Supporting Information

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SI Material and Methods

Bacterial Strains and Culture Conditions. The strains used in this study were *B. subitlis* NCBI3610, a prototrophic strain for our laboratory collection (1), and derivative mutants SSB488 (*epsA-O*::*tet*) (2), CA017 (*tasA*::*kan*) (3), SSB572 (*epsA-O*::*tet tasA*::*spc*) (2), DS92 (*sinR*::*spc*) (4), and SSB551 (*sinR*::*spc epsA-O*::*tet*). Luria–Bertani (LB) broth was: 1% tryptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl. MSgg broth: 100 mM morpholinepropane sulphonic acid (Mops) (pH 7), 0.5% glycerol, 0.5% glutamate, 5 mM potassium phosphate (pH 7), 50 μg/mL tryptophan, 50 μg/mL phenylalanine, 2 mM $MgCl_2$, 700 μM $CaCl_2$, 50 μM $FeCl_3$, 50 μM $MnCl_2$, 2 μM thiamine, 1 μM $ZnCl_2$ (1). MOLP broth was prepared as described. (5). Congo Red indicator plates were MSgg agar and YESCA-agar containing 20 μg/mL Congo Red and 10 μg/mL Coomassie Brilliant Blue G (6). Media were solidified through addition of agar to 1.5%.

Antibiotic concentrations (final) were MLS (1 μg/mL erythromycin, 25 μg/mL lincomycin); spectinomycin (100 μg/mL); tetracycline (500 μg/mL for broth, 10 μg/mL for agar); chloramphenicol (5 μg/mL); and kanamycin (10 μg/mL).

Assays for Colony and Pellicle Formation and Extracellular Complementation. Bacteria were grown in LB broth to an OD_{600} of 1.0. For colony architecture analyses, 3 μL of starting culture were spotted onto agar plates and incubated at 30 °C for 42–120 h, as indicated. For pellicle formation analysis, 12 μL of starting culture were added to 4 mL or 1 mL of MSgg or MOLP broth in a 6- or 24-well microtiter dish, respectively, and the dishes were incubated without agitation at 30 °C for 60 h. To test extracellular complementation, starting cultures were mixed with protein solutions and the mixtures were used to form pellicles, as described above. To test the ability of bacteria growing in colony to bind Congo Red, 3 μL of starting cultures were spotted in CR indicator agar, and the plates were incubated at 30 °C for 72 h.

Protein Expression and Purification. TasA was directly purified from the *B. subtilis* double mutant strain *sinR eps*. To this end, cells were grown in MSgg broth at 37 °C for 20 h. The culture was centrifuged at 10,000 × *g* for 15 min and the pellet containing the cells was extracted twice with saline extraction buffer [5 mM potassium phosphate (pH 7), 2 mM $MgCl_2$, 100 mM morpholinepropane sulphonic acid (Mops) (pH 7) and 1 M NaCl] supplemented with a protease inhibitor mixture (Complete; Roche). After centrifugation for 15 min at 10,000 × *g*, the supernatant was separated from the cells and clarified by filtration through a 0.4 μm polyethersulfone bottle-top filter. This crude extract was treated with a 30% ammonium sulfate solution at 4 °C and centrifuged at 20,000 × *g* for 10 min. The supernatant containing TasA was further purified using a HiLoad_26/60 Superdex S200 column (Pharmacia Biotech), equilibrated in 50 mM NaCl, 20 mM Tris (pH 7) buffer. Different fractions were collected, concentrated in amycon centrifugal filter devices (Millipore), and analyzed by SDS/PAGE for the presence of TasA. TasA was purified to homogeneity by this approach as evidenced by a single band on SDS/PAGE stained by Coomassie blue. The identity and integrity of the protein was checked by N-terminal sequencing (Edman degradation) and ESI-MS-MS analysis of the peptides obtained after trypsin digestion. The concentration of the purified protein was determined by the Bincichoninic Acid (BCA) Protein Assay (Pierce, Thermo Scientific) using BSA as a standard.

Molecular Weight Estimation. The molecular weight of TasA before and after treatment with formic acid was estimated using a Superdex-200 HR 10/30 column with an AKTA explorer FPLC system (Amersham Biosciences). The column was equilibrated with 20 mM Tris, 50 mM NaCl buffer, pH 7.5. The molecular weight of the eluted protein was estimated by a calibration curve prepared using the elution times measured for standards (Bio-Rad) ranging from 1.35 to 670 kDa [thyroglobulin (670 kDa), γ-globulin (158 kDa), albumin (67 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa)].

Immunoblot and Dot-Blot Analyses. For whole-culture (cells plus culture medium) immunoblot analysis, samples were taken from MSgg cultures at intervals, adjusted to equal cell numbers using OD600nm, and mixed with 2× SDS loading buffer before boiling for 5 min and SDS/PAGE analysis. In fractionation studies, the supernatants were TCA precipitated, the pellet washed twice in cold acetone and after complete evaporation the residue suspended in 1× SDS loading buffer. Samples were electrophoresed on a 12% SDS-polyacrylamide gel and blotted onto polyvinylidene difluoride membrane using standard techniques. Blots were probed with anti-TasA polyclonal antibody that was raised in rabbits against purified TasA (7) and used at a dilution of 1:20,000, or anti-sigmaA, (a gift from D. Rudner, Harvard Medical School, Boston, MA) used at a dilution of 1:10,000. For dot-blot analyses, TasA samples were spotted onto 0.4-μm Hybond-C, Nitrocellulose membranes (Amersham Life Science) and allowed to dry for 5 min. The membranes were blocked in 10% skim milk in TBST (20 mM Tris, 137 mM NaCl, and 0.01% Tween 20). The dot blots were washed three times in TBST and incubated with a 1:10,000 dilution of A11 primary antibody (Invitrogen). A secondary anti-rabbit IgG antibody conjugated to horseradish peroxidase (Sigma) was used at a dilution of 1:20,000. The blots were developed using the Pierce super signal detection system according to manufacturer's instructions (Pierce, Thermo Scientific).

Congo-Red and Thioflavin T Assays. Protein solutions were incubated in a 20 μM solution of Congo Red prepared in 10 mM Tris (pH 7.4) and 100 mM NaCl, for 20 min before spectrophotometric analysis (8, 9). Absorption spectra were acquired using a Cary 1Bio UV/visible spectrophotometer (Spectra Max M2, Molecular Devices) at 25 °C and a scan rate of 300 nm/min. For all samples, spectra of corresponding solutions without Congo Red were used as blanks.

Thioflavin T assay was carried out in a 96-well microtiter plate as previously described (10). Purified TasA (40 μg) was mixed with 20 μM Tht solution in a final volume of 200 μL. The fluorescence measurements were done with a Cary 1Bio UV/visible spectrophotometer (Spectra Max M2; Molecular Devices) fluorescence plate reader set up at 438-nm excitation and 495-nm emission with a 475-nm cutoff.

CD Spectroscopy. CD spectra were recorded at 25 °C on an Aviv 62DS spectropolarimeter equipped with a thermoelectric temperature controller. Samples of purified TasA were prepared in 10 mM phosphate buffer, pH 7.4. Spectra representing the average of five scans from 260 to 195 nm were measured in a 1-mm path length cuvettete, using a step size of 1 nm and a 3-s signal-averaging time. All spectra were corrected for the base line obtained with the buffer alone (11).

In Vitro Polymerization Assay. Freshly purified TasA was depolymerized by short (1 min) or long (5 min) treatment with 10% formic acid solution. After the treatment, formic acid was eliminated by evaporation in a speed-vac at 30 °C. The remaining product containing TasA was resuspended in 50 mM NaCl, 20 mM Tris (pH 7) buffer and incubated at room temperature. Samples were taken at different time intervals and mixed with 20 μM Thioflavin T for fluorescence analyses, as described above.

Transmission Electron Microscopy and Immunolabeling. Samples were diluted with distilled water and adsorbed onto a carbon or formvar/carbon coated grid. The grid surface was made hydrophilic before use with glow discharge in a vacuum evaporator. Once the specimen was adsorbed onto the film surface, the excess sample was blotted off on a filter paper (Whatman #1) and the grid was floated on 5 μL of stain solution (1–2% aqueous uranyl acetate) for a few minutes and then blotted off. The samples were dried and examined in a Tecnai G^2 Spirit BioTWIN microscope at an accelerating voltage of 80 KV. Images were taken with an AMT 2k CCD camera.

For immunolocalization of TasA, diluted samples on nickel grids were floated on blocking buffer consisting of 1% nonfat dry milk in PBS with 0.1% Tween 20 for 30 min, incubated for 2 h with anti-TasA primary antibody diluted 1:150 in blocking buffer, rinsed in PBST, exposed to goat-anti-rabbit 20-nm gold secondary antibody (Ted Pella, Inc.) for 1 h, and rinsed. All grids were stained with uranyl acetate and lead citrate and viewed as described above.

1. Branda SS, González-Pastor JE, Ben-Yehuda S, Losick R, Kolter R (2001) Fruiting body formation by Bacillus subtilis. *Proc Natl Acad Sci USA* 98:11621–11626.
2. Branda SS, Chu F, Kearns DB, Losick R, Kolter R (2006) A major protein component of the *Bacillus subtilis* biofilm matrix. *Mol Microbiol* 59:1229–1238.
3. Vlamakis H, Aguilar C, Losick R, Kolter R (2008) Control of cell fate by the formation of an architecturally complex bacterial community. *Genes Dev* 22:945–953.
4. Kearns DB, Chu F, Branda SS, Kolter R, Losick R (2005) A master regulator for biofilm formation by *Bacillus subtilis*. *Mol Microbiol* 55:739–749.
5. Ahimou F, Jacques P, Deleu M (2000) Surfactin and iturin A effects on Bacillus subtilis surface hydrophobicity. *Enzyme Microb Technol* 27:749–754.
6. Hammar M, Bian Z, Normark S (1996) Nucleator-dependent intercellular assembly of adhesive curli organelles in *Escherichia coli*. *Proc Natl Acad Sci USA* 93:6562–6566.
7. Stöver AG, Driks A (1999) Secretion, localization, and antibacterial activity of TasA, a *Bacillus subtilis* spore-associated protein. *J Bacteriol* 181: 1664–1672.
8. Klunk WE, Jacob RF, Mason RP (1999) Quantifying amyloid by congo red spectral shift assay. *Methods Enzymol* 309:285–305.
9. Chapman MR, et al. (2002) Role of *Escherichia coli* curli operons in directing amyloid fiber formation. *Science* 295:851–855.
10. LeVine H, III (1999) Quantification of beta-sheet amyloid fibril structures with thioflavin T. *Methods Enzymol* 309:274–284.
11. Tjernberg L, Hosia W, Bark N, Thyberg J, Johansson J (2002) Charge attraction and beta propensity are necessary for amyloid fibril formation from tetrapeptides. *J Biol Chem* 277:43243–43246.

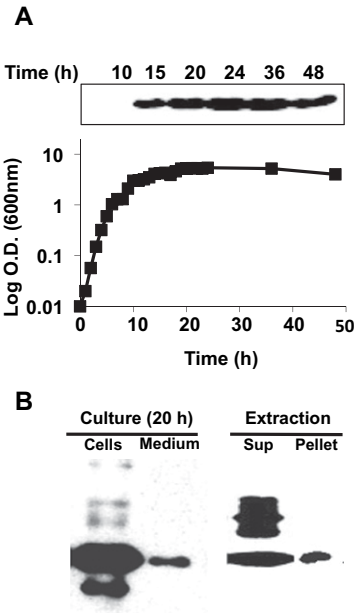

Fig. S1. Preparation of TasA extracts from a *B. subtilis sinR eps* mutant. (*A*) Kinetics of TasA production in cultures grown in MSgg. Top is an anti-TasA immunoblot of culture fractions (cells and medium) harvested at the corresponding times on the growth curve shown below. (*B*) Immunoblot of 20-h cultures showed most of the TasA associated with cells (*Left*). Most of the cell-associated TasA is released from the cells by treatment 1 M NaCl.

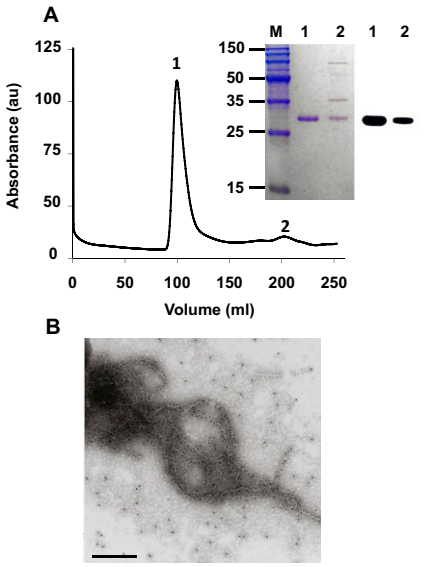

Fig. S2. Purification of TasA. (*A*) Protein elution profile from a HiLoad_26/60 Superdex S200 size exclusion column. TasA eluted mostly in the void fraction (1) and only a small fraction eluted after 200 mL (2). *Insets* show purity of TasA by SDS-page (*Left*) and immunoblot analysis using anti-TasA antibody (*Right*). Lane M, molecular marker. Molecular masses are indicated in kilodaltons. (*B*) Immunogold-EM micrographs of fraction 1 (void volume) containing TasA polymerized in the form of long fibrils. (Scale bar, 500 nm.)

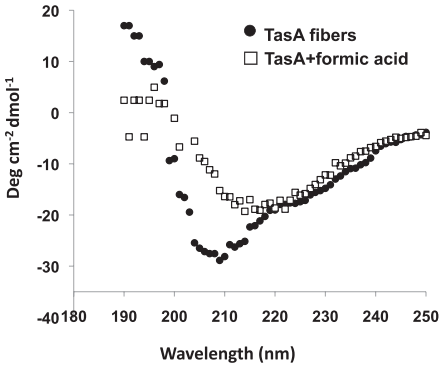

Fig. S3. Secondary structural changes of TasA on treatment with formic acid. Comparison of CD spectra of purified TasA in the form of fibers (●) and after 1-min treatment with 10% formic acid (□) in 10 mM phosphate buffer, pH 7.4.

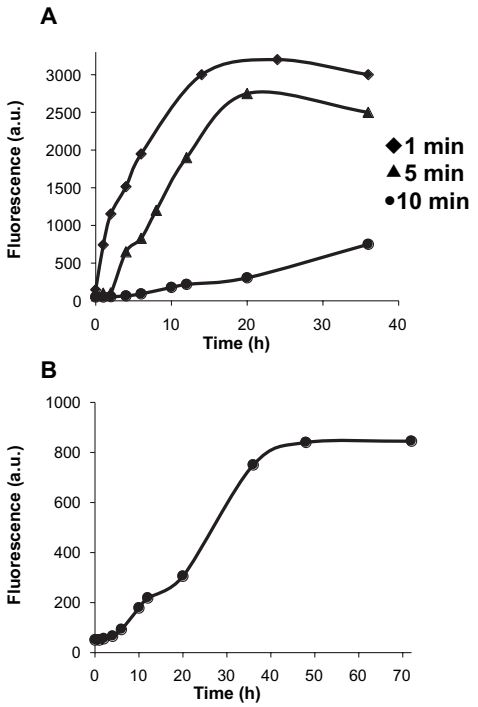

Fig. S4. Repolymerization of TasA in vitro. (*A*) Comparison of polymerization of TasA after 1-min (◆), 5-min (■), or 10-min (●) treatment with 10% formic acid. After evaporation of formic acid, the repolymerization was followed by the Thioflavin T assay. (*B*) Purified TasA treated for 10 min (●) with 10% formic acid.