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

Sun-Young Kang^{1,3*}, Anaya Pokhrel^{1,3*}, Sara Bratsch^{1,3*}, Joey J. Benson², Seung-Oh Seo^{1,3},
Maureen B. Quin^{1,3}, Alptekin Aksan^{2,3}, Claudia Schmidt-Dannert^{1,3**}

¹ Department of Biochemistry, Molecular Biology & Biochemistry, University of Minnesota, Minneapolis, MN 55455, USA

² Department of Mechanical Engineering, University of Minnesota, Minneapolis, MN 55455, USA

³ BioTechnology Institute, University of Minnesota, St. Paul, MN 55108, USA

* These authors contributed equally to this work.

**** Corresponding author:**

Prof. Claudia Schmidt-Dannert

140 Gortner Laboratory

University of Minnesota

1479 Gortner Avenue

St. Paul, MN 55108

E-mail: schmi232@umn.edu

Phone: +1 612 625 5782

ABSTRACT

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* was engineered to display SpyTags on polar flagella for cell attachment and cross-linking of SpyCatcher modified secreted scaffolds. Through deletion of the autolysis LytC, endospore limited *B. subtilis* cells become a structural component of the material with spores for long-term storage of genetic programming. Known silica biomineralization peptides were 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 silica material and that new functions can be readily 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 as functional, self-healing materials for use as responsive coatings and plasters.

Keywords

Engineered Living Materials, biocomposite, self-assembly, protein scaffolds, silica, Bacillus.

Introduction

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 ¹⁻⁴. The fabrication of most ELMs, however, has so far largely relied on physical methods for incorporating a living component in an external material ⁴⁻⁷. 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 *E. coli* to produce an extracellular matrix from curli fibers ⁸⁻²⁵. Other types of extracellular matrices for ELM fabrication were created from secreted bacterial cellulose to embed microbial cells ^{26,27} or from elastin-like polypeptides to attach *Caulobacter* cells via their protein S-layers²⁸. 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 structures 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. To broaden the ELM landscape, we therefore sought to engineer a resilient ELM biocomposite that uses the spore-forming bacteria *B. subtilis* as its living component for the secretion of 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. It forms spores that remain viable for a long time ²⁹⁻³¹, allowing it to persist in our ELM under unfavorable conditions until revival. This was recently demonstrated by bioprinting of *Bacillus* spores in an agarose matrix ⁶. For the secretion of a self-assembling protein matrix, we chose a highly robust scaffolding system that we have previously characterized and engineered for the attachment of different cargo proteins ³²⁻³⁵. 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 ^{34,35}. We therefore rationalized that *B. subtilis* could be engineered to secrete EutM-SpyCatcher (EutM-SpyC) building blocks and attach itself via 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 biomineralization peptide (EutM-BM),

scaffold-silicification could then yield a durable biocomposite material for future development into functional coatings or plasters (illustrated in **Fig. 1**).

In this work, we first optimized secretion by *B. subtilis* of our EutM scaffold building blocks which are not normally secreted and in addition, rapidly self-assemble both *in vivo* and *in vitro* into large structures³²⁻³⁸. We then show that *B. subtilis* can be engineered to display a SpyTag on cluster, polar flagellar 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 created an endospore forming strain. Screening of known silica biomineralization peptides then allowed us to design scaffolds for silica polymerization to fabricate biocomposite materials with enhanced mechanical properties. Finally, we confirmed that our ELM can be regenerated from a piece of silica material and that new functions can be incorporated into the material simply 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 as functional, self-healing materials such as responsive coatings and plasters.

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. 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 (**Table S1**)³⁹. Bacterial growth, media pH, flagella presence, and sporulation were then analyzed for 4 days in 24 h intervals at 20 and 30°C (**Fig. S1, S2 and S3**). Although pH is not typically considered in culture conditions, silica polymerization is optimal between pH 6.0-8.0 and at pH levels above and below this range polymerization is inhibited⁴⁰. During growth, *B. subtilis* increasingly alkalifies the culture medium 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)⁴¹. 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 (**Fig. S1**).

Peritrichous flagella were observed through 72 h of growth without significant sporulation (**Fig. S2**). 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. 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. (**Fig. S1**). 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 (**Fig. S3a**). However, spores were already observed after 48 h of growth at 20°C in LB media, indicating cells were stressed under this condition (**Fig. S3b**). 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 signals⁴²⁻⁴⁴. In contrast, our scaffolding protein EutM is cytoplasmic that also rapidly self-assembles into scaffolds when overexpressed in *E. coli*³²⁻³⁸. 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)⁴⁵⁻⁴⁹ by fusing them to the N-terminus of our scaffold protein (**Fig. S4a**).

Each recombinant protein was expressed in *B. subtilis* WT in SMM, at 20°C for 48h 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*^{33,35}. 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 **Fig. S5**). 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 (**Fig. S4b**). Compared to cultures expressing SacB-EutM-SpyCatcher, no scaffolds sedimented in the other cultures secreting low levels of EutM protein (**Fig. S4b**). 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^{29,31}. 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*⁵⁰ by in-frame deletion of *lytC* to generate a *B. subtilis* Δ *lytC* strain (**Fig. 3a** and **Fig. S6**).

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 **Fig. S6**) 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 poles⁵¹. The protein FlhG (also called YlxH, MinD2, FleN, and MotR) is essential to this flagellation pattern^{52,53}. The deletion of *flhG* results in the aggregation of basal bodies at the polar ends of cells⁵¹. 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* Δ *lytC* Δ *flhG* was then transformed with expression vectors (pCT-empty, pCT-EutMSpyC) 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 (**Fig. S2a, S3a, S6d**). Unlike the wild-type strain, no free spores were released by the autolysin deficient $\Delta lytC$ strain in SMM (**Fig. S2b, S3b, S6c**). Endospore formation by *B. subtilis* $\Delta lytC \Delta flhG$ harboring pCT-empty and pCT-EutMSpyC 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 after induction, 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-EutMSpyC (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, **Fig. 2d** and **3d**). We previously observed that EutM proteins do not migrate according to size and/or as double bands^{33,36,38}. 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 bacteria⁵⁴. LC-MS analysis showed that indeed the signal peptide was not cleaved from either EutM protein band (**Fig. S7**). This suggests that EutM likely folds rapidly upon emerging from the Sec translocase complex^{42,43}, 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 structures³²⁻³⁸. 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 process⁵⁵.

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* $\Delta lytC \Delta flhG$ cultures and His-EutM-SpyCatcher without signal sequence from recombinant *E. coli* cultures. After metal affinity chromatography and dialysis into SMM, scaffolds were observed by transmission electron microscopy (TEM). In comparison to the negative control of SMM which shows many nonhomogenously sized and poorly stained structures and a few heavily stained structures (**Fig. S8a, e**), scaffolds isolated from *E. coli* formed rolled-up tubes (**Fig. S8b**) 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 (**Fig. S8c, d, f-h**). These structures have also been previously observed for EutM homologs^{33,38}. 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. 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 sulfhydryl-specific (maleimide) dye⁵⁶. The modified flagellin gene was then expressed from its native promoter on a plasmid in wild-type *B. subtilis* and the $\Delta lytC \Delta flhG$ double deletion strain. The assembled flagella filaments are therefore composed of both engineered and native flagellin monomers. The recombinant Hag^{T209C} protein was then modified to include a SpyTag domain at nine different positions chosen inside the variable D2/D3-region of the flagellin protein (**Fig. 4a, b**)⁵⁷. 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 *hag* gene of the recovered plasmids (**Fig. 4c**). Fluorescence microscopy (**Fig. 4d**) of the remaining mutants revealed flagella located at the mid cell for *B. subtilis* WT and poles for *B. subtilis* $\Delta lytC \Delta flhG$ which adopt different filament conformations (**Fig. 4e, S9a**). Wild-type flagella form a left-handed helix⁵⁸, which was retained for insertions at positions 612 and 645 in Hag^{T209C}. 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. 4e**). One strain expressing Hag^{T209C} with a SpyTag at position 492 had both normal and curly flagella (**Fig. S9a**).

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. 4d**) to recombinant *B. subtilis* WT and $\Delta lytC \Delta flhG$ cultures that were then grown overnight for flagella expression. No attachment of tdTomato-SpyCatcher to flagella of strains with Hag^{T209C} SpyTag insertions at positions 492, 555, and 612 was observed by fluorescence microscopy (**Fig. 4f, S9b**). Some red fluorescent labeling was observed for SpyTag display at position 645 (**Fig. S9b**), but the best attachment of tdTomato-SpyCatcher was achieved on flagella that display a SpyTag at position 588 (Hag^{T209C::SpyTag}⁵⁸⁸), despite the shorter than wild-type flagella formed by the recombinant strains (**Fig. 4f**).

We then confirmed that Hag^{T209C::SpyTag}⁵⁸⁸ modified flagella on *B. subtilis* $\Delta lytC \Delta flhG$ are capable of forming isopeptide bonds with EutM-SpyCatcher proteins secreted during cultivation. For this, two *B. subtilis* $\Delta lytC \Delta flhG$ strains - one expressing Hag^{T209C-SpyTag}⁵⁸⁸ 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. 4g**). Based on these results, we therefore chose Hag^{T209C::SpyTag}⁵⁸⁸ expression for cross-linking *B. subtilis* $\Delta lytC \Delta flhG$ 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 biocomposite material. We selected four known silica biomineralization peptides (R5, CotB1p, SB7, and Synthetic⁵⁹⁻⁶³ for C-terminal fusion to His-EutM (**Fig. S10a**). We then characterized silica precipitation and confirmed scaffold formation with the fusion proteins overexpressed and purified from *E. coli*. We intentionally left the His-Tag on 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 sponges⁶⁴.

Silica precipitation by the modified EutM scaffolds was then quantified using a spectroscopic molybdate blue assay⁶⁵ (**Fig. S10a**). 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 **Fig. S10b**, left column). 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 (**Fig. S10b**).

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 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 **Fig. S10b**, center and right columns). Silica nanoparticle formation was most prominent on His-EutM and His-EutM-SB7 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.

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* expressed His-EutM-CotB with the additional SacB signal sequence (**Fig. 5b**). EutM-CotB expression and secretion by *B. subtilis* Δ lytC Δ flhG transformed with pCT-EutMCotB (SMM, 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 uncleaved 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 fusion⁶⁶. 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 (**Fig. S11**). 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 (**Fig. S11**).

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 **Fig. 5b**. For SpyTag-display on flagella of *B. subtilis* Δ lytC Δ flhG, 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. subtilis* Δ lytC Δ flhG 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* Δ lytC Δ flhG cultures transformed with the EutM co-expression constructs with and without Hag^{T209C}::SpyTag⁵⁸⁸. We observed large structures labeled with cysteine reactive dye that interfere with cell labeling (**Fig. S12**). In contrast, cells that expressed only the modified Hag^{T209C} or Hag^{T209C}::SpyTag⁵⁸⁸ contained clearly labeled flagella under the same growth conditions (**Fig. S12**). 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* Δ lytC Δ flhG transformed with an empty control plasmid or one of the EutM-SpyCatcher and EutM-CotB co-expression plasmids with or without Hag^{T209C}::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 1h (**Fig. 6a**, see **Fig. S13** for biological replicates). While all cultures show some silica condensation into clusters at charged surfaces present in the cultures, cultures that expressed and secreted EutM scaffolds (induced cultures) formed aggregated silica materials. Larger silica material aggregates 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 microstructure²⁷. 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 significant increases 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-Hag^{T209C}::SpyT⁵⁸⁸) compared to the empty plasmid control (pCT-empty) (**Fig. 6c**, **Table S6**). 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* Δ lytC Δ flhG pCT-EutMCotB-EutMSpyC-Hag^{T209C}::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 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. Cells 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**). 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 (**Fig. 7a**) and indicates that a material is physically taking up the space that does not stain after silica biomineralization. The same observations were made when the cell density in silica blocks was increased to an OD₆₀₀ of 10 and 20 by gentle centrifugation and resuspension of cells prior to biomineralization (**Fig. S14 b and c**). We therefore theorize that silica deposition on EutM scaffolds surrounding the cells prevents staining. Although no scaffolds were visualized in thin sections, silica gels made in the same manner above show protein bands of the expected sizes for both EutM-SpyCatcher and biomineralizing scaffolds on SDS-PAGE analysis (**Fig. S14a**) supporting our theory that silica biomineralization is interfering 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. 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 than 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 number plasmids in *Bacillus* is a well-known issue^{67,68} 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 *Bacillus* 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 but with two different microorganisms has recently been successfully explored for the fabrication of a living material that incorporates cellulose²⁷. For demonstration purposes, we chose the formation of colored material by expressing a purple chromoprotein⁶⁹. An additional plasmid pCT-Purple-Hag^{T209C::SpyT⁵⁸⁸} was constructed for transformation into *B. subtilis* Δ lytC Δ flhG and co-cultivation with our final ELM strain. Co-cultures (mixed at a 1:1 ratio in the induction step) were grown as before (**Fig. 6**) and biomineralization and silica material formation compared to co-cultures with an empty plasmid control (**Fig. 8d**). Only co-cultures that also expressed the EutM scaffolds formed a purple a cross-linked silica material due to the attachment of the purple protein expressing *Bacillus* cells (which also display SpyTags on their flagella) to the scaffolds. Likewise, a purple-colored silica block could be fabricated under the same conditions used above with monocultures of our engineered

Bacillus strain (**Fig. 8e**). These results demonstrate an easy and modular approach for the fabrication of living materials with tailored functions and properties ranging e.g. from color and antimicrobial properties⁷⁰ to more complex functions such as sensing and responding.

CONCLUSIONS

We successfully engineered *B. subtilis* to produce and secrete proteins that self-assemble into scaffolds capable of biomineralizing silica, forming a material with tailorable properties and functions via its ability to crosslink cells and covalent attachment of cargo proteins. Our ELM system does not rely upon special processing after cultivation and instead just requires the addition of silica. Microscopy and rheological data show this ELM system function effectively to secrete biomineralizing and cross-linking scaffold that produce a material with stronger mechanical properties than the non-scaffold secreting controls. Although silica gel formation requires a concentration of 100 mM silica, which is currently much higher than used by natural systems which can biomineralize silica through concentration mechanisms from less than 100 μ M available silica in their environments⁷¹, the use of hydrolyzed TEOS as a source of silica is inexpensive. Additional research to 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 peptides⁵⁹⁻⁶³ would give access to a range of other ELM biocomposite materials. We have shown that our ELM can be regenerated from a piece of silica material 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 be regenerated over many cycles. Finally, this work offers engineering strategies for expanding the repertoire of current ELM chassis organisms towards other spore forming and/or ultra-resilient bacteria that secrete and embed themselves into a genetically programmable protein matrix for the fabrication of a range of functional materials.

METHODS

Methods and materials are provided in the **Supplementary Information**.

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FIGURES

Fig. 1: Formation of a living silica biocomposite material.

B. subtilis is designed to secrete a protein matrix from self-assembling EutM scaffold building blocks. Cells display a SpyTag on engineered clustered, polar flagella for cross-linking with SpyCatcher domains fused to scaffold building blocks. Scaffolds are designed to enhance silica polymerization and cells are modified to retain spores as endospores to create a durable biocomposite material that can be regenerated. (BM, biomineralization peptide; SP, secretion signal peptide).

Fig. 2: Establishment of self-assembling scaffold building block secretion.

a Self-assembling EutM scaffold building blocks containing a C-terminal SpyCatcher (SpyC) domain and a SacB secretion signal sequence followed by a His-tag are expressed and secreted by *B. subtilis* under the control of a cumate inducible promoter.

b Optimal secretion of EutM-SpyCatcher scaffold building blocks was obtained in SMM at 20°C. Protein expression was induced after reaching OD₆₀₀ of 0.4-0.7. Growth and pH of cultures was observed for 96 h and compared to an empty plasmid control culture. Error bars represent the standard deviations of three biological replicate cultures.

c, d Protein expression and secretion after 48 h of cultivation were analyzed by SDS-PAGE. Self-assembling scaffolds settle with cells, requiring their solubilization from cell pellets by a gentle wash with 4 M urea (see Methods for details). Samples analyzed include: (A) Culture supernatant (10-fold concentrated by TCA precipitation), (B) Urea supernatant after scaffold solubilization, and (C) resulting cell pellet after lysozyme treatment for analysis of remaining protein. Arrows indicate EutM-SpyCatcher bands. Expected sizes are 24.4 kDa and 21.1 kDa with and without SacB secretion signal peptide, respectively.

Fig. 3: Development of a *Bacillus* strain for durable material fabrication.

a Construction of a *B. subtilis* Δ lytC Δ flhG 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* WT and *B. subtilis* Δ lytC Δ flhG transformed with pCT-EutMSpyC were compared to confirm endospore and polar flagella phenotype of the engineered strain under expression conditions. Representative samples show spore release for the wild-type strain (observed in LB, 48 h, 20°C shown) and endospore formation by the engineered strain (SMM, 72 h, 20°C shown) (see also **Fig. S2, S3** and **S6**). 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 **Fig. S2** and **S3**). Clustered polar flagella are observed in the engineered strain that are prevalent through 48 h of growth (SMM, 48 h, 20°C shown) (see also **Fig. S6**). 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.

c *B. subtilis* Δ lytC Δ flhG strains transformed with the pCT-empty (control) or pCT-EutMSpyC plasmids and cultured for 96 h (SMM, 20°C) show growth characteristics comparable to the *B. subtilis* wild-type strain in **Fig. 2**. Error bars represent the standard deviations of three biological replicate cultures.

d Expression and secretion of EutM-SpyCatcher by the engineered *B. subtilis* Δ lytC Δ flhG 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.

Fig. 4: Engineering of flagella for SpyTag display.

a Flagella of *B. subtilis* were engineered for SpyTag display by inserting the SpyTag sequence at different locations in the hypervariable regions of the flagellin protein (encoded by *hag*). Shown are conserved domains, including the solvent exposed D2/D3 domains⁵⁷.

b Renderings of the flagellar filament and the flagellin structure⁷² show the locations of the SpyTag insertions (orange, red) and the T209C mutation for staining with a cysteine reactive dye. The functional SpyTag insertion site is highlighted in red. Also shown are the SpyTag structure (PDB: 4MLI) and sequence.

c SpyTag insertions into the *hag* gene had different impacts on flagella phenotypes and SpyTag-display by *Bacillus* strains expressing plasmid born *hag*^{T209C}::SpyTag^{xxx} under the control of its native promoter. Several *hag* insertion mutants could not be transformed and seem to be lethal when expressed in trans of the genomic *hag* copy. (*) Indicates this *hag* mutant could be transformed into *Bacillus* but recovered plasmids were mutated. No or some attachment means that modified flagella could not or only be weakly labelled with tdTomato SpyCatcher.

d Flagella assemble from genomic flagellin (*hag*) and plasmid born, engineered flagellin (Hag^{T209C}SpyTag^{xxx}) subunits. Engineered flagellins are stained with a cysteine reactive dye (Alexa Fluor^{TM} 488 C₅ maleimide) and functional SpyTag display was tested by growing cultures with purified tdTomato-SpyCatcher protein.

e Fluorescence imaging of *B. subtilis* Δ lytC Δ flhG transformed with pCT-empty, pRBBm34-Hag^{T209C} or pRBBm34-Hag^{T209C}::SpyT⁵⁸⁸ (LB, 12 h, 30°C) show that expression of *hag*^{T209C}::SpyTag⁵⁸⁸ results in clustered, polar flagella that are shorter than the flagella assembled upon expression of *hag*^{T209C}. Cells are stained and images false colored as indicated. See Fig. S9 for other SpyTag positions.

f Functional SpyTag display by Hag^{T209C}::SpyTag⁵⁸⁸ was confirmed by growing the three strains under the same conditions in the presence of purified tdTomato-SpyCatcher. Cells were stained and images false colored as indicated.

g *In situ* attachment of secreted EutM-SpyCatcher via isopeptide bond formation with SpyTags displayed on flagella of *B. subtilis* Δ lytC Δ flhG expressing *hag*^{T209C}::SpyTag⁵⁸⁸ was verified by 1:1 co-cultivation with strains transformed with pCT-EutMSpyC or pCT-empty (control). After 4 days (SMM, 20°C, 10 μ M cumate), flagella were extracted and analyzed by SDS-PAGE. A faint band with the expected size of 55 kDa for a EutM-SpyCatcher (21.1 kDa) and Hag^{T209C}::SpyTag⁵⁸⁸ (34.1 kDa) protein complex was detected in co-

cultures secreting EutM-SpyCatcher. Genome encoded flagellin (32.6 kDa, encoded by *hag*) appears to be the major protein isolated from both co-cultures.

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 with and without 100 mM silica for 2 h at room temperature. His-EutM formed tubular nanostructures that appeared to be rolled-up planar arrays fused together at multiple locations. His-EutM-CotB formed similar scaffolds but with a greater surface roughness (left column). SEM images show an increase in scaffold surface roughness, surface porosity, and the presence of silica nanoparticles fused to scaffolds which are characteristics of silicification (center and right columns). Silica nanoparticle formation was most prominent on His-EutM suggesting that silicification may have been nucleated in the solution phase by this protein. In contrast, a significant increase in surface roughness seen for His-EutM-CotB scaffolds suggests surface catalyzed biomineralization by these structures.

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 Hag^{T209C}::SpyTag⁵⁸⁸ in *B. subtilis* Δ lytC Δ flhG. Each gene is expressed by its own promoter (cumate inducible P_{CT5} promoter, native *hag* promoter). **c** Expression and secretion of EutM-CotB by *B. subtilis* Δ lytC Δ flhG 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. EutM-CotB is present in the solubilized fractions, but at significantly lower levels than EutM-SpyCatcher.

Fig. 6: Formation of living, silica biocomposites with increased rigidity

a Generation of silica biocomposites was tested by cultivating *B. subtilis* Δ lytC Δ flhG 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). Only induced cultures that co-expressed EutM-CotB and EutM-SpyCatcher formed aggregated silica material upon induction. The aggregation appears to be more pronounced in cultures that also co-expressed SpyTag labeled flagella for cross-linking of cells and EutM scaffolds. Experiments were performed with three biological culture replicates (see **Fig. S13**).

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). The engineered strain, EutMCotB-EutMSpyC-Hag^{T209C}::SpyT⁵⁸⁸ provided the greatest cross-linking effect, resulting in a greater force required to deform the material and increased rigidity compared to the empty plasmid control.

Fig. 7: Microscopy characterization of *Bacillus* silica biocomposites.

a The location of assembled scaffolds was tested by cultivating *B. subtilis* ΔlytC ΔflhG transformed with EutMCotB-EutMSpyC-Hag^{T209C}::SpyT⁵⁸⁸ plasmid (uninduced as control). Cultures were first grown (SMM, 20°C, 48 h) with (induced) and without (uninduced) cumate induction of protein expression. 500 μL of culture was mixed with 0.125 mg eGFP or SpyTag-eGFP and mixed at RT for 30 minutes 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. This zone corresponds to the SpyTag-eGFP labeled areas above and indicates there is interference with staining the scaffolds after silica is biomineralized.

Fig. 8: ELM biocomposite regeneration and proof-of-concept for material functionalization.

a Silica biocomposite material regeneration was tested by re-inoculating cultures with a piece of a silica plug fabricated from *B. subtilis* Δ lytC Δ flhG cultures co-expressing EutM-CotB and EutM-SpyCatcher and Hag^{T209C}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 x 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.

SUPPLEMENTARY INFORMATION

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

Sun-Young Kang^{1,3*}, Anaya Pokhrel^{1,3*}, Sara Bratsch^{1,3*}, Joey J. Benson², Seung-Oh Seo^{1,3},
Maureen B. Quin^{1,3}, Alptekin Aksan^{2,3}, Claudia Schmidt-Dannert^{1,3**}

¹ Department of Biochemistry, Molecular Biology & Biochemistry, University of Minnesota, Minneapolis, MN 55455, USA

² Department of Mechanical Engineering, University of Minnesota, Minneapolis, MN 55455, USA

³ BioTechnology Institute, University of Minnesota, St. Paul, MN 55108, USA

* These authors contributed equally to this work.

**** Corresponding author:**

Prof. Claudia Schmidt-Dannert

140 Gortner Laboratory

University of Minnesota

1479 Gortner Avenue

St. Paul, MN 55108

E-mail: schmi232@umn.edu

Phone: +1 612 625 5782

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METHODS

1. Materials

Chemicals

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

Microscopy supplies

Remel RYU flagella stain, polysine microscope slides, plain precleaned glass microscope slides, Alexa Fluor™ 488 C₅ maleimide, Syto16, and Trump's Fixative were purchased from ThermoFisher Scientific (Waltham, MA, USA). 22 mm 1.5 cover glasses were purchased from Azer Scientific (Morgantown, PA, USA). 200 mesh copper grids with 10 nm formvar and 1 nm carbon, aqueous 2% uranyl acetate, and EM grade: 10% glutaraldehyde, 0.2 M sodium cacodylate trihydrate pH 7.4, 16% paraformaldehyde, and 4% osmium tetroxide were purchased from VWR International, an Electron Microscopy Sciences reseller (Radnor, Pennsylvania, USA). SynaptoRed™ C2 was purchased from Avantor delivered by VWR a Biotium reseller (Radnor, PA, USA). FluoroShield was used in fluorescent microscopy experiments and was purchased from AbCam (Cambridge, MA, USA). Silicon wafers were used for SEM and obtained from Ted Pella (Reeding, CA, USA). Schaeffer and Fulton Spore stain kit (containing malachite green 50 g/L and safranin O 5 g/L) was obtained from MilliporeSigma (Burlington, MA, USA).

Protein purification

Spectra/Por Dialysis Tubing (MWCO 6-8 kDa) used for dialysis of proteins was purchased from Spectrum LifeSciences (Rancho Dominguez, CA, USA). HisTrap™ FF columns (GE Healthcare, IL, USA) were used for all protein purifications except for proteins used for TEM that were purified from *E. coli* and secreted proteins from *Bacillus* that were purified using TALON metal affinity resin from TaKaRa Bio USA, Inc. (Mountain View, CA, USA). 2 mL of TALON metal affinity resin was used in a Batch/Gravity flow protein purification protocol following manufacturer's directions. Pierce™ BCA Protein Assay Kit used for protein concentration measurements was purchased from ThermoFisher Scientific (Waltham, MA, USA). Ultrapure water was prepared by filtering deionized water through a Milli-Q water purification system (Millipore, Billerica, MA, USA) to reach a final electrical resistance higher than 18.2 MΩ cm⁻¹.

Molecular biology kits and reagents

All supplies were purchased from New England Biolabs (NEB) (Ipswich, MA, USA). Q5® High-Fidelity DNA polymerase was used for PCR amplifications. The Q5® Site-Directed Mutagenesis kit was used for site directed mutagenesis and the NEBuilder® HiFi DNA Assembly Master Mix was used for Gibson assembly of DNA fragments, and restriction enzymes (*Bgl*II, *Bam*HI, and *Pst*I) and T4 DNA Ligase were used for general cloning. Primers were ordered from Integrated DNA Technologies (Coralville, IA, USA).

SDS-PAGE

40% acrylamide and bis-acrylamide solution (37.5:1), TEMED and Precision plus all blue prestained protein standard (catalog# 161-0373) were purchased from Bio-Rad (Hercules, CA, USA). Other chemicals for SDS-PAGE analysis and Coomassie brilliant blue staining were purchased from Sigma-Aldrich.

2. Bacterial strains, media and general molecular biology methods

Strains

Escherichia coli TOP10 (Invitrogen, Carlsbad, CA, USA) was used for cloning and plasmid propagation. *E. coli* T7 Express (C2566) (NEB) and used for protein expression and purification. *Bacillus subtilis* 168 (ATCC 23857) was used as a host strain (referred to as WT strain). All plasmids and strains used in this study are listed in **Table S1**.

Media

LB (Luria broth; Tryptone 10 g/L, NaCl 5 g/L, Yeast extract 5 g/L) medium was used for growth of *E. coli* and *B. subtilis* strains.

B. subtilis strains were also grown in modified Spizizen's minimal medium (SMM) ^{7,8}, containing, per liter: K₂HPO₄ - 17.5 g, KH₂PO₄ - 7.5 g, Na₃-Citrate 2 H₂O - 1.25 g, MgSO₄ 7 H₂O - 0.25 g, N-source (L-glutamine - 10 g, (NH₄)₂ SO₄ - 0.5 g), trace elements (CaCl₂ 2 H₂O - 7.35 mg, MnCl₂ 4 H₂O - 1 mg, ZnCl₂ - 1.7 mg, CuCl₂ 6 H₂O - 0.43 mg, CoCl₂ 6 H₂O - 0.6 mg, Na₂MoO₄ 2 H₂O - 0.6 mg), Fe-Citrate (FeCl₃ 6 H₂O - 1.35 mg, Na₃-Citrate 3 H₂O - 10 mg), glucose - 5 g, tryptophan - 50 mg.

SMM was prepared by mixing stock solutions which included, 5X SMM base (K₂HPO₄ - 87.5 g/L, KH₂PO₄ - 37.5 g/L, Na₃-Citrate·2H₂O - 6.25 g/L, MgSO₄·7H₂O - 1.25 g/L), N-source (sterilized using a 0.2 µm filter) (glutamine - 40g/L, (NH₄)₂SO₄ - 2 g/L), 100X trace elements (CaCl₂·2H₂O - 0.735 g/L, MnCl₂·4H₂O - 0.1 g/L, ZnCl₂ - 0.17 g/L, CuCl₂·6H₂O - 0.043 g/L, CoCl₂·6H₂O - 0.06 g/L, Na₂MoO₄·2H₂O - 0.06 g/L), 100X Fe-citrate (sterilized using a 0.2 µm filter) (FeCl₃ 6 H₂O - 0.135

g/L, Na₃-Citrate.3H₂O - 1 g/L), 50% glucose (500 g/L) and tryptophan (sterilized using a 0.2 µm filter) (5 g/L).

Fresh solution of sterile N-source and glucose were prepared every time while stocks of other solutions were stored at 4°C. Working solution of SMM was prepared by mixing 200 mL of 5X SMM, 10 mL of 100X trace element, 10 mL of 100X Fe-Citrate, 10 mL of 50% glucose, 10 mL from 5 mg/mL stock of Tryptophan and 250 mL of N-source into 510 mL of sterile water.

SM1 and SM2 media as described previously by Bennallack *et. al.*, 2014⁹ were used for the preparation of *B. subtilis* competent cells.

For plasmid maintenance, LB was supplemented with 100 µg/mL ampicillin for *E. coli* strains while LB and SMM were supplemented with 10 µg/mL tetracycline or 20 µg/mL erythromycin for *Bacillus* strains.

Transformation, strain storage

Transformation of *E. coli* with plasmids followed standard molecular biology techniques. Transformation of *B. subtilis* strains was done using a method previously described by Bennallack *et. al.*, 2014⁹. Transformants were screened by colony PCR using a method modified from Jamal *et. al.*, 2017¹⁰. To prepare samples for colony PCR, a single colony of *B. subtilis* was mixed in 300 µL sterile water. Cells were spun down (21,130 x g) and 200 µL of the supernatant was removed. Cells were resuspended in the remaining 100 µL of sterile water followed by 20 mins of sonication in a cold-water bath sonicator (Branson 3510R-MTH, CT, USA). After sonication, cells were spun down as above and 2 µL of the supernatant was used as a template for a 10 µL PCR reaction. Plasmids from positive *B. subtilis* transformants were isolated and their sequences verified by Sanger sequencing. Recombinant strains with verified plasmid sequences were stored as glycerol stocks and used in this work.

3. Construction of plasmids and in-frame deletion in *B. subtilis*

All plasmids and strains used in this study are described in **Table S1**. 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 **Table S2** and **Table S3**. Primer pairs for *Bacillus* genomic manipulations and flagellin engineering are listed in **Table S5**.

Plasmids for protein expression in *E. coli* and *B. subtilis*

Plasmids were constructed using a combination of Gibson Assembly (HiFi® DNA assembly kit, NEB) for fragment assembly, site directed mutagenesis (Q5® mutagenesis kit, NEB) for shorter

insertions, deletions and insertions, or restriction enzyme cloning and ligation for larger fragment assembly following manufacturers' instructions and as described previously^{1,11}.

Briefly, for the C-terminal fusion of biomineralization peptides to His-EutM in pCT5-His-EutM^{1,11}, primers were designed to insert the corresponding into the plasmid using the Q5® mutagenesis kit according to the manufacturers' instructions.

To construct the pCT-His-dtTomato-SpyCatcher plasmid, His-EutM was combined with the fluorescent protein in a previously constructed pCT5-His-EutM-SpyC plasmid² by HiFi Assembly. His-tdTomato-SpyCatcher was amplified from tdTomato-pBAD¹².

To create the EutM *Bacillus* expression vectors, His-EutM-SpyCatcher was amplified from pCT5-His-EutM-SpyC and inserted into our in-house, cumate inducible *Bacillus* expression vector pCT5-bac2.0¹¹ by HiFi Assembly. For a control plasmid, pCT-empty was created by deletion of the *sfgfp* gene from pCT5-bac2.0 using the the Q5® mutagenesis kit according to the manufacturers' instructions. All signal peptides (**Table S4**) were fused to His-EutM-SpyCatcher by site directed mutagenesis (Q5® mutagenesis kit, NEB) with appropriate primers. The pCT-EutMCotB plasmid for *Bacillus* expression was made by replacing the SpyCatcher sequence in pCT-EutMSpyC with the CotB sequence by site directed mutagenesis. To create pCT-EutMCotB-EutMSpyC, the complete SacB-His-EutM-SpyCatcher expression cassette (including cumate inducible promoter) from pCT-EutMSpyC was amplified and subcloned into pCT-EutMCotB. The hag^{T209C}::SpyTag⁵⁸⁸ expression cassette (including its native promoter) was added to pCT-EutMCotB-EutMSpyC by HiFi assembly with the hag expression module amplified from the corresponding pBBRm34 plasmid (see below). Finally, to construct pCT-Purple-Hag^{T209C}::SpyT⁵⁸⁸, the chromoprotein was obtained from pSB1C3-tsPurple¹³ and assembled into pCT-EutMCotB-EutMSpyC-hag^{T209C}::SpyT⁵⁸⁸ by replacing the EutM expression modules.

Plasmids for SpyTag display on *B. subtilis* flagella

The flagellin gene (*hag* gene) was amplified from *B. subtilis* genomic DNA using primer pair (NP-hag_fwd and NP-hag_rev) and cloned into the pRBBm34 backbone using HiFi assembly. The flagellin T209C mutation was introduced by Q5®-mutagenesis with primer pairs (NP-hag-T209C_fwd and NP-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 **Table S5**.

Plasmids for in-frame deletions of *lytC* and *flhG* in *B. subtilis*

To generate the Δ *lytC* marker-less deletion construct, the pH BintE vector carrying a temperature sensitive origin of replication, erythromycin and ampicillin resistance was used as the backbone. The *lytC* upstream region was amplified from *B. subtilis* genomic DNA using primer pair

d*lytC*_upstream_fwd and d*lytC*_upstream_rev while the *lytC* downstream region was amplified using primer pair d*lytC*_downstream_fwd and d*lytC*_downstream_rev (**Table S5**) and the deletion cassette was cloned into pHBintE vector using HiFi assembly. The plasmid for Δ *flhG* marker-less deletion was created in a similar manner: The *flhG* upstream region was amplified using primer pair d*flhG*_upstream_fwd and d*flhG*_upstream_rev while the *flhG* downstream region was amplified using primer pair d*flhG*_downstream_fwd and d*flhG*_downstream_rev (**Table S5**). The *flhG* deletion cassette was cloned into pHBintE vector using HiFi assembly.

In-frame deletion of *lytC* and *flhG*

The *lytC* deletion plasmid (temperature sensitive pHBintE-d*lytC*) was transformed into *B. subtilis* using the method described by Bennallack *et. al.*, 2014⁹. After transformation, plates were incubated at a temperature permissive for plasmid replication (30°C) and for selection of erythromycin resistant transformants. Colonies were then transferred into 4 mL LB medium without antibiotic added followed by several rounds of dilution and regrowth at a temperature non-permissive for plasmid replication (37°C). For screening of the deletion mutant appropriate dilutions of the cells were plated onto LB agar. Colonies were streaked on LB plates with and without erythromycin to identify erythromycin sensitive colonies that have successfully evicted the plasmid. Screening the erythromycin sensitive colonies for desired double homologous recombinants was performed using colony PCR with primer pair d*lytC*_confirmation_fwd and d*lytC*_confirmation_rev as shown in **Table S5**. Transformation of *flhG* deletion construct was done into *B. subtilis* Δ *lytC* host using the pHBintE-d*flhG* plasmid. In frame deletion of *flhG* followed by screening was performed with same method as above for *lytC* but the primer pair used for screening of colonies for *flhG* deletion mutants was d*flhG*_confirmation_fwd and d*flhG*_confirmation_rev.

4. Cultivation of recombinant *B. subtilis* strains

Glycerol stocks of recombinant *Bacillus* strains (WT and Δ *lytC* Δ *flhG* deletion strain) transformed with pCT expression plasmids (pCT-empty, pCT-EutMSpyC, pCT-EutMCotB, pCT-EutMCotB-EutMSpyC, pCT-EutMCotB-EutMSpyC-Hag^{T209C::SpyT⁵⁸⁸}) 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 flasks were cultured at 37°C, 180 rpm until OD₆₀₀ reached 0.4-0.7 and protein expression was induced with 10 µM cumate. The induced cultures were then grown at 20°C or 30°C, 100 rpm for 48 to 96 h as described in this study. Final scaffold building block expression and secretion was

done in SMM with 10 μ M cumate for induction and subsequent growth at 20°C, 100 rpm for 48 h (referred to as “standard conditions”).

For co-cultivation experiments, glycerol stocks of three *B. subtilis* Δ lytC Δ flhG strains transformed with pCT-empty, pCT-EutMCotB-EutMSpyC-Hag^{T209C}::SpyT⁵⁸⁸ or pCT-Purple-Hag^{T209C}::SpyT⁵⁸⁸ were first spread onto selective LB plates, and single colonies were used to inoculate 4 mL of selective LB. After 19 h of growth at 30°C, cultures were diluted 3% into 50 mL of selective SMM in a 250 mL flask. Individual cultures were then grown at 37°C until OD₆₀₀ 0.4-0.7. 25 mL of each culture was then mixed in a new 250 mL flask as follows: Co-culture 1: *B. subtilis* Δ lytC Δ flhG pCT-empty + *B. subtilis* Δ lytC Δ flhG pCT-Purple-Hag^{T209C}::SpyT⁵⁸⁸, Co-culture 2: *B. subtilis* Δ lytC Δ flhG pCT-EutMCotB-EutMSpyC-Hag^{T209C}::SpyT⁵⁸⁸ + *B. subtilis* Δ lytC Δ flhG pCT-Purple-Hag^{T209C}::SpyT⁵⁸⁸. Protein expression was then induced with 10 μ M 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).

5. Analysis of scaffold building block expression and secretion by *Bacillus* strains

Extracellular secretion and intracellular accumulation of scaffold building blocks by recombinant *Bacillus* strains was analyzed by preparing four different fractions 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 cells with sonication followed by lysozyme treatment. The preparation of all four protein fractions is shown as a flow chart in **Fig. S5a**. 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 **Fig. S5b**.

The following workflow was used for sample preparation: 5 mL of culture broth was centrifuged at 3,220 x g, at 4°C for 10 mins to separate culture supernatant from pelleted cells and co-precipitated scaffolds.

For SDS-PAGE analysis of soluble scaffolds in the culture supernatant (fraction i, or A in Fig. 1), proteins from 1 mL of supernatant were precipitated by adding 200 μ L of 100% Trichloroacetic acid (TCA) and 1 h incubation on ice. Precipitated proteins were centrifuged at 21,130 x g for 10 mins 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 mins at 4°C. The acetone

was removed and the washing step was repeated twice. The dried precipitate was resuspended in 100 μ L of 1X SDS loading buffer (2% w/v SDS, 0.1 M DTT) (6X standard 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 urea buffer (4 M urea, 50 mM Tris-HCl, pH 7.5). The resuspended solution was centrifuged at 3,220 x g at 4°C for 10 mins and the supernatant was analyzed by SDS-PAGE.

For SDS-PAGE of the remaining scaffolds in the urea washed pellet ([fraction iii](#)), cells were lysed with 200 μ g/mL final concentration of lysozyme in 500 μ L 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 mins) was analyzed by SDS-PAGE.

For SDS-PAGE of total protein ([fraction iv](#)), pelleted cells and co-precipitated scaffolds from 5 mL of culture was resuspended into 500 μ L of 50 mM Tris-HCl pH 7.5 in an eppendorf tube. The suspension was sonicated in a coldwater bath sonicator (Branson 3510R-MTH, 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 μ L 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.

6. *De novo* sequencing of secreted EutM-SpyCatcher proteins

For *de novo* sequencing of secreted EutM-SpyCatcher, protein bands separated by SDS-PAGE were cut 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 viewed using the PEAKS Xpro Studio 10.6 software (Bioinformatics Solutions Inc., Waterloo, ON, Canada).

7. Analysis of EutM-SpyCatcher attachment to Hag^{T209C}::SpyTag⁵⁸⁸ displaying flagella

Covalent isopeptide bond formation between secreted EutM-SpyCatcher and Hag^{T209C}::SpyTag⁵⁸⁸ displayed on flagella of *B. subtilis* Δ lytC Δ flhG was analyzed by SDS-PAGE of sheared flagella isolated from co-cultures. Glycerol stocks of three *B. subtilis* Δ lytC Δ flhG strains (pCT-empty, pCT-EutMSpyC, pRBBm34-Hag^{T209C}::SpyT⁵⁸⁸) 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* Δ lytC Δ flhG pCT-empty + *B. subtilis* Δ lytC Δ flhG pRBBm34-Hag^{T209C}::SpyT⁵⁸⁸, Co-culture 2: *B. subtilis* Δ lytC Δ flhG pCT-EutMSpyC + *B. subtilis* Δ lytC Δ flhG pRBBm34-Hag^{T209C}::SpyT⁵⁸⁸. Protein expression was induced with 10 μ M cumate and co-cultures grown at 20°C, 100 rpm for 96 h.

Cell pellets were collected from 50 mL cultures by centrifugation (3,220 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. Turbo^{TM} DNase (Invitrogen, Waltham, MA, USA) was then added at a 0.5 U/mL final concentration and incubated on ice for 10 mins. Cells were then removed by centrifugation at 3,220 x g for 10 mins 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 μ L 50 mM Tris-HCl (pH 7.5) for SDS-PAGE analysis.

8. 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 previously described^{1,2,14,15}. His-EutM and His-EutM-silica binding peptide fusion proteins were purified using the EutM lysis buffer, while EutM-SpyCatcher was purified using the EutM-SpyCatcher buffer described in². His-tdTomato-SpyCatcher protein was expressed in *E. coli* and purified using the EutM-SpyCatcher protocol².

For silica binding assays and SEM analysis of purified His-EutM 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 Batch/Gravity flow protein 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^{1,2,14,15}. EutM-SpyCatcher from *E. coli* was isolated from 125 mL cultures using the EutM-SpyCatcher protocol² but with a TALON gravity column instead of a HisTrap^{TM} FF column (GE Healthcare, IL, USA). For the purification of His-EutM-SpyCatcher proteins

secreted by *B. subtilis* $\Delta lytC \Delta flhG$, recombinant strains harboring pCT-EutMSpyC were grown and induced under standard conditions described above. Cells and scaffolds from twelve 50 mL cultures (total volume: 600 mL) were pelleted at 3,220 x g, at 4°C 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.

For GFP labeling of scaffolds in *B. subtilis* $\Delta lytC \Delta flhG$ transformed with EutMCotB-EutMSpyC-Hag^{T209C}::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 ².

9. Silica biomineralization and material characterization

Preparation of hydrolyzed TEOS

Silicic 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 ¹⁶.

Silica binding assays on EutM scaffolds

Silica precipitation was measured using the blue silicomolybdate method ^{17,18}. Silica was added to purified protein scaffolds, at a concentration of 1 mg/mL in deionized water, to a concentration of 100 mM and the suspension was mixed for 2 h at room temperature (RT). The precipitate was then collected by centrifugation (3 mins at 18,800 x g), and the resulting pellet was washed 3 times with deionized water. Precipitated silica in the scaffold pellet was depolymerized by adding 1 M NaOH for 10 mins. The samples were then diluted to 1.6 mL and 0.15 mL of solution A (containing 20 g/L ammonium molybdate tetrahydrate and 60 mL/L concentrated hydrochloric acid in deionized water) was added to the sample. After 10 mins, 0.75 mL of solution B (containing 20 g/L oxalic acid, 6.67 g/L 4-methylaminophenol sulphate, 4 g/L anhydrous sodium sulfite, and 100 mL/L concentrated sulphuric acid in deionized water) was added. The blue color was allowed to develop for 2 h at RT, and the absorbance was measured at 810 nm. All samples were run in triplicate.

6-well plate biomineralization experiments

5 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 biomineralization 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 tops removed and then tops 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-G2) with 15 mm compression disks. A frequency sweep was performed with a gap of 4.5 mm and an oscillation strain of 1%.

Larger silica gel blocks from “purple” co-cultures of *B. subtilis* Δ lytC Δ flhG pCT-EutMCotB-EutMSpyC-Hag^{T209C::SpyT⁵⁸⁸} + *B. subtilis* Δ lytC Δ flhG pCT-Purple-Hag^{T209C::SpyT⁵⁸⁸} were prepared in the same manner, except that 3 mL of culture was used and the material was cured for 24 h.

ELM regeneration

Small piece (~ 5 mm³) from silica plugs fabricated from *B. subtilis* Δ lytC Δ flhG pCT-EutMCotB-EutMSpyC-Hag^{T209C::SpyT⁵⁸⁸} cultures (biomineralized with 200 mM silica and cured for 24 h at 25°C) was used to inoculate fresh cultures for ELM fabrication under standard conditions (see above). Scaffold building block expression, secretion and biomineralization in 6-well plates by the regrown cultures was confirmed as described above.

Protein analysis of silica gels

Silica gel plugs (15 mm x 5 mm) from 1 mL cultures of *B. subtilis* Δ lytC Δ flhG pCT-EutMCotB-EutMSpyC-Hag^{T209C::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 μ L 6x SDS loading buffer at 100°C for 1 h and 8 μ L of spun down sample supernatant was loaded onto 15% SDS-PAGE gel.

10. Microscopy

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 μ L of sample was loaded onto a polysine microscope slide, covered with a coverslip, and 10 μ L Remel RYU flagella stain was applied to the edge of the coverslip.

Slides were examined using a Nikon Eclipse 90i microscope with a 100X 1.3 numerical aperture oil-immersion objective (University Imaging Center (UIC), University of Minnesota (UMN)). Images were captured using a Nikon D2-Fi2 color camera running on Nikon Elements software.

Spore staining

The Schaeffer and Fulton Spore stain kit was used for spore staining with a modified protocol. Briefly, 500 μ L of *Bacillus* culture was placed into a glass test tube. One drop ($\approx$ 150 μ L) 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 μ L of sample was spread by pipette onto a plain, precleaned glass slide. The slide was allowed to air dry and then heat fixed with flame. The slide was decolorized with distilled water, counterstained with safranin for $\approx$ 20 seconds and again decolorized.

Slides were examined using a Nikon Eclipse 90i microscope with a 100X 1.3 numerical aperture oil-immersion objective (UIC UMN). Images were captured using a Nikon D2-Fi2 color camera running on Nikon Elements software.

SEM imaging

EutM scaffolds (1 mg/mL purified proteins in water) were attached to silicon wafers by incubating the protein suspensions on a silicon 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 two min, and washed with water again. Samples were washed with increasing ethanol concentrations of 25%, 50%, 75%, and 100%, and then supercritically dried using a Tousimis Critical-Point Dryer (Model 780A). 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).

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 $\times g$ 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 μ L PBS, pH 7.2 with 5 μ g/mL Alexa Fluor^{TM} 488 C₅ maleimide (to stain cysteines in flagella with the Hag^{T209C} subunit) and incubated in the dark for five mins. 450 μ L PBS was added to the samples prior to centrifugation as above. The pellet was resuspended in 50 μ L PBS with 5 μ g/mL SynaptoRed^{TM} C2 (FM4-64) 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 10. Three μ L of sample were mixed with an equal volume of anti-fade fluorescent mounting medium FluoroShield and applied to a polysine microscope slide.

Slides were examined using a Nikon Eclipse 90i microscope with a 100X 1.3 numerical aperture oil-immersion objective (UIC UMN). Illumination was obtained using a Lumencor Sola Light Engine. SynaptoRed™ C2 was visualized using a dsRed fluorescence cube (excitation 530-560 nm, emission 590-650 nm) with a 3 second exposure time. Alexa Fluor™ 488 C₅ maleimide was visualized using a GFP fluorescence cube (excitation 450-490 nm, emission 500-550 nm) with a 5.6 second exposure time. Images were captured using a Hamamatsu Orca Flash 4.0 v2 CMOS monochrome camera in black and white using Nikon Elements software.

***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 His-tdTomato-SpyCatcher protein and grown overnight at 30°C and 220 rpm. One mL of culture was centrifuged at 2,000 x *g* for 10 mins and gently resuspended in one mL of PBS, pH 7.5. The samples were pelleted as above. The pellets were resuspended with 50 µL PBS pH 7.2 with 5 µg/mL Alexa Fluor™ 488 C₅ maleimide and incubated in the dark for five mins. 450 µL PBS was added to the samples prior to centrifugation as above. Cellular nucleic acid was stained by resuspending the pellets in 50 µL Tris-HCl, pH 8.0 with 10 µM Syto16 and incubated in the dark for 10 mins. 450 µL Tris-HCl, pH 8.0 was added and samples were centrifuged as above. Unused dye was removed by resuspension with 1 mL PBS and samples were centrifuged again. After resuspension to an OD₆₀₀ of 10, 3 µL of sample was mixed with an equal volume of FluoroShield and loaded onto a polysine microscope slide.

Slides were examined using a Nikon Eclipse 90i microscope with a 100X 1.3 numerical aperture oil-immersion objective (UIC UMN). Illumination was obtained using a Lumencor Sola Light Engine. tdTomato was visualized using a dsRed fluorescence cube (excitation 530-560 nm, emission 590-650 nm) with a 3 second exposure time. Alexa Fluor™ 488 C₅ maleimide and Syto™16 were visualized using a GFP fluorescence cube (excitation 450-490 nm, emission 500-550 nm) with a 0.3-5.6 second exposure time. Images were captured using a Hamamatsu Orca Flash 4.0 v2 CMOS monochrome camera in black and white using Nikon Elements software.

TEM of purified scaffolds from *E. coli* and *Bacillus*

Purified His-EutM-SpyCatcher protein scaffolds were diluted to 1 mg/mL. Ten µL was placed onto 200 mesh copper grids with 10 nm formvar and 1 nm carbon for 3 mins. The fluid was removed and 10 µL of Trump's fixative (4% formalin, 1 % glutaraldehyde, 0.1 M sodium cacodylate) was applied for 5 mins to fix the proteins. The fluid was removed and the grids were rinsed with 10 µL of deionized water three times. Negative staining was completed using 1% aqueous uranyl acetate which was applied and removed immediately to prevent over-staining. Grids were

examined using a JEOL-JEM-1400Plus transmission electron microscope with a LaB6 tungsten filament at 60 kV (UIC UMN). Images were acquired using an Advanced Microscopy Techniques XR16 camera using AMT capture Engine software version 7.0.0.187.

In situ labeling of secreted EutM-SpyCatcher scaffolds with His-SpyTag-eGFP

B. subtilis Δ lytC Δ flhG harboring pCT-EutMCotB-EutMSpyC-Hag^{T209C}::SpyT⁵⁸⁸ was grown under standard conditions with (induced) and without (uninduced) cumate added for induction of protein expression. 500 μ L of culture was mixed with 0.25 mg His-eGFP or His-SpyTag-eGFP (an estimated 2:1 ratio of SpyCatcher : eGFP established in ⁷) and mixed at RT for 30 mins. Ten μ L was applied to a glass slide and covered with a slip. Slides were examined using a Nikon Eclipse 90i microscope with a 40X 0.75 numerical aperture objective (UIC UMN). Illumination was obtained using a Lumencor Sola Light Engine. His-eGFP and His-SpyTag-eGFP were visualized using a GFP fluorescence cube (excitation 450-490 nm, emission 500-550 nm) with a 0.032-0.2 second exposure time. DIC images were captured with a 0.037-0.039 second exposure. Images were captured using a Hamamatsu Orca Flash 4.0 v2 CMOS monochrome camera in black and white using Nikon Elements software.

Silica material thin sections and TEM

B. subtilis Δ lytC Δ flhG harboring pCT-EutMCotB-EutMSpyC-Hag^{T209C}::SpyT⁵⁸⁸ 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 either directly silica biomineralized or were concentrated prior to silica gel formation. For concentration, cultures were centrifuged at 3,220 x g for 10 mins and the supernatant was removed. Pellets were resuspended to OD₆₀₀ 10 or 20 using the media removed from the pellet. Silica gel plugs were prepared as described above for mechanical testing using 200 mM silica. Gels were pushed out of the syringes and 2 mm³ gel portions were cut from the center of both induced and uninduced samples. The gel pieces were fixed at 4°C and 10 rpm overnight in 100 mM sodium cacodylate, 2% glutaraldehyde, and 2% paraformaldehyde. The gel pieces were then rinsed with 100 mM sodium cacodylate three times for 30 mins each at 4°C and 10 rpm. An overnight post-fixation using 4% osmium tetroxide in 100 mM sodium cacodylate was completed at 4°C and 10 rpm. The samples were extensively rinsed with water and subjected to an ethanol dehydration series (25%, 50%, 75%, 95%, 100%). Gel samples were trimmed into approximately 1 mm³ pieces, infiltrated with EMbed 812 resin (1:2 resin:ethanol, 1:1, 2:1, 100% resin without hardener, 100% resin with benzyl dimethyl amine hardener repeated once (2x total); all times 8 h-overnight), and polymerized in a 60°C oven for 48 h. Sections were cut using a diamond knife on a Leica Ultracut UCT microtome at a thickness of 70-100 microns and collected on 200-mesh formvar/carbon-

coated copper grids. Sections were stained with 3% aqueous uranyl acetate for 20 mins, rinsed in ultrapure water (5 sec, 5x), stained with Sato's triple-lead stain¹⁹ for 3 mins, and rinsed in ultrapure water (5 sec, 5x). Samples were examined using a JEOL-JEM-1400Plus transmission electron microscope with a LaB6 tungsten filament at 60 kV (UIC UMN). Images were acquired using an Advanced Microscopy Techniques XR16 camera using AMT capture Engine software version 7.0.0.187.

Image Analysis

Images were cropped, scale bars added, and channels were colorized and merged using Fiji v. 1.53²⁰. **Fig. S9** image edits for part A include *B. subtilis* $\Delta lytC \Delta flhG$ strain SpyTag 555 in which the red and green channels were multiplied by 1.25 before merging. **Fig. S12** image edits for part A include the Hag^{T209C} control in which the red channel was multiplied by 1.5 before merging with the green channel. In addition, for Hag^{T209C::SpyTag⁵⁸⁸} the red channel was multiplied by 1.25 before merging. **Fig. S12** image edits in part B include the Hag^{T209C} control in which the red channel was multiplied by 1.5 before merging. In addition, for Hag^{T209C::SpyTag⁵⁸⁸} the red channel was multiplied by 1.25 before merging.

Flagella RYU 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 (<https://www.gimp.org/>).

SUPPORTING FIGURES

Fig. S1: Establishing growth conditions suitable for silica biocomposite formation with *B. subtilis* pCT-empty and pCT-EutMSpyC.

Growth of *B. subtilis* WT strain transformed with the cumate inducible, pCT-empty expression plasmid was compared in two media (LB and SMM, 100 rpm) at (a) 20°C and (b) 30°C. Similarly, growth of *B. subtilis* WT strain transformed with pCT-EutMSpyC was compared in LB and SMM at (c) 20°C and (d) 30°C. OD₆₀₀ and pH of cultures were followed for 96 h. Cultures were induced with 10 µM cumate once they reached an OD₆₀₀ of 0.4-0.7. *B. subtilis* WT strain transformed with pCT-EutMSpyC (c) show changes in growth rates at 20°C suggesting diauxic growth and/or an increased metabolic burden caused by recombinant protein expression. Error bars represent standard deviations calculated for values from three biological replicate cultures.

Fig. S2: Analysis of spore formation and flagella phenotypes of *B. subtilis* pCT-empty under different growth conditions.

Spore formation and flagellation of *B. subtilis* WT pCT-empty was analyzed under different growth conditions. At 24 h time intervals, samples were removed from SMM and LB cultures grown at 20°C or 30°C (shown in **Figure S1**) for staining and imaging by light microscopy. Cells were stained with RYU to observe flagella (a) or malachite green and safranin red to visualize spores (b). Flagella were observed through 72 h of growth, with the most flagella present at 48 h of growth or less under all conditions. Only one, unreleased spore was observed at 48 h of growth in SMM at 30°C, corresponding to reaching stationary phase.

Fig. S3: Analysis of spore formation and flagella phenotypes of *B. subtilis* pCT-EutMSpyC.

Spore formation and flagellation of *B. subtilis* WT transformed with pCT-EutMSpyC was analyzed under different growth conditions. At 24 h time intervals, samples were removed from SMM and LB cultures grown at 20°C or 30°C (shown in **Fig. S1**) for staining and imaging by light microscopy. Cells were stained with RYU to observe flagella (**a**) or malachite green and safranin red to visualize spores (**b**). Flagella were observed through 72 h of growth, with the most flagella present at 48 h of growth or less under all conditions. Sporulation was observed after 48 h in LB medium at 20°C, correlating with a corresponding decrease in OD₆₀₀ due to cell lysis (see **Fig. S1**) while the few spores visible at 48 h in SMM at 30°C did not impact OD.

Fig. S4: Testing of additional secretion signal sequence for scaffold building block secretion by *B. subtilis*.

a Scaffold building block secretion levels by *B. subtilis* WT was compared with five commonly used secretion signal peptides placed upstream of the His-EutM-SpyCatcher region.

b Cultures expressing the EutM-SpyCatcher proteins were grown for 48 h in SMM at 20°C under induction condition to analyze protein secretion by SDS-PAGE. Culture supernatants were collected and concentrated 25-fold by TCA precipitation prior to analysis (left panel). Cell pellets were then treated with 4 M urea (see **Fig. 2**) to solubilize co-precipitated EutM-SpyCatcher scaffolds for analysis (right panel). *B. subtilis* transformed with a pCT-empty vector was included as negative control (N). Only the SacB signal sequence facilitated secretion of EutM-SpyCatcher in the supernatant and as co-precipitant in the cell pellet.

Fig. S5: Experimental workflow used for the analysis of EutM scaffold building block secretion and expression by engineered *B. subtilis*.

Different fractions of proteins were prepared by *B. subtilis* WT pCT-EutMSpyC after 48 h of induction in SMM at 20°C.

a Workflow for the preparation of the following fractions containing EutM-SpyCatcher protein: (i) culture supernatant, (ii) urea supernatant, (iii) urea solubilized pellet and (iv) total protein. Fractions i-iii correspond to A, B and C in **Fig. 2, 3 & 8**. Fraction iv represents total protein (intracellular and precipitated scaffolds) in sedimented pellet after complete lysis of cells.

b Comparison of proteins solubilized by urea treatment from pelleted cells and scaffolds (fraction ii) and total protein released from broken cells and sedimented scaffolds (fraction iv). Urea treatment does solubilize EutM scaffolds but does not break cells.

Fig. S6: Construction and phenotypal characterization of the double deletion strain *B. subtilis* Δ *lytC* Δ *flhG* as ELM fabrication chassis.

Deletions of genes encoding the autolysin LytC (**a**) and flagellar basal body protein FlhG (**b**) were made in *B. subtilis* 168 (WT strain) by consecutive rounds of homologous recombination with a temperature sensitive plasmid. In frame deletions were confirmed by PCR, yielding amplification products with the expected sizes. Endospore formation (**c**) and polar flagella clusters (**d**) were then confirmed for the double deletion strain transformed with different expression plasmids used in this work. *B. subtilis* Δ *lytC* Δ *flhG* strains were cultured in SMM at 20°C under induction condition. Spores were visualized with malachite green and safranin red at days after induction as indicated (when endospores formed). Red was replaced with magenta using FIJI software (**c**). Flagella of the same cultures RYU stained and imaged after 48 h of induction. Images were converted to grayscale using GIMP software (**d**).

Fig. S7: Analysis of EutM-SpyCatcher secreted by *B. subtilis* ΔlytC ΔflhG.

SDS-PAGE analysis and LC-MS *de novo* sequencing of EutM-SpyCatcher proteins produced by *B. subtilis* ΔlytC ΔflhG pCT-EutMSpyC after 48 h of induction in SMM at 20°C. Two bands (21.1 and 24.4 kDa) are visible in the urea supernatant (see **Fig. 3d**). Boxed bands were cut and subjected to *de novo* sequencing by LC-MS. Coverages of sequenced peptides were visualized by PEAKS Studio Viewer shown to the right. Under these expression conditions, both protein bands have an uncleaved SacB signal peptide. A segment after the signal peptide cleavage site (AFA) was not sequenced in addition to other sequence regions. To confirm that the LC-MS sequencing results were not due to plasmid mutations that occurred during cultivation in the signal peptidase cleavage site regions, plasmids isolated from 10 colonies obtained from the *B. subtilis* ΔlytC ΔflhG pCT-EutMSpyC were Sanger sequenced to cover the signal peptide and the EutM-SpyCatcher region. No mutations were found. Peptide fragments sequenced corresponding to the C-terminus of both protein bands indicate that the minor, smaller 21.1 kDa band corresponds to an isoform that migrates faster in the SDS-PAGE gel.

Fig. S8: Comparison of scaffold formation by EutM-SpyCatcher purified from recombinant *E. coli* and *B. subtilis* ΔlytC ΔflhG cultures.

His-tagged EutM-SpyCatcher from *E. coli* and urea solubilized scaffolds of *B. subtilis* ΔlytC ΔflhG pCT-EutMSpyC cultures were purified by metal affinity chromatography as described in the methods. The eluted proteins were dialyzed into SMM (**a**, **e** media blank used for *E. coli* and *B. subtilis* protein dialysis, respectively) to remove imidazole and urea for analysis of scaffold formation in this medium. The SMM dialyzed EutM-SpyCatcher proteins (*E. coli* 1.8 mg/mL, *B. subtilis* 2.3 mg/mL) were diluted to 1 mg/mL with sterile SMM and structures were visualized by TEM. **a-c** are magnified 10,000 x, **d** is 20,000 x, **e**, **f**, **h** are 6,000 x. **g** is enlarged 5x from **f**.

b Rolled up tubes were visible in *E. coli* protein samples and have been observed previously for EutM scaffolds^{1,2}. **c**, **d**, **f-h** Rolled up tubes, sheets, coral- and small spherical structures (that stain as donut-like structures) were visible in *Bacillus* samples and have also been previously observed for EutM scaffolds^{1,2,4-6}. The enlarged spherical structures from **f** are also present in **c**, **d**, and **h**.

Fig. S9: Characterization of phenotypes and functions of SpyTag-modified flagella.

B. subtilis WT and *B. subtilis* Δ lytC Δ flhG strains were transformed with pRBBm34-Hag^{T209C}::SpyT^{###} (dye reactive Cys, SpyTag insertions at indicated nucleotide locations) plasmids and flagella phenotypes and functions compared to strains transformed with pRBBm34-Hag^{T209C} and pCT-empty plasmid as positive and negative controls, respectively.

a Fluorescence microscopy to evaluate flagella morphologies due to SpyTag insertion. Individual colonies from plates were inoculated in 4 mL LB medium (supplemented with tetracycline) and grown overnight at 30°C and 220 rpm. The *hag* gene is expressed from its native promoter. Flagella were stained with Alexa Fluor^{TM} 488 C₅ maleimide and cell membranes with SynaptoRed^{TM} C2. Samples were examined using a Nikon Eclipse 90i microscope at 100X. dsRed (excitation 530-560 nm, emission 590-650 nm) and GFP (excitation 450-490 nm, emission 500-550 nm) channels were captured in black and white. Channels were false colored magenta for RFP and yellow for GFP and merged using FIJI.

b Fluorescence microscopy to evaluate SpyTag function when inserted at different locations of the Hag protein. Cultures were grown as above but with the addition of 0.15 mg purified tdTomato-SpyCatcher protein to label functionally displayed SpyTags on flagella. Flagella were stained with Alexa Fluor^{TM} 488 C₅ maleimide and cells visualized with the nucleic acid stain Syto^{TM} 16. Samples were imaged and channels false colored as above.

Fig. S10: Characterization of silica binding and precipitation by EutM biomineralization peptide fusion proteins.

a Four silica binding peptides were fused to His-EutM (top panel) and silica binding to the purified proteins (1 mg/mL protein incubated with 100 mM silica for 2 h) compared to His-EutM. Silica precipitation was quantified spectrophotometrically with the molybdate blue assay. Error bars represent standard deviations calculated for values from three sample replicates.

b The same protein scaffolds (1 mg/mL) incubated without (- silica; left column) and with (+ silica (100 mM); middle, and right) for 2 h were visualized by SEM. Deposition of silica increases surface roughness and porosity, and also forms silica nanoparticle aggregates on the surface. His-EutM-CotB shows the strongest silica precipitation. His-EutM also precipitates silica due to its His-tag and positive charge ³⁻⁶.

Fig. S11: Comparison and optimization of silica gel formation by *B. subtilis* WT cultures expressing EutM-CotB scaffolds or no scaffolds.

B. subtilis WT pCT-empty (control) or pCT-EutMCotB cultures were grown in SMM at 20°C for 48 h after induction of protein expression. Different silica concentrations (50-500 mM) were then added to 2.5 mL culture samples and silica gel formation followed for 24 h at 20°C. The addition of 100 mM silica created a soft solid silica gel after 24 h with EutM-CotB expressing culture samples while control culture samples did not yield a solidified gel. Lower silica concentration did not form a gel and higher concentrations rapidly formed a hard solid, regardless of culture sample (top panel). Bottom panel shows the solidification of these samples with increasing silica concentrations added (left to right). Cultures expressing EutM-CotB solidified with 100 mM silica (labeled with a star) while the control culture sample remained liquid with 100 mM silica.

Fig. S12: Visualization of cell phenotypes of *B. subtilis* ΔlytC ΔflhG cultures transformed with different plasmids.

B. subtilis ΔlytC ΔflhG was transformed with plasmids for the expression of modified flagella (pRBBm34-Hag^{T209C} and pRBBm34-Hag^{T209C}::SpyT⁵⁸⁸), EutM scaffold building blocks (pCT-EutMCotB-EutMSpyC and pCT-empty(control)), and SpyTagged flagella and EutM scaffold building blocks (pCT-EutMCotB-EutMSpyC-Hag^{T209C}::SpyT⁵⁸⁸). Strains harboring the pCT plasmids were grown for up to 48 h in SMM at 20°C under induction condition to express EutM scaffolds. Strains harboring the pRBBm34 plasmids were grown under the same conditions, but without induction. After 24 (a) and 48 h (b), samples were stained with the cysteine reactive dye Alexa Fluor^{TM} 488 C₅ maleimide and the cell membrane dye SynaptoRed^{TM} C2. Samples from EutM scaffold building block expressing cultures contained secreted scaffolds that appeared to be stained with the cysteine reactive Alexa Fluor dye, presumably due to labeling of one or both Cys residues present in the EutM protein.

Fig. S13: Silica biomineralization by engineered *B. subtilis* $\Delta lytC \Delta flhG$ cultures.

B. subtilis $\Delta lytC \Delta flhG$ strains harboring three different pCT plasmids (second row) were grown in SMM at 20°C for 48 h under uninduced (-) and induced (+) conditions for protein expression. 5 mL cultures were then transferred to 6-well plates for silica biomineralization with 100 mM silica at 20°C, 100 rpm for 1 h. Experiments were performed with three biological culture replicates that are shown. Only cultures that co-expressed EutM-CotB and EutM-SpyCatcher formed aggregated silica materials upon induction. The aggregation appears to be more pronounced in cultures that also co-expressed SpyTag labeled flagella for cross-linking of cells and EutM scaffolds.

Fig. S14: Additional characterization of *Bacillus* silica biocomposites.

a Silica gel blocks from 1 mL cultures (see **Fig. 6b**) were dissolved in SDS-PAGE loading buffer to confirm the presence of EutM scaffold proteins in the silica blocks by SDS-PAGE. Silica blocks made from empty vector control (-) cultures show no scaffold proteins while silica blocks from the EutMCotB-EutMSpyC-Hag^{T209C}::SpyT⁵⁸⁸ induced (+) cultures have bands with the expected sizes for EutM-SpyCatcher and EutM-CotB.

b, c Silica blocks generated from concentrated cultures with cell densities adjusted to OD₆₀₀ of 10 or 20 were viewed by TEM for comparison to silica gel blocks directly formed from cultures (**Fig. 7**). *B. subtilis* Δ lytC Δ flhG pCT-EutMCotB-EutMSpyC-Hag^{T209C}::SpyT⁵⁸⁸ was first grown under inducing (induced) or non-inducing (uninduced) conditions for protein expression (SMM, 20°C, 48 h). Cultures were centrifuged to collect secreted scaffolds and cells and resuspended in collected SMM supernatants to OD 10 (**b**) or 20 (**c**). Silica gel blocks were then formed by adding 200 mM silica 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 as was observed with unconcentrated cultures (**Fig. 7**).

SUPPORTING TABLES

Table S1: Plasmids and strains used in this study.

Plasmid	Description	Source
<i>E. coli</i> expression plasmids		
pCT5-His-EutM	<i>eutM</i> (NP_461400) containing a N-terminal 6xHis tag fused via a GS-linker to generate N-His-GS-EutM, P _{ct5} promoter, Amp ^R (originally named: pCT5BB-EutM)	1,2
pCT5-His-EutMSpyC	derived from pCT5-His-EutM: EutM shell protein with N-terminal His tag and C-terminal SpyCatcher	1,2
pCT5-His-EutMCotB	derived from pCT5-His-EutM: EutM shell protein with N-terminal His tag and C-terminal CotB silica binding tag	This study
pCT5-His-EutMR5	derived from pCT5-His-EutM: EutM shell protein with N-terminal His tag and C-terminal R5 silica binding tag	This study
pCT5-His-EutMSB7	derived from pCT5-His-EutM: EutM shell protein with N-terminal His tag and C-terminal SB7 silica binding tag	This study
pCT5-His-EutMSynthetic	derived from pCT5-His-EutM: EutM shell protein with N-terminal His tag and C-terminal Synthetic silica binding tag	This study
tdTomato-pBAD	His-tagged tdTomato cloned into pBAD expression vector	12
		Addgene #54856
pSB1C3-tsPurple	Constitutive expression of purple chromoprotein	13
		Addgene #117848
pET28a-eGFP	N-terminal 6xHis tag followed by a thrombin cleavage site fused to GFP, T7 promoter, Km ^R	2
pET28a-SpyTag-eGFP	N-terminal 6xHis tag followed by a thrombin cleavage site and GS linker fused to SpyTag-eGFP, T7 promoter, Km ^R	2
<i>B. subtilis</i> expression plasmids		
pCT5-bac2.0	Designed for cumate inducible gene expression in Bacilli, P _{xyIR} -CymR and P _{CT5} -cuO-sfGFP, Amp ^R , Tet ^R	11
		Addgene #119872
pCT-empty	sfGFP deleted from pCT5-bac2.0	This study
pCT-EutMSpyC	Derived from pCT5-bac2.0 by replacing sfGFP with levansucrase signal peptide (SacB), N-terminal His tag, EutM and C-terminal SpyCatcher	This study

Plasmid	Description	Source
pCT-XynAEutMSpyC	Derived from pCT5-bac2.0 by replacing sfGFP with xylanase A signal peptide (XynA), N-terminal His tag, EutM and C-terminal SpyCatcher	This study
pCT-YngKEutMSpyC	Derived from pCT5-bac2.0 by replacing sfGFP with fusion of glycosyl hydrolase signal peptide (YngK), N-terminal His tag, EutM and C-terminal SpyCatcher	This study
pCT-CelAEutMSpyC	Derived from pCT5-bac2.0 by replacing sfGFP with Cellulase A signal peptide (CelA), N-terminal His tag, EutM and C-terminal SpyCatcher	This study
pCT-LipAEutMSpyC	Derived from pCT5-bac2.0 by replacing sfGFP with lipase A signal peptide (LipA), N-terminal His tag, EutM and C-terminal SpyCatcher domain	This study
pCT-EutMCotB	Derived from pCT5-bac2.0 by replacing sfGFP with levansucrase signal peptide (SacB), N-terminal His tag, EutM and C-terminal CotB tag	This study
pCT-EutMCotB-EutMSpyC	Hybrid plasmid of SacB-His-EutM-CotB and SacB-His-EutM-SpyC modules, each gene expressed from a pCTbac2.0 cumate inducible promoter	This study
pCT-EutMCotB-EutMSpyC-Hag ^{T209C} ::SpyT ⁵⁸⁸	Hybrid plasmid of SacB-His-EutM-CotB, SacB-His-EutM-SpyC and Hag ^{T209C} ::SpyT ⁵⁸⁸ modules, EutM genes are expressed from a pCTbac2.0 cumate inducible promoter, the Hag gene is expressed from its native promoter	This study
pCT-His-tdTomatoSpyC	Fluorescent tdTomato with SpyCatcher expressed from cumated inducible promoter	This study
pCT-Purple-Hag ^{T209C} ::SpyT ⁵⁸⁸	Purple chromoprotein expression cassette combined with Hag expression cassette, chromoprotein expression from pCTbac2.0 cumate inducible promoter, Hag gene expression from native promoter	This study
pRBBm34	<i>Bacillus</i> expression vector, GFP expression under control of native xylose inducible promoter of <i>B. megaterium</i> , Amp ^R , Tet ^R	²¹ Addgene #48114
pRBBm34-Hag ^{T209C}	<i>hag</i> gene from <i>B. subtilis</i> WT (with T209C mutation in Hag protein) under control of native hag promoter (NP)	This study
pRBBm34-Hag ^{T209C} ::SpyT ³⁹⁹	hagT209C with SpyTag insertion after 399 nucleotides (counting from start codon) under control of native hag promoter (NP)	This study
pRBBm34-Hag ^{T209C} ::SpyT ⁴²⁶	hagT209C with SpyTag insertion after 426 nucleotides under control of native hag promoter (NP)	This study

Plasmid	Description	Source
pRBBm34-Hag ^{T209C} ::SpyT ⁴⁵⁹	hagT209C with SpyTag insertion after 459 nucleotides under control of native hag promoter (NP)	This study
pRBBm34-Hag ^{T209C} ::SpyT ⁴⁹²	hagT209C with SpyTag insertion after 492 nucleotides under control of native hag promoter (NP)	This study
pRBBm34-Hag ^{T209C} ::SpyT ⁵³⁷	hagT209C with SpyTag insertion after 537 nucleotides under control of native hag promoter (NP)	This study
pRBBm34-Hag ^{T209C} ::SpyT ⁵⁵⁵	hagT209C with SpyTag insertion after 555 nucleotides under control of native hag promoter (NP)	This study
pRBBm34-Hag ^{T209C} ::SpyT ⁵⁸⁸	hagT209C with SpyTag insertion after 588 nucleotides under native promoter	This study
pRBBm34-Hag ^{T209C} ::SpyT ⁶¹²	hagT209C with SpyTag insertion after 612 nucleotides under control of native hag promoter (NP)	This study
pRBBm34-Hag ^{T209C} ::SpyT ⁶⁴⁵	hagT209C with SpyTag insertion after 645 nucleotides under control of native hag promoter (NP)	This study
pHBintE	Cloning, expression and integration vector for <i>Bacillus</i> spp. with xylose-inducible promoter PxylA; Amp ^R , ori ^{ts}	²² Addgene #48136
pHBintE- <i>ΔlytC</i>	<i>lytC</i> upstream and downstream sequences cloned into pHBintE vector for in frame deletion of <i>lytC</i>	This study
pHBintE- <i>ΔflhG</i>	<i>flhG</i> upstream and downstream sequences cloned into pHBintE vector for in frame deletion of <i>flhG</i>	This study

Strain	Description	Source
<i>Escherichia coli</i> Top10	General cloning host	Invitrogen
<i>Escherichia coli</i> C2566	Protein expression	New England Biolabs
<i>Bacillus subtilis</i> WT	<i>Bacillus subtilis</i> wild-type strain 168	ATCC 23857
<i>Bacillus subtilis</i> <i>ΔlytC</i>	<i>B. subtilis</i> WT engineered to retain the endospore	This study
<i>Bacillus subtilis</i> <i>ΔlytC ΔflhG</i>	<i>B. subtilis</i> WT engineered to have polar flagella and to retain the endospore	This study

Table S2: Amino acid sequences of proteins and peptides in this study.

Name	Amino acid sequences
SacB	MNIKKFAKQATVLTFTTALLAGGATQAFAAST
EutM	EALGMIETRGLVALIEASDAMVKAARVKLVGVKQIGGGLCTAMVRGDVAACKAATDAGAAAAQRIGELVSV HVIPRPHGDLEEVFPISFKGDSNIVDGGSGSGG
SpyCatcher	DSATHIKFSKRDEDEGKELAGATMELRDSSGKTISTWISDGQVKDFYLYPGKYTFVETAAPDGYEVATAITFT VNEQGQVTVNG
SpyTag	AHIVMVDAKPTK
CotB	SGRARAQRQSSRGR
R5	SSKSGSYSGSKGSKRRIL
SB7	RQSSRGR
Synthetic	KFFEAAAKFFE
His-EutM	MHHHHHHGSGSGSGSGSGSEALGMIETRGLVALIEASDAMVKAARVKLVGVKQIGGGLCTAMVRGDVAA CKAATDAGAAAAQRIGELVSVHVIPRPHGDLEEVFPISFKGDSNI
His-EutM-CotB	MHHHHHHGSGSGSGSGSGSEALGMIETRGLVALIEASDAMVKAARVKLVGVKQIGGGLCTAMVRGDVAA CKAATDAGAAAAQRIGELVSVHVIPRPHGDLEEVFPISFKGDSNIVDGGSGSGSGRARAQRQSSRGR
His-EutM-R5	MHHHHHHGSGSGSGSGSGSEALGMIETRGLVALIEASDAMVKAARVKLVGVKQIGGGLCTAMVRGDVAA CKAATDAGAAAAQRIGELVSVHVIPRPHGDLEEVFPISFKGDSNIVDGGSGSGSSKSGSYSGSKGSKR RIL
His-EutM-SB7	MHHHHHHGSGSGSGSGSGSEALGMIETRGLVALIEASDAMVKAARVKLVGVKQIGGGLCTAMVRGDVAA CKAATDAGAAAAQRIGELVSVHVIPRPHGDLEEVFPISFKGDSNIVDGGSGSGGRQSSRGR
His-EutM-Synthetic	MHHHHHHGSGSGSGSGSGSEALGMIETRGLVALIEASDAMVKAARVKLVGVKQIGGGLCTAMVRGDVAA CKAATDAGAAAAQRIGELVSVHVIPRPHGDLEEVFPISFKGDSNIVDGGSGSGGKFFEAAAKFFE
SacB-His-EutM-SpyCatcher	MNIKKFAKQATVLTFTTALLAGGATQAFAASTHHHHHHHGSGSGSGSGSGSEALGMIETRGLVALIEASDAM VKAARVKLVGVKQIGGGLCTAMVRGDVAACKAATDAGAAAAQRIGELVSVHVIPRPHGDLEEVFPISFKGD SNIVDGGSGSGGDSATHIKFSKRDEDEGKELAGATMELRDSSGKTISTWISDGQVKDFYLYPGKYTFVETA PDGYEVATAITFTVNEQGQVTVNG
SacB-His-EutM-CotB	MNIKKFAKQATVLTFTTALLAGGATQAFAASTHHHHHHGSGSGSGSGSGSEALGMIETRGLVALIEASDAM VKAARVKLVGVKQIGGGLCTAMVRGDVAACKAATDAGAAAAQRIGELVSVHVIPRPHGDLEEVFPISFKGD SNIVDGGSGSGSGRARAQRQSSRGR
Hag ^{T209C}	MRINHIAALNTLNRLSSNNSASQKNMEKLSSGLRINRAGDDAAGLAISEKMRGQIRGLEMASKNSQDGIS LIQTAEGALTETHAILQRVRELVVQAGNTGTQDKATDLQSIQDEISALTDEIDGISRTEFNGKKLLDGTYKV DTATPANQKNLVFQIGANATQQISVNIEDMGADALGIKEADGSIAALHSVNDLDVTKFADNAADCADIGFDA QLKVVDIAINQVSSQRAKLGAVQNRLEHTINNLSASGENLTAAESRIRDVDMAKEMSEFTKNNILSQASQA MLAQANQQPQNVLQLLR

Name	Amino acid sequences
Hag ^{T209C::SpyT³⁹⁹}	MRINHNI AALNTLNRLSSNNSASQKNMEKLSSGLRINRAGDDAAGLAISEKMRGQIRGLEMASKNSQDGIS LIQTAEGALTETHAILQRVRELVVQAGNTGTQDKATDLQSIQDEISALTDEIDGISNRTEFN AHIVMVDAYKP TK GKKLLDGTYKVDATPANQKNLVFIQIGANATQQISVNIEDMGADALGIKEADGSIAALHSVNDLDVTKFA DNAAD C ADIGFDAQLKVVDEAINQVSSQRAKLGAVQNRLEHTINNLSASGENLTAAESRIRDVDMAKEMSE FTKNNILSQASQAMLAQANQQPQNVLQLLR
Hag ^{T209C::SpyT⁴²⁶}	MRINHNI AALNTLNRLSSNNSASQKNMEKLSSGLRINRAGDDAAGLAISEKMRGQIRGLEMASKNSQDGIS LIQTAEGALTETHAILQRVRELVVQAGNTGTQDKATDLQSIQDEISALTDEIDGISNRTEFN GK LLDGT Y AHI VMVDAYKPTK KVDATPANQKNLVFIQIGANATQQISVNIEDMGADALGIKEADGSIAALHSVNDLDVTKFAD NAAD C ADIGFDAQLKVVDEAINQVSSQRAKLGAVQNRLEHTINNLSASGENLTAAESRIRDVDMAKEMSEF TKNNILSQASQAMLAQANQQPQNVLQLLR
Hag ^{T209C::SpyT⁴⁵⁹}	MRINHNI AALNTLNRLSSNNSASQKNMEKLSSGLRINRAGDDAAGLAISEKMRGQIRGLEMASKNSQDGIS LIQTAEGALTETHAILQRVRELVVQAGNTGTQDKATDLQSIQDEISALTDEIDGISNRTEFN GK LLDGT Y KV DTATPANQ KAHIVMVDAYKPTK NLVFIQIGANATQQISVNIEDMGADALGIKEADGSIAALHSVNDLDVTKFA DNAAD C ADIGFDAQLKVVDEAINQVSSQRAKLGAVQNRLEHTINNLSASGENLTAAESRIRDVDMAKEMSE FTKNNILSQASQAMLAQANQQPQNVLQLLR
Hag ^{T209C::SpyT⁴⁹²}	MRINHNI AALNTLNRLSSNNSASQKNMEKLSSGLRINRAGDDAAGLAISEKMRGQIRGLEMASKNSQDGIS LIQTAEGALTETHAILQRVRELVVQAGNTGTQDKATDLQSIQDEISALTDEIDGISNRTEFN GK LLDGT Y KV DTATPANQKNLVFIQIGANAT AHIVMVDAYKPTK QQISVNIEDMGADALGIKEADGSIAALHSVNDLDVTKFA DNAAD C ADIGFDAQLKVVDEAINQVSSQRAKLGAVQNRLEHTINNLSASGENLTAAESRIRDVDMAKEMSE FTKNNILSQASQAMLAQANQQPQNVLQLLR
Hag ^{T209C::SpyT⁵³⁷}	MRINHNI AALNTLNRLSSNNSASQKNMEKLSSGLRINRAGDDAAGLAISEKMRGQIRGLEMASKNSQDGIS LIQTAEGALTETHAILQRVRELVVQAGNTGTQDKATDLQSIQDEISALTDEIDGISNRTEFN GK LLDGT Y KV DTATPANQKNLVFIQIGANATQQISVNIEDMGADAL AHIVMVDAYKPTK GIKEADGSIAALHSVNDLDVTKFA DNAAD C ADIGFDAQLKVVDEAINQVSSQRAKLGAVQNRLEHTINNLSASGENLTAAESRIRDVDMAKEMSE FTKNNILSQASQAMLAQANQQPQNVLQLLR
Hag ^{T209C::SpyT⁵⁵⁵}	MRINHNI AALNTLNRLSSNNSASQKNMEKLSSGLRINRAGDDAAGLAISEKMRGQIRGLEMASKNSQDGIS LIQTAEGALTETHAILQRVRELVVQAGNTGTQDKATDLQSIQDEISALTDEIDGISNRTEFN GK LLDGT Y KV DTATPANQKNLVFIQIGANATQQISVNIEDMGADALGIKEAD AHIVMVDAYKPTK GSIAALHSVNDLDVTKFA DNAAD C ADIGFDAQLKVVDEAINQVSSQRAKLGAVQNRLEHTINNLSASGENLTAAESRIRDVDMAKEMSE FTKNNILSQASQAMLAQANQQPQNVLQLLR
Hag ^{T209C::SpyT⁵⁸⁸}	MRINHNI AALNTLNRLSSNNSASQKNMEKLSSGLRINRAGDDAAGLAISEKMRGQIRGLEMASKNSQDGIS LIQTAEGALTETHAILQRVRELVVQAGNTGTQDKATDLQSIQDEISALTDEIDGISNRTEFN GK LLDGT Y KV DTATPANQKNLVFIQIGANATQQISVNIEDMGADALGIKEADGSIAALHSVND AHIVMVDAYKPTK LDVTKFA DNAAD C ADIGFDAQLKVVDEAINQVSSQRAKLGAVQNRLEHTINNLSASGENLTAAESRIRDVDMAKEMSE FTKNNILSQASQAMLAQANQQPQNVLQLLR
Hag ^{T209C::SpyT⁶¹²}	MRINHNI AALNTLNRLSSNNSASQKNMEKLSSGLRINRAGDDAAGLAISEKMRGQIRGLEMASKNSQDGIS LIQTAEGALTETHAILQRVRELVVQAGNTGTQDKATDLQSIQDEISALTDEIDGISNRTEFN GK LLDGT Y KV DTATPANQKNLVFIQIGANATQQISVNIEDMGADALGIKEADGSIAALHSVNDLDVTKFAD AHIVMVDAYKPT K NAAD C ADIGFDAQLKVVDEAINQVSSQRAKLGAVQNRLEHTINNLSASGENLTAAESRIRDVDMAKEMSE FTKNNILSQASQAMLAQANQQPQNVLQLLR

Name	Amino acid sequences
Hag ^{T209C} ::SpyT ⁵⁴⁵	MRINHNIAALNTLNRLSSNNSASQKNMEKLSSGLRINRAGDDAAGLAISEKMRGQIRGLEMASKNSQDGIS LIQTAEGALTETHAILQVRVRELVVQAGNTGTQDKATDLQSIQDEISALTDEIDGISNRTEFNGKKLLDGTYKV DTATPANQKNLVFQIGANATQQISVNIEDMGADALGIKEADGSIAALHSVNDLDVTKFADNAADCADIGFDA HIVMVDAKYPTKAQLKVVDEAINQVSSQRAKLGAVQNRLEHTINNLSASGENLTAAESRIRDVDMAKEMSE FTKNNILSQASQAMLAQANQQPQNVLQLLR
His-tdTomato- SpyCatcher	MHHHHHHGSGSGSGSGSGSVDVSKGEEVIKEFMRFKVRMEGSMNGHEFEIEGEGEGRPYEGTQTAKLK VTGGGLPFAWDILSPQFMYGSKAYVKHPADIPDYKKLSFPEGFKWERVMNFEDGGLVTVTQDSSLQDGT LIYKVKMRGTNFPDGPVMQKKTMGWEASTERLYPRDGLVKGEIHQALKLKDGGHYLVEFKTIYMAKKPV QLPGYYYVDTKLDITSHNEDYTIVEQYERSEGRHHLFLGHGTGSTGSGSSGTASSEDNNMAVIKEFMRFK VRMEGSMNGHEFEIEGEGEGRPYEGTQTAKLKVTGGGLPFAWDILSPQFMYGSKAYVKHPADIPDYKKL SFPEGFKWERVMNFEDGGLVTVTQDSSLQDGTLIYKVKMRGTNFPDGPVMQKKTMGWEASTERLYPR DGLVKGEIHQALKLKDGGHYLVEFKTIYMAKKPVQLPGYYYVDTKLDITSHNEDYTIVEQYERSEGRHHLFL YGMDELYKGSGSGSGDSATHIKFSKRDEDEGKELAGATMELRDSSGKTISTWISDGQVKDFLYPGKYTFV ETAAPDGYEVATAITFTVNEQQQVTVNG
His-Purple-native promoter- Hag ^{T209C} ::SpyT ⁵⁸⁸	MHHHHHHSSGLVPRGSMASLVKDMCVKMTMEGTVNGYHFKCVGEGEGKPFEGTQNMIRIRVTEGGPLP FAFDILAPCCMYGSKTFIKHVSGIPDYFKESFPEGFTWERTQIFEDGGVLTAHQDTSLEGNCILYKVKVLGT NFPANGPVMQKKTAGWEPCVEMLYPRDGLVCGQSLMALKCTDGNHLTSHLRTTYRSRKPSNAVNMPF HFGDHRIELKAEQKGKFEYQYESAVARYSDVPEKAT [untranslated region] MRINHNIAALNTLNRLSSNNSASQKNMEKLSSGLRINRAGDDAAGLAISEKMRGQIRGLEMASKNSQDGIS LIQTAEGALTETHAILQVRVRELVVQAGNTGTQDKATDLQSIQDEISALTDEIDGISNRTEFNGKKLLDGTYKV DTATPANQKNLVFQIGANATQQISVNIEDMGADALGIKEADGSIAALHSVNDHIVMVDAKYPTKLDVTKFA DNAADCADIGFDAQLKVVDEAINQVSSQRAKLGAVQNRLEHTINNLSASGENLTAAESRIRDVDMAKEMSE FTKNNILSQASQAMLAQANQQPQNVLQLLR

Table S3: Nucleotides sequences of proteins and peptides in this study.

Name	Nucleotide sequence
EutM	GAAGCATTAGGAATGATTGAAACCCGGGGCCTGGTTGCGCTGATTGAGGCCTCCGATGCGATGGTAA AAGCCGCGCGCGTGAAGCTGGTCGGCGTGAAGCAGATTGGCGGTGGCCTGTGTACTGCCATGGTGC GTGGCGATGTGGCGGCGTGCAAAGCCGCAACCGATGCTGGCGCCGCTGCGGCGCAGCGCATTGGC GAGTTGGTCTCCGTACACGTGATTCCACGCCCGCACGGCGATCTGGAAGAAGTGTTCCCGATCAGCT TCAAAGGCGACAGCAACATT
SpyCatcher	GATAGTGCTACCCATATTAATTTCTCAAACCGTGATGAGGACGGCAAAGAGTTAGCTGGTGCAACTAT GGAGTTGCGTGATTCATCTGGTAAACTATTAGTACATGGATTCAGATGGACAAGTGAAGATTTCTA CCTGTATCCAGGAAAATATACATTTGTGCAAACCGCAGCACCAGACGGTTATGAGGTAGCAACTGCTA TTACCTTTACAGTTAATGAGCAAGGTCAGGTTACTGTAAATGGC
SpyTag	GCCACATCGTGATGGTGGACGCCTACAAGCCGACGAAG
CotB	AGCGGAGGCAGCGGCCGTGCGCGAGCTCAGCGTCAAAGCTCACGTGGGCGC
R5	AGCTCTAAGAAAAGTGGCAGCTATTCAGGCAGCAAAGGTTCTGAAGCGACGCATTCTG
SB7	CGTCAAAGCTCACGTGGGCGC
Synthetic	AAGTTCTTTGAAGCTGCCGCGAAGAAATCTTTGAA
His-EutM	ATGCATCATCATCACCACCACGGTTCTGGTTCTGGTTCTGGTTCTGGTTCTGGTTCTGAAGCATTAGGA ATGATTGAAACCCGGGGCCTGGTTGCGCTGATTGAGGCCTCCGATGCGATGGTAAAAGCCGCGCGC GTGAAGCTGGTCGGCGTGAAGCAGATTGGCGGTGGCCTGTGTACTGCCATGGTGCCTGGCGATGTG GCGGCGTGCAAAGCCGCAACCGATGCTGGCGCCGCTGCGGCGCAGCGCATTGGCGAGTTGGTCTCC GTACACGTGATTCCACGCCCGCACGGCGATCTGGAAGAAGTGTTCCCGATCAGCTTCAAAGGCGACA GCAACATT
His-EutM-CotB	ATGCATCATCATCACCACCACGGTTCTGGTTCTGGTTCTGGTTCTGGTTCTGGTTCTGAAGCATTAGGA ATGATTGAAACCCGGGGCCTGGTTGCGCTGATTGAGGCCTCCGATGCGATGGTAAAAGCCGCGCGC GTGAAGCTGGTCGGCGTGAAGCAGATTGGCGGTGGCCTGTGTACTGCCATGGTGCCTGGCGATGTG GCGGCGTGCAAAGCCGCAACCGATGCTGGCGCCGCTGCGGCGCAGCGCATTGGCGAGTTGGTCTCC GTACACGTGATTCCACGCCCGCACGGCGATCTGGAAGAAGTGTTCCCGATCAGCTTCAAAGGCGACA GCAACATTGTCGACGGGAGTGGTGGCAGCGGAGGCAGCTCTAAGAAAAGTGGCAGCTATTCAGGCA CGTCAAAGCTCACGTGGGCGC
His-EutM-R5	ATGCATCATCATCACCACCACGGTTCTGGTTCTGGTTCTGGTTCTGGTTCTGGTTCTGAAGCATTAGGA ATGATTGAAACCCGGGGCCTGGTTGCGCTGATTGAGGCCTCCGATGCGATGGTAAAAGCCGCGCGC GTGAAGCTGGTCGGCGTGAAGCAGATTGGCGGTGGCCTGTGTACTGCCATGGTGCCTGGCGATGTG GCGGCGTGCAAAGCCGCAACCGATGCTGGCGCCGCTGCGGCGCAGCGCATTGGCGAGTTGGTCTCC GTACACGTGATTCCACGCCCGCACGGCGATCTGGAAGAAGTGTTCCCGATCAGCTTCAAAGGCGACA GCAACATTGTCGACGGGAGTGGTGGCAGCGGAGGCAGCTCTAAGAAAAGTGGCAGCTATTCAGGCA GCAAAGGTTCTGAAGCGACGCATTCTG
His-EutM-SB7	ATGCATCATCATCACCACCACGGTTCTGGTTCTGGTTCTGGTTCTGGTTCTGGTTCTGAAGCATTAGGA ATGATTGAAACCCGGGGCCTGGTTGCGCTGATTGAGGCCTCCGATGCGATGGTAAAAGCCGCGCGC GTGAAGCTGGTCGGCGTGAAGCAGATTGGCGGTGGCCTGTGTACTGCCATGGTGCCTGGCGATGTG

Name	Nucleotide sequence
	GCGGCGTGCAAAGCCGCAACCGATGCTGGCGCCGCTGCGGCGCAGCGCATTGGCGAGTTGGTCTCC GTACACGTGATTCCACGCCCCGACGGCGATCTGGAAGAAGTGTCCCGATCAGCTTCAAAGGCGACA GCAACATTGTCGACGGGAGTGGTGGCAGCGGAGGCCGTCAAAGCTCACGTGGGCGC
His-EutM- Synthetic	ATGCATCATCATCACCACCACGGTTCTGGTTCTGGTTCTGGTTCTGGTTCTGGTTCTGAAGCATTAGGA ATGATTGAAACCCGGGGCCTGGTTGCGCTGATTGAGGCCTCCGATGCGATGGTAAAAGCCGCGCGC GTGAAGCTGGTCGGCGTGAAGCAGATTGGCGGTGGCCTGTGTACTGCCATGGTGGTGGCGATGTG GCGGCGTGCAAAGCCGCAACCGATGCTGGCGCCGCTGCGGCGCAGCGCATTGGCGAGTTGGTCTCC GTACACGTGATTCCACGCCCCGACGGCGATCTGGAAGAAGTGTCCCGATCAGCTTCAAAGGCGACA GCAACATTGTCGACGGGAGTGGTGGCAGCGGAGGCCAAGTTCTTTGAAGCTGCCGCGAAGAAATTCTT TGAA
SacB-His-EutM- SpyCatcher	ATGAACATCAAAAAGTTTGCAAAACAAGCAACAGTATTAACCTTTACTACCGCACTGCTGGCAGGAGG CGCAACTCAAGCGTTTGCGGCTTCAACTCATCATCATCACCACCACGGTTCTGGTTCTGGTTCTGGTT CTGGTTCTGGTTCTGAAGCATTAGGAATGATTGAAACCCGGGGCCTGGTTGCGCTGATTGAGGCCTC CGATGCGATGGTAAAAGCCGCGCGCGTGAAGCTGGTCGGCGTGAAGCAGATTGGCGGTGGCCTGTG TACTGCCATGGTGGTGGCGATGTGGCGGCGTGCAAAGCCGCAACCGATGCTGGCGCCGCTGCGGC GCAGCGCATTGGCGAGTTGGTCTCCGTACACGTGATTCCACGCCCCGACGGCGATCTGGAAGAAGTG TTCCCGATCAGCTTCAAAGGCGACAGCAACATTGTCGACGGGAGTGGTGGCAGCGGAGGCCGATAGTG CTACCCATATTAAATTCTCAAACGTGATGAGGACGGCAAAGAGTTAGCTGGTGCAACTATGGAGTTG CGTGATTCATCTGGTAAACTATTAGTACATGGATTTCAGATGGACAAGTGAAAGATTTCTACCTGTAT CCAGGAAAATATACATTTGTCGAAACCGCAGCACCAGACGGTTATGAGGTAGCAACTGCTATTACCTTT ACAGTTAATGAGCAAGGTCAGGTTACTGTAAATGGCTAA
SacB-His-EutM- CotB	ATGAACATCAAAAAGTTTGCAAAACAAGCAACAGTATTAACCTTTACTACCGCACTGCTGGCAGGAGG CGCAACTCAAGCGTTTGCGGCTTCAACTCATCATCATCACCACCACGGTTCTGGTTCTGGTTCTGGTT CTGGTTCTGGTTCTGAAGCATTAGGAATGATTGAAACCCGGGGCCTGGTTGCGCTGATTGAGGCCTC CGATGCGATGGTAAAAGCCGCGCGCGTGAAGCTGGTCGGCGTGAAGCAGATTGGCGGTGGCCTGTG TACTGCCATGGTGGTGGCGATGTGGCGGCGTGCAAAGCCGCAACCGATGCTGGCGCCGCTGCGGC GCAGCGCATTGGCGAGTTGGTCTCCGTACACGTGATTCCACGCCCCGACGGCGATCTGGAAGAAGTG TTCCCGATCAGCTTCAAAGGCGACAGCAACATTGTCGACGGGAGTGGTGGCAGCGGAGGCGAGCGGC CGTGCGCGAGCTCAGCGTCAAAGCTCACGTGGGCGCTGA
native <i>hag</i> promoter - Hag ^{T209C} ::SpyT 588	AAGTAGTGTGCAGCAGCGTTATCCAGCGATGTGATCTCCGCATTATCCTCACAAAAAAGTGAGGATTT TTTTATTTTTGTATTAACAAAATCAGAGACAATCCGATATTAATGATGTAGCCGGGAGGAGGCGCAAAA GACTCAGCCAGTTACAAAATAAGGGCACAGGACGTGCCTTAACAACATATTCAGGGAGGAACAAAAC AATGAGAATTAACCACAATATTGCAGCGCTTAACACACTGAACCGTTTGTCTTCAAACAACAGTGCAG CCAAAAGAATGGAGAACTTTCTCAGGTCTTCGCATCAACCGTGCGGGAGATGACGCAGCAGGT CTTGCGATCTCTGAAAAATGAGAGGACAAATCAGAGGTCTTGAAATGGCTTCTAAAACTCTCAAGAC GGAATCTCTTATCAAACAGCTGAGGGTGCATTAACCTGAAACTCATGCGATCCTTCAACGTGTTTCTG GAGCTAGTTGTTCAAGCTGGAAACACTGGAACCTCAGGACAAAGCAACTGATTGCAATCTATTCAAGAT GAAATTTACAGCTTTAACAGATGAAATCGATGGTATTTCAAATCGTACAGAATTCAATGGTAAGAAATTGC TCGATGGCACTTACAAAGTTGACACAGCTACTCCTGCAAATCAAAGAAGCTTGGTATTTCAAATCGGAG CAAATGCTACACAGCAAATCTGTAAATATTGAGGATATGGGTGCTGACGCTCTTGAATTAAGAAG CTGATGGTTCAATTGCAGCTCTTCATTAGTTAATGATGCCCCACATCGTGATGGTGGACGCTTACAAG CCGACGAAGCTTGACGTAAACAAATTCGCAGATAATGCAGCAGATTGTGCTGATATCGGTTTCGATGC TCAATTGAAAGTTGTTGATGAAGCGATCAACCAAGTTTCTTCTCAACGTGCTAAGCTTGGTGGGTACA

Name	Nucleotide sequence
	AAATCGTCTAGAGCACACAATTAACAACCTTAAGCGCTTCTGGTGAAAACCTTGACAGCTGCTGAGTCTCG TATCCGTGACGTTGACATGGCTAAAGAGATGAGCGAATTCACAAAGAACAACATTCTTTCTCAGGCTTC TCAAGCTATGCTTGCTCAAGCAAACCAACAGCCGCAAAACGTAATTCAATTATTACGTTAA
His-tdTomato- SpyCatcher	ATGCATCATCATCACCACCACGGTTCTGGTTCTGGTTCTGGTTCTGGTTCTGGTTCTGTCGACGTGAG CAAGGGCGAGGAGGTATCAAAGAGTTTATGCGCTTCAAGGTGCGCATGGAGGGCTCCATGAACGG CCACGAGTTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCACCCAGACCGCCAAGC TGAAGGTGACCAAGGGCGGCCCTGCCCCTTCGCTGGGACATCCTGTCCCCCAGTTCATGTACGG CTCCAAGGCGTACGTGAAGCACCCCGCCGACATCCCCGATTACAAGAAGCTGTCTTCCCCGAGGGC TTCAAGTGGGAGCGCGTGATGAACCTTCGAGGACGGCGGTCTGGTGACCGTGACCCAGGACTCCTCC CTGCAGGACGGCACGCTGATCTACAAGGTGAAGATGCGCGGCACCAACTTCCCCCCCCGACGGCCCC GTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCACCGAGCGCCTGTACCCCGCGACGGCGTG CTGAAGGGCGAGATCCACCGAGCCCTGAAGCTGAAGGACGGCGGCCACTACCTGGTGGAGTTCAAG ACCATCTACATGGCCAAGAAGCCCGTGCAACTGCCCGGCTACTACTACGTGGACACCAAGCTGGACA TCACCTCCCACAACGAGGACTACACCATCGTGGAACAGTACGAGCGCTCCGAGGGCGGCCACACCT GTTCTGGGGCATGGCACCGGCAGCACCGGCAGCGGCAGCTCCGGCACCGCCTCCTCCGAGGACAA CAACATGGCCGTATCAAAGAGTTTATGCGCTTCAAGGTGCGCATGGAGGGCTCCATGAACGGCCAC GAGTTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCTACGAGGGCACCCAGACCGCCAAGCTGAA GGTGACCAAGGGCGGCCCTGCCCTTCGCTGGGACATCCTGTCCCCCAGTTCATGTACGGCTCC AAGGCGTACGTGAAGCACCCCGCCGACATCCCCGATTACAAGAAGCTGTCTTCCCCGAGGGCTTCA AGTGGGAGCGCGTGATGAACCTTCGAGGACGGCGGTCTGGTGACCGTGACCCAGGACTCCTCCCTGC AGGACGGCACGCTGATCTACAAGGTGAAGATGCGCGGCACCAACTTCCCCCCCCGACGGCCCCGTAAT GCAGAAGAAGACCATGGGCTGGGAGGCCTCCACCGAGCGCCTGTACCCCGCGACGGCGTGCTGAA GGGCGAGATCCACCGAGCCCTGAAGCTGAAGGACGGCGGCCACTACCTGGTGGAGTTCAAGACCAT CTACATGGCCAAGAAGCCCGTGCAACTGCCCGGCTACTACTACGTGGACACCAAGCTGGACATCACC TCCACAACGAGGACTACACCATCGTGGAACAGTACGAGCGCTCCGAGGGCGGCCACCACTGTTCC TGACGGCATGGACGAGCTGTACAAGGGGAGTGGTGGCAGCGGAGGCGATAGTGCTACCCATATTA ATTCTCAAAACGTGATGAGGACGGCAAAGAGTTAGCTGGTGCAACTATGGAGTTGCGTGATTCATCTG GTAAACTATTAGTACATGGATTTAGATGGACAAGTGAAGATTTCTACCTGTATCCAGGAAAATATA CATTTGTCGAAACCGCAGCACCAGACGGTTATGAGGTAGCAACTGCTATTACCTTTACAGTTAATGAG CAAGGTCAGGTTACTGTAAATGGCTGA
His-Purple- native promoter- Hag ^{T209C} ::SpyT 588	ATGCATCATCATCATCATCAGCAGCGGCCTGGTGCCGCGCGGCAGCATGGCGAGCTTGGTTAAGA AAGATATGTGTGTTAAGATGACGATGGAAGGTACTGTGAACGGTTATCACTTTAAGTGCCTTGGCGAG GGTGAAGGCAAGCCGTTTCGAGGGCACGCGAAGATGCGCATTCGTGTACCGAGGGCGGTCCGCTG CCTTTTGCAATTCGACATCCTGGCCCCGTGCTGTATGTACGGCTCTAAGACCTTCATTAACACGTGAG CGGTATCCCGGATTACTTTAAAGAGTCCTTTCCAGAGGGCTTCACTTGGGAACGTACCCAGATTTTGA GGACGGTGGTGTTCTGACCGCGCACCAAGACACCAGCCTGGAAGGTAATTGCCTGATCTATAAAGTG AAGGTTCTGGGTACCAATTTCCCGCGCAATGGTCCGGTGATGCAAAAGAAAACCGCGGGTTGGGAGC CGTGCGTCGAGATGCTGTATCCGCGTGACGGCGTCTTGTGTGTCAGAGCTTGATGGCGCTGAAGTG CACCGATGGCAATCATCTGACCAGCCACCTGCGCACGACGTATCGTAGCCGTAAACCGAGCAACGCC GTTAACATGCCGAGTTCCATTTTGGTGACCATCGCATCGAAATCCTGAAAGCTGAGCAGGGCAAATT CTACGAACAATACGAATCGGCTGTGCGACGTTACAGCGATGTGCCGAAAAAGCGACGTAAAGTTAAAA GTCTCGTAAAGCGTTCTATCAATAACCGGTTGGTGCCAGGCATCAAATAAAACGAAAGGCTCAGTCGA

Name	Nucleotide sequence
	AAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTGCGGTGAACGCTCTCTACTAGAGAGCTCATGCCGGC GGCACCTCGCTAACGGATTCACTACTCCAAGAATTGGAGCCAGTACAATGGTCCA AAGTAGTGTGCAG CAGCGTTATCCAGCGATGTGATCTCCGCATTATCCTCACAAAAAAGTGAGGATTTTTTTATTTTGTAT TAACAAAATCAGAGACAATCCGATATTAATGATGTAGCCGGGAGGAGGCGCAAAAGACTCAGCCAGTT ACAAAATAAGGGCACAAGGACGTGCCTTAACAACATATTCAGGGAGGAACAAAACA ATGAGAATTAAC CACAATATTGCAGCGCTTAACACACTGAACCGTTTGTCTTCAAACAACAGTGCGAGCCAAAAGAACAT GGAGAACTTTCTTCAGGTCTTCGCATCAACCGTGCGGGAGATGACGCAGCAGGTCTTGCATCTCT GAAAAAATGAGAGGACAAATCAGAGGTCTTGAAATGGCTTCTAAAACTCTCAAGACGGAATCTCTCTT ATCCAAACAGCTGAGGGTGCATTAAGTAACTCATGCGATCCTTCAACGTGTTGCTGAGCTAGTTGTT CAAGCTGGAAACACTGGAAGTCAAGGACAAAGCAACTGATTTGCAATCTATTCAAGATGAAATTTAGCT TTAACAGATGAAATCGATGGTATTTCAAATCGTACAGAATTCAATGGTAAGAAATTGCTCGATGGCACT TACAAAGTTGACACAGCTACTCCTGCAAAATCAAAAGAACTTGGTATTCCAAATCGGAGCAAATGCTACA CAGCAAATCTCTGTAAATATTGAGGATATGGGTGCTGACGCTCTTGAATTAAGAAGCTGATGGTTCA ATTGCAGCTCTTCATTAGTTAATGAT GGCCACATCGTGATGGTGGACGCCTACAAGCCGACGAAGCT TGACGTAACAAAATTCGAGATAATGCAGCAGATTG TGCT GATATCGGTTTCGATGCTCAATTGAAAGT TGTTGATGAAGCGATCAACCAAGTTTCTTCTCAACGTGCTAAGCTTGGTGCGGTACAAAATCGTCTAGA GCACACAATTAACAACCTTAAGCGCTTCTGGTGAAACTTGACAGCTGCTGAGTCTCGTATCCGTGACG TTGACATGGCTAAAGAGATGAGCGAATTCACAAAGAACAACATTCTTTCTCAGGCTTCTCAAGCTATGC TTGCTCAAGCAACCAACAGCCGCAAAACGTACTTCAATTATTACGTAA

Table S4: Amino acid sequences of signal peptides.

Signal peptides	Amino acid sequence	Source	Reference
SacB	MNIKKFAKQATVLTFTTALLAGGATQAFAAST	<i>B. subtilis</i>	[Gilbert, 2017] ²³
XynA	MFKFKKNFLVGLSAALMSISLFSATASAAST	<i>B. subtilis</i>	[Gilbert, 2017] ²³
YngK	MYIKKCIGSILFLLFCSSALPAKAAST	<i>B. megaterium</i>	[Stammen, 2010] ²⁴
CelA	MKRSISIFITCLLITLLTMGGMIASPASAAST	<i>B. subtilis</i>	[Gilbert, 2017] ²³
LipA	MKKVLMAFIICLSLILSVLAAPPSGAKAAST	<i>B. megaterium</i>	[Stammen, 2010] ²⁴

Table S5: List of primers.

Name	Sequence
PB_Back_fwd	gttaaaagtctcgtaaagcg
PB_Back_rev	gtttgtcctccttattagtaatc
PB_Ins_fwd	aactaataaggaggacaaacatgcatcatcatcatcac
PB_Ins_rev	cgccttacgagacttttaacttacgctgcttttccgg
pHBintE seq fwd	tcggaattctcatgtttgacagc
pHBintE seq rev	caagggttggttgcgcat
dlytC_upstream_fwd	tccaaactagaactgaacaattgtatgattg
dlytC_upstream_rev	aggaaatcaatcgaagagacaaatctaac
dlytC_downstream_fwd	tctcttcgattgattcctccttaaatgg
dlytC_downstream_rev	tcgaactagttctacttcaacagaattagtc
dlytC_fwd	tgaagtagaaactagttcgaagatctcc
dlytC_rev	ttgttcagttctagttggaccatttgc
dlytC_confirmation_fwd	tatctgcaccatcacccgctc
dlytC_confirmation_rev	caagtttatgggtgctacagttg
dflhG_upstream_fwd	tccaaactagtcgaaaaaattaaaagcgaaaatg
dflhG_upstream_rev	acgaatcaaaagcatcctcacaaaaccaag
dflhG_downstream_fwd	tgaggatgctttgattcgtgctgtgattg
dflhG_downstream_rev	tcgaactagtacccgctttaacagcagc
dflhG_fwd	taaagcgggtactagttcgaagatctcc
dflhG_rev	ttttttcgactagttggaccatttgc
dflhG_confirmation_fwd	aaagcattggagtggtggat
dflhG_confirmation_rev	gaagatcagtcgggtcttctc
NP-hag_fwd	caatggtccaaactagtgagcagcagc
NP-hag_rev	ccggcatgcattaacgtaataattgaagtacgtttgc
NP-hag-T209C_fwd	tgcagcagattgtgctgatcgc
NP-hag-T209C_rev	ttatctgcgaattttgttac
NP-hag-T209C-SPT399_fwd	cgctacaagccgacgaagggaagaaattgctcgatg
NP-hag-T209C-SPT399_rev	tccaccatcacgatgtggcattgaattctgtacgatttgaaatac
NP-hag-T209C-SPT426_fwd	cgctacaagccgacgaagaaagtgacacagctactc

Name	Sequence
NP-hag-T209C-SPT426_rev	tccaccatcacgatgtgggcgtaagtccatcgagcaatttc
NP-hag-T209C-SPT459_fwd	cgctacaagccgacgaagaacttggtattccaaatcg
NP-hag-T209C-SPT459_rev	tccaccatcacgatgtgggccttttgattgcaggagtag
NP-hag-T209C-SPT492_fwd	cgctacaagccgacgaagcagcaaatctctgtaaatattgag
NP-hag-T209C-SPT492_rev	tccaccatcacgatgtgggctgtagcattgtccgatttg
NP-hag-T209C-SPT537_fwd	cgctacaagccgacgaagggaattaagaagctgatggttcaattg
NP-hag-T209C-SPT537_rev	tccaccatcacgatgtgggcaagagcgtagcaccata
NP-hag-T209C-SPT555_fwd	cgctacaagccgacgaagggttcaattgcagctcttc
NP-hag-T209C-SPT555_rev	tccaccatcacgatgtgggcatcagcttcttaattccaag
NP-hag-T209C-SPT588_fwd	cgctacaagccgacgaagcttgacgtaacaaaattcgc
NP-hag-T209C-SPT588_rev	tccaccatcacgatgtgggcatcattaactgaatgaagagc
NP-hag-T209C-SPT612_fwd	cgctacaagccgacgaagaatgcagcagattgtgctg
NP-hag-T209C-SPT612_rev	tccaccatcacgatgtgggcatctgcgaattttgttacg
NP-hag-T209C-SPT645_fwd	cgctacaagccgacgaaggctcaattgaaagtgttgatgaag
NP-hag-T209C-SPT645_rev	tccaccatcacgatgtgggcatcgaaaccgatatcagcac

Table S6: Variation of storage modulus (G') and loss modulus (G'') with frequency of silica gels.

	Storage modulus (G')		
Angular frequency (rad/S)	pCT-empty	pCT-EutMCotB-EutMSpyC	pCT-EutMCotB-EutMSpyC-Hag ^{T209C} ::SpyT ⁵⁸⁸
100	1.05.E-03 ± 2.49.E-04	1.29.E-03 ± 7.29.E-05	1.36.E-03 ± 2.45.E-04
63.0957	1.01.E-03 ± 2.63.E-04	1.21.E-03 ± 7.88.E-05	1.36.E-03 ± 2.90.E-04
39.8107	9.60.E-04 ± 2.52.E-04	1.18.E-03 ± 5.22.E-05	1.31.E-03 ± 2.45.E-04
25.1189	9.38.E-04 ± 2.40.E-04	1.14.E-03 ± 4.60.E-05	1.27.E-03 ± 2.16.E-04
15.8489	9.06.E-04 ± 2.29.E-04	1.10.E-03 ± 4.87.E-05	1.24.E-03 ± 2.04.E-04
10	8.84.E-04 ± 2.19.E-04	1.07.E-03 ± 4.28.E-05	1.20.E-03 ± 1.77.E-04
6.30957	8.59.E-04 ± 2.07.E-04	1.02.E-03 ± 5.88.E-05	1.16.E-03 ± 1.52.E-04
3.98107	8.34.E-04 ± 1.59.E-04	1.01.E-03 ± 5.39.E-05	1.13.E-03 ± 1.19.E-04
2.51189	8.32.E-04 ± 1.82.E-04	9.70.E-04 ± 5.13.E-05	1.11.E-03 ± 1.31.E-04
1.58489	8.13.E-04 ± 1.57.E-04	9.11.E-04 ± 4.23.E-05	1.06.E-03 ± 1.10.E-04
1	7.82.E-04 ± 1.36.E-04	8.92.E-04 ± 5.50.E-05	1.06.E-03 ± 1.13.E-04
	Loss modulus (G'')		
Angular frequency (rad/S)	pCT-empty	pCT-EutMCotB-EutMSpyC	pCT-EutMCotB-EutMSpyC-Hag ^{T209C} ::SpyT ⁵⁸⁸
100	1.07.E-04 ± 2.81.E-05	9.03.E-05 ± 5.69.E-05	4.37.E-05 ± 1.23.E-04
63.0957	1.04.E-04 ± 6.01.E-05	1.12.E-04 ± 2.32.E-05	7.73.E-05 ± 3.30.E-05
39.8107	1.02.E-04 ± 4.18.E-05	9.05.E-05 ± 7.49.E-06	7.16.E-05 ± 1.50.E-05
25.1189	8.00.E-05 ± 2.92.E-05	9.48.E-05 ± 2.08.E-05	7.30.E-05 ± 1.52.E-05
15.8489	8.52.E-05 ± 3.16.E-05	9.18.E-05 ± 2.48.E-05	6.45.E-05 ± 2.34.E-05
10	8.24.E-05 ± 2.74.E-05	9.03.E-05 ± 1.17.E-05	7.18.E-05 ± 3.58.E-05
6.30957	9.71.E-05 ± 3.07.E-05	9.46.E-05 ± 2.48.E-05	8.12.E-05 ± 2.96.E-05
3.98107	9.86.E-05 ± 2.54.E-05	7.06.E-05 ± 3.59.E-05	7.15.E-05 ± 2.97.E-05
2.51189	9.03.E-05 ± 1.29.E-05	9.40.E-05 ± 5.46.E-05	9.47.E-05 ± 2.95.E-05
1.58489	8.31.E-05 ± 2.09.E-05	1.40.E-04 ± 3.93.E-05	9.31.E-05 ± 1.07.E-05
1	1.27.E-04 ± 2.82.E-05	1.34.E-04 ± 3.71.E-05	1.09.E-04 ± 2.25.E-05

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