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# Highlights of the Genetics and Importance of *Bacillus subtilis* Spore Formation

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***Author's contribution***

*The sole author designed, analyzed, interpreted and prepared the manuscript.*

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## ABSTRACT

*Bacillus* species are important producers of essential biomolecules such as antibiotics, insecticides and enzymes. However, the immense potential for commercial scale production of these biomolecules still remain largely unexplored. Optimization of the exploitation of these species has, therefore, become the major preoccupation of many laboratories worldwide. Success in this regard depends on a thorough understanding of the species gene regulatory mechanisms, metabolism and secretary pathways. *Bacillus subtilis* by virtue of its relatively simple cellular organization, experimental tractability and excellent genetics has become the principal paradigm for the study of the cellular processes of the *Bacillus* genus. The bacteria survive adverse environmental conditions by undergoing a complex process of spore formation termed sporulation. The process of spore formation involves cellular differentiation of an asymmetrically dividing mother cell, with each compartment undergoing distinct cell-specific gene expression. These processes eventually culminate in the formation of resistant spores. The molecular genetics, importance and industrial applications of these species as well as the historical perspective of *Bacillus* research have been reviewed. Furthermore, scientific advancements in the study of the spore formation process in *B. subtilis* have been presented with emphasis on the basic genetic regulatory mechanisms.

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## 1. INTRODUCTION

The genus *Bacillus* constitutes a diverse group of Gram-positive rod-shaped soil bacteria characterized by their ability to form spores in response to adverse environmental influences. *Bacillus* species are important source of antibiotics, insecticides and enzymes [1]. Over the years, scientists have focused on various approaches aimed at optimizing the exploitation of the industrial potential of these species to the benefit of man [2]. Success of such effort, however, depends on a thorough understanding of the gene regulatory mechanisms, metabolism and secretary pathways of the species. To achieve this, it was necessary to have a tractable experimental organism that is a representative of the *Bacillus* genus [3,4].

In that regard, *Bacillus subtilis* became the organism of choice for studying the physiology and cellular differentiation processes of *Bacillus* species due to the wealth of information documented on it over the past decades unlike any other organism of the genus. During adverse conditions, *B. subtilis* survives by forming resistant spores through processes of cellular differentiation of an asymmetrically dividing mother cell [5]. The spore formation process of *B. subtilis* offers an ideal biological system for studying the mechanism of cellular differentiation, physiology and gene regulation due to its relatively simple cellular organization, experimental tractability and excellent genetics. In addition, the sequencing of the whole genome of the bacterium and the experimental approaches made possible by the accessibility of the resulting sequences brought a new perspective to research in microbial genetics [6].

*B. subtilis* is potentially the most ideal cell for the commercial production of valuable industrial biomolecules. Despite the immense benefits, *Bacillus* species particularly *B. subtilis* is yet to attract much attention of microbiological research that it deserves. This review introduces the medical, industrial and biological importance of *B. subtilis* and the molecular genetics of its spore formation process.

## 2. HISTORICAL LANDMARKS OF *Bacillus* RESEARCH

The first scientists to experiment with *Bacillus* species as early as the mid-18$^{th}$ century were Louis Pasteur, who used heat-attenuated *Bacillus anthracis* as the first antibacterial vaccine, and Robert Koch who used anthrax as the test case for development of postulates relating infectious agents and specific diseases [7]. Their work served as the impetus for further research into the genus *Bacillus*. Spore formation by *Bacillus* species was recognized at the time to be an interesting biological phenomenon and a factor in pathogenesis. But serious research on *B. subtilis* for studying mechanisms of gene regulation, metabolism and differentiation did not pick up until major breakthroughs including the successful isolation of mutant strains, optimization of transformation protocol and the development of excellent microscopy were achieved [8,9].

In 1947, P. R. Burkholder and N. H. Giles used X-rays and UV light mutagenesis to isolate a number of *B. subtilis* mutants. These mutants became the basis for biochemical and genetic analysis and detailed studies of recombination. John Spizizen [8] successfully demonstrated transformation of *B. subtilis* by chromosomal DNA. Expert electron microscopy work by Phillip Fitz-James [9] established the normal sequence of sporulation related morphological changes and correlated particular mutations with blockages at specific stages in spore development. Following these successes, research on the isolation and mapping of non-sporulating mutants of *B. subtilis* commenced and progressed rapidly.

Over the years it became clear that no one bacterium is perfect representative of all others and that, at a minimum, the gram-positive bacteria is better represented by *B. subtilis* than any other Gram-positive organism [10]. Thus *B. subtilis* became the principal paradigm for the study of Gram-positive bacteria. A fairly detailed outline of *B. subtilis* physiology and the identification of genes for metabolic and developmental pathways were available by the late 1970s. RNA polymerase sigma factors were first discovered in *B. subtilis*. As DNA sequencing technology improved to the point that sequencing of whole genomes of organisms became possible, *B. subtilis* was among the first bacteria to be completely sequenced.

The *B. subtilis* genome sequencing project, an international collaborative project between the European Union and Japan, funded by the two governments, began in 1989. In all 34

laboratories were involved in the systematic sequencing of the entire genome of 4,215kb long chromosome. The project was completed July 19, 1997 [6] and the sequence and annotations were released in the international DNA sequence database SubtiList (http://www.pasteur.fr/Bio/SubtiList) on November 20, 1997.

## 3. SPORE FORMATION IN *Bacillus subtilis*

### 3.1 Sporulation Initiation Signals

*Bacillus subtilis* undergoes vegetative and sporulation life cycles. In the presence of favourable conditions, growth is vegetative in which case the cell divides almost precisely at mid-cell by means of a septum into two identical daughter cells. On the other hand, in adverse conditions, septum formation is redirected from mid-cell to an asymmetric polar position (Fig. 1).

Chromosome integrity during entry into sporulation is very essential for an efficient spore formation process. Chromosome integrity is ensured by the *Bacillus subtilis* DisA [11], a cyclic diadenosine monophosphate synthase [12]. Initiation of spore formation involves regulatory factors that activate sporulation specific gene expression as well as sporulation initiation signals that these regulatory factors recognize and respond to. Cell cycle-derived cues have been implicated to induce entry into sporulation. A major factor in the regulation of this process is Sda [13]. Expression of Sda is induced to prevent sporulation under conditions not favourable for initiation of DNA replication [13]. Besides serving as a control point for replication initiation, Sda inhibits initiation of sporulation when the cell contains actively replicating chromosomes. Such inhibition by Sda is important to prevent the formation of nonviable polyploid spores. The decision to initiate sporulation is also governed by the phosphorylation of the transcription factor Spo0A.

Overexpression of KinA (or KinB)—the key kinase responsible for initiation of sporulation during exponential growth—is sufficient to induce entry into sporulation [14]. These initiation signals result from nutrient deficiency, low cell density and anomalies in DNA synthesis during the cell cycle [15]). Starvation for carbon, nitrogen or phosphorus induces sporulation in

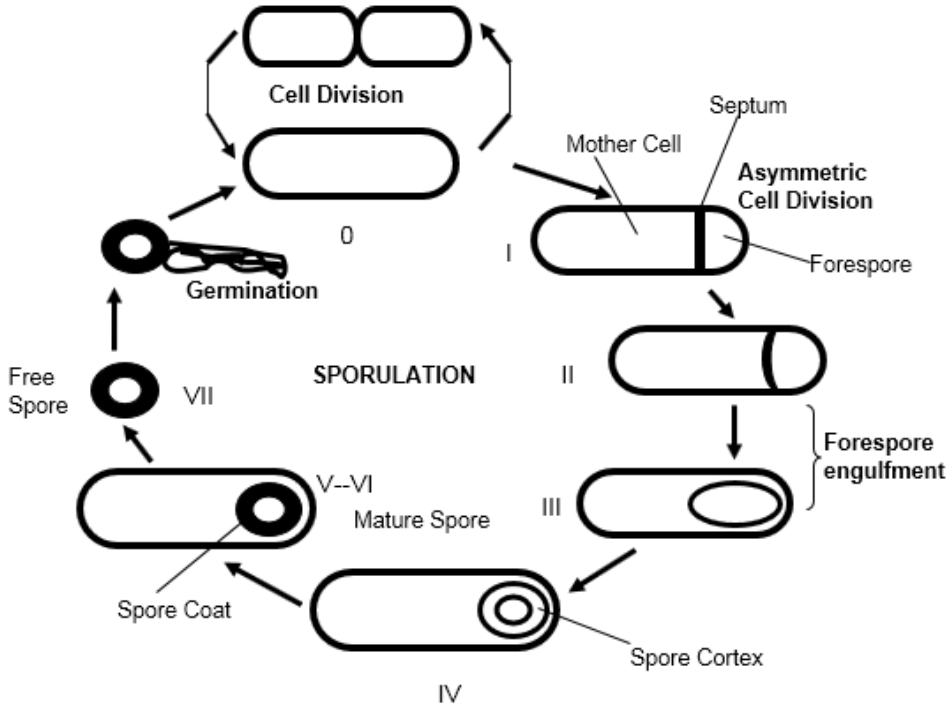


**Fig. 1. The sporulation process of *Bacillus subtilis*. The transition stages of the spore formation process are designated in Roman numerals**

*B. subtilis* whiles the availability of carbon sources such as glucose repress it. Moreover, it is also known that GTP deprivation due to depletion of readily metabolized carbon or nitrogen sources represent the primary inducer of nutritional signals. It is now known that commencement of the sporulation process requires that 1. The sporulating cells reach a stage in the cell cycle, 2. The tricarboxylic acid cycle is intact, and 3. At least one extracellular initiation signal activated by a phosphorylation complex is present in appropriate amount [16].

### 3.2 Morphological Transitions during Spore Formation

Pioneering electron-microscopic studies revealed well-characterized morphological transitions in *B. subtilis* sporulation. Stage 0 of sporulation comprises cells that have ceased exponential growth in a sporulation medium but are yet to exhibit overt changes in morphology. Sporulation proceeds with an asymmetric polar division of the vegetative cell by a polar septum into two unequal cells; a larger mother cell and a smaller forespore cell compartments [17] (Fig. 1). Sporulation continues with SpoIIIE — a large polytopic membrane protein belonging to the FtsK family of DNA transporters—controlling the translocation of the distal two-thirds of the chromosome into the forespore [18]. The completion of the specialized polar septum in the sporulating cell marks the end of stage I and signals the initiation of stage II. Later in stage II the edges of the septum begin to migrate toward the proximal pole of the cell [19].

At stage III, the edges of the septal membranes meet at the pole of the cell and fuse, completing the engulfment of the forespore within the cytoplasm of the mother cell. The proteins SpoIID, SpoIIM and SpoIIP located in the outer forespore membrane are necessary for the process of engulfment. Abanes-De Mello et al. [20] demonstrated that SpoIID is a peptidoglycan hydrolase and thus gave credence to the proposal that it acts as part of a complex, including SpoIIM and SpoIIP, to hydrolyze the peptidoglycan between the two membranes, thereby moving the mother cell membrane around the forespore to effect engulfment. The distinct ovoid shape of the spore begins to become apparent as a layer of cortex is synthesized between the forespore membranes. The synthesis of cortex layer, the main event of stage IV, is simultaneous with the beginning of stage V, which involves mainly the deposition of the proteinaceous spore coat on the outside surface of the spore. At stage VI, the stage of spore development and maturation, the characteristic properties of resistance, dormancy and germinability appear in sequence [21,22]. The mature dormant resistant spore is released by lysis of the mother cell at stage VII [23].

## 4. REGULATION OF GENE EXPRESSION DURING SPORE FORMATION

### 4.1 Sporulation-specific RNA Polymerase Sigma Factors

The sigma subunit of bacterial RNA Polymerase (RNAP) is a DNA binding protein that confers promoter specificity to the core subunits. The sigma subunits enable the holoenzyme to recognize specific promoter DNA sequences at transcription initiation. These subunits are classified into two main groups; the sigma 70 family and the sigma 54 family of enhancer-dependent sigma factors [24]. The sigma 70 family is further divided into the primary and alternative sigma factors.

The primary factors are essential proteins that direct the transcription of genes important for vegetative growth and maintenance functions of the cell whereas the alternative sigma factors are responsible for development and adaptive functions [25]. Notable among the alternative sigma factors are the sporulation-specific—expressed only during spore formation—sigma ($\sigma$) factors comprising SigF ($\sigma^F$), SigE ($\sigma^E$), SigG ($\sigma^G$), and SigK ($\sigma^K$) [26] (Fig. 2).

The organization of the compartment-specific sigma-factor regulatory network and the genes comprising this network are largely conserved in multiple endospore-forming bacteria species, in contrast with the structural sporulation genes [27]. The programme regulating transcription of sporulation genes essentially involves the synthesis and activation of these four sigma subunits. SigF and SigG control forespore gene expression while SigE and SigK regulate mother cell gene expression.

### 4.2 Compartment - Specific Gene Expression

Changes in pattern of gene expression is fundamental to morphological differentiation in both the pre-divisional sporangium and later in the two post-divisional compartments.

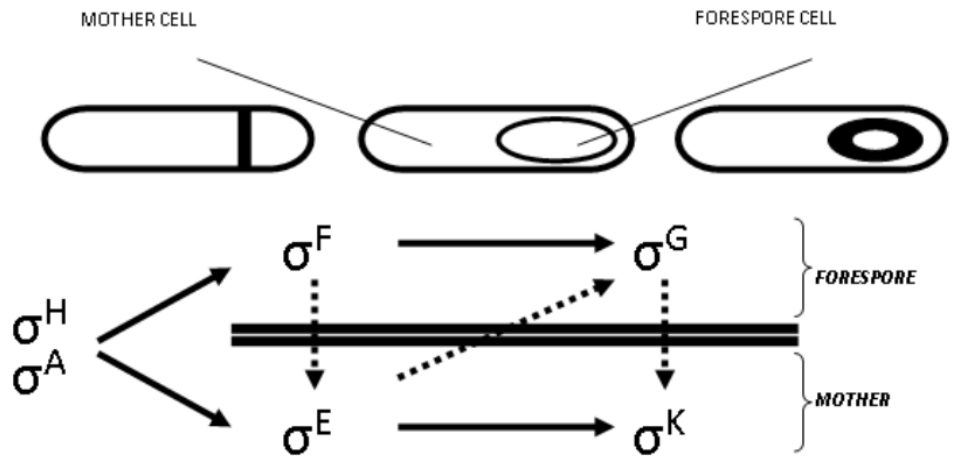


**Fig. 2. Compartment-specific activation of sigma factors. The central parallel lines represent the intercompartment boundary between the mother cell and the forespore**

Sporulation initiation signals in response to starvation induce the expression of several key sporulation genes and operons that bring about a switch in site of septum formation [27]. The formation of the polar septum is a landmark event in spore formation and directly contributes to the activation of the first compartment-specific transcription factor, SigF in the forespore. SigF governs the complex regulatory mechanism of sequential activation of three other compartment-specific transcription factors [28]. Therefore, the proper activation of SigF is crucial for all subsequent spore development and regulatory processes [29].

SigA the major sigma factor present in vegetative cells and SigH a minor sigma factor direct transcription of genes whose products redirect septum formation from the mid cell to polar position [30]. Soon after the septum forms, SigF becomes active in the forespore. Shortly after, SigE activity triggered by SigF is observed in the mother cell (Fig. 2). In *B. subtilis* sporulation, chromosomal asymmetry is important for the correct activation of SigE, the first mother cell-specific transcription factor. It is known that DNA segregation into the forespore is dependent on SpoIIIE, a polytopic membrane protein [31]. Report by Eichenberger et al. [32] indicates that another protein that is forespore-expressed, the SpoIIR protein crosses the first membrane of the polar septum and leads to the activation of SigE and the subsequent expression of approximately 300 genes. Products of both SigF and SigE controlled genes are required for the process of forespore engulfment.

After the engulfment of forespore by the mother cell is completed, SigE causes SigG to become active in the engulfed forespore where it directs transcription of genes encoding proteins that condition the spore for germination when nutrients become available [33]. Soon after SigG becomes active, it induces SigK activity in the mother cell [34]. SigE and SigK are required for the synthesis of the cortex and coat layers that encase the forespore. Simultaneously, the two compartments follow parallel pathways of transcription regulation where SigF directs the transcription of the gene that encodes SigG (Fig. 2). Likewise, SigE directs the expression of the structural gene for SigK [35]. The sequential appearance and mechanism of altering in a crisscross fashion, the transcriptional specificity of each sigma factor is fundamental in the regulation of gene expression during sporulation in *B. subtilis.*

## 5. ASSEMBLY OF SPORE CORTEX AND COAT PROTEINS

*Bacillus* is characterized by the ability to produce spores during adverse conditions. Spores are dormant and resistant cell types that serve as adaptive forms by which the bacteria survive extreme environmental stresses [36]. Some of the adverse conditions that the bacteria commonly encounter include bacteriocidal agents such as lysozyme, heat and ultraviolet radiation [37]. A large number of spores of a particular species in a habitat is a strong indication of previous or continuing growth and metabolism in that niche. The spore has the

capacity to germinate into a new cell in response to favourable growth conditions [38]. Besides enhancing the survival of the cell, the spore also serves as a means by which the species are disseminated by air currents, rain runoff or animals throughout the environment and hence enhance the colonization of diverse habitats [39].

In *B. subtilis* the spore coat is composed of over 20 polypeptides that are often highly cross-linked and organized into several morphologically distinct layers including a spore core, a lamellar lightly staining cortex, an amorphous inner coat, and an electron-dense outer coat (Fig. 3).

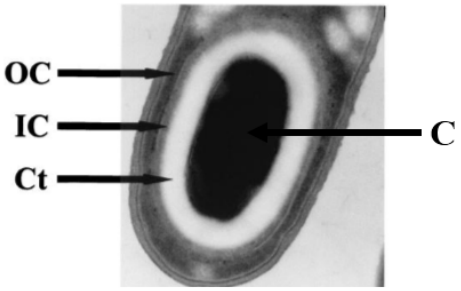


**Fig. 3. An electron micrograph showing multi-layer spore structure of *Bacillus subtilis* comprising spore cortex, Ct; Inner coat, IC; Outer coat, OC; spore core, C. Figure adopted from Amiteye et al. [40]**

Synthesis of the coat proteins is regulated by four mother cell transcription factors SigE, SpoIIID, SigK and GerE [41]. After septation of the sporulating cell, SpoIVA protein localizes at the mother cell side of the forespore membranes under the control of SigE activity. Wang et al. [42] showed that the assembling coat is synthesized in the mother cell and is targeted to the outer forespore membrane by SpoIVA. A basement layer that serves as a platform for coat assembly is formed through SpoIVA binding and hydrolyzing ATP, to allow it to self-assemble [43]. Other proteins involved in assembly are SpoVID that directly interacts with SpoIVA [44] and SafA, which is necessary for the encasement of the spore [45]. SafA was revealed to affect the localization of about 16 inner coat protein fusions [46,47], corroborating its central role in coat assembly. A pre-coat, consisting of the matrix protein components, CotJA, CotJC and a CotE protein layer, are then assembled over SpoIVA [48].

Shortly after SigK is activated by SigG in the mother cell (Fig. 2), the cortex appears followed by the expression of a range of coat protein genes. Amiteye et al. [40] demonstrated by an electron microscopy investigation that diacylglycerol kinase enzyme encoded by the gene *dgkA* is vital for efficient formation of the spore cortex. The assembly of the spore coat primarily involves the deposition of protein CotD, CotS and CotT into the inner coat while CotA and CotM are built into the outer coat. CotH and CotE proteins occur at the interface of the inner and outer coat layers [49]. At the final phase, under the control of the transcription factor GerE, protein CotB and CotG are synthesized and incorporated into the outer coat. Further coat modifications involving glycosylation, proteolysis and cross linking completes the spore [50]. Spore peptidoglycan assembly occurs in the space between the septal two membranes. This is controlled by the action of genes expressed in the mother cell compartment. One of such genes, SpoVE encodes the shape, elongation, division and sporulation protein. Real et al. [51] reported that SpoVE localizes to the outer forespore membrane and interacts closely both *in vivo* and *in vitro* with SpoVD, a protein that is also required for spore cortex synthesis [52]. It has also been proposed that another protein SpoVB is important in spore cortex synthesis and is necessary for Lipid II translocation [53].

## 6. MEDICAL AND INDUSTRIAL IMPORTANCE OF *Bacillus* SPECIES

A number of *Bacillus* species that inhabit complex ecological and continuously changing environments such as those within the soil and aquatic environments, produce an abundance of special metabolites that enhance their survival capabilities [54]. These metabolites occur in the form of antibiotics, insecticides and enzymes valued in medicine, agriculture and industry for their antimetabolic and pharmaceutical properties [1]. *Bacillus* species are generally not harmful to humans or animals except for a very few. Some species including *B. licheniformis, B. amyloliquefaciens* and *B. subtilis*, have long been applied safely on commercial scales in the production of many valuable industrial bioproducts. *B. subtilis* directly secretes proteins into the culture medium, and thus, shortening the filtration of the recombinant proteins. Several vectors have been recognized for the production process of proteins in *B. subtilis* [55,56]. *Bacillus* enzymes represent an estimated 60% of the global one billion-dollar industrial-enzyme market [57].

*B. subtilis* strains are also used in the production of bacterial vaccines either as antigen bearers or as antigen cellular manufactories. *B. subtilis*, as a probiotic organism, has beneficial effects on both animals and humans [58,59]. Bacteria that are probiotic—related to live organisms that are creatures beneficial to their host—have numerous applications. The basic interest in the *Bacillus* species, as probiotic organisms, has only come about since the last 15 years [60,61]. Besides, the consideration of *B. subtilis* spores as vaccine vehicles, delivered through mouth, has attracted considerable attention because of its probiotic effects and the extended shelf-life [62,63]. The health effects related to probiotic *B. subtilis* include, cholesterol-lowering, immune system excitation and prohibition of cancer aggression [60,61].

*B. subtilis* is one of the most widely used bacteria for the production of specialty chemicals and industrial enzymes. Besides, the safe status of the bacterium has made it a major source of amylase and protease enzymes for food use. Many *Bacillus* species have also proved to be effective against a broad range of plant pathogens [64]. The use of biological control agents for the management of plant pathogens and diseases is considered as a safer and more sustainable strategy for safe and profitable agricultural productivity compared to the application of agro-chemicals. *Bacillus*-based bio-control agents have been very useful in the field of bio-pesticides applications. They have been reported as plant growth promoter, systemic resistance inducer, and used for production of a broad range of antimicrobial compounds — such as lipopeptides, antibiotics and enzymes — and competitors for growth factors with other pathogenic microorganisms through colonization. Baidoo and Botchey [65] reported effective control of *Sesamia calamistis* in maize plants grown in screen house in Ghana using local isolates of *B. thuringiensis*.

*B. subtilis* has the ability to take up and recombine extracellular DNA into its genome, which makes it naturally useful for efficient genetic transformations [66]. DNA technology has also permitted the isolation of industrially important enzymes from microorganisms that either cannot be cultured or which naturally thrive in environments that are difficult to replicate on an industrial scale. Such enzymes often have superior performance characteristics in target industrial applications than enzymes traditionally used but their introduction into the marketplace necessitates large-scale production in a cell factory host organism [67]. *Bacillus* species are efficient at producing their native proteins but often show lower efficiency when secreting modified or heterologous proteins from other organisms [1]. Nevertheless, *Bacillus* species are the best available cell candidates and research into the mechanisms and control of their secretary pathways will directly enhance and diversify their industrial productivity.

Studies show that *B. subtilis* can be used to increase the production of acetone-butanol-ethanol from starch without an anaerobic remedy [68]. It may also be applied as a good bacterial source in the production of a high rate alkaline alpha-amylase. This *Bacillus* species is also generally applied for producing fermented products. Aerobic fermentation is a common method for producing enzymes in *B. subtilis* strains (e.g. amylase, protease), insecticides, antibiotics, purine nucleotides, polyglutamic acid, and D-ribose, polyhydroxybutyrate [69]. It also produces milk-clotting enzymes [70,71]. Fibrinolytic enzymes in the bacteria have been important in fibrin clots and are used as thrombolytic agents. These products are important for both medical and industrial uses.

## 7. CONCLUSIONS

A great deal of progress has been made in understanding the molecular genetic basis of spore formation in *B. subtilis* but there is still much scope of further work to be done. The whole genome sequence is complete and available thus, setting the stage for rapid advancement in addressing a wide range of questions of fundamental importance in developmental biology, including programmed gene expression, intercellular communication, and genetic control of morphogenesis. The understanding of these aspects of *B. subtilis* biology will pave the way for successful engineering of the *B. subtilis* secretory pathways that will enable large-scale commercial production of many beneficial biomolecules at optimum levels for various industrial uses.

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## COMPETING INTERESTS

Author has declared that no competing interests exist.

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