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DOI
10.1016/j.tibtech.2024.09.018

Publication date
2024

Document Version
Proof

Published in
Trends in Biotechnology

Citation (APA)
Kummetha, L. R., Oh, J. J., van der Linden, F. H., & Aubin-Tam, M. E. (2024). Leveraging the versatile properties of bacterial spores in materials. *Trends in Biotechnology*, *43*(4), 812-825. https://doi.org/10.1016/j.tibtech.2024.09.018

Trends in Biotechnology

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Review

Leveraging the versatile properties of bacterial spores in materials

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Inspired by biological functions of living systems, researchers have engineered cells as independent functional materials or integrated them within a natural or synthetic matrix to create engineered living materials (ELMs). However, the 'livingness' of cells in such materials poses serious drawbacks, such as a short lifespan and the need for cold-chain logistics. Bacterial spores have emerged as a game changer to bypass these shortcomings as a result of their intrinsic dormancy and resistance against harsh conditions. Emerging synthetic biology tools tailored for engineering spores and better understanding of their physical properties have led to novel applications of spore-based materials. Here, we review recent advances in such materials and discuss future challenges for the development of time- and cost-efficient spore-based materials with high performance.

Highlights

Living systems have inspired approaches to engineer cells as independent functional materials or integrate them within a natural or synthetic matrix to create engineered living materials (ELMs).

To address the principal challenges arising from the 'livingness' of cells, bacterial spores have emerged as a game changer in the field, enabling users to 'activate' cells on demand and to treat ELMs with harsh conditions.

The inherent physical properties of the structural components of the spores have led to diverse applications of spore-based materials.

Emerging synthetic biology tools and better understanding of bacterial spores might contribute to expanding the relevance of spores in various fields, such as biosensing, biocatalysis, and data storage, among others.

Bacterial spores in the realm of material science

Living organisms have acquired complex functions through evolution over a long period of time. Depending on the species, cells of an organism can function as independent biological units or can function collaboratively as a single, larger biological entity. In the latter, the cells are often embedded within a biological matrix, which the cells orchestrate the appearance and function of. For example, guard cells in plant leaves respond to environmental cues to regulate the opening and closing of stomatal pores, thereby contributing to gas exchange and hydration of the leaves. Inspired by such living systems, researchers have engineered cells as independent functional materials or have integrated them within a natural or synthetic matrix to create **engineered living materials (ELMs)** (see Glossary) [1]. However, the 'livingness' of cells presents several challenges for the application of such materials: the short lifespan of cells leads to fast expiration unless resources are regularly provided, and the susceptibility of the cells to harsh conditions impedes their practical use and distribution.

Some genetically engineerable bacterial species (e.g., *Bacillus subtilis*) can form **endospores** to survive in challenging environments (e.g., nutrient-free or dry environments). Endospores remain in a dormant state [2] in which they exhibit minimal metabolism, but can **germinate** and initiate a **vegetative cell** cycle under growth-permissive conditions. Thus, although requiring a germination step, spores or spore-integrated materials can overcome the aforementioned challenges. Bacterial spores can tolerate harsh conditions without significant loss of cell viability, and their dormancy enables users to control when to 'activate' the cells with programmable functions (Figure 1, Key figure). In addition, spores comprise several structural components (Box 1), each with unique physical properties. These properties inspire the development of unprecedented spore-based functional materials, without the need for germination, and whose functionality can be improved with genetic engineering.

In this review, we examine emerging spore-based materials with a primary focus on the model spore-forming bacterium *B. subtilis*. We categorize spore-based materials based on the origin

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Key figure

Leveraging properties of *Bacillus* spores to develop functional materials

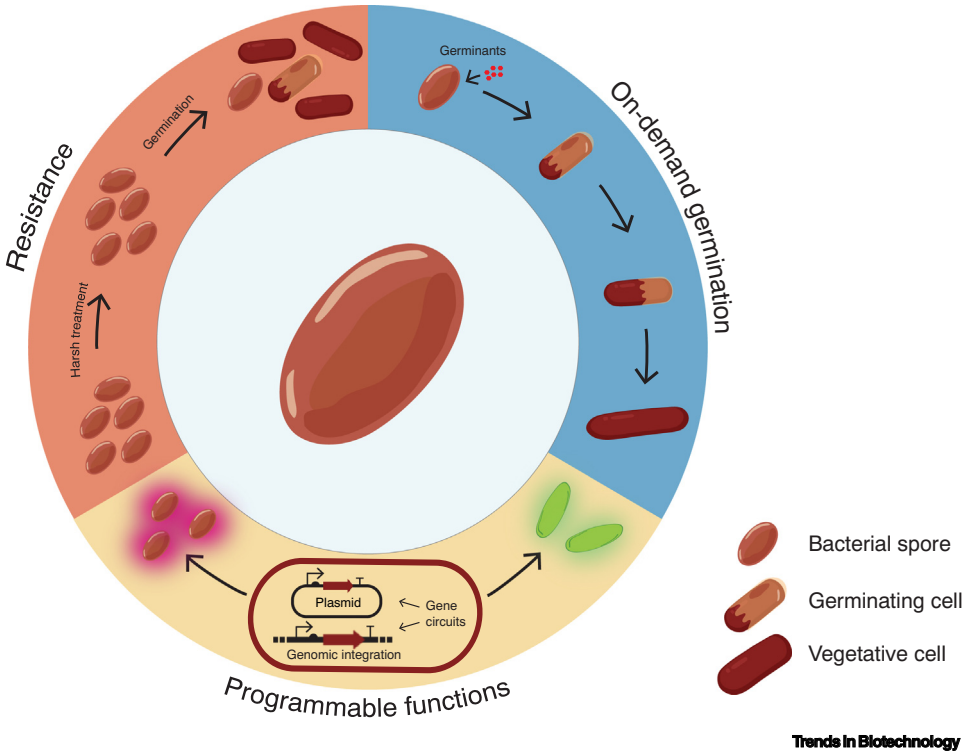

Figure 1. Spore-based materials primarily benefit from the resistance of spores to harsh conditions, such as high temperatures, desiccation, and acidic conditions. This makes them excellent candidates to overcome the limitations of vegetative cells. Furthermore, using spore-based materials, users can start germination on-demand. Finally, the genetic programmability of bacterial spores can lead to the development of spore-based materials for diverse applications either as spores or vegetative cells after germination.

of their functionality, namely materials that are active after germination (Figure 2) and materials that function through the inherent physical properties of the structural components of the spores, including core, cortex, and coat (Figure 3). In addition, we present important challenges to be faced for the realization of materials in the context of real-world needs.

Spore germination-based materials

Biosensors

Vegetative bacterial cells can be engineered with synthetic gene circuits to sense and respond to specific ions or molecules, producing discernible signals, such as fluorescence or color, through a reporter molecule. These whole-cell biosensor systems are more cost-effective and portable compared with conventional diagnostic tools based on macromolecules, such as enzymes, antibodies, and proteins [3]. Unfortunately, they have limited shelf-life and are susceptible to harsh environments due to the fragile nature of living cells. In such cases, bacterial spores can prove beneficial. First, users can choose when to germinate spores, thereby prolonging the storage period. Second, users can transport them easily outside laboratory environments,

Glossary

Endospore: a metabolically dormant cell that some bacterial species can form within a mother cell. Its formation is usually triggered by depletion of surrounding nutrients. After the nurturing by, and autolysis of, the mother cell, the endospore is released to the environment.
Energy density: amount of energy stored by the material per unit volume.
Engineered living materials (ELMs): materials containing living cells, either wild-type or genetically engineered, acting as the functional units of the material. The cells are embedded in a natural or synthetic matrix that is engineered to either act as a structural scaffold or to show dynamic responses based on cellular activity.
Exogenous proteins: non-native proteins expressed in a host cell by using a gene or part of a gene originating from other species.
Germination: complex process through which, under favorable conditions, the endospore undergoes a transition to a vegetative state.
Gut microbiome: human body is densely colonized by a consortium of bacteria. The collection of microbial community colonizing the gastrointestinal tract is termed the gut microbiome, which has multiple roles in human health.
Microbial chassis: microbial recipient of engineered biological systems.
Prebiotic: nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon.
Probiotic: microbes potentially beneficial to human health, often added to dietary supplements or dairy products.
Quorum sensing: type of communication and gene regulation process in response to cell population density, allowing regulation of cell density.
Short-chain fatty acids (SCFAs): simple carboxylic acids of one to six carbon atoms produced through fermentation of undigested polysaccharides and oligosaccharides by saccharolytic bacteria in the gut.
Sporulation: process resulting in the formation of spores (e.g., endospores, exospores, fungal spores, and akinete) starting from vegetative cells of microbes.

Box 1. Anatomy of *Bacillus* spores

Bacillus spores are ellipsoidal in shape, ranging in length from 0.8 to 2.3 μm, and in width from 0.4 to 1.2 μm [59]. They comprise (from inside out) the core, inner membrane, cortex, outer membrane, and coat (Figure I). Some *Bacillus* species (e.g., *Bacillus cereus*) have an additional layer outside the crust, called the exosporium, which is absent in *Bacillus subtilis* [60].

Core

The core is the central part of *Bacillus* spores, accounting for 27% (0.0896 μm^3, on average) of the spore volume [39]. The bacterial chromosome, ribosomes, and enzymes are localized within the core, along with large amounts of the spore-specific molecule dipicolinic acid (DPA), in a 1:1 chelate with divalent cations, predominantly Ca^{2+} (CaDPA). The core has a low water content (25–50% of wet weight) compared with vegetative cells (80%) [61].

Inner membrane

The spore core is encased within a largely immobile lipid membrane, called the inner membrane [62]. *Bacillus* spores can be germinated by the binding of nutrients acting as germinants (e.g., L-alanine) to germinant receptors (GRs), which are located in the inner membrane [63]. Some GRs were recently reported to act as nutrient-gated ion-channels [64].

Cortex

The cortex accounts for 51.7 % of the spore volume [39]. In both *Bacillus* vegetative cells and spores, this layer mainly comprises peptidoglycans (PG), which likely act as the skeleton of the cell. The PG layer helps to maintain shape integrity under external mechanical forces exerted on the cells (e.g., turgor pressure or bursting) and maintains cell viability [65]. This layer has an identical chemical composition to that of vegetative cells, with a few exceptions, which are discussed further in Box 3 in the main text.

Outer membrane

The exact role of the outer membrane remains elusive. It might contribute to selective permeability of small molecules (e.g., glycol) [66] or may simply be a vestigial structure, derived from the sporulating mother cell [63].

Coat

The coat is regarded as a molecular sieve that excludes large molecules (e.g., lysozymes) while allowing the passage of small-molecule germinants. In terms of ultrastructure, the *Bacillus* spore coat comprises four layers, with the innermost layer being the basement layer, followed by the inner coat, outer coat, and crust. Morphogenesis and protein components of each layer are discussed in Box 4 in the main text. Outside the coat, there is a halo-like layer of surface polysaccharides. The protein cgeA was recently identified as an important glycoprotein for attachment of the polysaccharide layer to the crust [67].

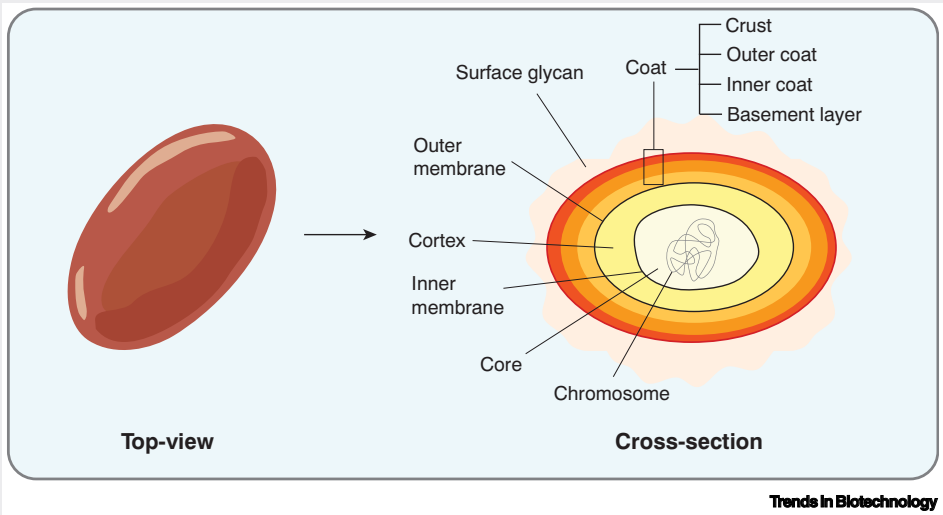

Figure I. Schematic of the structure of *Bacillus subtilis* spores.

Strain response: measure of how much an object deforms from an applied load.
Vegetative cell: bacterial cell that is active in terms of growth and metabolism.

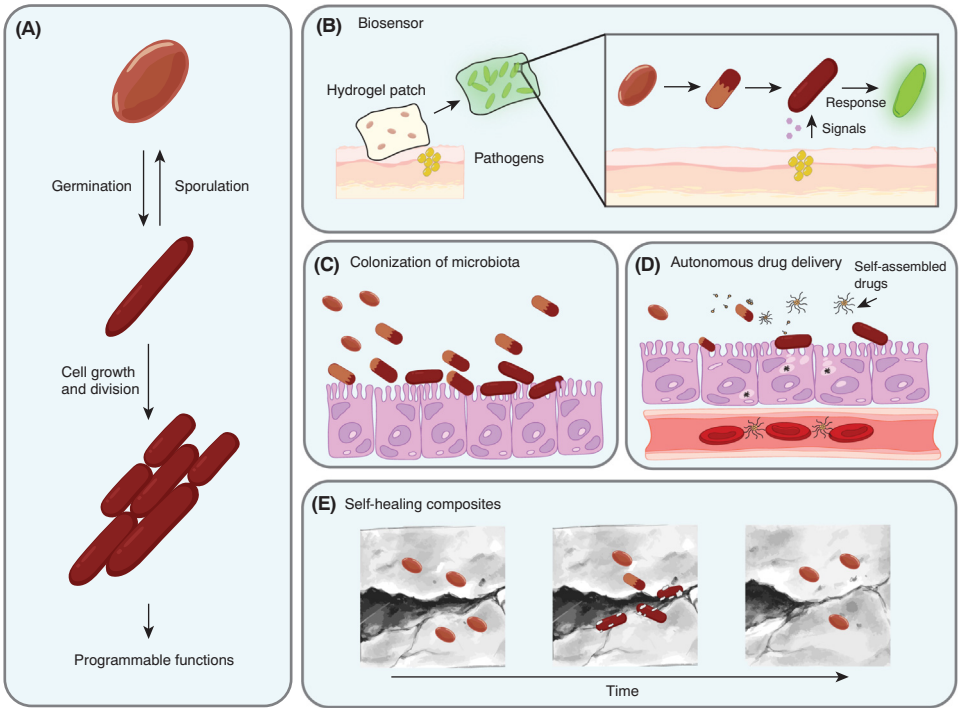

Figure 2. Spore germination-based functional materials. (A) Schematics of spore germination and outgrowth of vegetative cells, which is required for the functionality of the material. (B) Germinated cells sense molecules, such as from pathogens, and respond by producing signaling molecules, localized inside the cells or secreted to the environment (in green). (C) Probiotic spores survive the extreme pH condition in the gastrointestinal tract, and colonize the gut after germination. (D) Upon germination, hydrophobic coat proteins coated with therapeutic chemicals self-assemble into micellular nanoparticles, which are delivered into either adjacent cells or the bloodstream. (E) Cracks in composite materials allow nutrients and air to reach bacterial spores encased within, triggering germination and self-repair by precipitation of biominerals.

circumventing the need for cold-chain logistics typically required for living cell-based materials. Third, spore-based systems can be treated to remove biocontaminants while the spores remain viable. For example, Date and coworkers demonstrated the concept of spore germination-based whole-cell biosensors for sensing either arsenic using engineered *B. subtilis* or zinc using engineered *Bacillus megaterium* [4]. The spores from both strains germinated, and the resulting vegetative cells exhibited sensing capabilities, even after 6 months of storage or drying treatment. The detection limit of the *B. subtilis* arsenic sensor remained reproducible after multiple **sporulation**–germination cycles, proving their consistent analytical performance compared with nonsporulating species.

Whole-cell biosensors have been integrated within a matrix (e.g., hydrogel) to develop ELMs for sensing applications (Figure 2B). Schulz-Schönhagen and colleagues embedded spores from IPTG-sensing *B. subtilis* within a mechanically robust polyvinyl alcohol hydrogel [5]. A roll-to-roll solvent casting set-up allowed cheap, continuous, and scalable production of such sensor chips. Sensor chips subjected to a range of temperatures (–20 to 80°C) or long storage (up to 28 days) still had stable sensing abilities. Another study enhanced the resistance of an ELM biosensor by encapsulating *B. subtilis* spores within hollow ZIF-8, a metal-organic framework [6]. The biocompatible ZIF-8 layer provided additional protection for spores from harsh conditions [e.g., ultraviolet

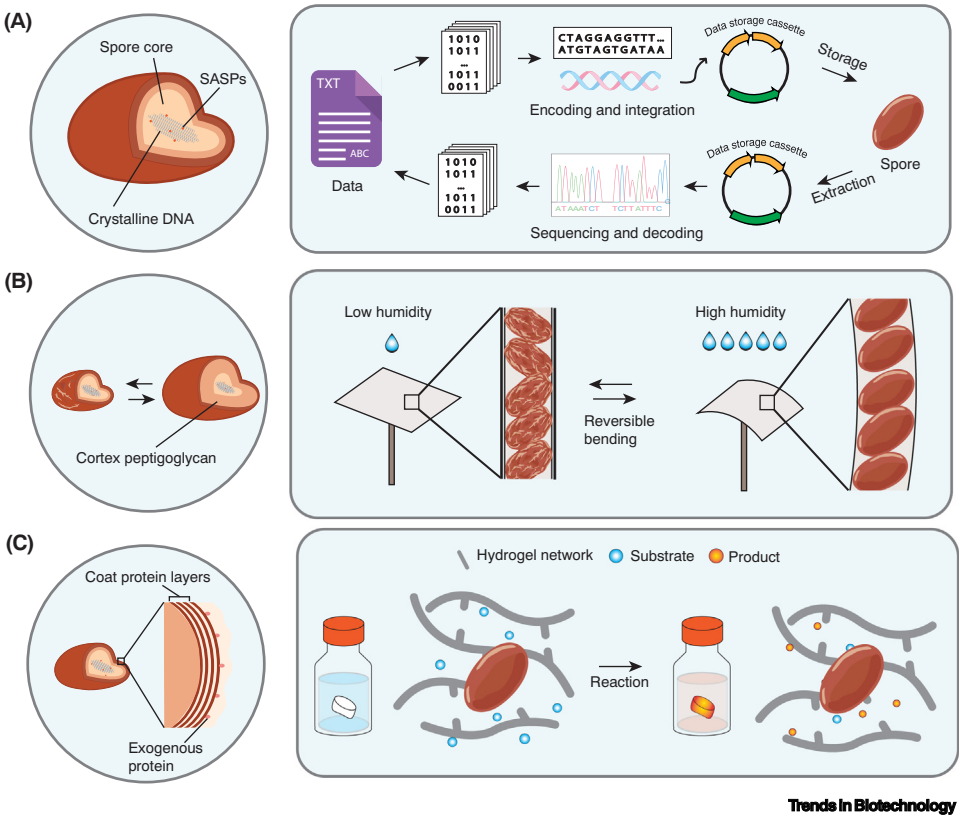

Figure 3. Functional spore-based materials without germination. (A) Genetic information is well protected in the spore core because of its low water content, the outer layers of the spore, and small acid-soluble proteins (SASPs) inducing a crystalline nucleoid (left). Data encoded in an artificial DNA sequence can also be stably stored inside the spore and retrieved later (right). (B) Spore cortex peptidoglycan induces rapid volumetric changes (left). Hygromorphic materials can be assembled with spores deposited onto a substrate. The material can bend in response to humidity changes, through expansion of the spore (right). (C) Exogenous proteins are expressed, anchored within the coat of spores (left), which can exert desired functions (e.g., enzymatic activity). These spores can contribute to the development of catalytic hydrogel materials (right).

(UV)-C] and guaranteed controlled release of spores based on the concentrations of nutrients. The released spores can germinate, and the resulting vegetative cells can detect specific molecules (e.g., IPTG or cuminic acid), depending on the gene circuit introduced within cells.

A recent study demonstrated that ELMs containing *B. subtilis* spores can also be bioprinted, allowing extensive control over the shape of the material [7]. Here, spores within a 3D-printed agarose matrix could still germinate when provided with nutrients, and the resulting vegetative cells emitted fluorescence in the presence of a signal-inducing molecule (e.g., vanillic acid or xylose). The spores survived and remained functional even after exposure to harsh conditions, such as 100% ethanol, high osmolarity (1.5 M NaCl), and radiation (UV-A, X-rays, and γ-radiation), although there was a significant drop in performance after exposure to high-intensity X-ray and γ-radiation. As an application, *B. subtilis* embedded within agarose was modified to sense *Staphylococcus aureus*-specific molecules and secrete lysostaphin in response, killing *S. aureus*, and making this material both a biosensor and a biotherapeutic. The 3D printing of agarose also facilitated shape customization to fit the wounds of patients.

Biotherapeutics

Probiotic spores or probiotic spore-based materials have contributed to the field of **gut microbiome** therapeutics. After oral administration, individual spores or spores embedded within such materials maintain their high viability under the extreme pH conditions of the gastrointestinal tract and can reach the gut environment. In the gut, these spores can germinate and initiate their beneficial roles, such as colonization of the gut environment and reduction of pathogen levels (Figure 2C) [8]. By contrast, since probiotics in the vegetative form struggle to survive the gastrointestinal tract, conventional probiotic treatments often require a high daily intake of viable cells [~10^9 colony-forming units (CFU)/day], or treatment of the probiotics to maintain their viability, for example via encapsulation in protective layers [9].

As a proof of concept, *B. subtilis* spores orally administered to mice colonized the gut and reduced the abundance of pathogenic bacterial strains, such as *S. aureus* and *Enterococcal faecalis* [8,10]. A previous study provided molecular evidence that *Bacillus* lipopeptides, fengycins, contributed to the decolonization of *S. aureus* in murine intestines by inhibiting *S. aureus* **quorum sensing**. Another study in mice proved that orally administered probiotic *B. subtilis* spores blocked translocation of *E. faecalis* from the gut to the bloodstream and the subsequent systemic infection, by inhibiting its quorum-sensing activity. Furthermore, a recent study encapsulated **probiotics** with **prebiotics** to improve the gut microbiota [11]. Here, the spores from *Clostridium butyricum*, a strain producing anticancer **short-chain fatty acids (SCFAs)**, were coated with dextran. The dextran coating helped adhesion of spores to intestinal surfaces and promoted fermentation by *C. butyricum* for SCFA production after germination in the gut. The resulting SCFA production successfully suppressed tumor growth in the murine intestine.

A few studies have developed probiotic spore-based materials for delivery of self-assembled therapeutic nanoparticles (Figure 2D). Two studies chemically modified spores from the probiotic bacteria *Bacillus coagulans* to deliver such drugs through the gastrointestinal tract. Yin and coworkers [12] covalently linked the coat proteins of probiotic spores with curcumin, an anticancer agent, and provided further protection to curcumin by covalent conjugation with folate. After spore germination in the gut and subsequent degradation of the outer coat, the coat protein–curcumin–folate complexes self-assembled into micellar nanoparticles. These were well absorbed in the colon and induced apoptosis of cancer cells. Similarly, Song and colleagues coated *B. coagulans* spores with deoxycholic acid, and used electrostatic adsorption to load the spores with the chemotherapeutics doxorubicin and sorafenib [13]. Following spore germination, the coat disassembled, and the hydrophobic proteins in the coat and the hydrophilic deoxycholic acid formed nanoparticles while encapsulating the chemotherapeutics. These nanoparticles resulted in improved endocytosis by epithelial cells.

Self-healing composites

The self-healing property of materials refers to the intrinsic capability of repair without human intervention [14]. Self-healing has drawn attention in the field of construction materials, especially for concrete. Concrete, one of the major components of construction projects, is a composite made of cement, water, and a variety of aggregates (e.g., sands). A persistent issue in concrete structures is crack formation. Cracks that are <0.2 mm wide can lead to permeation of chemicals, including chlorides, sulfates, and acids. This will lead to degradation of the concrete matrix and corrosion of the steel reinforcements within, thereby reducing the durability of concrete buildings. To reduce the efforts associated with the identification and repair of cracks by external means, an autonomous healing mechanism of the concrete is desirable. A promising strategy is the incorporation of ureolytic bacteria (e.g., *Sporosarcina pasteurii* and *B. megaterium*) in the concrete matrix [15,16]. These bacterial species can survive in the alkaline conditions associated with concrete

and induce production of minerals, such as calcium carbonate, which can plug the cracks. This process is called 'microbial-induced calcite precipitation' (MICP) (Box 2). In addition to self-healing, the addition of an optimal amount of vegetative bacteria to the concrete matrix improves its compressive strength [15].

To overcome the short lifespan of bacterial cells, spores are used as biohealing agents in concrete. The permeation of oxygen and moisture through the cracks, combined with pre-existing nutrients within the matrix, results in germination of spores into vegetative cells, followed by MICP (Figure 2E). However, the addition of spores directly to the concrete mixture showed that the spores were viable for only 2 months [17]. The lifespan of the spores in the concrete matrix was enhanced by loading the spores and nutrients in porous particles (e.g., expanded clay particles), which provided additional protection and thereby extended the viability of spores in the concrete to over 6 months [18].

Genetic engineering of bacteria could also be used to improve the healing properties of concrete. However, this has so far only been tested with vegetative cells. The anaerobic bacterium *Cupriavidus* sp. BHK2 expresses an extracellular protein that is responsible for biosilicification (i.e., leaching silica from silicate substrates by living organisms), and is stable at high pH and high temperatures. This protein can induce the precipitation of silicate into a gehlenite phase ($Ca_2Al_2SiO_7$), which enhances the mechanical strength of cementitious structures. Using genetic manipulation techniques, Sarkar and coworkers introduced the biosilicification gene from BHK2 to *B. subtilis,* because this bacterium shows higher viability in cement compared with BHK2 [19]. The addition of the engineered bacteria to concrete provided self-healing properties by precipitation of both calcite and gehlenite. The synergistic development of both minerals enhanced the compressive and flexural strength of the concrete compared with incorporation of unmodified *B. subtilis*. In addition, for noncementious materials, *B. subtilis* can be engineered to secrete scaffold proteins that assemble both cells and the protein as structural components of composite [20]. By appending a silica biomineralization peptide to the scaffold with further genetic engineering, regenerative silica composites were successfully fabricated with enhanced mechanical properties.

Box 2. Biomineralization by microbial-induced calcite precipitation

Biomineralization is the process of formation of minerals by various organisms. Calcium carbonate constitutes a major proportion of biominerals [68]. The ureolytic pathway observed in some bacterial species is one of the most studied process for calcium carbonate biomineralization. Bacteria (e.g., *Sporosarcina pasteurii*) produce urease that hydrolyzes urea [$CO(NH_2)_2$] to form ammonia (NH_3) and carbon dioxide (CO_2) (Equation I). Ammonia is converted to ammonium (NH_4^+) and hydroxide (OH^-) ions, leading to an increase in pH (Equation II). Hydration of CO_2 to form bicarbonate (HCO_3^-) is promoted by the alkaline conditions as well as carbonic anhydrase (Equation III) and is subsequently turned into carbonate ions (CO_3^{2-}) (Equation IV). The surface of the bacterium is negatively charged and attracts positively charged calcium ions (Ca^{2+}) and can act as a nucleation site. The increase in pH shifts the equilibrium toward the formation of CO_3^{2-} ions, and the availability of Ca^{2+} ions on the surface leads to the precipitation of $CaCO_3$ (Equation V) [69].

$$CO(NH_2)_2 + H_2O \rightarrow 2NH_3 + CO_2 \quad [I]$$

$$2NH_3 + 2H_2O \leftrightarrow 2NH_4^+ + 2OH^- \quad [II]$$

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+ \quad [III]$$

$$HCO_3^- \leftrightarrow CO_3^{2-} + H^+ \quad [IV]$$

$$Ca^{2+} + CO_3^{2-} \rightarrow CaCO_3 \quad [V]$$

As a counter-concept of healing, biodegradable plastics with embedded bacterial spores have been recently reported [21,22]. Kim and coworkers developed biodegradable polyurethanes through a hot melt extrusion process by incorporating heat-tolerant *B. subtilis* spores. These heat-tolerant spores were produced by adaptive laboratory evolution, and can degrade polyester-based plastics upon germination at the end of their lifespan [21]. Tang and coauthors leveraged the genetic programmability of *B. subtilis* to introduce inducible secretion of exogenous enzymes capable of hydrolyzing plastics after germinating spores, causing near-complete degradation [22]. It is also notable that the addition of spores in both studies did not compromise the mechanical properties of the plastics.

Functional spore-based materials without germination

DNA storage materials using the spore core

Living organisms use DNA as a storage medium for genetic information, enabling the transmission of their genetic blueprint throughout history. DNA boasts a high information density (up to ~700 TB/g), surpassing a magnetic chip-based data storage system [23]. It can remain readable for thousands of years, offering long-term data storage capabilities [24]. However, DNA stored in nonideal conditions is susceptible to mutation or degradation, possibly leading to inaccurate data [25–27].

While living cells can be used for DNA storage, constant maintenance and unexpected replication errors make them unsuitable. The *Bacillus* spore core might be an ideal alternative vehicle for data storage, given that the bacterial chromosome localized within the core is less susceptible to mutation or degradation for several reasons (Figure 3A) [28,29]: (I) the gel-like structure of the core combined with the low water content (~20%) is likely to induce minimal reactivity of enzymes. In the core, diverse macromolecules and calcium dipicolinate (CaDPA; Box 1) are immobilized, while water is highly mobile, similar to levels seen in the protoplast of normal cells [30,31]; (II) small acid-soluble proteins (SASPs) are formed during sporulation and saturate the core. These SASPs have nonspecific DNA-binding properties, protecting chromosomes from chemicals, UV, and heat [32,33]. These links to DNA are also known to induce a crystalline nucleoid, which stabilizes DNA fibrils and protects them against modification [28]; and (III) the core enclosing the bacterial chromosome has multiple well-designed layers on the outside, enabling selective permeability and screening for undesired chemicals or lytic enzymes. The immobile inner membrane that envelops the core has low permeability for ions and even for small nonionic solutes, hence protecting the core DNA from chemical attacks [34,35]. At the periphery of the coat, multiple layers of cross-linked proteins and surrounding glycoproteins filter macromolecules from outside [36,37].

A recent study demonstrated that localization of data encoded on DNA in the spore core enabled stable and long-term storage [38]. Liu and colleagues transformed three *Bacillus* species with a synthetic vector harboring a data-encoded sequence. Less than 1% error rates in the retrieved sequence were found after the spores underwent aging treatments based on heat, oxidative stress, and UV irradiation. Furthermore, by embedding the spores within a protective matrix (e.g., acacia gum), the DNA sequences can be preserved in principle for more than a few hundred years.

Hygromorphic materials using the spore cortex

Living bacteria exhibit osmolarity- and moisture-induced expansion and shrinkage. However, they cannot tolerate numerous fluctuations or extremes. Given the physical properties of the cortex peptidoglycan layer, *Bacillus* spores show rapid shape-changing behavior in response to relative humidity (RH) while staying dormant (Figure 3B) [39–42] (Box 3). A recent study using focused ion beam scanning electron microscopy (FIB-SEM) revealed that the cortex layer of *B. subtilis* spores is ~106.6 nm thick and contributes to 51.7% of the volume of the spore [39].

Box 3. What makes the spore cortex expand and shrink?

In terms of chemical structure, the PG is assembled from a glycan backbone of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid, and the glycan strands are cross-linked by short peptides of L-alanine, D-glutamic acid, and *meso*-diaminopimelic acid [70,71]. PG chains in *B. subtilis* spores exhibit less cross-linking (13–19%) compared with those in vegetative cells (~40%), owing to the absence of the main covalent linker, teichoic acids, and to the fewer peptide side chains in spores (Figure I) [72,73]. The PG layer can absorb water, and the amount of cross-linking is thought to influence the extent of absorption. For reference, loosely cross-linked PGs of other bacteria showed a notable volumetric change upon alteration of the ionic strength of the environment [74]. In addition, the PG layer of *B. subtilis* spores has a nanoporous structure varying from 6.8 to 38.4 nm in pore diameter [75]. These two characteristics are likely to influence the expansion under changing osmolarity conditions, as well as the reversible expansion and shrinkage dependent on the relative humidity (RH) [39].

The RH dependent behavior arises from the hydrogel-like sorption of water on the outer layers of the spores. The PGs present in the cortex of the spore have a cross-linked structure similar to that of rubber and can swell by the sorption of water, again similar to rubber in organic solvents. As the RH increases, so does the swelling of the cortex and the size of the pores, allowing for faster diffusion of water. Upon lowering the RH from equilibrium, the water molecules diffuse faster through pores in high RH and, therefore, the response time back to the equilibrium is shorter compared with lower RH conditions. This results in a RH-dependent response time of the spores [76,77].

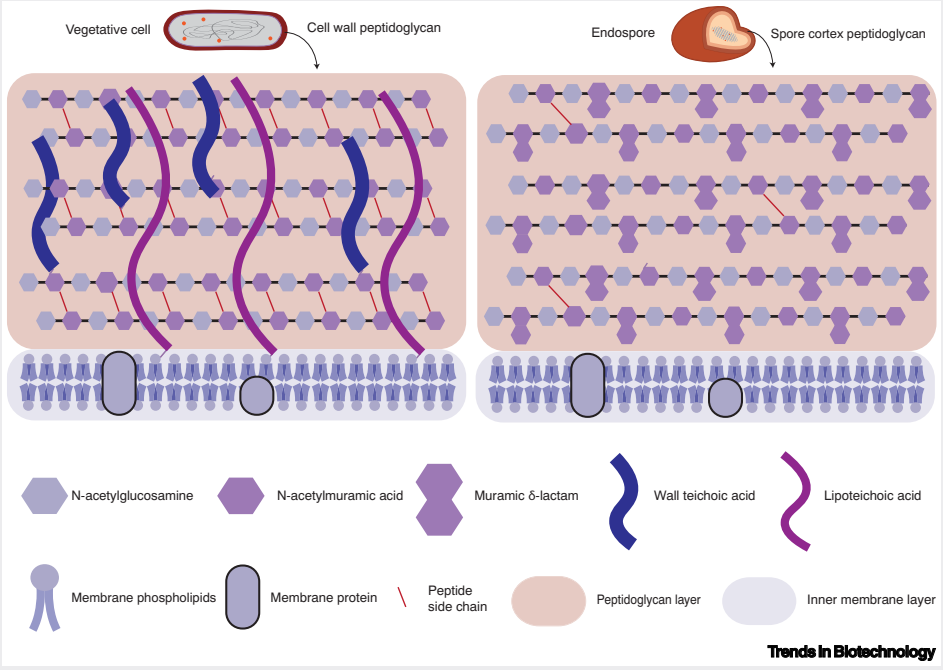

Figure I. Comparison of the *Bacillus subtilis* peptidoglycan layer in vegetative cells (left) versus endospores (right).

The same study revealed that the cortex layer expanded 43.2% in volume when varying the RH from 10% to 90%, resulting in the cortex contributing to 67.3% of the total volume change of the spore.

A previous study evaluated the **energy density** from **strain responses** of *Bacillus* spores under controlled cycles of RH using atomic force microscopy [40]. The response of spores to changing humidity conditions exerted a mechanical force on the microcantilever tip, measuring energy densities ranging from 10 to 20 MJ/m^3, highly comparable to actuator materials (e.g., shape memory alloys, such as TiNi). Taking advantage of the reasonably high energy densities, hygromorphic sheets were fabricated using *Bacillus* spores as functional units [40–42]. Spores deposited onto a latex rubber sheet induced differential strain and subsequent curvature changes under

controlled RH cycles (Figure 3B). Combined with an electromagnetic generator, the actuation performance of spore-coated rubber sheets could generate electricity when alternating the flow of dry and moist air, reaching a specific electrical power of 233 mW kg^{-1} [40].

The efficiency of spore-based hygromorphs can be improved in several ways. First, Chen and coworkers suggested that the selection of spores from a genetically modified strain or other promising strains with a higher strain response would lead to nanogenerators with improved performance. For instance, *B. subtilis* lacking genes encoding coat proteins (*cotE* or *gerE*) and *B. thuringiensis* exhibited enhanced strain (%) under exerted force, and a twofold increase in energy density compared with wild-type *B. subtilis* spores [43]. For the *B. subtilis* mutant, the improvement stemmed from a reduced spore volume and, thus, a higher relative cortex volume. Second, adhesives might help to transmit high mechanical forces between spores or between aligned spore layers by bonding them together or filling the space between them. Cakmak and colleagues demonstrated that using synthetic adhesives in a spore-based actuator led to 100-fold increased actuation speed and energy production [41].

Biocatalytic materials using the spore coat

The coat of *B. subtilis* spores comprises a multilayered proteinaceous structure, with each layer formed by distinct types of protein (Box 4). The coat has attracted extensive interest in the field of spore-based materials due to possibilities for molecular display; **exogenous proteins** can be fused to the coat proteins that are anchored within the layer, such that they are localized on the spore surface (Figure 3C) [44]. By doing so, exogenously expressed proteins do not require the secretion system of a **microbial chassis** and subsequent purification, while retaining their native form and activity. Not only enzymes (e.g., β-galactosidase and lipases), but also other proteins, such as fluorescent proteins, peptide-based toxins, and flagella cap proteins, have been successfully displayed on the coat [45–49]. These findings have mainly led to the development of spore-based immunization agents and catalytic materials. Since the application of engineered spores as immunization agents has been reviewed elsewhere [50], we focus here on their use in catalysis and on the developed related synthetic biology tools.

Some coat components of *B. subtilis* spores themselves show catalytic activity [51]; for example, the CotA protein, one of the components within the outer coat, exhibits laccase activity and biosynthesizes melanin-like pigments outside the coat, leading to higher fitness of spores under UV irradiation. Aside from this intrinsic catalytic activity, numerous studies on engineered spores have shown enzymatic activity of exogenous enzymes displayed on the spore surface [46,52]. Recently, research revealed that exogenous enzymes fused to the coat have a higher stability and shelf-life compared with the purified soluble versions. Bartels and coworkers introduced exogenous laccases from *Escherichia coli* and *Bacillus pumilis* to a CotA-deficient *B. subtilis* strain [46]. Laccase fused either N or C terminally to coat proteins exhibited enzymatic

Box 4. Morphogenesis of coat and subcellular localization of coat proteins

The proposed model of coat proteins in *Bacillus subtilis* comprises four distinct layers: the basement layer, inner coat, outer coat, and crust [60,78]. Assembly of each layer is regulated by specific morphogenetic proteins: SpoIVA, SafA, CotE and CotX/CotY/CotZ, respectively. The contributions of SafA, CotE, and CotX/CotY/CotZ proteins to the assembly of each layer were elucidated using a reverse genetic approach [60,78–81]. These underlying proteins are thought to facilitate coat assembly by providing binding sites for the other coat proteins. The crust, the outermost glycoprotein layer in *B. subtilis*, is assembled from at least six proteins: CotV, CotW, CotX, CotY, CotZ, and CgeA. Crust proteins are assembled during the prespore stage of sporulation. CotX, CotY and CotZ have crucial roles in crust formation, with CotY and CotZ serving as the main structural components. CotX and CotV are homologs and minor components of the crust, with CotV being dependent on CotX. CgeA proteins anchor polysaccharides to the coat by providing glycosylation sites [67,82,83]. CotW might have a role as a linker between the outer coat and the crust [82].

activity and a half-life of nearly 3 months, whereas the purified soluble enzyme had a half-life of <24 h. Furthermore, Hui and colleagues demonstrated that an anchored lipase had increased stability in harsh conditions (e.g., methanol or elevated temperature) compared with a free soluble protein, likely due to local environmental differences [45]. The same study highlighted that the spores can be reused for enzymatic reactions. These recent findings indicate that catalytic spores have practical advantages for industrial use compared with purified enzymes.

Spores have also been housed within a matrix to develop new catalytic materials. Recently, Kawada and coworkers demonstrated the manufacturing of self-assembled catalytic materials by integration of genetically engineered *B. subtilis* spores within phenylboronic acid (PBA)-functionalized polymers [53]. The diol groups of the surface glycan of *B. subtilis* spores autonomously form covalent bonds with boronic acid-functionalized polymers, resulting in mechanically robust hydrogels [54]. Within the hydrogels, lipase anchored in the *B. subtilis* spore coat exhibited enzymatic activity.

Several toolkits enabling protein fusion to the crust, which is one of the layers of the spore coat, accelerated the development of spore-based catalytic materials. A previous study standardized protein display on the *B. subtilis* spore crust, an approach known as 'Sporobeads' [46]. In this system, vectors constructed using the BioBrick cloning standard [55], enable exogenous protein fusion to the N or C terminus of various crust proteins under the P_{cotYZ} promotor, which is a promotor for coat proteins. Based on intensity measurements of GFP fusions, N-terminal fusions to CotY and CotZ exhibited the best anchoring capacities (up to ~2×10^4 molecules per spore), while fusions to CgeA showed the lowest. Another recent spore protein display system, 'TIED', features a T7 RNA polymerase (RNAP)-driven expression system inserted into two separate gene loci of the *B. subtilis* genome [45]. Expression of the T7 RNAP gene integrated in the *thrC* site, under control of a coat protein promoter, such as P_{cotZ}, induced expression of a crust-fused exogenous protein inserted in the *amyE* site, under control of the P_{T7} promoter. The TIED system improved the loading density of enzymes up to a range of 10^6 to 10^7 per spore.

Concluding remarks and future perspectives

To implement spore-based materials in practical applications, scientific, ethical, and environmental prospects should be taken into consideration. Among the related scientific challenges (see Outstanding questions), controllable germination is an essential goal to achieve in multiple applications. Bacterial spores are sensitive, and small amounts of nutrients or unexpected environmental conditions (e.g., cationic surfactants, such as dodecylamine, or very high pressure) can cause them to germinate. Uncontrolled germination is likely to impair the functionality and practical use of the material within the user-defined scheme. For materials requiring multiple sporulation and germination cycles (e.g., prolonged self-healing composites), it is imperative to demonstrate the viability of the spores and their ability to undergo multiple cycles. Further efforts are required to characterize germination/sporulation traits under various environmental conditions.

While the resistance and dormancy of spores enable their use in the field, such uses are associated with the risk of leakage into the environment. Unintentional release of genetically modified organisms has been an important topic of environmental and ethical debate in recent years. Their escape could lead to unpredicted effects on multiple organisms in the ecosystem, which can be particularly critical because of the resistance of bacterial spores to disinfectants and other sterilization methods. To prevent the spread of antibiotic resistance genes in applications using genetically modified spores, selection methods using specific amino acids, instead of antibiotics, could be put in place. Alternatively, implementation of auxotrophic biocontainment strategies, such as thymineless death complemented with controlled expression of gene of interest, can

Outstanding questions

Are bacterial spores time- and cost-efficient functional units for manufacturing materials, compared with vegetative cells? Sporulation takes longer time and the purification of spores from cells is laborious and requires chemicals. These factors might hinder the scalable production of spore-based materials, especially those requiring large amounts of spores (e.g., self-healing concrete or hygromorphs).

Are *Bacillus subtilis* spores the optimal resource for spore-based material development? *B. subtilis* has been used as model organism for biological studies and relevant applications, which provide a better understanding of the intrinsic properties of bacterial spores. However, *B. subtilis* might not be the best option in terms of material performance.

How can we regulate sporulation and germination of spores within materials? For germination-based materials, germination and sporulation within materials should be programmable following the users' scheme. For nongermination-based materials, the spores should be maintained in a dormant state.

How can we fabricate spore-based materials with minimal human intervention? While biohybrid materials offer promising avenues, biologically grown ELMs represent a self-assembled bottom-up approach toward achieving autonomous production. For instance, the utilization of cellular consortia comprising endospore-forming bacteria and engineered matrix-producing bacteria (such as those generating cellulose scaffolds) could facilitate autonomous biological production of materials.

How can we fairly compare the performance of spore-based materials and other alternative systems from various perspectives? For the development of spore-based materials, their performance with respect to cost, time, and functionality should be compared comprehensively with those of competing technologies.

prevent the dissemination of engineered bacteria [56,57]. In addition, physical containment methods, such as using a robust tough shell hydrogel, can prevent the escape of the bacteria [58]. Overall, thorough risk assessment, strict regulations, and proper control measures to prevent the spread of genetically modified spores will be necessary for the use of these materials in real-world applications.

How does the performance of spore-based materials compare with other competing systems? For instance, cell-free systems, using materials, such as enzymes and metabolites, derived from cells, are designed to perform biological functions *in vitro* (e.g., translation, transcription, and catalytic functions) and they are not limited by cell cytotoxicity, cellular complexity, and genomic mutations down the line. However, the shelf-life of these materials is limited by the stability of the enzymes. By comparison, spore-based materials can autonomously produce desired molecules and functions, which do not require laborious processes to obtain enzymes or cell extracts. The materials can be stored at ambient conditions for extended periods of time and can germinate on demand without compromising their functionality. However, strict comparisons in terms of cost, time, and performance with alternative technologies have not yet been performed, which are important milestones for the use of such materials in real-world applications.

The intrinsic dormancy, resistance, and unique physical properties of bacterial spores have already led to their diverse applications in materials. Nevertheless, distinctive properties of bacterial spores offer inspiration for the development of new spore-based materials. For instance, exploiting the selective binding properties of germinosomes for specific amino acids could lead to the creation of spore-based amino acid sensors. In addition, the genetic programmability of bacteria can improve the range of existing applications (e.g., biosensors or biocatalysts) or improve their functionality, both of which are highly dependent on emerging gene circuits. These two factors suggest that unexploited spore properties and synthetic biology tool development are crucial for the emergence of novel spore-based materials.

Author contributions

L.R.K., J-J.O., and M-E.A-T. conceptualized the review. L.R.K. and J-J.O. reviewed the literature and wrote the original draft. All authors reviewed and edited the manuscript.

Acknowledgments

This work was supported by the NextSkins project, funded by the European Union's Horizon Europe Research and Innovation Programme under grant agreement number 101071159.

Declaration of interests

The authors declare no competing interests.

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