Engineering *Bacillus subtilis* for the formation of a durable living biocomposite material

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Engineered living materials (ELMs) are a fast-growing area of research that combine approaches in synthetic biology and material science. Here, we engineer *B. subtilis* to become a living component of a silica material composed of self-assembling protein scaffolds for functionalization and cross-linking of cells. *B. subtilis* is engineered to display SpyTags on polar flagella for cell attachment to SpyCatcher modified secreted scaffolds. We engineer endospore limited *B. subtilis* cells to become a structural component of the material with spores for long-term storage of genetic programming. Silica biomineralization peptides are screened and scaffolds designed for silica polymerization to fabricate biocomposite materials with enhanced mechanical properties. We show that the resulting ELM can be regenerated from a piece of cell containing silica material and that new functions can be incorporated by co-cultivation of engineered *B. subtilis* strains. We believe that this work will serve as a framework for the future design of resilient ELMs.
The design of cells capable of producing self-organizing, biocomposite materials has the potential to enable the fabrication of new types of functional living materials with the ability of self-fabrication and self-repair. Engineered living materials (ELMs) are therefore a new and fast-growing area of research that combines approaches in synthetic biology and material sciences. The fabrication of most ELMs, however, has so far largely relied on physical methods for incorporating a living component in an external material. Engineering of ‘truly’ living materials where the living component actively facilitates material fabrication and organization is much more challenging. True ELMs have been created by engineering *Escherichia coli* to produce an extracellular matrix from curli fibers or from elastin-like polypeptides to attach *Caulobacter* cells via their protein S-layers. Among these examples, the secretion of a polypeptide-based scaffolding system or matrix offers greater control over material assembly and functionalization due to the genetic programmability of polypeptide structure and functions.

Despite the advances in ELM design, the diversity of different ELM types capable of autonomous self-fabrication and regeneration is still small. In addition, ELM fabrication is currently limited to common chassis organisms that lack the resilience and long-term viability capabilities to withstand conditions outside of the laboratory. Here, we sought to broaden the ELM landscape by engineering a resilient ELM biocomposite that uses the spore-forming bacteria *Bacillus subtilis* as its living component for the secretion of self-assembling protein scaffolds for cell cross-linking and silica biomineralization.

*B. subtilis* is an industrially used GRAS (Generally Recognized as Safe) bacteria known to have excellent protein secretion capabilities. Importantly, it forms spores that remain viable for a long time and allow the bacteria to survive extreme conditions. *B. subtilis* cells will therefore be able to enter a dormant spore state in our ELM under unfavorable environmental conditions, allowing it to persist until favorable conditions induce germination and cell revival. This was recently demonstrated by bioprinting of *Bacillus* spores in an agarose matrix. In addition, because spores contain the genetic information that was programmed into engineered vegetative cells, living materials may be autonomously fabricated at the sites of use from stored spores. For the secretion of a self-assembling protein matrix, we chose a highly robust 2D-scaffolding system from bacterial microcompartment shell proteins that we have characterized and engineered for the attachment of different cargo proteins. We rationalized that 2D-scaffold forming proteins rather than the commonly engineered bacterial biofilm-associated amyloid fibers (e.g. CsgA from *E. coli* or TasA from *B. subtilis*) will form different matrix architectures and surfaces for functionalization. Demonstration of extracellular matrix formation from nonamyloid protein building blocks will also lay the foundation for the design of new types of ELM matrices from the many other protein building blocks currently assembled into functional bioanomaterials. Our 2D-scaffolds self-assemble from hexameric units of the bacterial microcompartment shell protein EutM. EutM scaffold building blocks are highly amenable to engineering and tolerate N- and C-terminal fusions, including a C-terminal SpyCatcher domain for the covalent linkage of SpyTag-modified proteins to scaffolds. We therefore rationalized that *B. subtilis* could be engineered to secrete EutM-SpyCatcher (EutM-SpyC) building blocks and attach itself covalently via iso-peptide bond formation by the SpyTag-SpyCatcher system to the formed scaffolds to become both a structural and the living component of the formed material. Further, by engineering the scaffolds to display a bio-mineralization peptide (EutM-BM), scaffold mineralization could then yield a durable biocomposite material for future development into functional coatings or plasters. As a proof-of-concept, we chose silica, as one of the most abundant earth minerals that is inexpensive and the main ingredient of many building materials. A number of proteins are also known to control the biomineralization orthosilicic acid Si(OH)4 (the soluble form of silica), which can be used as a source for biomineralization peptides.

In this work, we first optimize secretion by *B. subtilis* of our EutM-SpyCatcher scaffold building blocks, which are not normally secreted and in addition, rapidly self-assemble both in vivo and in vitro into large structures. We then show that *B. subtilis* can be engineered to display SpyTags on clusters of polar flagella for cell attachment and cross-linking of EutM scaffolds that constitute the protein matrix of our ELM. To allow our production strain to remain a structural component of the formed material even in its dormant state, we create an endospore-forming strain. Screening of known silica biomineralization peptides then allows us to design scaffolds for silica polymerization to fabricate biocomposite materials with enhanced mechanical properties. Finally, we confirm that our ELM can be regenerated from a piece of cell containing silica material and that new functions can be incorporated into the material simply by co-cultivation of engineered *B. subtilis* strains. Figure 1 illustrates this workflow from strain engineering to material fabrication and regeneration. We believe that this work will serve as a framework for the future design of resilient ELMs as functional, self-healing materials for use as coatings and plasters that can respond to external stimuli due to the functions provided by the engineered cells in such materials.

### Results and discussion

**Identification of growth conditions for ELM fabrication.** Several factors need to be considered for the fabrication of a living silica biocomposite material. *Bacillus* growth conditions must be identified that are permissible for silica biomineralization and preferably, do not create an overly discolored material for aesthetic and functional (for example for sentinel or stealth applications) reasons. These conditions must also support scaffold building block expression and secretion during exponential to early stationary phase growth when cells are flagellated and have not yet initiated sporulation. Growth conditions for *B. subtilis* 168 (referred to as WT) were therefore evaluated using two different media; a Luria Bertani (LB) and a clear, buffered Spizizen minimal medium (SMM). *Bacillus* cells were transformed with a plasmid (pCT-empty) derived from our previously developed cumate (*p*-isopropyl benzoate) inducible *Bacillus* expression vector (Supplementary Data 1). Bacterial growth, media pH, flagella presence, and sporulation were then analyzed for 4 days in 24 h intervals at 20 and 30 °C (Supplementary Figs. 1, 2, and 3). Although pH is not typically considered in culture conditions, silica polymerization is optimal between pH 6.0 and 8.0 and at pH levels above and below this range polymerization is inhibited. During growth of *B. subtilis*, the culture medium turns increasingly alkaline by switching from sugar to amino acid catabolism - releasing ammonia from tryptophan (SMM) or hydrolyzed amino acids from tryptone and yeast extract (LB) (along with the secretion of alkaline proteases). In buffered SMM at 20 °C, the pH was maintained around 7.0 until 48 h of growth, while under all other conditions tested and during longer growth, media pH quickly increased above 7.0 (Supplementary Fig. 1). Peritrichous flagella (uniformly distributed flagella) were observed through 72 h of growth without significant sporulation (Supplementary Fig. 2). Buffered SMM and growth for 48 h at
20 °C therefore appeared to be optimal for our ELM production by engineered *B. subtilis*. We then repeated these growth experiments with *B. subtilis* WT transformed with a plasmid (pCT-EutMSpyC) for the secretion of the scaffolding protein EutM-SpyCatcher modified with the N-terminal secretion peptide SacB from *B. subtilis* (Fig. 1 and Fig. 2a). As before, pH increased above 7.5 regardless of medium at 30 °C after 24 h in LB and after 48 h in SMM. (Supplementary Fig. 1). Again, only cultures grown in SMM at 20 °C maintained a stable pH around 7.0 for 48 h and even until 96 h after induction of protein expression. Notably, growth was significantly reduced at 20 °C in SMM and LB during 48 h after induction compared to the empty vector control cultures. This was most notable in SMM at 20 °C (Fig. 2b), suggesting that EutM-SpyCatcher protein overexpression and secretion may burden cells, slowing cell growth. Like the empty vector cultures above, EutM-SpyCatcher expressing cultures had peritrichous flagella through 72 h of growth, with the most flagella present until 48 h (Supplementary Fig. 3a). However, spores were already observed after 48 h of growth at 20 °C in LB media, indicating cells were stressed under this condition (Supplementary Fig. 3b). Based on these results, we chose to perform all subsequent cultivations for ELM production in SMM at 20 °C for 48 h as our standard condition.

Establishing EutM scaffold building block secretion. The majority of industrially produced recombinant proteins by *B. subtilis* are extracellular enzymes (e.g. hydrolases) and achieving high-level secretion typically requires the identification of efficient secretion signal peptides to replace their native secretion signals. In contrast, our scaffolding protein EutM is cytoplasmic that also rapidly self-assembles into scaffolds when overexpressed in *E. coli*. We therefore screened different signal peptides and investigated secretion of EutM-SpyCatcher by *Bacillus*. In addition to SacB, we tested four other, commonly used secretion peptides (XynA, YngK, CelA, and LipA) by fusing them to the N-terminus of our scaffold protein (Supplementary Fig. 4a).

Each recombinant protein was expressed in *B. subtilis* WT in SMM, at 20 °C for 48 h as the optimal condition confirmed above. Protein secretion levels were then compared between the different cultures by SDS-PAGE analysis. We analyzed total protein in the culture, and after centrifugation of cultures, protein in the resulting culture supernatant and pellet. However, a very low amount of secreted protein was observed in the supernatant compared to visible bands in the pellet, and we suspected that secreted and scaffolded EutM proteins were pelleting together with the cells. We therefore solubilized scaffold proteins from pelleted cells with 4 M urea, which we have used previously to solubilize EutM scaffolds produced by *E. coli*. At this concentration, urea does not lyse cells which we verified by comparing total protein released from completely lysed cells after urea treatment and protein solubilized from sediment (see Supplementary Fig. 5). We compared by SDS-PAGE three fractions as shown in Fig. 2c: (A) culture supernatants (concentrated by trichloroacetic acid (TCA)), (B) urea supernatant after scaffold solubilization from pellets, and (C) total protein released from remaining pellet after urea solubilization and cell lysis. Only the SacB secretion signal peptide yielded significant yields of secreted protein in the culture supernatant and the solubilized fraction (Fig. 2d). Only small amounts of secreted EutM protein (only after 25x concentration) could be detected in culture supernatants with the other four signal peptides (Supplementary Fig. 4b). Compared to cultures expressing SacB-EutM-SpyCatcher, no scaffolds sedimented in the other cultures secreting low levels of EutM protein (Supplementary Fig. 4b). From these results, we concluded that SacB is the best secretion signal peptide for EutM-SpyCatcher.

Engineering *B. subtilis* cells as integral material component. With scaffold building block secretion confirmed, we next set out to engineer our host strain to persist as a component within the matrix it fabricates by providing structure to the material and for material regeneration when favorable growth conditions are provided (Fig. 1). *B. subtilis* spores are extremely durable and can remain dormant indefinitely until proper environmental conditions return whereupon the spores will germinate. During
sporulation, endospores are formed in the mother cell which then undergoes lysis for spore release. To retain *Bacillus* cells as structural material components while also allowing spore formation for long-term viability of our material, we deleted the cell wall autolysin LytC (N-acetylmuramoyl-L-alanine amidase) in *B. subtilis* by in-frame deletion of *lytC* (Fig. 1), we also introduced a second in-frame deletion of *lytH* to generate a *B. subtilis* ΔlytC strain (Fig. 3a and Supplementary Fig. 6).

As our objective is to link *B. subtilis* cells to secreted EutM-SpyCatcher scaffold via SpyTag display on its flagella (Fig. 1), we also introduced a second in-frame deletion of *flhG* (Fig. 3a and Supplementary Fig. 6) to change the flagellation pattern on cells from peritrichous to polar flagella clusters. Normally, *B. subtilis* produces flagella around the midcell and lacks flagella at the poles. The protein FlhG (also called YlhH, MinD2, Flen, and MotR) is essential to this flagellation pattern. The deletion of *flhG* results in the aggregation of basal bodies at the polar ends of cells. We reasoned that reducing flagellation density and directing flagella to the poles will enable more spaced-out cross-linking on scaffolds and less ‘clumping’ of cells (which we later serendipitously discovered appears to be deleterious for *B. subtilis*, see below).

The resulting double deletion strain *B. subtilis* ΔylytC ΔflhG was then transformed with expression vectors (pCT-empty, pCT-EutM-SpyC) for confirmation of expected cell phenotypes and scaffold building block secretion (Fig. 3b–d). Comparison of flagella pattern between wild-type *B. subtilis* and the double deletion strain confirmed the presence of clustered polar flagella and cell wall enclosed endospores in the deletion strain (Fig. 3b). Clustered polar flagella were most prevalent at 48 h or less after induction, and the most flagella both for the wild-type and the double deletion strain were observed in cultures grown at 20 °C (Supplementary Figs. 2a, 3a, 6d). Unlike the wild-type strain, no free spores were released by the autolysin deficient ΔlytC strain in SMM (Supplementary Figs. 2b, 3b, 6c). Endospore formation by *B. subtilis* ΔylytC ΔflhG harboring pCT-empty and pCT-EutM-SpyC was not observed until 72 h of growth under the optimal growth conditions (SMM, 20 °C) identified above (Fig. 3b).

Following the confirmation of clustered flagella and no sporulation during growth in SMM at 20 °C for 48 h, we next evaluated growth and protein expression under these conditions (Fig. 3c, d). Interestingly, the growth rate of the double deletion strain was steadier and higher than the wild-type strain when transformed with either the empty control plasmid or pCT-EutM-SpyC (compare Fig. 2b and Fig. 3c). The pH of the culture was also maintained at around 7.0 for 48 h. Expression and secretion of scaffold building blocks by the engineered strain was unchanged from the wild-type strain (Fig. 3d, compare to Fig. 2d) despite reaching a slightly higher optical density after 48 h.

We noticed that the secreted EutM-SpyCatcher protein appears as a major higher (24.4 kDa) and a minor lower (21.1 kDa) molecular weight band on SDS-PAGE gels in all three analyzed fractions (culture supernatant, urea solubilized scaffolds and lysed
cell pellet, Figs. 2d and 3d). We previously observed that EutM proteins do not migrate according to size and/or as double bands. Here, we wondered whether the differences in apparent molecular weights might be due to uncleaved SacB signal peptide. Secretion of native proteins with uncleaved Sec signal sequences into culture supernatants has been documented for bacteria. LC-MS analysis showed that indeed the signal peptide was not cleaved from either EutM protein band (Supplementary Fig. 7). This suggests that EutM likely folds rapidly upon emerging from the Sec translocase complex, preventing access for the signal peptidase and pulling the folding protein into the extracellular space. EutM and its homologs are known to rapidly fold and self-assemble in vitro and in vivo to form higher-order structures. In contrast, the release and folding of the B. subtilis levansucrase, which is the source of the SacB secretion signal sequence, has been shown to be a slow process.

To determine whether the remaining SacB signal peptide effects EutM-SpyCatcher assembly and to compare EutM-SpyCatcher protein assembly in SMM medium to previous observations in Tris buffer, we purified uncleaved and secreted protein from recombinant B. subtilis ΔlytC ΔflhG cultures and His-EutM-SpyCatcher without signal sequence from recombinant E. coli cultures. After metal affinity chromatography and dialysis

Fig. 3 Development of a Bacillus strain for durable material fabrication. a Construction of a B. subtilis ΔlytC ΔflhG strain deficient in spore release and exhibiting polar, clustered flagella was achieved by deleting the autolysin LytC and the flagella basal body protein FlhG. b Sporulation and flagella phenotypes of B. subtilis strains transformed with pCT-EutMSpyC were compared to confirm endospore and polar flagella phenotype under expression conditions. Representative samples show spor release for the wild-type strain (LB, 48 h, 20 °C shown) and endospore formation by the engineered strain (SMM, 72 h, 20 °C shown) (see also Supplementary Figs. 2, 3 and 6). Peritrichous flagella are observed for the wild-type strain (LB, 48 h, 20 °C shown) under all tested growth conditions through 72 h and flagellation decreases after 48 h (see also Supplementary Figs. 2 and 3). Clustered polar flagella are observed in the engineered strain through 48 h of growth (SMM, 48 h, 20 °C shown) (see also Supplementary Fig. 6). Samples were stained with either malachite green and safranin red (top) or RYU (bottom) and examined by light microscopy at 100x. Spore images had red replaced with magenta and flagella images were converted to grayscale. Images shown are representative three independent biological replicate cultures. c B. subtilis ΔlytC ΔflhG transformed with the pCT-empty (control) or pCT-EutMSpyC plasmid (SMM, 20 °C) show growth characteristics comparable to the B. subtilis wild-type strain in Fig. 2. Data are shown as mean values ± SD and error bars represent the standard deviations of three independent biological replicate cultures. Colored bars and lines represent mean values. Black symbols represent data points and error bar. d Expression and secretion of EutM-SpyCatcher by the engineered B. subtilis ΔlytC ΔflhG strain was verified in SMM medium at 20 °C after 48 h of induction by SDS-PAGE. Arrows indicate EutM-SpyCatcher bands that are not present in the empty plasmid control. A strong band corresponding to urea solubilized EutM-SpyCatcher scaffolds (B) from co-pelleted cells confirm comparable protein expression and secretion levels of the engineered and the wild-type strains (Fig. 2). Samples (A) and (C) corresponding to 10-fold concentrated culture supernatant and cell lysate after scaffold solubilization also show comparable protein bands to the wild-type strain. Shown data is representative of three independent biological replicate cultures. Source data are provided as a Source Data file.
into SMM, scaffolds were observed by transmission electron microscopy (TEM). In comparison to the negative control of SMM which shows many nonhomogeneously sized and poorly stained structures and a few heavily stained structures (Supplementary Fig. 8a, e), scaffolds isolated from E. coli formed rolled-up tubes (Supplementary Fig. 8b) which have been observed previously for EutM proteins expressed in E. coli[33]. Bacillus secreted protein assembled into fewer large, rolled-up tubes and smaller, spherical structures (that stain as donut-like structures) as well as sheets and less-ordered coral-like scaffolds (Supplementary Fig. 8c, d, f-h). These structures have also been previously observed for EutM homologs[33,40]. This greater variety of scaffold assemblies may be due to the SacB sequence, modifying surface charges and assembly. These results confirm that B. subtilis secreted EutM-SpyCatcher forms scaffolds and that the SMM growth medium does not inhibit self-assembly.

**Linking B. subtilis cells to secreted EutM-SpyCatcher scaffolds by SpyTag display.** We next set out to engineer the display of a SpyTag on flagella for cell attachment to secreted scaffolds (Fig. 1). For characterization of flagella SpyTag display, a cysteine variant (T209C) of the flagellin Hag protein was first created for labeling of flagella filaments with a sulphydryl-specific (maleimide) dye[57]. The modified flagellin gene was then expressed from its native promoter on a plasmid in wild-type B. subtilis and the ΔlytC ΔflhG double deletion strain. The assembled flagella filaments are therefore composed of both engineered and native flagellin monomers. The recombinant HagΔT209C protein was then modified to include a SpyTag domain at nine different positions chosen inside the variable D2/D3-region of the flagellin protein based on the assumption that mutations in these locations would least likely interfere with filament assembly and function (Fig. 4a, b)[58]. Four Hag insertions (bp positions 399, 426, 459, 537) were deleterious for both B. subtilis strains, meaning that either no transformants could be obtained or transformants contained missense mutations or deletions in the modified haq gene of the recovered plasmids (Fig. 4a). Fluorescence microscopy (Fig. 4c) of the remaining mutants revealed flagella located at the mid cell for B. subtilis WT and poles for B. subtilis ΔlytC ΔflhG which adopt different filament conformations (Figs. 4d, 9a). Wild-type flagella form a left-handed helix[59], which was retained for insertions at positions 612 and 645 in HagΔT209C. Curly, right-handed forms (with half the pitch and amplitude) were observed for flagella expressing SpyTags at positions 555 and 588. SpyTag insertion at position 588 also resulted in shortened flagella compared to unmodified flagella (Fig. 4d). One strain expressing HagΔT209C with a SpyTag at position 492 had both normal and curly flagella (Supplementary Fig. 9a).

Functional display of the SpyTag on B. subtilis flagella by the different insertion mutants was then evaluated by adding purified red fluorescent tdTomato-SpyCatcher protein (Fig. 4c) to recombinant B. subtilis WT and ΔlytC ΔflhG cultures that were then grown overnight for flagella expression. No attachment of tdTomato-SpyCatcher to flagella of strains with HagΔT209C SpyTag insertions at positions 492, 555, and 612 was observed by fluorescence microscopy (Fig. 4e, Supplementary Fig. 9b). Some red fluorescent labeling was observed for SpyTag display at position 645 (Supplementary Fig. 9b), but the best attachment of tdTomato-SpyCatcher was achieved on flagella that display a SpyTag at position 588 (HagΔT209C::SpyTag588), despite the shorter than wild-type flagella formed by the recombinant strains (Fig. 4e).

We then confirmed that HagΔT209C::SpyTag588 modified flagella on B. subtilis ΔlytC ΔflhG are capable of forming isopeptide bonds for covalent attachment to EutM-SpyCatcher proteins secreted during cultivation. For this, two B. subtilis ΔlytC ΔflhG strains - one expressing HagΔT209C::SpyTag588 and the other EutM-SpyCatcher – were co-cultured under the identified conditions for scaffold building block secretion but extended to 96 h of induction (SMM, 20 °C, 96 h induction). Flagella were then isolated from cells and analyzed by SDS-PAGE, confirming the presence of a higher-molecular weight protein complex (55 kDa) that was not present in the control (Fig. 4f). This band is weaker compared to the major, genome encoded flagellin band as the modified flagella only contain a small number of engineered, SpyTag-displaying flagellin monomers. Based on these results, we therefore chose HagΔT209C::SpyTag588 expression for cross-linking B. subtilis ΔlytC ΔflhG cells to EutM-SpyCatcher scaffolds.

**Engineering of silica biomineralization.** Our next objective was to modify EutM scaffolds such that they would biomineralize silica for the fabrication of a resilient living bio composite material (Fig. 1). We selected four known silica biomineralization peptides (R5, CotB1p, SB7, and Synthetic60–64) for C-terminal fusion to His-EutM (Supplementary Fig. 10a). We confirmed scaffold formation with the fusion proteins overexpressed and purified from E. coli and characterized silica precipitation. We intentionally left the His-Tag on all of our scaffold building blocks as histidine residues are enriched in proteins involved in natural silica biomineralization processes such as e.g. in the histidine-rich glassin protein present in the siliceous skeletons of sponges[65].

Silica precipitation by the biomineralization peptide modified His-EutM and the unmodified His-EutM scaffolds was then quantified using a spectroscopic molybdate blue assay (Supplementary Fig. 10a). The CotB1p (hereafter referred to as CotB) peptide fusion was most efficient at precipitating silica at 7.44 ± 0.36 mmol/g protein and was also the most cationic peptide, rich in arginine residues. As expected, His-EutM scaffolds also precipitated silica at 1.98 ± 0.01 mmol/g protein. The structures (scaffolds) formed by the fusion proteins and His-EutM were then visualized by SEM (Fig. 5a and Supplementary Fig. 10b, center and right columns). His-EutM formed tubular nanostructures that appeared to be rolled-up tubes fused together at multiple locations. In contrast, His-EutM-R5 formed globular structures fused into chains, while His-EutM-CotB formed similar scaffolds but with a greater surface roughness. Fusion of the SB7 and Synthetic peptide to EutM resulted in the formation of dense scaffolds comprised of fused together globular and poorly-formed tubular structures (Supplementary Fig. 10b).

Silica precipitation on scaffolds was investigated by incubating 1 mg/mL scaffolds for 2 h at room temperature with 100 mM silica. SEM imaging of scaffolds (Fig. 5a, center and right) showed that silica addition resulted in an increase in scaffold surface roughness, surface porosity, and the presence of silica nanoparticles fused to the scaffolds which are characteristics of silicification (Fig. 5a and Supplementary Fig. 10b, center and right columns). Silica nanoparticle formation was most prominent on the surface of His-EutM scaffolds, suggesting that biosilicification may have been nucleated in the solution phase by these proteins. In contrast, a significant increase in surface roughness seen for His-EutM-R5 and His-EutM-CotB scaffolds suggests surface catalyzed biomineralization by these structures. TEM imaging of silica precipitation on TEM grids coated with purified His-EutM and His-EutM-CotB proteins also showed the deposition of a more granular layer on His-EutM-CotB coated surfaces compared to His-EutM (Supplementary Fig. 10c), despite the formation of curved, fibrillar structures by His-EutM-CotB as opposed to the uniform structures made by His-EutM.

Based on these results, we chose the CotB biomineralization peptide for fusion to scaffold building blocks for secretion by B.
subtilis. An expression construct was made where the SpyCatcher domain in pCT-EutMSpyC was replaced with CotB to yield plasmid pCT-EutMCotB, replicating the E. coli His-EutM-CotB with the additional SacB signal sequence (Fig. 5b). EutM-CotB expression and secretion by B. subtilis ΔlytC ΔflhG transformed with pCT-EutMCotB (SM, 20 °C, 48 h induction) was confirmed by SDS-PAGE (Fig. 5c, lane 3). As in the case of EutM-SpyCatcher, the SacB signal sequence appears to be unceiled for EutM-CotB based on the size of the observed protein band (16.8 kDa vs 13.8 kDa for the uncleaved and cleaved proteins, respectively). Secretion of EutM-CotB however was much lower than EutM-SpyCatcher, probably due to the added positive charged by the CotB peptide fusion. Yet, despite the low amount of secreted EutM-CotB, cultures of B. subtilis strains transformed with pCT-EutMCotB significantly increased silica gel formation at low silica concentrations compared to empty vector control cultures (Supplementary Fig. 11). After 48 h induction of protein expression (SMM, 20 °C), samples of cultures were incubated with different concentrations of silica (50–500 mM) and silica gel formation followed for 24 h at 20 °C. With 100 mM silica added, the EutMCotB expressing cultures solidified into a gel, while the control cultures only formed a liquid soft gel (Supplementary Fig. 11).

We then proceeded with combining the expression and secretion of our EutM-SpyCatcher building block for cell cross-linking with the EutM-CotB scaffold building block for enhanced biomineralization, yielding the expression vectors as shown in Supplementary Fig. 11. For SpyTag display on flagella of B. subtilis ΔlytC ΔflhG, a plasmid for co-expression of EutM scaffold building blocks with the modified flagellin subunit Hag T209C::SpyTag588 was also constructed. Each expression module contains its own promoter as shown. EutM building block expression and secretion by B.
Δ. For Biocomposite ELM fabrication and characterization residues of the EutM proteins are labeled. represent secreted EutM scaffold where one or both cysteine Fig. 12). We therefore presume that the dye-labeled structures fl. for measurements with a rheometer. Small, but statistically significant increases (p < 0.05) in storage moduli (G’) were measured for cultures co-expressing EutM scaffold building blocks (1.23-fold for pCT-EutMCotB-EutMSpyC and 1.37-fold for pCT-EutMCotB-EutMSpyC-HagT209C::SpyTag588 compared to the empty plasmid control (pCT-empty)) (Fig. 6c, Supplementary Table 2 and 3 for statistical analysis). The loss modulus (G’’)

Fig. 5 Implementing silica biomineralization by scaffolds. a Silica biomineralization by His-EutM and His-EutM-CotB scaffolds expressed and purified from E. coli was investigated by SEM following the incubation of 1 mg/mL protein without (left column) and with (center columns) 100 mM silica for 2 h at room temperature. Right columns show 10-fold magnification of marked regions. Images shown are representative of one set of purified proteins. b Different plasmids were constructed for EutM-CotB expression and secretion in B. subtilis alone or together with EutM-SpyCatcher as shown. For final ELM fabrication, a consolidated plasmid was designed for the co-expression of EutM-SpyCatcher and EutM-CotB with HagT209C::SpyTag588 in B. subtilis ΔlytC ΔfliH. Each gene is expressed by its own promoter (cumate inducible P_{CTS} promoter, native hag promoter). c Expression and secretion of EutM-CotB by B. subtilis ΔlytC ΔfliH was investigated and compared to EutM-SpyCatcher. Urea solubilized fractions were prepared from cultures transformed with different expression plasmids and grown as in Fig. 2. Except for pCT-EutMSpyC (lane 2), all urea fractions from the different recombinant cultures were concentrated 10-fold by TCA precipitation prior to SDS-PAGE analysis. Data shown are representative of three independent experiments. Source data are provided as a Source Data file.

subtilis ΔlytC ΔfliH) transformed with the different plasmids was then confirmed and compared, showing again lower secretion levels for EutM-CotB compared to co-expressed EutM-SpyCatcher in SMM at 20 °C after 48 h of induction (Fig. 5c). We also attempted to visualize by fluorescence microscopy the flagella phenotypes of B. subtilis ΔlytC ΔfliH cultures transformed with the EutM co-expression constructs with and without HagT209C::SpyTag588. We observed large structures labeled with cysteine reactive dye that interfere with cell labeling (Supplementary Fig. 12). In contrast, cells that expressed only the modified HagT209C or HagT209C::SpyTag588 contained clearly labeled flagella under the same growth conditions (Supplementary Fig. 12). We therefore presume that the dye-labeled structures represent secreted EutM scaffold where one or both cysteine residues of the EutM proteins are labeled.

Biocomposite ELM fabrication and characterization. For the final fabrication of our biocomposite ELM we performed biomineralization experiments with induced and uninduced (control) cultures of B. subtilis ΔlytC ΔfliH transformed with an empty control plasmid or one of the EutM-SpyCatcher and EutM-CotB co-expression plasmids with or without HagT209C::SpyTag588. After 48 h of cultivation, 5 mL culture samples were transferred and further incubated with and without 100 mM silica in 6-well plates at 20 °C, 100 rpm for 1 h (Fig. 6a, see Supplementary Fig. 13 for biological replicates). While all cultures show some silica condensation into clusters at charged surfaces present in the cultures, only cultures that expressed and secreted EutM scaffolds (induced cultures) formed aggregated silica materials. Larger silica material aggregates (about twice the size) were formed in cultures that also displayed SpyTagged flagella for material cross linking.

To test whether the silica materials made by the engineered Bacillus strains above have different mechanical properties due to scaffold secretion and cross-linking, we optimized conditions for the formation silica blocks suitable for the rheological analysis (Fig. 6b). Rheological measurements have also been performed for other types of living materials, such as for example a cellulose-based material of a bacterial and yeast co-cultures with programmable weakening of material microstructure.27 For measurements of our materials, induced culture samples from Fig. 6a were incubated in a syringe with 200 mM silica at 20 °C or 25 °C for up to 5 h until solid gel blocks were obtained (25 °C, 5 h) for measurements with a rheometer. Small, but statistically significant increases (p < 0.05) in storage moduli (G’) were measured for cultures co-expressing EutM scaffold building blocks (1.23-fold for pCT-EutMCotB-EutMSpyC and 1.37-fold for pCT-EutMCotB-EutMSpyC-HagT209C::SpyTag588 compared to the empty plasmid control (pCT-empty)) (Fig. 6c, Supplementary Table 2 and 3 for statistical analysis). The loss modulus (G’’)
remained the same for all samples and was lower than $G'$ as would be expected for materials with lower viscous dissipation. The increases in $G'$ shows that the silica gel blocks from scaffold secreting cultures have a higher degree of intermolecular bonding giving them greater mechanical rigidity. The engineered Bacillus strain with the SpyTag displaying flagella yielded the most robust material due to cross-linking of cells and scaffolds.

Although we have shown that our final ELM Bacillus strain (B. subtilis ΔlytC ΔflhG pCT-EutMColB-EutMSpyC-HagT209C::SpyT588) improved material strength, we wanted to determine where the secreted scaffolds are located in the material. Uninduced and induced cultures used for silica material formation in Fig. 6 were incubated with SpyTag-eGFP or eGFP (control) to label SpyCatcher domains on EutM scaffolds not linked to SpyTag.
Fig. 6 Formation of living, silica biocomposites with increased rigidity. a Generation of silica biocomposites was tested by cultivating B. subtilis ΔlytCΔflhG transformed with plasmids (empty as control) for expression of the shown proteins and their functions. Cultures were first grown (SMM, 20 °C, 48 h) with (+ induced) and without (- uninduced) induction of protein expression. 5 mL cultures were transferred to 6-well plates (0 h, 0 mM silica) and incubated (20 °C, 100 rpm) for 1 h after addition of 100 mM silica (1 h, 100 mM). Experiments were performed with three biological culture replicates (see Supplementary Fig. 13). b 3D blocks of silica biocomposite materials were then fabricated from cultures of the same three strains grown under the same conditions with induction. After 48 h of growth, 200 mM silica was added into 1 mL cultures in syringes as molds and the aliquots of the cultures were cured at 20 °C or 25 °C. At different time points, silica gel plugs were removed from their molds to evaluate their hardness. After 5 h of curing at 25 °C, solid gel plugs suitable for rheology testing were obtained. c Rheological properties of the cured gel plugs were measured using an extensional DMA Rheometer with 15 mm compression disks. A frequency sweep was performed with a gap of 4.5 mm and an oscillation strain of 1%. Three biological replicate gel blocks were measured for each strain. Variation was observed in the storage modulus (G' solid line) and loss modulus (G″, dashed line). Data are shown as mean values ± 5D and error bars represent the standard deviations of three independent biological samples. Source data are provided as a Source Data file.

Fig. 7 Microscopy characterization of Bacillus silica biocomposites. a The location of assembled scaffolds was tested by cultivating B. subtilis ΔlytCΔflhG transformed with EutMCotB-EutMSpyC-HagT209C-SpyT588 plasmid (uninduced as control). Cultures were first grown (SMM, 20 °C, 48 h) with (induced) and without (uninduced) cumatate induction of protein expression. 500 μL of culture was mixed with 0.125 mg eGFP or SpyTag-eGFP and mixed at RT for 30 min before acquiring DIC and GFP images which were merged and GFP channel colored blue. Induced cultures have EutM scaffolds assembled outside and between cells, with available binding sites for additional SpyTagged proteins. b 3D blocks of silica biocomposite materials were then formed from cultures of the same sample examined in a. 200 mM silica was added after 48 h of growth and 1 mL aliquots of the cultures were transferred to syringes as molds for curing at 25 °C. After 5 h of curing at 25 °C, solid gel plugs were cut into 2 mm3 pieces and prepared for thin sectioning. Cells were observed in clusters for both the uninduced and induced cultures, however only the induced culture contained clear zones around cells (see Supplementary Fig. 14a for a close-up). This zone corresponds to the SpyTag-eGFP labeled areas above and indicates there is interference with staining the scaffolds after silica biomineralization. (Images shown are representative of two independent experiments.).

displaying flagella of cells (Fig. 7a). No labeling was observed with uninduced or eGFP control samples. In contrast, regions outside and in between cells were labeled in the induced samples, suggesting that EutM proteins are secreted and assemble into scaffolds between the cells. The relatively high binding of SpyTag-eGFP indicates that there are many available binding sites for future incorporation of additional, secreted SpyTagged cargo proteins for the fabrication of functional ELMs.

The same cultures examined by GFP labeling were then transformed into a silica blocks as in Fig. 6b for TEM visualization of cells in the biocomposite material. After curing, solid gel plugs were cut into 2 mm3 pieces and processed for thin sectioning. Cell were observed in clusters for both the induced and uninduced cultures in relatively low density which corresponds to a final OD600 of ~3.5 (induced) or ~6 (uninduced) of the cultures prior to silica material formation (Fig. 7b, Supplementary Fig. 14a). Unexpectedly, no scaffolds could be visualized outside or in between cells in the material from the induced cultures. Instead, only thin sections from the induced cultures contained large clear zones around cells. These zones correspond to the SpyTag-eGFP labeled areas above and indicates there is interference with staining scaffolds during thin sectioning.

Regeneration and functional augmentation of biocomposite ELM. Ultimately, a living composite material for application as e.g. functional coating or plaster should be self-regenerating and integrate desired functions through their living components (Fig. 1). We therefore first tested whether our ELM could be regenerated from a small plug (~5 mm3) taken from silica blocks generated from cultures of our final ELM strain. Blocks fabricated with 200 mM silica were cured for 1 day at 25 °C and then small pieces were used to inoculate fresh cultures for expression and secretion of scaffold building blocks (Fig. 8a). After 48 h of growth with and without induction (control), secretion of EutM proteins at similar levels as the parent culture could be confirmed in the induced cultures (Fig. 8b). Similarly, we could reproduce the formation of cross-linked silica material in 6-well plates with the induced cultures (Fig. 8c). These results show that the recombinant Bacillus cells remain viable in the silica material and retain their engineered functions. We repeated the same experiments with silica blocks that were cured for 2 weeks at 25 °C. Although cultures could be regrown in selective media, meaning that the plasmid is still present, no scaffold building block secretion could be detected. Due to plasmid-based expression of our proteins, we theorized this issue might be caused by mutations in their coding sequences. Sequencing of plasmids isolated from individual colonies derived from the revived culture indeed confirmed that all of them contained multiple mutation in the EutM gene regions. The rapid mutability of high copy plasmids in Bacillus is a well-known issue and can be solved in the future by integrating the expression cassettes currently located on a plasmid into the genome of our engineered Bacillus strain. Nevertheless, the ability to regrow the engineered strain with its plasmid after 2 weeks in a silica gel speaks to the resilience of B. subtilis as ELM chassis organism.
As a proof-of-concept, we sought to demonstrate the incorporation of another property into our material. Rather than adding another genetic burden to our engineered *B. subtilis* strain, we opted to co-cultivate our engineered strain with a second engineered *Bacillus* strain that would provide the new property. Such a co-cultivation strategy has recently been successfully explored for the fabrication of a living material that incorporates cellulose. For demonstration purposes, we chose the formation of colored material by expressing a purple chromoprotein. An additional plasmid was constructed for transformation into *B. subtilis* strain ΔlytC ΔflhG and co-cultivation with our final ELM strain. Co-cultures (mixed at a 1:1 ratio in the induction step) were grown as before and biomineralization and silica formation compared to co-cultures with an empty plasmid control. Only co-cultures that co-expressed EutM-CotB and EutM-SpyCatcher formed an aggregated silica material with embedded purple cells. A block of a purple, silica biocomposite (15 mm × 30 mm) was then fabricated from co-cultures of engineered strains co-expressing scaffold building blocks and the purple protein together with SpyTagged flagellin. A solid material was formed with 200 mM silica after curing for 24 h at 25 °C. All results shown are representative of three independent biological samples. Source data are provided as a Source Data file.

![Fig. 8 ELM biocomposite regeneration and proof-of-concept for material functionalization.](https://nature.nature.com/naturecommunications/article/10.1038/s41467-021-27467-2/)

**a** Silica biocomposite material regeneration was tested by re-inoculating cultures with a piece of a silica plug fabricated from *B. subtilis* ΔlytC ΔflhG cultures co-expressing EutM-CotB and EutM-SpyCatcher and HagT209C::SpyTag588, biomineralized with 200 mM silica (see Fig. 6) and cured for 24 h at 25 °C. Cultures were regrown (SMM, 20 °C, 48 h) with (+ induced) and without (- uninduced) cumate induction of protein expression. **b** Expression and secretion of EutM scaffold building blocks by the induced, regenerated cultures was confirmed by SDS-PAGE analysis of culture supernatant (A) and urea solubilized scaffolds (B). All fractions were concentrated 10-fold by TCA precipitation prior to SDS-PAGE analysis. **c** Silica biomineralization into a cross-linked biocomposite material was confirmed for the induced, regenerated cultures after 1 h at 20 °C with 100 mM silica in 6-well plates as in Fig. 6. **d** Proof-of-concept incorporation of additional functions into the silica ELM was demonstrated by the co-cultivation of two strains, one strain co-expresses scaffold building blocks and SpyTagged flagellin, while a second strain co-expresses a purple chromophore protein and SpyTagged flagellin. In the control co-culture, the plasmid for scaffold building block secretion in one strain was replaced with an empty plasmid. Strains were inoculated at a 1:1 ratio and cultures grown under inducing conditions for biomineralization as described above. Only co-cultures that co-expressed EutM-CotB and EutM-SpyCatcher formed an aggregated silica material with embedded purple cells. **e** A block of a purple, silica biocomposite (15 mm × 30 mm) was then fabricated from co-cultures of engineered strains co-expressing scaffold building blocks and the purple protein together with SpyTagged flagellin. A solid material was formed with 200 mM silica after curing for 24 h at 25 °C. All results shown are representative of three independent biological samples. Source data are provided as a Source Data file.
enhance biomineralization by engineering and increasing the density of biomineralization peptides on scaffolds could reduce silica concentrations, and the incorporation of different types of biomineralization peptides would give access to a range of other biomimetic materials. We have shown that our ELM can be regenerated from a piece of silica material containing cells after 24 h of curing. But extended material storage and regeneration attempts resulted in the accumulation of mutations in plasmids recovered from regrown cells. We utilized plasmids for easy optimization of protein expression, but these expression constructs once designed in their final configurations will need to be integrated into the genome to ensure stable, genetic programming of ELMs that can regenerate over many centuries. Finally, this work offers engineering strategies for expanding the repertoire of current ELM chassis organisms towards other spore-forming and/or ultra-robust bacteria that secrete and embed themselves into a genetically programmable protein matrix for the fabrication of a range of functional materials.

Methods

Materials and chemicals. Reagent grade tetraethyl orthosilicate (TEOS), ammonium molybdate tetrahydrate, 4-methylaminoprophyl sulfide, anhydrous sodium sulfate, all acids and bases, and other chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich Corp., MO, USA).

R.M. Flagella stain, polyvinyl microscope slides, plain precleaned glass microscope slides, Alexa Fluor™ 488 C, maleimide, Syto16, and Trump microscope slides were purchased from ThermoFisher Scientific (Waltham, MA, USA). 22 nm 1.5 cm-1.

Methods.

Construction of plasmids and in-frame deletion in B. subtilis. All plasmids and strains used in this study are described in Supplementary Data 1. Plasmid sequences were verified by Sanger sequencing (ACGT, Inc. Wheeling, IL, USA).

Amino acid sequences and nucleotide sequences for all proteins and genes reported in this work are found in Supplementary Data 2 and 3. Primer pairs for plasmid modifications and flagellar engineering are listed in Supplementary Data 4.

Plasmids were constructed using a combination of Gibson Assembly (HiFi DNA assembly kit, NEB) for fragment assembly, site-directed mutagenesis (Q5 site-directed mutagenesis kit, NEB) for shorter insertions, deletions, and insertions, and restriction enzyme cloning and ligation for larger fragment assembly following manufacturers’ instructions and as described previously33,34.

Briefly, for the C-terminal fusion of biomineralization peptides to His-EutM in pCT5-His-EutM3,34,35, primers were designed to insert the corresponding DNA sequences into the plasmid using the Q5 mutagenesis kit according to the manufacturers’ instructions.

To construct the pCT-His-dTomato-SpyCatcher plasmid, His-EutM was constructed with the fluorescent protein in a previously constructed pCT5-His-EutM3,34,35, His-EutM3 was amplified by HiFi Assembly. His-tdTomato-SpyCatcher was amplified from pCT5-hpBD7.

To create the EutM Bacillus expression vectors, His-EutM-SpyCatcher was amplified from pCT5-His-EutM3,34,35, and inserted into our in-house, cationic inducible Bacillus expression vector pCT5-bac2.0 by HiFi Assembly. For a control plasmid, pCT-empty was created by deletion of the sfcp gene from pCT5-bac2.0 using the Q5 mutagenesis kit according to the manufacturers’ instructions.

All signal peptides (Supplementary Table 1) were fused to His-EutM-SpyCatcher by site-directed mutagenesis (Q5 mutagenesis kit, NEB) with appropriate primers. The pCT-EutM-CotB plasmid for Bacillus expression was made by replacing the SpyCatcher sequence in pCT5-EutM-CotB by the complete SpyCatcher expression cassette (including cationic inducible promoter) from pCT5-EutM3,34,35, and amplified by HiFi Assembly. The pCT-EutM-CotB plasmid was amplific and subcloned into pCT-EutM-CotB.

The hag1209C-SpyTag38 expression cassette (including its native promoter) was added to pCT-EutM-CotB by HiFi Assembly with the hag expression module amplified from the corresponding pBR322 plasmid (see below). Finally, to construct pCT-Purple-Hag2.109C-SpyTag38, the chromoprotein was obtained from pSBC3-tpSPurple70 and assembled into pCT-EutM-CotB by HiFi Assembly.

The flagellin gene (hag gene) was amplified from B. subtilis genomic DNA using polymerase chain reaction (PCR) (HP-hag-fw and HP-hag-rev) and cloned into the pBBr34 backbone using HiFi Assembly. The Flagellin T209C mutation was introduced by Q5 mutagenesis with primer pairs (HP-hag-T209C-fw and HP-hag-T209C-rev). A SpyTag was inserted into nine different sites between D1b-N and D1-C domains using Q5 mutagenesis with primer pairs listed in Supplementary Data 3.

Phage T209C mutation was introduced by Q5 mutagenesis with primer pairs (HP-hag-T209C-fw and HP-hag-T209C-rev). A SpyTag was inserted into nine different sites between D1b-N and D1-C domains using Q5 mutagenesis with primer pairs listed in Supplementary Data 4.
To generate the lytC marker-less deletion construct, the pHBinE vector carrying a temperature-sensitive origin of replication, erythromycin and ampicillin resistance cassette, with the marker flanked by the S. aureus 5’ and 3’ promoter, was amplified from B. subtilis genomic DNA using primer pair dlytC upstream fwd and dlytC upstream rev, while the lytC downstream region was amplified using primer pair dlytC downstream fwd and dlytC downstream rev (Supplementary Data 4) and the deletion cassette was cloned into pHBinE vector using HiHi assembly. The plasmid for dlytC marker-less deletion was created in a similar way; the flibg upstream region was amplified using primer pair dflbg upstream fwd and dflbg upstream rev while the flibg downstream region was amplified using primer pair dflbg downstream fwd and dflbg downstream rev (Supplementary Data 4). The flibg deletion construct was done into B. subtilis lytC host using the pHBinE- dflbg plasmid. In-frame deletion of flibg followed by screening performed with identical method as above but the primer pair used for screening on colonies for flibg deletion mutants was dflbg confirmation fwd and dflbg confirmation rev.

Cultivation of recombinant B. subtilis strains. Glycerol stocks of recombinant B. subtilis strains (WT and ΔlytC Δflbg deletion strain) transformed with pCT expression plasmids (pCT-empty, pCT-EutMSpyc, pCT-EutMsilica, pCT-EutMsilica)* were streaked onto LB plates supplemented with tetracycline (10 µg/mL) and grown overnight at 30 °C. A single colony was used to inoculate 4 mL of selective LB. After 19 h of growth at 30 °C, 220 rpm strains were diluted 3% into 50 mL of LB or SMM supplemented with tetracycline (10 µg/mL) in a 250 mL flask. The cultures were centrifuged at 3220 x g, 30 °C, 100 rpm for 40 min, 4 °C and protein expression was induced with 10 µM cumate. The induced cultures were then grown at 30 °C or 37 °C until OD600 was 0.4–0.7. 25 mL of each culture was then used to inoculate a new 250 mL flask as follows: Co-culture 1: B. subtilis ΔlytC Δflbg pCT-empty + B. subtilis ΔlytC Δflbg pCT-Purple—HagT209C::SpyTag588, Co-culture 2: B. subtilis ΔlytC Δflbg pCT-EutMsilica—HagT209C::SpyTag588, Co-culture 3: B. subtilis ΔlytC Δflbg pCT-EutMsilica—HagT209C::SpyTag588, Co-culture 4: B. subtilis ΔlytC Δflbg pCT-Purple—HagT209C::SpyTag588. Protein expression was then induced with 10 µM cumate and co-cultures grown at 20 °C, 100 rpm for 48 h.

All expression and cultivation experiments were performed with three biological replicates (i.e. three independent cultures).

Analysis of scaffold building block expression and secretion by Bacillus strains. Extracellular secretion and intracellular accumulation of scaffold building blocks were assessed by SDS-PAGE. B. subtilis strains were analyzed by SDS-PAGE fractionated from cultures for SDS-PAGE analysis: (i) culture supernatant, (ii) urea supernatant containing urea solubilized scaffolds that co-precipitated with cells, (iii) lysed cell pellets after solubilization of co-precipitated scaffolds, and (iv) total protein (secreted-precipitated and intracellular) from completely lysed sonications by lysozyme treatment. The preparation of all four protein fractions is shown as a flow chart in Supplementary Fig. 5a. Fractions i-iii correspond to samples A, B, and C in Figs. 2, 3, and 8. Comparison of protein bands in fraction ii (i.e., absence of bands except for solubilized EutM protein) and iv indicates that urea does not break open cells Supplementary Fig. 3b. The following workflow was used for sample preparation: 5 mL of culture broth centrifuged at 21,130 x g for 10 min at 4 °C and the supernatant was removed. The precipitate was washed with 1 mL of pre-chilled acetone and the tube was centrifuged again at 21,130 x g for 10 min at 4 °C. The acetone was removed and the washing step was repeated twice. The precipitated protein was resuspended in 100 µL of 1X SDS-loading buffer diluted with 50 mM Tris-HCl, 4 M urea, pH 7.5) for a 10-fold concentrated sample.

For SDS-PAGE analysis of secreted scaffolds that co-precipitate with cells after centrifugation of 5 mL cultures (fraction ii), the collected pellet was carefully resuspended in 1 mL of 100% TCA and 1 h incubation on ice. The dried precipitate was resuspended in 1 mL of 50 mM Tris-HCl, pH 7.5 with incubation at 37 °C for 1 h. The clarified supernatant after centrifugation (3,220 x g, 10 min) was analyzed by SDS-PAGE.

For SDS-PAGE of the remaining scaffolds in the urea washed pellet (fraction iii), cells were lysed with 200 µg/mL final concentration of lysozyme in 500 µL of 50 mM Tris-HCl, pH 7.5 with incubation at 37 °C for 1 h. The clarified supernatant after centrifugation (3,220 x g, 10 min) was analyzed by SDS-PAGE. For SDS-PAGE of total protein (fraction iv), pelletted cells and co-precipitated scaffolds from 5 mL of culture was resuspended into 500 µL of 50 mM Tris-HCl pH 7.5 in an eppendorf tube. The suspension was sonicated in a coldwater bath sonicator (Branson 3510R, CT, USA) followed by lysis with lysozyme as above. The resulting suspension was mixed with SDS-loading buffer.

All protein samples were denatured by boiling for 10 mins at 100 °C and 8 µL of each sample was loaded onto a 15% SDS-PAGE gel. A PowerPAC 300 power supply (Bio-Rad) was used for SDS-PAGE analysis. Coomassie brilliant blue staining was then used for visualization of protein bands.

De novo sequencing of secreted EutM-SpyCatcher proteins. For de novo sequencing of secreted EutM-SpyCatcher, protein bands separated by SDS-PAGE were excised from gels and submitted to the Center for Mass Spectrometry & Proteomics, University of Minnesota for analysis by LC-MS using a Thermo Scientific LTQ Orbitrap Velos mass spectrometer. Data were analyzed and visualized using the PEAKS Xpro Studio 10.6 software (Bioinformatics Solutions Inc., Waterloo, ON, Canada).

Analysis of EutM-SpyCatcher attachment to HagT209C::SpyTag588 displaying flagella. Covalent isopeptide bond formation between secreted EutM-SpyCatcher and HagT209C::SpyTag588 displayed on flagella of B. subtilis ΔlytC Δflbg was analyzed by SDS-PAGE of sheared flagella isolated from co-cultures. Glycerol stocks of three B. subtilis ΔlytC Δflbg strains (pCT-empty, pCT-EutMsilpyC, pRBBm34·HagT209C:SpyTag588) were first spread onto selective LB plates, and single colonies were used to inoculate 4 mL of selective LB liquid cultures. After 19 h of growth at 30 °C, cultures were diluted 3% into 50 mL of fresh, selective SMM and grown at 37 °C until OD600 of 0.4–0.7 was reached. 25 mL of each culture was then mixed in a new 250 mL flask as follows: Co-culture 1: B. subtilis ΔlytC Δflbg pCT-empty + B. subtilis ΔlytC Δflbg pRBBm34·HagT209C:SpyTag588, Co-culture 2: B. subtilis ΔlytC Δflbg pCT-EutMsilpyC + B. subtilis ΔlytC Δflbg pRBBm34·HagT209C:SpyTag588, Protein expression was induced with 10 µM cumate and co-cultures grown at 20 °C, 100 rpm for 96 h.

Cell pellets were collected from 50 mL cultures by centrifugation (3220 x g, 30 mins) and resuspended in 20 mL PBS (pH 7.5). The suspension was then sonicated at low power (30% power, 5 sec on and 5 sec off, three times) (Branson 450 Digital Sonifier, CT, USA) to shear off flagella. TurboTM DNase (Invitrogen, Waltham, MA, USA) was then added to a 0.5 U/mL, final concentration, followed by incubation on ice for 10 mins. Cells were then removed by centrifugation at 3220 x g for 10 min at 4 °C, followed by an ultra-centrifugation step (39,800 x g for 2 h at 4 °C) to collect sheared flagella which were then resuspended in 100 µL 50 mM Tris-HCl (pH 7.5) for SDS-PAGE analysis.

Protein purification. His-EutM, His-EutM-silica binding peptide fusion proteins and His-EutM-SpyCatcher were expressed in E. coli and purified by metal affinity chromatography as described in detail. 33–36 Briefly, proteins were isolated from 500 mL recombinant E. coli C3566 LB cultures (37 °C, 220 rpm) transformed with pT7-CT plasmids encoding EutM and His-EutM-silica binding peptide fusion proteins (Supplementary Data 1). Gene expression was induced with 50 µM cumate at OD600 of 0.4–0.7 and cultures continued to be incubated overnight. Cells were collected by centrifugation (4000 x g, 30 min, 4 °C) and resuspended into either 30 mL EutM purification buffer (20 mM Tris-HCl, 250 mM NaCl, 5 mM imidazole, 4 M urea, pH 5.7) for His-EutM-SpyCatcher or 400 mL EutM-SpyCatcher purification buffer (50 mM Tris-HCl 250 mM NaCl, 20 mM imidazole, pH 8.0)35. Resuspended cells were lysed by sonication on ice (30 min, power 50%, pulse on 10 s, pulse off 20 s with a Branson Sonifier) followed by centrifugation (12,000 x g, 40 min, 4 °C) and passing of the supernatant through 0.2 µm ultrafilter. The soluble protein was loaded onto a 5–25% gradient gel (GE Healthcare Life Sciences, Pittsburgh, PA) using an AKTA FPLC system. Bound protein was eluted with 250 mM imidazole added to the purification buffer. His-tId Tomato-SpyCatcher protein was expressed and purified using the EutM-SpyCatcher protocol.35

For silica binding assays and SEM analysis of purified His-EutM-Silica and His-EutM-silica binding fusion protein, purified proteins were dialyzed against deionized water.
For TEM analysis of EutM-SpyCatcher scaffold formation in SMM, proteins expressed in *E. coli* or secreted by *Bacillus* cultures were purified with a 2 mL TALON metal affinity resin using a Bacterial Metal Affinity Purification protocol following manufacturer’s directions (TaKaRa Bio USA, Inc., see above). EutM-SpyCatcher protein from *Bacillus* was eluted with the same elution buffer (50 mM Tris-HCl, 250 mM NaCl, 250 mM imidazole pH 8.0) used for His-EutM-SpyCatcher as previously described. EutM-SpyCatcher from *E. coli* was isolated using the EutM-SpyCatcher protocol but with a TALON gravity column instead of a HisTrap FF column (GE Healthcare, IL, USA). For the purification of His-EutM-SpyCatcher proteins secreted by *B. subtilis* ΔJlyC ΔJhFs, recombinant strains harboring pCT-EutMSpyC-Hag were grown and induced under standard conditions described above. Cells and scaffolds from ten 50 mL cultures (total volume: 600 mL) were pelleted at 3,220 x g, at 4 °C for 10 mins and scaffolds solubilized in 30 mL of 50 mM Tris-HCl, pH 7.5 containing 4 M urea as described above. The solubilized scaffolds were then purified by TALON metal affinity chromatography as described above for *E. coli*. Purified protein from *E. coli* or *Bacillus* cultures were then dialyzed against SMM after elution from the TALON metal affinity resin.

For GFP labeling of scaffolds in *B. subtilis* ΔJlyC ΔJhFs transformed with EutMCorb-EutM-SpyC-HagΔ209C-SpyT588 plasmid, His-tagged eGFP and SpyTag-eGFP were expressed in *E. coli* from pET28 expression plasmids and then purified by metal affinity chromatography as described in 35.

**Preparation of hydrolyzed TEOs as silica source.** Silica acid solutions (i.e. silica) were prepared by hydrolyzing TEOS. TEOS was hydrolyzed by adding water and 1 M hydrochloric acid at a 1.5:3:0.0013 molar ratio of TEOS:water:HCl and stirring vigorously. Under these conditions, hydrolysis is complete after 80 mins for use as silica source in silica binding and biominalization experiments.

**Silica binding assays on EutM scaffolds.** Silica precipitation was measured using the blue silicinolysate method. Silica was added to purified protein scaffolds, at a concentration of 1 mg/mL in deionized water, to a concentration of 100 mM hydrochloric acid at a 1:5.3:0.0013 molar ratio of TEOS:water:HCl and stirring vigorously. The blue color was allowed to develop for 2 h at RT, and the absorbance was measured at 690 nm ± 0.02. Then, 2.5 mL culture aliquots were transferred into sterile 6-well plates. Silica was added to cultures to a concentration of 1 mg/mL and stirred at 200 rpm for 10 mins and scaffolds solubilized into 30 mL of 50 mM Tris-HCl, pH 7.5 containing 4 M urea as described above. The solubilized scaffolds were then purified by TALON metal affinity chromatography as described above for *E. coli*. Purified protein from *E. coli* or *Bacillus* cultures were then dialyzed against SMM after elution from the TALON metal affinity resin.

**Optimization of silica concentration for EutM-CoT mediated biominalization.** To test and optimize conditions for silica gel formation by secreted EutM-CoT scaffolds, recombinant *Bacillus* cultures were incubated with six different concentrations of silica. *B. subtilis* WT strain harboring pCT-EutMCorb or pCT-empty (control) were first cultured in 50 mL SMM under optimal scaffold-secreted conditions described above (20 °C, 100 rpm, for 48 h with 10 μM cumate added for induction of protein expression). Cultures were grown as three independent biological replicates. OD600 and pH measured after 48 h were for *B. subtilis* pCT-empty: OD600 = 1.73 ± 0.09, pH = 6.94 ± 0.01, and for *B. subtilis* pCT-EutMCorb: OD600 = 1.26 ± 0.16, pH = 6.90 ± 0.02. The culture aliquots were transferred to fresh culture glass tubes, incubated with 50–500 mM silica at 20 °C for 24 h and finally inspected for gel formation.

**Six-well plate biominalization experiments.** Five mL of *Bacillus* cultures were transferred into sterile 6-well plates. Silica was added to cultures to a concentration of 100 mM. Samples were allowed to gel at 20 °C, 100 rpm for 1 h. The biominalization experiments were performed with three biological replicates (i.e., three independent cultures for each experiment).

**Silica block formation and mechanical testing.** Silica gel plugs were prepared in 10 mL syringes (ID = 15 mm) with the tips removed and then tips were covered instead with sterilized caps to maintain sterile conditions. Silica was added to 1 mL cultures to a concentration of 200 mM. Samples were incubated at 25 °C for 5 h, and the gel plugs were pushed out of the syringe. Mechanical properties of the gel plugs were measured using an extensional DMA Rheometer (TA Instruments RSA-1). Samples were sputter-coated with 8 nm of iridium and viewed with a Hitachi SU8230 field emission gun scanning electron microscope (University of Minnesota Characterization Facility).

**Protein analysis of silica gels.** Silica gel plugs (15 mm x 5 mm) from 1 mL cultures of *B. subtilis* ΔJlyC ΔJhFs pCT-EutMCorb-EutM-SpyC-HagΔ209C-SpyT588 or pCT-empty as a control were fabricated in the same manner as described above. After 24 h curing at 25 °C, the entire 1 mL solidified silica gels were dissolved in 200 μL 6x SDS-loading buffer at 100 °C for 1 h and 8 µL of spun down sample supernatant was loaded onto 15% SDS-PAGE gel.

**Flagella light microscopy.** One mL of *Bacillus* culture was centrifuged at 2,000 x g for 5 mins and gently resuspended in 1 mL of PBS, pH 7.5. The sample was pelleted as above, and the pellet was resuspended to an OD600 of 10. Five µL of sample was loaded onto a polylysine microscope slide, covered with a coverslip, and 10 µL Remel RYU flagella stain was applied to the edge of the coverslip.

**Spore staining.** The Schaeffer and Fulton Spore stain kit was used for spore staining with a modified protocol. Briefly, 500 µL of *Bacillus* culture was placed into a test tube. One drop (~150 µL) of malachite green was added to the tube and immersed into boiling water for 10 min. If samples were dehydrating before 10 min, deionized water was added dropwise. Following boiling, 50 µL of the sample was spread by pipette onto a plain, precleaned glass slide. The slide was allowed to air dry and then heat fixed with a flash dryer at ~100 °C. A drop of distilled water, counterstained with safranin for ~20 s and finally air dried. Slides were examined using a Nikon Eclipse 90i microscope with a 100 x 1.3 numerical aperture oil-immersion objective (UIC, University of Minnesota (UMN)). Images were captured using a Nikon D2-F2 color camera running on Nikon’s NIS-Elements software version 5.2.1.00.

**SEM and TEM imaging of silica precipitation.** For SEM imaging, EutM scaffolds (1 mg/mL purified proteins in water) were attached to silica wafers by incubating the protein suspensions on a silica wafer for 1 h. In samples with silica, the wafers with attached protein were incubated in 100 mM silica for 2 h. Samples were washed with water, fixed with Trump’s Fixative for 2 min, and washed with water again. Samples were washed with increasing ethanol concentrations of 25, 50, 75, and 100%, and then supercritically dried with a Tousimis Critical Point Dryer (Model 780 A). Samples were sputter-coated with 8 nm of iridium and viewed with a Hitachi SU8230 field emission gun scanning electron microscope (University of Minnesota Characterization Facility).

For TEM imaging, TEM grids were coated with EutM scaffolds by incubating grids in a protein solution (0.1 mg/mL of purified His-EutM or His-EutM-CoT in water) for 1 h at room temperature. Then, a small amount of 100 mM silica was added to the grid and incubated for 1 h. Grids were then washed with deionized water, fixed with Trump’s fixative for 2 mins, rewashed and stained with 2% uranyl acetate for 10 s. Grids were imaged as described below for other purified protein scaffolds.

**T209C flagella staining.** Glycerol cultures were inoculated into 4 mL of selective LB medium for plasmid maintenance and grown overnight at 30 °C and 220 rpm. One mL of culture was centrifuged at 2,000 x g for 10 mins. The supernatant was discarded and pelleted cells were gently resuspended in 1 mL of PBS, pH 7.5. The samples were pelleted as above and pellets resuspended with 50 µL PBS, pH 7.2 with 5 µg/mL Alexa Fluor™ 488 C3 maleimide (to stain cysteines in flagella with the HagΔ209C subunit) and incubated in the dark for five mins. 450 µL PBS was added to the samples prior to centrifugation as above. The pellet was resuspended in 50 µL PBS with 5 µg/mL Synaptolactin C2 (FM-64) and incubated for five mins in the dark. Samples were pelleted as above and then resuspended with 1 mL of PBS. After another centrifugation step, the pellets were resuspended to an OD600 of ~1. Three µL of sample were mixed with an equal volume of anti-fade fluorescent mounting medium Fluoroshield™ and applied to a polycarbonate microscope slide.

Slides were examined using a Nikon Eclipse 90i microscope with a 100 x 1.3 numerical aperture oil-immersion objective (UIC, UMN). Illumination was obtained using a Lumenar Sola Light Engine. Synaptolactin C2 was visualized using a diRed fluorescence cube (excitation 530–560 nm, emission 590–650 nm) with a 2-second exposure time. Alexa Fluor™ 488 C3 maleimide was visualized using a GFP fluorescence cube (excitation 450–490 nm, emission 500–530 nm) with a 1-second exposure time. Images were captured on a Hamamatsu Orca Flash 4.0 v2 CMOS monochrome camera in black and white using Nikon’s NIS-Elements software version 5.2.1.00.
In situ labeling of SpyTagged flagella with His-tdTomato-SpyCatcher. Glycerol cultures were inoculated into 4 mL of selective LB medium containing 0.15 mg of purified SpyCatcher protein and incubated overnight. The cultures were resuspended at 2000 x g for 10 mins and gently resuspended in one mL of PBS, pH 7.5. The samples were pelleted as above. The cultures were inoculated into 4 mL of selective LB medium containing 0.15 mg of SpyTag555 in which the red and green channels were multiplied by 1.25 before merging. Supplementary Fig. 12 image edits for part A include the HagT209C control in which the red channel was multiplied by 1.5 before merging with the green channel. In addition, for HagT209C-SpyTag555 the red channel was multiplied by 1.25 before merging. Supplementary Fig. 12 image edits for part B include the HagT209C control in which the red channel was multiplied by 1.5 before merging. In addition, for HagT209C-SpyTag555 the red channel was multiplied by 1.25 before merging.

Flagella RUV staining images were converted to grayscale and had the contrast and brightness increased by 10 using GNU Image Manipulation Program (GIMP) v. 2.8.18.

Statistical analysis. Statistical analysis of OD and pH data was performed using Origin v. 9.1. Statistical analysis of rheology data was performed using Microsoft Excel 2016. Mean and standard deviations from three biological replicates were calculated using Average and STDEV functions. Standard deviations from three biological replicates are shown as error bars in graphs and plots.

Statistical significance (p-values) of rheology data (Supplementary Table 2) was determined by performing T-test (Paired Two Sample for Means) and ANOVA (One-way, Single-factor) analysis with G’ (storage modulus) and G” (loss modulus) angular frequency averages. T-Test analysis was used to determine the statistical significance of rheology data between two groups of angular frequency data (G’ or G”) from silica gels fabricated with B. subtilis strains transformed with three different plasmids (pCT-empty, pCT-EutMCotB-EutMSPyc, pCT-EutMCotB-EutMSPyc-HagT209C::SpyTagTS88). ANOVA analysis was performed on three groups of angular frequency data (G’ or G”) from silica blocks fabricated by the same three B. subtilis strains. Data were considered statistically significant when p < 0.05.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All data generated or analyzed in this study are included in this article and accompanying Supplementary Information, Supplementary Data and Source Data files. Engineered Bacillus strains and plasmids created in this study can be made available subject to an agreement with the corresponding author Prof. Schmid-Dannert (schmid23@umn.edu) who will respond to requests within a week. Protein Data Bank data sets (PDB: 5WJX, PDB: 6GOW, PDB: 4MLI) were used to generate protein structure representations. Source data are provided with this paper.

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In situ labeling of secreted EutM-SpyCatcher scaffolds with His-SpyTag-eGFP. B. subtilis ΔlytC ΔflhA harboring pCT-EutMCotB-EutMSPyc-HagT209C::SpyTagTS88 was grown under standard conditions with (induced) and without (uninduced) cumate added for induction of protein expression. Cultures with cell densities of OD600 ~3.5 (induced) or ~6 (uninduced) were used for Western Blot analysis. Using a Sypro Ruby stain, Western Blot analysis was performed on 12% pre-cast SDS-PAGE gels stained with Sypro Ruby. Figure 3 shows representative sypro Ruby images.

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References

1. Chen, A. Y., Zhong, C. & Lu, T. K. Engineering living functional materials. ACS Synth. Biol. 4, 8–11 (2015).
2. Tang, T.-C. et al. Materials design by synthetic biology. Nat. Rev. Mat. 6, 332–350 (2020).
3. Srubar, W. V., 3rd. Engineered living materials: Taxonomies and emerging technologies. Trends Biotechnol. 39, 574–583 (2020).
4. Rivera-Tarazona, L. K., Campbell, Z. T. & Ware, T. H. Stimuli-responsive engineered living materials. Soft Matter 17, 785–809 (2021).
5. Liu, X. et al. 3D printing of living responsive materials and devices. Adv. Mater. 30, 1704821 (2018).
6. Gonzalez, L. M., Mukhitov, N. & Voigt, C. A. Resilient living materials built by printing bacterial spores. Nat. Chem. Biol. 16, 126–133 (2020).
7. Yu, K. et al. Photosynthesis-assisted remodeling of three-dimensional printed structures. Proc. Natl. Acad. Sci. USA 118, e2016524118 (2021).
8. Lovley, D. R. & Yao, J. Intrinsically conductive microbial nanowires for environmental electronics with novel functions. Trends Biotechnol. 39, 946–952 (2020).
9. Saldana, J. D., Abdali, Z., Modafferi, D., Janeshf, B. & Dorval Courchesne, N. M. Fabrication of fluorescent pH-responsive protein-textile composites. Sci. Rep. 10, 13052 (2020).
10. Levkovitch, S. A., Gazit, E. & Laor Bar-Yosef, D. Two decades of studying functional amyloids in microorganisms. Trends Microbiol. 29, 251–265 (2020).
11. Balistreri, A., Kahana, E., Janakiram, S. & Chapman, M. R. Tuning functional amyloid formation through disulfide engineering. Front Microbiol 11, 944 (2020).
12. Abdali, Z. et al. Curli-mediated self-assembly of a fibrous protein scaffold for hydroxyapatite mineralization. ACS Synth. Biol. 9, 3334–3343 (2020).
13. Mao, X. et al. Directing curli polymerization with DNA origami nucleators. Nat. Commun. 10, 1395 (2019).
14. Kalloniatis, E., Alexiou, G., Ozçelik, C. E. & Seker, U. O. S. Genetic logic gates enable patterning of amyloid nanofibers. Adv. Mater. 31, e1902888 (2019).
15. Duraj-Thatte, A. M. et al. Genetically programmable self-regenerating bacterial hydrogels. Adv. Mater. 31, e1901826 (2019).
16. Cohen, N., Zhou, H., Hay, A. G. & Radian, A. Curli production enhances clay- E. coli aggregation and sedimentation. Collid Surf. B Inter faces 182, 110361 (2019).
17. Wang, X. et al. Programming cells for dynamic assembly of inorganic nano-objects with spatiotemporal control. Adv. Mater. 30, e1705968 (2018).
18. Dorval Courchesne, N. M. et al. Biomimetic engineering of artificial curli protein films. Nanotechnology 29, 454002 (2018).
19. Amy, E. et al. Fabrication of amyloid curli fibers-alginate nanocomposite hydrogels with enhanced stiffness. ACS Biomater. Sci. Eng. 4, 2100–2105 (2018).
20. Tay, P. K. R., Nguyen, P. Q. & Joshi, N. S. A synthetic circuit for mercury bioremediation using self-assembling functional amyloids. ACS Synth. Biol. 6, 1841–1857 (2017).
21. Seker, U. O., Chen, A. Y., Citorik, R. J. & Lu, T. K. Synthetic biogenesis of bacterial amyloid nanomaterials with tunable inorganic-organic interfaces and electrical conductivity. ACS Synth. Biol. 6, 266–275 (2017).
22. Dorval Courchesne, N. M., Duraj-Thatte, A., Tay, P. K. R., Nguyen, P. Q. & Joshi, N. S. Scalable production of genetically engineered nanofibrous multimacromolecular materials via filtration. ACS Biomater. Sci. Eng. 3, 733–741 (2017).
23. Botyanszki, Z., Tay, P. K., Nguyen, P. Q., Nussbaumer, M. G. & Joshi, N. S. Engineered catalytic biofilms: Site-specific enzyme immobilization onto E. coli curli nanofibers. Biotechnol. Bioeng. 112, 2016–2024 (2015).
24. Nguyen, P. Q., Botyanszki, Z., Tay, P. K. & Joshi, N. S. Programmable biofilm- building materials from engineered curli nanofibres. Nat. Commun. 5, 4945 (2014).
25. Drogue, B., Thomas, P., Balvay, L., Prigent-Combaret, C. & Dorel, C. Engineered adhesion bacteria by creating a single synthetic curli operon. J. Vis. Exp. 16, e4176 (2012).
26. Caro-Astorga, J., Walker, K. T., Herrera, N., Lee, K. Y. & Ellis, T. Bacterial cellulose spheroids as building blocks for 3D and patterned living materials and for regeneration. Nat. Commun. 12, 5027 (2021) https://www.nature.com/articles/s41467-021-25350-8.
27. Gilbert, C. et al. Living materials with programmable functionalities grown from engineered microbial co-cultures. Nat. Mater. 20, 691–700 (2021).
28. Orozco-Hidalgo, M. T. et al. Engineering high-yield biopolymer secretion creates an extracellular protein matrix for living materials. mSystems 6, e00930–e00920 (2021).
29. Cano, R. J. & Borucki, M. K. Revival and identification of bacterial spores in 25–40 million-year-old Dominican amber. Science 268, 1060–1064 (1995).
30. Schallmey, M., Singh, A. & Ward, O. P. Developments in the use of Bacillus megaterium for cellulose biodegradation. Bioresource Technol. 128, 1–31 (2013).
31. Ulrich, N. et al. Experimental studies addressing the longevity of Bacillus subtilis spores - The first data from a 500-year experiment. PLoS One 13, e0208425 (2018).
32. Choudhary, S., Quin, M. B., Sanders, M. A., Johnson, E. T. & Schmidt-Dannert, C. Engineered protein nanocompartments for targeted enzyme immobilization. Appl Microbiol Biotechnol. 102, 8373–8388 (2018).
33. Schmidt-Dannert, S., Zhang, G., Johnstone, T., Quin, M. B. & Schmidt-Dannert, C. Building a toolbox of protein scaffolds for future immobilization of biocatalysts. Appl Microbiol Biotechnol. 102, 5059–5071 (2018).
34. Zhang, G. Q., Quin, M. B. & Schmidt-Dannert, C. Self-assembling protein scaffold system for easy in vitro immobilization of biocatalytic cascade enzymes. Acta Catal. 8, 5611–5620 (2018).
35. Zhang, G., Schmidt-Dannert, S., Quin, M. B. & Schmidt-Dannert, C. Protein- based scaffolds for enzyme immobilization. Methods Enzymol. 617, 323–362 (2019).
36. Zeng, R., Lv, C., Wang, C. & Zhao, G. Biomaterials based on protein self-assembly: Design and applications in biotechnology. Biotechnol. Adv. 52, 107835 (2021).
37. Belton, D. J., Deschaume, O. & Perry, C. C. An overview of the fundamentals of the chemistry of silica with relevance to biosilicification and technological advances. FEBS J. 279, 1710–1720 (2012).
38. Held, M. et al. Engineering formation of multiple recombinant Eut protein nanocompartments in E. coli. Sci. Rep. 6, 24359 (2016).
39. Quin, M. B., Perdue, S. A., Hsu, S. Y. & Schmidt-Dannert, C. Encapsulation of multiple cargo proteins within recombinant Eut nanocompartments. Appl Microbiol Biotechnol. 100, 9187–9200 (2020).
40. Zhang, G., Johnstone, T., Quin, M. B. & Schmidt-Dannert, C. Developing a protein scaffolding system for rapid enzyme immobilization and optimization of enzyme functions for biocatalysis. ACS Synth. Biol. 8, 1867–1876 (2019).
69. Alonso, J. C., Viret, J. F. & Tailor, R. H. Plasmid maintenance in Bacillus subtilis recombination-deficient mutants. Mol. Gen. Genet. 208, 349–352 (1987).

70. Liljeruhm, J. et al. Engineering a palette of eukaryotic chromoproteins for bacterial synthetic biology. J. Biol. Eng. 12, 8 (2018).

71. Vera-Gonzalez, N. & Shukla, A. Advances in biomaterials for the prevention and disruption of Candida biofilms. Front Microbiol 11, 538602 (2020).

72. Treuner, P. et al. The silica balance in the world ocean: a reestimate. Science 268, 375–379 (1995).

73. Anagnostopoulos, C. & Spizizen, J. Requirements for transformation in Bacillus subtilis. J. Bacteriol. 81, 741–746 (1961).

74. Hauser, P. M. & Karamata, D. A rapid and simple method for Bacillus subtilis transformation on solid media. Microbiol. (Reed.) 140(Pt 7), 1613–1617 (1994).

75. Bennallack, P. R., Burt, S. R., Heder, M. J., Robison, R. A. & Griffiths, J. S. Characterization of a novel plasmid-borne thioprotein gene cluster in Staphylococcus epidermidis strain 115. J. Bacteriol. 196, 4344–4350 (2014).

76. Jamal, M. et al. Ultra-high efficient colony PCR for high throughput screening of bacterial genes. Indian J. Microbiol 57, 365–369 (2017).

77. Shaner, N. C. et al. Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma sp. red fluorescent protein. Nat. Biotechnol. 22, 1567–1572 (2004).

78. Benson, J. J., Sakkos, J. K., Radian, A., Wackett, L. P. & Aksan, A. Enhanced biodegradation of atrazine by bacteria encapsulated in organically modified silica gels. J. Colloid Inter. Sci. 510, 57–68 (2018).

79. Cha, J. N. et al. Silicatein filaments and subunits from a marine sponge direct the polymerization of silica and silicones in vitro. Proc. Natl Acad. Sci. USA 96, 361–365 (1999).

80. Sato, T. A modified method for lead staining of thin sections. J. Electron Microsc (Tokyo) 17, 158–159 (1968).

81. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682 (2012).

82. Altegoer, F. et al. FliS/flagellin/FliW heterotrimer couples type III secretion agellin homeostasis. Sci. Rep. 8, 11552 (2018).

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Author contributions
S.-Y.K. created and tested expression constructs for scaffold secretion and silica biomineralization in B. subtilis, designed and performed silica biomineralization experiments and analyzed data, wrote manuscript and Supplementary information. A.P. engineered constructs for endospore formation and SpyTag display into B. subtilis strain, optimized and analyzed scaffold secretion, wrote manuscript and Supplementary information. S.B. developed and optimized methods for strain phenotype analysis by light/fluorescence microscopy, performed the microscopic analysis of engineered B. subtilis cultures to visualize flagella and endospore. S.B. performed Bacillus silica material analysis by electron microscopy, wrote manuscript and Supplementary information. J.J.B. characterized silica condensation by protein scaffolds using spectrophotometric assays and by SEM and TEM and wrote methods for this characterization work. J.J.B. proof-read and commented on manuscript. S.O.S. selected locations for the flagella SpyTag display and designed initial set of constructs for B. subtilis engineering. S.S. proof-read and commented on manuscript. M.B.Q. designed and purified scaffolds for silica biomineralization and contributed to design, planning, and supervision of the work, M.B.Q. proof-read and commented on manuscript. A.A. contributed to the design, planning, and supervision of the silica biomineralization experiments. A.A. provided feedback and comments on the manuscript. C.S.-D. conceived, devised, conceptualized, and directed the overall project, analyzed data and wrote the manuscript draft together with S.-Y.K., A.P., S.B.

Competing interests
The authors declare no competing interests.

Additional information
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