Expanded View Figures

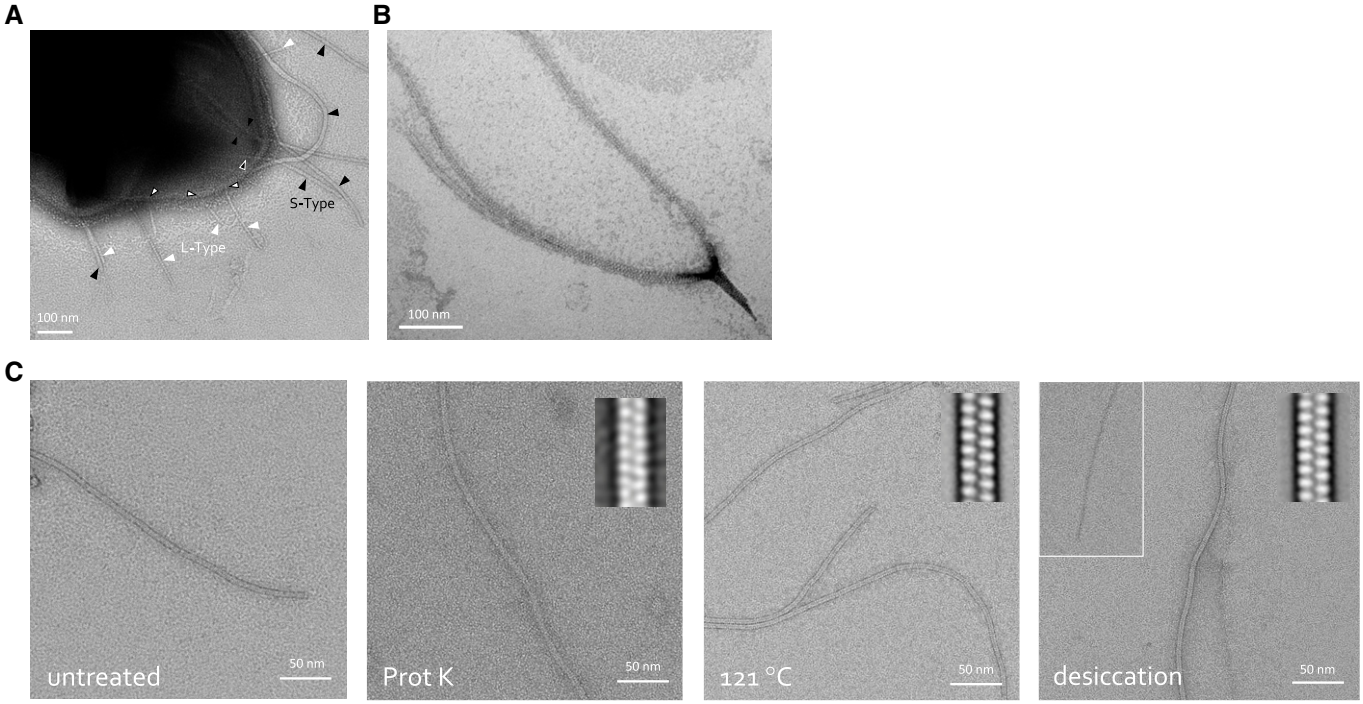

Figure EV1. Ena morphology and robustness.

A, B Negative stain TEM of *B. cereus* NVH 0075-95 endospore with indication of the two Ena morphologies: S-type (black arrowheads) and L-Enas (white arrowheads) (A), and closed-up view of a dislodged S-Ena bundle splitting into individual Ena fibers (B).

C Negative stain TEM images of isolated *ex vivo* S-Ena. To test Ena stability under different stresses, samples were treated, from left to right, with: (1) untreated control, (2) 1 h of 1 mg/ml proteinase K, (3) autoclaving (i.e., 20 min at 121°C) or (4) a 4 h of desiccation at 43°C. Inset shows 2D class averages to assess the structural integrity of the treated Ena. S-Enas are found to be resistant to Proteinase K treatment, autoclaving and desiccation at 43°C, although some fibers appear to lose subunit integrity upon desiccation (inset). Desiccation at 43°C may mimic conditions encountered by *Bacillus* spores during drought.

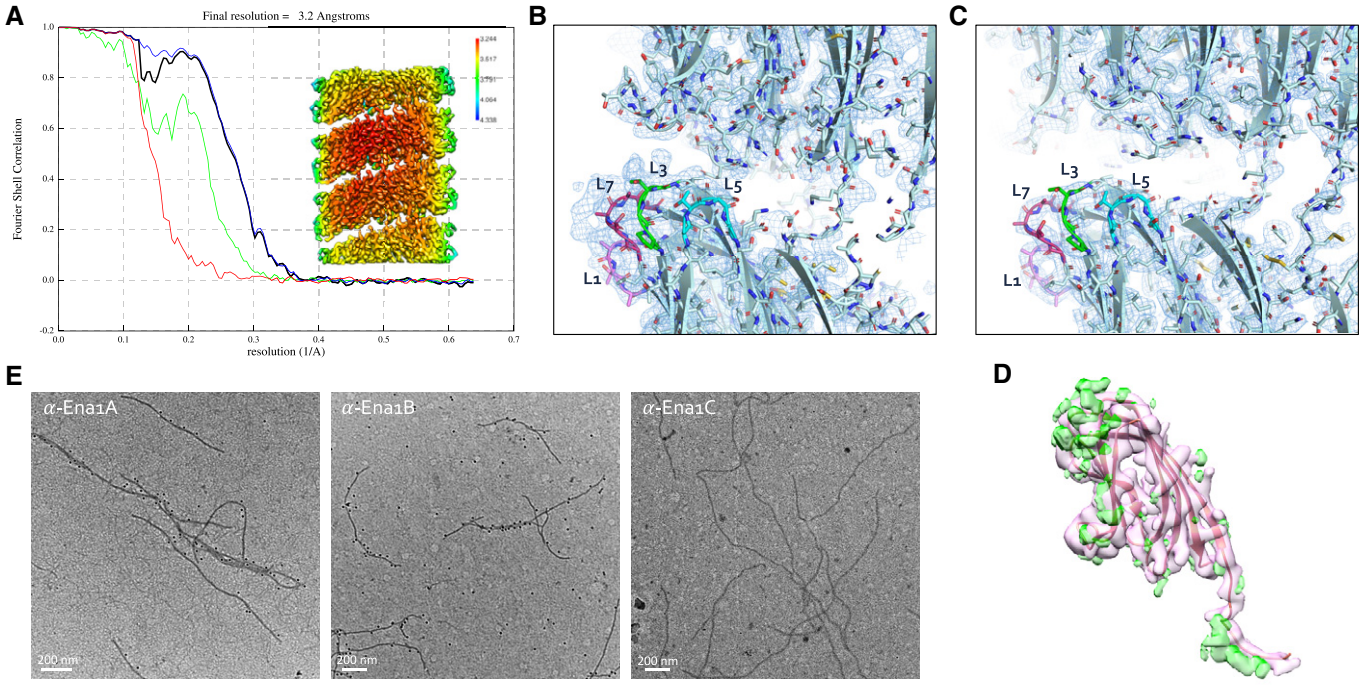

Figure EV2. S-Ena is composed of both Ena1A and Ena1B subunits.

A FSC curve and local resolution heatmap (inset) of the *rec*Ena1B helical reconstruction, indicating a final resolution of 3.2 Å at a cutoff of 0.143. FSC curve and local resolution were calculated by postprocessing in RELION3.0 using a solvent mask consisting of 3 helical turns.

B, C Side-by-side comparison of cryoEM maps calculated from *ex vivo* (B) and *rec*Ena1B filaments (C), with the refined Ena1B model docked into the maps. The *ex vivo* Ena map shows features unaccounted for by the Ena1B model near loops 3 (L3) and 7 (L7), corresponding to regions of amino acid insertions in the Ena1A sequence (Fig EV2B).

D *rec*Ena1B map (pink) and *rec*Ena1B—*ex vivo* Ena1 difference map (green) masked over a single Ena1B subunit and calculated by TEMPy:Diffmap (Farabella *et al*, 2015) from the CCPEM package (Burnley *et al*, 2017). Difference in both maps locates to L3, L7 and the conformation of Ntc.

E Immunogold TEM of *ex vivo* S-Enas, stained with, from left to right, anti-Ena1A, anti-Ena1B, and anti-Ena1C sera, each with gold-labeled (10 nm colloidal gold) anti-rabbit IgG as secondary antibody. Specific staining with Ena1A and Ena1B sera confirms the presence of both subunits in native Enas. No staining was seen with Ena1C serum.

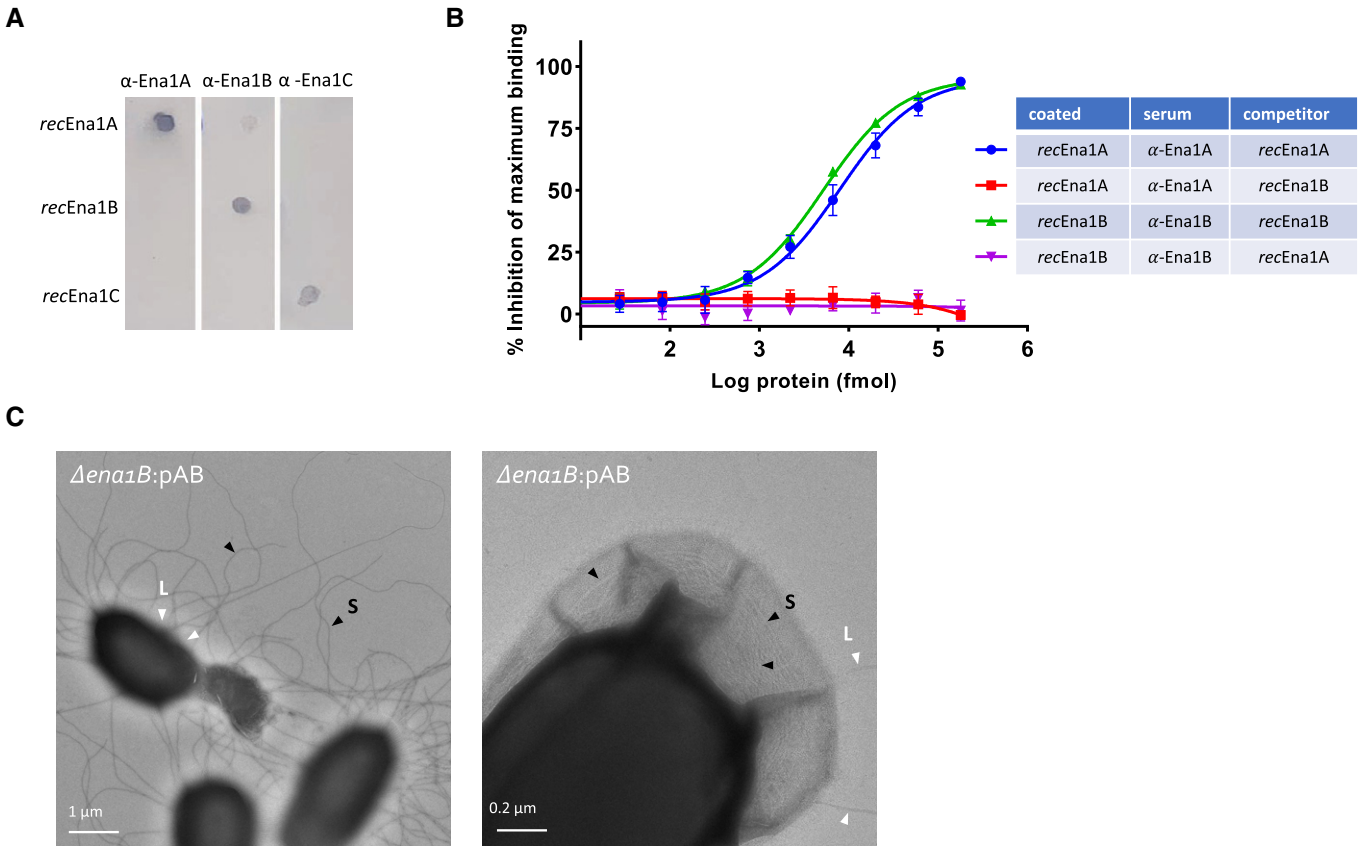

Figure EV3. Ena1 sera and Ena1A–Ena1B overexpression.

A Evaluation of residual cross-reactivity in anti-Ena1A, anti-Ena1B, and anti-Ena1C sera by dot blot. 100 ng of purified *rec*Ena1A, *rec*Ena1B, or *rec*Ena1C was coated on PVDF membrane, blocked, washed, and probed with the three different anti-sera at 1:1,000 in TBST. Dot blot shows robust cognate binding with good selectivity against other Ena1 subunits.

B Evaluation of residual cross-reactivity of anti-Ena1A and anti-Ena1B sera with *rec*EnaB and *rec*EnaA proteins, respectively, assayed using competitive ELISAs. The percentage inhibition of maximum binding was calculated using the formula (1-(S-B))/ (MA-B) × 100, where S, B, and MA are the average absorbance of the sample (sera + *rec*Ena competitor added to the *rec*Ena-coated wells), blank (only PBST added to the *rec*Ena-coated wells), and maximum activity (only sera added to the *rec*Ena-coated wells), respectively. The values presented are the averages of three independent experiments, and error bars depict ±SD of the averages. The lines are regression curves from three-parameter logistic regression analyses (GraphPad prism version 9).

C Negative stain TEM images of endospores of NVH 0075-95 mutant *Δena1B* complemented with pena1AB. Complementation with pena1AB results in aberrantly long and numerous S-Ena (Fig 6). This overexpression of S-Ena results in the frequent rupture of the exosporium (left panel), or the encapsulated of the S-Ena into the exosporium (right panel). Selected S-Ena and L-Ena are labeled with black and with arrows, and labeled S and L, respectively.

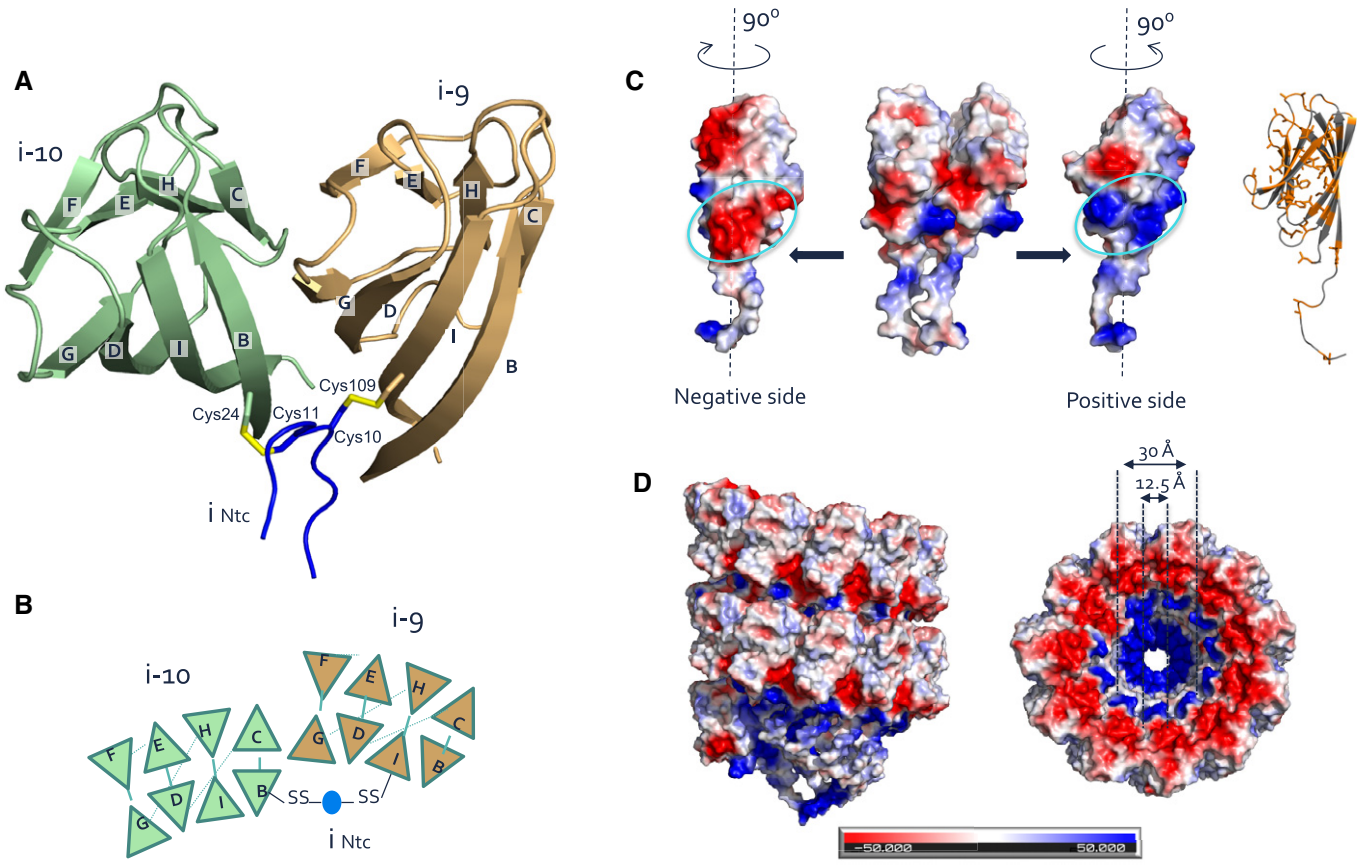

Figure EV4. Inter-subunit interactions in S-Ena.

A, B Ribbon (A) and schematic (B) representation of lateral subunit–subunit contacts in S-Ena. Strand G of BIDG sheet of each subunit is augmented with strand C of CHEF β-sheet of the succeeding subunit. Both subunits are covalently cross-linked via the Ntc (blue) of a subunit located, respectively, 9 or 10 subunits above. Cys11 and Cys10 go into a disulfide bond with residues 24 in the B strand of subunit i-10 and Cys109 in strand I of subunit i-9.

C, D Coulomb potential maps (calculated in PyMOL) of two adjacent subunits (C) and two helical turns of the S-Ena showing the distribution of charge on the atomic model surface. Each subunit possesses complementary positive and negatively charged patches of residues at the inter-subunit surface that are responsible for electrostatic stabilizing interactions between the subunits. Similarly, stacked helical rings in the S-Ena show a charge complementary interface (D).

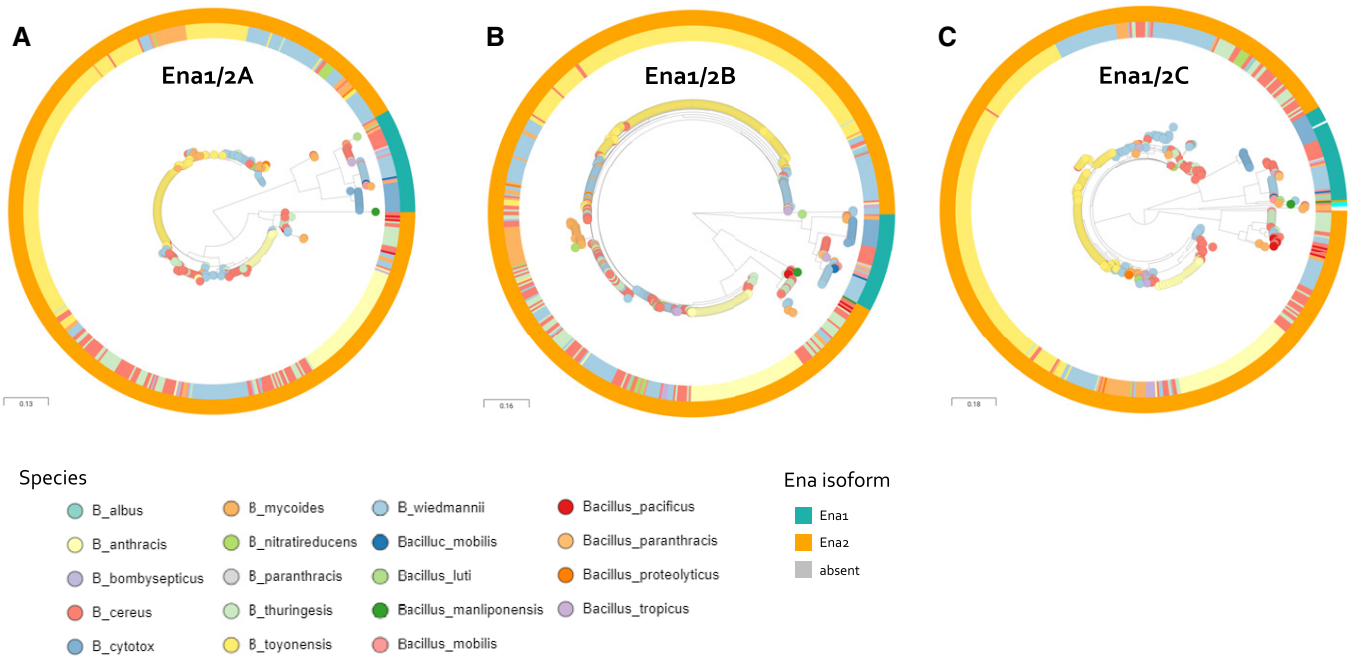

Figure EV5. Phylogenetic relationship between EnaA-C protein sequences among *Bacillus* spp.

Approximate likelihood trees generated by FastTree v.2.1.8 (Price *et al*, 2010), visualized in Microreact (Argimon *et al*, 2016). Trees are rooted on midpoint. Nodes are colored according to annotated species. See Methods for further details.

A Relationship between Ena1A and Ena2A isoforms of 593 isolates. Ena1A and Ena2A are defined as ortho- or homologues having >90% coverage and >80% and 50–65% sequence identity, respectively, with Ena1A_GCF_001044825; KMP91697.1 protein sequence defined in Appendix Table S5. Interactive tree accessible at: https://microreact.org/project/5UixxEY9vr2AVzXDVwa5t/1a8558fd.

B Relationship between Ena1B and Ena2B isoforms of 591 isolates. Ena1B, Ena1B_candidate, and Ena2B are defined as ortho- or homologues with > 90% coverage and > 80%, 60–80%, and 40–60% sequence identity to Ena1B_NM_Oslo protein sequence defined in Appendix Table S5, respectively. Interactive tree accessible at: https://microreact.org/project/jJ4pARvqf9gyT916sTar5u/1332f3b3.

C Relationship between Ena1C and Ena2C isoforms of 591 isolates. Ena1C, Ena1C_candidate, and Ena2C_candidates are defined as ortho- or homologues with > 90% coverage and > 80%, 60–80%, and 40–60% sequence identity to Ena1C_NM_Oslo protein sequence defined in Appendix Table S5, respectively. Furthermore, isolates in which an ortho- or homologue was found elsewhere in the genome than the usual EnaA-B locus are colored cyan. Isolates that lacked an Ena1C homo- or orthologue are colored gray. Interactive tree accessible at: https://microreact.org/project/aQaqCUCJoj2mw55KQujbGY/099d7885.