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SYNTHETIC BIOLOGY

Bacterially grown living materials with resistant and on-demand functionality

Jeong-Joo Oh^{1†}, Franka H. van der Linden^{1†}, Koray Malcı^{2,3}, Ramon A. van der Valk¹, Tom Ellis^{2,3}, Marie-Eve Aubin-Tam^{1*}

Inspired by naturally occurring biomaterials, autonomously grown engineered living materials (ELMs) feature cell-driven growth and programmable biological functions. However, the “livingness” of cells poses a short life span and low tolerance to harsh conditions, limiting the practical use of such materials. Here, we developed materials with programmable and dormant functionalities, grown from a mixture of *Komagataeibacter rhaeticus* and *Bacillus* endospores under engineered medium conditions. *K. rhaeticus* produces the bacterial cellulose (BC) matrix that integrates *Bacillus* spores within, whereas the confined spores keep dormant and are resistant to harsh conditions in the environment. *Bacillus* spores can germinate and confer desired functions to the materials. Modulating the binding affinity of spores to the BC matrix with genetic engineering can improve cell loading and therefore enhance the material functionality. These materials can serve as a versatile on-demand platform for applications as biosensors, biocatalytic materials, and in situ transformation of mechanically robust cellulose-based composites.

INTRODUCTION

Living materials in nature exhibit autonomous growth and complex functions, driven by the orchestration of cells confined within or adhered to biological matrices. These properties boost the emergence of “biologically grown” engineered living materials (ELMs) built with living microbes (1–4). In such materials, living cells produce self-assembling building molecules that form a mechanically robust matrix, avoiding the labor of integrating living cells when using man-made matrices (e.g., synthetic hydrogels). The cells also confer programmable functions to the material, including sense-and-respond function, biocatalysis, and local patterning (1–4). For example, *Escherichia coli* naturally secretes the amyloid protein CsgA, which self-assembles extracellularly into nanofiber networks to form biofilms (5). By appending functional peptide domains to CsgA, such biofilms exhibit programmable functions (2). However, the “livingness” of cells within biologically grown ELMs naturally comes with certain challenges. The short life span of cells limits the use of material unless nutrients are continuously provided. The susceptibility of cells to harsh conditions precludes postprocessing options (e.g., heat and drying) of materials and so requires cold-chain logistics for global distribution. These qualities render them unsuitable for on-site and on-demand applications outside controlled laboratory environments. One important aim of biologically grown ELMs is the creation of materials with a long life span and resistant functionalities to meet the requirements of real-world applications.

Living microbes can improve their fitness and survival by synthesis of an extracellular protective matrix. A representative example of this is the cellulose producing bacterium, *Komagataeibacter rhaeticus* (6), which produce highly pure bacterial cellulose (BC) fibers that surrounds cells and forms a mat at the air-liquid interface (6). The BC matrix plays a role as a physical barrier against various stress factors (6), and its water-holding ability may support the vegetative cell

cycle of bacteria. Furthermore, the matrix has great structural integrity due to the high crystallinity of BC fibers and their interconnected networks, enabling manual manipulation and postprocessing (7). These properties of BC have contributed to emerging cases of BC-based living materials (3, 7–9).

Some bacterial species (e.g., *Bacillus subtilis*) can keep their viability even in challenging environments (10) (e.g., nutrient free and high temperature) in the form of endospores, a dormant and metabolically inactive state that is initiated when conditions are unfavorable. Endospores can germinate when exposed to germinants and initiate a vegetative cell cycle under growth-permissive conditions. Therefore, incorporating spores instead of vegetative cells into ELMs can overcome the concerns typically associated with living cells (11, 12). Furthermore, given the variety of endospore-forming species and the current possibilities for genetic modification with synthetic gene circuits, such spore-based living materials have broad potential in terms of applications (11–14).

Here, we fabricate materials with programmable, resistant, and dormant functionalities, autonomously grown from mixtures of *K. rhaeticus* and *Bacillus* endospores, using an engineered medium. This approach leverages a division of labor; *K. rhaeticus* forms and grows a BC pellicle at the air-liquid interface, encapsulating the surrounding floating spores (Fig. 1A), and the integrated spores can germinate under growth-permissive conditions and conduct programmable functions. By genetically modifying spore surface properties, the incorporation efficiency and functionality of the materials were enhanced. Last, we demonstrate that these BC-spore living materials find applications in biosensing, biocatalysis, and in situ composite formation, by integrating spores with the desired function, gained either through genetic engineering or natively present in the species of choice.

RESULTS

Engineering culture conditions to grow BC-spore living materials

To enable the incorporation of *Bacillus* spores within a growing BC pellicle, we engineered medium compositions to meet the following

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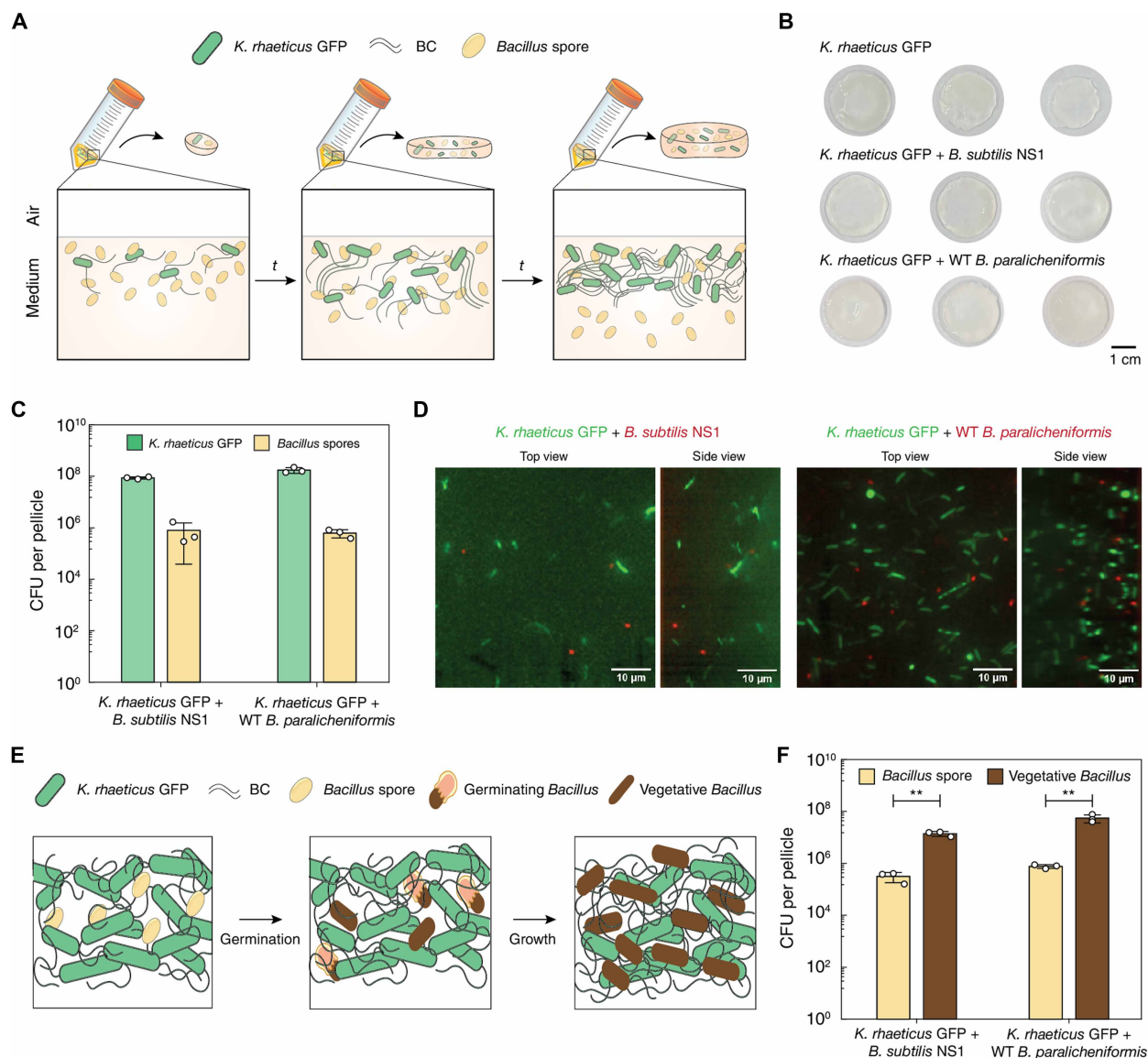


Fig. 1. BC-spore living materials autonomously grown from a mixture of *K. rhaeticus* vegetative cells and *Bacillus* endospores. (A) Schematic depicting the growing BC-spore living material. (B) Photographs of washed BC pellicles with and without *Bacillus* spores, grown by *K. rhaeticus* GFP. (C) Number of viable *K. rhaeticus* GFP and *Bacillus* spores within a pellicle. (D) Confocal imaging of pellicles composed of *K. rhaeticus* GFP and *B. subtilis* NS1 spores (left) or WT *B. paralicheniformis* spores (right). For visualization, *Bacillus* spores were labeled with a fluorescent lipid probe, di-4-ANEPPS. (E) Schematic showing the germination of *Bacillus* spores and reproduction within a BC pellicle. (F) Number of viable *Bacillus* cells within a pellicle after germination for 24 hours in LB. Asterisks indicate statistical significance by Student's *t* tests (***P* < 0.01). In (C) and (F), CFU counts were determined by plating serial dilutions of hydrolyzed pellicles. The BC-spore pellicles were prepared in triplicate (dots); error bars indicate the SD.

requirements: (i) the spores stay dormant; (ii) the spores need to float and be incorporated within the pellicle, which intrinsically grow at the air-liquid interface; and (iii) *K. rhaeticus* still forms robust BC pellicles that can be easily manipulated manually (with, e.g., tweezers) and posttreated (e.g., washing and drying). To satisfy these requirements, we modified a yeast extract, peptone, and dextrose (YPD) medium by lowering the pH and increasing the density of the liquid. Low pH conditions can inhibit the initial step of *Bacillus* spore germination (15, 16) but are still compatible with *K. rhaeticus* growth and cellulose production (17). Increasing the medium's density can prevent the sedimentation of *Bacillus* spores, which settle quickly in normal medium due to the high wet density of spores (18, 19).

By fine-tuning such medium parameters, we optimized a culture condition for the bacteria mixture of wild-type (WT) *K. rhaeticus* cells and *Bacillus* spores to form BC-spore living materials within a few days (figs. S1 to S7). Briefly, the bacteria mixture was cultured in YPD medium acidified to pH 4.0 and supplemented with 40% Nycodenz [5-(*N*-2,3-dihydroxypropylacetamido)-2,4,6-triiodo-*N,N'*-bis(2,3-dihydroxypropyl)isophthalamide]. *Bacillus* spores from two species (*B. subtilis* NS1 and WT *B. paralicheniformis*) stayed dormant at this pH (figs. S1 and S2). The Nycodenz supplementation increased the number of spores incorporated by 4.5-fold for *B. subtilis* NS1 and 49-fold for WT *B. paralicheniformis*, by preventing the sedimentation of spores (figs. S3 and S4). Modifications to the medium reduced

the pellicle yield. For instance, reducing the pH from 6.5 to 4.0 decreased the dry weight of cellulose pellicles by ~30%, from 28 mg (SD = 2.2 mg) to 20 mg (SD = 2.0 mg), after 3 days. Also, the addition of 40% Nycodenz further reduced the yield by ~50%, bringing it down to 11 mg (SD = 2.0 mg) (figs. S5 to S7). Despite these reductions, the BC pellicles remained physically durable and suitable for handling in subsequent experiments (Fig. 1B).

The cell number and their spatial distribution within BC-spore living materials were characterized, using a green fluorescent protein (GFP) expressing strain of *K. rhaeticus* (*K. rhaeticus* GFP) and fluorescently dyed spores (fig. S8; see the Supplementary Materials). *K. rhaeticus* GFP cells were dominant in total number (10^8) compared to *B. subtilis* NS1 (8×10^5) and WT *B. paralicheniformis* (6×10^5) spores (Fig. 1C). Confocal imaging revealed that *Bacillus* spores were incorporated separately, and they were predominantly detected in the outer 20 μm on both sides of the pellicle (Fig. 1D and fig. S9). Scanning electron microscopy (SEM) imaging of dried pellicles confirmed the presence of spores at the outside of the pellicles, encapsulated by cellulose fibers (figs. S10 and S11).

Once growth-permissive conditions are available, the encapsulated spores should be able to germinate and reproduce (Fig. 1E). To test this, we incubated BC-spore living materials in LB medium and counted the viable *Bacillus* cells per pellicle by plating serial dilutions after enzymatic degradation of the cellulose. After 24 hours of incubation, viable cell counts revealed a substantial increase in colony-forming units (CFU) per pellicle for both *B. subtilis* NS1 (3.1×10^5 to 1.4×10^7 CFU) and WT *B. paralicheniformis* (7.6×10^5 to 5.5×10^7 CFU) (Fig. 1F). These results indicated that *Bacillus* spores within BC can switch to a metabolically active state and initiate a vegetative cell cycle. Germinated cells can also undergo an additional sporulation cycle within the material (table S1; see the Supplementary Materials). Through a germination-sporulation cycle, the number of *B. subtilis* NS1 spores embedded within the material increased by 450- to 2100-fold, depending on the conditions of sporulation.

BC-spore living materials exhibit programmable, resistant, and dormant functionality

Having established the production of BC-spore materials, we investigated whether *Bacillus* cells after germination can endow programmable functionalities to the pellicles. As a proof of concept, we introduced a sense-and-respond function to the pellicles by integrating spores with inducible GFP expression in pellicles grown by WT *K. rhaeticus* (Fig. 2A). To this end, two *Bacillus* strains, *B. subtilis* NS1 and *B. paralicheniformis* GFP_{ind}, were used (fig. S12). The former strain contains a *GFPmut2* sequence under control of an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible promoter (*P_{spac-hy}*), which is inserted into the *amyE* site in the genome (20). The latter strain was prepared by transforming WT *B. paralicheniformis* with a plasmid, pAD-sfGFP-*P_{man}*, harboring a superfolder GFP (*sfGFP*) sequence under the control of a mannose-inducible promoter (21). The GFP expression conditions of both strains were optimized for the concentration of the inducers; accordingly, 1 mM IPTG and mannose (20 mg/ml) were chosen for *B. subtilis* NS1 and *B. paralicheniformis* GFP_{ind}, respectively, for inductions in further experiments (fig. S13).

We germinated the spores within pellicles, added the inducer, and recorded the fluorescence of the pellicles. The results reveal a clear signal from the pellicles for both strains compared to controls (no induction) (Fig. 2B). Consistent with those results, confocal

imaging exhibited that individually distributed *Bacillus* cells clearly expressed GFP within the pellicles (Fig. 2C). The fold change in fluorescence intensity was notably higher for pellicles containing *B. paralicheniformis* GFP_{ind} (Fig. 2, B and D), consistent with the tests for inducer concentrations (fig. S13). This was most likely due to (i) multiple copies of the *GFP* gene in *B. paralicheniformis* GFP_{ind} (expression from a plasmid) but a single copy integration in the genome of *B. subtilis* NS1, (ii) differences in *GFP* versions (*sfGFP* in *B. paralicheniformis* GFP_{ind} and *GFPmut2* in *B. subtilis* NS1), and (iii) differences in promoter strength (mannose or IPTG induction). These controlled gene expression studies confirmed that programmable functions can be conferred to the materials by integrating the spores from engineered *Bacillus* cells, using either plasmid-based expression or genomic integration. To demonstrate real-world bio-sensing applicability, we generated a glycerol-responsive BC-spore material to detect the presence of glycerol. Glycerol is a well-known food additive (e.g., hydration agent in sports drinks) and a by-product of fermentation (e.g., winemaking), both of which require continuous quality monitoring. A glycerol-inducible expression system based on the *glpD* promoter (named as *P_{gly}*) was used, which is regulated by the antiterminator protein GlpP (22). A plasmid containing this regulatory module was assembled with standardized transcriptional modules from STK as described in a previous study (23) and then introduced into the *B. subtilis* NS4 strain, creating a glycerol-sensing strain (*B. subtilis* NS4 GFP_{gly}). Spores from the strain were then incorporated into a BC matrix to produce a functional glycerol-responsive ELM. This ELM successfully sense glycerol in drinking samples for the purpose of food quality monitoring (fig. S14; see the Supplementary Materials).

Next, we investigated whether the resistant and dormant properties of the *Bacillus* spores are maintained in the BC-spore material. We first exposed pellicles with GFP-inducible spores to harsh conditions and evaluated the fold change in green fluorescence intensity (Fig. 2D). The functionality of the materials was mostly maintained after 1-hour treatment with 70% ethanol or 30 min of illumination with UV-A (wavelength around 365 nm and intensity of 21.8 mW/cm²) but not after 1 hour of illumination with UV-A. It should be noted that all pellicles were heated to 70°C during 10 min before germination to eliminate *K. rhaeticus* cells. In addition, pellicles were air-dried or freeze-dried, transforming them to thin paperlike materials (fig. S10). After germination, freeze-dried pellicles exhibited fold changes comparable to untreated ones, whereas the fold change was reduced for air-dried pellicles. The air-drying method caused less porosity and therefore less swelling of the cellulose fiber networks compared to freeze-drying (3, 24). Thus, freeze-drying is likely to favor medium diffusion into the BC and access to nutrients for the spores. To test long-term dormancy, air-dried or freeze-dried pellicles with *B. paralicheniformis* GFP_{ind} or *B. subtilis* NS1 spores were stored for up to 6 months at room temperature. The pellicles still displayed spore germination, and a GFP signal was successfully detected (Fig. 2E). These results confirm that the integrated spores can survive under the tested conditions and keep their dormancy, enabling various treatments and on-demand use of the BC-spore material.

Enzyme functionalization of the BC matrix using spores from engineered *B. subtilis*

Functionality of living materials mainly relies on the specialties of their living components. *B. subtilis* is well characterized as microbial

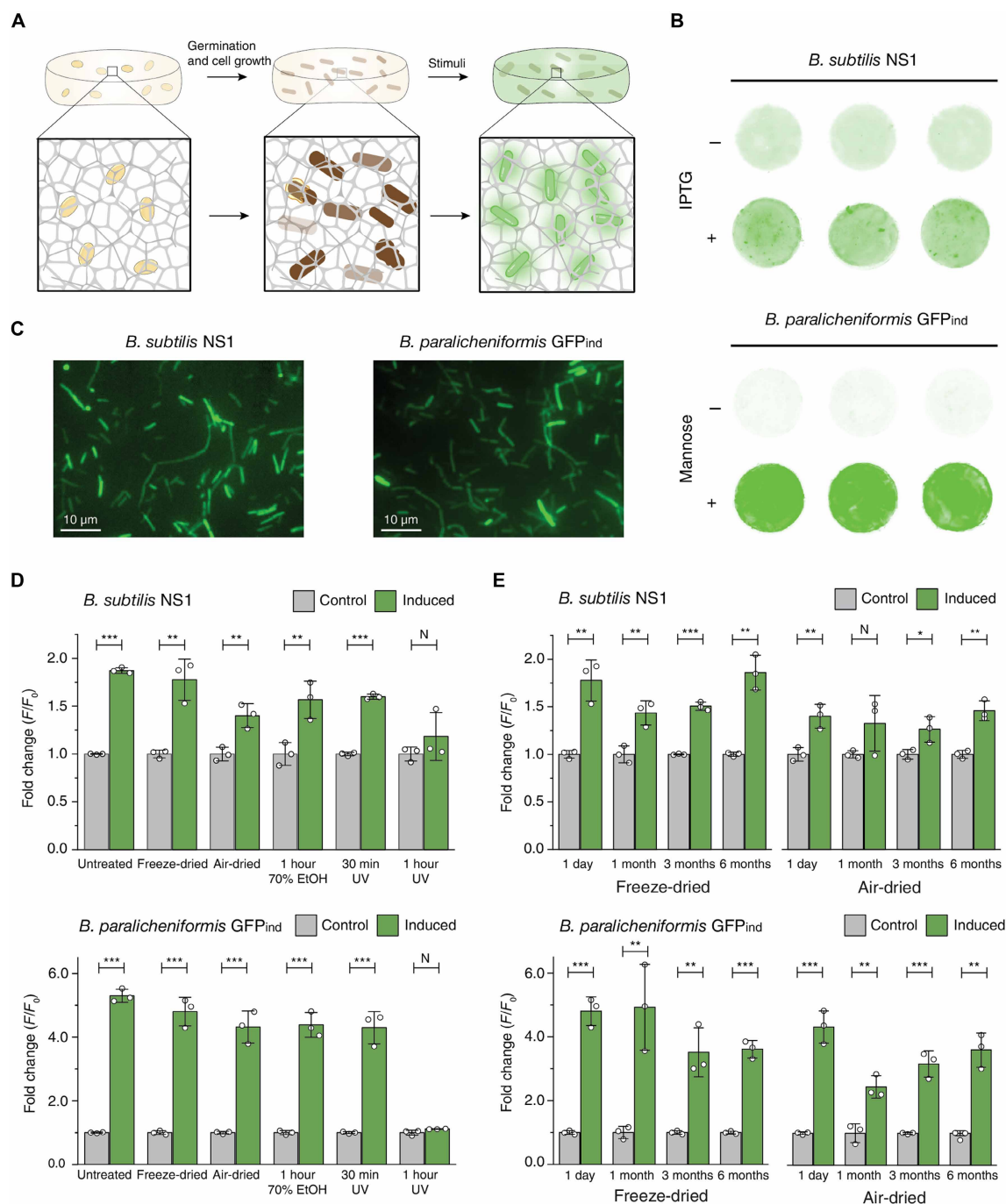


Fig. 2. BC-spore living materials exhibit programmable, resistant, and dormant functionalities. (A) Schematic illustrating how the incorporated *Bacillus* spores confer the sense-and-respond function to the pellicles. The spores undergo germination under growth-permissive conditions, and the resulting vegetative cells produce GFP after specific stimulation. (B) GFP expression in freshly formed, nondried pellicles containing vegetative cells of *B. subtilis* NS1 (top) and *B. parlicheniformis* GFP_{ind} (bottom). After germination, samples ($n = 3$) were incubated with or without inducer molecules (IPTG or mannose, respectively) and imaged for GFP fluorescence after 24 hours. Fluorescence is presented in green color on a white background using ImageJ, and different scaling was applied to each species to emphasize the difference between induction and control. When measured, the fluorescence in the control samples was similar for both species. (C) Confocal imaging of pellicles containing vegetative cells of *B. subtilis* NS1 (left) or *B. parlicheniformis* GFP_{ind} (right), after germination as shown in (B). (D) Environmental resistance examination of BC-spore living materials. The GFP expression from the pellicles containing *B. subtilis* NS1 or *B. parlicheniformis* GFP_{ind} was measured after various treatments: freeze-drying or air-drying, incubation in 70% ethanol (1 hour), and UV-A radiation (30 min and 1 hour). (E) Dormancy examination of dried BC-spore living materials. The GFP expression from the pellicles containing *B. subtilis* NS1 and *B. parlicheniformis* GFP_{ind} was measured after germination and induction. The pellicles were air-dried or freeze-dried and stored for 1, 3, or 6 months. In (D) and (E), GFP intensity was measured with a transilluminator after 24 hours of induction, and the fold changes (divided over noninduced samples; $F/F_0 = 1$ represents no fold change) were calculated. Asterisks indicate statistical significance by Student's t tests (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). N indicates no significant difference between groups based on the same test.

specialist for high-level protein secretion (25). By leveraging the protein secretion system of *B. subtilis*, we functionalize pellicles as catalytic materials by enzyme secretion (Fig. 3A). As a proof of concept, we first engineered *B. subtilis* NS1 to secrete β -galactosidase (β -Gal) fused to the PhoD secretion peptide under a strong constitutive promoter, P_{3P} (26, 27), from a newly designed plasmid pFJ08- P_{3P} -*lacZ* (*B. subtilis* NS1 β -Gal) (Fig. 3B and figs. S15 and S16). The enzyme activity within cellulose can be confirmed with the chromogenic substrate X- β -Gal (Fig. 3C), which is degraded into a dark blue compound.

Next, we prepared pellicles using a mixture of WT *K. rhaeticus* and the spores from *B. subtilis* NS1 β -Gal. Following the germination and growth of *B. subtilis* NS1 β -Gal within the pellicle, we confirmed enzyme activity from pellicles, as indicated by the color change (Fig. 3, D and E). Furthermore, it was observed that prolonged incubation of pellicles in medium increases the catalytic activity (Fig. 3, D and E). This is likely due to accumulation of secreted enzymes within pellicles. These results indicate that the BC can be functionalized by integrating the spores from enzyme-secreting *Bacillus* cells.

Engineering the binding affinity of spores to the matrix for material functionality enhancement

We hypothesized that increasing the number of spores incorporated in the pellicles would allow faster biosensing detection and enzymatic

functionalization. Higher initial cell numbers might reduce the incubation time to achieve a high biomass, allowing an earlier programmed output. Thus, we modulated the binding affinity of the spores to BC by both modifying the crust layer of coat protein and reducing the outermost polysaccharide layer (Fig. 4A and fig. S17). *B. subtilis* spores have hierarchical proteinaceous layers (basement layer, inner coat, outer coat, and crust), in which CgeA plays as a linker between the crust and the encapsulating polysaccharide layer (28, 29). We displayed the cellulose binding module (CBM) from *Clostridium thermocellum* on the crust proteinaceous layer by fusing the CBM with CotY, which is one of the main crust components, and mScarletI, a red fluorescent protein for confirmation of expression. The genetic construct was integrated into the *ThrC* ectopic site, yielding strain *B. subtilis* NS2 (fig. S17). To reduce the polysaccharide layer around the spore, the *cgeA* deletion in *B. subtilis* NS2 was conducted with the insertion of kanamycin resistance cassette into the *cgeAB* gene cluster, creating strain *B. subtilis* NS3. As a control, *cgeA* was deleted in *B. subtilis* NS1, yielding strain *B. subtilis* NS4 (fig. S17). We confirmed that the spores of engineered strains *B. subtilis* NS2 and *B. subtilis* NS3 expressed CBM and that the halolike structure of polysaccharides was barely visible on the surface of *B. subtilis* NS3 and *B. subtilis* NS4 (Fig. 4B). The engineered strains displayed similar spore density, buoyancy, germination properties, and resistance to

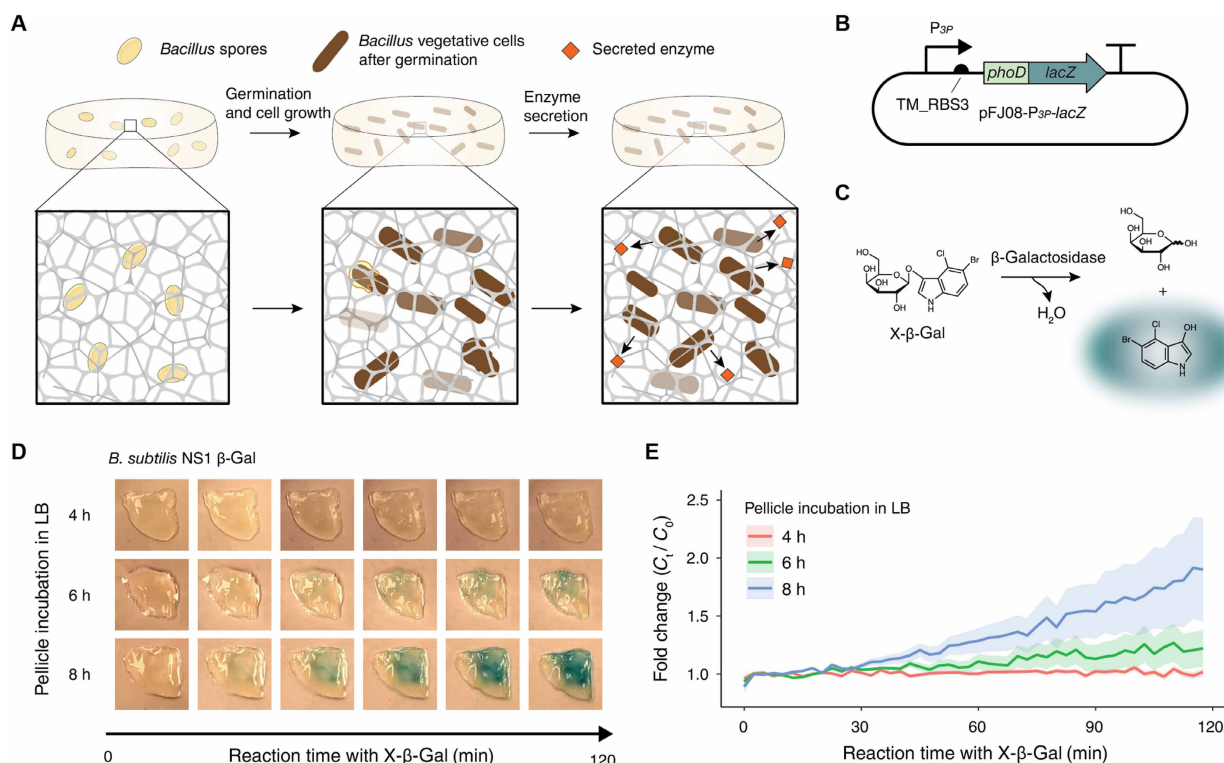


Fig. 3. BC-spore living materials can be enzyme-functionalized. (A) Schematic illustrating how the incorporated *Bacillus* spores confer the catalytic function to the pellicles. The spores undergo germination under growth-permissive conditions, and the resulting vegetative cells secrete enzymes. (B) Genetic construct for β -Gal secretion from *B. subtilis*. P_{3P} , a synthetic promoter optimized for *B. subtilis*; TM_RBS3, a ribosomal binding site optimized for *B. subtilis*; PhoD signal peptide, N-terminal native peptide tag for protein secretion; *lacZ*, gene for β -Gal, fused to the PhoD signal peptide. (C) X- β -Gal is converted from a transparent substrate to a dark blue precipitate by β -Gal secreted by *B. subtilis* NS1 β -Gal. (D) Visual confirmation of β -Gal activity from BC containing *B. subtilis* NS1 and *B. subtilis* NS1 β -Gal. BC containing spores were incubated 4 to 8 hours in medium, the medium was removed, and X- β -Gal was added to the pellicles. h, hours. (E) Reaction activity of enzyme-functionalized pellicles at different incubation times, as indicated by a fold change in red pixel intensity (blue coloration is reflected by a reduction of red color; $C_t/C_0 = 1$ represents no fold change). Experiments were performed in triplicate, and average (lines) and SD are indicated (bands).

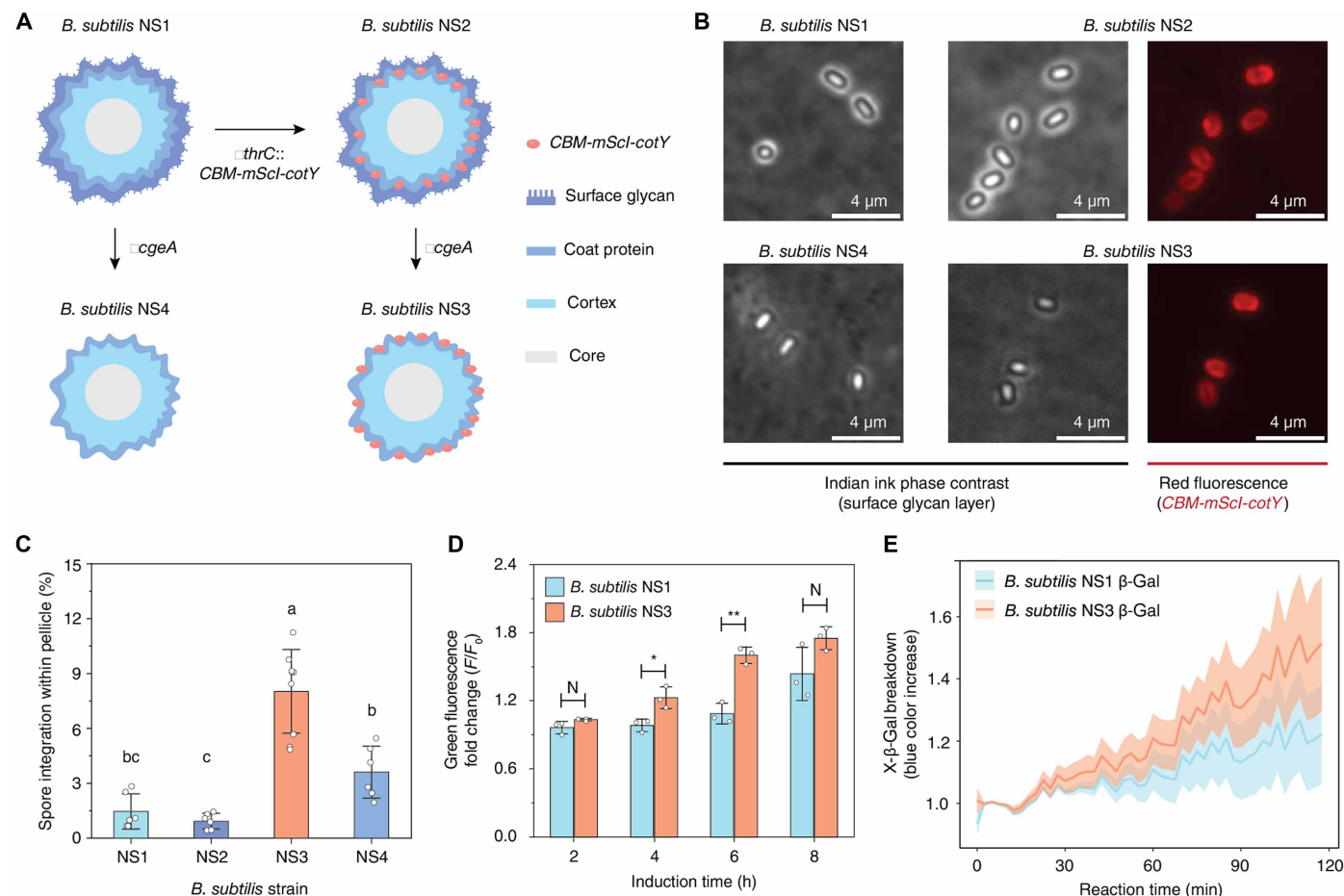


Fig. 4. Engineering the surface of *B. subtilis* spore for functionality enhancement of BC-spore materials. (A) Schematic drawing of CBM expression in the outermost layer of the proteinaceous coat by fusion with CotY and polysaccharide layer reduction by *cgeA* gene deletion. (B) Confirmation of CBM expression on the spore surface evaluated by mScarlet1 fluorescence (red) and polysaccharide layer reduction by phase contrast microscopy with Indian ink staining. (C) Incorporation efficiency of engineered *B. subtilis* spores within BC pellicles. Statistical significance was calculated using one-way analysis of variance (ANOVA) using Tukey's multiple comparison test. Significant differences between groups are indicated by different letters over error bars ($P < 0.05$). (D) Comparison of the intensity fold change of green fluorescence signals from BC pellicles containing *B. subtilis* NS1 or *B. subtilis* NS3 in relation to induction time in LB with IPTG (divided over control pellicles without IPTG induction). Asterisks indicate statistical significance by Student's *t* tests (* $P < 0.05$; ** $P < 0.01$). N indicates no significant difference between groups based on the same test. In (C) and (D), dots indicate individual measurements, bars are the mean, and whiskers are the SD. (E) Comparison of the reaction activity of enzyme-functionalized BC pellicles containing *B. subtilis* NS1 β-Gal or *B. subtilis* NS3 β-Gal spores, indicated by a fold change in red pixel intensity, after addition of X-β-Gal to the pellicles. The pellicles were tested for enzyme activity after 6 hours of incubation in LB medium, and the experiment was performed in triplicate; averages are shown by the lines, and the band indicates the SD.

various treatments except 70% ethanol exposure (figs. S18 and S19), and the vegetative cells showed no difference in growth kinetics compared to their parental strain NS1 (fig. S18).

B. subtilis NS2 spores (CBM expression) did not show a notably different integration efficiency in BC compared to its parental strain NS1 (NS1: $1.5 \pm 1.0\%$ and NS2: $0.9 \pm 0.4\%$, means $\pm$ SD). However, when the polysaccharide layer was reduced in strain NS3, a notable improvement was observed, reaching an incorporation efficiency of $8 \pm 2.3\%$ (Fig. 4C). The control strain *B. subtilis* NS4 also showed improved integration efficiency in BC ($3.6 \pm 1.4\%$) but notably less compared to *B. subtilis* NS3. Investigation of the spore distribution with confocal microscopy revealed that spores of *B. subtilis* NS3 and *B. subtilis* NS4 were often integrated in small clusters within BC (fig. S20). It was recently found that the reduction of the polysaccharide layer increased the surface hydrophobicity, which generally induce clumping behavior of spores due to hydrophobic interactions

(28–32). A relative hydrophobicity of 80% was measured for spores from *B. subtilis* NS3 and *B. subtilis* NS4, and these strains showed clumping behavior (30–32). Spores of *B. subtilis* NS1 and *B. subtilis* NS2 did not show clumping, and the relative hydrophobicity was only 20% (fig. S20). Together, these results indicate that the integration efficiency can be improved by changing the surface properties of the spores and that it can be further enhanced by displaying a binding module with affinity to the matrix. The lack of improvement with CBM alone (strain NS2) also suggests that the polysaccharide layer might physically block the interaction between the displayed CBM and BC or influence the local environment around the displayed CBM.

Next, we investigated whether higher numbers of incorporated spores would shorten the response time of pellicles. As a first experiment, GFP expression was induced by IPTG in pellicles containing NS1 or NS3 spores as described above. Within 4 hours, GFP expression was already reliably detected from pellicles containing

spores from strain NS3, as indicated by a fluorescence intensity fold change from 1.00 ± 0.04 (no IPTG control) to 1.23 ± 0.09 (IPTG induction), which increased after 8 hours to a fold change of 1.75 ± 0.10 . Pellicles containing strain NS1 exhibited noticeable GFP signals after at least 8 hours, with a fold change from 1.00 ± 0.02 (control) to 1.43 ± 0.23 (IPTG induction) (Fig. 4D). In a second experiment, we tested whether the enzyme functionalization by β -Gal secretion was improved, comparing strains NS1 and NS3 both transformed with the plasmid pFJ08-P_{3P}-LacZ. After 6 hours of incubation, pellicles containing strain NS3 β -Gal showed a clear stronger catalytic activity compared to pellicles containing NS1 β -Gal (Fig. 4E). After 2 hours of incubation with X- β -Gal, blue coloration of pellicles containing strain NS3 β -Gal reached a fold change of 1.5 ± 0.2 , whereas pellicles containing strain NS1 β -Gal reached 1.2 ± 0.2 . These experiments show that increasing the number of spores in materials by improving their binding affinity to the matrix is a valid strategy for improving the functionality of materials.

In situ transformation of BC-spore materials

Among the genus *Bacillus*, *B. paralicheniformis* is highly specialized in producing gamma-polyglutamic acid (γ -PGA) (33–35), a biopolymer of growing commercial interest. This polymer can play multiple beneficial roles in the food industry (36) and can contribute to mechanical properties of composite materials (37–39). We aimed to make BC-PGA composites by leveraging the PGA production capability of WT *B. paralicheniformis* (Fig. 5A). First, we investigated whether spores of WT *B. paralicheniformis* can germinate and the resulting vegetative cells produce PGA in medium E, known as γ -PGA production media (33). WT *B. paralicheniformis* spores germinated in medium E (Fig. 5B), with L-glutamic acid identified as being an important medium component for efficient germination (fig. S21). Next, growth kinetics of vegetative cells in medium E were recorded. The cells grew slowly compared to growth in LB medium or medium E with 2% glucose, but the germinated cells did use L-glutamic acid to produce γ -PGA, as indicated by daily monitoring of L-glutamic acid consumption (fig. S21; see the Supplementary Materials).

Having set the conditions for γ -PGA production using spores, we subjected BC containing WT *B. paralicheniformis* spores to similar conditions. The pellicles were first incubated with medium E allowing medium infiltration and spore germination. They were taken out of the medium, and in situ γ -PGA production was monitored over multiple days. The L-glutamic acid remaining within the BC was consumed and completely depleted after 3 days of incubation (Fig. 5C). We first noticed that the BC matrix lost its elasticity during γ -PGA production, as confirmed by changes in storage moduli (G'), loss moduli (G''), and yield stress (τ) of the wet pellicle (Fig. 5, C and D, and fig. S22). This can be explained by the presence of cellulase enzyme produced by WT *B. paralicheniformis* in the BC matrix (40), causing slight degradation of the matrix. A temporary increase in residual reducing sugars was detected (fig. S22; see the Supplementary Materials). The cellulose network remained stable enough to handle and manipulate, allowing us to proceed with further characterization.

BC-PGA composites were dried, and the mechanical properties were characterized by tensile testing (Fig. 5, E and F). Impressively, the BC-PGA films showed a remarkable increase in elongation at break (%) and toughness compared to BC films while exhibiting no substantial difference in tensile strength (Fig. 5, F to H). The BC films had low elongation at break (<1%), consistent with previous

studies (3, 41), whereas the γ -PGA-infused BC enabled elongation up to 30%. We measured a low toughness of BC of ~ 0.02 MJ/m³, due to the different culture conditions required for BC-spore formation (e.g., low pH and culture medium with increased density) (figs. S5 to S7). However, the BC-PGA composite shows an increased toughness of ~ 1 MJ/m³. This, together with the high elongation, endows high flexibility to BC-PGA materials. The flexibility of the BC-PGA films grants rolling that is unachievable with pure BC films (fig. S23). This flexibility, combined with the ability of γ -PGA to prevent food aging (42), can make the BC-PGA composites particularly suitable for applications such as food packaging (fig. S23; see the Supplementary Materials) (36).

The flexibility of the BC-PGA composites might stem from moisturizing effects of γ -PGA within composites. It is widely known that moisturized BC films have better elongation properties compared to fully dried sheets (43). Although partial degradation of BC by cellulase could weaken the composite (3), this may have been offset by the mechanical reinforcement effect of γ -PGA, resulting in no notable change in tensile strength.

DISCUSSION

In this work, we present the fabrication of BC-spore materials through a single culture approach. Integrating *Bacillus* spores into a BC matrix enables the material to be metabolically activated on demand. Hence, BC is changed from a nonresponsive material that can be easily stored for prolonged periods of time, into a functional one that can have a wide array of applications. As examples, spores from genetically modified strains conferred enzymatic production and sense-and-respond functions to the material. In addition, we showed that WT endospores from *B. paralicheniformis* can transform the BC matrix in situ, resulting in a biopolymer-infused composite with enhanced mechanical properties.

Biological ELMs feature living cells as functional units. Cell viability concerns often call for prompt use of the material after fabrication due to their limited life span or susceptibility to environmental changes. The BC-spore materials presented here have the advantage of long-term survival and resistance to harsh conditions thanks to the nature of *Bacillus* spores. The materials also retained their functionality after storage under dried conditions for up to 6 months or after exposure to harsh treatments (-80° to 70° C, 70% ethanol treatment, and drying). In laboratory settings, dried bacterial spores have been shown to survive for 10 years without compromising cell viability (10). Furthermore, several reports indicate that *Bacillus* spores can remain viable for more than 100 years (44). Not only can spores survive and resist harsh conditions, but their encoding DNA also retains high sequence fidelity after various treatments (45). The resistance to harsh conditions combined with the on-demand activation makes the presented platform of “dormant ELMs” suitable for practical use outside the laboratory environment.

This system could provide distinct operational advantages compared to cell-free systems or catalytic materials geared with enzymes (e.g., dried enzymes on paper). These materials are designed to perform biological functions in vitro, but the shelf life and activity of materials are highly dependent on the stability of incorporated enzymes (46). BC-spore materials, once activated, can autonomously produce fresh enzymes, which do not require laborious processes to obtain enzymes or cell extracts. Before activation, BC-spore materials can be stored under ambient conditions for extended periods of

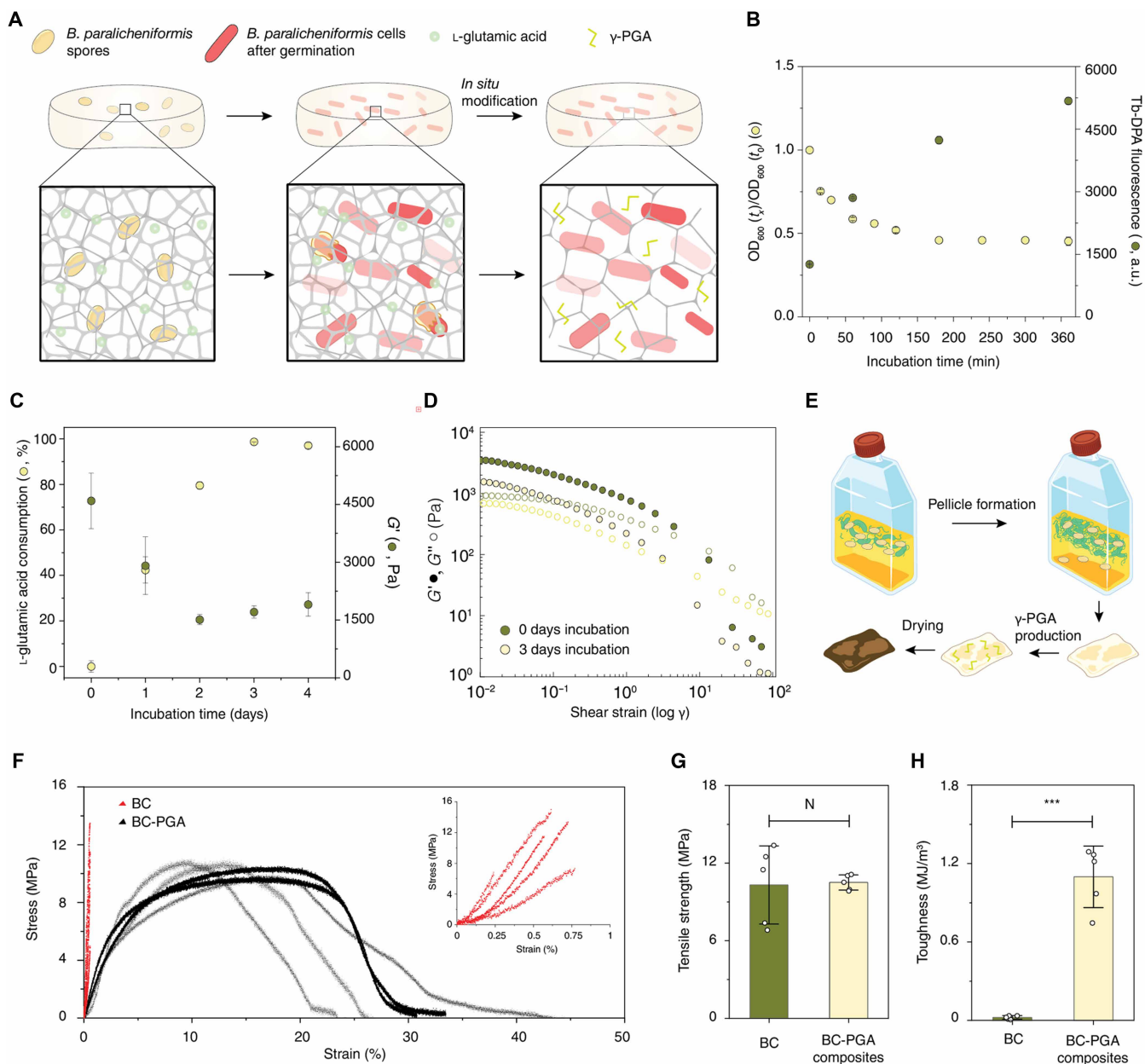


Fig. 5. In situ transformation of BC-spore pellicles into flexible BC-PGA composites. (A) Schematic drawing of germination and in situ γ -PGA production by WT *B. paralicheniformis* within BC. (B) Germination of WT *B. paralicheniformis* spores within medium E, as indicated by relative OD_{600} change of medium and Tb-DPA fluorescence in the supernatant. a.u., arbitrary units. (C) Consumption of L-glutamic acid (%) in pellicles and changes in storage moduli (G' , Pa) of pellicles as a function of incubation time. In (B) and (C), experiments were performed in triplicate; averages are shown as the dots, and whiskers indicate the SD. (D) Comparison of rheological properties of a BC pellicle after 0- and 3-day incubation with medium E as a function of oscillatory shear strain. (E) Schematics exhibiting preparation of BC-spore materials for characterization of mechanical properties. The brown color of dried BC-PGA composites comes from remaining $FeCl_3 \cdot 6H_2O$ of medium E. (F) Stress-strain curves of dried BC and BC-PGA films. Five replicate measurements were conducted on each specimen type. The inset shows the zoomed-in stress-strain curve of dried BC films. (G and H) Tensile strength (MPa) and toughness (MJ/m^3) of BC and BC-PGA films. Bars indicate the average of five replicates, and whiskers indicate the SD. Asterisks indicate statistical significance by Student's *t* tests (** $P < 0.001$). N indicates no significant difference between groups based on the same test.

time without compromising their functionality. It should be noted that BC-spore materials require a certain amount of time to become fully activated and enzymatically functional before use.

Bacterial spores have been incorporated in synthetic matrices for similar purposes. For example, both spores encased within 3D-printed agarose rods (11) or polyvinyl alcohol-based hydrogels (14)

exhibit programmable functionality, environmental resistance, and survival up to a few months, which is quite comparable with BC-spore materials. Here, however, the growth of mechanically strong BC matrices relies on autonomous fabrication driven by bacteria in nutrient media, avoiding human intervention and petrol-based resources. The shapes and size of BC-spore material could be controlled

by adjusting the cultivation vessel (6, 9) without compromising the mechanical properties. This broadens the potential use of BC-spore materials and enables two-dimensional scalable production.

The number of viable cells is generally an important factor for controlled functionality of ELMs (11, 47). Previous biological ELMs depend on self-assembly of cell populations within a medium or on a carefully balanced engineered cell consortium, limiting the control over the number of functional cells in the final product (2–4, 48). The number of cells within BC-spore material could be controlled through various means, such as density of culture medium and DNA-encoded surface modification of the spores, leading to a controlled level in functionality.

The development of more diverse ELMs, using different biological matrices and different species as functional units, broadens the overall possibilities of ELMs. The BC-spore materials presented here show a unique platform for autonomous material fabrication with high resistance and on-demand programmable functions. Further research can broaden the applications of spore-based materials by introducing de novo gene circuits for a wide range of purposes.

MATERIALS AND METHODS
Strains and genetic engineering

Two strains of *Komagataeibacter rhaeticus* were used in this study:

- 1) WT *K. rhaeticus*: *K. rhaeticus* iGEM strain (Table 1) (6).
- 2) *K. rhaeticus* GFP: WT *K. rhaeticus* transformed with the plasmid pKM_kr01, enabling constitutive expression of sfGFP under chloramphenicol selection (34 µg/ml). Plasmid pKM_kr01 was constructed using BsaI-mediated Golden Gate assembly, incorporating parts from the *Komagataeibacter* Tool Kit (KTK) (49): a strong constitutive Anderson promoter (J23104), ribosome binding site (RBS_BBa_B0035), *sfGFP*, and a transcription terminator (BBa_B0010) (fig. S8A).

For *B. paralicheniformis*, experiments were performed with two strains:

- 1) WT *B. paralicheniformis*: a WT strain (Table 1) [American Type Culture Collection (ATCC) 9945a].
- 2) *B. paralicheniformis* GFP_{ind}: the WT strain transformed with plasmid pAD-sfGFP (Sp)-P_{man} [a gift from O. Kuipers (21), Add-gene plasmid #133833], encoding for mannose-inducible sfGFP expression (fig. S12D).

For *B. subtilis*, eight different strains were used:

- 1) WT *B. subtilis*: a WT strain (Table 1) (*B. subtilis* 168, ATCC 23857).
- 2) *B. subtilis* NS1: containing a genomic integration in the *amyE* locus (20), including a spectinomycin resistance gene, a gene for GFPmut2 expression under control of the P_{spac-hy} promoter, and a gene for the Lac inhibitor (fig. S12A).
- 3) *B. subtilis* NS1 β-Gal: strain *B. subtilis* NS1 carrying a plasmid (pFJ08-P_{3P-lacZ}; fig. S14A) coding for β-Gal under control of the P_{3P} promoter (P_{amyL}-P_{amyQ}-P_{cryIIIA}-*cryIIIA*_{stab}) (26). First, the DNA sequence of *phoD* secretion peptide fused to the *lacZ* gene was placed in the previously mentioned pAD-sfGFP (Sp)-P_{man} plasmid by Gibson assembly, yielding plasmid pFJ06. To this end, two fragments were generated by polymerase chain reaction: an insert of 3284 base pairs (bp) (primers F11 and F12, Table 2) from plasmid pDR111_mannose_Bgal no NLS [a gift from C. Contag (50), Add-gene plasmid #188399] and a backbone fragment of 5398 bp from plasmid pAD-sfGFP (Sp)-P_{man} (primers F13 and F14, Table 2). Second, the promoter region in pFJ06 was replaced by Gibson assembly, yielding pFJ08-P_{3P-lacZ}. Here, the pFJ06 backbone was amplified (primers F23 and F24, Table 2), yielding a 8392-bp fragment, and the P_{3P} promoter and TM_RBS3 region was amplified from the plasmid pKM_bs01 (primers F25 and F26, Table 2), yielding a 882-bp fragment. To evaluate the gene expression efficiency of the promoter-RBS combination, the plasmid pKM_bs01 was constructed using BsaI-mediated Golden Gate assembly. The construct

Table 1. Strains used in this study.			
Strain	Genotype/plasmid	Selection	Reference
WT <i>K. rhaeticus</i>	<i>K. rhaeticus</i> iGEM strain		(6)
<i>K. rhaeticus</i> GFP	Plasmid pKM_kr01	Chloramphenicol (34 µg/ml)	This study
WT <i>B. paralicheniformis</i>	ATCC 9945a		ATCC 9945a
<i>B. paralicheniformis</i> GFP _{ind}	Plasmid pAD-sfGFP (Sp)-P _{man}	Chloramphenicol (5 µg/ml)	This study
WT <i>B. subtilis</i>	<i>B. subtilis</i> 168		ATCC 23857
<i>B. subtilis</i> NS1	Δ <i>amyE</i> ::(SpcR P _{spac-hy} -GFPmut2 LacI)	Spectinomycin (100 µg/ml)	(20)
<i>B. subtilis</i> NS1 β-Gal	Δ <i>amyE</i> ::(SpcR P _{spac-hy} -GFPmut2 LacI), plasmid pFJ08-P _{3P-lacZ}	Spectinomycin (100 µg/ml), chloramphenicol (5 µg/ml)	This study
<i>B. subtilis</i> NS2	Δ <i>amyE</i> ::(SpcR P _{spac-hy} -GFPmut2 LacI), Δ <i>thrC</i> ::(ErmR P _{cotYZ} -CBM-mScl-cotY)	Spectinomycin (100 µg/ml), erythromycin/lincomycin (1 and 25 µg/ml)	This study
<i>B. subtilis</i> NS3	Δ <i>cgeA</i> :: <i>kan</i> , Δ <i>amyE</i> ::(SpcR P _{spac-hy} -GFPmut2 LacI), Δ <i>thrC</i> ::(ErmR P _{cotYZ} -CBM-mScl-cotY)	Kanamycin (10 µg/ml), spectinomycin (100 µg/ml), erythromycin/lincomycin (1 and 25 µg/ml)	This study
<i>B. subtilis</i> NS3 β-Gal	Δ <i>cgeA</i> :: <i>kan</i> , Δ <i>amyE</i> ::(SpcR P _{spac-hy} -GFPmut2 LacI), Δ <i>thrC</i> ::(ErmR P _{cotYZ} -CBM-mScl-cotY), plasmid pFJ08-P _{3P-lacZ}	Kanamycin (10 µg/ml), spectinomycin (100 µg/ml), erythromycin/lincomycin (1 and 25 µg/ml), chloramphenicol (5 µg/ml)	This study
<i>B. subtilis</i> NS4	Δ <i>cgeA</i> :: <i>kan</i> , Δ <i>amyE</i> ::(SpcR P _{spac-hy} -GFPmut2 LacI)	Kanamycin (10 µg/ml), spectinomycin (100 µg/ml)	This study
<i>B. subtilis</i> NS4 GFP _{gly}	Δ <i>cgeA</i> :: <i>kan</i> , Δ <i>amyE</i> ::(SpcR P _{spac-hy} -GFPmut2 LacI), plasmid pKM_bs02	Kanamycin (10 µg/ml), spectinomycin (100 µg/ml), chloramphenicol (5 µg/ml)	This study

Table 2. Primers used in this study. Primers are given 5' to 3'; uppercase letters indicate annealing regions.	
No.	Sequence
F11	gaattgccgaccttgactagtgtctcattaCTATTTTGTACACCAGACCAACTGGTAATGG
F12	ctagatgattaactaataaggaggacaaacATGGCGTATGACTCAAGATTTGACGAATGG
F13	ccattaccagttggtctggtgtcaaaaatagTAATGAGCACTAGTCAAGGTCGGCAATTC
F14	ccattcgtcaaatcttgagtcatacgcCATGTTTGTCTCTTATTAGTTAATCATCTAG
F15	AACCCAGCGAACCATTTGAGG
F16	CTAGAAAGATTCATTGGGTCTCGTAG
F17	GAGATTAAGTAACGAACGGCGATG
F18	cctcaaatggttcgctgggttCATTACACACACCTCTATTTCGATAGTG
F19	ctacgagaccaatgaatctttctagCGTTACGTTCTTTTCATAACATCCGATG
F20	CATAAACAGGTAATAATCCGAGGAAGC
F23	cataggagactagccttATGGCGTATGACTCAAGATTTGACG
F24	ctgtgtgaaattgttatccgcGTTTCTTAGACGTCAGGTGGCAC
F25	cctgacgtctaagaacGCGGATAACAATTTACACAGGAGG
F26	caaatcttgagtcatacgcCATAAGGCTAGTCTCTATGTATAATACATAATTTCAAAC

included the P_{3p} promoter, TM_RBS3, *GFPmut3b*, and the L3S2P21 terminator, sourced from the SubtiToolKit (STK) (23).

4) *B. subtilis* NS2: To create spores with CBM expression on the coat, a gene encoding for CBM-mScarletI-CotY was integrated into the *thrC* locus of *B. subtilis* NS1 (fig. S16B). A gene block was ordered (IDT DNA) containing the promoter P_{cotYZ} and a gene fusion of a CBM from *C. thermocellum*-mScarletI (51)-*cotY*, inspired by the Sporobeads technique (52). The gene block was placed in the plasmid pDG1664 (53) by digestion (BamHI/EcoRI) and ligation, creating pFJ03-CBM-m*Scl*-*cotY*. After successful construction verified by sequencing, pFJ03-CBM-m*Scl*-*cotY* was linearized before integration.

5) *B. subtilis* NS3: The gene *cgeA* was deleted in the genome of strain *B. subtilis* NS2 (fig. S17B). A deletion cassette was constructed by overlap extension PCR as described in a previous study (54), using three fragments: an 1111-bp fragment containing the region upstream of *cgeA* amplified from genomic DNA of *B. subtilis* NS1 (primers F17 and F18, Table 2); a 1457-bp fragment harboring a kanamycin resistance cassette, amplified from the plasmid pJet1.2 *kanR* cassette [a gift from S. Gruber (55), Addgene plasmid #117122; primers F15 and F16, Table 2]; and a 1123-bp fragment amplified from genomic DNA of *B. subtilis* NS1 containing the region downstream of *cgeA* (primers F19 and F20, Table 2). Sequencing of the *cgeA* locus confirmed disruption of the gene as intended. Because of an unintended point deletion in the *cgeC* gene, this gene is truncated to 78 amino acids of the normal 102.

6) *B. subtilis* NS3 β-Gal: *B. subtilis* NS3 carrying the plasmid pFJ08-P_{3p}-*lacZ*.

7) *B. subtilis* NS4: The gene *cgeA* was deleted in the genome of strain *B. subtilis* NS1, similarly as how *B. subtilis* NS3 was constructed. The unintended point mutation in the *cgeC* gene that appeared in *B. subtilis* NS3 was also included in *B. subtilis* NS4.

8) *B. subtilis* NS4 GFP_{gly}: *B. subtilis* NS4 carrying the plasmid pKM_{bs02}.

All PCRs were performed with Phusion-HF polymerase (New England Biolabs) or, for fragments > 5000 bp, KOD Xtreme polymerase (Merck). All primers were ordered from Integrated DNA

Technologies (USA). All plasmids in this study were assembled in *E. coli* DH5α or in *E. coli* Turbo (New England Biolabs). Genomic DNA was extracted from *Bacillus* strains using a DNA purification kit (Roboklon GmbH, Berlin, Germany).

Gibson reactions were performed in a 20-μl volume containing 0.08 U T5 Exonuclease (New England Biolabs), 0.5 U Phusion DNA polymerase (New England Biolabs), 80 U Taq DNA ligase (New England Biolabs), and 10 to 100 ng of DNA fragments in equimolar amounts, in isothermal reaction buffer [0.1 M tris-HCl (pH 7.5), 10 mM MgCl₂, 0.2 mM dNTP mix, 10 mM dithiothreitol, 1 mM NAD, and PEG-8000 (polyethylene glycol, molecular weight 800; 50 mg/ml)]. The mixture was incubated at 55°C for 60 min and directly used for transformation of *E. coli*.

Golden Gate reactions were performed in a 10-μl volume containing 0.5 μl of T4 DNA ligase (New England Biolabs), 0.5 μl of BsaI-HF (New England Biolabs), 1 μl of T4 ligase buffer, equimolar amounts of DNA parts (50 fmol/μl per part), and water to adjust the final volume. Reactions were cycled 30 times in a thermocycler with digestion at 37°C (2 min) and ligation at 16°C (5 min), followed by heat inactivation at 60°C (10 min).

The assembled plasmids were transformed into *E. coli* using the standard heat-shock protocol. For *K. rhaeticus*, transformations were carried out via electroporation using 1-mm path-length electrocuvettes and a MicroPulser Electroporator (Bio-Rad) set at 2.5 kV and a pulse length of 5.9 ms, following a previously described method (6). Transformants were screened on 10× chloramphenicol (340 μg/ml) on YPD plates.

All *Bacillus* mutants in this study were obtained using a natural-competence induction protocol as previously described (55). In short, *B. subtilis* or *B. paralicheniformis* strains were grown overnight (37°C, 200 rpm) in 10 ml of competence medium. Ten milliliters of fresh broth was inoculated with 0.6 ml of overnight culture and incubated until OD₆₀₀ (optical density at 600 nm) = 0.8, and 10 ml of prewarmed starvation medium was added. The incubation was continued for an additional hour. A plasmid or DNA template (~1 μg) for homologous recombination was then mixed with 0.4 ml of cell culture and shaken at 37°C for 2 hours. Then, cells were plated onto

an appropriate selective agar plate and grown overnight at 37°C. If required, antibiotics or essential amino acids were separately added to competence medium [e.g., threonine (50 mg/liters) for strain *B. subtilis* NS2 and *B. subtilis* NS3]. To confirm genomic integration in the *thrC* locus for *B. subtilis* NS2 and *B. subtilis* NS3, transformed colonies were reselected using threonine auxotrophy as described in a previous study (56).

Sporulation of *Bacillus* strains

B. subtilis strains were grown overnight (37°C, 200 rpm) in 4 ml of LB medium [tryptone (10 g/liter), yeast extract (5 g/liter), and NaCl (5 g/liter)], with antibiotics if required. A culture of 10-ml LB medium was inoculated with the overnight culture to a starting OD₆₀₀ of 0.2 and grown to OD₆₀₀ = 0.8 (incubation at 37°C, 200 rpm). Cells were collected by centrifugation (15 min, 3005g), resuspended in phosphate-buffered saline (PBS) (pH 7.4), and incubated for 30 min at room temperature. The suspension was centrifuged again, and the cells were resuspended in 5 ml of Schaeffer's sporulation medium (57) [Difco nutrient broth (8 g/liter), KCl (1 g/liter), 1 mM MgSO₄·7H₂O, 10 μM MnCl₂·4H₂O, 0.5 mM CaCl₂, and 1 μM FeSO₄; the latter two components were added after autoclaving] and grown for 3 days (37°C, 200 rpm). Next, the culture was checked for sporulation by phase contrast microscopy, and the spores were purified by a Histodenz gradient. For details, see hereafter. Spores were stored in Milli-Q at 4°C and used for up to 2 months.

B. paralicheniformis strains were grown overnight (37°C, 200 rpm) in 50 ml of LB medium, with antibiotics if required. The next day, cells were collected by centrifugation (15 min, 3005g) and resuspended in 50 ml of Schaeffer's sporulation medium and grown for 3 days (37°C, 200 rpm). Next, the culture was checked for sporulation by phase contrast microscopy, and the spores were purified by a Histodenz gradient as described above. For details, see below. Spores were stored in Milli-Q at 4°C and used for up to 2 months.

To obtain stained spores of either *B. subtilis* or *B. paralicheniformis*, 7.7 μM di-4,6-ANEPPS was added to Schaeffer's sporulation medium before incubation. This dye is known to be incorporated into the inner membrane of spores (58).

For Histodenz purification, the culture was centrifuged (15 min, 3005g, 4°C) and the pellet was washed with 50 ml of cold Milli-Q and centrifuged again. The pellet was resuspended in 500 μl of cold 20% Histodenz (Sigma-Aldrich, D2158, Burlington, USA) and carefully pipetted on top of 750 μl of cold 50% Histodenz. After centrifugation (15 min, 21,000g, 4°C), the supernatant containing vegetative cells and cell remnants was removed, and the pellet was washed twice with 1.5 ml of cold Milli-Q, using centrifugation at 4270g of 5 min at 4°C. If required, the Histodenz purification was repeated to obtain samples of >99% spores.

Wide-field microscopy

For phase contrast microscopy, a sample containing spores was taken and immobilized under a 3% agarose pad. Cells were imaged using an inverted Olympus IX81 microscope equipped with an ORCA-Flash 4.0 camera, with a ×100 UplanSApo oil objective in combination with a phase contrast ring, controlled by INSCOPER software (Cesson-Sévigné, France).

For visualization of the polysaccharide layer around the spores, a spore sample was mixed 1:1 with Indian ink (Talens, Apeldoorn, The Netherlands), immobilized under a 3% agarose pad, and imaged with phase contrast microscopy.

For imaging of mScarletI red fluorescence in *B. subtilis* NS2 and NS3, the same Olympus IX81 microscope was used, in combination with 580-nm excitation from a CoolLED pE-4000 light source (CoolLED, Andover, UK) and an mCherry-40LP-A filter cube (Semrock, part of IDEX Health & Science, Rockster, USA: 562/40-nm excitation filter, 593-nm dichroic mirror, and 593-nm long-pass emission filter).

BC pellicle production

A preculture of *K. rhaeticus* or *K. rhaeticus* GFP was grown in 5 ml of YPDD [YPD with double glucose concentration: peptone (10 g/liter), yeast extract (5 g/liter), and glucose (40 g/liter)] with 1% cellulase at 30°C for 2 to 3 days to an OD₆₀₀ of 0.6, supplemented with chloramphenicol (34 μg/ml) if required. The cells were collected (10 min, 3220g, 4°C), washed twice with cold sterile PBS (pH 7.4), and resuspended in YPD (pH 4.0) [peptone (10 g/liter), yeast extract (5 g/liter), and glucose (20 g/liter), adjusted to pH 4.0 by HCl] to an OD₆₀₀ of 0.4 to create the inoculum.

A volume of 10 ml of YPD media (pH 4.0) with 40% (w/v) Nicodenz (Axis Shield) was inoculated with 0.2 ml of the inoculum in a 50-ml conical tube, supplemented with chloramphenicol (34 μg/ml) if required. A spacer was placed in the lid of the tube to ensure proper aeration. If required, 50 μl of *Bacillus* spore suspension (OD₆₀₀ = 5) was gently pipetted on the surface. Normally, pellicles were grown for 3 days at 30°C, followed by washing twice (for 8 and 16 hours) at 4°C with saline citrate buffer [50 mM, pH 4.0, 0.9% (w/v) NaCl] under gentle shaking conditions. If needed, pellicles were washed with 10 ml of Milli-Q for 1 hour at 4°C.

Bacterial counts in BC-spore materials

For counting both *Bacillus* spores and *K. rhaeticus* cell counts in pellicles, BC pellicles were grown using *K. rhaeticus* GFP and washed as described above, with or without *Bacillus* spores. After washing with Milli-Q, the pellicles were placed in 10 ml of PBS (pH 7.4) with 4% cellulase and incubated at 4°C while shaking for 2 days and with intermittent vortexing of the solution. Next, 1 ml of the suspension was centrifuged (4°C, 3200g) and resuspended in 1 ml of cold Milli-Q. For *K. rhaeticus* counts, the solution was diluted to 1000×, 10,000× and 100,000× in cold Milli-Q. A volume of 100 μl was plated on HS-agar plates supplemented with chloramphenicol (34 μg/ml), and colonies were counted after 3 days of incubation at 30°C. For *Bacillus* counts, the solution was heated for 10 min at 70°C to kill vegetative cells and diluted 1×, 10×, and 100× in cold Milli-Q. A volume of 100 μl was plated on LB-agar plates, and colonies were counted after overnight incubation at 30°C. For each condition, three pellicles were grown and analyzed. The CFU/pellicle was determined from plates with >10 colonies.

For all other experiments where only *B. subtilis* cell numbers were compared, the same procedure was followed, but pellicles were grown from WT *K. rhaeticus*. Under each condition, 50 μl of the *B. subtilis* suspension of OD₆₀₀ = 5 was added to the culture. The vial number of *Bacillus* spores in this volume was determined by plating serial dilutions on LB-agar in triplicate. Integration efficiency was calculated as “number of integrated spores”/“number of added spores” × 100%.

Confocal imaging

For confocal imaging of *K. rhaeticus* GFP and *Bacillus*, a Nikon Eclipse Ti-2 inverted microscope with Crest X-light V3 spinning

disc unit (CrestOptics, Rome, Italy) was used, equipped with an SR HP Plan Apo λ S 100 $\times$ C Silicon objective and a Photometrics Kinetic sCMOS detector. GFP expressing *K. rhaeticus* GFP, *B. subtilis* NS1, and *B. paralicheniformis* GFP_{ind} were imaged with a 488-nm solid-state laser, a full-multiband pentaband filter set (MXR00543-CELESTA-DA/FI/TR/Cy5/Cy7-A, Semrock, part of IDEX Health & Science, Rochester, USA), and a 511/20-nm emission filter (Semrock). *Bacillus* spores dyed with di-4,6-ANEPPS were imaged with 561-nm excitation, the same full-multiband pentaband filter set, and a 685/40-nm emission filter (Semrock). Pellicles were placed in a dish with glass bottom and covered with a coverslip for imaging. Z-stacks were recorded with 0.3- μ m step size.

Activity and resilience testing of BC-spore materials

The resilience of BC pellicles with incorporated spores was tested by the ability of *Bacillus* spores to germinate and produce GFP after various treatments of the pellicles. Before resilience testing, the measurement of the GFP induction was optimized. Therefore, 200 μ l of LB in the wells of a 96-well black wall microplate (Imaging Plate CG, Zell-Kontakt GmbH, Nörten-Hardenberg, Germany) was inoculated with *B. subtilis* NS1 or *B. paralicheniformis* GFP_{ind} spores at an initial OD₆₀₀ of ~0.5 and incubated at 30°C. After 2 hours, IPTG (0, 1, 10, and 100 μ M and 1, 10, and 100 mM) or mannose (0, 20, 40, 60, 80, and 100 mg/ml) was added to *B. subtilis* NS1 or *B. paralicheniformis* GFP_{ind}, respectively. The GFP fluorescence was measured continuously by a BioTek Synergy H1 plate reader (Biospx, LA Abcoude, The Netherlands) with 420/50-nm excitation and 516/20-nm emission and averaging 10 $\times$ per well while shaking (double orbital, 425 rpm) at 30°C. Also, the absorbance at 600 nm was measured. The experiment was performed in triplicate.

BC pellicles were grown by WT *K. rhaeticus*, with incorporated *B. subtilis* NS1 or *B. paralicheniformis* GFP_{ind} spores. The following treatments were applied: no treatment (freshly produced pellicles); air-drying (30°C, 24 hours) on regular baking paper; freeze-drying on lens cleaning tissue (Thorlabs MC-5, Newton, USA); 1-hour soaking in 70% ethanol followed by twice washing with Milli-Q, 1 hour each; 30-min or 1-hour illumination with UV-A (21.8 mW/cm², E Sevenneonlighting, Shenzhen, China); 1-, 3-, or 6-month storage as freeze-dried or air-dried pellicles at room temperature. Directly before germination, the pellicles were heated to 70°C for 15 min to eliminate *K. rhaeticus* cells, with the exception of the ultraviolet (UV)-treated pellicles: These were heated to 70°C before UV treatment. Pellicles were placed in LB medium (7 ml in a 6-well plate, 30°C, 80 rpm) to induce germination of the spores. After 2 hours, IPTG (1 mM) or mannose (20 mg/ml) was added to *B. subtilis* NS1 or *B. paralicheniformis* GFP_{ind}, respectively, or Milli-Q as a negative control. After 24 hours, the GFP expression of the full pellicles was measured using the Amersham Typhoon scanner (Cytiva, Marlborough, USA), measuring with 488-nm excitation and 525/20-nm emission. The average intensity of each pellicle was measured in ImageJ (version 1.54f) and divided over the mean of the control pellicles for that condition. The experiment was performed in triplicate.

β -Gal measurements

Pellicles were grown in 50-ml tubes using WT *K. rhaeticus* with incorporated spores of *B. subtilis* NS1, *B. subtilis* NS1 β -Gal, or *B. subtilis* NS3 β -Gal. The pellicles were cut in four, and each piece was incubated in 5 ml of LB in a 6-well plate for up to 8 hours, at 37°C and 80 rpm. Pellicle fragments were placed on a petri dish, the supernatant was

removed, and 10 μ l of X- β -Gal (40 mg/ml) in dimethyl sulfoxide (DMSO) was added to each piece. Blue coloration was imaged with a camera (Canon EOS M50 Mark II, Japan), for 2 hours with 2.5-min interval. Each experiment was performed in triplicate. Red pixel intensity values per pellicles were extracted using FIJI (ImageJ 2.14.0/1.54f) and corrected for background intensity (area without pellicle) by division over the background. The values were divided over the values in the first frame(s) and inverted to gain reaction kinetic curves showing the increase in blue coloration.

Characterization BC-PGA composites

A BC pellicle was grown for 3 days by WT *K. rhaeticus*, with incorporated WT *B. paralicheniformis* spores in a 50-ml tube. The grown pellicles were washed twice (8 and 16 hours) with 50 mM citrate buffer (pH 4.0) supplemented with 0.9% (w/v) NaCl. Each pellicle was placed in 10 ml of medium E [L-glutamic acid (20 g/liter), sodium citrate monobasic (13.6 g/liter), glycerol (80 g/liter), NH₄Cl (7 g/liter), KH₂PO₄ (0.5 g/liter), MgSO₄ (0.244 g/liter), FeCl₃·6H₂O (0.04 g/liter), CaCl₂·2H₂O (0.15 g/liter), and MnSO₄·H₂O (0.1 g/liter), pH adjusted to 7.5 with NaOH] in 60-mm petri dishes and incubated at 30°C for 6 hours, for both spore germination and medium exchange within pellicles. After removal of the medium, the dishes were sealed with parafilm and statically incubated at 30°C. After incubation, pellicles were randomly selected for measurement of residual glutamate and for characterization of wet pellicle rheological properties.

For residual glutamate measurement, individual pellicles were placed into a 50-ml tube and shaken with 10 ml of Milli-Q for glutamate diffusion into the liquid. The supernatant was filtered through a 0.2- μ m syringe filter, and the glutamate content was measured with a colorimetry-based glutamate measurement kit (MAK330, Sigma-Aldrich), following the manufacturer's guidelines.

The viscoelastic properties of BC-PGA wet composites were characterized using a strain-controlled and stress-controlled rheometer (Thermo Fisher Scientific, HAAKE MARS III). The pellicles were shaped into a diameter of 20 mm and were mounted onto a serrated plate for plate-plate method measurements. The gap size between the plates was 0.2 mm, and the strain for measurement was in the range of 0.1 to 10,000%. Storage (G') and loss moduli (G'') were determined as a function of shear strain via oscillatory amplitude sweeps at a fixed frequency of 1 Hz.

Mechanical properties of BC-PGA composites

For mechanical testing of BC-PGA pellicles, BC pellicles were grown for 3 days by WT *K. rhaeticus*, with incorporated WT *B. paralicheniformis* spores in a mammalian culture cell flask (surface dimension of BC pellicles: 30 mm by 80 mm). The formed pellicles were washed two times with 50 mM citrate buffer with 0.9% NaCl and once with Milli-Q. Each pellicle was placed in 10 ml of medium E in 90-mm petri dishes and incubated at 30°C for 6 hours, for both spore germination and medium exchange within pellicles. After removal of the medium, the dishes were sealed with parafilm and statically incubated at 30°C for up to 3 days. The pellicles were taken out, mounted onto the Teflon-coated film, and dried at 70°C for 16 hours. The samples were cut to 10 mm by 70 mm, and their thickness was measured with a digital micrometer. The tensile properties of composites were analyzed with a universal testing machine (Instron 34SC, USA), following the requirements from the ASTM D882 standard (59) with a pulling speed of 1 mm/min.

Supplementary Materials

This PDF file includes:

Supplementary Results

Supplementary Methods

Figs. S1 to S23

Table S1

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