunst et al. (1997)
Spo0M (synonym ygaI) Encodes a *ca.*29.5 kDa protein.	Member of arrestin gene family Stops pass from 0 to II stages Phosphorylates >500 genes Member of SigH and SigW regulons	953373–954149	Alvarez (2008) Sonoda et al. (2015) Vega-Cabrera et al. (2018)
comA (synonyms srfB, comAA) Encodes a 23.98 kDa protein	Activates transcription and *quorum sensing* Activates phrA	279°	Guillen et al. (1989) Wolf et al. (2016)
sinI (second gene of a two-gene operon) Encodes a 6.47 kDa protein	Antagonist of sinR. Represses binding of SinR to aprE[b] and stage II sporulation genes	219°	Bai et al. (1993) Lewis et al. (1996)
kipA (synonym pxpC) Encodes a 36.92 kDa subunit of 5-oxoprolinase, antagonist of KipI[c]	Detoxification of 5-oxoproline, control of the phosphorelay, initiation of sporulation	460592–461599	Wang et al. (1997)
phrA (synonym gsiAB) Encodes a 4.66 kDa protein	Suppresses dephosphorylation activity of RapA (aspartate phosphatase). Inhibits, control of the phosphorelay	1316995–1317129	Perego et al. (1996) McQuade et al. (2001)
phrE Encodes a 4.72 kDa protein	Regulator aspartate phosphatase (RapE); does not affect rapA and rapB. Controls the sporulation phosphorelay	2660330–2330464	Jiang et al. (2000b) McQuade et al. (2001)

(continued)

Table 1 (continued)

Locus/gene/ protein activators	Effect/regulation	Map position Degrees/ coordinates[e]	Reference
phrH Encodes a 6.3 kDa Protein	Response regulator aspartate phosphatase (RapH), dephosphorylates Spo0F-P, control of the phosphorelay, sequestration of ComA activity	752079–752252	Mirouze et al. (2011)

[a]Phr pentapeptide (six aminoacids in the case of PhrH) inhibits Rap proteins. Processing of the Phr precursor proteins into active pentapeptides is a key event in the initiation of sporulation and competence (i.e., PhrA (ARNQT) and PhrE (SRNVT) peptides inhibit the RapA and RapE phosphatases, respectively (Stephenson et al. 2003)

[b]aprE gene product is a major extracellular alkaline serine protease (subtilisin E) of 39.37 kDa

[c]Kip I is a potent inhibitor of the autophosphorylation reaction of kinase A (inhibits SpoA-P), but does not inhibit phosphate transfer to Spo0F. The inhibitory activity of KipI is counteracted by KipA (Wang et al. 1997)

[d]Autophosphorylation occurs in *trans* (one subunit of the multimer phosphorylates the other subunit) within the homotetramer complex, instead of *cis* (one subunit of kinase phosphorylating itself within the multimer) (Devi et al. 2015)

[e]When possible, gene mapping is expressed as degrees to honor the efforts in transducing-mapping, since interrupted mating-mapping cannot be carried out in *Bacillus*

[f]Lack of rho factor or a defective one leads in *B. subtilis* to activate spoA, thus initiating sporulation cascade (Bidnenko et al. 2017)

thanks to proteins such as RacA, Soj, Spo0J, and MinD (Wu and Errington 2003; Willis et al. 2020). In this way, when the prespore is finally formed, it tapes *ca.* 30% of one chromosome and the remaining 70% of the chromosome relays on the feeding tube, and particularly on the translocase SpoIIIE (Bath et al. 2000; Willis et al. 2020), which is a hexameric protein that embraces the double-stranded DNA, and translocates each arm into the prespore, presumably through the formation of small pores. It is known that the terminus chromosomal region in *B. subtilis* is comprised between 152 and 187°, and that this region is the last one to be translocated into the prospore (Willis et al. 2020). The feeding tube, therefore, is crucial for spore formation and maturation, as this process requires many gene products expressed by the mother cell genes that are transferred to the forespore through this tunnel. The genes involved in this stage and their function are summarized in Table 3.

Sporulating stage III is characterized by the engulfment of the forespore by the mother cell; this results in the forespore being covered by a double-membrane, inner and outer membranes (McKenney et al. 2013), within the mother cell cytosol. This phase is accompanied by a simultaneous synthesis of modified peptidoglycan, which contains the modified sugar muramic-δ-lactam and a low level of peptide cross-links between the glycan strands (Popham 2002), located between the inner and outer membranes. Deposition of a proteinaceous layer takes place mainly externally, thus completing the formation of the spore "cortex," that constitutes the characteristic structure of Stage IV (see Tables 4 and 5 for its main components and functions).

Table 2 Main repressors involved in *Bacillus* (*subtilis*) initiation of sporulation

Locus/gene/prot repressors	Effect/regulation	Map position Degrees/ coordinates	Reference
sda Encodes a 6.02 kDa protein	Blocks autophosphorylation of KinA. Controls the phosphorylation status of Spo0A	2647456–2647614	Rowland et al. (2004)
kipI (synonyms pxpB, ycsJ) Encodes a 26.57 kDa protein	Blocks autophosphorylation of KinA	459867–460589	Jacques et al. (2011a, b)
spo0A (synonyms spo0C, spo0G, spoIIL, sof-1) Encodes a 29.5 kDa protein	Main component in *Bacillus* sporulation. Phosphorelay regulator, initiation of sporulation, coordinates DNA replication and initiation of sporulation by binding to sites close to the oriC Negatively controls transcription of abrB interacts with two sigma factors (A and H)	217°	Fujita and Sadaie (1998) Baldus et al. (1995) Strauch et al. (1990)
sinR (synonym sin, flaD) Encodes a binding protein of 111 aa (binds $aprE^{b}$ gene)	Represses the key sporulation gene spo0A A pleiotropic late growth regulator	219°	Bai et al. (1993) Lewis et al. (1996) Mandic-Mulec et al. (1995)
rapA (synonym gsiAA, spo0L), rapB (synonym spo0P, ywmE), rapE (synonym yqcH), and **rapH (synonyms yeeH, yzqA)** Encode 44.81 kDa, 44.88 kDa, 44.40 kDa, and 49.96 kDa proteins, respectively	Response regulator aspartate phosphatase, dephosphorylates Spo0F~P, control of the phosphorelay	115°	Perego et al. (1996) Tzeng et al. (1998) Jiang et al. (2000b) Hayashi et al. (2000) Parashar et al. (2011)
spo0E, yisI, and ynzD Encode 9.65 kDa, 13.08 kDa, and 6.55 kDa proteins, respectively	Dephosphorylation of Spo0A~P, control of the phosphorelay	1430684–1430941 1153265–1153621 1922841–1923014 respectively	Kunst et al. (1997) Perego (2001)
GTP-bound **codY** Encondes a 28.86 kDa protein (regulates more than 100 genes and operons)	Inhibits rapA-phrA Regulation of a large regulon (more than 100 genes and operons) in response to branched-chain amino acid limitation	141°	Belitsky and Sonenshein (2008) Sonenshein (2005) Brinsmade et al. (2014)

(continued)

Table 2 (continued)

Locus/gene/prot repressors	Effect/regulation	Map position Degrees/ coordinates	Reference
abrB Encodes a 10.63 kDa protein (transcriptional regulator)	Epistatic to spo0A and spo0B mutations General repressor of spo0H	328°	Zuber and Losick (1987) Perego et al. (1988)
hpr Encodes a 23,718 kDa protein	Transcriptional regulator; overexpression inhibits sporulation	76°	Perego and Hoch (1988) Biaudet et al. (1996)
dnaA Encodes a 50.70 kDa protein (AAA+ ATPase)	Overexpression of Sda[a] Affects expression of transcription factors Spo0A, AbrB, PhoP, SinR, and RemA[b]	410–1750	Fukuoka et al. (1990) Washington et al. (2017)

[a]An inhibitor of histidine kinases that regulates initiation of sporulation in *Bacillus subtilis*.
[b]Transcriptional regulators of the extracellular matrix genes, acts in parallel to SinR, AbrB (Winkelman et al. 2009)

A peculiarity of *B. subtilis* sporulation is that there is a temporal dissociation between the occurrence of late events and the expression of genes that determine them (Jenkinson et al. 1980), as the proteins responsible for the changes during stages V and VI are already synthesized by the end of stage IV. Stage V is characterized by the formation of the spore coat, which contains approximately 70 proteins, originated from the mother cell, many of which started migrating to the spore surface at the time of engulfment (Popham 2002). Stage VI (maturation and sporangium lysis; summarized in Table 6) starts with the synthesis of dicarboxylic dipicolinic acid (derived from L-aspartate, see Fig. 5), that chelates high amounts of Ca^{++} and transforms the spore into a refractile structure containing the coat proteins (Fig. 6), selectively stainable by malachite green at high temperature, and thus forming a spore crust (which is the outer most layer of spores lacking sporangium). The crust structure is composed of several coat proteins such as CotV, Cot W, CotX, CotY, CotZ, and CgeA (Bartels et al. 2019), being CotY the most important in the crust structure in terms of scaffolding and morphogenetic functions (Shuster et al. 2019; Dubois et al. 2020) along with CoX and CotZ. In addition to these coat proteins, the crust contains a variety of glycans with functions largely unknown, although at least two different glycans have been proposed: one linked to the outer coat proteins and another strictly linked to the crust (Shuster et al. 2019; Dubois et al. 2020). In this sense, the genes *spsM, spsABCDEFGIJKL, yfnHGFED, ytdA-ytcABC, and cgeAB-cgeCDE* have been involved in the synthesis of the surface proteins (Cangiano et al. 2014). Lately, it has been demonstrated (Dubois et al. 2020) that these *sps* genes encode the legionaminic acid pathway that is required for crust assembly. The legionaminic acid is a 9 carbon, beta-neuraminic acid derivative

Table 3 Main genes and proteins involved in sporulation stage II

Locus/gene/ protein	Effect/regulation	Map position Degrees/ coordinates	Reference
spoIIAA Encodes a 12.85 kDa protein	Controls sigF activity (anti-anti-sigma factor) Inhibitory feedback on Spo0A	211°	Najafi et al. (1995) Duncan et al. (1996) Arabolaza et al. (2003)
spoIIAB Encodes a 16.21 kDa protein	Controls sigF (anti-sigma factor). Also functions as a phosphokinase on spoIIAA	211°	Schmidt et al. (1990) Duncan and Losick (1993) Najafi et al. (1995)
sigF (synonym spo II AC) Encodes a 29.22 kDa protein	RNA polymerase forespore-specific (early) sigma factor Sig. Turns on approximately 48 genes, including the gene for RsfA, which represses a gene in the sigma(F) regulon	2443429–2444196	Clarkson et al. (2004) Camp and Losick (2009) Camp et al. (2011)
spoIID (synonym spoIIC) Encodes a 37.25 kDa protein	Cell wall hydrolase (lytic transglycosylase), required for the complete dissolution of the asymmetric septum	316°	Gutierrez et al. (2010)
spoIIE (synonyms poIIH, spoIIK) encodes a 91.78 kDa protein	A membrane serine phosphatase. Controls SigF activity, required for normal formation of the asymmetric septum. Interacts with morphogenic protein rodZ and GpsB and involved in early stages of asymmetric septum formation.	8°	Guzmán et al. (1988) Barák et al. (1996) Muchová et al. (2016, 2020)
spoIIF (synonyms kinA, spoIIJ, gsiC, scoD)	Two-component sensor kinase Controls spoIID	118°	Louie et al. (1992)
spoIIGA Encodes a 34.70 kDa protein	Maturation of SigE (σ^E)	1603779–1604708	Jonas et al. (1988) Peters and Haldenwang (1994) Schyns et al. (1997)
spoIIG (spoIIGB sigma E) Encodes a 27.5 kDa protein	Sigma factor 29 Produced as pre E and processed by SpoIIGA membrane protease	135°	Trempy et al. (1985) Imamura et al. (2008) Eichenberger et al. (2003)

(continued)

Table 3 (continued)

Locus/gene/ protein	Effect/regulation	Map position Degrees/ coordinates	Reference
spoIIJ (synonyms kinA, spoIIF, gsiC, scoD	Two-component sensor kinase Acts on SpoOA and/or SpoOF polypeptide It is a "sensor" class of signal-transducing systems in bacteria	118°	Antoniewski et al. (1990)
spoIIN (synonym ftsA) Encodes a 47.94 kDa protein	Controls spoIID Cell division protein, membrane anchor for FtsZ	1596474–1577796	Louie et al. (1992) den Blaauwen et al. (2017)
ftsZ (synonym ts-1) Encodes a 40.20 kDa protein	Cell division initiation protein (septum formation)	1597832–1598980	Adams and Errington (2009)

(5,7-diamino-3,5,7,9-tetradeoxy-D-glycero-beta-D-galacto-non-2-ulopyranosonic acid), also found on the flagellin of *Helicobacter pylori* and *Campylobacter jejuni*.

Finally, the mature spore is normally released, as a dormant resistant cell, by lysis of the sporangium wall (old mother cell's). The spore can remain dormant for a long period of time, many years until reactivation (germination) takes place when environmental conditions, such as food and temperature, permit it. During the first phases of germination, efflux of ions occurs and step by step also disassembly of the coat proteins and the cortex; most of the previously captured calcium is released. During all the time the spore was a dormant structure, well within the spore core there were a variety of unaltered mRNAs. The question is, are these mRNAs functional during germination?. This question and several others have been recently proposed by Setlow and Christie in their recent review of 2020. The existence of fully functional mRNAs in spores would indeed speed up the germination processes, since the germinating spores would pass directly to translation as the ribosomes became, in turn, functional.

Futuristically, it would be interesting if *B. subtilis* had receptors for 4,5-dihydroxy-2,3-pentanedione derivatives, collectively known as "autoinducers AI-2" and involved in *quorum sensing* responses. This, without a doubt, would facilitate the coordination of sporulation in an otherwise asynchronous culture (a recent communication on the role of autoinducer AI-2 may be found in Zhang and colleagues in 2020).

Table 4 Main genes and proteins involved in sporulation stage III

Locus/gene/protein	Effect/regulation	Map position Degrees/ coordinates	Reference
spoIIIA Locus of a polycistronic operon Encodes eight proteins, SpoIIIAA to SpoIIIAH (contain the ring-building motif) plus the additional SpoIIQ	Minor role in the regulation of prespore-specific gene expression controlled by sigmaE Forms the "feeding tube" between mother cell and forespore Required for SigG activation	218°	Illing and Errington (1990) Illing and Errington (1991) Guillot and Moran (2007) Zeytuni et al. (2018) Mastny et al. (2013)
spoIIIC (Synonym sigmaKC-terminal half) Encodes a 16 kDa protein	RNA polymerase sporulation-specific sigma factor (SigK) (3′ region of the interrupted sigK gene), with sigK	230°	Errington et al. (1988) Eichenberger et al. (2004)
spoIIIG (synonym sigG)	RNA polymerase sporulation forespore-specific (late) sigma factor SigG activated by SigmaF	135°	Strauch et al. (1988)
spoIIID Encodes a 10.66 kDa protein	Transcriptional regulator (repressor or activator) of a subset of sigma E-dependent genes	317°	Chen et al. (2014)
spoIIIE Encodes an 86.96 kDa protein Member of the sigA regulon	ATP-dependent dsDNA translocase. Transports the forespore chromosome across the sporulation septum	149°	Butler and Mandelstam (1987) Wu and Errington (1994) Cattoni et al. (2014)
spoIIIJ (synonym spo0J87) Encodes a 29.37 kDa protein	Sec-independent membrane protein translocase, essential for SigG activity at stage III, involved in the assembly of the SpoIIIAH-SpoIIQ complex	360°	Errington et al. (1992) Serrano et al. (2003)
spoIIIL (synonym yqzE) Encodes a 9.62 kDa protein	Component of the SpoIIIA-SpoIIQ trans-envelope complex, required for the activation of SigG	2555887–2556066	Meeske et al. (2016)
gerE Encodes an 8.43 kDa	Transcriptional regulator (repressor or activator) of a subset of SigK-dependent late spore coat genes	2904727–2904951	Crater and Moran (2002)

Table 5 Main genes and proteins involved in sporulation stage IV

Locus/gene/prot	Effect/regulation	Map position Degrees/ coordinates	Reference
spoIVA (synonym spoVP) Encodes a 55.01 kDa protein	ATPase, spore coat morphogenetic protein, anchors the spore coat to the spore surface via SpoVM and SpoVID	205°	Roels et al. (1992) Stevens et al. (1992) Driks et al. (1994) Ramamurthi et al. (2006)
spoIVB Encodes a 45.81 kDa protein	Serine protease. Cleaves SpoIVFA resulting in pro-SigK processing. Activates spoVT. Involved in regulation of sigma F and G	217°	Van Hoy and Hoch (1990) Gómez (1996) Wakeley et al. (2000)
spoIVC (synonym cisA) Encodes a 57.31 kDa protein	Site-specific DNA recombinase. Involved in activation of SpoIIID and generation of active sigma E.	226°	Fujita and Kobayashi (1985) Sato et al. (1990)
spoIVD Encodes 30 kDa and 43.07 kDa proteins	SpoVID guides SafA to the spore coat ATPase binding. Cortex-located	230°	Ozin et al. (2001)
safA (synonym yrbA) Encodes a 43.07 kDa protein	Morphogenetic protein associated with SpoVID, a major organizer of the inner spore coat	2844675–2845838	Setlow (2012) Fernandes et al. (2018)
spoIVE (synonym spoIIIC)	Sporulation	230°	Piggot (1973) Piggot and Coote (1976) Stragier and Losick (1996)
SpoIVFA (synonyms bofB, spoVL) Encodes a 29.46 kDa protein	Inhibitor of SpoIVFB metalloprotease resulting in control of SigK activation	241°	Ricca et al. (1992) Cutting et al. (1990)
spoIVFB Encodes a 33.49 kDa protein	Intramembrane metalloprotease that processes pro-sigma-K to active SigK	241°	Lu et al. (1995) Yu and Kroos (2000) Halder et al. (2017)
spoIVG	Sporulation	97°	Piggot (1973)

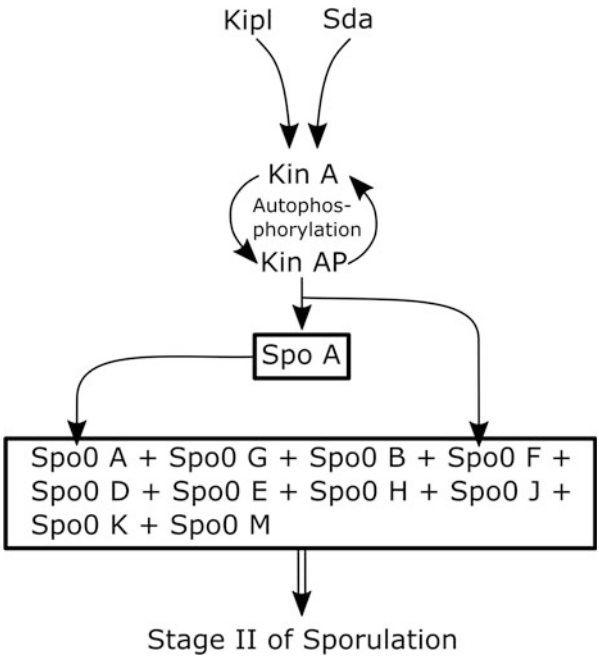

Fig. 4 Early events in endospore formation, including sporulation stages 0 and II

3 Secondary Metabolites Produced During Endospore Formation

3.1 Antibiotics

Spore-forming bacteria are excellent secondary metabolite producers, including antibiotics. Bu'Lock already described in 1961 the relationship between intermediary metabolism and antibiotic synthesis (Bu'Lock 1961), while Weinberg summarized the main characteristics of secondary metabolites (Weinberg 1964). According to this author, a secondary metabolite has a restricted distribution (best if species-specific), does not play an obvious role in general metabolism, and is rapidly synthesized even when bacterial growth is minimal or non-existent. Sermonti concluded in 1980 that secondary metabolism is a primitive type of metabolism. Kalenova et al. (2017) recently reported that secondary metabolites produced by *Bacillus* sp., isolated from late Neogene permafrost, have a very potent effect on cytokine production by human peripheral blood mononuclear cells. These metabolites induced the production of both proinflammatory (TNF-α, IL-1β, IL-8, IL-2, and IFNγ) and anti-inflammatory (IL-4 and IL-10) cytokines, and the secretion levels of cytokines were far higher than those induced by *B. cereus*, medicinal strain IP5832, metabolites. These results propound a putative role for these secondary metabolites in the development of immunomodulating drugs.

Table 6 Main genes and proteins involved in sporulation stages V and VI/VII

Locus/gene/prot	Effect/regulation	Map position Degrees/ coordinates	Reference
spoVAA Encodes a 23.03 kDa protein	Uptake of dipicolinic acid and Ca^{++} into developing spores, required for spore maturation	211°	Fort and Errington (1985) Tovar-Rojo et al. (2002) Vepachedu and Setlow (2007) Li et al. (2012)
spoVAC Encodes a 15.97 kDa protein	Uptake of dipicolinic acid and Ca^{++} into developing spores, required for spore maturation	211°	Tovar-Rojo et al. (2002)
spoVAD Encodes a 35.84 kDa protein	Uptake of dipicolinic acid and Ca^{++} into developing spores, required for spore maturation	211°	Tovar-Rojo et al. (2002)
spoVAEA Encodes a 22.00 kDa protein	Uptake of dipicolinic acid and Ca^{++} into developing spores, required for spore maturation	2439804–2440415	Li et al. (2012)
spoVB (synonym IIIF) Encodes a 55.91 kDa protein	Involved in spore cortex peptidoglycan synthesis (member of the MurJ superfamily, lipid II flippase)	236°	Popham and Stragier (1991) Meeske et al. (2015)
spoVC (synonym pth) Encodes a 20.73 kDa protein	Peptidyl-tRNA hydrolase, involved in spore coat formation.	7°	Menez et al. (2002)
spoVD Encodes a 71.08 kDa protein	Penicillin-binding protein (spore cortex) Transpeptidase activity	133°	Daniel et al. (1994) Bukowska-Faniband and Hederstedt (2013)
spoVE Encodes a 39.97 kDa	Peptidoglycan glycosyltransferase, required for spore cortex peptidoglycan synthesis	134°	Bugaichuk and Piggot (1986) Theeragool et al. (1993)
spoVF (divergon containing (operons spoVFAB, asd, dpaG, and dapA)	Cortex formation Involved in dipicolinic acid synthesis	148°	Chen et al. (1993) Takahashi et al. (2015)
spoVG Encodes a 10.75 kDa protein	RNA-binding regulatory protein, negative effector of asymmetric septation at the onset of sporulation. Also described in *B. anthracis*	6°	Matsuno and Sonenshein (1999) Chen et al. (2020)
spoVK (synonym spoVJ Encodes a 36.52 kDa protein	Spore maturation	153°	Fan et al. (1992)

(continued)

Table 6 (continued)

Locus/gene/prot	Effect/regulation	Map position Degrees/ coordinates	Reference
spoVM Encodes a 2.88 kDa protein	Required for normal spore cortex and coat synthesis inhibits the proteolytic activity of FtsH	140°	Levin et al. (1993) Kim et al. (2017)
spoVN (synonym of ald) Encodes a 39.53 kDa protein	L-alanine dehydrogenase. Required for normal sporulation	3278325–3279461	Siranosian et al. (1993)
spoVR Encodes a 55.46 kDa protein	Involved in spore cortex synthesis. Expression of spoVR initiates during the second hour of sporulation from a sigma E-dependent promoter	72°	Beall and Moran (1994)
spoVS Encodes an 8.66 kDa protein	Spore coat assembly and spore core dehydration	150°	Resnekov et al. (1995) Rigden and Galperin (2008)
spoVT (synonym of yabL) Encodes a 19.60 kDa protein	Transcription activator and repressor of SigG-dependent genes Essential sporulation gene for *Bacillus cereus*	64099	Asen et al. (2009) Ramirez-Peralta et al. (2012) Eijlander et al. (2016)
spoVV (synonym ylbJ) Encodes a 44.68 kDa protein	Transport of dipicolinic acid across the outer forespore membrane	1570574–1571800	Ramírez-Guadiana et al. (2017)
spoVID Encodes a 64.80 kDa protein	Spore coat morphogenetic protein that promotes encasement of the spore. Involved in assembly of the inner and outer spore coat layers. Interacts with SafA and CotE	244°	Ozin et al. (2000) Nunes et al. (2018)
safA (synonym yrbA) Encodes a 43.07 kDa protein	Morphogenetic protein associated with SpoVID, major organizer of the inner spore coat	2844675–2845838	Nunes et al. (2018)
cotA (synonym pig) Encodes a 58.33 kDa protein	Spore coat protein (outer), laccase, bilirubin oxidase	683462–685003	Imamura et al. (2010)
cotB Encodes a 42.81 kDa protein	Spore coat protein (outer)	3714739–3715881	Imamura et al. (2010)

(continued)

Table 6 (continued)

Locus/gene/prot	Effect/regulation	Map position Degrees/ coordinates	Reference
cotC Encodes a 14.64 kDa protein	Spore coat protein (outer)	1904995–1905195	Imamura et al. (2010)
cotE Encodes a 20.83 kDa protein	Outer spore coat morphogenetic protein	1775067–1775612	Nunes et al. (2018)
ytxO Encodes a 16.41 kDa protein	Spore coat protein (outer) Protection of the spore	3159258–3159689	Imamura et al. (2010)
cotD	Spore coat protein (inner)	200°	Henriques et al. (1995)
cotF Encodes an 18.58 kDa protein	Spore coat protein (inner)	4167110–4167592	Imamura et al. (2010)
cotS Encodes a 40.93 kDa protein	Spore coat protein (inner)	3159691–3160746	Takamatsu et al. (1998)
cotT Encodes a 15 kDa protein	Spore coat protein (inner)	114°	Takamatsu et al. (2000)
gerQ (synonyms ywdL, ipa-62r) Encodes a 2013 kDa protein	Spore coat protein, necessary for the proper localization of CwlJ	3893441–3893986	Ragkousi et al. (2003)
cwlJ Encodes a 16.22 kDa protein	Spore germination Spore coat protein, cell wall hydrolase. Requires SafA (member of the spore's proteinaceous coat) for activity	282469–282897	Ishikawa et al. (1998) Bagyan and Setlow (2002) Amon et al. (2020)
YaaH (synonym sleL) Encodes a 48.47 kDa protein	General stress protein, survival during ethanol stress, SafA-dependent protein in inner spore coat, spore cortex lytic protein. Involved in the germination of spores *N*-acetylglucosaminidase	23868–25151	Kodama et al. (1999) Lambert and Popham (2008) Üstok et al. (2015)
YeeK Encodes a 15.78 kDa protein	Spore coat protein (inner)	753265–753702	Takamatsu et al. (2009)
YsnD Encodes a 11.58 kDa	Protection of the spore. Spore coat protein	2897788–2898123	Imamura et al. (2010)
YxeE Encodes a 14.57 kDa protein	Inner coat protein	4065597–4065962	Kuwana et al. (2007)

(continued)

Table 6 (continued)

Locus/gene/prot	Effect/regulation	Map position Degrees/ coordinates	Reference
cotJB Encodes a 11.61 kDa protein	Polypeptide composition of the spore coat	756139–756402	Henriques et al. (1995) Seyler et al. (1997)
spoVIF (synonym yjcC) Encodes a 11.45 kDa protein	Required for spore coat assembly and resistance	1256436–1255866	Kuwana et al. (2003)

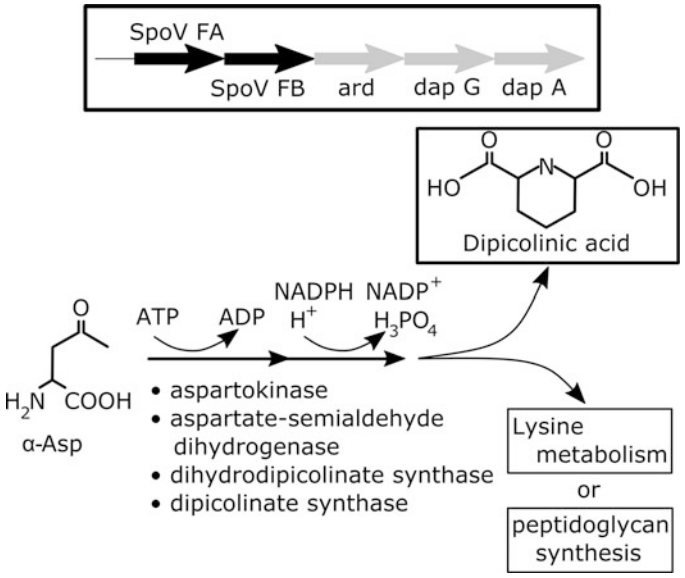

Fig. 5 Mechanism of synthesis of dipicolinic acid by *Bacillus subtilis*

Manganese and copper are two transition metals that appear to be important both in endospore formation (Weinberg 1964; Krueger and Kolodziej 1976) and in secondary metabolite synthesis (i.e., iron for mycobacillin or cobalt for D-glutamyl peptide; Jansen and Hirschmann 1944; Foster and Woodruff 1946). Manganese, in particular, appears to be essential as, according to Weinberg, no other biologically active element can substitute it. Apart from transition metals, starvation (depletion of a usable nitrogen source) triggers both sporulation and secondary metabolism (including synthesis of antibiotics), originating a metabolic state known as the "stringent response" that involves GTP and active ribosomes (Lukin et al. 1983; Ochi and Ohsawa 1984).

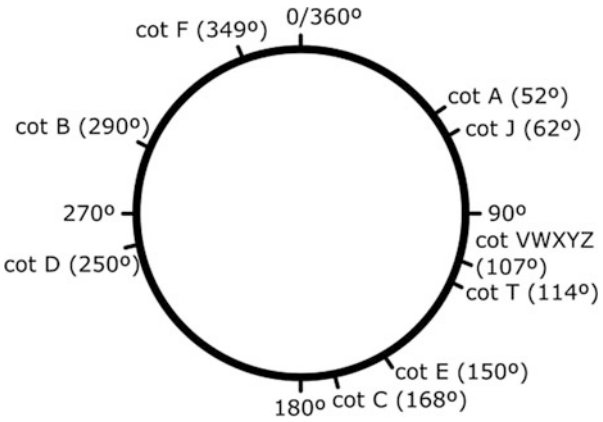

Fig. 6 Summary of the different *cot* genes involved in coat formation in *Bacillus subtilis* spores

We envisage that research into novel sources of antibiotics and secondary metabolites (as well as other pharmaceutically relevant compounds) in the near future will involve the study of yet unknown microorganisms isolated from insects, plants, or animals. Indeed, insects represent the most diverse group of animals and should constitute an excellent source of microorganisms capable of producing bioactive molecules as secondary metabolites. In his review, Bode provides prime examples of entomopathogenic bacteria as sources of secondary metabolites, these include *Bacillus thuringiensis*, *Pseudomonas entomophila*, *Xenorhabdus*, and *Photorhabdus* (Bode 2009).

The genus *Bacillus* is an eminent antibiotic producer (mostly polypeptidic), with already 167 peptides described by Berdy in 1974 and a number of new ones characterized since (see review by Katz and Demain 1977). The classical antibiotics produced by ***B. subtilis*** include mycobacillin, subtilin, bacilysin, bacillomycin, fungistatin, bulbiformin, bacillin, bacillaene, subsporin, bacillocin, mycosubtilin, fungocin, iturin, neocidin, and eumycin. ***B. brevis*** secretes gramicidin S, tyrocidine, linear gramicidin, brevin, edeine, eseine, bresseine, and brevistin. ***B. pumilus*** synthesizes micrococcin P, pumilin, and tetain, while ***B. mesentericus*** produces esperin, and ***B. licheniformis*** generates bacitracin, licheniformin, and proticin. Antibiotic production in ***B. polymyxa*** includes polymyxin, colistin, gatavalin, and jolipeptin, while ***B. circulans*** secretes butirosin, circulin, polypeptin, EM-49, and xylostatin. ***B. cereus*** makes biocerin, cerexin and thiocillin, and ***B. laterosporus*** synthesizes laterosporamine and laterosporin.

Those described above are all peptide antibiotics, listed by Katz and Demain in 1977, and all share the following basic properties: (1) their size is much smaller than "normal" antibiotics; (2) they are usually produced as a close family of peptides; (3) they can be constituted by either amino acids only, or be complexed with other compounds, such as polymyxins, that contain either 6-methyloctanoic acid or 6-ethylheptanoic acid as a fatty acid residue; (4) frequently contain D-amino acids

not found in proteins, and (5) they are mainly resistant to hydrolysis by peptidases and proteases.

The peptide antibiotic synthesis requirements are the same for all of them; they all require amino acids, ATP, the appropriate synthase (that can be purified from cell-free extracts), Mg^{2+} ion, and a reducing agent. The antibiotic extends from the N-terminal to the C-terminal end, as is the case in protein synthesis, and only enzyme-bound intermediates are involved (Katz and Demain 1977). Lipmann and collaborators proposed a mechanism for the synthesis of cyclic peptide antibiotics, such as gramicidin S, which involves peptidyl transfers from enzyme-bound thioester intermediates (Gevers et al. 1969; Lipmann 1973). When the peptide antibiotic is linear (i.e., gramicidins) the pentadecapeptide remains thioester-linked, and formylation occurs after completion of the polypeptide synthesis (Bauer et al. 1972). Despite all the advances in our knowledge of the genetics, biochemistry, and synthesis of sporulation-related antibiotics, little is known about the role (or roles) that these compounds play in the producing organism. The suggested function as a biochemical sink has its merit, although, as indicated by Katz and Demain (1977), these antibiotics are produced specifically when the cell detects harsh conditions and could either be packaged in the *Bacillus* spore to provide a favorable environment (by eliminating competitors) during germination or inhibit spore germination until environmental conditions are favorable.

Antifungal antibiotics produced by *Bacillus* are somehow linked to sporulation, as they are secondary metabolites. They are not common in these bacteria, although there are some lipopeptides (Hamley 2015) with antifungal action, such as fengycin, surfactin, and iturin family compounds (Dunlap et al. 2013); and more recently, Knight and coworkers described one secreted by *B. subtilis* subsp *inaquosorum* (Knight et al. 2018). All these antibiotics are synthesized by synthetases not linked to ribosomes, they exhibit different types of cyclization and varied length of the fatty acid chain. Fengycin was the first antifungal identified (Vanittanakom et al. 1986), although surfactin is perhaps the most powerful biosurfactant and the iturin family displays a broad-spectrum antifungal activity (Knight et al. 2018).

Iturins are a group of lipopeptide antifungal amphiphilic antibiotics that act on the cytoplasmic membrane altering K^+ permeability. Iturins increase membrane permeability by forming ion-conducting pores, due to their interaction with sterols and phospholipids present in the membrane. The antifungal activity of these compounds increases with the number of aggregates formed and depends on the type of amino acids contained by the lipopeptide, as well as the type of sterols present in the cytoplasmic membrane.

Iturin A (Fig. 7) is the archetype for *B. subtilis* lipopeptide (Besson et al. 1976). It is encoded by the iturin A operon, which spans over 38 kb and contains four open reading frames, ituD, ituA, ituB, and ituC (Tsuge et al. 2001). Recently (Zhou et al. 2020) have reported on the isolation from deep sea, of a new bacterial strain, tentatively classified within the *Bacillus* genus, that synthesizes two new iturin-like lipopeptides, designated as C_{14} iturin W, and C_{15} iturin W, with fungicidal activity by introducing damage into the fungal plasmalemma. Mycosubtilin, also produced by some *B. subtilis* strains, is similar to iturin, although there are minor

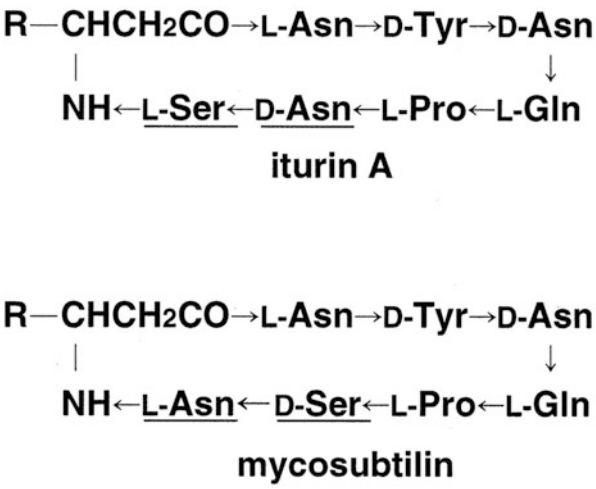

Fig. 7 Comparison of the structures of Iturin A and mycosubtilin. Although the two compounds are very similar, the amino acids at positions 6 and 7 in the mycosubtilin sequence are D-Ser→L-Asn, while in iturin A these amino acids are inverted

differences between the two antibiotics, both in the conformation of serine and asparagine and in the order the two amino acids are found on the lipopeptides.

Iturins may have additional roles as biocontrol agents. It has been reported lately (Wang et al. 2020), that iturin A directly extracted from *B. subtilis* strain WL-2 readily exerts a controlling role on the fungus *Phytophthora infestans* (potato late blight disease that shapes a threat worldwide for *Solanum tuberosum* culture) through disruption of the cellular membrane and oxidative stress.

Plipastatin (A and B) are potent *Bacillus* antimicrobial lipopeptides (inhibitors of phospholipase A2; Volpon et al. 2000), thought to replace shortly conventional treatments in plant–fungal infections. *B. subtilis* synthesizes this antibiotic directed by the operon *ppsABCDE* operon (Vahidinasab et al. 2020); the authors accomplished the construction of a new strain able to produce in a constitutive manner, increased amounts of plipastatin.

Interestingly, recently it has been reported that some fungal–bacterial interactions are able to select mutants able to synthesize increased levels of compounds with antifungal activity (Albarracín-Orio et al. 2020). Surprisingly, the authors found that interactions of *B. subtilis* with the fungus *Setophoma terrestris*, originated bacterial variants which had lost the ability to form lipopeptides and instead had gained the capability to synthesize compounds with antifungal activity.

Genome mining applied to *B. subtilis* NCD-2 is giving positive results as far as unraveling the potential to find antimicrobial compounds in this strain, and also to determine the specificity of respective gene clusters (Su et al. 2020). The strain is a good one to fight soil-borne plant pathogenic fungi, since it has been described as producer of broad-spectrum antifungal compounds. Additional species of the *Bacillus* genus, such as *B. velezensis* have been described as bein g good sources of L ipopeptides and polyketides (Ruiz-García et al. 2005; Rabbee and Baek 2020), that allows the bacterium to exert quite positive antagonistic effects against plant pathogens, such as *Verticillium dahlia* that causes wilt in olive trees (Castro et al. 2020),

or to promote the growth of *Malus hupehensis* Rehd (Wang et al. 2019) while related to *B. subtilis,* is different in that it contains nine gene clusters (namely, *srf*, *bmy*, *fen*, *dhb*, *bac*, *mln*, *bae*, *dfn*, and *nrs*) by which the bacterium produces a large variety of antimicrobial compounds (Rabbee and Baek 2020).

Table 7 summarizes the most relevant antibiotics produced by *Bacillus*.

3.2 Alkanes

Alkanes, with a general molecular formula of C_nH_{2n+2}, represent the simplest organic molecules that are widely distributed in nature; they are stable due to their backbone carbon atoms, having attained their octet of electrons through the formation of four covalent bonds.

Alkanes can be used as an advanced biofuel because of their high-energy content, which is 30% higher than ethanol. Although it has been reported that recombinant *E. coli* strains can produce a different range of alkanes, such as pentadecane and heptadecane (Choi and Lee 2013), the use of these compounds is far from being industrially exploited, and this includes the alkanes produced as secondary metabolites in Gram-positive sporulating bacteria. However, most sporulating bacteria appear to be good alkane degraders. Efficient microbial biosynthesis of alkanes with long carbon chains is difficult to achieve in a single organism (Lehtinen et al. 2018), as this process requires a two-step pathway. Hence, the first step of CO_2 reduction to acetate should be carried out by a homoacetogenic bacterium following the Wood–Ljungdahl pathway. Transformation into long-chain hydrocarbons, on the other hand, would be best achieved by a second engineered microorganism expressing the enzymes acyl-ACP reductase (AAR) and aldehyde deformylating oxygenase (ADO); ADO is regarded as the bottleneck for the alkane biosynthesis, due to the low activity of the enzyme.

The available data indicate that aerobic Gram-positive sporulating bacteria do not naturally exhibit the ability to generate alkanes, at least not in enough quantities to be industrially relevant. In fact, some results suggest that these microorganisms are totally unable to do so unless they are genetically engineered. However, this appears not to be the case for anaerobic clostridia; Bagaeva and Zinurova reported in 2004 that *Clostridium pasteurianum* could in fact synthesize alkanes (C_{25}-C_{35} intracellular and C_{11}-C_{24} extracellular) at the end of its logarithmic growth phase, in an atmosphere formed by a mixture of CO_2+H_2/ argon. A particularity of this bacterial species, not present in Gram-negative bacteria, is its ability to produce branched alkanes. Despite the aforementioned, there have been recent papers describing the ability of certain strains of *B. subtilis* to form a "volatilome" formed by secondary metabolites that include hydrocarbons, ketones, alcohols, aldehydes, ester, acids, among many others (up to 231), and some having the property to control the fungal population in the rhizosphere (Kai 2020).

Table 7 Main antibiotics produced by *Bacillus* species

Bacillus species	Antibiotic	Main structure	Active against	Reference
B. silvestris	Bacillistatins 1,2	Cyclodepsipeptides	Cancer cells	Pettit et al. (2009) Mondol et al. (2013)
B. subtilis	Mycobacillin	Cyclic peptide	Fungi	Majumdar and Bose (1958)
B. subtilis	Subtilin	32-amino acid peptide	Bacteria	Chan et al. (1992)
B. subtilis	Bacilysin (tetaine)	Dipeptide: *N*-L-Alanyl-3-(5-oxo-7-oxabicyclo (4.1.0)hept-2-yl)-L-alanine	Bacteria, Fungi, HeLa cells	Newton (1949) Özcengiz and Öğülür (2015)
B. subtilis	Bacillomycin	Lipoheptopeptide.	Fungi	Peypoux et al. (1981)
B. subtilis	Fungistatin	Polypeptide	Fungi	Lewis et al. (1946)
B. subtilis	Bulbiformin	Polypeptide	Fungi	Vasudeva et al. (1958)
B. subtilis	Bacillin (synonym bacilysin)	Dipeptide	Bacteria Fungi	Foster and Woodruff (1946) Atsumi et al. (1975)
B. subtilis	Bacillaene	Conjugated hexaene	Bacteria	Patel et al. (1995)
B. subtilis	Bacilosarcin	Isocoumarin	Plant growth factor	Azumi et al. (2008)
B. subtilis	Bacitracin	Cyclic peptide	G+ bacteria	Johnson et al. (1945) Waterman et al. (2017)
B. subtilis	Subsporin A	Repeated tetrapeptide (14 aa′). L-Asp_4, D-Asp_2, D-Tyr_2, L-Glu_2, L-Pro_2	Fungi	Ebata et al. (1969)
B. subtilis	Bacillocin	Lipopeptide	Fungi	Zheng and Slavik (1999)
B. subtilis	Mycosubtilin	Lipopeptide	Fungi	Walton and Woodruff (1949)

(continued)

Table 7 (continued)

Bacillus species	Antibiotic	Main structure	Active against	Reference
B. subtilis	Fengycin A, B	Lipopeptide	Fungi	Lin et al. (1998) Ma et al. (2016)
B. subtilis	Iturins	Lipopeptides	Fungi	Delcambe (1952)
B. subtilis	Eumycin	Lipopeptide	Fungi and Bacteria	Johnson and Burdon (1946)
B. subtilis	Bacilotetrins A, B	Cyclic-lipotetrapeptides	MRSA[a]	Tareq and Shin (2017)
B. subtilis	Plipastatin A, B	Lipopeptide	Fungi	Volpon et al. (2000) Vahidinasab et al. (2020)
B. subtilis 109GGC020		Linear lipopeptide	Magnaporthe oryzae Triticum	Chakraborty et al. (2020)
B. brevis	Gramicidin S (Soviet)	Cyclic polypeptide	Bacteria	Katz and Demain (1977)
B. brevis	Tyrocidine	Cyclic decapeptide	Bacteria	Katz and Demain (1977)
B. brevis	Linear gramicidin (A, B, C) = Gramicidin D (Dubos)	Linear pentadecapetides	Bacteria, some fungi and viruses	Manwaring (1940) Dubos and Hotchkiss (1941) Katz and Demain (1977)
B. brevis	Brevin	Peptide	Bacteria	Barnes and Newton (1953) Katz and Demain (1977)
B. brevis	Edeine	Polypeptide	Bacteria, fungi, viruses	Kurylo-Borowska (1959) Wojciechowska et al. (1983)

B. brevis	Esein	Peptide	Bacteria	Zharikova et al. (1972) Katz and Demain (1977)
B. brevis	Bresein	Peptide	Bacteria	Zharikova et al. (1972) Katz and Demain (1977)
B. brevis	Brevistin	Acylpeptide	Bacteria (G+)	Shoji et al. (1976a, b)
B. pumilus	Micrococcin P	Polypeptide. Azamacrocycle and a lactam.	Bacteria. Protists (including malaria parasite)	James and Watson (1966) Rogers et al. (1998)
B. pumilus	Pumilin (subtenolin)	Nd	Bacteria	Bhate (1955) Howell and Tauber (1948)
B. pumilus	Tetain (bacilysin)	Dipeptide	Bacteria, fungi	Borowski (1953) Walker and Abraham (1970)
B. licheniformis	Licheniformin	Polypeptide	Bacteria (including *Corynebacterium diphteriae)*	Callow et al. (1947)
B. licheniformis	Proticin	Phosphorus-containing conjugated triene	Bacteria, particularly *Proteus* spp	Präve et al. (1972) Vértesy (1972)
B. licheniformis and *B. subtilis*	Bacitracin	Peptide. The synthesis is drastically increased by the supplement of *S*-Adenosylmethionine	Bacteria	Cai et al. (2020)
B. polymyxa (*Paenibacillus polymyxa*)	Polymyxins A, B	Cyclic peptide	Bacteria	Stansly and Schlosser (1947)
P. polymyxa	Colistin (Polymyxin E)	Cyclic peptide	Bacteria (G–)	Koyama et al. (1950)

(continued)

Table 7 (continued)

Bacillus species	Antibiotic	Main structure	Active against	Reference
Paenibacillus kobensis	Polymyxin M (Mattacin)	Cyclic peptide	Bacteria (G–)	Martin et al. (2003)
P. polymyxa	Gatavalin	Polypeptide	Bacteria, fungi	Nakajima et al. (1972)
P. polymyxa	Jolipeptin	Polypeptide	Bacteria	Ito and Koyama (1972)
B. circulans	Butirosin	Aminoglycoside	Bacteria	Howells et al. (1972)
B. circulans	Circulin	Cyclic decapeptide	Bacteria	Fujikawa et al. (1965)
B. circulans	Polypeptin	Cyclic lipopeptide	Bacteria, fungi	Garson et al. (1949) Howell (1950) Mountford et al. (2017)
B. circulans	EM-49 (octapeptin)	Polypeptide	Bacteria	Rosenthal et al. (1977)
B. circulans	Xylostatin (Ribostamycin)	Aminocyclitol	Bacteria	Akita et al. (1970)
B. cereus	Biocerin	Structure undetermined	Bacteria	Johnson et al. (1949)
B. cereus	Cerexin	Lipopeptide	Bacteria	Shoji et al. (1975)
B. cereus	Thiocillin I, II, III	Macrocyclic thiazole peptide	Bacteria	Shoji et al. (1981)
B. laterosporus	Laterosporamine	Non-peptidic	Bacteria	Shoji et al. (1976a, b)
B. laterosporus	Laterosporin A	Polypeptide	Bacteria	Barnes (1949)
B. laterosporus	Basiliskamides A, B	Polyketides	Fungi	Barsby et al. (2002)
B. laterosporus	Tupuseleiamides A, B	Acyldipeptides	Fungi	Barsby et al. (2002)
B. amyloliquefaciens	Macrolactin S, V	Polyene macrolide	Bacteria	Gao et al. (2010)

Bacillus sp (SY-1)	Bacillamide	2-acetylthiazole-4-carboxylic acid [2-(1H-indol-3-yl)ethyl]amide.	Algae Dinoflagellates	Jeong et al. (2003) Mondol et al. (2013)
Bacillus sp	Loloatin B	Cyclic decapeptide	MRSA[a], VRE[b]	Gerard et al. (1996)
Bacillus sp	Bogorol	Polypeptide	MRSA[a]	Barsby et al. (2001)
Bacillus sp	Mixirin	Acylpeptide	Cancer cells	Zhang et al. (2004)
Bacillus sp	Hallobacillin	Cyclic acylpeptide	Cancer cells	Trischman et al. (1994) Mondol et al. (2013)
B. thuringiensis	No name	Acyl homoserine lactone lactonase	Plant pathogenic bacteria	Zhou et al. (2008)
B. thuringiensis	Zwittermicin	Linear aminopolyol	Fungi	Kevany et al. (2009)
B.velezensis		Lipopeptides and polyketides		Ruiz-García et al. (2005) Rabbee and Baek (2020)

[a]Methicillin-resistant *Staphylococcus aureus*
[b]Vancomycin-resistant *Enterococcus*

3.3 Parasporal Crystals

Parasporal crystals constitute one of the few examples in Biology in which a cell contains a crystallized structure with biological activity. The archetypes for these structures are the bipyramidal parasporal crystals of *B. thuringiensis*, a Gram-positive, endospore-forming bacterium closely related to both *B. cereus* and *B. anthracis*, the causative agent of anthrax. The crystals are synthesized during endospore formation and are hence associated with the secondary metabolism of *Bacillaceae.* This microorganism was initially described by Ishiwatari Shigetane (1901) in the silkworm and named *Bacillus sotto*. It was later renamed as *B. thuringiensis* after Berliner (1915) isolated it from the gut of the flour moth caterpillar in Thuringia, Germany (Milner 1994). There are currently several known *B. thuringiensis* subspecies (all producing parasporal crystals) that display different toxicity towards insects, such as *Lepidoptera*, *Coleoptera*, *Diptera*, *Hymenoptera*, and *Nematoda* (Schnepf et al. 1998; Wei et al. 2003; Soberón et al. 2013). The proteinaceous nature (δ-endotoxin or cry proteins) of the parasporal crystal was described by Hannay and Fitz-James in 1955, while the crystal-specific toxicity towards caterpillars of the lepidopteran species *Pieris brassicae* was known since 1965 (Lecadet and Martouret 1965). This research defined the type subspecies, *Berliner*, while further subspecies, such as *kurstaki*, *israelensis*, and *aizawa*, were later described. In 1968 de Barjac and Bonnefoi carried out the first attempt to rationalize the taxonomy of *B. thuringiensis* subspecies and varieties. Cry proteins are encoded by cry genes, which are located on a plasmid in most *B. thuringiensis* strains. In 1979 both Robert A. Zakharian and coworkers and Miteva independently reported the plasmid location of the cry genes, suggesting a role for the plasmid in both endospore and crystal formation (Zakharian et al. 1979; Miteva 1979)

There are multiple studies on the mode of action of *B. thuringiensis* toxins (i.e., Koch et al. 2015) which, unlike chemical pesticides, are effective only after being ingested by the insect. The parasporal Cry proteins are approximately 70–140 kDa and, once within the gastrointestinal tract of insects, they become activated by proteases and specifically bind to epithelial cells receptors (mostly cadherin-like glycoproteins); they create pores, formed by oligomers of six Cry molecules (this is essential for lethality), that cause a dramatic cellular osmotic imbalance which eventually leads to the death of the insect.

Since the cry genes were cloned in 1981 (Schnepf and Whiteley 1981) there have been many successful attempts to express them in transgenic crop plants, such as corn, some of which involved biotechnological companies such as Monsanto. The initial concerns about the possible negative effects of the thuringiensis toxins, either released into the environment through the roots of the transgenic plants, or present in the foodstuffs, resulted in the experiments being concealed from the public, such as the work by Saxena and Stotzky in 2000. In fact, there was no need for such concern, as indicated by Koch and coworkers in 2015: "*Cry proteins are very limited in their duration of effectiveness because they can be washed off the plant (e.g., by rain) or inactivated by sunlight within days after application, and they require considerable*

water, heat, and feedstock to produce, and must be manually applied, either by hand sprayer on small plots or by machine if applied to large tracts." Because of their safety of use, a variety of Cry proteins have been approved for use in at least one country to protect against lepidopteran pests, and these include: Cry1Ab inserted into maize by Monsanto; Cry1Ac expressed in cotton, corn, brinjal, and soy by Monsanto; Cry1A.105 + Cry2Ab2 and Cry1Ac + Cry2Ab2 were introduced in maize varieties by Monsanto; Cry1Ac + Cry1F in cotton and soy by Dow; Cry1Fa2 in maize by Dow; Cry1Ac + Cry1F in cotton and soy by Dow; Cry1Ab + Cry2Ae in cotton by Bayer. In addition, Cry34Ab1 + Cry35Ab1 were expressed in maize by Dow and DuPont to protect from *Coleoptera* (Koch et al. 2015). The economic importance of Cry proteins in crop protection was reviewed by Marques and coworkers in 2019. As for the price for the production of these proteins, it passes through the obtention of Cry protein-overproducing strains. An easy way of doing this was recently reported by Quan and coworkers in 2020. The authors, by simply deleting the *leu B* gene (encodes for the 3-isopropylmalate dehydrogenase in the leucine synthesis pathway) in a conditionally asporogenous *B. thuringiensis*, were able to overexpress such a protein.

The isolation of new and natural strains of *B. thuringiensis* must proceed at whatever pace, since Nature has always provided new useful mutations for human industrial applications. In this sense, Liu and colleagues reported in 2020 the isolation of a new strain *B. thuringiensis*, X023, which exhibits enhanced insecticidal (against *Plutella xylostella*) activity by copper ions. This ion promoted the expression of *cry1Ac* and *vip3Aa*, the synthesis of aminoacids, the glyoxalate pathway, as well as the poly-β-hydroxybutyrate accumulation; all these compounds are necessary for the synthesis of parasporal crystals (Liu et al. 2020).

Concerning the safety of use of these biocides, they are generally considered as safe, as they are quite specific in their mode of action against lepidopteran or Diptera insects; however, their use may disturb the general metabolism of other insects initially thought not to be susceptible to the *cry* toxins. In this sense, Nawrot-Esposito and colleagues reported in 2020 that these bioinsecticides cause defects in the larval development of *Drosophila melanogaster*, by reducing the protein digestion. Differential side-effects of thuringiensis biocides have also been reported on this fly by Babin and coworkers in 2020 non-target *Drosophila* flies.

Late reports (Ursino et al. 2020) have shown that *B. subtilis* may be directly used to produce mosquitocidal toxins against species of *Aedes*, known to transmit some arbovirus-caused diseases. Some of these diseases include Dengue fever and Yellow fever (transmitted by *Aedes aegypti*), Japanese Encephalitis and Rift Valley fever (transmitted by *Culex tritaeniorhynchus*), among others. It is clear that the genetic background of *B. subtilis* is by far better known than that of *B. thuringiensis*; so any genetic manipulation with projection in the industry (i.e., increase production of lepidopteran or dipteral toxins, or obtention of altogether different toxins) should have a better outcome if developed in *B. subtilis.* The deepest study on this topic follows in the next chapter.

3.4 Lanthipeptides

Lanthipeptides constitute "natural products," ribosomally synthesized by bacilli as secondary metabolites, and are posttranslationally modified peptides (RiPPs) (Nolan and Walsh 2009; Dias et al. 2015). These modifications include the formation of meso-lanthionine and 3-methyllanthionine, as well as dehydrated amino acids. Xin and coworkers classified lanthipeptides into four groups in 2015, depending on the enzymes involved in post-translational processing. In group I, amino acid dehydration is carried out by a dedicated lanthipeptide dehydratase, and cyclization is catalyzed by a lanthipeptide cyclase; in group II, the lanthipeptide is modified by specific proteins; whereas in groups III and IV, lanthipeptide dehydration and cyclization reactions are carried out by multifunctional enzymes. *B. thuringiensis* and *B. cereus* are able to produce more than 20 bacteriocins, many with potential usage both in the Food Science industry and in the clinical control of pathogenic bacteria (Rea et al. 2010). Cerecidins merit a special citation among the lanthipeptides produced by the cereus group, for their prospective usefulness in controlling pathogenic bacteria (Wang et al. 2014). In fact, cerecidins A1 and A7 are known to be active against Gram-positive bacteria, displaying remarkable efficacy against both multidrug-resistant *S. aureus* (MRSA strains) and vancomycin-resistant *Enterococcus faecalis*.

As a general rule, lanthipeptides are encoded by structural genes (lanA), normally synthesized as non-active precursors that are later hydrolyzed into an N-terminal peptide and a C-terminal peptide; the N-terminal leader peptide is important for post-translational modifications (Yang and van der Donk 2013; Dias et al. 2015). The structural genes for these peptides (lanA) frequently cluster with genomic islands, this is the case for lanthipeptides synthesized by *Bacillus methylotrophicus* (Dias et al. 2015), and this supports the notion that their production might be the result of evolutionary adaptation to best achieve their in vivo function, either as controllers of other microorganisms (Wang et al. 2014) or as plant growth promoters (Hao et al. 2012). It appears that Gram-positive spore-forming bacteria require antimicrobial lanthipeptides to conquer harsh environments, as the strains and bacterial species isolated from harder habitats seem to produce novel lanthipeptides with new characteristics (Othoum et al. 2018). The structural lanthipeptide genes have been cloned (Ongey et al. 2018) and are in the process of being genetically modified in order to both increase production of these compounds, that are normally produced in low amounts by their "natural" bacterial species, and broaden their application. Lanthipeptides are very promising bioactive compounds with a great potential use not only in human and veterinary medicine but also in the control of bacteria that cause food spoilage.

4 Secondary Metabolites in the Environment

Microbiologists are still blatantly ignorant concerning the number of bacterial species on earth and can only hypothesize to estimate the enormous number (perhaps up to 80%) of bacteria that cannot yet be grown in axenic conditions in the laboratory. This is either due to the lack of appropriate culture media or because microorganisms are rarely found in nature in pure culture (only pathogenic microorganisms constitute a monoculture when causing a disease), and to flourish, they need to be in contact with other microorganisms, often through "quorum sensing" mechanisms, or may require secondary metabolites such as antibiotics or lanthipeptides. Zengler and coworkers researched this topic in their interesting publication entitled "Cultivating the uncultured" (2002), putting forward a proposal for a universal method to detect, or at least estimate, the numerous unculturable microorganisms present in the environment. According to Nai and Meyer (2018) "*Only a paradigm shift in cultivation techniques—from axenic to mixed cultures—can allow a full comprehension of the (chemical) communication of microorganisms, with profound consequences for natural product discovery, microbial ecology, symbiosis, and pathogenesis*." This means that it is essential to develop the microbial co-culture technology, as well as understand the effects of secondary metabolites produced by a given microbial specimen on the biological development of neighboring organisms. Despite our lack of knowledge in these basic research areas, some advances are slowly taking place, among them are the early reports by Johnson and colleagues and Patel and Roth, both in 1978. More recently, Shank (2013) studied bacterial co-cultures to examine the influence of secondary metabolites on microbial interspecies interactions in the natural environment. In addition, Nai and Meyer (2018) reported that the three technical approaches currently used (3D-bioprinting, single-cell metabolomics, and microfluidics) can allow systematic co-culture of three or more microorganisms. Hopefully, the next few decades would bring a much better understanding of the complex microbial relationships that occur in "natural" environments.

This knowledge and understanding could revitalize the search for novel natural compounds with antimicrobial activity, such as antibiotics, a task currently practically abandoned by pharmaceutical companies throughout the world. Some authors estimate that there are still up to 1000 novel antimicrobials awaiting discovery, as well as a great number of yet unknown enzybiotics (Veiga-Crespo et al. 2007). Production of novel drugs could be attained by microbial co-cultures in which the secondary metabolites secreted by one species induce expression of antibiotics or antimicrobials in another species (Bertrand et al. 2014). Gram-positive organisms and spore-forming bacteria, together with members of the *Pseudomonadaceae* family, are prime candidates to use in co-culture experiments, as they are among the best secondary metabolite producers. Although the number of combinations for laboratory co-culture experiments is high, the family *Bacillaceae* (*B. subtilis, B. cereus, B. licheniformis, B. thuringiensis, or B. brevis*) can be anticipated as good candidates for co-culture with antibiotic-producing fungi, such as *Penicillium*,

Aspergillus, or *Acremonium*. These co-cultures could result in the production of novel, improved β-lactams. Other good contenders for co-culture experiments are members of the *Streptococcaceae* and *Myxococcaceae* families, as they constitute well known antibiotic producers. This opens up the exciting possibility of obtaining new and improved antibacterials in the near future, as long as both governments and private companies are willing to invest in this new venture. This research is essential for the future of antibiotic development and must be done now to find new antimicrobials to counteract the threat of poly-resistant bacterial strains. Antibiotic resistance was described by the World Health Organization in 2018 as "one of the biggest threats to global health, food security, and development" facing humanity today.

5 Toxins

The ability of spore-forming Gram-positive bacilli (such as *Bacillus or Clostridium*) to produce toxins is very high and, in most bacteria, it is linked to secondary metabolism. These compounds include some of the most potent neurotoxins known in nature (i.e., *C. botulinum, C. perfringens*, *C. sordellii*, or *Cl. tetani*). Although the toxigenic phenotype has mainly been assigned to the strict anaerobic *Clostridium* genus, this ability is also displayed by some species of the mostly aerobic *Bacillus* genus, such as *B. cereus* and *B. anthracis*. *Clostridium botulinum* was named *Bacillus botulinus* by Emile van Ermengem, who originally isolated it from spoiled ham (1897). The American bacteriologist Ida Albertina Bengtson (1881–1952), the first woman hired to work at the National Institutes of Health (Lindenmann 2005), renamed it as *Clostridium* in 1924, as it is an anaerobic organism, hence restricting the genus *Bacillus* to aerobic spore-forming rods. Despite this, the bacterium was still referred to as *Bacillus* in publications well into the 1950s, such as in the article by Bulatova and Matveev (1957) concerning clostridial species. Finally, Collins et al. (1994) reorganized and redefined the species included in the genus *Clostridium*.

These neurotoxins produced by these bacteria are proteinaceous in nature and composed of two subunits (α and β). Botulism toxin was originally purified and crystallized by Lamanna et al. (1946), and is classified into eight types, referred to as A to H (Dover et al. 2014); A and B are the most important to humans. This toxin prevents the release of the neurotransmitter acetylcholine from axon endings at the neuromuscular junction and causes flaccid paralysis. The botulinic toxin is currently used in a number of medical applications, ranging from wrinkle reduction to the treatment of limb spasticity after a stroke (Sun et al. 2019); it is also applied in esthetic plastic surgery to treat facial sagging (Zhou et al. 2019), as well as in the treatment of Parkinson's disease (Cardoso 2018), bruxism (Tinastepe et al. 2015) and strabism (Scott 1981).

Eklund et al. demonstrated in 1971 that, when *C. botulinum* type C is cured of its prophage, the bacteriophage Ceβ, it ceases to produce toxin and becomes nontoxigenic *C. novyi* type A. This discovery could open the possibility of toxin

gene movilization among different clostridial species (Eklund et al. 1974). In the late twentieth century, a neurotoxigenic *Clostridium butyricum* strain, isolated from food, was found to be involved in an outbreak of food-borne type E botulism (Aureli et al. 1986; Meng et al. 1997). In addition, Cassir and coworkers recently demonstrated (2016) that *Clostridium butyricum*, normally used as a probiotic, could become a new emerging pathogen. *Enterococcus faecium* has also been reported as a potential producer of botulinum toxin, presumably due to horizontal transmission of the toxic gene from a clostridial strain (Zhang et al. 2018)

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