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UNIVERSITY OF NAPLES “FEDERICO II”



Ph.D. COURSE IN BIOLOGY XXXVI CYCLE

**Exploring novel *Bacillus* spore-forming strains from
the human gut: probiotic potential, therapeutic
applications, and molecular insights**

PhD student: Maria Vittoria

Tutor: Prof. Ezio Ricca

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OUTLINE OF THE THESIS

This PhD thesis was conducted in the Biology Department of “Federico II” University of Naples under the supervision of Prof. Ezio Ricca as part of the XXXVI cycle of the Doctorate in Biology. The PhD scholarship is framed within the National Research and Innovation Operational Program 2014-2020 for 'Innovative Doctorates of Industrial Character'.

In recent years, understanding of the complex relationship between the human gut microbiota and human health has increased, leading to growing interest in harnessing the therapeutic potential of probiotics. Among the countless microbial inhabitants of the human gut, spore-forming bacteria belonging to the genus *Bacillus*, have emerged as promising candidates for probiotic applications. These resilient organisms, capable of forming endospores, show remarkable survival under harsh environmental conditions, offering advantages in probiotic formulations and delivery. The quest to identify and characterize novel *Bacillus* spore-forming organisms from the human gut is an important goal in the field of probiotic research. Not only do these organisms have the potential to provide health benefits, but they also have unique physiological properties that deserve to be explored.

The thesis is structured into three chapters and two appendices and undertakes a comprehensive investigation to elucidate the probiotic properties, genomic characteristics and therapeutic applications of novel *Bacillus* spore-forming strains isolated from the human gut environment. The study emphasizes the significance of exploring new strains as probiotics to identify distinctive properties to meet the increasing demand for non-traditional therapeutic approaches and microbiome-based therapies.



OUTLINE OF THE THESIS

In Chapter 1 are presented the results of the initial isolation and characterization of spore-forming bacteria inhabiting the human intestinal environment. According to the general guidelines for the safety evaluation of microorganisms isolated from human microbiota, four strains out of the thirty-two isolates were chosen for their safety and for their beneficial properties e.g. biofilm production, antimicrobial activity, presence of gene clusters potentially encoding novel antimicrobial peptides, antioxidant activity, induction of a cytoprotective stress response in human intestinal cells *in vitro*. The results were published on July 31, 2023: *Probiotics as an Alternative to Antibiotics: Genomic and Physiological Characterization of Aerobic Spore Formers from the Human Intestine*. Microorganisms, 11(8), Article 8.

Chapter 2 focuses on the *in vivo* evaluation of the probiotic properties of two intestinal isolate strains, *Bacillus subtilis* SF106 and *Bacillus clausii* (recently renamed *Alkalihalobacillus clausii*) SF174. Probiotic treatments and microbiota-targeted approaches have been shown to be beneficial in several pathologies, including chronic inflammation of the intestine. This chapter reports the results obtained by administering spores of these two strains in a mouse model of chemically induced ulcerative colitis. The spores were found to attenuate overall inflammation and symptoms, showed immunomodulatory trend, improved the integrity of the intestinal barrier and remodeled the gut microbiota in favor of beneficial microbes. The results of this research have been included in an article currently under review.

Chapter 3 is dedicated to the study of a human intestinal isolate that differed from all other isolates due to its production of cytoplasmic granules and the presence of spores with unusual curved morphology. The isolate, MV19, was assigned to the species *B. cereus sensu stricto*.



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Analysis of growth and sporulation at different temperatures revealed that at the sub-optimal growth temperature of 25 °C sporulation was slow and less efficient but a high total number of fully functional spores was produced. At the optimal growth temperature of 42 °C sporulation was faster and some of the released spore were curved, structurally and functionally defective. This suggested an important adaptation characteristic of these bacteria to environmental conditions. The results of this chapter were published on February 3, 2023: *Sporulation efficiency and spore quality in a human intestinal isolate of Bacillus cereus*. Research in Microbiology, Volume 174, Issue 6, 2023, 104030, ISSN 0923-2508.

Appendix I and Appendix II describe the side experiments conducted during the three years of my PhD, focusing on elucidating the mechanisms governing spore structure and permeability. This adds valuable insight into how *Bacillus* spores interact with their environment, which is also linked to their probiotic functionality.

Appendix I describes a molecular study of protein assembly in the surface layers of spores of the model organism *Bacillus subtilis*. The study focuses on the protein CotG, an important component of the spore surface. Under canonical conditions, CotG is phosphorylated by the thermolabile kinase CotH and assembles on the surface of the spores; our results showed that, depending on the sporulation temperature, CotG tends to aggregate in the cytoplasm of the mother cell, dragging along other coat proteins. This gives the spores a different structure and consequently different physiological properties. Results were published on March 7, 2022: *CotG controls spore surface formation in response to the temperature of growth in Bacillus subtilis*. Environmental Microbiology, 24: 2078-2088.



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Appendix II discusses the ability of CotG protein to regulate spore permeability through its central region of positively charged tandem repeats. These repeats facilitate the interaction and aggregation of CotG with other coat proteins, resulting in a protein layer that controls the permeability of external molecules. The level of permeability of the spore structure appears to be determined by the distance between the N and C termini of the CotG protein. This model can be applied to most spore formers of the *Bacillus* genus because they all possess CotG-like proteins. These results were published on November 10, 2022: *CotG Mediates Spore Surface Permeability in Bacillus subtilis*. American Society for Microbiology, mBIO, Volume 13, Issue 6.

In conclusion, this PhD thesis presents a comprehensive investigation into the isolation and characterization of endospore-forming *Bacillus* spp. that contributes to the advancement of knowledge in the field of probiotics and offers potential applications for these bacteria in various healthcare contexts. The overall results shed light on the probiotic potential for human health, but also on the intricate molecular mechanisms that regulate spore structure and function. These results are promising for the development of new probiotic applications and contribute to the broader field of microbiology.



INTRODUCTION

1.1 Probiotics: features and impact on human health

The original observation of the beneficial role of certain selected bacteria is attributed to the Nobel Prize winner Eli Metchnikoff, who suggested that the dependence of intestinal microbes on food makes it possible to take measures to modify the flora in our bodies and replace the harmful microbes with beneficial ones. Since then, probiotic bacteria have become the undisputed synonym for beneficial microbes, gaining popularity over the years and finding application in various clinical and general health scenarios (Elshagabee *et al.*, 2017).

Probiotics are “*live microorganisms, which when administered in adequate amounts confer health benefits to the host*” (FAO/WHO, 2002). Probiotic formulations are developed and standardized for both human and animal consumption. Various dairy products, fermented foods, functional foods, dietary supplements and pharmaceutical formulations harbor probiotic strains for various human health benefits (Elshagabee *et al.*, 2017). They have also found application in feeds for the prevention of animal gastrointestinal infections, with extensive use in the poultry and aquaculture industry (Hong *et al.*, 2005).

The search for safe and cost-effective alternative treatments and concerns about the development of antibiotic resistance have led researchers to look for alternatives to the current treatment regimes, which are mainly based on the use of antibiotics. Probiotic therapy appears to be the most viable option proposed, with a long history of use and guaranteed safety (Sanders *et al.*, 2019).

PROBIOTICS: FEATURES AND IMPACT ON HUMAN HEALTH

Several species are currently being used as probiotics. *Lactobacillus* and *Bifidobacterium* spp. are the two most commonly consumed groups of bacteria globally due to their well-established health benefits. The expansion of research in this field has led to the inclusion of other bacteria, such as members of the *Enterococcus*, *Streptococcus*, and *Bacillus* spp., in the list of probiotics (Sanders *et al.*, 2019). This field is still expanding. Recent proposals of *Faecalibacterium prausnitzii* (Martín *et al.*, 2017) and *Akkermansia muciniphila* (Depommier *et al.*, 2019) as next-generation probiotics are examples of this. Most of our understanding of probiotic mechanisms of action comes from research using *in vitro* cell cultures, *in vivo* animal models or *ex vivo* human models. Currently, several mechanisms have been proposed to explain how probiotic bacteria can positively affect human and animal health. The key points of their mechanism of action are listed below and illustrated in **Figure 1**.

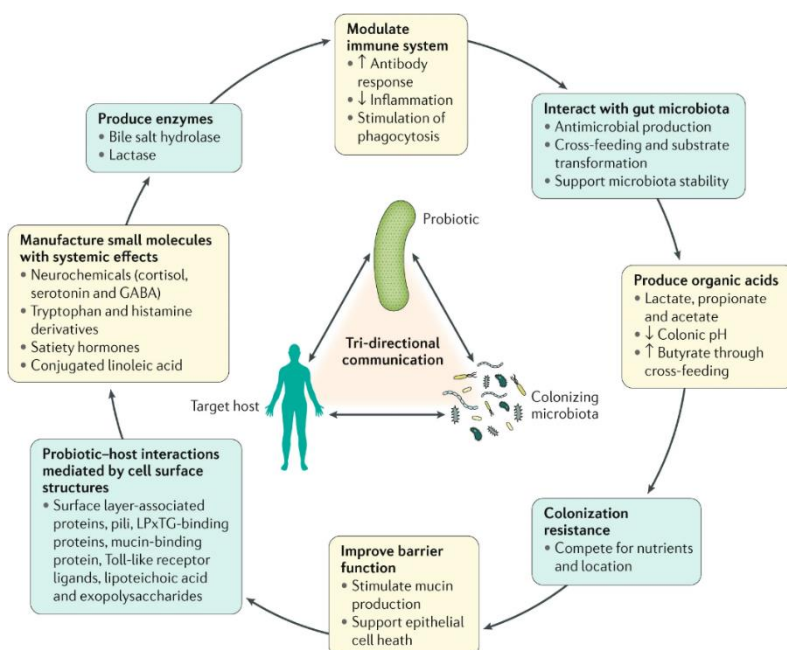


Figure 1 | Mechanisms of action of probiotic bacteria (Sanders *et al.*, 2019)



PROBIOTICS: FEATURES AND IMPACT ON HUMAN HEALTH

- I. Interaction with gut microbiota:** probiotics can affect the balance and diversity of the gastrointestinal microbiota through various mechanisms. These mechanisms include antagonism against pathogenic bacteria through competition for nutrients or the production of a wide range of antimicrobial compounds, such as bacteriocins (Hegarty *et al.*, 2016) and non-ribosomally synthesized peptides like surfactin (Rani *et al.*, 2021). On the other hand, probiotic bacteria can also provide a mechanism for cross-feeding with the commensal microbiota. Cross-feeding refers to the exchange of metabolites, such as energy and nutrients, between different species or strains of microorganisms (Culp & Goodman, 2023). Bacterial metabolic processes can release vitamins, amino acids and organic acids (such as lactate, propionate, butyrate and other Short-Chain Fatty Acids), which can have a beneficial effect on the growth of commensal bacteria and various physiological processes in the host (**Figure 2**).

- II. Improvement of barrier function:** studies conducted *in vitro* and in animal models have shown that a variety of probiotic strains can improve intestinal barrier function by increasing the expression of tight junction proteins such as occludin (ZO-1 and ZO-2) or claudin (Claud-1, Claud-3, Claud-5) (Mennigen & Bruewer, 2009) or by regulating the mucus biosynthesis/degradation process in the gut by increasing the expression of mucin-coding genes (such as MUC-2) (Aliakbarpour *et al.*, 2012). Additionally, reducing inflammation and preventing oxidative stress damage can improve the intestinal barrier (**Figure 2**).



PROBIOTICS: FEATURES AND IMPACT ON HUMAN HEALTH

- III. Modulation of immune system:** probiotic bacteria can modulate the host's immune response through direct (cell-cell interaction) or indirect mechanisms (production of metabolites) (**Figure 2**). There are several mechanisms involved: (1) probiotics can inhibit the inflammatory response of the intestinal immune system by inhibiting NF- κ B activation and have an anti-apoptotic effect on intestinal epithelial cells; (2) they can increase the activity of Natural Killer (NK) cells; (3) probiotic bacteria have been shown to induce the secretion of cytokines and the expression of co-stimulatory molecules by Antigen-Presenting Cells (APCs); (4) additionally, some strains can interact with Dendritic Cells (DCs), allowing for their activation to drive the T-cell response and stimulate the secretion of IL-10 and IL-12 (Aureli *et al.*, 2011).
- IV. Manufacture of small molecules with local and non-local effects:** bacteria produce numerous small molecules that benefit the host and microbiota (**Figure 2**). Among these molecules are neurochemicals, including oxytocin, gamma-aminobutyric acid, serotonin, tryptamine, norepinephrine, dopamine, and acetylcholine, which are known to affect brain function. In this context, a developing and innovative area of research is the study of Psychobiotics, which are microorganisms with the potential to modulate bidirectional communication between the intestine and the brain (Sharma *et al.*, 2021).

PROBIOTICS: FEATURES AND IMPACT ON HUMAN HEALTH

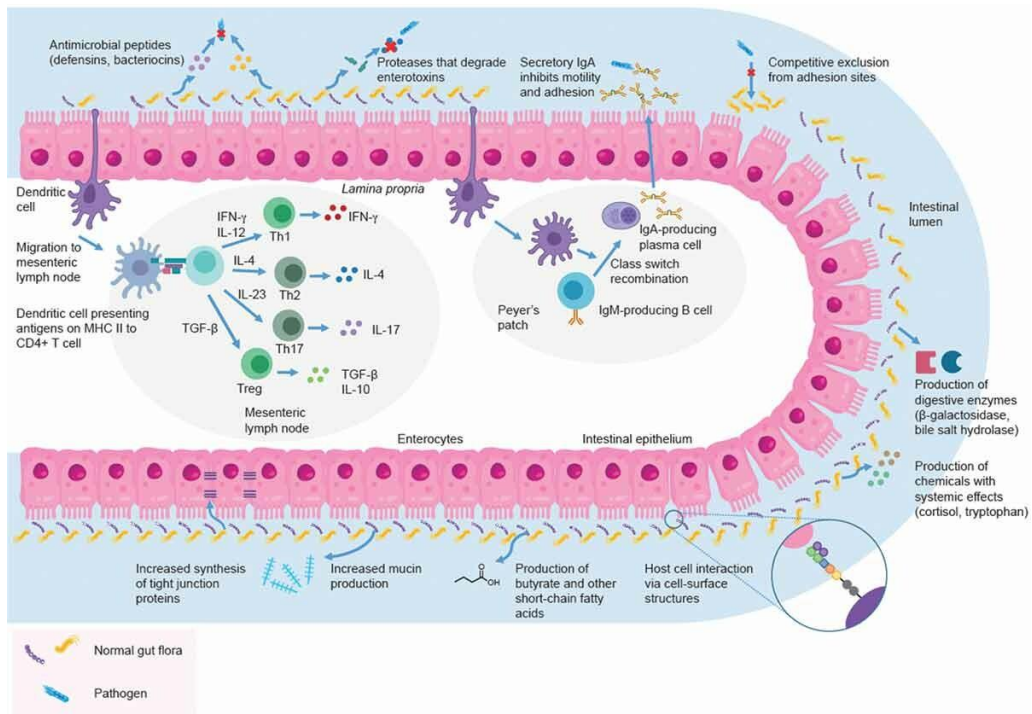


Figure 2 | Probiotics can modulate intestinal homeostasis in several ways: production of antimicrobial peptides (such as defensins and bacteriocins); production of proteases that counteract enterotoxins; competitive exclusion for epithelial cell adhesion sites; interaction with host cells via cell-surface structures; production of digestive enzymes; production of chemicals with systemic effects (cortisol, tryptophan); production of short-chain fatty acids; increased production of mucins and tight junctions; modification of the balance between Th1/Th2/Th17 and Treg cells; modulation of the release of pro- and anti-inflammatory cytokines (Wong-Chew *et al.*, 2022)

- V. Production of enzymes:** microbial enzymes, such as β -galactosidase and bile-salt hydrolases, produced and released by some probiotic strains, can improve the digestion of lactose and other biological macromolecules. For instance, *Streptococcus thermophilus*, which is present in yogurt, facilitates lactose digestion, its susceptibility to being permeabilized by bile when it enters the small intestine, and promotes the release of microbial β -galactosidase to break down lactose into digestible glucose and galactose. This results in a clinical benefit for lactose intolerant individuals.



***Bacillus* GENUS: SPORE FEATURES**

1.2 *Bacillus* genus: spore features

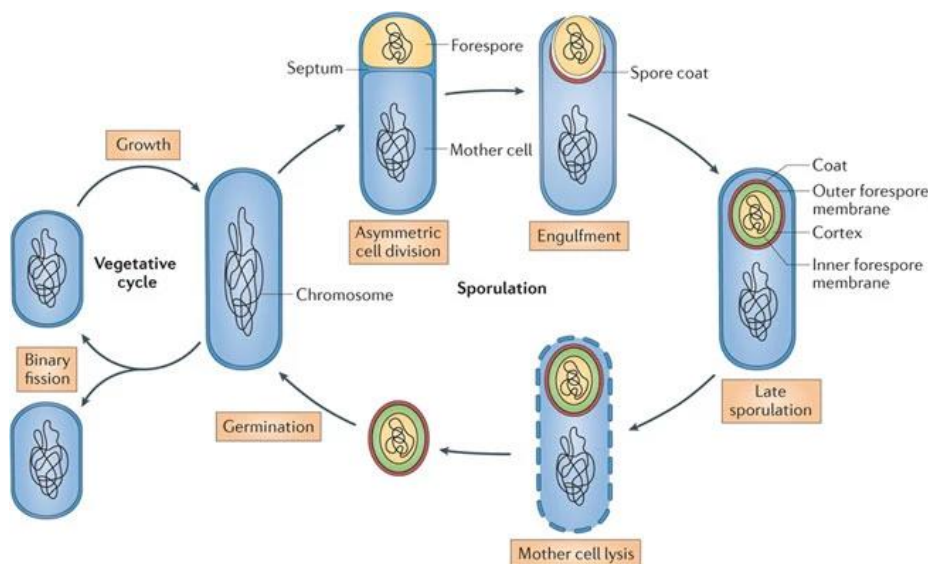
Spore-forming bacteria mainly belong to the *Bacillus* and *Clostridium* genera, which share the ability to differentiate into a metabolically quiescent cell (the spore), in response to environmental conditions. *Bacillus* are Gram-positive, rod-shaped, aerobic or facultative anaerobic bacteria, that are generally designated as a group of soil inhabitants. However, their wide distribution allows them to be isolated from various sources, including air, water, the human and animal gut, as well as vegetables and food (Nicholson *et al.*, 2000). Spores are thought to be present in animal intestines as a result of their introduction into the body through contaminated food, water, and air. They temporarily colonize the new environment and become allochthonous inhabitants in the gut (Saggese *et al.*, 2021).

The sporulation process in these microorganisms is a survival strategy regulated by hundreds of genes and various metabolic pathways (Errington, 2003) and it is an example of cell differentiation. It is activated when environmental conditions no longer support the life of the vegetative cell, such as in cases of nutrient deficiency.

Sporulation starts with an altered asymmetric cell division that produces a small forespore within a larger mother cell (**Figure 3**). Chromosome segregation in the forespore occurs after septation, which requires a DNA transport protein called SpoIIIE.

Following septation, the cell wall material in the septum is hydrolyzed and the mother cell engulfs the forespore, which becomes a protoplast in the mother cell cytoplasm. Both cells contribute to the maturation of the spore by progressive dehydration of the forespore cytoplasm and by forming a series of protective layers. At the end of the process, the mature spore is released in the environment through the lysis of the mother cell (**Figure 3**).

Bacillus GENUS: SPORE FEATURES



Nature Reviews | Microbiology

Figure 3 | Key stages of sporulation process (McKenney *et al.*, 2013)

The process of engulfment of the mother cell is what distinguishes the “endospores” of these bacteria from all others, because they form inside the mother cell and are released when it is lysed. Once released, the free spore can survive almost indefinitely without water or nutrients, even in adverse environmental conditions such as extreme temperatures, pH, radiation, chemical agents, including antibiotics and lytic enzymes (McKenney *et al.*, 2013). While in a dormant state, the spores continually monitor their surroundings using receptors embedded in their inner membrane. These receptors are activated by L-amino acids or D-sugars, which signal the spore the return to a favorable environmental condition. This triggers the process of germination, leading the spore to return to its active vegetative form (Christie & Setlow, 2020).

The high resistance of spores is due to their unique structure (**Figure 4**).

***Bacillus* GENUS: SPORE FEATURES**

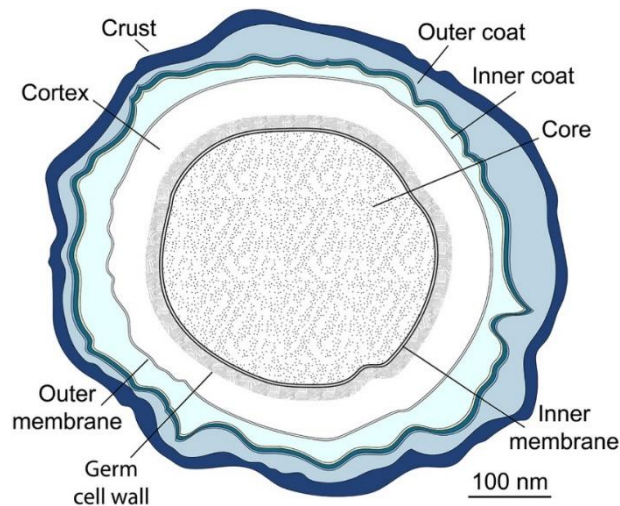


Figure 4 | Spore structure

The spore's structure comprises multiple concentric layers including a central *core*, that contains the dehydrated cytoplasm with all cellular components, such as cytoplasmic proteins, ribosomes, and DNA; the *core* is surrounded by the inner membrane that has low permeability due to the limited mobility of lipids; next is the germ cell wall and the *cortex* which is formed by a peptidoglycan layer that plays a crucial role in spore *core* dehydration and heat resistance; the *cortex* is surrounded by another multi-layered protein structure called the *coat* divided into an *inner* and *outer coat*, which primarily serve to shield the inner layers from toxic solvents, reactive oxygen species, and UV radiation.

Additionally, the *coat* ensures an appropriate response of the spore to signals from the external environment, such as the presence of nutrients or other factors that promote germination (Christie & Setlow, 2020). The *crust*, the outermost layer of the spore, is composed of glycoproteins. Its functions in *Bacillus subtilis* have not yet been fully clarified, but preliminary experiments suggest that it plays a role in compacting the underlying layers of the *coat*.



SPORE-BASED PROBIOTICS AND ATTRIBUTES IN THE GUT

1.3 Spore-based probiotics and attributes in the gut

Bacillus spp. represents a very heterogeneous group in terms of phenotypic and genotypic features. Some species belonging to the *Bacillus cereus* group are well known for their pathogenic potential such as *B. anthracis* causative agent of anthrax, *B. cereus* commonly recognized as food poisoning agents and *B. thuringiensis* commonly known for being entomopathogen (Ehling-Schulz *et al.*, 2019).

However, bacteria of the *Bacillus* genus also contain several species known for their beneficial properties such as *B. subtilis*, *B. clausii*, *B. coagulans*, *B. licheniformis* and others. Some of these have been used as probiotics for at least 50 years. For instance, the Italian product Enterogermina® based on four different strains of *B. clausii* spores was registered in 1958 in Italy as an over-the-counter medicinal supplement (Cutting, 2011).

The use of *Bacillus* as probiotics, however, has deeper roots and appears to be originally based on traditional foods containing spores, which are common in many cultures and are considered to have beneficial effects on health. One well-known example in this context is Natto, a traditional Japanese health food made from soy fermented with *B. subtilis* var. natto. Similar fermented foods are traditionally used in various countries, including Korea (Chongkukjang), India (Kinema), Thailand (Thuo nao), Myanmar (Pepok), Cambodia, and Laos (Sieng) (Saggese *et al.*, 2021). These foods contain spores of different *Bacillus* species, such as *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens*. Nowadays, spores are commonly used as dietary supplements in humans, growth promoters in animals and as an alternative to antibiotics, and in aquaculture to enhance the growth and disease resistance of cultured shrimp (Cutting, 2011).

SPORE-BASED PROBIOTICS AND ATTRIBUTES IN THE GUT

Spore-based probiotics offer several advantages compared to non-spore-forming probiotics due to their unique characteristics: (1) they can be stored for long periods at room temperature, even when dried, without affecting their vitality; (2) they can survive the low pH of the gastric barrier, which is not the case for all *Lactobacillus* species; (3) they have low production costs and are easy to prepare; (4) spore-based probiotics also have the ability to resist production processes and (5) can be preserved over a wide temperature range (Barbosa *et al.*, 2005).

Bacillus spores are capable of completing their life cycle in the intestine, providing benefits to the host in both spore and vegetative form (Lu *et al.*, 2022; Tam *et al.*, 2006; Williams & Weir, 2024). The spores enter the body orally or nasally and overcome the gastric barrier to reach the small intestine (Figure 5).

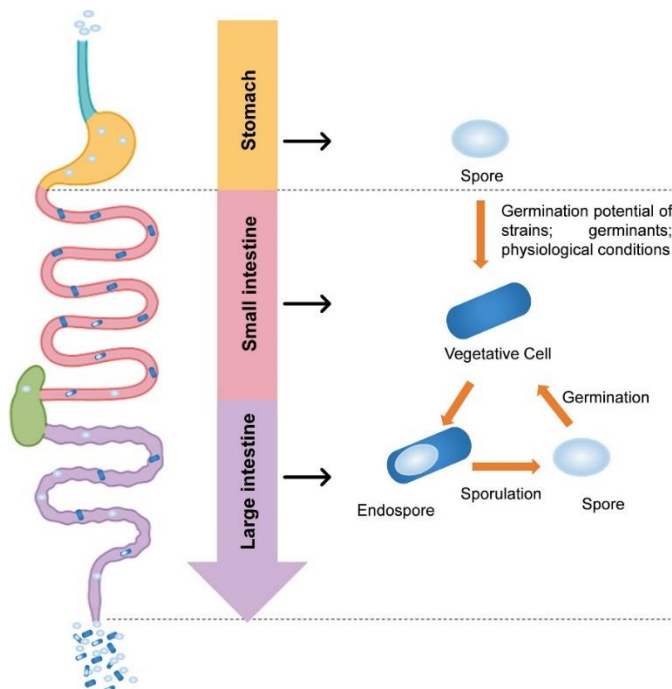


Figure 5 | Transit and life cycle of spores through the gastro-intestinal tract

SPORE-BASED PROBIOTICS AND ATTRIBUTES IN THE GUT

Here, under favorable conditions they germinate into the vegetative cells that can proliferate and potentially resporulate in the colon when conditions are no longer favorable. Efficacy of *Bacillus* spp. as probiotic has been screened in several *in vitro* and *in vivo* animal models and a few have also been validated in human clinical trials (Elshaghabe *et al.*, 2017). The beneficial properties of probiotic products based on *Bacillus* spores are numerous and are summarized in **Figure 6**. In this section I will only discuss a subset of these properties, which are more related to my PhD project.

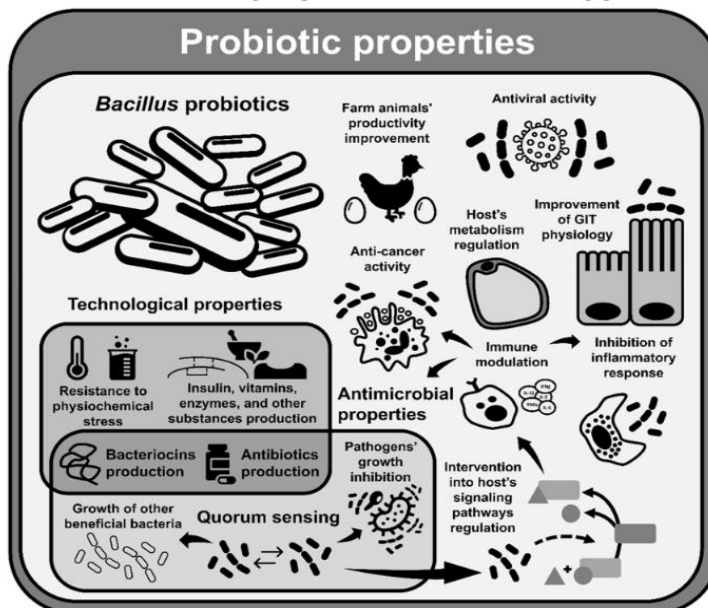


Figure 6 | Beneficial properties of *Bacillus* based probiotics in the gut (Todorov *et al.*, 2022)



SPORE-BASED PROBIOTICS AND ATTRIBUTES IN THE GUT

1.3.1. Ribosomal and non-ribosomal synthesis of antimicrobial peptides

Antimicrobials produced by *Bacillus* are generally antimicrobial peptides (AMPs) with broad-spectrum antibacterial activity and highly rigid, hydrophobic and/or cyclic structures. They are synthesized via two different pathways: (1) Non-ribosomal synthesis of compounds, such as subtilin, surfactin, iturin, mersacidin, sublancin, subtilisin, fengycin, and others, and (2) Ribosomal synthesis of linear precursor peptides, such as bacilysin, amicoumacin, difficidin, rhizocticin, cerein, and coagulin (Lu *et al.*, 2022).

Ribosomally synthesized peptides (RPs) are typically derived from short precursors of around 100 amino acids and are converted into mature compounds through post-translational modifications. These peptides, commonly referred to as “bacteriocins”, are divided into three classes based on their chemical structure, modifications, thermos-stability and mechanisms of action (Kawulka *et al.*, 2004).

Class I includes lanthipeptides described as small peptides (<5 kDa), consisting of unusual lanthionine amino acids with several post-translational modifications useful to acquire the active forms. Lanthipeptides carry out their mechanism by acting on the bacterial cell envelope, cell wall synthesis or inhibition of protein synthesis (**Figure 7**). An example is the Subtilosin A produced by *B. subtilis* strains (Thennarasu *et al.*, 2005) which is a cyclic peptide with unusual Cys-Phe bonds that showed antimicrobial properties against *L. monocytogenes* and *G. vaginalis* (Abriouel *et al.*, 2011) whose mechanism of action is to disrupt lipid bilayers.

Class II includes small (<10 kDa), linear, and unmodified peptides that are resistant to heat and acid-base treatments and do not undergo post-translational modifications. They act by forming pores in the target membrane (**Figure 7**).

ANTIMICROBIAL PEPTIDES

Class III molecules are generally large, thermolabile molecules (>30 kDa) with active -SH group that exhibit phospholipase activity (Tajbakhsh *et al.*, 2017).

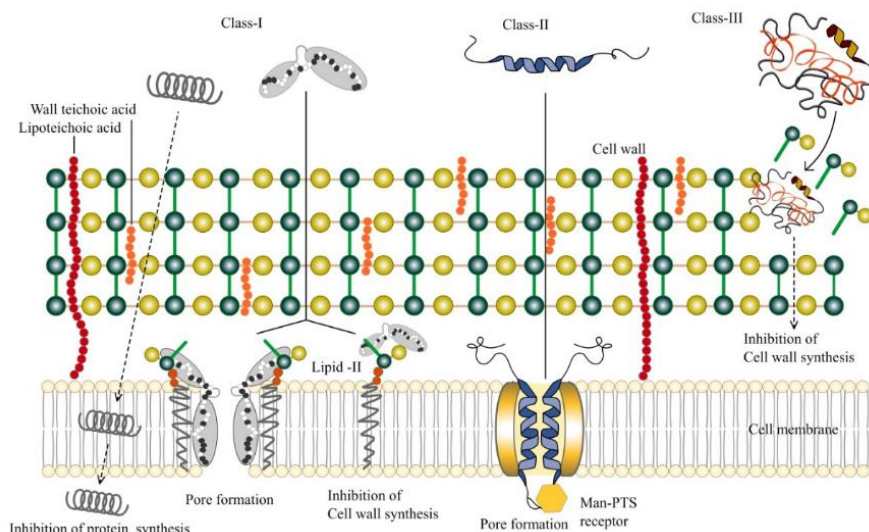


Figure 7 | Mode of action of the different classes of bacteriocins

Non-ribosomal peptides (NRPs) and polyketides (PKs) are a large group of natural products with linear, cyclic, and branched structures with a wide range of biological activities and pharmacological properties, such as anticancer, immunosuppressive, and antibacterial (Tajbakhsh *et al.*, 2017). They are synthesized on large non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) multienzyme complexes that show a modular organization in which each module can be further divided into domains. The domains represent the enzymatic units that catalyze the individual steps of the non-ribosomal synthesis (Schwarzer *et al.*, 2003).

An example of NRPs are lipopeptides. Lipopeptides are a class of cyclic amphiphilic biosurfactants that consist of a hydrophobic fatty acid chain linked to a hydrophilic peptide ring.

ANTIMICROBIAL PEPTIDES

The lipopeptides produced by *Bacillus* spp. are mainly iturin, surfactin and fengycin (Yang *et al.*, 2020) and they can have multiple activities, such as inhibition of fungi, bacteria, viruses and antitumoral activity. It has been shown that secreted fengycin/surfactin lipopeptides can eliminate pathogens indirectly by inhibiting the quorum-sensing system of the pathogens *S. aureus* and *E. faecalis* (Lu *et al.*, 2022).

Polyketides show the same biological activities of the NRPs; examples of these compounds are bacillaene, difficidin and macrolactin.

Although many *Bacillus* strains are capable of producing bioactive secondary metabolites, the biosynthetic arsenal of *B. velezensis* is reported to be more potent and diverse than that of *B. subtilis*, which is the most studied species in the entire genus.

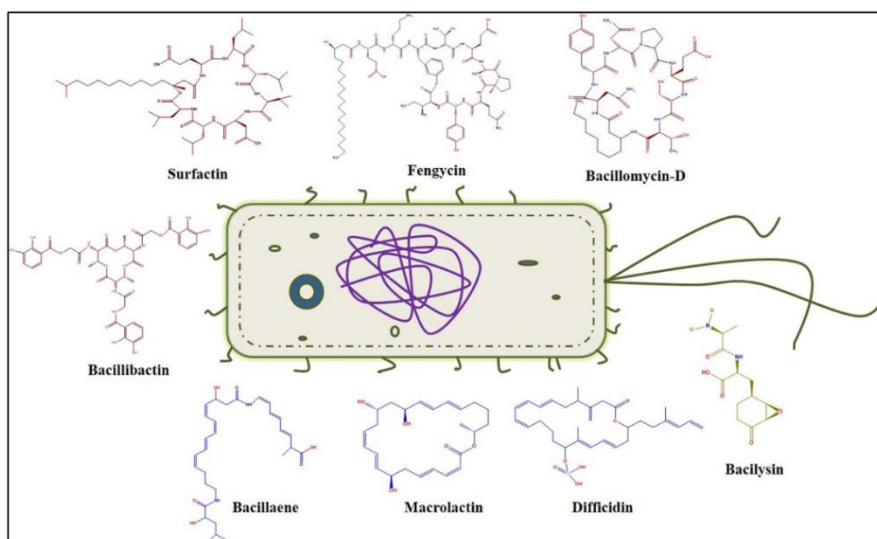


Figure 8 | Antimicrobial compounds synthesized by *B. velezensis*. The compounds highlighted in red are NRPs; blue compounds are PKs; green color compound bacilysin is synthesized by a ribosome independent pathway



ANTIMICROBIAL PEPTIDES

Recently NRPs and PKs synthesized by *B. velezensis* (**Figure 8**) have demonstrated promising antimicrobial activities suitable for agricultural applications and biocontrol (Fazle Rabbee & Baek, 2020).

Recent advancements in sequencing technology, coupled with the accessibility of bioinformatic pipelines, have significantly streamlined the identification of numerous biosynthetic gene clusters (BGCs) responsible for secondary metabolite production. Notably, tools like ANTismash (Antibiotics and Secondary Metabolite Analysis Shell) software facilitate a comprehensive search for all biosynthetic clusters within bacterial genomes (Puan *et al.*, 2023). This software not only proves instrumental in discovering new antimicrobial agents but also enables the assessment of the potential of a probiotic strain to produce valuable bioactive compounds.

1.3.2. Quorum-sensing signal molecules

Bacterial cell-to-cell signaling, also known as Quorum Sensing, is a mechanism that enables cells to communicate with each other through the secretion and sensing of small, diffusible signaling molecules. This process allows bacteria to communicate and modify the behavior of the same or other species in response to environmental conditions and perturbations. Both Gram-positive and Gram-negative bacteria use quorum-sensing molecules, which in the former are usually bioactive peptides, while in the latter include non-peptide molecules such as acyl-homoserine lactone (Bassler & Losick, 2006). As they play a role in determining the diversity and composition of the enteric microbiome, the profile of QSMs reflects the state or impending changes in the microbiota (Fujiya *et al.*, 2007).



QUORUM-SENSING SIGNAL MOLECULES

The quorum-sensing signal mechanism exerted by probiotic *Bacillus* occurs via two pathways. One is via the quorum-sensing signal molecules (QSSM) associated with immunomodulation and cytoprotection, and the other is via the quorum-sensing inhibition molecules (QSIMs) that have antimicrobial activity by inhibiting the quorum-sensing mechanism of pathogenic bacteria (Lu *et al.*, 2022).

An example of QSSM is the Competence and Sporulation Factor (CSF) produced by *B. subtilis*, which is a secreted pentapeptide that once internalized, is able to stimulate the quorum response and sporulation. Researches have shown that CSF activates important survival pathways in Caco-2 intestinal epithelial cells, including p38 MAP kinase and protein kinase B (Fujiya *et al.*, 2007). Additionally, it can induce cytoprotective Heat-Shock Proteins (Hsps), which help to prevent cell damage caused by oxidants and maintain barrier function. It has also been shown to induce anti-inflammatory cytokine IL-10 and reduce the release of pro-inflammatory mediators IL-4, IL-6 and CXCL-1 in Caco-2 cells, and to significantly reduce gut inflammation in mice when tested *in vivo* with synthetic CSF (Okamoto *et al.*, 2012).

CSF is produced exclusively by members of the *B. subtilis* species. However, recent studies have shown that other species, such as *B. megaterium*, *B. pumilus*, *B. clausii*, produce different quorum sensing peptides (CSF-like peptides) which are also capable of inducing a cytoprotective stress response with a similar mechanism in human intestinal cells *in vitro* (Di Luccia *et al.*, 2016; Vittoria *et al.*, 2023).

EXTRACELLULAR MATRIX

1.3.3. Extracellular matrix and exopolysaccharides

Biofilm formation is the process through which microbial cells aggregate to form an extracellular matrix that encapsulates the cells and facilitates their attachment to surfaces, enabling their persistence in diverse environments, including the gastrointestinal tract (GIT) of mammals (Yahav *et al.*, 2018). The extracellular matrix (ECM) is primarily composed of polysaccharides, proteins, and extracellular DNA (eDNA). Its formation begins with the initial adsorption of bacteria to surfaces, followed by the activation of quorum sensing signaling, which leads to cellular attachment and polysaccharide production. Once the microcolony is formed, the biofilm undergoes a process of maturation and dispersion into the environment (**Figure 9**).

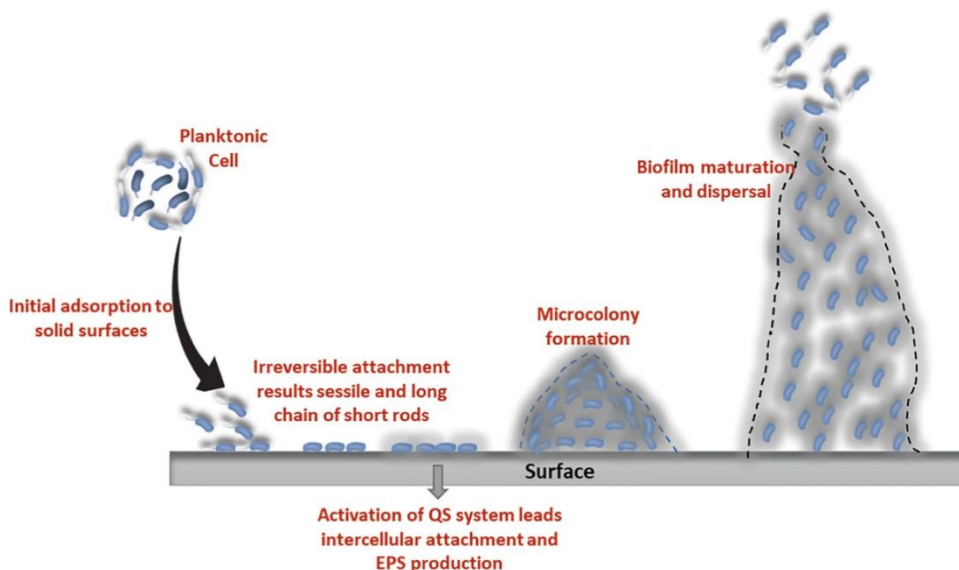


Figure 9 | Different stages of biofilm formation



EXTRACELLULAR MATRIX

Matrix production in *Bacillus* spp. mainly depends on the transcription of two operons: *epsA-O* and *tapA-sipW-tasA*. The *epsA-O* operon consists of 15 genes responsible for exopolysaccharide (EPS) synthesis, the primary component of biofilm (Sowmiya *et al.*, 2024). EPS helps bacterial cells adhere to each other and shields them from environmental stress. The *tapA-sipW-tasA* operon is responsible for the secretion of protein TasA that organizes into long amyloid-like fibers connected to the cell wall by an anchoring another protein, TapA.

Biofilm formation by *Bacillus* species in the intestinal tract can have beneficial effects. The biofilm provides a protective barrier against harmful bacteria, viruses, and other pathogens in the gut. These biofilms can also aid in the breakdown of complex carbohydrates and other nutrients, making them more accessible for absorption by the host (Sowmiya *et al.*, 2024).

EPS purified from different *Bacillus* sources have shown diverse functions, including anticancer, antioxidant, antibacterial, anti-inflammatory, and immunomodulatory activities (Lu *et al.*, 2022). A mouse model study showed that wild-type *B. subtilis* provided protection against *Citrobacter rodentium*, an enteric pathogen that causes acute colitis. In contrast, an EPS-deficient *B. subtilis* strain did not exhibit the same effect (Jones & Knight, 2012).

However, some studies suggest that EPS can protect by modulating the host's immune response rather than solely preventing pathogen colonization or epithelial destruction. Research has shown that TLR-4 signaling is essential for EPS-mediated protection. In fact, purified EPS from *B. subtilis* induces anti-inflammatory M2 macrophages both *in vitro* and *in vivo* in a TLR4-dependent manner. When TLR-4 is bound, EPS causes M2 macrophages to inhibit CD4⁺ and CD8⁺ T cells by producing TGF- β and PD-L1 (Paynich *et al.*, 2017).



INTERACTION WITH HOST CELLS

1.3.4. Interaction of spores and vegetative cells with epithelial and immune cells

The outer layers of the bacterial cell wall and other cell components play a crucial role in bacteria-host interactions and immunomodulatory responses. Both spores and vegetative cells of different *Bacillus* spp. can interact with intestinal and immune cells (**Figure 10**) through complex mechanisms that are not yet fully understood. *Bacillus* spores are believed to primarily act through a “passive effect” as they are metabolically inert. This effect is achieved either by direct interaction of the surface components of the spores, eliciting a response from the intestinal cells, or indirectly through modification of the commensal microbiota. On the other hand, vegetative cells, with their active metabolism, can influence the gastrointestinal environment in the same way, but through an “active effect” (Williams & Weir, 2024).

The specific interaction between spores and host cells has been studied with killed spores or with extracted spore coat proteins (Lu *et al.*, 2022). It has been shown that when administered to mice infected with H5N1 influenza virus, heat-killed (121 °C) spores of the *B. subtilis* laboratory strain PY79 enhanced both humoral and cell-mediated immune responses. Additionally, they promoted maturation of DC cells and retrieval of NK cells, while enhancing Th1-type cytokines expression in DC cells (Song *et al.*, 2012).

In a separate study, it was found that heat-inactivated spores of *B. subtilis* PY79 enhanced the response of antigen-specific CD4⁺ and CD8⁺ T cells and antibody responses when co-administered with tetanus toxin C fragment (TTFC). Additionally, they increased antigen-specific IgA and IgG antibodies in both the draining lymph nodes and blood (Barnes *et al.*, 2007).



INTERACTION WITH HOST CELLS

Another study demonstrated that *B. subtilis* PY79 and its isogenic strain unable to germinate in the GIT, induced cell proliferation in the spleen and mesenteric lymph nodes, as well as the production of IFN γ , but not IL-4 and IL-10. These results indicate that spores preferentially stimulate a robust cell-mediated immune response with a Th1 phenotype, regardless of their ability to germinate in the GIT (Mauriello *et al.*, 2007).

A recent study showed that *B. subtilis* spores protected human keratinocytes from oxidative stress and chemical injury *in vitro*, by adhering to the keratinocyte surface and inducing nuclear translocation of the transcription factor Nrf-2, which activates stress-response pathways (Petruk *et al.*, 2018).

Antioxidant activity has also been associated with spores of other species, such as *B. megaterium* spores. These spores have shown good antioxidant properties both *in vitro*, by reducing intracellular ROS in Caco-2 cells, and *in vivo*, in a mouse model of dextran sodium sulphate (DSS)-induced oxidative stress, where pre-treatment with spores protected mice from chemically induced damage (Mazzoli *et al.*, 2019). The interaction between *Bacillus* vegetative cells and the GIT has been extensively studied over the years because, once ingested, spores are known to germinate, as discussed in paragraph 1.3.

A recent work showed that vegetative cells of *B. subtilis* Bs29784 were able to upregulate the expression of tight junction proteins of Caco-2 cells exposed to IL-1 β and significantly reduce IL-8 production by limiting nuclear translocation of NF- κ B and preventing I κ B degradation (Rhayat *et al.*, 2019). Similar activity was noted for other strains of *B. subtilis*, but to varying degrees depending on the strain used. The effects were observed in both the supernatants and the cells, indicating that they can be attributed to both secreted metabolites and molecules associated with the cell surface.



INTERACTION WITH HOST CELLS

Another study evaluated the ability of probiotic candidate *B. amyloliquefaciens* SC06 to influence obesity and associated liver injury (Wang *et al.*, 2019). It was found that *B. amyloliquefaciens* SC06 cells ameliorated body weight gain, glucose metabolism and lipid accumulation in mice, as well as liver injury, by regulating the antioxidant capacity and gut microbiota of the host.

In vitro analysis of the four strains of *B. clausii* present in Enterogermina® demonstrated their immunomodulatory properties. The vegetative form of *B. clausii* strains can induce the activity of nitric oxide synthase II (NOS II), IFN- γ production, and CD4⁺ T cell proliferation (Urdaci *et al.*, 2004), while other *Bacillus* strains, including *B. licheniformis* 31, *B. cereus* IP 5832, *B. cereus* NT, *B. cereus* DM-423, and *B. subtilis* 168, had a lower stimulatory effect than *B. clausii* strains.

Bacillus species play a crucial role in the development of the gut-associated lymphoid system (GALT), as demonstrated by Huang *et al.* (2008). In this study, three species (*B. subtilis*, *B. licheniformis*, and *B. flexus*) were found to induce cell proliferation in Peyer's patches, stimulate APC cells and T lymphocytes, modulate the release of pro-inflammatory cytokines, and up-regulate the levels of Toll-like receptors TLR-2 and TLR-4 (Huang *et al.*, 2008).

Overall, *Bacillus* species, in both vegetative and spore forms, can interact with cells and exhibit immunomodulatory properties that can differ from species to species and often even between strains of the same species. The **Figure 10** illustrates the mechanisms contributing to the phenomenon described in this paragraph

INTERACTION WITH HOST CELLS

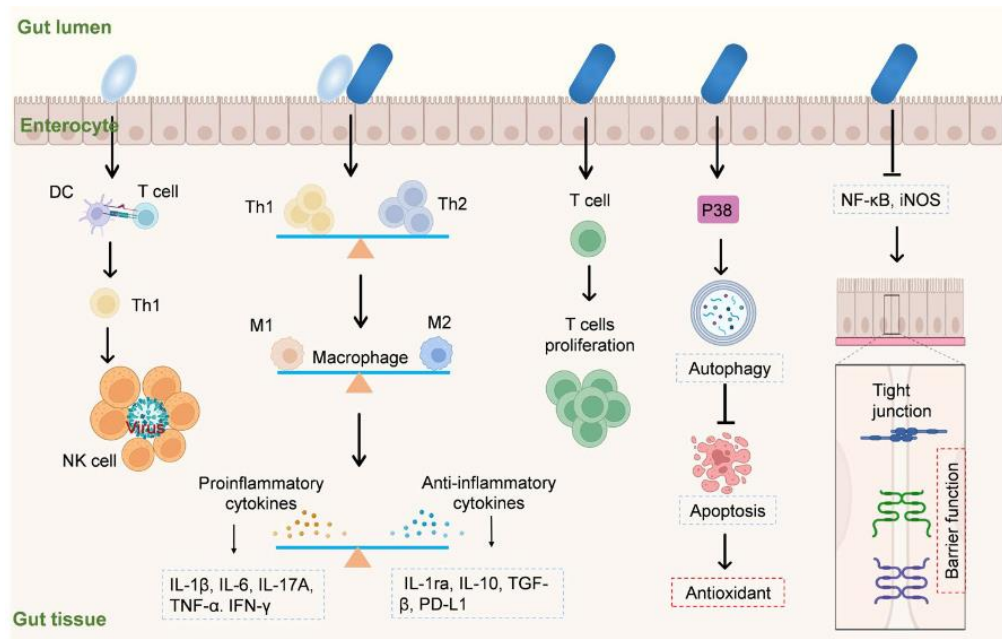


Figure 10 | Interaction between spores and vegetative cells with host cells and their mechanisms of immunoregulation

1.3.5. Effect on gut commensal microbiota

An increasing number of studies indicate that probiotic treatments may effectively treat or prevent certain illnesses by maintaining or rebalancing the microbiota. Dysbiosis, or an imbalance in the microbial community, can lead to the colonization and invasion of opportunistic pathogens in the gut, increasing the host's immune response and the risk of developing Inflammatory Bowel Diseases (IBDs) (Qiu *et al.*, 2022), such as Ulcerative Colitis (UC) or Chron's Disease (CD). Several metagenomic and meta-analysis studies have shown changes in the gut microbiota of patients with these diseases. In some cases, variations in microbial community composition have also been found between UC and CD microbiota (Pittayanon *et al.*, 2020; Sankarasubramanian *et al.*, 2020).



EFFECT ON GUT COMMENSAL MICROBIOTA

Recent research has shown that administering a *B. subtilis* HH2 strain, isolated from healthy animals, to rabbits with chemically induced colitis had numerous beneficial effects. These included improving barrier function by increasing occludin expression, modulating cytokine expression (TNF- α , IL-1 β and IL-10), and increasing microbial diversity that had been reduced by the induction of the pathology. The administration of spores resulted in an increase in the abundance of the *Bifidobacteriaceae* family and a reduction in *Bacteroidetes* and *Proteobacteria* (Luo *et al.*, 2020).

Similarly, the administration of *B. coagulans* FCYS01 spores supplemented with chito-oligosaccharides to mice with DSS-induced UC, improved the overall inflammatory status and positively affected the composition of the gut microbiota through the enrichment of SCFA-producing bacteria, such as *Akkermansia* and *Ruminococcus* species (Liu *et al.*, 2022).

In a previous study, the probiotic strain *B. clausii* UBBC-07 was administered to IBD patients and was found to modulate the gut microbiota in the treated group compared to the placebo group. Specifically, there was an increase in the *Firmicutes* and a decrease in the *Bacteroidetes* phyla. At the genus level, there was an increase in the abundance of *Lactobacillus*, *Bifidobacterium*, and *Faecalibacterium* compared to the placebo group (Bamola *et al.*, 2022).

It is now widely accepted that *Bacillus* spp. play a key role in the remodeling of the commensal microbiota, not only in humans but also in animals, such as in aquaculture, where *Bacillus*-based probiotics can be used to improve nutrient uptake, regulate the microbiota by promoting anti-pathogenic activity as an alternative to the massive use of antibiotics (Olmos Soto, 2021).

EFFECT ON GUT COMMENSAL MICROBIOTA

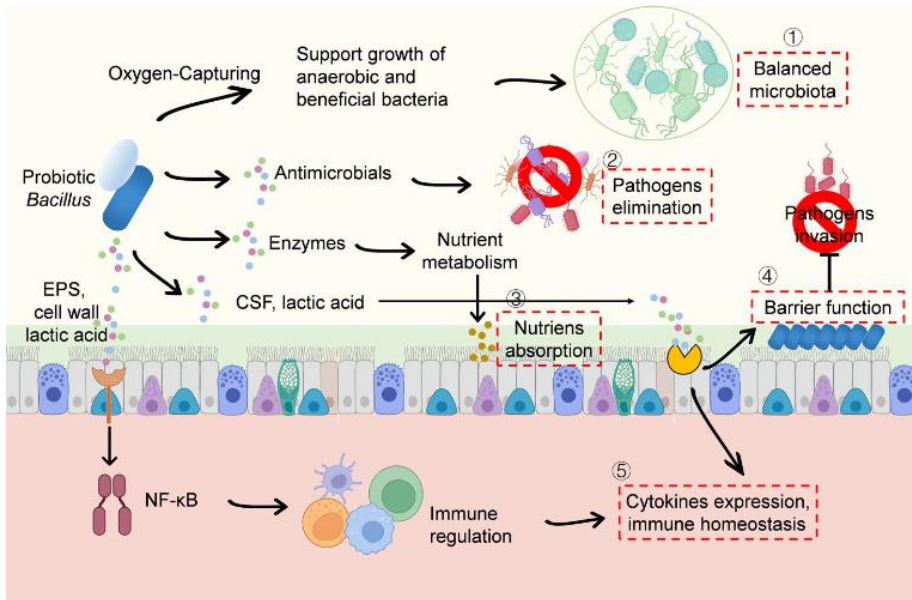


Figure 11 | Overview of the main mechanisms of action of spores and vegetative cells of the probiotic *Bacillus* species

In summary, **Figure 11** illustrates the various mechanisms by which *Bacillus* probiotics can be involved in bacterium-host interactions: (1) Supporting the growth of beneficial bacteria to balance the gut microbiota; (2) Eliminating pathogens through the production of antimicrobials which exhibit both direct inhibitory activity and indirect quorum-sensing inhibition activity; (3) Producing bioactive compounds, such as digestive enzymes (e.g. galactosidase, amylase, lipase, and protease), that increase nutrient absorption; (4) Improvement of barrier function by directly stimulating tight junctions/mucin production or producing compounds that prevent oxidative damage, such as CSF; (5) Improvement of immune homeostasis through the interaction of bacterial cellular components with intestinal and immune cells or the production of compounds that exert immune stimulation, such as EPS.



EFFECT ON GUT COMMENSAL MICROBIOTA

Despite the widespread use of bacterial spores in commercial probiotic products, ongoing research is actively exploring the beneficial effects of *Bacillus*-based probiotics. Specifically, this research is focused on isolating novel strains that may exhibit unique properties or produce novel compounds. In addition, an effort is underway to clarify the mechanisms underlying their beneficial functions, which still remain not fully understood. Some of these studies are opening up new avenues in the world of *Bacillus*-based probiotics, proposing *Bacillus* spores as a versatile platform for the delivery of beneficial molecules. This approach combines the recognized benefits of probiotics with the potential of additives, thereby expanding the scope of their applications. Moreover, emerging research suggests that *Bacillus* probiotics show promise in addressing neurodegenerative diseases. These studies highlight the potential of probiotics as innovative therapeutic interventions targeting previously unexplored pathways.

As our understanding of *Bacillus* probiotics continues to evolve, their diverse applications and therapeutic potential are increasingly recognized, opening up new directions for research and clinical translation in the field of microbiome-based therapeutics.



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CHAPTER 1

Probiotics as an Alternative to Antibiotics: Genomic and Physiological Characterization of Aerobic Spore Formers from the Human Intestine

Maria Vittoria^{1†}, Anella Saggese^{1†}, Rachele Isticato^{1,2}, Loredana Baccigalupi^{2,3} and Ezio Ricca^{1*}

1 Department of Biology, Federico II University of Naples, 80125
Naples, Italy;

2 National Biodiversity Future Center (NBFC), 90133 Palermo,
Italy;

3 Department of Molecular Medicine and Medical Biotechnology,
Federico II University of Naples, 80131 Naples, Italy

*Correspondence: ericca@unina.it;

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Article

Probiotics as an Alternative to Antibiotics: Genomic and Physiological Characterization of Aerobic Spore Formers from the Human Intestine

Maria Vittoria ^{1,†}, Anella Saggese ^{1,†}, Rachele Istitato ^{1,2}, Loredana Baccigalupi ^{2,3} and Ezio Ricca ^{1,*}

¹ Department of Biology, Federico II University of Naples, 80125 Naples, Italy; maria.vittoria@unina.it (M.V.); anella.saggese@unina.it (A.S.); istitato@unina.it (R.I.)

² National Biodiversity Future Center (NBFC), 90133 Palermo, Italy; loribacci@unina.it

³ Department of Molecular Medicine and Medical Biotechnology, Federico II University of Naples, 80131 Naples, Italy

* Correspondence: ericca@unina.it; Tel.: +39-0816-79-036

[†] These authors contributed equally to this work.

Abstract: A total of thirty-two aerobic spore former strains were isolated from intestinal samples of healthy children and analyzed for their hemolytic and antibiotic-resistant activities. Four strains selected as non-hemolytic and sensitive to all antibiotics recommended as relevant by regulatory agencies were short-listed and evaluated for their *in silico* and *in vitro* probiotic potentials. The four selected strains were assigned to the *Bacillus velezensis* (MV4 and MV11), *B. subtilis* (MV24), and *Priestia megaterium* (formerly *Bacillus megaterium*) (MV30) species. A genomic analysis indicated that MV4, MV11, and MV24 contained a homolog of the gene coding for the *f* brinolytic enzyme nattokinase while only MV30 encoded a glutamic acid decarboxylase essential to synthesize the neurotransmitter GABA. All four strains contained gene clusters potentially coding for new antimicrobials, showed strong antioxidant activity, formed biofilm, and produced/secreted quorum-sensing peptides able to induce a cytoprotective stress response in a model of human intestinal (HT-29) cells. Altogether, genomic and physiological data indicate that the analyzed strains do not pose safety concerns and have *in vitro* probiotic potentials allowing us to propose their use as an alternative to antibiotics.

Keywords: probiotics; antimicrobials; beneficial bacteria; *Bacillus velezensis*; *Bacillus subtilis*; *Priestia megaterium*; antibiotic resistance; antioxidants; CSF



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1. Introduction

Antibiotic resistance is a global health threat, predicted to cause, by 2050, a million annual deaths due to an increased incidence of infectious diseases that will, in turn, affect many medical and surgical procedures [1]. So far, the identification and design of new antibiotics, and the development of novel vaccines and probiotics have been proposed as alternatives to current antibiotics [2]. Probiotics, live bacteria that exert a beneficial health effect, are recognized as potential candidates to substitute antibiotics for the capacity of some bacteria to protect the animal body against bacterial and viral infections. Inhibition of bacterial adhesion, enhanced mucosal barrier function, modulation of the immune systems, and secretion of antimicrobial metabolites are the main mechanisms proposed to explain the anti-infectious activity of probiotics [3].

Several species of the *Bifidobacterium*, *Lactobacillus*, and *Bacillus* genera have long been used as commercial probiotics [4], while other bacteria, such as *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* have been recently proposed as next-generation probiotics [5,6]. In this context, the case of *Bacillus*-based probiotics is peculiar since all commercial preparations do not contain vegetative cells but metabolically quiescent spores [7]. For these organisms, spore formation is a survival strategy since spores are extremely stable cells,



resistant to harsh conditions such as high temperatures, low pH, absence of water and nutrients, presence of lytic enzymes, toxic chemicals, and UV irradiations [7]. In response to the renewed presence of water, nutrients, and of favorable environmental conditions, spores germinate, originating vegetative cells able to grow and eventually sporulate again [8]. Also, due to their resistance properties, *Bacillus* spores are ubiquitous in nature and can be isolated from common and extreme environments [7,8]. *Bacillus* spores are also abundantly present in the gut of animals, where they enter as contaminants of food and water and safely transit the gastric barrier. In the intestine, some of the ingested spores germinate [9–12], and the germination-derived cells grow and temporarily colonize that niche, before re-sporulating in the terminal part of the intestine and leaving the body as spores [13,14].

Ingested spores of various *Bacillus* species have health-beneficial effects [9,11] and provide protection against bacterial [15] and viral infections [16]. Such probiotic effects have been ascribed to various mechanisms, including the promotion of the development of the Gut-Associated Lymphoid Tissue (GALT) [17], the induction of the production of cytokines in mesenteric lymph nodes (MLN) (IL-1, IL-5, IL-6, IFN- γ , and TNF- α) and spleen (IFN- γ and TNF- α) [18], the upregulation of the expression of the Toll-like receptors TLR-2 and TLR-4 [19], and the protection of human intestinal cells from oxidative stress [20] by inducing the nuclear translocation of the transcriptional factor Nrf-2 that, in turn, activates stress-response genes [21]. More recently, *B. subtilis* cells have been shown to have regulatory effects on epithelial differentiation by inhibiting the Notch pathway and, as a consequence, shifting differentiation toward a secretory cell fate [22]. Such a Toll-like receptor 2-dependent effect causes an increased number of Paneth and goblet cells in the intestine that, in turn, results in the production of antimicrobial peptides [22]. An additional mechanism by which spores exert their beneficial effects is the modulation of the microbial composition of the gut, exerted by favoring the prevalence of other potentially beneficial bacteria such as *Faecalibacterium prausnitzii* [23], *Akkermansia muciniphila*, and *Bifidobacterium* spp. [24].

In the intestine, germination-derived cells produce a variety of metabolites that mediate the interaction with the host's intestinal and immune cells. Examples of such beneficial molecules produced by several *Bacillus* strains are enzymes, such as the NattoKinase (NK), a serine protease characterized by a potent thrombolytic activity and proposed for the treatment of multiple cardiovascular diseases [25], and the Glutamic Acid Decarboxylase (GAD), able to catalyze the synthesis of γ -aminobutyric acid (GABA), the most common inhibitory neurotransmitter in the central nervous system that also regulates cardiovascular functions such as blood pressure and heart rate [26]. In addition, peptides and lipopeptides with antimicrobial activity [27,28] and quorum-sensing peptides [29] are also produced by several *Bacillus* strains. An example is the Competence and Sporulation Factor (CSF), a quorum-sensing pentapeptide secreted by *B. subtilis* cells that is also recognized by human epithelial cells in which it induces the synthesis of the heat-shock (HS) proteins [29]. In a murine model, CSF prevents oxidant-induced intestinal epithelial injuries and loss of barrier function, exerting a cytoprotective activity [30]. CSF is only produced by members of the *B. subtilis* species but other species produce different peptides (CSF-like) similarly able to induce the synthesis of the heat-shock (HS) proteins in human cells in vitro [31]. More recently, CSF has been recognized as a key factor for the anti-neurodegenerative activity of *B. subtilis* in the model organism *Caenorhabditis elegans* [32]. *B. subtilis* strains able to produce biofilm (bf), CSF, and nitric oxide (NO) were shown to extend the longevity of *C. elegans* by downregulating the insulin-like signaling system, thus opening the possibility that ingested spores of *B. subtilis* could contribute to delay the development of age-related diseases [33,34].

In this work, several *Bacillus* strains were isolated from intestinal samples of healthy children not under probiotic or antibiotic treatment. Four of these strains which were not hemolytic and not resistant to antibiotics, were assigned to the *B. velezensis* (MV4 and



MV11), *B. subtilis* (MV24), and *Priestia megaterium* (formerly *B. megaterium*) (MV30) species and characterized at the genomic and physiological level.

2. Materials and Methods

2.1. Bacterial Isolation and Characterization

Fecal samples of healthy children (from ten months to ten years old) that had not been under antibiotic or probiotic treatment in the previous three months were collected and about 1 g of feces homogenized in sterile Phosphate-buffered saline (PBS) and heat-treated at 80 °C for 20 min. The samples were then serially diluted and plated on Difco Sporulation Medium (DSM, for 1 L: 8 g/ L Nutrient Broth, 1 g/ L KCl, 1 mM MgSO₄, 1 mM Ca(NO₃)₂, 10 µM MnCl₂, 1 µM FeSO₄, Sigma-Aldrich, Darmstadt, Germany) and incubated overnight at 37 °C. The colonies were selected and isolated as previously described [35]. All isolates were tested for hemolytic activity by spotting 10 µL of growing cells on Columbia Agar plates supplemented with 5% defibrinated horse blood (ThermoScientific, Waltham, MA, USA) and, after 24 h of incubation at 37 °C, the clear halo around the colonies indicated a positive result. The non-hemolytic strains were then tested for their antibiotic susceptibility by plating 0.5 McFarland of bacteria on LB agar plates (for 1 L: 10 g/ L Bacto-Tryptone, 5/ L g Bacto yeast extract, 10 g/ L NaCl, pH 7.0). Antibiotic-impregnated MIC test strips (Liof lchem®, Roseto, Italy) were then placed on the plates and incubated overnight at 37 °C. The minimum inhibitory concentration (MIC) was measured at the intersection of the inhibition halo and the antibiotic strip. The strains were indicated as resistant (R) if the antibiotic concentration exceeded the threshold level set by the EFSA for *Bacillus* strains [36].

2.2. Whole-Genome Sequencing and Bioinformatic Analysis

Bacterial genomic DNA was extracted from exponentially growing cells as previously reported [37]. Genome sequencing of MV4, MV11, MV24, and MV30 was performed with Illumina MiSeq Sequencing System by GenProbio (Parma, Italy). Genome assemblies were performed with SPAdes v3.14.0 by means of a MEGAnnotator pipeline [38]. The genomes of MV4, MV11, MV24, and MV30 have been deposited in GenBank as BioProject numbers PRJNA977871, PRJNA977867, PRJNA977868, PRJNA977866, and accession numbers CP127102, JASNWD000000000, JASPFH000000000, JASPGF000000000, respectively. For phylogenetic analyses, all genomic sequences were imported into the KBase system to create a genome set that was subsequently employed to build the tree, using Insert Genome Into SpeciesTree-v2.2.0. The phylogenomic trees were then visualized on the Interactive Tree of Life (iTOL, platform v6). Average Nucleotide Identity (ANI) values between the sequenced genomes and the closest bacteria were obtained using the Ezbiocloud tool ([https:// www.ezbiocloud.net/ tools/ ani](https://www.ezbiocloud.net/tools/ani) (accessed on 3 October 2022)).

For the detection of toxin-associated genes, the genome of the strains was analyzed using the VF analyzer pipeline [39] as described above [35].

The presence of homologs of nattokinase and glutamate decarboxylase was investigated in amino acid sequences of MV strains using protein BLAST alignment ([https:// blast.ncbi.nlm.nih.gov/ Blast.cgi?PAGE=Proteins](https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) (accessed on 3 October 2022)).

Biosynthetic gene clusters for the secondary metabolites and predicted core structure peptides were obtained using the web-based genome mining tool antiSMASH ([http:// anti smash.secondarymetabolites.org](http://antismash.secondarymetabolites.org) (accessed on 7 November 2022)).

Functional annotations of MV genomes were obtained using EggNOG-mapper v2 ([http:// eggnog-mapper.embl.de](http://eggnog-mapper.embl.de), (accessed on 7 November 2022)). Similarly, the genomes were also scanned using the dbCAN2 server ([https:// bcb.unl.edu/ dbCAN2/ blast.php](https://bcb.unl.edu/dbCAN2/blast.php) (accessed on 7 November 2022)) that integrated three tools/ databases for automated CAZyme annotation [40].

2.3. Antimicrobial and Biofilm Production Assays

Antimicrobial activity was assessed as previously described [40]. Briefly, 10 µL of supernatant was spotted onto LB agar plates and the plates were air dried. Then, 100 µL of an exponential culture of each of the target strains was mixed with 10 mL of 0.7% LB agar and plated. Fresh medium was used as a negative control. The plates were incubated overnight at 37 °C and the antimicrobial activity was determined as the diameter of the inhibition halo.

Biofilm formation was tested as previously reported [41]. Briefly, growing cells were inoculated into 24-well culture plates with three different media: LB, DSM [42], and S7 minimal medium (for 1 L: 50 mM morpholine-propane-sulfonic acid (MOPS) (adjusted to pH 7.0 with KOH), 10 mM (NH₄)₂SO₄, 5 mM potassium phosphate (pH 7.0), 2 mM MgCl₂, 0.9 mM CaCl₂, 50 µM MnCl₂, 5 µM FeCl₃, 10 µM ZnCl₂, 2 µM thiamine hydrochloride, 20 mM sodium glutamate, 1% glucose, 0.1 mg/mL phenylalanine, and 0.1 mg/mL tryptophan) [43]. The plates were incubated for 48 h at 37 °C, and then biofilm production was assessed by crystal violet assay as previously described in [41]. The experiment was performed in triplicate.

2.4. Antioxidant Activity Tests

2.4.1. Hydrogen Peroxide Scavenging Assay

The hydrogen peroxide stability was measured by following absorbance at 240 nm of 1 mL of fresh hydrogen peroxide solution (50 mM Potassium Phosphate Buffer, pH 7.0; 0.036% (w/w) H₂O₂). Quantitative determination of H₂O₂ scavenging activity of cells of MV strains was measured by the loss of absorbance at 240 nm as previously described [20]. Briefly, 10⁷, 10⁸, and 10⁹ cells/mL of selected strains were incubated at room temperature in 1 mL of 0.036% of hydrogen peroxide solution. After 30 min, the hydrogen peroxide concentration in the supernatant was determined by measuring the absorbance at 240 nm. The percentage of peroxide removed was calculated as reported below:

$$\text{H}_2\text{O}_2 \text{ removed (\%)} = (1 - A_{\text{sample}} / A_{\text{control}}) \times 100$$

2.4.2. DPPH Assay

The α,α-diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging method was used to evaluate the potential antioxidant activity of MV strains [20]. Different concentrations of the cells of each strain (10⁷, 10⁸, and 10⁹ cells/mL) were incubated in a final volume of 1 mL of 50 mM of citrate phosphate buffer (pH 7) containing 0.1 mM of freshly prepared DPPH (giving absorbance ≤ 1.0). The reaction was allowed to proceed for 30 min in the dark at room temperature. The DPPH free radical scavenging activity was then monitored by determining the absorbance at 515 nm and calculated according to the following equation:

$$\text{DPPH radical scavenging activity (\%)} = (1 - A_{\text{sample}} / A_{\text{control}}) \times 100$$

where A_{sample} is the absorbance of the reacted mixture of DPPH with the extract sample, and A_{control} is the absorbance of the DPPH solution.

2.5. Growth and Treatment of HT-29 Cells with Bacterial Conditioned Medium

HT-29 cells (ATCC HTB-38), derived from colon adenocarcinoma, were grown in RPMI 1640 medium (EuroClone S.p.A., Pero, Italy) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. HT-29 cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂ [40]. At 80% confluence, cells were harvested and diluted in 6-well plates to a total concentration of 5.5×10^6 cells per well. Bacterial conditioned medium (CM), required for the treatment of the HT-29 cells, was obtained by growing bacterial cells in minimal S7 medium, aerobically, for 16 h at 37 °C [44]. The culture was then centrifuged (7000 rpm for 10 min) and the supernatant (CM) was filtered through a 0.22 µm low protein binding filter (Millex, Millipore, Bedford, MA, USA). The obtained CM was used for HT-29

cells treatment at a concentration of 20% v/v in a complete growth medium as previously reported [44]. After 24 h incubation of HT-29 cells with 20% CM, the cells were harvested, lysed, and cell extracts were prepared for Western blot analysis as described below. A heat-shock treatment (45 °C for 30 min) was performed prior to cell lysis for heat-shock proteins induction in HT-29 cells (not treated with CM), used as a positive control. The experiment was repeated in triplicate.

2.6. Protein Extraction, SDS-PAGE, and Western Blot Analysis

Cells were scraped and harvested in lysis buffer (50 mM Tris-HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate and protease inhibitors) and the cell lysates were incubated on ice for 40 min followed by centrifugation at 13,000 rpm for 30 min to remove cellular debris. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Segrate, Milano, Italy). Subsequently, 2X Laemmli buffer (Sigma Aldrich, Milan, Italy) was added and proteins were boiled at 100 °C for 5 min and separated on a 12% SDS-PAGE gel. Proteins were transferred (2 h at 100 V, 4 °C) to polyvinylidene fluoride membranes (Millipore, Burlington, MA, USA). The membranes were blocked in a PBS-Tween20 (0.2%) buffer, with 5% (w/v) Bovine Serum Albumin (BSA, PanReac AppliChem, Darmstadt, Germany) and incubated with the primary antibodies anti-HSP27 and anti- β -Actin (Elabscience®, Wuhan China), diluted 1:500 and 1:1000, respectively, in a PBS-Tween20-2.5% BSA solution overnight at 4 °C. After several washes with PBS-Tween20 buffer, the membranes were incubated with 1:7000 anti-rabbit secondary antibody (SantaCruz, Santa Cruz, CA, USA) for 2 h and then visualized by enhanced chemiluminescence (ECL; GE-Healthcare, Little Chalfont, UK) using horseradish peroxidase-conjugated secondary antibody (SantaCruz Biotechnology) and analyzed using Quantity One® software from the ChemiDoc™ XRS system (version 4.62, Bio-Rad, Hercules, CA, USA). Band intensity was evaluated using ImageQuant analysis. Values were normalized to the β -Actin and band intensity was measured as follows: [Reference sample β -Actin/ Treated sample β -Actin] $\times$ Band quantity (<https://www.bio-rad.com/it-it/applications-technologies/image-analysis-quantitation-for-western-blotting?ID=PQEERM9V5F6X> accessed on 6 February 2023)).

2.7. β -Galactosidase Activity

The β -galactosidase activity was evaluated as previously described [31]. Briefly, growing cells of strain ED5 (comQ::kan phrC::cat srlA::lacZ) were supplemented with 50% (v/v) of the CM of each intestinal isolated *Bacillus* strain (MV4, MV11, MV24, MV30). The growth was followed at 37 °C under aerobic conditions, until the stationary phase of growth. Then, 1 mL of these cultures were harvested and centrifuged at 4000 rpm for 5 min. The pellet was resuspended in 1 mL of Z-buffer (0.06 M Na₂HPO₄, 0.04 M NaH₂PO₄, 0.01 M KCl, 0.001 M MgSO₄, and 0.05 M β -mercaptoethanol) and 10 μ L of toluene. Tubes were incubated at 30 °C for 20 min and vortexed every 5 min. The enzymatic reaction was started by adding 200 μ L of Ortho-nitrophenyl- β -galactoside (ONPG) solution (4 mg/mL ONPG in Z-Buffer without β -mercaptoethanol) and incubated at 30 °C. When the color was clearly yellow (A 420 nm ~0.3), the reaction was stopped by adding 500 μ L of 1 M Na₂CO₃, and the reaction time was recorded. Tubes were then centrifuged at 10,000 rpm for 5 min and the absorbance at 420 nm was recorded for each sample immediately. The β -galactosidase activity was expressed in Miller Units, as described in [45].

3. Results

3.1. Preliminary Characterization and Selection of Strains with Probiotic Potentials

Fecal samples of healthy children (Mat. Meth) were collected, heat-treated as previously reported [35], plated on Difco Sporulation (DS) medium, and incubated aerobically at 37 °C for 24–48 h. Putative siblings, colonies of apparently identical morphology originating from the same intestinal sample, were eliminated. All colonies that did not show a free spore or a forming spore still within a sporangium were also eliminated.

A total of thirty-two aerobic spore formers (MV2-MV33) were selected for further characterization. Cells of all thirty-two strains showed a rod-shaped morphology under the light microscope and were able to form phase-bright ellipsoidal endospores localized in central or sub-terminal positions. Only strain MV19 formed unusual spores that have been described elsewhere [41]. In addition to spores, strains MV19 [41], MV21, MV26, and MV30 formed multiple parasporal granules (possibly inclusion bodies) (Supplementary Materials Figure S1).

All thirty-two isolates were screened for hemolytic activity on blood agar plates. Isolates appearing greenish were considered able to oxidize the iron of the hemoglobin molecules within red blood cells and indicated as alpha-hemolytic; isolates forming a clear halo around their colonies were considered able to lyse red blood cells and indicated as beta-hemolytic; isolates that did not appear greenish and did not form any halo were considered not hemolytic and indicated as gamma-hemolytic. As shown in Supplementary Materials Table S1, ten out of thirty-two isolates were not hemolytic.

The ten not hemolytic strains were analyzed for their resistance to the panel of eight antibiotics indicated as relevant for members of the *Bacillus* genus by the European Food Safety Authority, (EFSA, 2012). As shown in Table 1, four of the ten considered strains (MV4, MV11, MV24, and MV30) were sensitive to all tested antibiotics, four (MV6, MV14, MV20, and MV26) were resistant to a single antibiotic and the remaining two strains were resistant to three (MV15) and four (MV17) antibiotics over the EFSA breakpoint values, respectively. The four strains sensitive to all antibiotics (MV4, MV11, MV24, and MV30) were considered as potential probiotics and used for further characterization.

Table 1. Minimum inhibitory concentration (MIC) values in mg/L.

Strain	Van	Gen	Kan	Strep	Ery	Clin	Tet	Chl
MV4	1.500	0.500	0.750	8.000	0.094	0.750	8.000	1.000
MV6	0.500	0.125	2.000	R ¹	0.125	0.190	1.000	0.250
MV11	0.190	0.750	0.500	1.000	0.047	0.125	2.000	2.000
MV14	3.000	0.125	0.750	8.000	0.250	4.000	R ¹	5.000
MV15	0.380	0.250	R ¹	2.000	0.380	R ¹	0.250	R ¹
MV17	0.047	0.500	R ¹	R ¹	R ¹	0.750	0.750	R ¹
MV20	0.190	0.250	0.500	1.000	0.125	R ¹	1.000	0.750
MV24	1.500	0.500	1.500	3.000	0.380	0.380	0.094	4.000
MV26	0.125	0.047	0.125	1.000	0.064	R ¹	0.250	2.000
MV30	0.125	0.047	0.190	8.000	0.064	4.000	0.500	1.500
EFSA								
Breakpoints	4.000	4.000	8.000	8.000	4.000	4.000	8.000	8.000

¹ R = resistant (over the EFSA breakpoint value).

3.2. Species Assignment and Phylogenetic Analysis

The four isolates were used to extract total DNA and the genomic sequences were obtained with a coverage of about 30×. The genomes were approximately 4.0 Mbp long for MV4, MV11, and MV24, and about 5.8 Mbp for MV30 (Table 2). The total number of ORFs was 3860, 3956, and 4380 for MV4, MV11, and MV24, respectively, and higher for MV30 (6003) (Table 2). No plasmid sequences were detected in any of the strains (not shown). All genome sequences were deposited in the GenBank database (Methods).

Based on the analysis of the sequence of the 16S RNA genes, MV4 and MV11 were tentatively considered as belonging to the *B. velezensis* species while MV24 and MV30 were to the *B. subtilis* and *Priestia megaterium* (formerly *Bacillus megaterium*) species, respectively. The Average Nucleotide Identity (ANI) of the four genomes was determined against the genome of a type strain of each of the putative species and confirmed the species assignment suggested by the 16S RNA gene analysis (Table 3).

Table 2. General features of the MV4, MV11, MV24, and MV30 genomes.

	MV4	MV11	MV24	MV30
Size (bp)	3,987,695	4,035,847	4,116,400	5,853,282
Number of contigs	36	58	112	35
Average Coverage	58	23	41	133
Number of predicted ORFs	3860	3956	4380	6003
Average GC (percentage)	46.35	46.2	43.26	37.46

Table 3. Average Nucleotide Identity (ANI) (%) based on whole genome alignments.

	<i>B. velezensis</i> FZB42	<i>B. subtilis</i> 168	<i>P. megaterium</i> QM B1551
MV4	98.36	77.3	68.77
MV11	98.36	77.04	68.44
MV24	77.3	98.54	68.97
MV30	68.31	68.58	97.42

The phylogeny of the four strains was further characterized by comparing forty-nine universally conserved genes (Mat.&Meth) with those of several other strains of related species. A phylogenetic tree was generated by Insert Genome into Species Tree app in KBase (Figure 1).

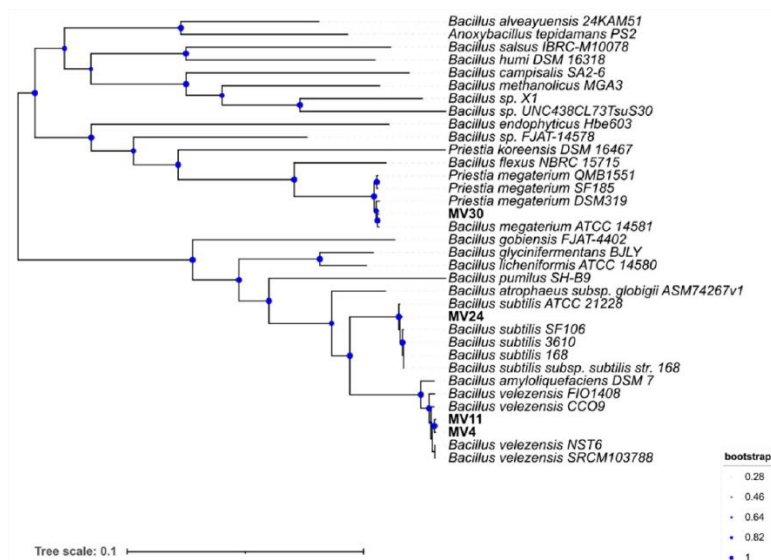


Figure 1. Phylogenetic tree. The genomes were imported into the Kbase system and the phylogenetic tree was constructed using Insert Genome Into SpeciesTree-v2.2.0. Bootstrap confidence values were generated using 1000 permutations.

3.3. Genome Analyses

MV4, MV11, MV24, and MV30 genomes were screened for the presence of toxin genes previously found in various other *Bacillus* strains [31,41]. As reported in Supplementary Materials Table S2, homologs of most of the genes coding for virulence factors were not found in MV4, MV11, MV24, or MV30. Homologs of the *hlyIII* gene, coding for the channel-forming protein Hemolysin III, were found in all four genomes as well as in the non-pathogenic strains *B. clausii* KSM-K16 and *B. subtilis* 168 (Supplementary Materials Table S2). The two *B. velezensis* strains and MV30 also contained homologs of the *cysC* gene coding for an adenylyl-sulfate-kinase similar to the *Pseudomonas* adenylyl-sulfate-kinase phytotoxin (Supplementary Materials Table S2). Only the genome of MV30 contains two homologs of the *cylR2* gene, coding for a DNA binding protein of *Enterococcus* acting as a repressor of the cytotoxin operon (Supplementary Materials Table S2).

The functional annotation of the four genomes was performed by using the EggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups) database, a public resource classification database able to provide Cluster Orthologous Groups (COGs) of proteins with functional annotations (Supplementary Materials Figure S2). For all four strains, the majority of the annotated genes were classified into the “Unknown Category” (S category in Supplementary Materials Figure S2). According to this annotation system, the next most represented classes for all four strains were “amino acid transport and metabolism”, “transcription”, and “carbohydrate transport and metabolism” (respectively, E, K, and G categories in Supplementary Materials Figure S2).

The genomes of the four isolates were also annotated using the Carbohydrate-Active enZymes (CAZymes) database in comparison with the genome of a reference strain of the respective species. MV4 and MV11 showed a number of genes putatively coding for glycosyl-hydrolase (GH) and a total number of CAZymes bigger than a reference strain of *B. velezensis* (Table 4). MV24 and MV30 showed, respectively, a slightly bigger and smaller number of CAZymes than their reference strains (Table 4). The GH category contains various hydrolases that act on the glycosidic bond and is divided into families. Some families such as GH1, GH4, GH5, GH16, and GH32 contain enzymes involved in the degradation of cellulose, starch, and sucrose while the GH26, GH30, GH43, GH51, and GH53 contain enzymes specialized in the degradation of hemicellulose, xylan, and cellobiose [46]. Figure 2 reports the abundance of the GH families mainly involved in the degradation of plant polysaccharides found in the four MV genomes. With respect to a reference strain of the same species, MV4, MV11, and MV30 contain more genes for the GH13 family and fewer genes for the GH1 family, respectively, containing enzymes specialized in the degradation of starch or monosaccharides (Figure 2).

Table 4. Number of genes putatively coding for the six CAZyme categories.

Species and Strain	GH	GT	PL	CE	CBM	AA	Total
<i>B. velezensis</i> MV4	47	37	3	11	16	3	117
<i>B. velezensis</i> MV11	48	37	3	11	16	3	118
<i>B. velezensis</i> FZB42	40	35	3	13	14	5	110
<i>B. subtilis</i> MV24	56	39	7	14	22	3	141
<i>B. subtilis</i> 168	56	34	7	14	23	4	138
<i>P. megaterium</i> MV30	49	42	1	20	23	5	140
<i>P. megaterium</i> QM B1551	53	42	1	24	21	5	146

MV30 also does not contain GH26 and GH43, present in the *P. megaterium* reference strain and coding for enzymes involved in the degradation of mannans or arabinose/ xylose, respectively. The GH profile of the *B. subtilis* strain MV24 was very similar to that of the reference strain with the only difference in the number of GH26 (degradation of mannans) higher in MV24 than in *B. subtilis* 168 (Figure 2).

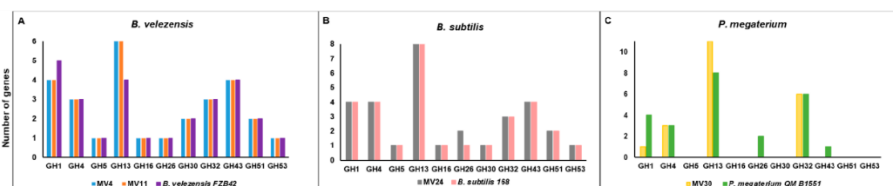


Figure 2. Number of predicted genes encoding cellulose/hemicellulose degradation enzymes found in the genome of MV4, MV11 (A), MV24 (B), and MV30 (C) compared to the indicated reference strains.

The genome sequences of the four strains were also analyzed to search for homologs of NK (EC 3.4.21.62), using as a query the protein sequence found in *Bacillus subtilis* subsp. natto BEST195 (accession No. BAI84580.1). Homologs were present in MV4 (85.86% identity, MV4_3335), MV11 (86.13%, Contig_6_158), and MV24 (99.21%, MV24_0328), while no homologs of the NK coding gene were present in the MV30.

The presence of homologs of the GAD, an enzyme that converts glutamate to GABA, was also investigated and a homolog with an identity of 77.9% with the GAD of other *Bacillus* species (WP_049107856.1) was found only in the MV30 genome (MV30_2943).

3.4. Antimicrobial and Antioxidant Activities

The four genomes were analyzed to identify genes coding for antimicrobials and antioxidants. The antiSMASH (Antibiotics and Secondary Metabolite Analysis Shell) database was used to assess the presence of clusters of genes associated with the synthesis of antimicrobials and secondary metabolites. As reported in Supplementary Materials Table S3 and schematically summarized in Figure 3, various gene clusters were identified in all four strains. In particular, seven, five, and four clusters with at least 80% similarity with known genes were found, respectively, in MV4, MV11, and MV24 while no homologous clusters were found in MV30 (Supplementary Materials Table S3). Based on this, both *B. velezensis* strains potentially produce macrolactin H, bacillaene, fengycin, bacillibactin, and bacilysin while only MV4 also codes for diffucidin and surfactin while the genome of the *B. subtilis* strain MV24 codes for bacillibactin, bacilysin, subtilosin A, and fengycin (Supplementary Materials Table S3). In addition, several gene clusters of all four strains showed low or no similarity with previously identified genes, suggesting that they may code for variants of known molecules or totally new antimicrobials (Supplementary Materials Table S3). As an example, clusters coding for surfactin homologs of MV4 and MV11 were compared with the well-characterized surfactin locus of *B. velezensis* FZB42 [47]. The surfactin cluster of MV4 (82% similarity with that of FZB42) and the two surfactin clusters of MV11 (47 and 39% of similarity with that of FZB42) were predicted to produce very different molecules (Figure 4), confirming that the exploitation of these genomes may provide useful information for the identification of novel antimicrobials.

To validate the genomic predictions on the potential production of antimicrobials, the MV strains were tested against a panel of target microorganisms by agar diffusion assay with cell-free supernatants. MV30 did not show any antimicrobial activity against the target bacteria while the other three strains were active against *L. monocytogenes* and *B. cereus*. Only strains of the *B. velezensis* species (MV4 and MV11) were weakly active against some other bacteria and a strain of *Candida albicans* (Table 5).

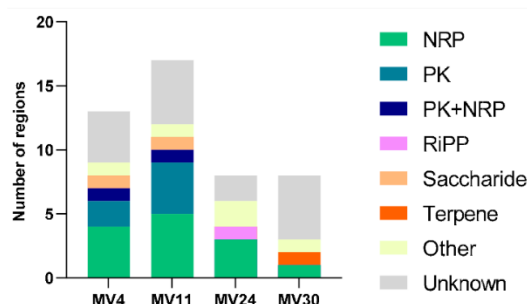


Figure 3. Abundance of clusters of genes coding for antimicrobials and other secondary metabolites in MV4, MV11, MV24, and MV30. The identified clusters were divided into functional classes. NRPS = Non-ribosomal peptide synthetase; PK = polyketide synthase; RiPP = Ribosomally synthesised and post-translationally modified peptide product; Saccharide = possible saccharide; Terpene = possible terpene; Other = Cluster containing a secondary metabolite-related protein that does not fit into any other category; Unknown = unknown BCG clusters.

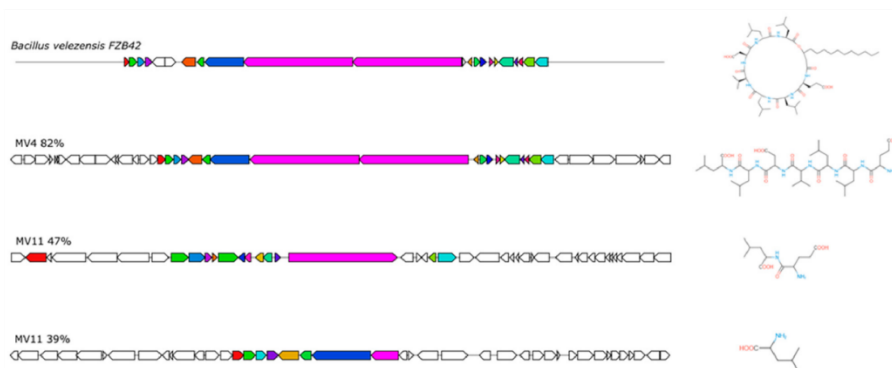


Figure 4. Surfactin loci of MV4, MV11, and the reference strain FZB42. The same colors indicate genes coding for products with similar functions. On the right is reported the predicted structure of each gene cluster.

All four genomes were screened for the presence of genes coding for antioxidants. Homologs of the enzymes catalase (CAT; EC:1.11.1.6) and superoxide dismutase (SOD; EC:1.15.1.1) were present in all four genomes. In particular, MV4, MV11, and MV24 contain three CAT (MV4_0856, MV4_0864, MV4_1319 in MV4; Contig_4_193; Contig_4_217 Contig_6_12 in MV11; MV24_3061, MV24_0468, MV24_1926 in MV24) and three SOD (MV4_0856, MV4_1319, MV4_0864 in MV4; 1_870, 2_130, 1_878 in MV11; MV24_2302, MV24_1406, MV24_2295) homologs. MV30 genome only contains one homolog of each enzyme (CAT: MV30_1299 and SOD: MV30_1877).

Table 5. Antimicrobial activity of MV4, MV11, MV24, and MV30 against a panel of target strains.

	MV4	MV11	MV24	MV30
<i>Listeria monocytogenes</i> ATCC 7644	+	+	+	-
<i>Bacillus cereus</i> ATCC 10987	++	++	+	-
<i>Enterococcus faecalis</i> ATCC 29212	-	-	-	-
<i>Streptococcus faecalis</i> ATCC 33186	+/-	+/-	-	-
<i>Escherichia coli</i> ATCC 25922	+/-	+/-	-	-
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhimurium ATCC 14028	+/-	+/-	-	-
<i>Citrobacter rodentis</i> ATCC 14580	+/-	+/-	-	-
<i>Shigella sonnei</i> ATCC 25931	-	-	-	-
<i>Candida albicans</i> ATCC 14028	+/-	+/-	-	-
<i>Lactocaseibacillus rhamnosus</i> GG ¹	-	-	-	-

+/- inhibition halo < 1 cm; + inhibition halo > 1 cm e < 2 cm; ++ inhibition halo > 2 cm. ¹ This representative probiotic strain was used to validate the selectivity of the antimicrobial properties of the isolated bacteria.

The four strains were then tested for their antioxidant activity. Vegetative cells (collected after 24 h of aerobic growth at 37 °C) (Methods) were tested for the scavenging activity against hydrogen peroxide (H₂O₂) or free radicals (by α,α -diphenyl- β -picrylhydrazyl (DPPH) method), as previously reported [20]. As shown in Figure 5, the H₂O₂-scavenging activity was stronger than the anti-free radicals activity with between 80 and 90% of H₂O₂ elimination with 1×10^8 cells and a total H₂O₂ elimination with 1×10^9 cells of all four strains. Over 60% of free radical elimination was observed with 1×10^9 cells of MV4, MV11, and MV24 (Figure 5A–C), while about 40% of free radicals reduction was observed with the same number of MV30 cells (Figure 5D).

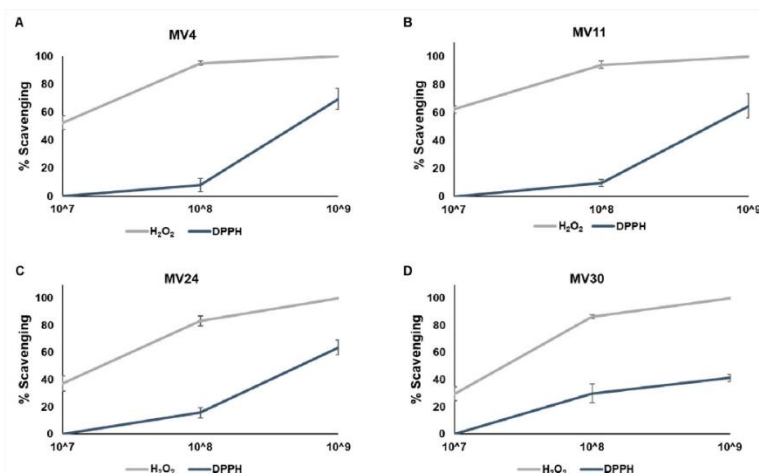


Figure 5. Evaluation of the ability of MV4 (A), MV11 (B), MV24 (C), and MV30 (D) cells to reduce hydrogen peroxide (H₂O₂, grey line) and the radical 2,2-diphenyl-1-picrylhydrazyl (DPPH, blue line). The assay was performed using suspensions of 10⁷, 10⁸, and 10⁹ cells and was repeated in triplicate.

3.5. Matrix Formation

In *B. subtilis*, the model system for spore formers, the biofilm matrix components include secreted proteins, extracellular DNA (eDNA), and an exopolysaccharide (EPS) [48].

The genes coding for the secreted proteins and the EPS are clustered in the *epsA-O* and *tapA-sipW-tasA* operons that are controlled by the master regulators *SinI-SinR* and *Spo0A* [48]. Also, in *B. cereus* products of the *pur* operon, coding for enzymes needed for purine biosynthesis is required for biofilm formation [49]. The genomes of MV4, MV11, MV24, and MV30 were analyzed for the presence of all these genes involved in matrix formation. As reported in Supplementary Materials Table S4, all four genomes contain homologs of the *epsA-O* and *tapA-sipW-tasA* operons, of their regulators, and of the *pur* operon.

All four strains were analyzed for their ability to produce biofilm in different media in comparison with a *B. subtilis* laboratory collection strain producing minimal amounts of biofilm (PY79) and a natural strain of the same species known to produce large amounts of biofilm (NCIB3610) [50]. In our experimental conditions (aerobic growth at 37 °C) all four strains produced different amounts of matrix in different media (Figure 6). The two *B. velezensis* strains produced a low amount of biofilm in rich (LB) medium and higher amounts in sporulation-inducing (DSM) or minimal (LB) media (Figure 6). Similarly, to the reference strain of *B. subtilis* NCIB3610, *B. subtilis* MV24 produced the highest amount of matrix in a minimal (S7) medium. However, while the amount of biofilm produced by the two strains was almost identical in LB and DSM media, in minimal medium MV24 produced significantly more biofilm than NCIB3610 (Figure 6). MV30 showed a different pattern of biofilm formation with the highest amount produced in the LB medium and the lowest in the sporulation-inducing medium (Figure 6).

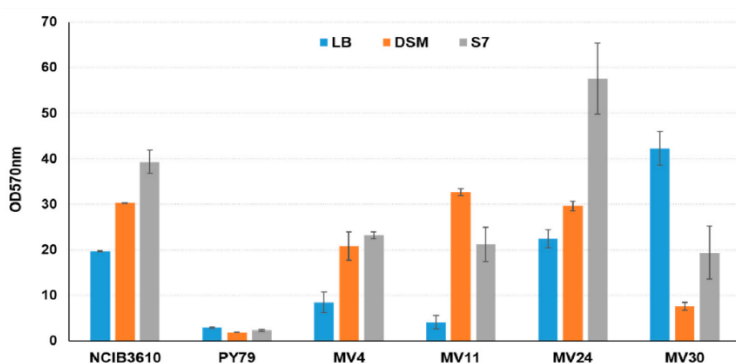


Figure 6. Biofilm production assay performed on the four bacterial strains in three different media: LB (blue bars), DSM (orange bars), and S7 (grey bars). The assay was performed in triplicate. *B. subtilis* strains NCIB3610 and PY79 were used as positive and negative controls, respectively.

3.6. Production of CSF or CSF-like Peptides

Several *Bacillus* strains produce and secrete peptides sensed by human epithelial cells, such as the CSF pentapeptide of *B. subtilis* which is able to induce a cytoprotective heat-shock response [28,30]. The four MV strains were evaluated for their ability to induce a heat-shock response in human epithelial cells. HT-29 colon carcinoma cells were either heat-shocked (30 min at 45 °C) or treated with 20% v/v of cell-free conditioned medium (CM) from the four MV strains and Hsp27 induction evaluated by Western blotting with anti-Hsp27 antibody (Figure 7).

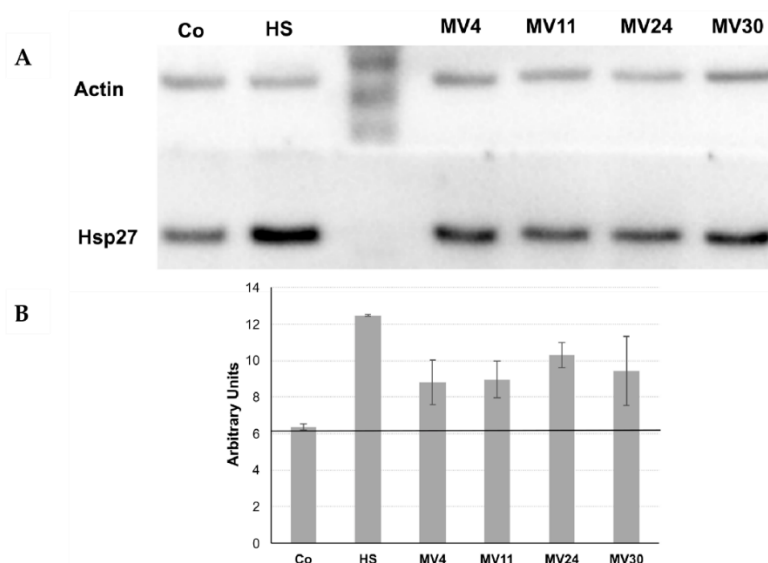


Figure 7. Effect of the conditioned medium (CM) of the four strains on Hsp27 production in HT-29 intestinal cells. Representative Western blot was performed with anti-Hsp27 antibodies (A) and a densitometric analysis was obtained by the average of three different experiments (B). Untreated (Co) and heat-shocked (HS) cells were used as a negative and positive control, respectively. The band intensity of each sample was evaluated by Image Quant analysis and normalized for the β -Actin intensity of the same sample. The black continuous line indicates the uninduced level of Hsp27 in HT-29 cells.

After normalization of the intensity of the obtained signals with those of the β -actin extracted from the same cells, the levels of Hsp27 after the treatment with the CM of each of the four strains appeared higher than the threshold level obtained with uninduced HT-29 cells (Figure 7). Although the experiment was not quantitative, it suggested that the CM of all four MV strains was able to induce the expression of the heat-shock protein Hsp27 in the HT-29 cells and, therefore, that all four strains secreted CSF or CSF-like peptides.

A quantitative evaluation of the amount of secreted CSF was obtained by using a recombinant strain of *B. subtilis* carrying a null mutation in the gene coding for CSF (phrC) and a gene fusion between the promoter of the CSF-controlled gene *rapA* and the coding part of the reporter gene *lacZ* of *Escherichia coli* [30]. The recombinant strain ED5, not producing CSF, does not express the gene fusion but responds to exogenous CSF producing β -galactosidase (Figure 8A) [30]. Cells of ED5 growing in LB medium were supplemented (20% v/v) with the CM of the four MV and control strains, and tested for β -galactosidase production, as previously reported [30]. The *B. subtilis* isolate MV24 induced β -galactosidase at levels about two-fold higher than the laboratory collection strain of the same species (PY79) (Figure 8B). As expected, the three strains not belonging to the *B. subtilis* species did not induce β -galactosidase, suggesting that they produce CSF-like peptides able to induce a heat-shock response in epithelial cells [30] (Figure 5) but are unable to activate *rapA* transcription in *B. subtilis* (Figure 8B).

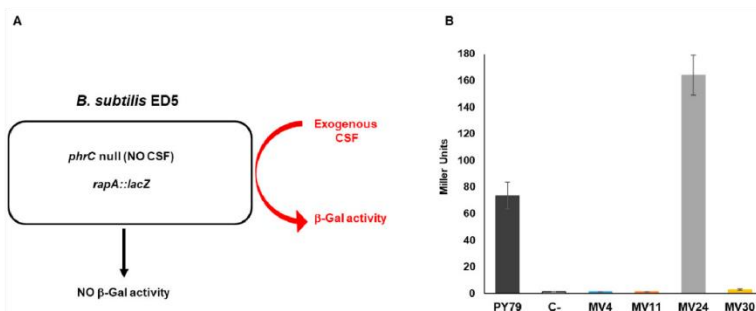


Figure 8. Production of CSF pentapeptide. (A) Schematic representation of the mutant strain of *B. subtilis* ED5 unable to produce CSF (*phrC* null) carrying the *rapA::lacZ* gene fusion. The mutant alone does not produce endogenous CSF but responds to exogenous CSF producing β -galactosidase. (B) Beta-galactosidase activity was measured with ED5 cells treated with the supernatants of MV4, MV11, MV24, and MV30. Supernatants of PY79 (*B. subtilis* wild-type) and ED5 (isogenic *phrC* deletion mutant) were used as a positive and negative control, respectively. The β -galactosidase is expressed in Miller Units and data are the average of three independent experiments.

4. Discussion

Four aerobic spore former strains isolated from the intestinal samples of healthy children were selected as potential probiotics for not being hemolytic and sensitive to all antibiotics indicated as relevant for spore formers by the EFSA. The four strains were unambiguously assigned to species that are in the Qualified Presumption of Safety (QPS) list of the EFSA (<https://www.efsa.europa.eu/en/topics/topic/qualif-ed-presumption-safety-qps> (accessed on 5 September 2022)) and are marketed as probiotics for human or animal use [9,12], therefore indicating that they could be considered as safe for human use. This assumption is supported by the in-silico observation that the four MV genomes do not carry putative genes coding for known *Bacillus* toxins. Indeed, the only gene present in all four genomes coding for a putative toxin is the *hlyIII* gene, common to pathogenic and non-pathogenic bacteria. The product of *hlyIII* was, however, not sufficient to cause an α - or β -hemolytic phenotype in the four MV strains. A gene coding for a putative phytotoxin, *cysC*, is carried by MV4, MV11, and MV30 while only the latter strain contains the *cylR2* gene, a homolog of a transcriptional repressor of a cytotoxin operon of *Enterococcus*.

All four strains formed biofilm and showed antioxidant activity in vitro. Both these properties are relevant for a probiotic strain, favoring the colonization of the intestinal environment and protecting the intestinal cells [20]. In addition, MV4, MV11, and MV30 have a gene coding for an NK homolog while MV30 has a gene coding for the enzyme GAD, able to convert glutamate into GABA. NK is a serine protease of the subtilisin family that when orally ingested exhibits fibrinolytic, antithrombotic, and antihypertensive effects [50]. GABA is an inhibitory neurotransmitter that improves various physiological illnesses, including diabetes, hypertension, and depression in humans [51]. Therefore, the potential to produce NK and/or GABA is considered a relevant property for a potential probiotic strain.

The four MV strains considered in this study also produced and secreted CSF (MV24) or CSF-like (MV4, MV11, and MV30) peptides. In addition to inducing anti-inflammatory and cytoprotective responses both in vitro and in vivo [28,29], these molecules have a role in the anti-neurodegenerative activity of *B. subtilis* in the model organism *C. elegans* [31–33]. Ingested spores of strains producing such molecules could have beneficial effects for the treatment of inflammatory and age-related neurodegenerative diseases.

Although the production of antimicrobials is not the main mechanism of action of probiotics against infectious diseases, the observed production of an array of secondary metabolites with potential antimicrobial activity by the four strains is certainly an additional relevant point for a probiotic to be used as an alternative to antibiotics. In this context, the identification of genes coding for products with low or no similarity with known antimicrobials is particularly promising. Such genes may encode either variants of previously identified molecules with different targets and mechanisms of action or totally new antimicrobials. The detailed characterization of these genes, which will be a future research challenge, is expected to provide useful information for the identification of novel antimicrobials.

MV4, MV11, MV24, and MV30 strains do not pose safety concerns and have beneficial properties, therefore they can be considered for in vivo experiments aimed at testing their role as an alternative to antibiotics for humans and animals.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/microorganisms11081978/s1>.

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CHAPTER 2

***Bacillus subtilis* SF106 and *Bacillus clausii* SF174 spores reduce the inflammation and modulate the gut microbiota in a colitis model**

Maria Vittoria¹, Edward Horwell², Deborah Bastoni¹, Anella Saggese¹,
Loredana Baccigalupi³, Simon M. Cutting² and Ezio Ricca^{1*}

1 Department of Biology, Federico II University, Naples Italy;

2 Department of Biological Sciences, Royal Holloway University of
London, Egham, Surrey, UK;

3 Department of Molecular Medicine and Medical Biotechnology,
Federico II University, Naples Italy

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RESEARCH ARTICLE

Bacillus subtilis SF106 and *Bacillus clausii* SF174 spores reduce the inflammation and modulate the gut microbiota in a colitis model

M. Vittoria¹ , E. Horwell² , D. Bastoni¹ , A. Saggese¹ , L. Baccigalupi³ , S.M. Cutting² and E. Ricca^{1*}

¹Department of Biology, Federico II University, via Cinthia 21, 80126 Naples, Italy; ²Department of Biological Sciences, Royal Holloway University of London, Egham, Surrey, United Kingdom; ³Department of Molecular Medicine and Medical Biotechnology, Federico II University, Naples, Italy; *ericca@unina.it

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Abstract

Chronic intestinal inflammation is associated with strong alterations of the microbial composition of the gut. Probiotic treatments and microbiota-targeting approaches have been considered to reduce the inflammation, improve both gut barrier function as well as overall gastrointestinal health. Here, a murine model of experimental colitis was used to assess the beneficial health effects of *Bacillus subtilis* SF106 and *Bacillus clausii* (recently renamed *Shoucheella clausii*) SF174, two spore-forming strains previously characterised *in vitro* as potential probiotics. Experimental colitis was induced in BALB/c mice by the oral administration of dextran sodium sulphate (DSS) and groups of animals treated with spores of either strain. Spores of both strains reduced the DSS-induced inflammation with spores of *B. clausii* SF174 more effective than *B. subtilis* SF106. Spores of both strains remodelled the mouse gut microbiota favouring the presence of beneficial microbes such as members of the *Bacteroidetes* and *Akkermansia* genera.

Keywords

probiotics – spores – intestinal inflammation – microbiota

1 Introduction

The intestinal microbiota and its host form a symbiotic relationship that strongly contributes to the host metabolic health (Fan *et al.*, 2021). Alterations of the gut microbial composition (dysbiosis) are associated in the pathogenesis of inflammatory bowel diseases (IBD), characterised by an abnormal production of inflammatory cytokines by host cells and disruption of the epithelial integrity, as observed in Ulcerative Colitis (UC) and Crohn's Disease (CD) (Fan *et al.*, 2021; Piazzesi *et al.*, 2022). As a consequence, a series of microbiota-directed

intervention strategies have been proposed to restore a healthy gut microbiota, including the oral administration of: (1) prebiotics, that once fermented by the intestinal microbiota, improve gut barrier functions; (2) probiotics, live bacteria that exert a beneficial health effect; (3) symbiotic, a combination of prebiotics and various probiotic strains; and (4) postbiotics, heat-inactivated probiotics or molecules secreted by probiotic strains (Ewaschuk *et al.*, 2008; Laval *et al.*, 2015; Sanders *et al.*, 2019; Yan *et al.*, 2007).

The oral use of probiotics is widely accepted and is based on the capacity of some bacteria to regu-



late cytokine expression, reduce inflammation and protect gut barrier integrity (Fan *et al.*, 2021; Laval *et al.*, 2015; Piazzesi *et al.*, 2022; Sanders *et al.*, 2019). Several strains of Lactobacilli, Bifidobacteria and Bacilli have long been used as commercial probiotics (Da Silva *et al.*, in press; Saggese *et al.*, 2021), while various members of the *Bacteroidetes* genus and the *Faecalibacterium prausnitzii* (Martin *et al.*, 2017) and *Akkermansia muciniphila* (Depommier *et al.*, 2019) species have recently been proposed as next-generation probiotics.

Of all bacteria commercially used as probiotics the case of members of the *Bacillus* genus is peculiar. These are spore-forming bacteria and all *Bacillus*-based commercial probiotic preparations contain spores, metabolically quiescent and extremely stable cells produced when the environmental conditions no longer allow cell growth (McKenney *et al.*, 2013; Saggese *et al.*, 2021). The quiescent spore responds to the presence of nutrients by germinating, thereby allowing metabolically active cells to grow and eventually to sporulate again (Christie *et al.*, 2020). Spore formers of the *Bacillus* genus are ubiquitous in nature and are also common inhabitant of the animal gut (Egan *et al.*, 2021; Fakhry *et al.*, 2008; Hong *et al.*, 2009). It has been shown that *Bacillus* spores can conduct their entire life cycle in the animal gut, entering as spores through the oral route, safely passing the gastric barrier (Spinosa *et al.*, 2000) and germinating in the intestine (Casula *et al.*, 2002). In the small intestine, the germination-derived cells proliferate and temporarily colonise the upper intestinal tract, then re-sporulate in the colon and exit the body as spores (Hoa *et al.*, 2001; Tam *et al.*, 2006).

Both spores and germination-derived cells of various *Bacillus* species interact with intestinal and immune cells through complex mechanisms poorly understood. *In vitro* studies have shown that *Bacillus subtilis* spores are taken up and rapidly eliminated by both murine (Duc *et al.*, 2004) or human (Ceragioli *et al.*, 2009) macrophages. A different *in vitro* study has shown that spores protect human epithelial cells from oxidative stress by inducing the nuclear translocation of the transcriptional factor Nrf-2, that activates stress-response genes (Petruk *et al.*, 2018). Also vegetative cells of various *Bacillus* species have been shown to interact with model intestinal cells. Examples include: (1) the contribution exerted by a combination of *B. subtilis* and *Bacteroides fragilis* cells to the maturation of the gut associated lymphoid tissue (GALT) in rabbits (Rhee *et al.*, 2004); (2) the induction of the synthesis of the heat-shock proteins (HSPs) in intestinal epithelial cells stimulated by the competence and sporulation factor (CSF),

a pentapeptide produced and secreted by *B. subtilis* cells (Fujita *et al.*, 2007). CSF-induced synthesis of HSPs contributes to the prevention of intestinal cell injury, loss of intestinal barrier function and it has been shown to have immunomodulatory and cytoprotective activities (Okamoto *et al.*, 2012). Although CSF is a *B. subtilis*-specific molecule, other *Bacillus* species, such as *Bacillus megaterium*, *Bacillus pumilus* and *Bacillus clausii*, produce and secrete peptides similarly able to induce HS proteins (CSF-like) (Di Luccia *et al.*, 2016; Vittoria *et al.*, 2023).

In addition, spores have been shown able to exert indirect beneficial effects by modulating the microbial composition of the gut and favouring the prevalence of potentially beneficial bacteria such as *Faecalibacterium prausnitzii* (Ji *et al.*, 2022), *Akkermansia muciniphila*, and *Bifidobacterium* spp. (Marzorati *et al.*, 2021).

A recent human trial has highlighted the safety, tolerance and positive health impact of the co-administration of *B. clausii*, *B. megaterium* and of a cocktail of probiotics containing *B. subtilis*, *B. megaterium*, *Bacillus coagulans* and *B. clausii* (Rea *et al.*, 2023). In addition, a double-blind, placebo-controlled trial indicated that a *B. subtilis*-based probiotic alleviates gastrointestinal symptoms (Garvey *et al.*, 2022).

Here, we used a dextran sodium sulphate (DSS)-induced colitis model (Chassaing *et al.*, 2014; Li *et al.*, 2019), to analyse the probiotic potential of spores from the strains SF106 and SF174, respectively, belonging to the *B. subtilis* and *B. clausii* species. The two strains have been previously characterised at the genomic and physiological level and shown to secrete vitamins, antimicrobial and antibiofilm molecules and to efficiently bind mucin and intestinal epithelial cells *in vitro*, without posing safety concerns (Saggese *et al.*, 2022).

2 Materials and methods

Spores production and purification

B. subtilis SF106 and *B. clausii* SF174 were isolated from ileal biopsies of healthy human volunteers and characterised in a previous work (Saggese *et al.*, 2022). For spores production, both strains were grown in Difco sporulation (DS) medium (for 1 l: 8 g Nutrient Broth, 1 g KCl, 1 mM MgSO₄, 1 mM Ca(NO₃)₂, 10 µM MnCl₂, 1 µM FeSO₄, Sigma-Aldrich, Taufkirchen, Germany) at 37 °C with vigorous shaking (150 rpm), for 30 h, to allow complete sporulation (Saggese *et al.*, 2016). Spores were then harvested (10,000×g for 10 min) and purified on a step gradient of 20 to 50% of Gastrografin (Bayer S.p.A,



Leverkusen, Germany), as previously reported (Maia *et al.*, 2020). Spore preparations were routinely checked for purity under the light microscope and by colony forming units (cfu) determination of heat-treated and untreated samples. Spores were considered pure when less than 1% of vegetative cells were present in the sample. For animal trial, the spore-pellet stock was washed three times with sterilised phosphate buffer solution (PBS, pH 7.4) and diluted prior mice gavage.

Animal trial and induction of acute colitis in mice

The animal trial was approved by the Animal Welfare Ethical Review Body (AWERB) of the Royal Holloway University of London, and project license (PB9FA6ABB) was granted by the Home Office (UK). Twenty 'pathogen-free' BALB/c mice (age 8-10 weeks) were purchased from Charles River, UK. Mice were housed in HEPA-filtered individually ventilated cages and all the animals were kept in the Experimental Animal Center of Royal Holloway University of London under standard conditions (constant temperature of 18 ± 2 °C, humidity of $50 \pm 5\%$, and 12-h light-dark cycle) and given free access to sterile standard mouse chow (5LF5, LabDiet) and water. Balb/c mice were randomly divided into four experimental groups ($n = 5$) and acclimatised to their social group and environment for one week before the study start. Two groups (106 and 174) received by oral gavage either 5×10^8 spores of SF106 or 5×10^8 spores of SF174 suspended in 200 μ l of PBS buffer while the other two groups (Control and DSS) received the same volume of PBS buffer. After 7 days in all groups, except for the Control group (naïve mice), colitis was induced by daily treatment for 7 days with 4% (w/v) of DSS, added to the drinking water. Fresh DSS was replaced every 2-3 days. On day 14 all the mice were euthanised, colorectum and anus were excised and colon tissues were isolated for further analysis.

Assessment of colitis severity and histological analysis

During the DSS challenge, body weight, stool consistency and rectal bleeding were monitored daily, starting one day before the addition of 4% DSS to the water (day 6). The presence of occult blood in the faeces was also analysed using a faecal occult blood test kit (Hema-Screen, Immunostics Inc, Eatontown, NJ, USA). The scores assigned according to symptom severity were used to obtain the disease activity index (DAI), calculated as previously reported (Hidalgo-Cantabrana *et al.*, 2016). On the day mice were euthanised, the distal colon was collected and fixed overnight at 4 °C in a 10% (v/v) formalin solution. The samples were pro-

cessed and stained with haematoxylin and eosin and for histological analysis, several slides were analysed under the microscope and scores were assigned according to the severity of the inflammatory state, as previously reported (Koelink *et al.*, 2018); two investigators evaluated each blinded sample separately and the mean of the two was used to obtain the mouse colitis histological index (MCHI) for each sample.

RNA extraction, retro-transcription and qPCR analysis

About 100 mg of rectosigmoid colon for each sample was placed in 1 ml of TRIzol™ reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) and homogenised for 30 s for RNA extraction. To avoid contamination of DSS in colonic tissue, which has been reported to inhibit the reverse transcription reaction and amplification of mRNA (Viennois *et al.*, 2013), the extracted total RNA was purified with lithium chloride using the RNA purification protocol described previously (Oldak *et al.*, 2018). Residual gDNA was removed and reverse transcription was performed using the QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany). The Real-Time was performed according to manufacturer's protocol using the QuantiNova RT-PCR kit (Qiagen) in a Corbett Rotor-Gene 6000 thermocycler. The primer sequences used in this study are listed in the Supplementary Table S1 and their amplification efficiency was validated before performing the qPCR analyses. The $2^{-\Delta\Delta C_t}$ method was used to determine the fold change, which was normalised to the β -actin expression levels of each gene. The relative quantification of each analysed gene expression was normalised to that of the naïve control mice (considered zero).

Bioinformatic analyses

Faeces was collected from each mouse on day 14. DNA was extracted using the Quick-DNA Fecal/Soil MicroPrep Kit (Zymo Research, Tustin, CA, USA), as per the manufacturer's guidelines. Blank extractions were systematically included to check for potential cross-contaminations. DNA quality and quantity were checked as described previously (Buglione *et al.*, 2022).

16S rDNA was amplified in the V3-V4 region, and sequenced with Illumina's TruSeq DNA library (Novogene Co., Ltd., Beijing, China) for bioinformatic analysis. All analyses were performed using the R software (R-Cran project, <http://cran.r-project.org/>) within a jupyter notebook instance (Loizides *et al.*, 2016). Sequences were analysed using the DADA2 package (Callahan *et al.*, 2016), after removing primers. All sequences with an average call quality for each base between 20 and 40

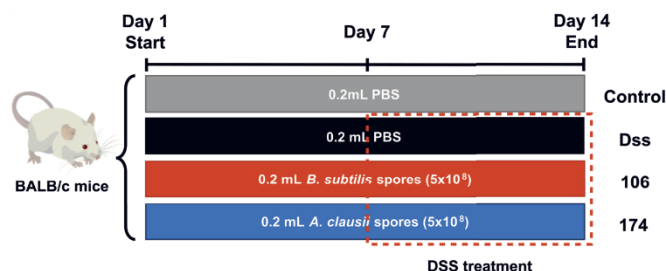


FIGURE 1 | Experimental design. Grey rectangle: control (naïve mice); black rectangle: mice treated with DSS only; red rectangle: mice treated with SFI06 spores; blue rectangle: mice treated with SFI74 spores; red dotted line: 4% DSS-treatment.

were retained for downstream processing. DADA2's end product was an amplicon sequence variant (ASV) table, and the Silva database release 138 was used as a comparison to assign the taxonomy to the sequence variants (<https://www.arb-silva.de/>). The end product of DADA2 was used to investigate the prokaryotic diversity using the phyloseq package (McMurdie *et al.*, 2013), as previously reported (Cordone *et al.*, 2023). The alpha diversity was calculated using the Shannon diversity index while the beta diversity was analysed using weighted Jaccard dissimilarity index, and related to different variables using non-metric multidimensional scaling (nMDS) with the vegan package (Oksaen *et al.*, 2009).

Statistical analysis

Statistical analyses were performed with GraphPad Prism (GraphPad Prism, La Jolla, CA, USA; version 9.1.0 for Windows). All graphs are presented as mean $\pm$ standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used to detect differences between the groups compared to the DSS group, followed by Dunnett's multiple comparison test. A two-way ANOVA was used to determine the effect of different treatment groups over time on weight change and DAI, followed by Tukey's multiple comparison test with individual variances computed for each comparison. A value of $P \leq 0.05$ was considered statistically significant.

3 Results

SFI06 and SFI74 spores protect mice from the DSS-induced inflammation

Balb/c mice were randomly subdivided into four experimental groups ($n = 5$) as schematically reported in Figure 1. Two groups, 106 and 174, received, respectively, 5×10^8 spores of strain SFI06 or SFI74 every day for fourteen days by oral gavage. In addition, from day 7 to day 14, 4%

DSS was added to their drinking water to induce symptoms of ulcerative colitis (UC). In parallel, two control groups received an oral gavage of PBS for fourteen days with the positive control having 4% DSS (via drinking water) from day 7 to day 14 (DSS) (Figure 1). All animals were monitored daily from the day before the beginning of the DSS treatment (day 6) to the end of the treatment (day 14) to assess the weight variations and the Disease Activity Index (DAI). As reported in Figure 2A, a weight gain was recorded in mice of the Control group (grey line, Figure 2A) while the treatment with DSS (black line, Figure 2A) caused a drastic weight loss. Mice of both spore-treated groups (106 in red and 174 in blue in Figure 2A) showed a slight initial weight loss (day 7) followed by a significant weight recovery from day 7 to day 10 and by a final weight reduction that was, however, significantly lower than that observed in animals of the DSS group ($P < 0.0001$) (Supplementary Table S2).

To assess disease progression typical symptoms of UC, including stool consistency and rectal bleeding (see Materials and methods) were monitored. Scores were assigned based on the severity of symptoms (see Materials and methods) and used to generate a DAI graph (Figure 2B). At day 11 mice of the DSS group (black line, in Figure 2B) showed severe symptoms ($DAI = 4.0 \pm 1.1$). In contrast, animals of both spore-treated groups (red and blue line, Figure 2B) did not show significant symptoms until day 13 of the treatment, indicating a delay in disease progression. On day 14, 106 mice reached a DAI score of 7.0 ± 1.0 , while 174 mice maintained a score of 5.0 ± 1.5 , significantly lower than the value recorded for mice of the DSS group ($P < 0.0001$) (Supplementary Table S2).

SFI06 and SFI74 spores protect the intestinal integrity

On day 14 all mice were euthanised and distal colon samples collected, fixed and stained with H&E for histological analysis (see Materials and methods). Histo-

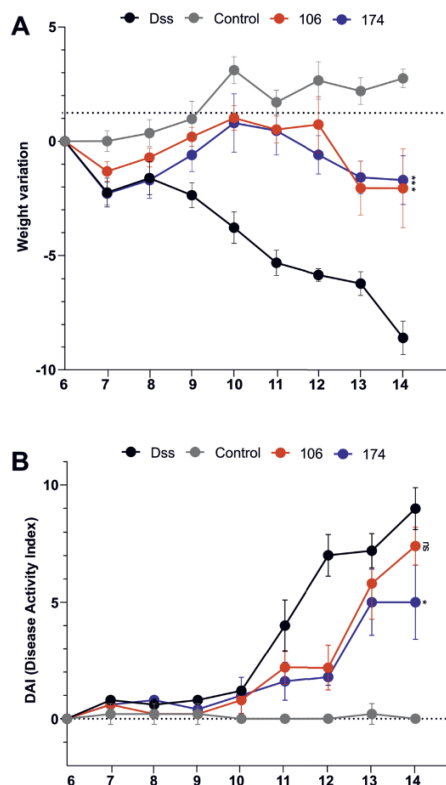


FIGURE 2 Effect of SF106 and SF174 spores on disease progression. Weight change (A) and disease activity index (B) were compared from the day before the DSS treatment (day 6) to day 14. Grey line: control (naïve mice); black line: mice treated with DSS only; red line: DSS-treated mice receiving SF106 spores; blue line: DSS-treated mice receiving SF174 spores. Data are reported as mean $\pm$ standard error of the mean ($n = 5$ per group). Two-way ANOVA was used to assess statistically significant differences between groups ($P < 0.0001$ for both analyses), followed by Dunnett's multiple comparison test. Only the significance of the SF106 and SF174 groups compared to the DSS group on the last day of treatment are reported. **** $P < 0.0001$; *** $P < 0.001$; ns = not significant.

logical samples of the Control group (top left panel of Figure 3A) showed an intact epithelial tissue, with intact crypts without inflammatory cell infiltration, numerous goblet cells (red arrows in Figure 3A) and a normal muscularis propria (black arrows in Figure 3A). In contrast, severe histological damages were observed in the colon of mice of the DSS group (lower left panel of Figure 3A), with erosion and destruction of the intestinal epithelium, ulceration of the mucosa (black star in Fig-

ure 3A), destruction of the intestinal crypts and villi, depletion of the goblet cells and abundant infiltration of inflammatory cells in the mucosa and submucosa (blue arrows in Figure 3A). In spore-treated mice (right panels in Figure 3A), there was minimal tissue damage, with visible crypts and goblet cells and reduced muscular hypertrophy (black arrows in Figure 3A). Furthermore, leucocyte inflammatory infiltrates (blue arrows in Figure 3A) were not observed in animals of the 174 group and were minimal in mice of the 106 group. To better evaluate the severity of the colitis, an objective histological scoring system was employed. The un-treated naïve mice showed no evidence of histological abnormality (not shown), whereas the histology from the DSS group resulted in a cumulative score of 13.0 ± 0.3 (Figure 3B). In mice treated with either spore type, a statistically significant reduction in the severity of histological inflammation was seen, with scores being of 11.6 ± 0.5 and 8.2 ± 0.6 for 106 and 174 groups, respectively. Although the protective effect was observed with spores of both strains, it was, however, stronger with SF174 than with SF106 spores (Figure 3B).

A qPCR approach was used to analyse the expression levels of the *muc-2* (a secretory mucin whose expression has been associated with several intestinal diseases (Liu *et al.*, 2020)) and *claud-1* (a tight junction protein altered in several IBDs (Pope *et al.*, 2014)) genes. Considering the level of expression in naïve mice as zero, the treatment with DSS drastically reduced the expression of the *muc-2* and *claud-1* genes (black boxes in Figure 3C,D), while the treatment with either spore type (red and blue boxes in Figure 3C,D) restored the expression of the two genes at levels similar to those observed in control animals. These results, consistent with histological analyses, indicate the beneficial effect of the treatment with either type of spores on maintaining and improving intestinal barrier function.

SF106 and SF174 spores reduce DSS-induced inflammation markers

To evaluate the effect of the spore treatment on the DSS-induced inflammatory response, the expression levels of genes coding for some of the major cytokines were analysed by qPCR. Normalising for the values of the Control group (equal to 0 in the graphs), clear variations were observed in animals of the DSS group with an increased expression of the pro-inflammatory cytokine tumour necrosis factor (TNF)- α , interleukin (IL)-6 and IL-1 β genes and a decrease of the anti-inflammatory cytokine IL-10 (Figure 4A-D). The TNF- α increase and IL-10 decrease were partially reverted by SF174 spores

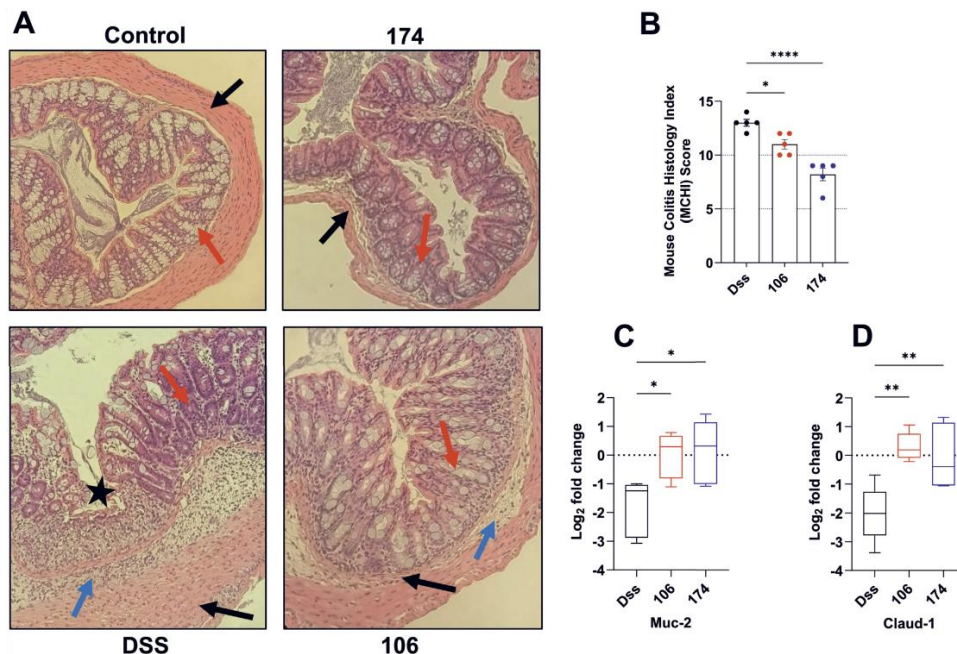


FIGURE 3 Effect of SF106 and SF174 spores on DSS-induced colonic injuries and gut integrity. (A) Microscopic pictures of proximal colon tissue stained with H&E. Red arrows: goblet cells; black arrows: muscularis propria; blue arrows: submucosal inflammatory infiltrate; black star: mucosa ulceration. (B) Mouse Colitis Histology Index scores assigned after microscopic analysis of colon sections (Methods). qPCR analysis of *muc-2* (C) and *claud-1* (D) genes. Data are shown as mean ± SEM (n = 5). One-way ANOVA was performed to determine the statistically significant differences between groups followed by Dunnett's multiple comparisons test against the DSS-only group. **** $P < 0.0001$; *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

(blue boxes in Figure 4A and 4D), while the effects of SF106 spores (red boxes in Figure 4A-D) were statistically significant only on the TNF- α levels (Figure 4A). The increased expression levels of the IL-6 (Figure 4B) or IL-1 β (Figure 4C) genes were not affected by either spore treatment.

Expression levels of the genes coding for the Toll-like receptor 2 and 4 (TLR-2 and TLR-4), recognising, respectively, Gram-positive or Gram-negative bacteria, were also analysed by qPCR. As shown in Figure 4E and 4F, the DSS treatment down-regulated TLR-2 and did not affect TLR-4. While the treatment with SF106 spores tended towards the value of naive animals that with SF174 spores completely subverted ($P < 0.01$) the DSS-induced down-regulation (red and blue boxes, respectively, Figure 4E). No statistically significant differences were observed for the expression levels of the TLR-4 gene (Figure 4F).

Gut microbial composition

The effects of SF106 and SF174 spores on the composition of the mice gut microbiota was analysed by 16S

rRNA gene sequencing of faecal samples collected on day 14. With the exception of the DSS group (n = 5), analyses for the other groups (Control, 106 and 174) were performed on four samples (n = 4) due to lack of material at the time of sampling. The alpha-diversity by the Shannon Index showed that the diversity was overall similar between the four experimental groups with a slightly higher and lower diversity for the 106 and Control group, respectively (Figure 5A). The DSS treatment strongly reduced the heterogeneity of the microbial community observed between the various animals of the group that was instead higher in the 106 group (Figure 5A). In the beta diversity analysis using non-metric multidimensional scaling (nMDS) based on weighted and unweighted Jaccard dissimilarity index, all groups formed clear clusters. The 106 and 174 clusters (respectively, red and blue symbols in Figure 5B) were well separated from Control and DSS clusters (Figure 5B). Consistently with the results of Figure 5A, all animals of the DSS group formed a small cluster close to that of the Control group (Figure 5B).

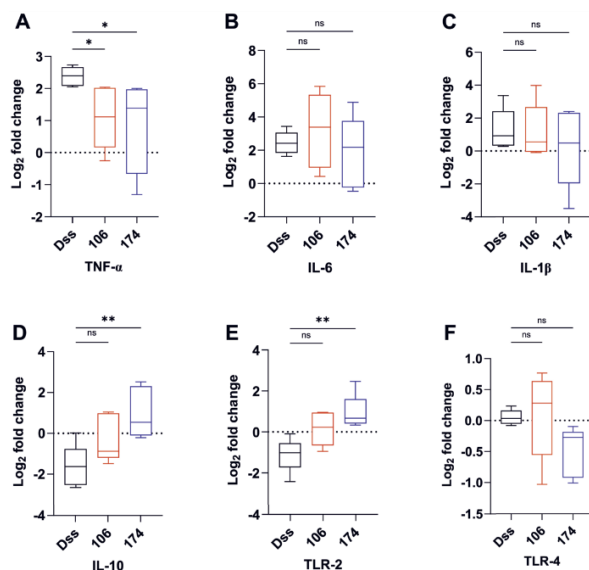


FIGURE 4 Effect of SF106 and SF174 spores on the expression of inflammatory and anti-inflammatory markers: (A) tumour necrosis factor (TNF)- α , (B) interleukin (IL)-6, (C) IL-1 β , (D) IL-10, (E) Toll-like receptor (TLR)-2 and (F) TLR-4. Data are shown as mean $\pm$ standard error of the mean ($n = 5$). Black boxes: mice treated with DSS only; red boxes: DSS-treated mice receiving SF106 spores; blue boxes: DSS-treated mice receiving SF174 spores. One-way ANOVA was performed to determine the statistically significant differences between groups followed by Dunnett's multiple comparisons test against the DSS-only group. ** $P < 0.01$; * $P < 0.05$; ns = not significant.

The taxonomy analysis of the ten most represented phyla and genera is reported in Figure 6A and Figure 6B, respectively. In all four groups *Firmicutes* and *Bacteroidota* were the most abundant phyla but while they represented over 90% of the total phyla in the control and DSS groups, in the spore-treated groups were less abundantly represented (64.3 and 81.5% of the total, in SF106 and SF174, respectively) and replaced by members of the *Verrucomicrobiota*, *Proteobacteria* and *Actinobacteria* phyla (Table 1).

At the genus level the abundance of several genera was significantly altered by the spore treatment. In comparison with the microbiota of the DSS animals, the abundance of thirteen genera was reduced by both SF106 and SF174 spores in a statistically significant way (Table 2). Of those thirteen genera only one, the *Lactobacillus* genus, was reduced by the DSS treatment with respect to the control group and further reduced by both spore treatments (Table 2). All other twelve genera were increased by the DSS treatment with respect to the control group and rescued by the SF106 or SF174 spore treatment at the level observed in the control group or even at lower levels (Table 2).

The abundance of some other genera was, instead, increased by the spore treatment. With respect to mice

of the DSS group, SF174 spores caused a statistically significant increase in the abundance of members of the *Bacteroides* and *Akkermansia* genera, while SF106 had a similar but not statistically significant effect (Figure 7). Only SF106 spores positively effected in a statistically significant way the abundance of the low represented genera *Hyphomicrobium*, *Devosia*, *Terrimonas* and *LWQ8* (uncultured family of *Saccharimonadales*) (Table 3).

4 Discussion

The main result of this study is that a preventive treatment with spores of *B. subtilis* SF106 or *B. clausii* SF174 has a beneficial health effect by reducing the DSS-induced inflammation in a murine model. Spores of the two strain behaved similarly but those of strain SF174 were more effective and SF174 pre-treated animals showed a lower disease activity and histology indices than those observed with mice pre-treated with SF106 spores. In addition, SF174 spores induced the expression of the anti-inflammatory cytokine IL-10 at levels statistically higher than those observed in the DSS-treated group while spores of the SF106 showed a slight, not statistically significant, increase of IL-10 expression.

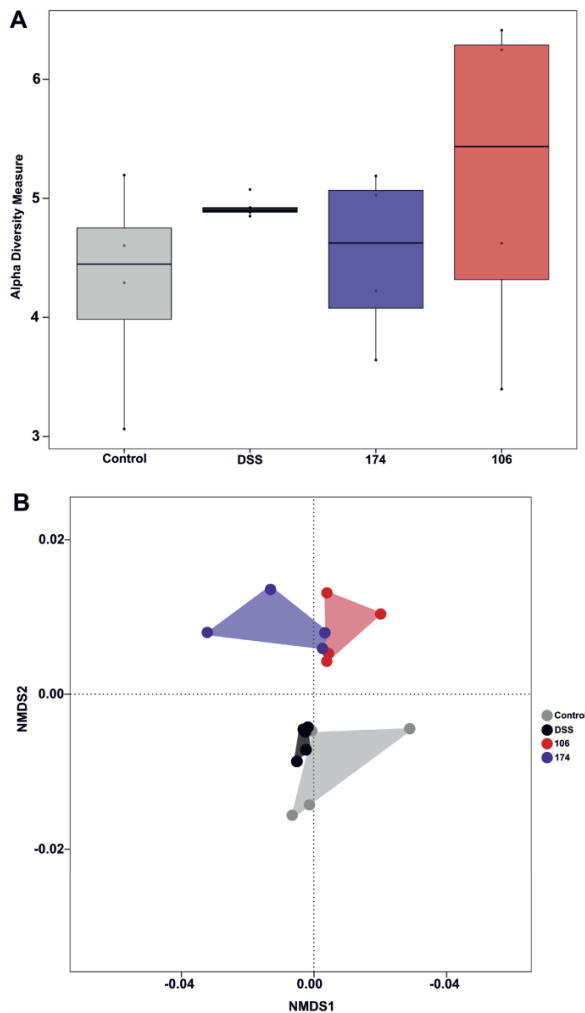


FIGURE 5 Gut microbial community diversity. Alpha diversity measure across the different groups by the Shannon index (A) and non-metric multidimensional scale (nMDS) plot based on weighted Jaccard dissimilarity index (B). Grey symbols: control (naïve mice); black symbols: mice treated with DSS only; red symbols: DSS-treated mice receiving SFI106 spores; blue symbols: DSS-treated mice receiving SFI174 spores.

The analysis of the expression of cytokines and Toll-like receptors 2 and 4 (TLR-2 and TLR-4) suggested that both spore-based treatments have an immunomodulatory trend in mice with DSS-induced colitis. Both spores reduced the expression of the pro-inflammatory cytokine $\text{TNF-}\alpha$ while only SFI174 spores induced a statistically significant increase of the expression of the anti-inflammatory cytokine IL-10. Such increase was at levels higher than those observed in the Control (naïve)

animals indicating a general anti-inflammatory activity independent of the DSS-induced inflammation.

TLR-2 and TLR-4 recognise commensal and pathogenic bacteria, with TLR-2 mainly active on Gram-positives and TLR-4 on Gram-negatives. Such recognition is essential for the release of pro- and anti-inflammatory cytokines and, therefore, for the maintenance of the intestinal homeostasis (Latorre *et al.*, 2018). The balance of TLR-2 and TLR-4 receptors can

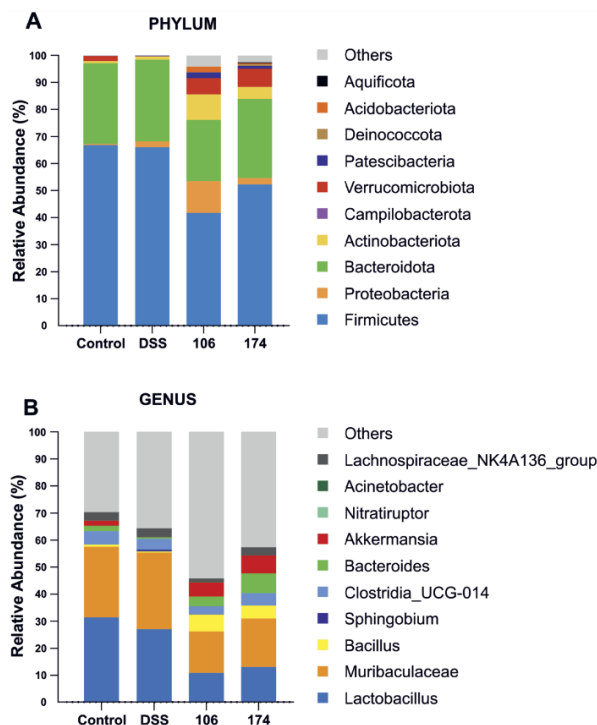


FIGURE 6 Taxonomic analysis. Bar plot of the relative abundance of the ten most abundant phyla (A) and genera (B) in the different groups.

TABLE 1 Relative abundance (%) of the ten most abundant Phyla in the four experimental groups

Phylum	Control	DSS	SF106	SF174
<i>Firmicutes</i>	66.79	66.03	41.71	52.24
<i>Bacteroidota</i>	29.84	30.22	22.59	29.26
<i>Verrucomicrobiota</i>	1.92	0.06	5.99	6.75
<i>Proteobacteria</i>	0.43	2.17	11.80	2.38
<i>Actinobacteriota</i>	0.82	1.17	9.43	4.40
<i>Patescibacteria</i>	0.08	0.29	2.13	1.09
<i>Campilobacterota</i>	0.00	0.00	0.03	0.01
<i>Deinococcota</i>	0.01	0.00	0.11	0.50
<i>Acidobacteriota</i>	0.00	0.00	2.01	0.51
<i>Aquificota</i>	0.00	0.00	0.03	0.21
Others	0.12	0.06	4.17	2.64

trigger intracellular signalling cascades and modulate the expression of cytokines (Jia *et al.*, 2020; Latorre *et al.*, 2018). In the present work the analysis of the expression of TLR-2 and TLR-4 indicated a down-regulation of TLR-2 and a slight up-regulation of TLR-4 in the DSS group, probably contributing to the increased production of

pro-inflammatory cytokines and to the decreased production of the anti-inflammatory cytokine IL-10 observed as consequences of the DSS treatment. Such effects were reverted in a statistically significant way (TLR-2) or close to statistically significance ($P = 0.08$) (TLR-4) by SF174 spores. SF106 spores induced a similar but weaker effect on TLR-2 expression while the effect on TLR-4 was unclear due to the high variability of the expression observed in the mice of the SF106 group. The different balance of expression of TLR-2 (up-regulated) and TLR-4 (down-regulated) caused by the spore pre-treatment is a likely cause of the observed IL-10 increase and indicates that the oral administration of SF174 spores promotes an anti-inflammatory response in mice.

The analysis of the microbial composition of the mice gut indicated that both spore treatments modified the gut microbiota. With respect to animals treated only with DSS, both spores reduced the abundance of the same thirteen genera. In twelve of those thirteen genera the relative abundance was increased by the DSS treatment with respect to naïve animals and

TABLE 2 Relative abundance (%) of genera negatively affected by SF106 or SF174 spores

Genus	Control	DSS	SF106	SF174
<i>Lactobacillus</i>	31.34 ± 7.25	26.95 ± 7.21	10.80 ± 4.2 ^b	13.00 ± 4.20 ^a
<i>Lachnospiraceae UCG-001 group</i>	0.40 ± 0.34	1.32 ± 0.57	0.19 ± 0.14 ^b	0.08 ± 0.09 ^b
<i>Enterorhabdus</i>	0.56 ± 0.44	1.04 ± 0.30	0.25 ± 0.17 ^b	0.17 ± 0.13 ^b
<i>Clostridioides</i>	0.08 ± 0.10	0.80 ± 0.45	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b
<i>Roseburia</i>	0.26 ± 0.25	0.71 ± 0.26	0.06 ± 0.05 ^c	0.01 ± 0.02 ^c
<i>Tyzzarella</i>	0.12 ± 0.04	0.37 ± 0.11	0.13 ± 0.04 ^c	0.12 ± 0.05 ^c
<i>Eubacterium xylanophilum group</i>	0.09 ± 0.10	0.35 ± 0.15	0.02 ± 0.02 ^b	0.00 ± 0.01 ^c
<i>Incertae sedis</i>	0.12 ± 0.03	0.25 ± 0.04	0.07 ± 0.01 ^d	0.07 ± 0.04 ^d
<i>Gemella</i>	0.12 ± 0.07	0.21 ± 0.05	0.09 ± 0.03 ^a	0.08 ± 0.04 ^a
<i>Eubacterium nodatum group</i>	0.13 ± 0.05	0.18 ± 0.04	0.05 ± 0.03 ^b	0.07 ± 0.04 ^b
<i>Intestinimonas</i>	0.07 ± 0.02	0.11 ± 0.03	0.05 ± 0.02 ^a	0.07 ± 0.03
<i>Chryseomicrobium</i>	0.02 ± 0.03	0.04 ± 0.00	0.00 ± 0.00 ^b	0.00 ± 0.00 ^b
<i>Eubacterium brachy group</i>	0.03 ± 0.02	0.03 ± 0.01	0.01 ± 0.01	0.00 ± 0.00 ^a

^a $P \leq 0.05$.

^b $P \leq 0.01$.

^c $P \leq 0.001$.

^d $P \leq 0.0001$ by a one-way ANOVA analysis performed comparing groups SF106 or SF174 vs DSS.

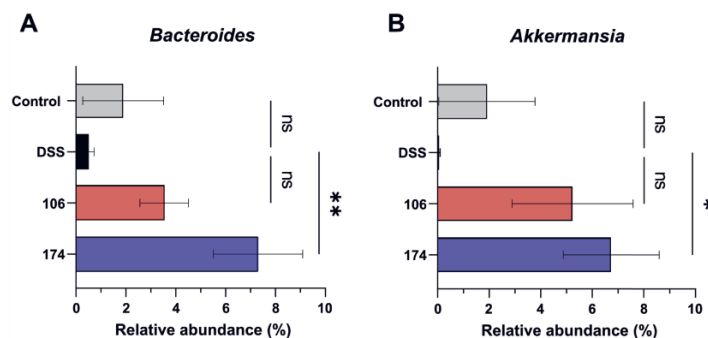


FIGURE 7 Effect of SF106 and SF174 spores on the abundance of *Bacteroides* and *Akkermansia* genera. Data are shown as mean ± standard error of the mean. One-way ANOVA was performed to determine the statistically significant differences between groups followed by Tukey's multiple comparison test. ** $P < 0.01$; * $P < 0.05$; ns = not significant.

rescued by both spore types. Only the abundance of members of the *Lactobacillus* genus was decreased by DSS and further decreased by both spore types. Both spore types had a positive effect on the abundance of members of the *Bacteroidetes* and *Akkermansia* genera, although the increase was statistically significant only with SF174. Similar effects have been previously reported with spores of other *Bacillus* strains able to increase the abundance of those bacteria *in vitro* (Ji et al., 2022) or *in vivo* (Marzorati et al., 2021). *Bacteroidetes* and *Akkermansia* are beneficial microbes, considered as 'next-generation probiotics' (Depommier et al., 2019). Indeed, *Akkermansia muciniphila* has been shown to increase colonic mucin production, providing a thicker

mucosal barrier to the underlying epithelial layer, perhaps explaining the upregulation of MUC-2 seen in this study and, in part, the protective effects of SF174.

Supplementary material

Supplementary material is available online at: <https://doi.org/10.6084/m9.figshare.25913347>

Table S1. Sequence list of primers used for qPCR reactions.

Table S2. Statistics weight and DAI.



TABLE 3 Relative abundance (%) of genera positively affected by SF106 spores

Genus	Control	DSS	SF106	P-value ¹
<i>Hyphomicrobium</i>	0.00 ± 0.00	0.00 ± 0.00	0.11 ± 0.07	0.049
<i>Devosia</i>	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.03	0.040
<i>Terrimonas</i>	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.00	0.002
LWQ8	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.01	0.038

¹ One-way ANOVA analysis performed comparing group 106 vs DSS.

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Authors' contribution

MV performed most of the experiments; EH contributed to the animal experiments; DB contributed to the bioinformatic analysis; AS, LB and SMC contributed to the analysis of the experimental results and to the text writing; ER supervised the work and wrote the manuscript.

Conflict of interest

E.R. acts as a consultant for Gruppo Savio that has the rights for the commercialization of the strains SF106 and SF174.

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CHAPTER 3

Sporulation efficiency and spore quality in a human intestinal isolate of *Bacillus cereus*

Maria Vittoria^{a,1}, Anella Saggese^{a,1}, Giovanni Di Gregorio Barletta^a,
Stefany Castaldi^a, Rachele Isticato^a, Loredana Baccigalupi^b and Ezio
Ricca^{a*}

a Department of Biology, Federico II University, Naples Italy;

b Department of Molecular Medicine and Medical Biotechnology,
Federico II University, Naples Italy

*Correspondence: ericca@unina.it;

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Maria Vittoria ^{a,1}, Anella Saggese ^{a,1}, Giovanni Di Gregorio Barletta ^a, Stefany Castaldi ^a,
Rachele Isticato ^a, Loredana Baccigalupi ^b, Ezio Ricca ^{a,*}

^a Department of Biology, Federico II University of Naples, Italy

^b Department of Molecular Medicine and Medical Biotechnology, Federico II University of Naples, Italy



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abstract

Bacteria classified as *Bacillus cereus sensu stricto* cause two different type of gastrointestinal diseases associated with food poisoning. Outbreaks of this opportunistic pathogen are generally due to the resistance of its spores to heat, pH and desiccation that makes hard their complete inactivation from food products. *B. cereus* is commonly isolated from a variety of environments, including intestinal samples of infected and healthy people. We report the genomic and physiological characterization of MV19, a human intestinal strain closely related (ANI value of 98.81%) to the reference strain *B. cereus* ATCC 14579. MV19 cells were able to grow in a range of temperatures between 20 and 44 °C. At the optimal temperature the sporulation process was rapidly induced and mature spores efficiently released, however these appeared structurally and morphologically defective. At the sub-optimal growth temperature of 25 °C sporulation was slow and less efficient but a high total number of fully functional spores was produced. These results indicate that the reduced rapidity and efficiency of sporulation at 25 °C are compensated by a high quality and quantity of released spores, suggesting the relevance of different performances at different growth conditions for the adaptation of this bacterium to diverse environmental niches.

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1. Introduction

The *Bacillus cereus* group (*B. cereus sensu lato*) consists of several spore-forming Gram-positive bacterial species, widespread in nature as spores and vegetative cells [1,2]. The spores greatly contribute to the wide distribution of these bacteria in many environments for their resistance to extremes of temperatures and pH, to UV radiations and to the presence of lytic enzymes and toxic chemicals. The quiescent spore germinates when the environmental conditions allow cell growth and originates new vegetative cells able to grow by binary fission and eventually to sporulate again [3].

The taxonomy of the *B. cereus* group is complex and rapidly evolving. Based on recent studies [1,4], the group includes three well-characterized and several far less studied species. *B. cereus*

sensu stricto (*B. cereus*), responsible for two types of foodborne intoxications, *Bacillus thuringiensis*, an entomopathogen producing parasporal crystal inclusions made of proteins with insecticidal activity and *Bacillus anthracis*, the agent of anthrax in humans and animals, are well-known species, genetically and physiologically characterized in details. The less studied species of the group include *Bacillus mycoides* and *Bacillus pseudomycoides*, forming rhizoidal colonies on solid media, the psychrotolerant *Bacillus weihenstephanensis*, the animal probiotic *Bacillus toyonensis*, the psychrotolerant and cytotoxic *B. wiedmannii*, the thermotolerant and occasionally pathogen *Bacillus cytotoxicus* and several, recently identified other species [1,4].

B. cereus is a major cause of foodborne outbreaks [5] and is responsible of two types of intoxications: the emetic gastrointestinal syndrome and the diarrheal syndrome. In addition, *B. cereus* is also involved in nosocomial non-gastrointestinal infections in immunosuppressed patients [6]. These are often caused by biofilm formation on biomedical devices [7] and include septicemia, meningitis, brain abscesses, gas gangrene like infections, pneumonia, severe ocular infections and bacteremia in preterm

* Corresponding author. Department of Biology, Federico II University of Naples, via Cintia - complesso MSA, Naples 80126, Italy. Fax: +39 081679233.

E-mail address: ericca@unina.it (E. Ricca).

¹ These two authors contributed equally.

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neonates [8]. The severity of the diseases caused by some *B. cereus* strains makes it important to discriminate between pathogenic and non-pathogenic strains. Such discrimination is generally based on the analysis of several genetic loci that, coding for known toxins, have been selected as markers. These include the heat-resistant, emetic toxin cereulide, synthesized by a Non-Ribosomal Protein Synthase (NRPS) encoded by the *ces* gene locus, the hemolysin BL encoded by the *hbl* genes, the non-hemolytic enterotoxin encoded by the *nhe* genes and the cytotoxin K encoded by the *cytK* gene [8,9].

Toxin-producing strains of *B. cereus* are of particular concern for public health due to their ability to form biofilms and spores [10]. Both biofilm and spore formation are survival strategies for *B. cereus* in facing adverse environmental conditions. The biofilm increases both the resistance properties of cells and spores and their adhesion to surfaces, therefore playing an important role in the contamination of foods, food processing and clinical equipment [10]. *B. cereus* can form different types of biofilm under different growth conditions [11] and is generally formed by proteins, carbohydrates and DNA [12,13]. Cells within the established biofilms can produce spores [11,14,15] and these have distinct properties with respect to spores derived from planktonic cells [16], appearing larger, more heat resistant and less efficient in germination [17]. The structure and the functional properties of the spore are also strongly affected by other growth and sporulation conditions such as medium composition, temperature, pH and water activity [16,18]. In *B. cereus*, that generally grows within a temperature range of 10–50 °C with a temperature optimum between 30 and 40 °C, the growth temperature influences the size and the morphology of the produced spores [19]. Size and morphology, in turn, influence the functional properties of the spore with the largest ones showing the least resistance to chemicals and those surrounded by long appendages appearing more adhesive than those lacking such structures [20]. The spore surface hydrophobicity is also affected by the growth conditions and a high hydrophobicity has been associated with a strong adhesion to surfaces [21,22]. In the *B. cereus* type strain ATCC 14579 the sporulation temperature acts on spore coat formation through the morphogenetic protein CoteE, that is more abundantly represented in spores formed at 20 °C than at 37 °C [23,24].

In the frame of a study aimed at the isolation and characterization of spore formers of the *Bacillus* genus from intestinal samples of healthy children not under antibiotic or probiotic treatment, strain MV19 was selected for some peculiar morphological properties. In particular, MV19 differed from all other isolated strains for the production of cytoplasmic granules and the presence of spores of unusual, curved morphology. MV19 was unambiguously assigned to the *B. cereus sensu stricto* species and the presence of the genes associated with virulence, biofilm formation, cytoplasmic granules and spore coat formation analyzed by whole-genome sequencing. Analyses of the growth and sporulation temperatures, the efficiency of sporulation, the yield and quality of the produced spores at different temperatures indicated that MV19 produced fully functional spores at the sub-optimal temperature of 25 °C and functionally and morphologically defective spores at the optimal temperature of 42 °C. These results suggest that producing different spores at different growth conditions is an important feature for the adaptation of spore formers to diverse environments.

2. Materials and methods

2.1. Bacterial isolation, spore production and purification

Faecal samples were collected from healthy children not under antibiotic or probiotic treatment in the frame of a project aimed at

collecting spore formers of the *Bacillus* genus of intestinal origin. About 1 g of each sample was homogenized in PBS and heat-treated for 20 min at 80 °C, serially diluted, plated on Difco sporulation medium (DSM) (for 1 L: 8 g/L Nutrient Broth, 1 g/L KCl, 1 mM MgSO₄, 1 mM Ca(NO₃)₂, 10 mM MnCl₂, 1 mM FeSO₄, Sigma Aldrich, Germany) and incubated overnight at 37 °C, under aerobic conditions. The selected colonies were re-streaked on DSM agar plates to isolate single colonies that were then checked for the formation of phase-bright spores under the light microscope. Strain MV19 was isolated from a sample of a 4-year-old child and considered for this study for its peculiar features. Spores were prepared at the required temperature by the exhaustion method [25] using liquid DSM. Before purification, spores were washed four times with cold sterile distilled water and centrifuged at 8000 g for 20 min and then purified using an ethanol treatment in order to kill vegetative cells, as described by [26].

2.2. Physiological properties of the isolated strain

Hemolytic activity was assessed by spotting 5 µL of growing cells on Columbia agar plates supplemented with 5% defibrinated horse blood (Thermo Scientific), incubated for 24 h at 37 °C and analyzed for the presence of a lysis halo around the colony.

Biofilm formation was tested by growing bacterial cells in 24-well culture plates. The experiment was performed either in LB medium (10 g Bacto-Tryptone, 5 g Bacto-yeast extract, 10 g NaCl, pH 7.0), or in S7 minimal medium (50 mM morpholine-propane-sulfonic acid (MOPS) (adjusted to pH 7.0 with KOH), 10 mM (NH₄)₂SO₄, 5 mM potassium phosphate (pH 7.0), 2 mM MgCl₂, 0.9 mM CaCl₂, 50 mM MnCl₂, 5 mM FeCl₃, 10 mM ZnCl₂, 2 mM thiamine hydrochloride, 20 mM sodium glutamate, 1% glucose, 0.1 mg/mL phenylalanine, and 0.1 mg/mL tryptophan) or in DSM, for 48 h without shaking, at 25 and 42 °C. Then, biofilm production was assessed by crystal violet assay as previously described in [27]. Data were normalized by total growth estimated by OD_{590nm}, and the experiment was performed in triplicate.

The growth rate at different temperatures (25, 37 and 42 °C) was measured by OD_{590nm} readings of cells growing in LB medium.

2.3. Physiological properties of MV19 spores

Spore resistance to chemical and physical treatments was tested as previously reported by [23], with some modifications. Lysozyme resistance was tested by following changes in CFU counts on LB agar plates after 1 h of incubation of the spores (1.0 × 10⁷ CFU mL⁻¹) with Lysozyme (7U mL⁻¹) at 37 °C.

Hydrogen peroxide (H₂O₂) resistance was tested by following changes in CFU counts on LB agar plates after 7.5 and 15 min of incubation of heat-activated (10 min at 70 °C) spore suspension (1.0 × 10⁷ CFU mL⁻¹) in a 5% H₂O₂ solution.

Similarly, 0.2 mL of heat-activated spores (1.0 × 10⁷ CFU mL⁻¹) were incubated at 90 °C for 15 and 30 min and then spores resistance to heat treatment was measured by following changes in CFU counts on LB agar plates [28].

Spore hydrophobicity was assessed by mixing 1.9-mL volume of spore suspension (0.4e 0.5 OD_{590nm}) in water with 0.1 mL of n-hexadecane and following changes in optical density at 600 nm after separation of the spores between the aqueous and apolar phases as previously reported by [23]. The ability of spores to form clumps was evaluated as described by [29].

Two methods were used to evaluate spore germination. Spores (1.0 × 10⁷ CFU mL⁻¹) activated at 70 °C for 20 min, were resuspended in LB medium, serially diluted and plated on LB agar plates for CFU counts. Then, the germination efficiency was assessed by flow cytometry as previously described in [29]. Heat-activated



spores were stained with 0.5 mM Syto 16 (Life Technologies, Waltham, MA) and incubated in the dark for 15 min at 30 °C. I max values for the absorption and fluorescence emissions of the complex with DNA are 488 and 518 nm, respectively. Then, spores were analysed in a flow cytometer (BD Accuri C6; BD Biosciences, San Jose, CA) and detected in FL1 by using the standard C6 filter configuration (FL1 453/30 BP) separating germination-unspecific and germination-specific fluorescence (threshold 10^4) [27].

The sporulation efficiency was measured by growing cells in DSM 25 and 42 °C and counting at various time points the total number of cells, sporangia, and spores under the phase-contrast microscope and measuring OD_{600nm} at spectrophotometer. All experiments were carried out in triplicate from independently purified spores.

2.4. Whole-genome sequencing and phylogenetic analysis

Exponentially growing cells were used to extract chromosomal DNA as previously reported [30]. Genome sequencing of MV19 genome was performed by GenProBio (Parma, Italy) with Illumina MiSeq Sequencing System. Genome assembly was performed with SPADs v3.14.0 by means of MEGANotator pipeline [31]. Open reading frames (ORFs) prediction was performed with RAPSearch2 against NCBI RefSeq database and HMMER against PFAM database. Ribosomal RNA genes prediction was performed with RNAmmer v1.2. Transfer RNA gene prediction was performed with tRNAscan-SE v1.21. The 16 S rRNA gene of the isolated strain was extracted from the sequenced genome and was compared to 22 reference 16 S rRNA genes of closely related strains identified using the Genome Taxonomy Database (GTDB) taxonomy and retrieved from the NCBI database. All sequences were aligned using Seaview 5.0.5 software [27] and the phylogenetic tree was created using the Maximum-likelihood algorithm with model GTR þ I þ G4. Statistical support was evaluated by the approximate likelihood-ratio test (aLRT) and is indicated at each node in the tree. *Bacillus subtilis* subsp. *subtilis* 168 was used as an outgroup to root the tree. Average Nucleotide Identity (ANI) value between the sequenced genome and the closest bacteria was obtained using GTDB-Tk Classify v1.6.0 toolkit [32]. The genome of the isolated strain has been deposited in GenBank as BioProject PRJNA846192 whose accession number is CP098734.

2.5. Whole-genome typing of MV19

The presence of putative toxins and virulence factor-associated genes was evaluated by analyzing the MV19 genome by using the VF analyzer pipeline via a comparison to the Virulence Factor Database (VFDB) [33]. Nucleotide sequences of genes involved in biofilm formation and the proteins involved in the formation of coat and exosporium were found in [34] and the alignments with the MV19 genome were performed using BLASTN and BLASTP, respectively.

2.6. Microscopy analyses

Five microliters of freshly purified spores were spotted on microscope slides and covered with coverslips and observed under phase-contrast light microscope by using an Olympus BX51 microscope fitted with a 100 objective UPlanFL1. Images were captured using an Olympus DP70 digital camera equipped with Olympus U-CA Magnification Changer.

For SEM analysis, MV19 spores (1.0×10^8 /mL) prepared at different temperatures were cut from subapical parts using a sharp razor blade, fixed with 3% glutaraldehyde in phosphate buffer (65 mM, pH 7.2-7.4) for 2 h at room temperature, post-fixed with

1% osmium tetroxide in the same phosphate buffer for 1.5 h at room temperature, and completely dehydrated with ethanol. Both samples were then mounted on aluminium stubs, coated with a thin gold film using an Edwards E306 Evaporator, and observed with a FEI (Hillsboro, OR, USA) Quanta 200 ESEM in high vacuum mode (P 70 Pa, HV 30 kV, WD10 mm, spot 3.0) [35].

For thin sectioning transmission electron microscopy (TEM) analysis, MV19 purified spores were processed as described previously [18] and imaged on a Philips EM 208 S (FEI) microscope equipped with digital camera and Image Analysis Software.

3. Results and discussion

3.1. Phylogenetic analysis and functional annotations of MV19 genome

The general genomic features of MV19 were summarized in Suppl. Mat. Table S1 and Fig. S1. MV19 was initially assigned to the *B. cereus* group by 16 S rRNA gene sequence alignment and a phylogenetic tree constructed by comparison with 22 type strains representative of the *B. cereus* group (Fig. 1). Since the sequence of the 16 S gene is often not sufficient to allow a correct species assignment [36], a whole genome comparison was performed (by GTDB-Tk Classify - v1.6.0) and an Average Nucleotide Identity (ANI) value of 98.81% was measured with its closest relative, the *B. cereus* ATCC 14579 strain, therefore, allowing an unambiguously taxonomic classification as *B. cereus sensu stricto* [4]. In addition, the analysis of the panC gene sequence through a bioinformatic tool available on <https://tool.cereusid.shinyapps.io/Bcereus/> [37], indicated MV19 as a group IV *B. cereus* (not shown).

Functional annotation of the MV19 genome was performed using the KO (KEGG Orthology) database using BlastKOALA tools (available at KEGG Web site <https://www.kegg.jp/>). A total of 5814 protein-coding sequences, 11 rRNAs, 68 tRNAs and 1 tmRNA were assigned. As reported in Suppl. Mat. Fig. S2A Kegg analysis showed that only 45% of the annotated genes could be assigned to subsystems. Among the subsystem categories present in the genome, "genetic information processing" and "signaling and cellular processing" had the highest feature counts, followed by "carbohydrate metabolism". The other less represented categories are reported in Suppl. Mat. Fig. S2B with their relative percentages. MV19 genome was further classified into orthologous groups based on their function (COG), by using eggNOG (<http://eggnoG-mapper.embl.de/>) (Suppl. Mat. Fig. S3). The majority of the annotated genes (1353 genes) were classified into "Function unknown" (S category in Suppl. Mat. Fig. S3), the next most represented class was "Amino acid transport and metabolism" (E category, 467 genes) followed by "Transcription" (K category, 451 genes).

3.2. Genome-wide detection of toxin genes and other virulence factors in MV19

Pathogenic strains of *B. cereus* are characterized by the presence of genes coding for toxins and other virulence-related factors [8,9]. To evaluate their putative presence in MV19, the genome was analyzed with the VFAnalyzer tool (<http://www.mgc.ac.cn/VFs/>), based on the VFDB database. As shown in Suppl. Mat. Table S2, the MV19 genome contains genes coding for the Non-hemolytic enterotoxin (nheA, nheB, nheC), the hemolytic enterotoxin (hlyA, hlyC, hlyD), the single protein cytotoxin K (cytK) and the hemolysin Cereolysin O (alo). In addition, it contains genes for the Hemolysin III (hlyIII) and for a protein belonging to the Hemolysin-III family proteins, while a gene coding for the Hemolysin II (hlyII) was not found. The MV19 genome contains only one of the genes required for Cereulide synthesis, *cesH* (Suppl. Mat. Table S2). The Cereulide

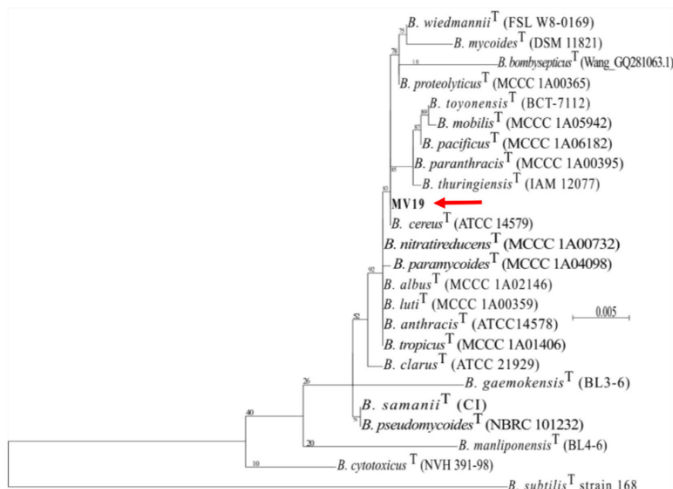


Fig. 1. The phylogenetic tree was constructed using the Maximum likelihood algorithm with model GTR + I + G4, based on 16 S rRNA gene sequences. The genome sequence of MV19 was aligned to the type strains of the "Bacillus cereus group" according to Genome Taxonomy Database (GTDB). Node support represents the approximate likelihood-ratio test (aLRT) and is shown at the corresponding node of the tree. *Bacillus subtilis* strain 168 is used as an outgroup.

biosynthetic genes are organized in a cluster of seven genes (*cesH*, *cesP*, *cesT*, *cesA*, *cesB*, *cesC*, and *cesD*) that are located on a megaplasmid. Six genes form an operon (*cesPTABCD*) while the seventh (*cesH*) is transcribed by its own promoter and is located at the 5' end of the operon in the same reading frame [38]. In MV19 the *cesPTABCD* operon is not present while the single gene present (*cesH*) is found on the chromosome.

In addition, genes coding for virulence-related factors such as phospholipases, sphingomyelinases, proteinases and peptidases were also found in the MV19 genome (Suppl. Mat. Table S3). In particular, the MV19 genome contains two ORFs encoding the Immune Inhibitor A metalloproteinase (*inhA*), needed to escape the immune surveillance and promote germination inside the host [39], the Phosphatidylcholine preferring phospholipase C (*plcA*), the Phosphatidylinositol-specific phospholipase C (*plpC*) and the Sphingomyelinase (*sph*), all involved in mammalian membrane-damaging (Suppl. Mat. Table S3). A gene coding for a homolog of the Streptococcal enolase (*eno*) was also found in the genome sequence of MV19 (Suppl. Mat. Table S3). This is a secreted protein also found in different *B. anthracis* strains, where it may contribute to pathogenicity by binding the infected host's plasminogen [40].

The ability to acquire iron is also considered a virulence-related factor, as it is crucial for bacterial growth and colonization of host tissues [41]. Genes coding for two important siderophores useful for iron acquisition from ferritin, bacillibactin (*bhbABCE*) and petrobactin (*asbABCE*), and the Iron-regulated leucine rich surface protein type A (*ilsA*), that acts as ferritin surface receptor, were all found in the MV19 genome (Suppl. Mat. Table S3).

Regulation of virulence gene expression occurs through a precise regulatory circuit, which in *B. cereus* is provided by a quorum sensing mechanism controlled by the transcriptional factor PlcR and the signalling peptide PapR [42]. As shown in Suppl. Mat.

Table S3, genes coding for their homologs are present in MV19 genome.

Altogether, MV19 contains genes for most known virulence factors of pathogenic *B. cereus* and should therefore be considered as a potentially virulent strain.

3.3. Physiological characterization

MV19 was isolated from a faecal sample of a healthy, four-year old boy not under antibiotic or probiotic treatment. The sample was heat-treated at 80 °C for 20 min to eliminate all vegetative cells, plated on DSM and incubated aerobically to isolate *Bacillus* spore formers. Purified colonies appeared after 16e18 h of incubation at 37 °C and appeared creamy, mucoid and of irregular shape. Under the light microscope cells of MV19 were rod shaped and formed phase bright, ellipsoidal spores localized in central or sub-terminal position (respectively white or yellow arrows in Fig. 2A). In addition to spores, some MV19 cells also produced one or two phase bright structures per cell (red arrows in Fig. 2B). Production of such structures, resembling inclusion granules, was independent on the growth medium, being produced in rich (LB), minimal (S7) or sporulation-inducing (DS) media, and on the growth phase (not shown). Several species of the *Bacillus* genus produce polyhydroxyalkanoates (PHA), polyesters used to store energy and carbon [43]. Genes putatively involved in PHA formation were identified in the MV19 genome (Suppl. Mat. Table S4). Some forming spores (Fig. 2C) and free spore (white arrows in Fig. 2D) showed an unusual curved morphology (see below).

A panel of relevant antibiotics was used to assess the susceptibility of MV19 cells. As reported in Table 1, MV19 cells showed MIC for vancomycin higher than the breakpoint levels indicated for

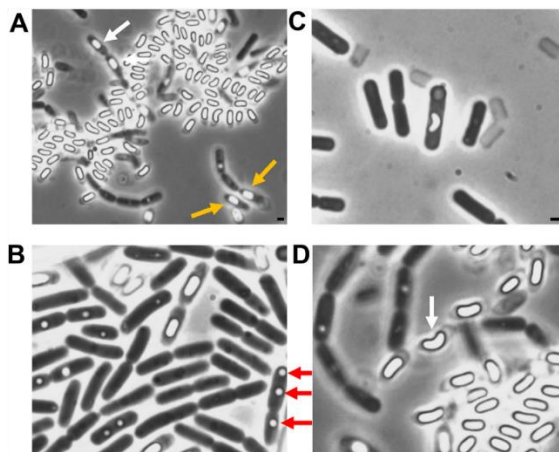


Fig. 2. Cells of MV19 grown on solid DSM at 42 °C and observed under the phase-contrast microscope. (A) Within sporangia spores have central (white arrows) or sub-terminal (yellow arrows) position. (B) Some cells contain phase bright structures (red arrows) resembling inclusion granules. Some forming spores (C) and free spore (D, white arrow) showed an unusual curved morphology. Scale bars correspond to 1 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Minimal Inhibitory Concentration (MIC)^a of selected antibiotics against MV19.

Antibiotic	B. cereus MV19 ^b	Bacillus EFSA breakpoint ^c
Vancomycin	12.00	4
Gindamycin	0.25	4
Gentamicin	0.38	4
Erythromycin	0.38	4
Streptomycin	2.00	8
Tetracycline	0.25	8
Chloramphenicol	4.00	8
Kanamycin	1.50	8

^a Analysis performed with paper strips with a concentration range of 0.016e 256 μ g mL⁻¹ for all the antibiotics tested.

^b Values in μ g mL⁻¹.

members of the *Bacillus* genus [44] and a weak resistance to streptomycin and chloramphenicol (Table 1).

MV19 cells were able to completely lyse red blood cells on plate and were, therefore, indicated as b-hemolytic (not shown). Cells were able to grow in a temperature range between 20 and 44 °C (not shown) and produced biofilm both on plate and on liquid cultures. The amount of biofilm formed was higher in minimal (S7) than in rich (LB) or sporulation-inducing (DS) medium after 48 h of growth (Fig. 3), as previously reported in other *Bacillus* species [45]. Biofilm formed in all tested media contained both cells and spores and was characterized by the presence of a pellicle and a ring (not shown), as previously reported for other *B. cereus* strains [11]. Genes putatively involved in biofilm formation were identified in the MV19 genome (Suppl. Mat. Table S5). In particular, the pur genes, required for purine biosynthesis, the eps genes, coding for the exopolysaccharide, the tapA gene needed for biofilm assembly [46,47], and the sinR gene coding for the master regulator of biofilm formation [48] were all present in the MV19 genome (Suppl. Mat. Table S5).

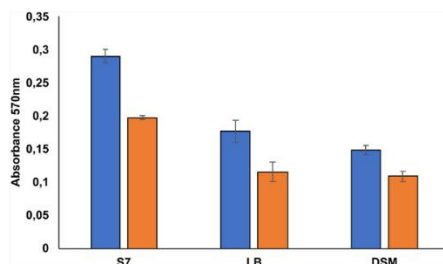


Fig. 3. Biofilm production in minimal (S7), rich (LB) and sporulation-inducing (DS) medium at 25 (blue) and 42 °C (orange). The graph shows the OD_{570nm} values of the samples stained with crystal violet. The graph reports the average of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Cell growth was followed at 25, 37 and 42 °C in LB medium. At 37 and 42 °C the generation time was identical (33 min) while at 25 °C it was longer (50 min). Moreover, at 37 and 42 °C cells very rapidly started their exponential growth and quickly entered into the stationary phase of growth. At 25 °C cells experienced a long lag phase, independently from the temperature used to obtain the starting inoculum, before entering into the exponential phase of growth. Therefore, 37 and 42 °C were considered as optimal growth temperatures and 25 °C as a sub-optimal condition in LB medium. Based on this, all further experiments were performed at the highest optimal temperature of 42 °C and at the sub-optimal temperature of 25 °C.

3.4. Sporulation and spore properties

MV19 were grown in DSM to induce spore formation. As in LB also in DSM cells grew more rapidly at 42 °C than at 25 °C (Fig. 4A). At the latter temperature entry into the exponential phase of growth was preceded by a long lag phase and a precise initiation of the sporulation cycle (T0) was not clearly identified. Indeed, after 18 h of growth at 25 °C, during the exponential phase of growth, a large number of sporulating cells was already present (see below).

Spore formation was followed by collecting samples from the growth cultures at various times and counting vegetative cells, sporangia and free spores under the light microscope. As reported in Table 2, at 42 °C few free spores (5%) were produced after 18 h of growth. These became 15% after 24 h and after 42 h only free spores were observed in the culture. At 25 °C no free spores were observed after 18 and 24 h and after 42 h about 25% of sporangia were still present (Table 2). Spore production occurred more rapidly and efficiently at 42 °C than at 25 °C but at 25 °C an over 15-fold higher number of spores was obtained (3.7×10^8 and 60.0×10^8 spores/ml

at 25 and 42 °C, respectively) (Fig. 5A). As previously reported for *B. subtilis*, a higher number of spores produced by slow-than fast-growing cells is due to a prolonged growth. This allows for additional cell divisions [48] as also occurs for MV19 (Fig. 4A).

Table 2
Efficiency of spore formation at 25 and 42 °C.

Temperature	Hours	Vegetative cells (%)	Sporangia (%)	Free spores (%)
25 °C	0	100	e	e
	18	50	50	e
	24	e	100	e
	42	e	25	75
42 °C	0	100	e	e
	18	20	75	5
	24	15	70	15
	42	e	e	100

^a Cells were grown aerobically in DS medium. At the indicated times aliquots were collected and analyzed under the light microscope. The % of vegetative cells, sporangia and free spores was calculated by counting over 300 bacteria in different microscopy fields.

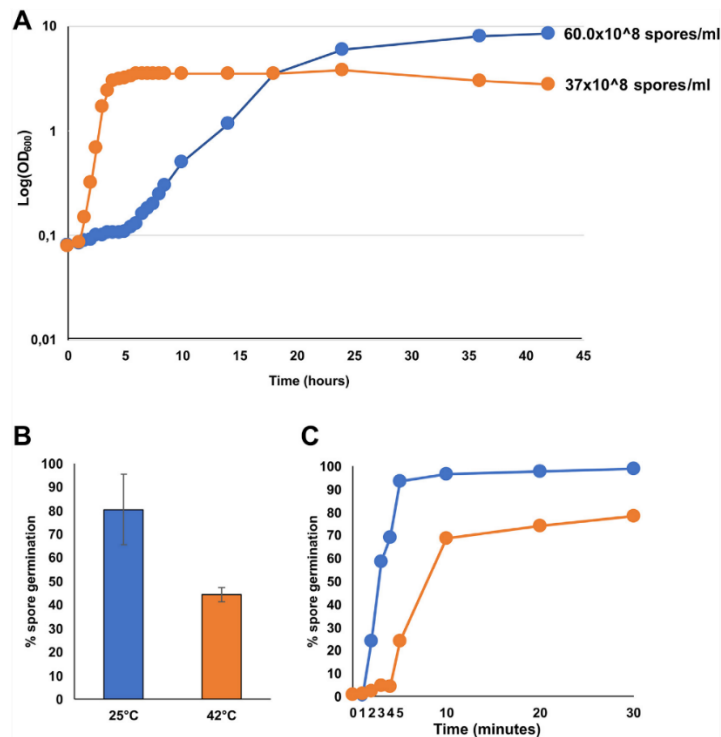


Fig. 4. (A) MV19 growth at 25 (blue) or 42 °C (orange) in sporulation-inducing medium. Germination efficiency measured by CFU counts (B) or flow cytometry (C) with spores produced at 25 (blue) and 42 °C (orange). Panel C reports the percentage of germination based on the flow cytometry data (Supplementary Material Fig. S4). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

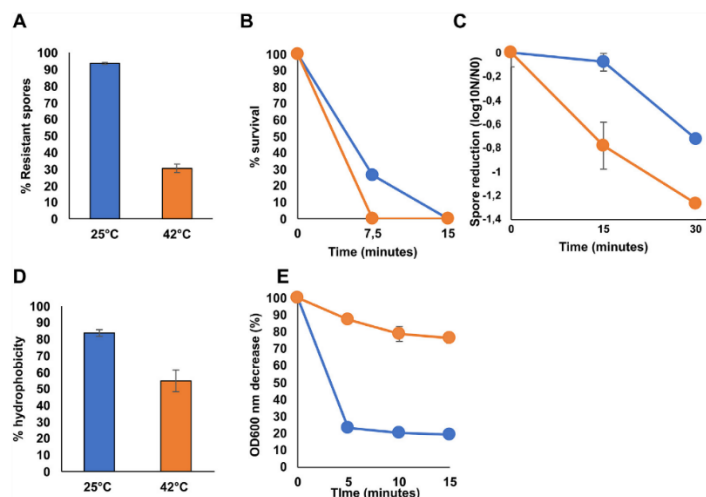


Fig. 5. Physiological properties of MV19 spores produced at 25 (blue) and 42 °C (orange). (A) Percentage of lysozyme resistant after 1 h of incubation with 7 U μml^{-1} of lysozyme (Sigma). (B) Percentage of hydrogen peroxide resistance of spores exposed for 7.5 and 15 min to 5% H_2O_2 (vol/vol). (C) $\log_{10}$ reduction of heat resistance of spores exposed for 15 and 30 min at 90 °C. (D) Percentage of hydrophobicity indicated as the proportion ($\frac{\text{OD}_{600\text{nm}}}{\text{OD}_{600\text{nm}} + \text{OD}_{400\text{nm}}}$) of spores in n-hexadecane after the separation of the spores into water and solvent phases. (E) Clumping assay: percentage of $\text{OD}_{600\text{nm}}$ decrease of spores suspended in distilled water. For all panels each value is the mean from three replicate experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Spores produced at 25 or 42 °C were then evaluated for their resistance properties and the germination efficiency. The efficiency of germination was assessed by plate count after suspension of purified spores in LB medium (Fig. 4B) and by flow cytometry (Fig. 4C and Suppl. Mat. Fig. S4), as previously described [29,49]. The two methods measure different aspects of spore germination with the plate count assay assessing the ability to form colonies by germinated spores and the flow cytometry analysis focusing on the initial steps of germination by measuring the rupture of spore surface and the subsequent entry of the fluorescent dye in the spore core. In spite of this, the two approaches showed a similar trend (Fig. 4BC) and indicated that spores produced at the sub-optimal temperature of 25 °C germinated more efficiently than those produced at the optimal growth temperature.

Spores produced at the sub-optimal temperature were also more resistant than those produced at 42 °C to lysozyme (Fig. 5A), H_2O_2 (Fig. 5B) and heat (Fig. 5C) and were more hydrophobic (Fig. 5D). Due to the high hydrophobicity of 25 °C spores, when suspended in water they tended to form clumps that precipitated to the bottom of the cuvette causing a decrease of $\text{OD}_{590\text{nm}}$ readings over time (Fig. 5E). Such effect was not observed when spores were resuspended in 50% methanol (not shown), confirming that it was due to the spore hydrophobicity.

3.5. Spore morphology

Spore morphology appeared dependent on the growth temperature. Spores produced at the sub-optimal temperature (25 °C) observed at the phase contrast microscope showed a regular and organized distribution (Fig. 6). All spores appeared phase bright, of elliptical shape and organized into shoulder-to-shoulder filaments

(Fig. 6). Such organization was not observed when spores were produced at 42 °C (Fig. 6). Since growing and sporulating cells produce more biofilm at 25 than at 42 °C (Fig. 3), such organized distribution of spores might depend on cells entering into sporulation while entrapped in the extracellular matrix. The released spores would then keep the organized distribution of the sporulating cells.

Some of the spores produced at 42 °C showed an unusual curved morphology (see Fig. 2CD and yellow arrows in Fig. 6), that was never observed in 25 °C spores. The unusual morphology was strictly dependent on the growth temperature with no curved spores at 25 °C and increasing numbers of curved spores raising the growth temperature from 37 (not shown) to 42 °C. Counting over 300 spores in different microscopy fields, at 42 °C about 30% of the spores showed the unusual morphology.

To further characterize the unusual morphology, purified spores were used for scanning and transmission electron microscopy analyses. As shown in Fig. 7, 25 °C but not 42 °C spores were enveloped into a matrix (yellow arrows in Fig. 7A), supporting the hypothesis that such a matrix could be responsible for the organized spore distribution at 25 °C observed in Fig. 6. In addition, the surface of spores appeared smooth at 25 °C and wrinkled at 42 °C (Fig. 7A), with curved spores (bottom left and bottom central panels of Fig. 7A) more wrinkled than normal ones (bottom right panel of Fig. 7A) produced at 42 °C. Spore morphology was also analyzed by thin-sectioning transmission electron microscopy (TEM). As shown in Fig. 7B, a continuous exosporium entirely surrounding the spore was formed at both 25 or 42 °C. Spores produced at 25 °C showed a well-defined coat, similar to that previously reported for the *B. cereus* strain ATCC 14579 [23]. Spores produced at 42 °C showed a different ultrastructure with an irregularly shaped coat (Fig. 7B).

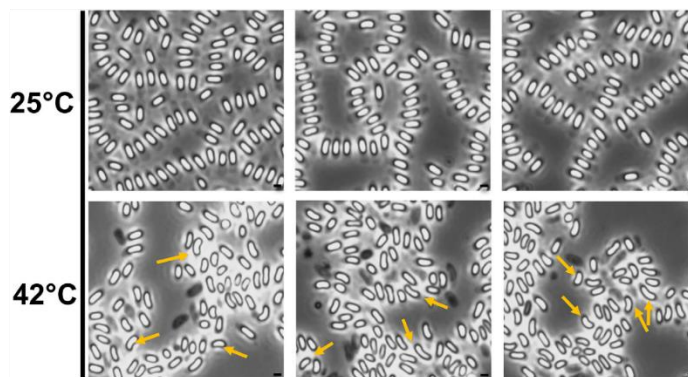


Fig. 6. Morphology of spores produced at 25 and 42 °C observed under phase-contrast microscope. Arrows indicate the curved shape observed for spores produced at 42 °C. Scale bars correspond to 1 μm.

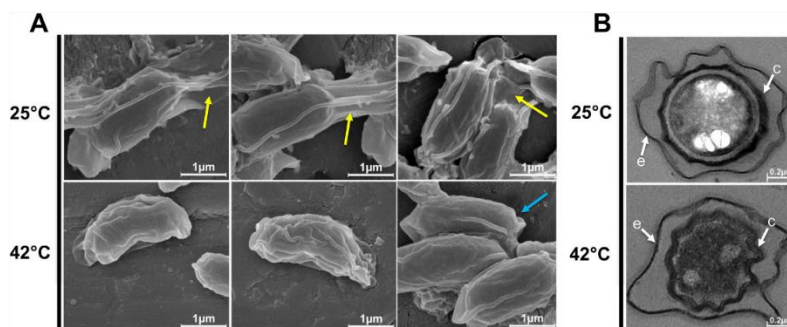


Fig. 7. (A) Scanning electron microscopy of spores produced at the two different temperatures (25 and 42 °C). Yellow arrows indicate the matrix enveloping the spores at 25 °C. The blue arrow indicates a non-curved spore produced at 42 °C. Scale bar 1 μm. (B) Transmission electron microscopy of spores produced at the two different temperatures. White arrows: e = exosporium, c = coat. Scale bar 0.2 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

The unusual curved morphology observed in about 30% of the 42 °C spores could be dependent on the assembly of the spore coat and exosporium proteins. The MV19 genome was analyzed for the presence of homologs of coat/exosporium genes by using as a "query" the list of the spore coat and exosporium proteins identified in the *B. cereus* reference strain ATCC 14579 [34]. As reported in the Suppl. Mat. Table S6, the majority of the putative MV19 proteins showed a high similarity with homologs of the type strain *B. cereus* ATCC 14579. Six proteins of the reference strain (BC_0944, BC_2426, BC_2427, BC_2569, BC_3515, BC_p0002) were not found in MV19 (Suppl. Mat. Table S6). These proteins are not characterized with the exception of BC_2569 annotated as "collagen triple helix repeats" and of BC_p0002 located on a plasmid of strain ATCC 14579.

4. Conclusions

MV19 is a human intestinal isolated strain belonging to the *B. cereus sensu stricto* species. Its genome contains genes coding for most known toxins and virulence-related factors of *B. cereus* with the exception of the genes required for Cereulide synthesis.

MV19 cells grow within a temperature range of 20 and 44 °C with optimal growth at 37 and 42 °C. At the optimal growth temperature of 42 °C also spore production is faster than at the sub-optimal temperature of 25 °C. However, the slow and prolonged growth at 25 °C delayed the entry into sporulation allowing more cell divisions and the production of over 15-fold more free spores than at 42 °C. The 25 °C spores are also more resistant (to lysozyme, H₂O₂ and heat), more efficient in germination and more hydrophobic than those produced at 42 °C.



These results were unexpected since it has been previously reported that spores produced at low temperatures are usually less resistant to heat and chemicals than those produced at high temperatures [50], and may represent a peculiarity of the MV19 strain.

Spores produced by slow- (25 °C) or fast-growing (42 °C) cells have different coat ultrastructures. Slow-growing cells produce spores structurally similar to those produced by *B. cereus* ATCC 14579 at 20 or 37 °C [23], while an irregular shape characterizes the coat of fast-growing cells. About 30% of the spores produced by fast growing cells have an unusually curved morphology and a wrinkled surface, suggestive of a defective structure that presumably affects the spore traits. Therefore, although at the optimal growth temperature of 42 °C spore formation occurs faster and with a high efficiency, some of the produced spores are structurally and functionally defective. This conclusion suggests that the reduced rapidity and efficiency of sporulation at 25 °C are compensated by a high quality and quantity of the released spores. These results point at the temperature of growth and sporulation as a key environmental factor influencing the structure and functionality of the produced spores. Being extremely stable cell forms that do not need nutrients or water, spores are produced as a survival strategy when the environmental conditions do not allow cell growth. Therefore, producing different spores at different environmental conditions can be considered as a "risk-spreading" strategy to allow the survival/adaptation of spore formers to diverse environmental conditions.

In *B. subtilis* a delay in spore formation negatively affects spore germination but increases the amount of spores produced and a quality-quantity trade-off has been proposed to occur and contribute to the evolutionary adaptation of spore formers [48,51]. In *B. cereus* MV19 at the sub-optimal temperature of growth and sporulation more cells and more fully functional spores are produced than at the optimal growth temperature, compensating the faster growth and efficient sporulation observed at 42 °C.

Although the molecular mechanisms regulating such balancing are totally obscure, some spore quality determinants have been proposed in *B. subtilis*. Examples are the electrical polarization of the outer membrane of developing spores [52], the enzyme alanine dehydrogenase [48] and the isoleucyl-tRNA synthase [53] have all been proposed as quality control checkpoints. Homologs of both the alanine dehydrogenase (MV19_2153) and the isoleucyl-tRNA synthase (MV19_0519) are present in the MV19 genome and share, respectively, 72% and 73.86% of identity with the *B. subtilis* proteins. Addressing the role of these spore quality determinants in *B. cereus* would be a relevant future research challenge.

Authors' contributions

AS, GDGB, SC and MV executed the experiments, AS, MV, SC, LB, RI and ER analyzed the data, interpreted the results. ER and AS wrote the manuscript, ER, RI and LB designed the study and provided the funding.

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Author agreement

Authors approve the final version of the submitted manuscript.

Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.resmic.2023.104030>.

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CONCLUSIONS

This PhD thesis highlights the importance of isolating and characterizing new spore-forming bacteria from the human gut, with the aim of exploring their potential use as probiotics. Although spore-based probiotic products are already on the market, the main species currently used in these products are *B. clausii*, *B. subtilis* or *B. coagulans*. However, many *Bacillus* species are being studied for their beneficial properties, so much so that the list of *Bacillus* considered safe for human use has been extended to include other species such as *B. velezensis*, *B. megaterium* (renamed *Priestia megaterium*), *B. pumilus* and others (EFSA Panel on Biological Hazards (BIOHAZ), 2024). The study and typification of different species is very important, as already explained in the introductory chapter, for the determination of new properties, including the production of new metabolites, or for a more detailed understanding of the mechanisms by which these microorganisms interact with the host organism.

Through my thesis work, I was able to isolate, identify, and select spore-forming bacterial strains that have the potential to provide health benefits to the host. I also characterized their functional properties both *in vitro* and *in silico*. Of the 32 aerobic spore-forming strains isolated from intestinal samples of healthy children, four strains (MV4, MV11, MV24, MV30) were selected as potential probiotics as they were non-hemolytic and sensitive to all antibiotics indicated by EFSA as relevant for bacteria of the genus *Bacillus* (EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2012).

The four strains were assigned to species on the EFSA Qualified Presumption of Safety (QPS) list, indicating that they could be considered safe for human use (*B. velezensis* MV4 and MV11, *B. subtilis* MV24 and *P. megaterium* MV30).



CONCLUSIONS

This hypothesis is supported by the *in silico* observation that the four MV genomes do not carry putative genes encoding known *Bacillus* toxins. In fact, the only gene present in all four genomes that encodes a putative toxin is the *hlyIII* gene, which is common to pathogenic and non-pathogenic bacteria. However, the *hlyIII* product was not sufficient to cause an alpha- or beta-hemolytic phenotype in the four MV strains. A gene encoding a putative phytotoxin, *cysC*, is carried by MV4, MV11 and MV30, while only the latter strain contains the *cylR2* gene, a homologue of a transcriptional repressor of an *Enterococcus* cytolysin operon. Additionally, the genomes of MV4, MV11, and MV30 contain homologous genes of Nattokinase, with over 85% identity in all three genomes. Nattokinase is a serine protease of the subtilisin family that exhibits fibrinolytic activity, as well as antithrombotic and antihypertensive effects when ingested orally (Nijland *et al.*, 2010). Furthermore, the genome of MV30 includes a homologous gene (77.9% identity) that encodes the enzyme glutamic acid decarboxylase (GAD), responsible for converting glutamate into γ -aminobutyric acid (GABA), which is the most prevalent inhibitory neurotransmitter in the central nervous system.

All four strains formed biofilms and exhibited antioxidant activity *in vitro*. Both of these properties are important for a probiotic strain to favor colonization of the intestinal environment and protect intestinal cells from damage (Mazzoli *et al.*, 2019). The four MV strains considered in this study were also tested for the production of CSF and CSF-like peptides which in addition to inducing anti-inflammatory and cytoprotective responses both *in vitro* and *in vivo*, also play a role in the anti-neurodegenerative activity of *B. subtilis* in the model organism *C. elegans* (Donato *et al.*, 2017, Cogliati *et al.*, 2020). Ingested spores of strains that produce such molecules may have potential benefits for treating inflammatory and age-related neurodegenerative diseases.



CONCLUSIONS

The tested bacteria secreted CSF (MV24) or CSF-like (MV4, MV11, and MV30) peptides that induced the expression of the heat shock protein Hsp27 in HT-29 cells that were treated with bacterial conditioned medium, resulting in cytoprotective effects.

Although antimicrobial production is not the primary mechanism of action of probiotics against infectious diseases, it is certainly a relevant characteristic for a probiotic strain to be used as a potential alternative to antibiotics. *In vitro* experiments demonstrated that MV strains, except MV30, exhibited antimicrobial activity against selected target strains, including *L. monocytogenes*, *B. cereus*, *S. faecalis*, *E. coli*, and others. In addition to the *in vitro* approach, an *in silico* analysis was conducted to evaluate the presence of gene clusters encoding antibacterial molecules and secondary metabolites. The analysis revealed that both *B. velezensis* strains have the potential to produce macrolactin H, bacillaene, fengycin, bacillibactin, and bacilysin. Only MV4 encodes difficidin and surfactin, whereas the genome of *B. subtilis* strain MV24 encodes bacillibactin, bacilysin, subtilosin A, and fengycin.

Genes encoding products dissimilar to known antimicrobials have been identified in all four genomes. This is promising as these genes may encode variants of previously identified molecules with different targets and mechanisms of action, or entirely new antimicrobials. Further research is required to characterize these genes, which will provide valuable information for identifying new antimicrobials. This preliminary characterization indicates that the MV4, MV11, MV24 and MV30 strains are safe and have beneficial properties, and can be considered for *in vivo* testing as alternatives to antibiotics in both humans and animals.



CONCLUSIONS

The second phase of the research evaluated the probiotic activity of *Bacillus* spores through *in vivo* testing in animal models. These tests were carried out during my 6-month term as a Visiting Research Student at Royal Holloway University of London, where a study was already underway to evaluate the efficacy of probiotics in mouse models of inflammatory bowel disease, allowing my research to be integrated with the expertise and resources of the host university. To conduct these experiments, I used purified spores of bacteria isolated from the human gut, previously validated in my laboratory, to test their probiotic activity *in vivo*.

In this context, purified spores of two strains *B. subtilis* SF106 and *B. clausii* SF174 (recently renamed *Alkalihalobacillus clausii*) were administered orally to mice before and after chemical induction of Ulcerative Colitis using Dextran Sodium Sulphate (DSS). The spores of the two strains behaved similarly, but those of the SF174 strain were more effective and, in fact, animals pretreated with SF174 showed lower disease rates and histological indices than those observed in mice pretreated with SF106 spores. Treatment with both types of spores showed an immunomodulatory trend in mice with DSS-induced colitis, as shown by qPCR analyses of key genes involved in intestinal inflammatory processes. Both spores significantly reduced the expression of the pro-inflammatory cytokine TNF- α , while only SF174 spores induced a statistically significant increase in the expression of the anti-inflammatory cytokine IL-10. This increase was higher than that observed in control (*naïve*) animals, suggesting a general anti-inflammatory effect independent of DSS-induced inflammation.



CONCLUSIONS

Toll-like receptors TLR-2 and TLR-4 recognize commensal and pathogenic bacteria, with TLR-2 primarily active on Gram-positive bacteria and TLR-4 on Gram-negative bacteria. This recognition is essential for the release of pro- and anti-inflammatory cytokines and thus for the maintenance of intestinal homeostasis (Latorre *et al.*, 2018). The balance between TLR-2 and TLR-4 receptors can trigger intracellular signaling cascades and modulate cytokine expression (Latorre *et al.*, 2018; Jia *et al.*, 2020). In my experiments, analysis of TLRs expression showed a down-regulation of TLR-2 and a slight up-regulation of TLR-4 in the DSS group, likely contributing to the increased production of pro-inflammatory cytokines and decreased production of the anti-inflammatory cytokine IL-10 observed as a result of DSS treatment. These effects were restored to statistical significance for TLR-2, and almost significant ($p=0.08$) for TLR-4 by SF174 spores. SF106 spores induced a similar but weaker effect on TLR-2 expression, while the effect on TLR-4 was unclear due to the high variability of expression observed in the SF106 group of mice. The different balance of TLR-2 (upregulated) and TLR-4 (downregulated) expression caused by spore pretreatment is a likely cause of the observed increase in IL-10 and suggests that oral administration of SF174 spores promotes an anti-inflammatory response in mice.



CONCLUSIONS

The analysis of the gut microbial composition in mice revealed that both spore treatments modified the gut microbiota community. Compared to animals treated with DSS only, both spore treatments reduced the abundance of the same thirteen genera (e.g. *Lachnospiraceae* UCG-001, *Clostridioides*, *Enterorhabdus*, *Roseburia* and others). In twelve of these thirteen genera, relative abundance was increased by DSS treatment compared to naïve animals and was restored by both types of spores. Only the genus *Lactobacillus* was reduced by treatment with DSS and further reduced by treatment with SF106 and SF174 spores. Both types of spores had a positive effect on the abundance of *Bacteroidetes* and *Akkermansia* genera. However, the increase was only statistically significant with SF174. Previous studies have reported similar effects with spores of other *Bacillus* strains, which were able to increase the abundance of these bacteria *in vitro* (Ji *et al.*, 2022) or *in vivo* (Marzorati *et al.*, 2021). *Bacteroidetes* and *Akkermansia* are microorganisms that are considered to be the "next generation of probiotics" and are thought to have beneficial effects (Depommier *et al.*, 2019). It has been demonstrated that *Akkermansia muciniphila* enhances colonic mucin production, resulting in a thicker mucosal barrier to the underlying epithelial layer. This may explain the increased expression of MUC-2 gene observed in this study and, to some extent, the protective effects of SF174.



CONCLUSIONS

As part of the study aimed at isolating and characterizing the *Bacillus* spore-forming strains mentioned above, strain MV19 was selected due to its unique morphological properties. Specifically, MV19 was distinguished from all other isolated strains by the presence of cytoplasmic granules and spores with an unusual curved morphology. MV19 is a strain that has been isolated from the human intestine and assigned to the species *B. cereus sensu stricto* through genome sequencing and phylogenetic analysis. Its genome contains genes that code for most of the known toxins and virulence factors of *B. cereus*, with the exception of the genes required for Cereulide synthesis. MV19 cells can grow in a temperature range of 20 to 44 °C, with optimum growth at 37 and 42 °C. At the optimal growth temperature of 42 °C, spore production occurs faster than at the sub-optimal temperature of 25 °C. However, the slow and prolonged growth at 25 °C delays entry into sporulation, allowing for more cell divisions and the production of over 15 times more free spores than those produced at 42 °C. Physiological assays showed that spores produced at 25 °C were more resistant to lysozyme, H₂O₂, and heat, more efficient in germination, and more hydrophobic than those produced at 42 °C. The results were surprising because previous research has shown that spores produced at low temperatures are typically less resistant to heat and chemicals than those produced at high temperatures (Melly *et al.*, 2002). This finding may be a unique feature of strain MV19.



CONCLUSIONS

Transmission electron microscopy (TEM) analysis revealed that spores produced by slow-growing cells (25 °C) and fast-growing cells (42 °C) have different coat ultrastructures. Spores produced by slow-growing cells have a structure similar to those produced by the reference strain *B. cereus* ATCC14579 at 20 °C or 37 °C (Bressuire-Isoard *et al.*, 2016). In contrast, the coat of spores produced by fast-growing cells is irregular in shape and around 30% of spores produced by fast-growing cells exhibit an atypical curved morphology and wrinkled surface, indicating a structural defect that likely affects spore characteristics. Therefore, although spore formation is faster and more efficient at the optimal growth temperature of 42 °C, some of the spores produced are structurally and functionally defective. This suggests that the lower speed and efficiency of sporulation at 25 °C is compensated by a higher quality and quantity of spores released.

These observations suggest that temperature during growth and sporulation is a critical environmental factor influencing the structure and functionality of the spores produced, which are extremely stable cellular forms that do not require nutrients or water and are produced as a survival strategy when environmental conditions do not allow cell growth. Therefore, the production of different spores under varying environmental conditions can be considered a 'risk-spreading' strategy to enable the survival and adaptation of spore-formers to diverse environments.



CONCLUSIONS

In *B. subtilis*, delaying spore formation has a negative impact on germination but increases the number of spores produced. This quality-quantity trade-off is believed to contribute to the evolutionary adaptation of spore formers (Mutlu *et al.*, 2018; Mutlu *et al.*, 2020). In *B. cereus* MV19, more cells and fully functional spores are produced at suboptimal growth and sporulation temperatures than at the optimal growth temperature, compensating for the faster growth and efficient sporulation observed at 42 °C. Although the molecular mechanisms regulating this balance are not yet fully understood, several determinants of spore quality have been proposed in *B. subtilis*. These include the electrical polarization of the outer membrane of developing spores (Sirec *et al.*, 2019), the enzyme alanine dehydrogenase (Mutlu *et al.*, 2018), and isoleucyl-tRNA synthase (Kermgard *et al.*, 2017). The genome of MV19 contains homologues of both alanine dehydrogenase (MV19_2153) and isoleucyl-tRNA synthase (MV19_0519), which share 72% and 73.86% identity with *B. subtilis* proteins, respectively. Addressing the role of these spore quality determinants in *B. cereus* would be a relevant future research challenge.



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APPENDIX I

CotG controls spore surface formation in response to the temperature of growth in *Bacillus subtilis*

Giovanni Di Gregorio Barletta[‡], Maria Vittoria[‡],
Mariamichela Lanzilli[†], Claudia Petrillo, Ezio Ricca* and Rachele
Isticato

Department of Biology, Federico II University, Naples Italy;

[†] Institute of Biomolecular Chemistry, National Research
Council of Italy, Pozzuoli, Naples, Italy

*Correspondence: ericca@unina.it;

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CotG controls spore surface formation in response to the temperature of growth in *Bacillus subtilis*

Giovanni Di Gregorio Barletta,[‡] Maria Vittoria,[‡] Mariamichela Lanzilli,[‡] Claudia Petrillo, Ezio Ricca^{✉*} and Rachele Istitico[✉]
Department of Biology, Federico II University, Naples, Italy.

Summary

Bacterial spores of the *Bacillus* genus are ubiquitous in nature and are commonly isolated from a variety of diverse environments. Such wide distribution mainly reflects the spore resistance properties but some *Bacillus* species can grow/sporulate in at least some of the environments where they have been originally isolated. Growing and sporulating at different conditions is known to affect the structure and the resistance properties of the produced spore. In *B. subtilis* the temperature of growth and sporulation has been shown to influence the structure of the spore surface throughout the action of a sporulation-specific and heat-labile kinase CotH. Here we report that CotG, an abundant component of the *B. subtilis* spore surface and a substrate of the CotH kinase, assembles around the forming spore but also accumulates in the mother cell cytoplasm where it forms aggregates with at least two other coat components. Our data suggest that the thermo-regulator CotH contributes to the switch between the coat of 25 °C and that of 42 °C spores by controlling the phosphorylation levels of CotG that, in turn, regulates the assembly of at least two other coat components.

Introduction

Spore formers are mostly Gram-positive bacteria of the *Bacillus* and *Clostridiales* genera sharing the ability to differentiate into a quiescent and resistant cell, the spore, in response to environmental conditions no longer allowing cell growth. The spore can survive almost indefinitely

without water and nutrients and at conditions of extreme temperature and pH, and in the presence of toxic chemicals and lytic enzymes (McKenney et al., 2013). Although quiescent, the spore senses the environment and responds to the presence of nutrients by germinating and forming a new cell able to grow and eventually sporulate (Christie and Setlow, 2020). Spore resistance is in part due to the spore structure, characterized by a dehydrated cytoplasm surrounded by protective layers: a peptidoglycan-like cortex and a proteinaceous and multi-layered coat (McKenney et al., 2013). In some species, such as *Bacillus anthracis* or *Clostridioides difficile*, the outer coat is surrounded by the exosporium, an additional layer formed by proteins and glycoproteins (McKenney et al., 2013). In *B. subtilis*, the model system for spore formers, the coat is surrounded by the crust, an additional layer formed by proteins and glycoproteins, in some respect resembling the exosporium of other species (McKenney et al., 2013).

Spores are ubiquitous in nature and are commonly isolated from almost all environments where they have been searched for, from desert sands and deep sediments, to the gut of animals and insects (Istitico et al., 2020). In some of these diverse environments, such as the animal gut (Cutting, 2011), the rhizosphere of plants (Timmusk et al., 2011) and the deep marine sediments (Cupit et al., 2019), spores of some *Bacillus* species germinate, proliferate and sporulate, indicating a physiological role of aerobic spore formers in those specific environments. The conditions of growth and sporulation influence the structure of the spore (Garcia et al., 2010; Abhyankar et al., 2016) and recent reports have shown that the temperature of sporulation modifies the structure of the spore coat and of the exosporium in *B. cereus* (Bressuire-Isaard et al., 2016) and induces a switch between two alternative types of spore surfaces in *B. subtilis* (Istitico et al., 2020). The latter study identified CotH, an atypical Ser/Tyr kinase, as a major thermo-regulator of the spore surface, more abundant and active at low (25 °C) than at high (42 °C) temperatures and able to induce a switch from the 25 °C to the 42 °C spore surface (Istitico et al., 2020).

In *B. subtilis* the gene coding for the kinase CotH is clustered with the genes coding for two coat proteins that

Received 27 December, 2021; accepted 25 February, 2022. *For correspondence. E-mail: ericca@unina.it; Tel. (+39)081679036; Fax (+39)081679233. †These two authors contributed equally. ‡Present address: Institute of Biomolecular Chemistry, National Research Council of Italy, Pozzuoli, Naples, Italy.

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are the only two CotH substrates, CotB and CotG (Nguyen et al., 2016; Freitas et al., 2020). While the cotB gene follows the 3' end of cotH and is independently transcribed (Donovan et al., 1987), the cotG gene is at the 5' end of cotH but divergently transcribed and entirely contained between the promoter and the coding region of cotH (Giglio et al., 2011). Both cotG and cotH genes are transcribed by SigK-controlled RNA polymerase but are differently regulated by the transcription factor GerE, which acts as an activator of cotG (Sacco et al., 1995) and a repressor of cotH (Naclerio et al., 1996). Although cotG mRNA is complementary to the 5' un-translated part of the cotH mRNA, it does not have a role in cotH expression (Giglio et al., 2011).

CotG is an abundant coat protein of 195 amino acids with a strong positive charge (Sacco et al., 1995) and is listed in the top-20 supercharged proteins present in data banks (Ma et al., 2020). Such positive charge is, at least

Regulation of the *B. subtilis* spore surface 2079

partially, balanced by its extensive and CotH-dependent phosphorylation (Saggese et al., 2014; Nguyen et al., 2016; Freitas et al., 2020). In a *B. subtilis* mutant lacking CotH, unphosphorylated CotG affects the coat assembly of other proteins, such as CotC and CotU (Saggese et al., 2014).

It has been previously reported that cotG is transcribed about twofold more efficiently in cells sporulating at 25 C or 37 C than at 42 C, while cotC, cotU and cotH have similar transcription levels at the three temperatures (Nicolas et al., 2012). In addition, the temperature also controls the stability of the kinase CotH, which is more stable at low than high temperatures (Isticato et al., 2020). As a consequence, at a low temperature (25 C), when the kinase CotH is abundantly present and active, its substrates CotB and CotG are efficiently phosphorylated and assembled on the coat surface contributing to the lamellar-like and electron-dense appearance of 25 C spores (Isticato et al., 2020). At a high temperature (42 C) CotH is less active (or totally inactive), CotB and CotG are less efficiently phosphorylated and assembled on the coat where are replaced by other proteins, including CotC and CotU, which contribute to the granular and thick appearance of the coat of 42 C spores (Isticato et al., 2020). The focus of this study is CotG and its negative effect on the assembly of CotC and CotU. We report that such negative effect is due to the unphosphorylated form of CotG that accumulates in the mother cell cytoplasm and forms aggregates with CotC and CotU. It is proposed that the negative effect observed in a mutant lacking CotH is the exacerbation of a regulatory role of CotG, that in a wild type cell controls the amounts of CotC and CotU assembled around the forming spore in response to the temperature of growth and sporulation.

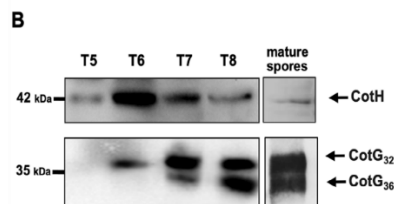
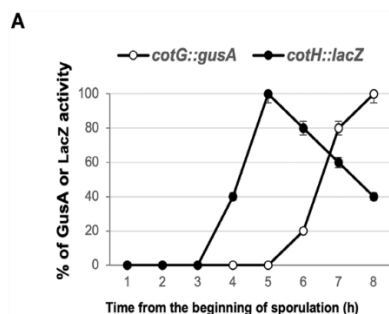


Fig. 1. Timing of production of CotG and CotH during sporulation. **A.** Temporal expression of the cotH (closed symbols) and cotG (open symbols) genes in *B. subtilis* strain GC224, carrying the cotG::gusA and cotH::lacZ fusions. **B.** Detection of CotH and CotG proteins by Western blotting analysis of proteins extracted from wild-type sporulating cells and mature spores reacted against anti-CotH or anti-CotG primary antibodies. Peroxidase-conjugated anti-rabbit secondary antibodies were used to visualize the recognized proteins.

Result and discussion

CotG accumulates in the mother cell cytoplasm

The cotG and cotH genes are both controlled by the sporulation-specific sigma factor of the RNA polymerase SigK in conjunction with the transcriptional factor GerE that activates cotG (Sacco et al., 1995) and represses cotH (Naclerio et al., 1996), presumably regulating the timing of cotG and cotH expression during sporulation. We monitored the expression of both genes simultaneously in sporulating cells of a strain carrying the cotH and cotG genes translationally fused to the lacZ and gusA genes of *E. coli* respectively. As expected, the cotH::lacZ fusion was expressed earlier than the cotG::gusA and its highest levels of expression were observed 5 h after the beginning of sporulation (T5) when cotG expression had not yet started (Fig. 1A). At late sporulation times, cotH expression

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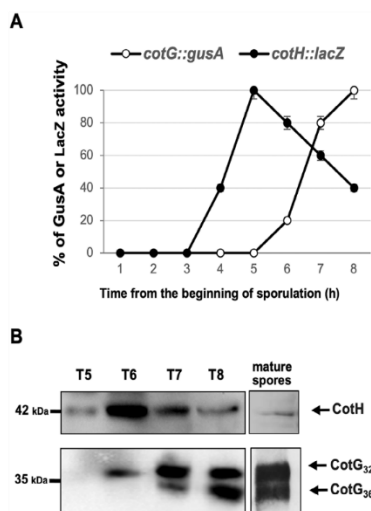


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Result and discussion

CotG accumulates in the mother cell cytoplasm

The cotG and cotH genes are both controlled by the sporulation-specific sigma factor of the RNA polymerase SigK in conjunction with the transcriptional factor GerE that activates cotG (Sacco et al., 1995) and represses cotH (Naclerio et al., 1996), presumably regulating the timing of cotG and cotH expression during sporulation. We monitored the expression of both genes simultaneously in sporulating cells of a strain carrying the cotH and cotG genes translationally fused to the lacZ and gusA genes of *E. coli* respectively. As expected, the cotH::lacZ fusion was expressed earlier than the cotG::gusA and its highest levels of expression were observed 5 h after the beginning of sporulation (T5) when cotG expression had not yet started (Fig. 1A). At late sporulation times, cotH expression

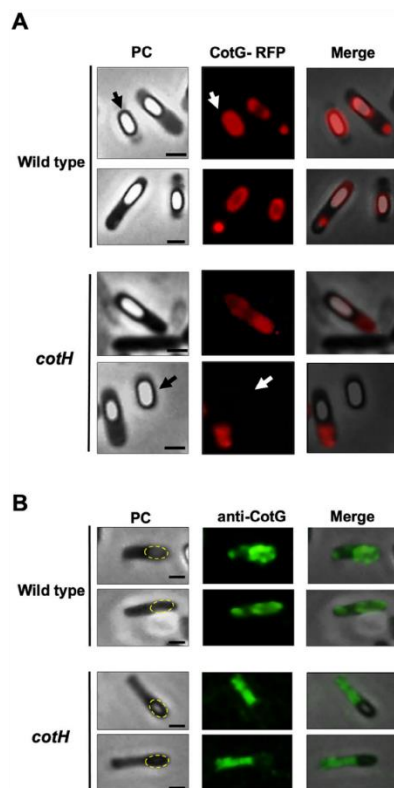


Fig. 2. Fluorescence microscopy detection of CotG. A. Fluorescence of the CotG-RFP fusion was analysed in an otherwise wild type (RH296) and isogenic mutant lacking *coth* (RH298). Arrows indicate mature spores observed by phase-contrast (black arrows) and fluorescence (white arrows) microscopy. B. Immunofluorescence analysis of wild type (PY79) and isogenic *coth* (ER220) mutant sporangia with anti-CotG antibody. For both panels, the percentage of cells showing the indicated fluorescence is reported in Supplementary Material Table S3. Representative microscopy fields are reported for each experiment and strain. PC: phase contrast. The exposure time was 500 ms in all cases. Scale bar 1 μ m.

rapidly declined while *cotG* expression increased (Fig. 1A). Consistently, in a wild type strain [PY79 (Youngman et al., 1984)] the kinase *CotH* was detected in extracts from sporulating cells 5 h after the beginning of sporulation (T5), reached its maximal abundance 1 h later (T6) and then rapidly decreased while *CotG* appeared at T6 and became

abundant at later times (Fig. 1B). As previously reported (Freitas et al., 2020), *CotG* was extracted from prespores and mature spores in two forms, a slower migrating form of about 36 kDa (*CotG*₃₆) and a faster migrating form of about 32 kDa (*CotG*₃₂) (Fig. 1B). *CotG*₃₆ appeared first at T6, and *CotG*₃₂ 1 h later, although was less abundant than *CotG*₃₆ (Fig. 1B). Then, at T8 and in mature spores the two *CotG* forms were similarly represented (Fig. 1B).

The observation that *CotG* is produced and accumulates at late time of sporulation, when the amount of the specific kinase *CotH* is decreasing (Fig. 1), raised the possibility that some *CotG* molecules could be present in sporangia in an unphosphorylated form. Repeated attempts to detect *CotG* by Western blotting in the mother cell cytoplasm were unsuccessful, therefore a fluorescence microscopy approach was followed using strain RH296 of *B. subtilis* carrying the *cotG* gene fused to the gene coding for the red fluorescent protein (RFP) of the coral *Discosoma* sp. (Donadio et al., 2016; Isticato et al., 2020). In an otherwise wild type strain most *CotG*-RFP molecules were localized around the forming spore (Fig. 2A); however, some *CotG*-RFP molecules were also observed in the mother cells, concentrated in well-defined small spots (Fig. 2A). In an isogenic strain lacking the kinase *CotH* (RH298), *CotG* is not phosphorylated and not assembled around the forming spore (Freitas et al., 2020); as a consequence a strong fluorescence signal (*CotG*-RFP) almost completely filled the mother cell cytoplasm (Fig. 2A). The dependency of *CotG*-RFP accumulation in the mother cell cytoplasm on *CotH* (Fig. 2A) makes it unlikely that the fluorescence observed in the mother cell of a wild type strain (Fig. 2A) was due to RFP alone (i.e. due to RFP cleaved off from *CotG*). However, to confirm this point, an immunofluorescence microscopy analysis was performed with anti-*CotG* antibody. As shown in Fig. 2B, *CotG*-specific fluorescence signals were observed around the forming spore and in the mother cell cytoplasm of a wild type strain, where they formed well-defined spots similar to those observed with *CotG*-RFP (Fig. 2A). In a mutant lacking *CotH* all *CotG*-dependent fluorescence was found in the mother cell cytoplasm (Fig. 2B).

Results of Figs 1 and 2 indicate that in a wild type strain some *CotG* molecules do not assemble around the spore and accumulate in the mother cell cytoplasm in well-defined spots. In a mutant lacking *CotH* such accumulation is massive and obviously formed by unphosphorylated *CotG*.

CotG is mostly unphosphorylated in the mother cell cytoplasm of wild type sporangia

To verify whether the *CotG* molecules present in the mother cell of a wild type strain were phosphorylated,

immunofluorescence experiments were performed with anti-PKC antibody (Cell Signalling Technology), recognizing phosphorylated serine residues surrounded by a lysine or an arginine. In a wild type strain (PY79) a strong fluorescence signal was observed associated to the forming spore while only a modest and diffuse signal was visible in the mother cell cytoplasm (Fig. 3). No fluorescence was observed in a mutant lacking *CotH* (AZ607), indicating that the fluorescence in wild type sporangia was only due to the kinase activity of *CotH* (Fig. 3). In a mutant lacking *CotB* (AZ668), the other substrate of the kinase *CotH*, fluorescence signals were only due to the *CotH*-dependent phosphorylation of *CotG* and appeared mainly present around the forming spore (Fig. 3). In sporangia of strain AZ668 only a minimal part of the fluorescence (due to phosphorylated *CotG* molecules) was found in the cytoplasm, never forming the well-defined fluorescence spot observed in Fig. 2.

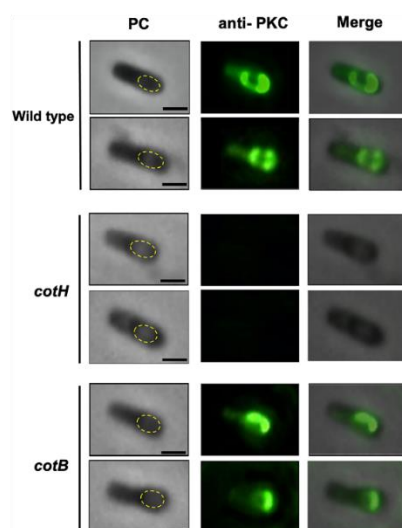


Fig. 3. Fluorescence microscopy detection of phosphorylated *CotG*. Immunofluorescence analysis of sporangia of wild type (PY79) and isogenic mutants lacking *CotH* (AZ607) and *CotB* (AZ668) with anti-PKC antibody. For all panels, the percentage of cells showing the indicated fluorescence is reported in Supplementary Material Table S3. Representative examples are reported by phase-contrast (PC) and fluorescence microscopy and by merging the images (Merge). In PC panels dotted yellow ovals indicate the position of the forming spore. The exposure time was 200 ms in all cases. Scale bar 1 μ m.

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Based on these results, we infer that in a wild type strain most *CotG* molecules are phosphorylated and assembled around the spore, the remaining *CotG* molecules are mostly unphosphorylated and form well-defined spots in the mother cell cytoplasm.

Unphosphorylated *CotG* forms aggregates with *CotC* and *CotU*

Accumulation of massive amounts of unphosphorylated, supercharged *CotG* in the mother cell cytoplasm of a mutant strain lacking *CotH* (Fig. 2A) could be the cause of the mis-assembly of the coat proteins *CotC* and *CotU* previously observed in mature spores of a *CotH* mutant strain (Saggese et al., 2014). To verify such hypothesis, immunofluorescence microscopy experiments were performed with anti-*CotC* antibody, known to recognize both *CotC* and *CotU* proteins (Isticato et al., 2004; Isticato et al., 2008). In a wild type strain (PY79), fluorescence signals were mostly observed around the forming spore and only a weak fluorescence was localized in the mother cell (Fig. 4). Fluorescence signals similar to those observed in wild type sporangia but of reduced intensity were also observed in mutants lacking *CotC* (RH101) or *CotU* (RH202) (Fig. 4), indicating that the fluorescence observed in wild type sporangia was due to both *CotC* and *CotU*.

In a mutant lacking *CotH* (AZ607) the *CotC/CotU*-dependent fluorescence signals appeared differently distributed between the forming spore and mother cell with respect to the wild type strain, with a stronger signal in the mother cell cytoplasm and weaker signal around the spore than in the isogenic wild type strain (Fig. 4). The weak assemblage of *CotC/CotU* around the forming spore is however transient, since no *CotC/CotU* are then observed around mature spores of a mutant lacking *CotH* (Saggese et al., 2014). Therefore, in a mutant lacking *CotH* both unphosphorylated *CotG* (Fig. 2) and *CotC/CotU* (Fig. 4) accumulated in the mother cell cytoplasm in well-defined spots. Such effect is *CotG*-dependent since it was not observed in a mutant lacking *CotG* (AZ604) (Fig. 4).

A fluorescence microscopy analysis of the *B. subtilis* strain RH406, carrying the RFP fused to *CotG* and the green fluorescent protein (GFP) fused to *CotC* (Isticato et al., 2020) confirmed that both *CotC* and *CotG* behave similarly and showed that the two proteins co-localize in the mother cell cytoplasm (Fig. 5). In an otherwise wild type strain both *CotG*-RFP and *CotC*-GFP were mostly localized around the forming spores but were also present in the mother cell cytoplasm (Fig. 5). When *CotH* was not present, only few *CotG*-RFP and *CotC*-GFP molecules were around the forming spore while most of them failed to assemble around the spore and were co-localized in aggregates in the mother cell cytoplasm (Fig. 5).

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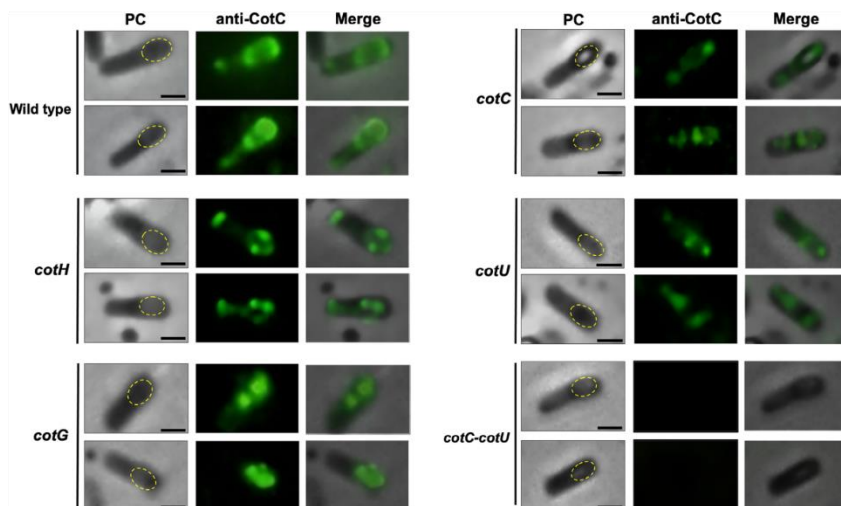


Fig. 4. Fluorescence microscopy detection of CotC and CotU. Immunofluorescence analysis of sporangia of wild type and isogenic mutants lacking *cotH* (AZ607), *cotG* (AZ604), *cotC* (RH101), or *cotU* (RH202) with anti-CotC antibody. For all panels, the percentage of cells showing the indicated fluorescence is reported in Supplementary Material Table S3. Representative examples are reported by phase-contrast (PC) and fluorescence (anti-CotC) microscopy and by merging the images (Merge). In PC panels dotted yellow ovals indicate the position of the forming spore. The exposure time was 1000 ms in all cases. Scale bar 1 μ m.

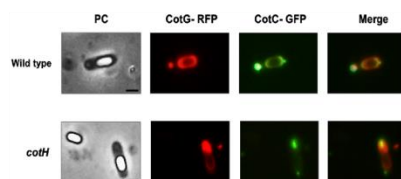


Fig. 5. Fluorescence microscopy detection of CotG-RFP and CotC-GFP. The two gene fusions, *cotG::rfp* and *cotC::gfp*, were analysed in an otherwise wild type (PY79) and in an isogenic mutant lacking *cotH* (AZ607). For all panels, the percentage of cells showing the indicated fluorescence is reported in Supplementary Material Table S3. Representative examples are reported for each experiment and strain. The exposure time was 500 ms for both fusions. Scale bar 1 μ m.

The behaviour observed with CotC/CotU was not observed with other coat proteins, independently from their dependence on CotG in a mutant lacking CotH (Saggese et al., 2014). Indeed, CotS (affected by CotG), CotA and CotZ (not affected by CotG) (Saggese et al., 2014) were all found assembled around the

forming spore with no fluorescence signals in the mother cell cytoplasm, both in a wild type and in a strain bearing a *cotH* null mutation (Fig. 6). Results of Fig. 6 then suggest that the formation of CotG-dependent aggregates is not common to all coat proteins but rather a specific property of CotC and CotU.

CotG controls the assembly of CotC and CotU in wild type sporangia in response to the temperature

A fluorescence microscopy analysis of *B. subtilis* strains carrying the *cotC::gfp* or the *cotG::rfp* fusion was performed on sporangia grown at 25 C and 42 C. Since the temperature affects the growth rate, the entry into the sporulation cycle and the time of spore formation, sporangia of similar age were produced at the different temperatures as previously described (Isticato et al., 2020). As shown in Fig. 7A, CotC and CotG molecules showed an opposite behaviour in sporangia grown at 25 C or 42 C. At 25 C CotG-RFP was abundant around the spore and formed well-defined spots in the cytoplasm while CotC-GFP was almost absent around the spore and localized in well-defined spots in the cytoplasm (Fig. 7A). At 42 C CotG-RFP was almost absent

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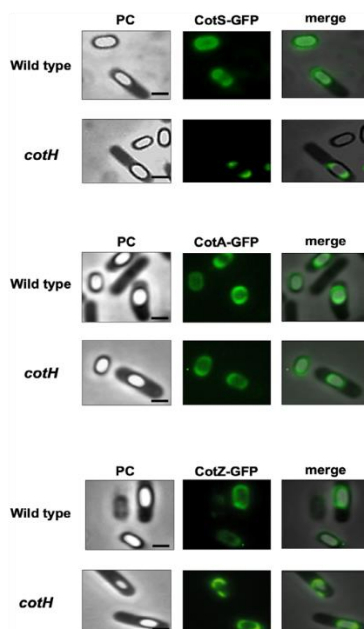


Fig. 6. Fluorescence microscopy detection of CotS, CotA and CotZ. *cotS::gfp*, *cotA::gfp* and *cotZ::gfp* fusions in an otherwise wild type strain (AZ644, AZ565 and AZ573 respectively) and in an isogenic mutant lacking *coth* (AZ647, RH240 and RH278 respectively) analysed by fluorescence microscopy. The percentage of cells showing the indicated fluorescence is reported in Supplementary Material Table S3. Representative examples are reported for each experiment and strain. The exposure time was 500 ms in all cases. Scale bar 1 μ m.

around the spore and formed well-defined spots in the cytoplasm while CotC-GFP was abundant around the spore and in minimal amounts in the cytoplasm (Fig. 7A). At 42 C CotG-RFP appeared less abundant than at 25 C, suggesting a reduced production of the protein. To clarify this point an isogenic strain carrying a translational *cotG::lacZ* fusion (Sacco et al., 1995) was grown at 25 C, 37 C and 42 C. As shown in Fig. 7B, the expression of the *cotG* genes was highest at 25 C, about two-fold lower at 37 C and about threefold lower at 42 C, in agreement with a previous transcriptomic study (Nicolas et al., 2012).

To evaluate the phosphorylation status of CotG at different temperatures, an immunofluorescence analysis with anti-PKC on sporangia growing at 25 C and 42 C was performed. At low temperature CotG around the spore appeared mostly phosphorylated while in the mother cell cytoplasm was mostly unphosphorylated (Fig. 7C), similarly to what was observed at 37 C (Fig. 3). As expected by the low activity of the kinase CotH at 42 C (Isticato et al., 2020), at high temperature only a minimal amount of CotG was phosphorylated and exclusively found around the forming spore (Fig. 7C).

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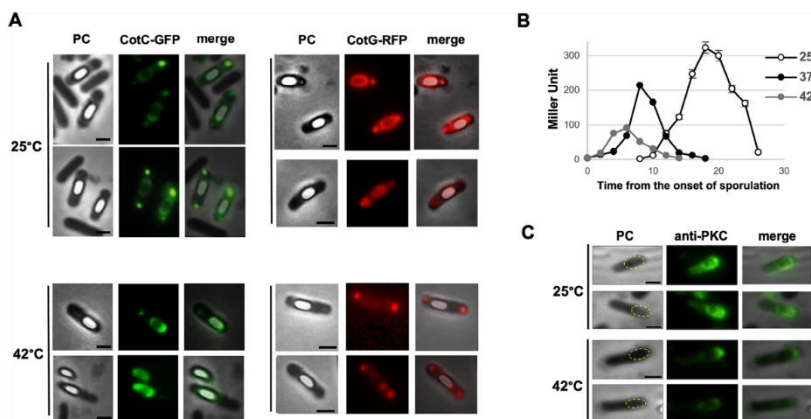


Fig. 7. Effect of the sporulation temperature on CotG and CotC assembly. A. Fluorescence microscopy analysis of strains carrying the *cotC::gfp* (RH238) or *cotG::rfp* (RH296) fusion in sporulating cells produced at 25°C or 42°C. The percentage of cells showing the indicated fluorescence is reported in Supplementary Material Table S3. Representative examples are reported for each experiment and strain. The exposure time was 500 ms in all cases. Scale bar 1 μ m. B. *cotG*-directed β -galactosidase synthesis during sporulation at 25°C, 37°C and 42°C. Due to the reduced growth rate at 25°C, cells were collected at late times according to Isticato et al. (2020). C. Immunofluorescence analysis of wild type (PY79) sporangia growing at 25°C or 42°C with anti-PKC antibody. The percentage of cells showing the indicated fluorescence is reported in Supplementary Material Table S3. Representative examples are reported by phase-contrast (PC) and fluorescence microscopy and by merging the images (Merge). In PC panels dotted yellow ovals indicate the position of the forming spore. The exposure time was 200 ms in all cases. Scale bar 1 μ m.

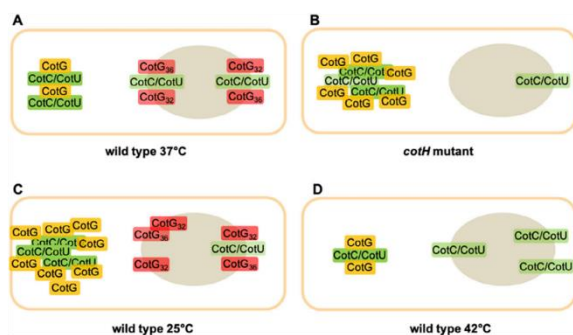


Fig. 8. Working model of CotG, CotC and CotU assembly at 37°C (A), in a mutant lacking CotH (B) and in a wild type strain at 25°C (C) or 42°C (D). Phosphorylated and unphosphorylated CotG molecules are boxed in red and yellow respectively.

Altogether, results of Fig. 7 suggest that at 25°C unphosphorylated CotG molecules accumulate in the cytoplasm sequestering most CotC (and presumably CotU) molecules in well-defined cytoplasmic spots and

thus impairing their assembly around the spore. At 42°C less CotG molecules are produced, a minimal amount is phosphorylated and assembled around the spore while the remaining part is unphosphorylated and forms



aggregates with CotC (and presumably CotU), slightly reducing their assembly around the spore.

A model to explain the assembly of CotG and its regulatory role on the assembly of CotC/CotU is proposed in Fig. 8. In a wild type strain growing at 37 °C, CotG is readily phosphorylated by CotH originating CotG₃₆ and CotG₃₂ (boxed in red in Fig. 8A). Some CotG molecules produced later in sporulation, when the kinase is no longer synthesized, are not phosphorylated and accumulate in the mother cell cytoplasm (boxed in yellow in Fig. 8A), where they form aggregates with CotC/CotU. As a result, both CotG and CotC/CotU are found around the spore and in the cytoplasm with CotG localization depending on its phosphorylation level (and therefore on CotH) and CotC/CotU localization depending on the level of unphosphorylated CotG (Fig. 8A). Under unphysiological conditions (in a mutant lacking CotH), all CotG molecules are unphosphorylated, massively accumulate in the cytoplasm sequestering all CotC/CotU molecules (Fig. 8B), and causing the previously reported negative effect of CotG on CotC/CotU assembly (Saggese et al., 2014). At 25 °C CotG is abundant both around the spore and in the cytoplasm, where it sequesters a large part of the CotC/CotU molecules limiting their assemblage around the spore (Fig. 8C). At 42 °C less CotG is produced but being almost completely unphosphorylated, does not assemble and accumulates in the cytoplasm sequestering some CotC/CotU molecules and thus regulating their assembly around the spore (Fig. 8D).

Conclusions

The main results in this manuscript are that *B. subtilis* controls by CotH-mediated phosphorylation the assembly of CotG and that unphosphorylated CotG accumulates in the mother cell cytoplasm forming aggregates with CotC and CotU. These results provide a possible mechanism to explain how *B. subtilis* builds different spores at different temperatures with those produced at 25 °C being lamellar and heavily electron dense and those produced at 42 °C appearing granular and thick (Isticato et al., 2020).

It has been previously proposed that CotH, a thermo-labile kinase, phosphorylates CotG and CotB that are then readily assembled on the forming spores and contribute to the structure/function of the 25 °C spore (Isticato et al., 2020). At 42 °C CotH is almost totally absent/inactive, its phosphorylation targets, CotG and CotB, are almost undetectable on the spore surface where are replaced by other coat components, including CotC and CotU (Isticato et al., 2020). Results reported here suggest that unphosphorylated CotG forms aggregates with CotC and CotU, sequesters them in the mother cell cytoplasm and renders them unavailable for coat

assemblage. Since the level of phosphorylated/assembled or unphosphorylated/unassembled CotG depends on the action of the thermo-sensor CotH, a hierarchical model is proposed to explain the effects of the temperature on the formation of the spore surface, with the temperature acting on cotG transcription (by still unexplored mechanism) and on the thermo-labile kinase CotH that controls its substrate CotG that, in turn, controls the assembly of CotC/CotU.

The unphosphorylated CotG accumulated in the cytoplasm has a strong positive charge [Net Charge Density (NCD) = 0.30 as defined by Ma et al., 2020] that may be responsible for the formation of the cytoplasmic aggregates. This hypothesis is supported by the previous observation that a mutant form of CotG, deleted of the central region where most of the positively charged amino acids are concentrated, and showing an NCD = 0.15, does not have any negative effect on the assembly of CotC/CotU (Saggese et al., 2016).

CotG of *B. subtilis* is not highly conserved but a CotG-like protein, with a positive charge and a modular structure similar to those of CotG, is common in almost all *Bacillus* species containing a CotH orthologue (Saggese et al., 2016). CotG and CotH are functionally linked to each other and their coding genes have a conserved organization with cotG entirely contained between the promoter and the coding part of cotH (Giglio et al., 2011), suggesting that the two proteins may have a similar role in the structure and function of the *Bacillus* spore. Understanding whether the CotH-CotG system responds to the temperature and influences the spore surface structure also in other *Bacillus* species, confirming the role of the positive charge of CotG in the formation of aggregates and clarifying the mechanisms by which cotG transcription is controlled by the temperature will all be challenging tasks of future research projects.

Experimental procedures

Bacterial strains and molecular procedures

Bacillus subtilis strains are listed in Table S1 and are all isogenic derivatives of strain PY79 (Youngman et al., 1984). Plasmid amplification for nucleotide sequencing, subcloning experiments, and transformation of *Escherichia coli* competent cells were performed with *E. coli* strain DH5α. Bacterial strains were transformed by previously described procedures: CaCl₂-mediated transformation of *E. coli* cells (Sambrook et al., 1989), competent cells and two-step transformation of *B. subtilis* (Cutting and Vander Horn, 1990). DNA ligation, isolation of plasmids and restriction digestion were performed by using standard methods. Chromosomal DNA extraction



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of *B. subtilis* was carried out as described previously (Cutting and Vander Horn, 1990).

B. subtilis strain construction

- strain expressing cotG::gusA and coth::lacZ fusions

A 406 bp DNA fragment containing the cotG promoter region and the region coding for 40 amino acids (from the N-terminal end) was PCR amplified using chromosomal DNA of the PY79 strain as a template and priming the reaction with oligonucleotides H2 and G2 (Table S2). The PCR product was cloned in the pTOPO vector (Invitrogen) yielding plasmid pFV200. The gusA gene (1812 bp) was excised from the pMLK83 plasmid (Karow and Piggot, 1995) by EcoRI digestion and cloned in pFV200 linearized by EcoRI digestion in frame with cotG codons, yielding plasmid pFV201. The gene fusion was then moved into plasmid pER82 (Ricca et al., 1992) by subcloning into the BamHI restriction site. The resulting plasmid, pFV202, carried the gene fusion and a kanamycin-resistance gene between two parts of the amyE gene of *B. subtilis*. This plasmid was analysed by restriction analysis, linearized by digestion with PstI and used to transform competent cells of the *B. subtilis* strain PY79. Kanamycin-resistant clones were the result of a double-crossover recombination event, resulting in the interruption of the non-essential amyE gene on the *B. subtilis* chromosome. All Kanamycin-resistant clones were tested by PCR using chromosomal DNA as a template and oligonucleotides AmyS and AmyA (Table S2) to prime DNA amplification. One positive clone (GC223) was used to extract the chromosomal DNA that was used to transform competent cells of strain GC222 carrying the coth::lacZ fusion integrated at the coth locus (Baccigalupi et al., 2004) and yielding strain GC224 (coth::lacZ cotG::gusA).

- strain expressing the cotG::rfp fusion in a mutant lacking CotG and Coth

Chromosomal DNA-mediated transformation of competent cells of the *B. subtilis* strain AZ603 (Δ cotG Δ coth::neo) (Saggese et al., 2014) with chromosomal DNA of *B. subtilis* strain RH296 (amyE::cotG::rfp) (Petrillo et al., 2020) was used to yield strain RH298 (Δ cotG Δ coth::neo amyE::cotG::rfp).

- strain expressing the cotG::rfp and cotC::gfp fusions in a mutant lacking CotG and Coth

Chromosomal DNA-mediated transformation of competent cells of the *B. subtilis* strain RH406 (amyE::cotG::rfp cotC::gfp) (Petrillo et al., 2020) with chromosomal DNA of *B. subtilis* strain AZ603 (Δ cotG Δ coth::neo) (Saggese et al., 2014) was used to yield strain AZ720 (Δ cotG Δ coth::neo amyE::cotG::rfp cotC::gfp).

- strain expressing the cotA::gfp in a mutant lacking Coth

Chromosomal DNA-mediated transformation of competent cells of the *B. subtilis* strain AZ565 (cotA::gfp) (Isticato et al., 2013) with chromosomal DNA of *B. subtilis* strain AZ607 (Δ cotG Δ coth::neo amyE::cotG) (Saggese et al., 2014) was used to yield strain RH240 (Δ cotG Δ coth::neo amyE::cotG cotA::gfp).

β -Galactosidase and β -glucuronidase assay

The specific β -galactosidase activity was determined using o-Nitrophenyl- β -Galactoside as substrate. One millilitre of coth::lacZ-bearing cells was collected, during sporulation, at the times indicated, and assayed as previously described (Ricca et al., 1992). The specific β -glucuronidase activity was determined using p-Nitrophenyl- β -D-Glucuronide as substrate, as previously reported (Melville et al., 1994). One millilitre of cotG::gusA-bearing cells was collected, during sporulation, at the times indicated, and assayed as previously described (Melville et al., 1994).

Spore coat proteins extraction and Western blot analysis

Sporulation of wild-type and recombinant strains was induced in Difco sporulation medium at 25 C, 37 C or 42 C, with vigorous shaking. During sporulation, at different times, forespores or mature spores were collected as described in Isticato et al. (2004) and washed four times with distilled water and purified as described in Nicholson and Setlow (1990). Spore coat proteins were extracted from a suspension of 1×10^9 mature spores or from a suspension of forespores fractions, by treatment at 65 C in SDS-DTT. The extracted proteins were quantified through a Bio-Rad DC protein assay kit (Bio-Rad), then 20 μ g of total spore coat proteins were fractionated on 15% polyacrylamide gels and electro-transferred on a nitrocellulose filter (Bio-Rad) for Western blot analysis. Monoclonal antibodies were used at working solution of 1:7.000 for CotG or 1:150 for Coth specific detection. Then, a horseradish peroxidase-conjugated anti-rabbit secondary antibody was used (Santa Cruz). Western blot filters were visualized by the SuperSignal West Pico chemiluminescence (Pierce) method as specified by the manufacturer.

Fluorescence and immunofluorescence microscopy

Fluorescence microscopy analyses were carried out using an Olympus BX51 fluorescence microscope fitted with a 100 objective UPlanF1. FITC (U-MNIB2) and TRITC (U-MWIG2) filters were used to detect the green or red fluorescence signals respectively. Images were captured using an Olympus DP70 digital camera equipped with Olympus U-CA Magnification Changer and



processed with Image Analysis Software (Olympus) for minor adjustments of brightness, contrast and colour balance and for creation of merged images. SmillaEnlarger 0.9.0 software was used to increase images quality. Immunofluorescence assay was performed as described in Manzo et al. (2013), with some modifications. One millilitre of sporulating cells was fixed for 1 h at room temperature in 80% methanol, then washed and incubated overnight at 4 °C in 100% methanol. Fixed sporangia were permeabilized by incubating samples with a GTE (5% glucose, 0.01 M EDTA pH 8.0, 20 mM Tris-HCl pH 7.5) – 0.2 mg ml⁻¹ lysozyme solution and rapidly placed on a coverslip previously treated for 5 min with poly-L-lysine. The coverslips were air dried, and cells were pretreated with 1% (wt/vol.) dried milk in phosphate-buffered saline (PBS, pH 7.4), prior to 2 h incubation at 4 °C with the monoclonal anti-CotC (1:200), anti-CotG (1:500) or anti-PKC (1:500) primary antibodies. After four washes with PBS, the samples were incubated with a 64-fold diluted anti-rabbit secondary antibody, conjugated with Fluorescein isothiocyanate, FITC (BETHYL) for 2 h at room temperature in the dark. After four washes, the coverslips were mounted onto microscope slides, adding one drop of PBS, and analysed by fluorescence microscopy, as described below. Sporangia with only addition of the secondary-FITC conjugated antibody were used as a control of the specificity of this technique.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supplementary Information.



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APPENDIX II

CotG mediates spore surface permeability in *Bacillus subtilis*

Anella Saggese^{a,1} Giovanni Di Gregorio Barletta^{a,1} Maria Vittoria^a
Giuliana Donadio^c, Rachele Isticato^a, Loredana Baccigalupi^b,
Ezio Ricca^{a*}

a Department of Biology, Federico II University, Naples, Italy

b Department of Molecular Medicine and Medical Biotechnology,
Federico II University, Naples, Italy

c Department of Pharmacy, University of Salerno, Italy

*Correspondence: ericca@unina.it;

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RESEARCH ARTICLE



CotG Mediates Spore Surface Permeability in *Bacillus subtilis*

Anella Saggese,^a Giovanni Di Gregorio Barletta,^a Maria Vittoria,^a Giuliana Donadio,^{a,*} Rachele Isticato,^a Loredana Baccigalupi,^b Ezio Ricca^a

^aDepartment of Biology, Federico II University, Naples, Italy

^bDepartment of Molecular Medicine and Medical Biotechnology, Federico II University, Naples, Italy

Anella Saggese and Giovanni Di Gregorio Barletta contributed equally to this work. Anella Saggese was placed first in the byline since she worked on the project the longest.

ABSTRACT Proteins and glycoproteins that form the surface layers of the *Bacillus* spore assemble into semipermeable arrays that surround and protect the spore cytoplasm. Such layers, acting like molecular sieves, exclude large molecules but allow small nutrients (germinants) to penetrate. We report that CotG, a modular and abundant component of the *Bacillus subtilis* spore coat, controls spore permeability through its central region, formed by positively charged tandem repeats. These repeats act as spacers between the N and C termini of the protein, which are responsible for the interaction of CotG with at least one other coat protein. The deletion but not the replacement of the central repeats with differently charged repeats affects the spore resistance to lysozyme and the efficiency of germination—probably by reducing the coat permeability to external molecules. The presence of central repeats is a common feature of the CotG-like proteins present in most *Bacillus* species, and such a wide distribution of this protein family is suggestive of a relevant role for the structure and function of the *Bacillus* spore.

IMPORTANCE Bacterial spores are quiescent cells extremely resistant to a variety of unphysiological conditions, including the presence of lytic enzymes. Such resistance is also due to the limited permeability of the spore surface, which does not allow lytic enzymes to reach the spore interior. This article proposes that the spore permeability in *B. subtilis* is mediated by CotG, a modular protein formed by a central region of repeats of positively charged amino acid acting as a “spacer” between the N and C termini. These, in turn, interact with other coat proteins, generating a protein layer whose permeability to external molecules is controlled by the distance between the N and C termini of CotG. This working model is most likely expandable to most sporeformers of the *Bacillus* genus, since they all have CotG-like proteins, not homologous to CotG of *B. subtilis* but similarly characterized by central repeats.

KEYWORDS *Bacillus subtilis*, endospores, germination, permeability, spore coat

Bacteria of the *Bacillus* genus produce (endo)spores, metabolically quiescent and extremely resistant cells (1). Spores persist almost indefinitely in environments without nutrients, under conditions of extreme temperature and pH, and in the presence of toxic chemicals and lytic enzymes (2). Although quiescent, spores constantly sense the environment and respond to the presence of nutrients by germinating and forming new vegetative cells (3).

In *Bacillus subtilis*, the spore core, the dehydrated cytoplasm and its membrane, is surrounded and protected by a peptidoglycan-like cortex, a proteinaceous and multilayer coat and the crust, formed by proteins and glycoproteins (2). The coat and crust form a rigid but semipermeable shell around the core, providing mechanical integrity, excluding large molecules from reaching the spore interior but allowing the transit of germinants (4). Some heterologous molecules can adsorb the spore surface and infiltrate through its surface layers without reaching the cortex or core (5). This property, which has biotechnological potential (6), depends on the physicochemical properties of both the heterologous molecules and

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Address correspondence to Ezio Ricca, ericca@unina.it.

*Present address: Giuliana Donadio, Department of Pharmacy, University of Salerno, Italy.

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TABLE 1 Structural properties of CotG of *B. subtilis* and *B. licheniformis* and the hybrid protein

Species	Protein	No. (%) of aa			Value by:		
		Total	Positively charged	Negatively charged	Total charge ^a	Instability index ^b	Unfoldability index ^c
<i>B. subtilis</i>	CotG full protein	195	92 (47.8)	13 (6.6)	1 57.0	77.5	2 0.722
	CotG with internal repeats deleted	126	68 (53.9)	0	1 52.9	90.5	2 0.908
<i>B. licheniformis</i>	CotG full protein	168	67 (39.8)	32 (19.0)	1 20.0	26.2	2 0.536
	CotG with internal repeats deleted	73	36 (49.3)	16 (21.9)	1 13.8	2 6.9	2 0.815
<i>B. subtilis</i> (recombinant)	CotGHyb full protein	142	60 (42.2)	29 (20.4)	1 17.8	22.5	2 0.72

^aAt pH 7.4 (<https://www.protpi.ch/Calculator/ProteinTool/>).

^bThe instability index is an estimate of the stability of a protein. A protein with an index smaller than 40 is predicted as stable in vitro. The reported values were calculated with an online tool (<https://web.expasy.org/protparam/>).

^cDisorder prediction was done by using an online tool (<https://fold.proteopedia.org/cgi-bin/findex>). Positive values indicate a structured polypeptide, whereas negative values indicate a disordered protein.

the spore structure (7). Indeed, structurally different spores, such as, for example, those produced by *B. subtilis* at different temperatures, show different surface permeabilities (7).

B. subtilis spore coats produced at low or high temperature are remarkably different, with 25°C coats appearing lamellar and heavily electron dense and 42°C spores appearing granular and thick (8). The switch between the different structures is mediated by CotH, a heat-labile (8) regulatory protein known to control at least nine other coat proteins (9) and to directly interact with the major coat morphogenetic protein CotE (10). CotH is an atypical Ser/Tyr kinase that specifically phosphorylates two major outer coat proteins, CotB and CotG (11, 12). The latter is a highly abundant coat protein (13) localized in the outermost spore surface layer (14) and characterized by a modular structure with a central part formed by tandem repeats of positively charged amino acids (15).

CotG has a high net charge and is listed in the top 20 positively supercharged proteins present in data banks (16). Such a high charge is partially balanced by CotG's extensive, CotH-dependent phosphorylation (12, 17), essential for CotG assembly on the spore coat (18). Unphosphorylated, and presumably unfolded, CotG molecules do not assemble on the spore and form inclusion body-like aggregates in the mother cell cytoplasm (18). In a mutant strain lacking the kinase CotH, all CotG molecules are unphosphorylated, form massive aggregates that sequester other coat proteins, and as a consequence, cause the production of defective spores (17, 18). Such altered spores are not observed in a mutant lacking the kinase CotH but also with the central repeats of CotG deleted, indicating that the formation of cytoplasmic aggregates is due to the central part of CotG (17, 18).

The modular organization of CotG, with a central region formed by 19 tandem repeats of 6 and 7 residues, is an outcome of multiple rounds of gene elongation events of an ancestral minigene (19). Similar events presumably started from different ancestral minigenes in different *Bacillus* species, since most species contain a CotG-like protein that is not homologous to CotG of *B. subtilis* but shares with it the chromosomal organization, the modular structure, the presence of central repeats, and in most cases, the positive charge (19).

The focus of this report is the function of the central repeats of CotG. The analysis of mutant forms of CotG, in which the central repeats were either deleted or replaced by the central repeats of CotG of *Bacillus licheniformis*, suggests that the positively charged central region regulates the permeability of the spore surface, acting as a spacer between the N and C termini of the protein.

RESULTS

The positive charge of CotG is mostly due to its central repeats. The net positive charge of CotG is due to the presence of 92 (47%) positively charged (K, R, and H) amino acids and only 13 (6%) negatively charged (D and E) residues. In addition, CotG has a low number (18 [9%]) of hydrophobic residues (A, G, I, L, M, P, F, W, and V), which contribute to making it an intrinsically disordered (IDP) and unstable protein (Table 1) (15). The extremely



high positive net charge of CotG is partially compensated for in vivo by the extensive phosphorylation occurring at 15 residues, mostly localized in the central repeats of the protein, which are likely relevant also for CotG folding (see Fig. S1A in the supplemental material) (12, 17). The central repeats of CotG have a strong role in the total positive charge and in the disordered status of the entire protein. Indeed, the 19 repeats are formed by 126 amino acids (aa), with 68 (53.9%) positive residues and no negatively charged ones (Table 1).

While most members of the *Bacillus* genus have a modular CotG-like protein that is positively charged and unstable (15), *B. licheniformis* has a weakly positive and stable CotG-like protein, as suggested by its instability index of , 40 (Table 1). The central repeats of the *B. licheniformis* protein (Fig. S1B) contribute to these parameters, with 16 negatively charged amino acids and a negative instability index (Table 1).

In order to analyze the role of the central repeats of CotG of *B. subtilis*, the central repeats of *B. licheniformis* were used to replace the repeats of the *B. subtilis* protein (see Materials and Methods). The resulting hybrid protein of 142 aa (17.0 kDa) (Fig. S1C) is characterized by a modestly positive net charge and is predicted to be a stable protein (instability index of 22.5) (Table 1). The gene fusion coding for the hybrid protein (GHyb), constructed in *Escherichia coli* (Materials and Methods) was integrated on the chromosome of *B. subtilis* strain AZ604, lacking the wild-type (WT) copy of *cotG*; therefore, in the resulting strain, AZ717, GHyb was the only form of CotG present and its expression was controlled by transcription and translation signals of the *B. subtilis* *cotG* gene (Materials and Methods).

The hybrid CotG is functional. To verify whether GHyb was functional, Western blotting experiments were performed on proteins extracted from purified spores of a wild-type strain and strains expressing GHyb with and without the kinase CotH. Anti-CotG antibody, raised against the N-terminal 14 residues of *B. subtilis* CotG (18), and anti-protein kinase C (anti-PKC) antibody, recognizing phosphorylated serine residues (18), were reacted against the extracted proteins. As previously reported, CotG was extracted from wild-type spores in two forms (CotG₃₅ and CotG₃₂), and both proteins were not found in extracts of spores of a strain lacking CotH (12, 20) (Fig. 1A). GHyb was instead extracted from spores independently from the presence of the kinase CotH (Fig. 1A). A faster-migrating protein corresponding to the predicted size of GHyb (17 kDa) was extracted from spores of both strains (Fig. 1A). Slower-migrating proteins were also extracted from both strains: a single protein with an apparent molecular weight of 20 kDa from a strain lacking the kinase (red star in Fig. 1A) and two proteins, both bigger than 20 kDa, in the strain expressing the kinase (yellow stars in Fig. 1A). When the anti-PKC antibody was used, no signals were observed in spores of strains lacking the kinase, indicating that all observed signals were due to CotH activity (Fig. 1B). The wild type and a *cotG* null mutant of *B. subtilis* showed a pattern of phosphorylated proteins consistent with that previously reported (12), with mature CotB (CotB₆₆) and CotG in the wild-type strain and with the immature form of CotB (CotB₃₆) in the *cotG* mutant (Fig. 1B). From the strain expressing the hybrid protein (and CotH), three proteins corresponding in size to those reacting with anti-CotG antibody in Fig. 1A and a protein corresponding in size to the mature form of CotB were observed (Fig. 1B). The additional proteins, smaller than CotB and CotG, which were also observed in Fig. 1B, were considered degradation products of CotB or CotG, since they were only recognized by anti-PKC antibody. In addition, similar proteins have been previously identified as degradation products (12).

The preliminary indication that the GHyb allowed CotB maturation (Fig. 1B) was confirmed by Western blotting with anti-CotB antibody. As previously established (12), the conversion of the immature form of CotB (CotB₃₆) into its mature form (CotB₆₆) requires the kinase activity of CotH and the presence of CotG. CotGHyb was able to complement the absence of CotG and allowed CotB maturation (Fig. 1C). Therefore, like a CotG form with its central repeats deleted (C^{central}) (15), GHyb also allows CotB maturation, indicating that the central repeats of CotG are dispensable for this function, which is, therefore, due to the N- and C-terminal regions of the protein.

GHyb spores were functional also in complementing the heat-sensitive phenotype of spores lacking CotG. Similar amounts of purified spores (between 0.77 × 10⁸ and 1.10 × 10⁸) were incubated at 80°C for 15 and 30 min, and their viability was assessed by plate counts. As

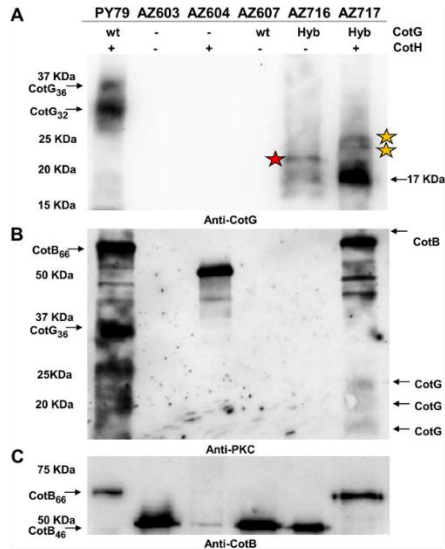


FIG 1. Assembly (A) and phosphorylation (B) of GHyb and (C) its effects on CotB. Western blot analyses of coat proteins extracted from purified spores of the indicated strains were performed on proteins fractionated on 12.5% SDS-PAGE gels. Electrotransferred proteins were then reacted with anti-CotG (A), anti-PKC (B), and anti-CotB (C) antibodies. The type of CotG form and the presence (1) or absence (2) of CotH are indicated. Red and yellow stars represent different GHyb forms.

shown in Fig. 2A, spores lacking CotG (orange line) showed decreased viability, while wild-type and GHyb spores were fully resistant. Since spores lacking CotG also lack CotB (20, 21), while GHyb spores normally assemble CotB (Fig. 1C), it is possible that the heat sensitivity observed in Fig. 2A is at least in part due to the absence of CotB. An isogenic strain carrying a null mutation in *cotB* (RH201) was then analyzed and shown to be fully resistant to the heat treatment (green line in Fig. 2A), indicating that the heat sensitivity is exclusively due to the absence of CotG and is fully complemented by GHyb. The reduced heat resistance of spores lacking CotG was consistent and most probably due to an increased release of dipicolinic acid (DPA) upon heat treatment. As shown in Fig. 2B, spores lacking CotG released 2-fold more DPA than wild-type and GHyb spores, suggesting that upon heat treatment, spores of a CotG-lacking strain are somehow leaky, release DPA, and as a consequence, show reduced viability.

GHyb does not have negative effects on the assembly of other coat proteins and does not form cytoplasmic aggregates. CotC and CotU, two highly homologous small coat proteins that assemble on the spore coat in multiple forms (22, 23), fail to assemble in a strain lacking CotH (17). Such an effect, due to the accumulation of unphosphorylated CotG in the mother cell cytoplasm (18), is not observed when the central repeats of CotG ($G^{central}$) were deleted (15). Western blotting experiments showed that all forms of CotC and CotU were present in coat extracts of strains carrying GHyb as the only CotG form, independently from the presence of CotH (Fig. 3A). A fluorescence microscopy analysis of strains carrying the *cotC::gfp* fusion indicated proper assembly of CotC around forming and mature spores (red and white arrows in Fig. 3B, respectively), independently from the presence of CotH (Fig. 3B). Although not directly tested, CotU is likely to behave similarly based on its presence in coat extracts (Fig. 3A) and its high homology with CotC (22, 23). When GHyb was present together with a wild-type version of *cotG* (strains AZ759 and AZ760), some CotC molecules were properly assembled around forming and mature spores, while

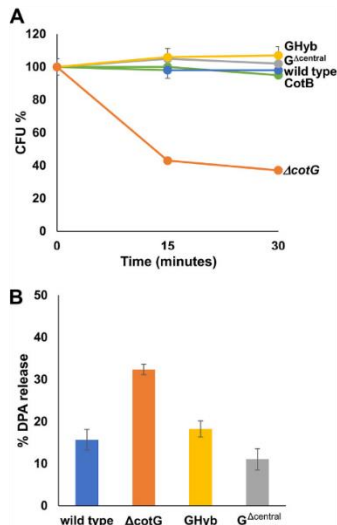


FIG 2. Functional analysis of spores expressing different forms of CotG. (A) Resistance to heat treatment (20 min at 80°C) and (B) DPA release (after 15 min at 100°C) of spores of the wild type (blue symbols) and mutant strains carrying a *cotG* null mutation ($\Delta cotG$ [orange symbols]), a *cotG* gene with the internal repeats deleted ($G^{central}$ [gray symbols]), the hybrid *cotG* gene (GHyb [yellow symbols]), or carrying a *cotB* null mutation (*CotB* [green symbols]). One hundred percent is considered the amount of DPA released from spores autoclaved at 120°C for 30 min. Error bars represent standard deviations. Each percentage is the mean of results from three replicate experiments, each performed with a different prepared spore suspension.

others were not assembled and found in the mother cell cytoplasm (yellow arrows in Fig. 3B). The latter result confirms that unphosphorylated CotG sequesters CotC in the mother cell cytoplasm (18) and suggests that the N- and/or the C-terminal regions of CotG (present in GHyb) are needed for CotC assembly on the spore coat. Altogether, the results of Fig. 3 indicate that, like $G^{central}$ (15), GHyb also does not have negative effects on the assembly of CotC.

This result suggests that both mutant forms of the protein do not form cytoplasmic aggregates, recently shown to sequester CotC and CotU and impair their coat assembly (18). To verify such a hypothesis, immunofluorescence microscopy experiments were performed with anti-PKC and anti-CotG antibodies. As shown in Fig. 4A and B, most GHyb and $G^{central}$ molecules were assembled around the forming spore and only a minimal part of them were found in the mother cell cytoplasm, where they never formed the well-defined spots typical of unphosphorylated CotG of *B. subtilis* (arrows in Fig. 4B) (18). In a mutant lacking *CotH*, CotG of *B. subtilis* failed to assemble on the spore and massively accumulated in the mother cell (arrows in Fig. 4C) (13). In the same genetic background, the majority of the GHyb and $G^{central}$ proteins were still able to assemble around the forming spore and the few molecules accumulated in the mother cell cytoplasm, never forming well-defined spots in the mother cell cytoplasm (Fig. 4C). Therefore, in agreement with the results of Fig. 3 and previously reported data (15), the deletion of the positively charged central repeats of CotG or their replacement with not positively charged repeats abolishes the dependency of CotG assembly on its phosphorylation and impairs the formation of CotG-dependent cytoplasmic aggregates.

The central repeats of CotG modulate the spore functions. The positively charged central repeats of CotG are not involved in *CotB* maturation (described above and see reference 15), and their only role in *B. subtilis* seems to be limited to the regulation of CotC-CotU

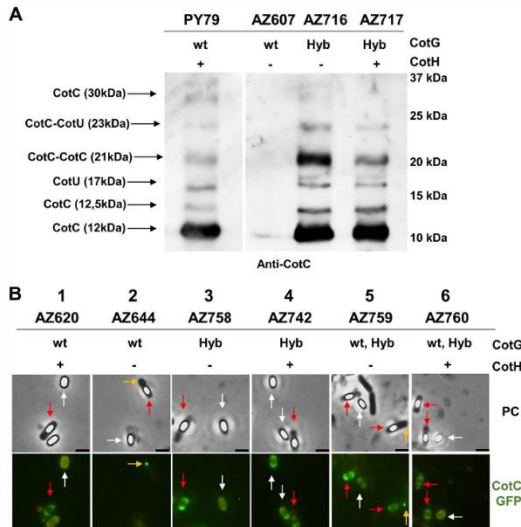


FIG 3 Effect of G-Hyb on the spore coat assembly of CotC and CotU. (A) Western blot analysis of coat proteins fractionated on 12.5% SDS-PAGE gels, electrotransferred onto a membrane, and incubated with anti-CotC antibody; (B) fluorescence microscopy analysis of spores carrying the fusion protein CotC-GFP. The type of CotG form and the presence (1) or absence (2) of CotH are indicated. Red and white arrows indicate forming and mature spores, respectively. Yellow arrows indicate CotC aggregates in the mother cell cytoplasm. Scale bar, 1 μ m.

assembly on the spore coat (18). However, most *Bacillus* species that have a CotG-like protein do not have CotC-CotU homologs (Table S1), suggesting that the conserved CotG protein family and their repeats might have an additional but still obscure role. To further investigate the role of CotG repeats, several spore properties were analyzed in a wild-type strain (WT) and in isogenic mutants either lacking CotG (herein indicated as DcotG) or containing $G^{Dcentral}$ or G-Hyb, as described above. The efficiency of germination was assessed by using either Asn or Ala as germinants and by measuring the decrease in optical density at 590 nm (OD_{590}) (Fig. 5A and B) and by flow cytometry (Fig. 5C and D and Fig. S2A and B) as previously described (24, 25). The two approaches gave similar results (Fig. 5): $G^{Dcentral}$ spores were less efficient than those of the wild type, while DcotG and G-Hyb spores showed an intermediate germination efficiency. The germination defect of $G^{Dcentral}$ spores was stronger with Asn than with Ala as the germinant, in particular at early times after the induction of germination.

The resistance to lysozyme was analyzed by treating purified spores with 100 nM lysozyme (Sigma) for 360 min and measuring the cell viability on the plate (CFU). While DcotG and G-Hyb spores were slightly less resistant than wild-type spores, $G^{Dcentral}$ spores were clearly more resistant (Fig. 6). Resistance to lysozyme is due to the spore coat, which acting as a sieve prevents the enzyme from reaching its target (peptidoglycan) (4). Therefore, the increased resistance of $G^{Dcentral}$ spores suggests that the internal repeats of CotG affect the spore permeability to the lytic enzyme.

The central repeats of CotG modulate the permeability of the spore surface. Heterologous proteins, when abundantly concentrated outside the spore, can cross the spore surface (5, 6). Such permeability increases the amount of protein adsorbed

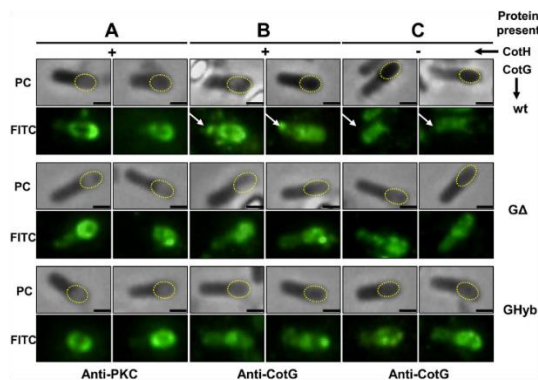


FIG 4 Immunofluorescence analysis of different strains carrying different forms of CotG with anti-PKC (A) and anti-CotG (B and C) antibodies in the presence (A and B) or absence (C) of CotH. In phase-contrast (PC) panels, the position of the forming spore is indicated by dotted red ovals. The exposure times were 200 ms for panel A and 500 ms for panels B and C. Scale bar, 1 μ m.

to the spore and varies with the chemical properties of the heterologous protein and with the coat structure (7). The adsorption of heterologous lysozyme was then used to evaluate the permeability of the surface layers of wild-type, DcotG, GHyb, and $G^{Dcentral}$ spores. Lysozyme was fluorescently labeled with rhodamine (Lys-Rd) as previously described (7), and 10 mM labeled protein used to treat 5.0×10^8 purified spores of the four strains. The reactions were carried out for 1 h at 37°C in 50 mM sodium citrate buffer (pH 4.0) as previously described for other heterologous proteins (26). Spores were collected by centrifugation and analyzed by fluorescence microscopy and flow cytometry as previously described (7). The labeled lysozyme adsorbed to all four types of spores, and the intensity of the fluorescence signal was weaker with $G^{Dcentral}$ spores than with all other spores (Fig. 7A). The relative fluorescence signals were analyzed by the ImageJ software (NIH) as previously reported (5) and appeared high and heterogeneous in wild-type spores, while the signals were low and homogeneous in $G^{Dcentral}$ spores (Fig. 7B). Fluorescence signals in DcotG and GHyb spores were slightly stronger and less heterogeneous than those of wild-type spores. The fluorescence heterogeneity observed with wild-type spores, and in part with DcotG and GHyb spores, is due to a combination of highly and weakly fluorescent spores, suggestive of a porous spore surface easily crossed by the labeled lysozyme molecules. As a consequence, different numbers of fluorescent molecules localize at different positions within the coat (6), producing signals of heterogeneous intensities. The fluorescence homogeneity, as well as the low red emission intensity, observed with $G^{Dcentral}$ spores is instead suggestive of a compact, less porous surface that cannot be easily crossed. As a consequence, all lysozyme molecules localize in the same position and produce homogeneous fluorescent signals.

The fluorescence signals were quantitatively analyzed in duplicate on 100,000 spores each by flow cytometry. The analysis confirmed the fluorescence microscopy results and indicated that the only statistically significant difference was between wild-type and $G^{Dcentral}$ spores ($P < 0.001$) (Fig. 8).

To support the hypothesis that the reduced adsorption of $G^{Dcentral}$ spores was due to a reduced permeability to lysozyme, the gene coding for the green fluorescent protein (GFP) fused to *B. subtilis* genes coding for proteins known to localize in the inner coat (CotS) (14, 27) or in the crust (CotZ) (14) were moved by chromosomal DNA-mediated transformation into wild-type, CotG, GHyb, and $G^{Dcentral}$ strains. Purified spores of the resulting strains (see Materials and Methods) were treated with the rhodamine-labeled lysozyme and observed by fluorescence microscopy. More than 80 free spores from each strain were used to measure

Spore Permeability in *B. subtilis*

mbio

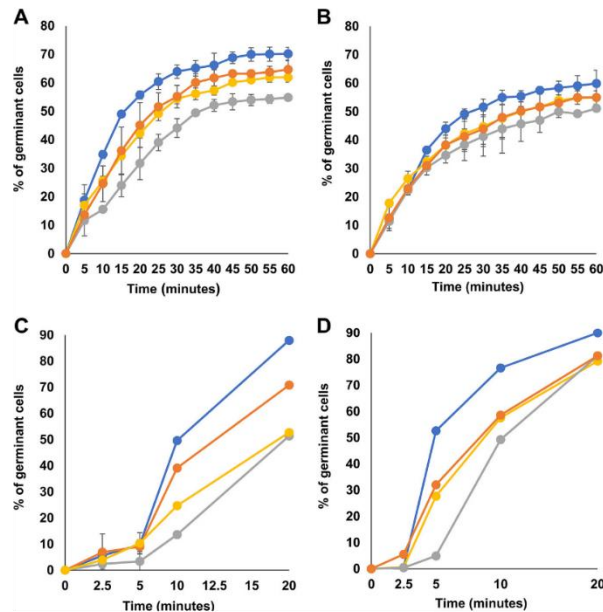


FIG 5 Germination efficiency of spores of the wild type (blue symbols) and mutant strains carrying a *cotG* null mutation (ΔcotG [orange symbols]), a *cotG* gene with the internal repeats deleted (G^{central} [gray symbols]), and the hybrid *cotG* gene (GHyb [yellow symbols]). The germination was induced using L-Ala-GFK (A and C) or L-Asn-GFK (B and D) and measured as the percentage of loss of the OD_{600} and by flow cytometry (C and D). Error bars (A and B) are based on the standard deviations of values from four independent experiments. Panels C and D report the percentage of germination obtained from flow cytometry data (Fig. S2).

the intensity of GFP and Lys-Rd on the spore long axis (indicated by the x axis in Fig. 9) by using ImageJ software as previously described (5). Averages of red and green fluorescence intensities of the various strains are plotted in Fig. 9. Although the size of the observed structures is below the resolution limit of the fluorescence microscope, the averaged results suggested a

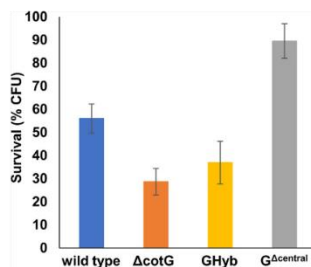


FIG 6 Lysozyme resistance of spores of the wild type (blue symbols) and mutant strains carrying a *cotG* null mutation (ΔcotG [orange symbols]), a *cotG* gene with the internal repeats deleted (G^{central} [gray symbols]), and the hybrid *cotG* gene (GHyb [yellow symbols]). Spores were incubated with lysozyme for 6 h at 37°C, and survival was estimated by CFU count. Error bars are based on the standard deviation of values from 4 independent experiments.

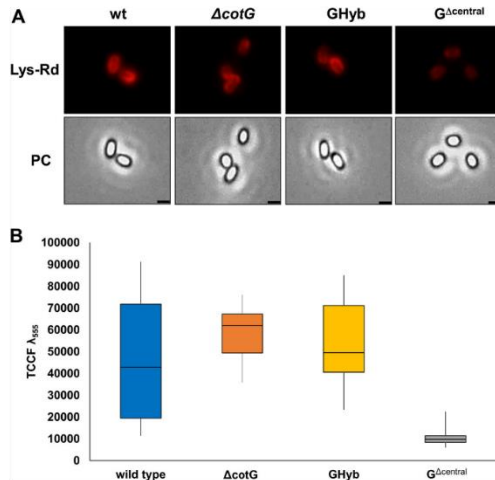


FIG 7 (A) Fluorescence microscopy analysis of spores of the wild type and mutant strains carrying a *cotG* null mutation ($\Delta cotG$), a *cotG* gene with the internal repeats deleted ($G^{\Delta central}$) and the hybrid *cotG* gene (GHyb) after treatment with lysozyme conjugated with rhodamine (Lys-Rd). The exposure time was 100 ms for each panel. Scale bar, 1 μm . PC, phase contrast. (B) Box plots displaying the total corrected cellular fluorescence (TCCF) for 50 different spores of each strain. The limits of each box represent the first and the third quartiles (25 and 75%), and the values outside the boxes represent the maximum and minimum values.

different permeability to the lytic enzyme for the spores of the various strains. The red fluorescence signal of the labeled lysozyme was always external to the inner coat (*cotS::gfp*) and internal to the crust (*cotZ::gfp*) for spores of all strains but $G^{\Delta central}$ spores (red and green arrows in Fig. 9).

DISCUSSION

The CotG-like protein family is conserved in sporeformers of the *Bacillus* genus. Members of the family do not have a homologous primary sequence but share a similar modular structure with a central part formed by several repeats containing, in most cases, a high percentage of positively charged amino acids (15). In *B. subtilis*, CotG is present in sporulating cells as a phosphorylated protein that is readily assembled around the forming spore and as a presumably unfolded unphosphorylated protein that accumulates in the mother cell cytoplasm, where it forms aggregates able to sequester other coat proteins (i.e., CotC and CotU) (18). On the spore, CotG is localized in the outermost layer (14) and interacts with CotB, and such interaction is strictly required for the CotH-dependent conversion of the immature form of CotB (CotB_{imm}) into its mature form (CotB_{mat}) (12, 21). Results of this study clarify that when the central repeats of CotG are replaced by repeats that are not as positively charged (GHyb), the assembly of the protein on the spore surface no longer depends on its phosphorylation, pointing to the massive CotH-dependent phosphorylation of CotG as an essential step needed to balance the high positive charge of the protein and probably to allow its proper folding as schematically represented in Fig. 10A.

When the internal repeats of CotG are either lacking ($G^{\Delta central}$) or have been replaced (GHyb), the protein is able to interact with CotB (Fig. 1) and allows CotC assembly (Fig. 3B), indicating that the central repeats are not involved in such interaction and may have different functions. Although we cannot rule out the possibility that the deletion of the central repeats of CotG alters the coat structure, causing pleiotropic effects, the reduced efficiency

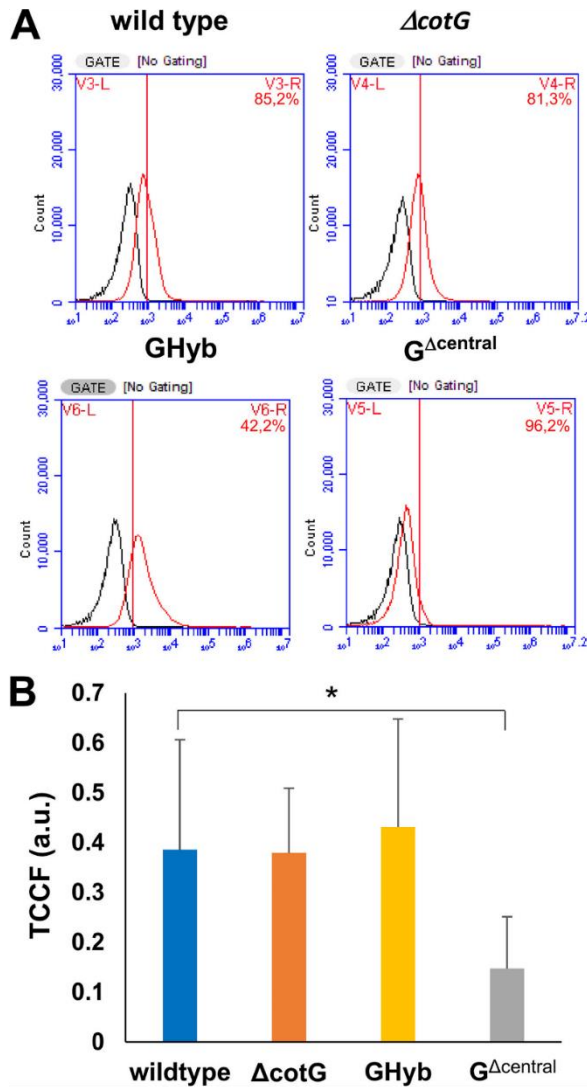


FIG 8 (A) Flow cytometry analysis of 10,000 spores of the wild type and mutant strains carrying a *cotG* null mutation ($\Delta cotG$), a *cotG* gene with the internal repeats deleted ($G^{\Delta central}$), and the hybrid *cotG* gene (GHyb) after treatment with lysozyme conjugated with rhodamine (Lys-Rd). The black curves represent the nonspecific fluorescence signal of the spores (no Lys-Rd), whereas the red curves represent the fluorescence signal after treatment with Lys-Rd. (B) Quantitative analysis of the fluorescence of over 50 spores by the ImageJ software. The y axis describes the total corrected cellular fluorescence (TCCF) value. The fluorescent intensity threshold is 1×10^3 (red line). a.u., arbitrary units.

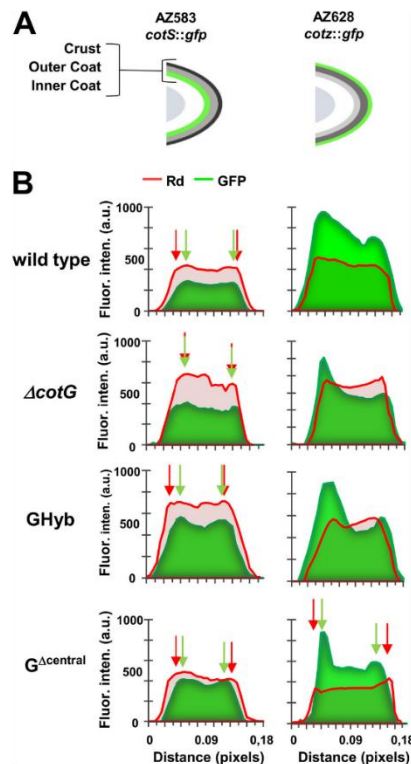


FIG 9 (A) Schematic representation of the localization of GFP in the spore coat of strains carrying GFP fused to inner coat (*cotS::gfp*), outer coat (*cotC::gfp*), or crust (*cotZ::gfp*) proteins; (B) plots of green (GFP) and red (Lys-Rd) fluorescence intensities along the long axis of spores of the strains indicated in panel A. Green and red arrows indicate peaks of GFP and Rd fluorescence intensities and are represented only when the Rd fluorescence signals colocalize or Rd signal is more external than the GFP signal. One pixel corresponds to 1.18 nm. a.u., arbitrary units.

of germination and the increased resistance to lysozyme together point to the central repeats of CotG as a modulator of the permeability of the spore surface. Such a conclusion is also indirectly supported by the reduced adsorption of lysozyme to the spore surface and by its localization outside the spore crust in a strain lacking the central repeats of CotG. A working model developed on the basis of the reported results hypothesizes that the N and C termini of CotG, involved in the interaction with another coat protein (CotB), are separated by the internal repeats acting as spacers (Fig. 10B). When the central repeats of CotG are deleted ($G^{\Delta central}$), the N and C termini are close to each other, the connection with CotB (and possibly with other coat proteins) is tight, and the spore is not permeable and highly resistant to lysozyme (Fig. 10B). When the central repeats of CotG are replaced by repeats that are not as positively charged (GHyb), the permeability of the spore surface is more similar to that of wild-type spores than $G^{\Delta central}$ spores, indicating that the presence of the repeats allows a distance between the N and C termini and, as a consequence, the formation of a less compact and more permeable spore surface. When CotG is totally lacking, the interaction with CotB cannot occur, causing the formation of a completely different spore

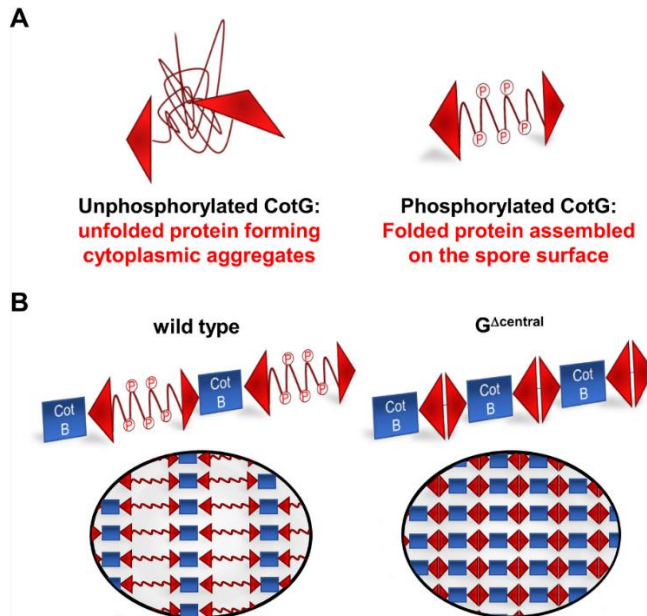


FIG 10 (A) Schematic representation of unphosphorylated and phosphorylated forms of CotG protein; (B) working model of the role of the central repeats of CotG in controlling spore permeability.

surface. Indeed, when CotG (and therefore CotB) is lacking (12) or when the protein's abundance is strongly reduced (8), the spore surface has a totally different structure. Several attempts to purify CotG after overexpression in heterologous hosts have been so far unsuccessful, suggesting the toxicity of the unphosphorylated protein. Low levels of CotG were produced in *E. coli* also when the kinase CotH was coexpressed (12). However, such levels were not sufficient to purify enough protein for structural studies, impairing the possibility to propose a more definite model so far.

MATERIALS AND METHODS

Bacterial strains and molecular procedures. Strains are listed in Table S2 in the supplemental material. Plasmid amplification for nucleotide sequencing, subcloning experiments, and transformation of *Escherichia coli* competent cells were performed with *E. coli* strain DH5α (28). Bacterial strains were transformed by previously described procedures: CaCl₂-mediated transformation of *E. coli* competent cells (28) and two-step transformation of *B. subtilis* (29). DNA ligation, isolation of plasmids, and restriction digestions were performed by using standard methods (28). Chromosomal DNA extraction of *B. subtilis* and *B. licheniformis* was carried out as described previously (29). The replacement of the antibiotic resistance gene cassettes was performed as described previously (30).

***B. subtilis* strain construction.** The gene fusion coding for GHyb was constructed in *E. coli*. The 59 (including the promoter region) and 39 portions of cotG of *B. subtilis* were PCR amplified using *B. subtilis* PY79 as a template and oligonucleotide pairs G1-H19/Glich1 (for 59 region) and G3-Glich4/X2 (Table S3). DNA coding for the internal repeats of the CotG-like of *B. licheniformis* was PCR amplified by using chromosomal DNA of strain ATCC 14580 as a template and the oligonucleotide pairs Glich2/Glich3 (Table S3). The partially overlapping PCR products were fused by using the gene SOEing (splicing by overlap extension) technique (31) to obtain a fusion DNA fragment that was cloned into the pGEM-T Easy vector (Promega). The cloned fragment was analyzed by nucleotide sequencing. The fusion fragment was then digested with the restriction enzymes BamHI and HindIII and inserted into pDGL731, an integrative vector commonly used to integrate cloned genes at the thrC gene (31), previously digested with the same enzymes. The obtained plasmid, pOSL6, was linearized



with *ScaI* and used to transform competent cells of P79 to yield strain AZ715 (thrCcotGhyb). The gene fusion was then moved by chromosomal DNA-mediated transformation into competent cells of strains AZ603 and AZ604 (Table S2) to yield strains AZ716 (DcoTG DcoTH thrCcotGhyb) and AZ717 (DcoTG DcoTH amyEcoTG_{neo}coth thrCcotGhyb).

The chloramphenicol resistance gene cassette of strains RH238 (cotCgfp), AZ644 (cotSgfp), and AZ573 (cotZgfp) was replaced with tetracycline resistance gene cassette by transformation with plasmid pTTC (27), obtaining AZ640 (cotCgfp), AZ583 (cotSgfp), and AZ628 (cotZgfp), respectively. Chromosomal DNA of AZ583 (cotSgfp) was used to transform competent cells of strains AZ604, AZ613, AZ717 to yield strains AZ681 (DcoTG DcoTH amyEcoTGatopcoth cotSgfp), AZ694 (DcoTG DcoTH amyEcoTGatopcoth thrCcotGDcentral cotSgfp) and AZ752 (DcoTG DcoTH amyEcoTGatopcoth thrCcotGhyb cotSgfp) respectively. Chromosomal DNA of AZ628 (DcoTG DcoTH amyEcoTGatopcoth cotZgfp), AZ695 (DcoTG DcoTH amyEcoTGatopcoth thrCcotGDcentral cotZgfp) and AZ751 (DcoTG DcoTH amyEcoTGatopcoth thrCcotGhyb cotZgfp) respectively. Chromosomal DNA of AZ640 (cotCgfp) was used to transform competent cells of AZ607, AZ717, AZ716, AZ715 to obtain AZ623 (DcoTG DcoTHneo amyEcoTG cotCgfp), AZ742 (DcoTG DcoTHneo amyEcoTGatopcoth thrCcotGhyb cotCgfp) AZ758 (DcoTG DcoTH thrCcotGhyb cotCgfp) and AZ760 (thrCcotGhyb cotCgfp), respectively.

The spectinomycin resistance gene cassette of strain ER220 (DcoTH:spec) was replaced with the neo-mycin resistance gene cassette by transformation with plasmid pTneo (27), obtaining AZ664 (DcoTHneo). Then, chromosomal DNA of AZ664 was used to transform competent cells of strain AZ715 (thrCcotGhyb) to obtain AZ757 (DcoTHneo thrCcotGhyb). Finally, chromosomal DNA of AZ640 (cotCgfp) was used to transform competent cells of AZ757 to obtain AZ759 (DcoTHneo thrCcotGhyb cotCgfp) (Table S2).

Spore purification, coat protein extraction, and Western blot analysis. Sporulation of all strains was induced by growing cells in Difco sporulation medium (DSM) at 37°C with vigorous shaking. After 30 h, spores were analyzed under the light microscope, collected by centrifugation, washed four times with distilled water, and purified as described previously (32). In particular, spores were purified by a lysozyme treatment (29) when they were prepared for coat protein extraction and by overnight incubation in H₂O at 4°C to lyse residual sporangial cells (25, 32) when prepared for functional analysis or lysozyme adsorption. Spore purity was checked by microscopic inspection, and the preparation was considered pure when less than 5% of sporangia were present. Spore coat proteins were extracted from a suspension of 1×10^9 spores by SDS-dithiothreitol (DTT) treatment (29). The extracted proteins were quantified with a Bio-Rad DC protein assay kit (Bio-Rad), and 20 μ g of total proteins was fractionated on 12.5 or 15% polyacrylamide SDS-PAGE gels. For Western blotting, SDS-PAGE gels were electrotransferred on a nitrocellulose filter (Bio-Rad), and filters were reacted with polydonal antibodies. Anti-CotC, anti-CotB, and anti-CotG antibodies were diluted 1:7,000, while commercial anti-PKC antibody (Cell Signaling Technology) was diluted 1:10,000. Then, a horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Santa Cruz) was used to recognize specific bands that were visualized by the SuperSignal West Pico chemiluminescence (Pierce) method as specified by the manufacturer.

Physiological analysis. (i) Heat resistance. Suspensions of 1.0×10^7 spores were incubated at 80°C for 0, 15, and 30 min, and then the suspensions were serially diluted and plated on LB agar (2%) plates and incubated overnight at 37°C for CFU count.

(ii) DPA release. Suspensions of 1.0×10^9 spores were incubated at 100°C for 15 min or autoclaved at 120°C for 30 min and centrifuged for 10 min at 13,000 $\times$ g, and then 150 μ l of supernatant was placed in a 96-well microtiter plate with 150 μ l 0.1 M Tris-HCl, 400 mM sodium acetate buffer (pH 5) and analyzed with a BioTek Synergy H4 microplate reader. One hundred percent was considered the amount of DPA released from spores autoclaved at 120°C for 30 min (total DPA content). Spores of all strains contained similar amounts of DPA ranging between 108 and 146 nM. For all samples, the DPA content was measured as described previously (8).

(iii) Lysozyme resistance. Spores were prepared as described above, omitting the lysozyme step and eliminating vegetative cells by heat treatment (10 min at 80°C). Purified spores were heat treated (20 min at 80°C), suspended in 10 mM Tris-HCl (pH 7.0) buffer containing lysozyme (0.01 mg/mL), and incubated at 37°C for 0 h and 6 h. Finally, the suspension was diluted and plated on LB agar (2%) and incubated overnight at 37°C for the CFU count.

(iv) Germination efficiency. Purified spores were heat activated (30 min at 70°C), and germination was induced by adding the indicated germinants. Germination was measured by OD₆₀₀ decrease (25) and by flow cytometry (24, 25) by using as the germinant 10 mM L-asparagine or 10 mM L-alanine in a buffer containing 10 mM Tris-HCl (pH 8.0) and a buffer containing 1 mM fructose, 1 mM glucose, and 10 mM KCl. The flow cytometry approach was based on the use of a nucleic acid stain, SYTO 16, that was added to spores before the induction of germination and the fluorescence followed by a BD Accuri C6 flow cytometer. Since nucleic acids of dormant spores are not accessible to SYTO 16, while those of germinating spores are efficiently stained (24), different numbers of highly fluorescent cells are indicative of different levels of germination (24).

Fluorescence and immunofluorescence microscopy. Fluorescence analyses were carried out using an Olympus BX51 fluorescence microscope fitted with a 100 $\times$ objective UPlanFL1. Fluorescein isothiocyanate (FITC) (U-MNIB2) and tetramethyl rhodamine isocyanate (TRITC) (U-MWIG2) filters were used to detect the green and red fluorescence signals, respectively. Images were captured using an Olympus DP70 digital camera equipped with an Olympus U-CA magnification changer and processed with Image Analysis software (Olympus) for minor adjustments of brightness, contrast, and color balance and for creation of merged images. Fluorescence intensities and the distance between two fluorescent peaks were measured using unadjusted merged images with ImageJ processing software (version 1.48; NIH) as previously described (5). One pixel corresponds to 1.18 nm in our detection system. ImageJ was also used to draw an outline around 80 spores for each strain, prior to area, integrated density, and the mean fluorescence measurements being recorded, together with several adjacent background readings. The total corrected cellular fluorescence



(TCOF) was calculated by subtracting (area of selected cell – mean fluorescence of background readings) from integrated density values.

Immunofluorescence experiments were performed as described previously (18). In brief, 1 mL of sporulating cells was fixed for 1 h at room temperature in 80% methanol and then washed and incubated overnight at 4°C in 100% methanol. Fixed sporangia were permeabilized by incubating samples with a solution containing GTE (5% glucose, 0.01 M EDTA [pH 8.0], 20 mM Tris-HCl [pH 7.5]) plus 0.2 mg/mL lysozyme and rapidly placed on a coverslip previously treated for 30 s with poly-L-lysine. The coverslips were air dried, and cells were pretreated with 1% (wt/vol) dried milk in phosphate-buffered saline (PBS [pH 7.4]) prior to 2 h of incubation at 4°C with the monoclonal anti-CotC primary antibody (raised in rabbit; 200-fold dilution). After four washes with PBS, the samples were incubated with a 64-fold-diluted anti-rabbit secondary antibody, conjugated with fluorescein isothiocyanate (FITC) (Bethyl Laboratories) for 2 h at room temperature in the dark. After four washes, the coverslips were mounted onto microscope slides, one drop of PBS was added, and the samples were analyzed by fluorescence microscopy (18). Sporangia with only addition of the secondary FITC-conjugated antibody were used as a control of the specificity of this technique.

Lysozyme labeling and treatment of *B. subtilis* spores. Lysozyme was fluorescently labeled with rhodamine as previously described (5). Labeled lysozyme (Lys-Rd) was used to treat spores. In brief, 10 mM Lys-Rd was added to a suspension of 5.0×10^8 spores in 50 mM sodium citrate pH 4.5 in a final volume of 200 μ L. After 1 h of incubation at 25°C, the binding mixtures were washed and centrifuged (10 min at 13,000 $\times$ g) to fractionate adsorbed spores (pellet) from unbound protein (supernatant). Collected spores were analyzed by fluorescence microscopy or flow cytometry as described in reference 7.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, PDF file, 0.05 MB.

FIG S2, PDF file, 0.2 MB.

TABLE S1, DOCX file, 0.01 MB.

TABLE S2, DOCX file, 0.02 MB.

TABLE S3, DOCX file, 0.01 MB.

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