Directed Evolution of Acoustic Reporter Genes Using High-Throughput Acoustic Screening

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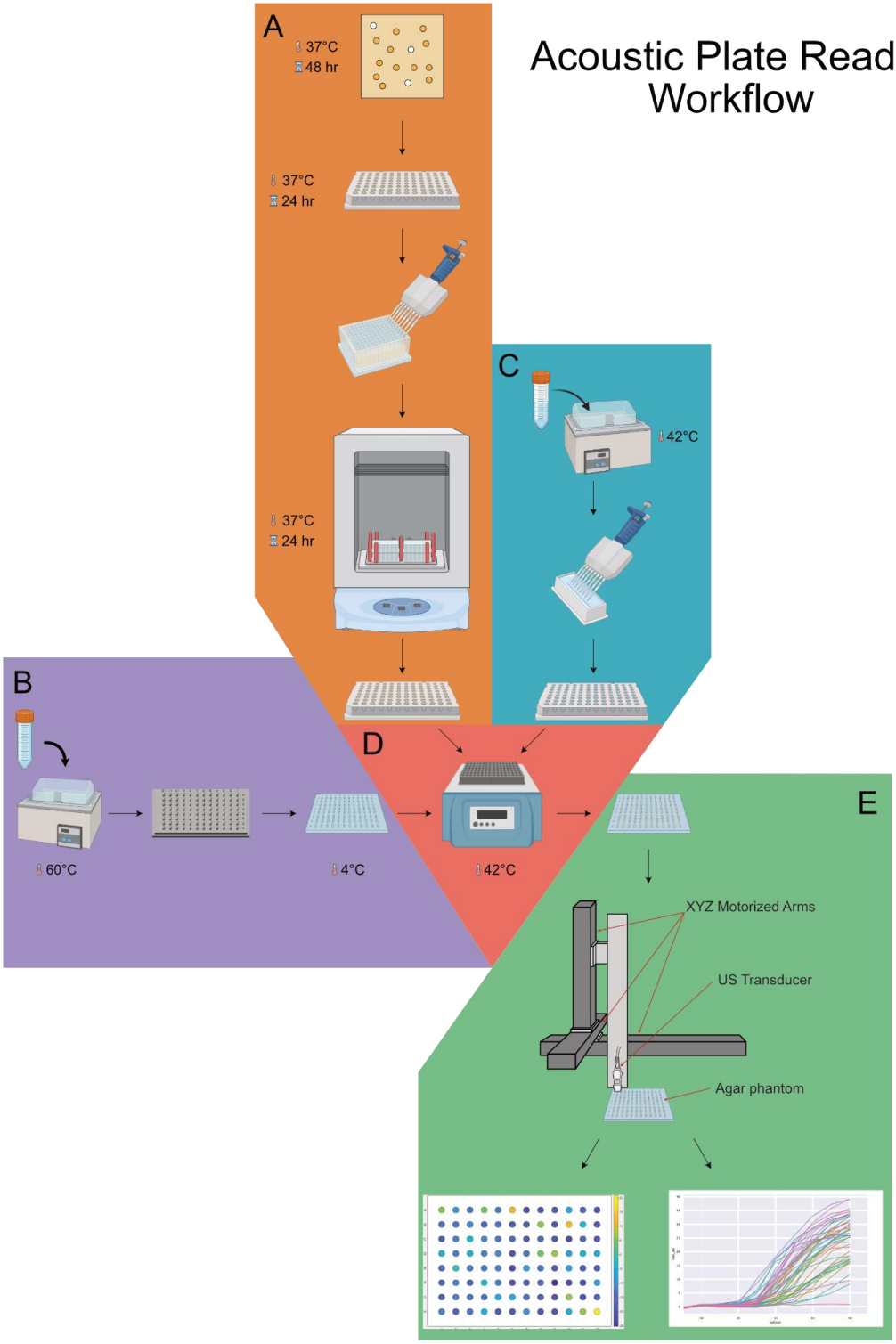

Figure S1. Detailed diagram of the Acoustic Plate Reader workflow. (A) GVs are expressed in *E. coli* as colonies on Petri dishes for 48 hr at 37°C, then colonies are picked into LB and grown to saturation in liquid culture for 24 hr at 37°C. These saturated liquid cultures are then diluted 1:100 into autoinduction LB and expressed for 24 hr at 37°C in 500 uL cultures in deep-well 96-

well plates (square wells are used for maximum culture aeration; USA Scientific 1896-2800). Aliquots of these cultures are aliquoted into an un-skirted 96-well PCR plate for subsequent loading into phantoms. (B) A solution of 2% Ultrapure Agarose (Invitrogen, 16500500) is prepared in 1X PBS and incubated at 60°C for at least 12 hr to degas. Agarose phantoms are then made by pouring 75 mL of this solution into a 96-well phantom mold and incubating at 4°C for 10 min. (C) A solution of 1% low-melting-temperature agarose (Goldbio, A-204-100) is prepared in 1X PBS and incubated at 60°C for at least 12 hr to degas. This solution is then aliquoted into an un-skirted 96-well PCR plate to be used for phantom loading. (D) Phantoms from B are loaded by placing the 96-well PCR plates from A and C into 96-well heat blocks at 42°C, and combining equal volumes of culture and agarose before pipetting into the empty phantom. (E) Phantoms from D are scanned using the acoustic plate reader, which generates US data for each sample and can image up to 12 96-well phantoms in a single scan.

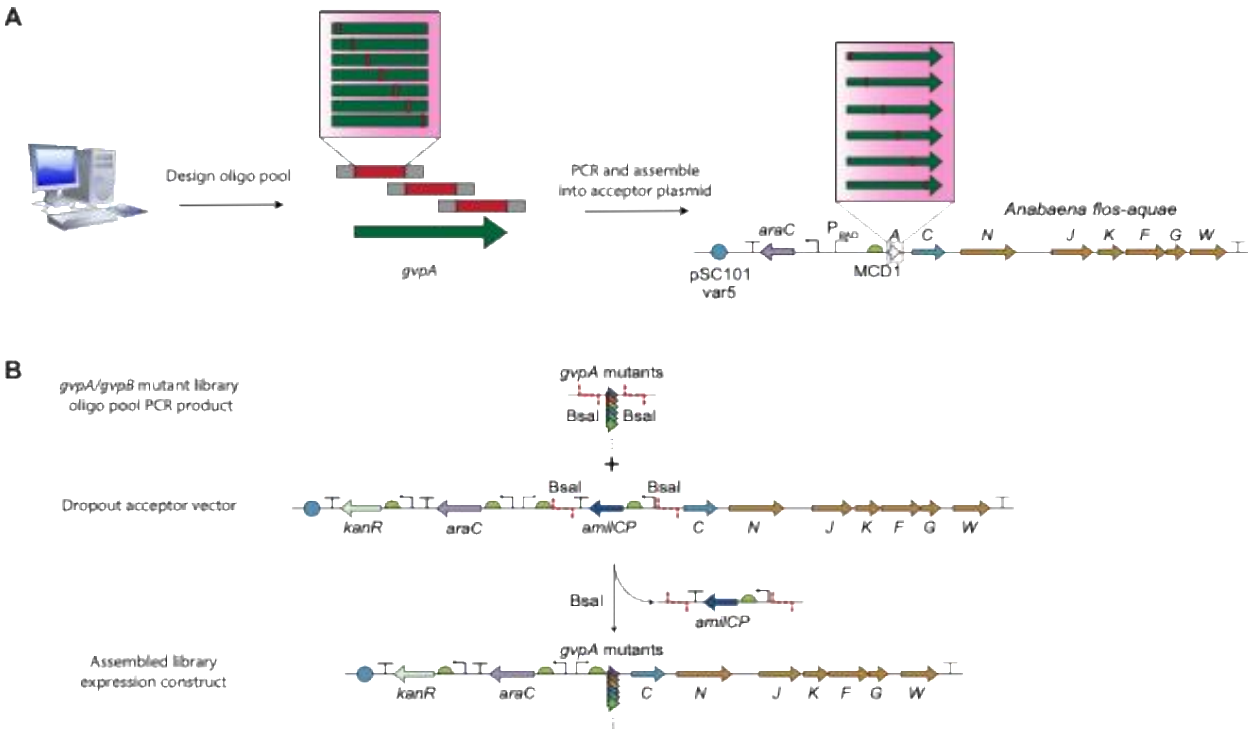

Figure S2. Details of *gvpA*/*gvpB* mutant library construction. (A) Overview of workflow for creating either scanning site saturation or recombination libraries. (B) Details of library assembly via a Golden Gate-based version of cassette mutagenesis (see Methods).

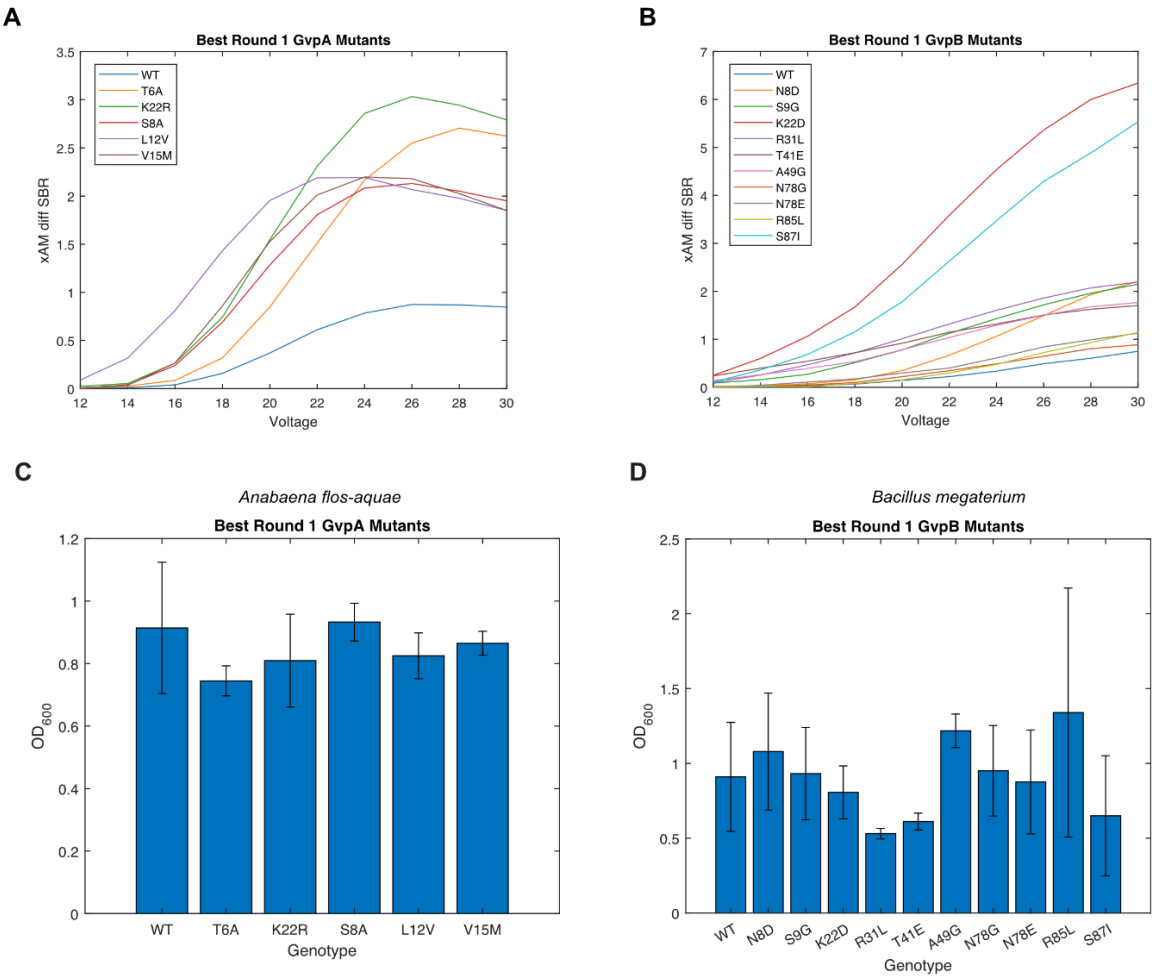

Figure S3. Characterization of the top mutants from Round 1 of evolution. (A-B) xAM difference SBR as a function of pressure for each of the top mutants. N=4 biological samples (each an average of 3 technical replicates). (C-D) OD600 measurements for the mutants shown in A-B. N=4 biological samples.

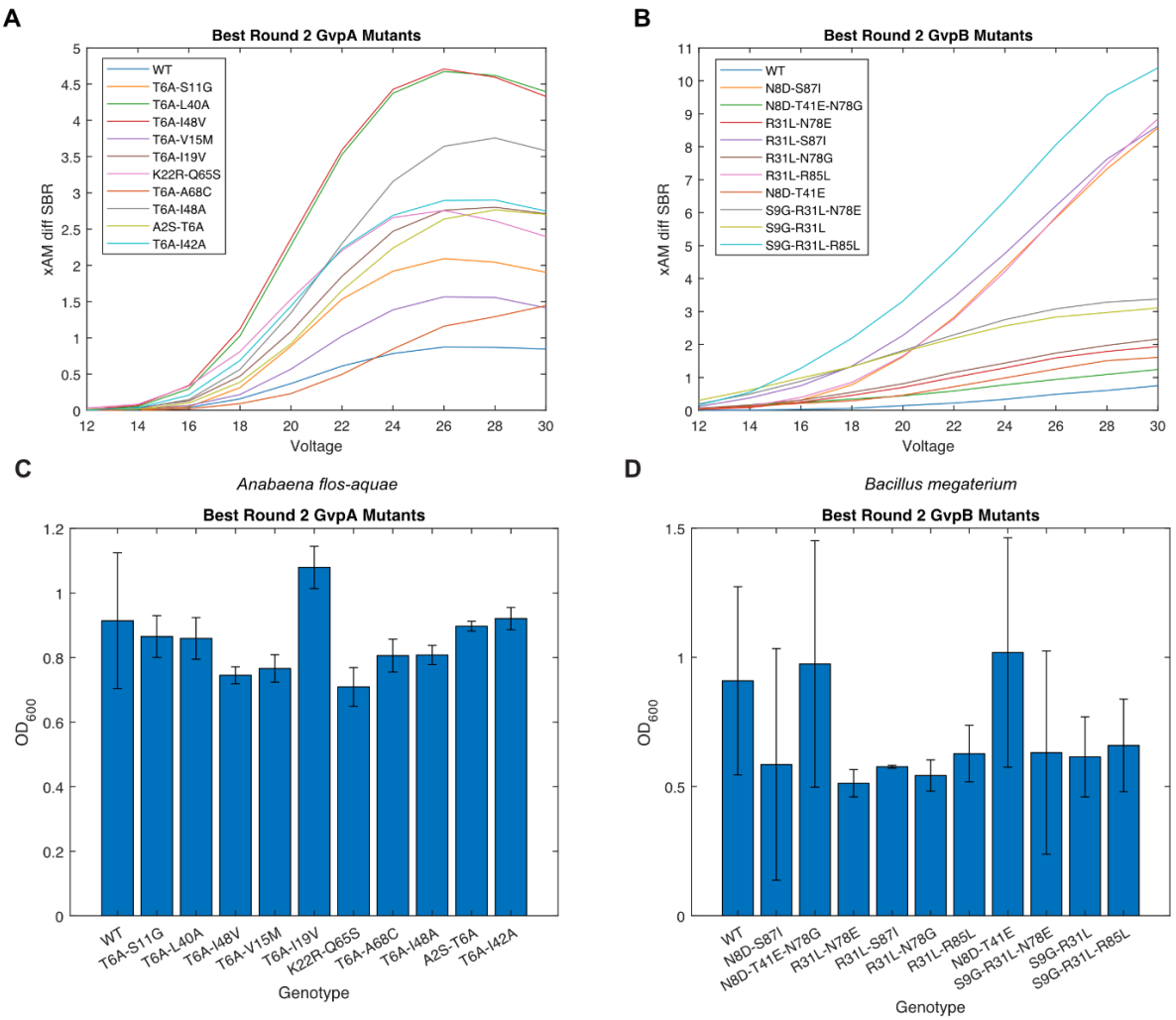

Figure S4. Characterization of the top mutants from Round 2 of evolution. (A-B) xAM difference SBR as a function of pressure for each of the top mutants. N=4 biological samples (each an average of 3 technical replicates). (C-D) OD600 measurements for the mutants shown in A-B. N=4 biological samples.

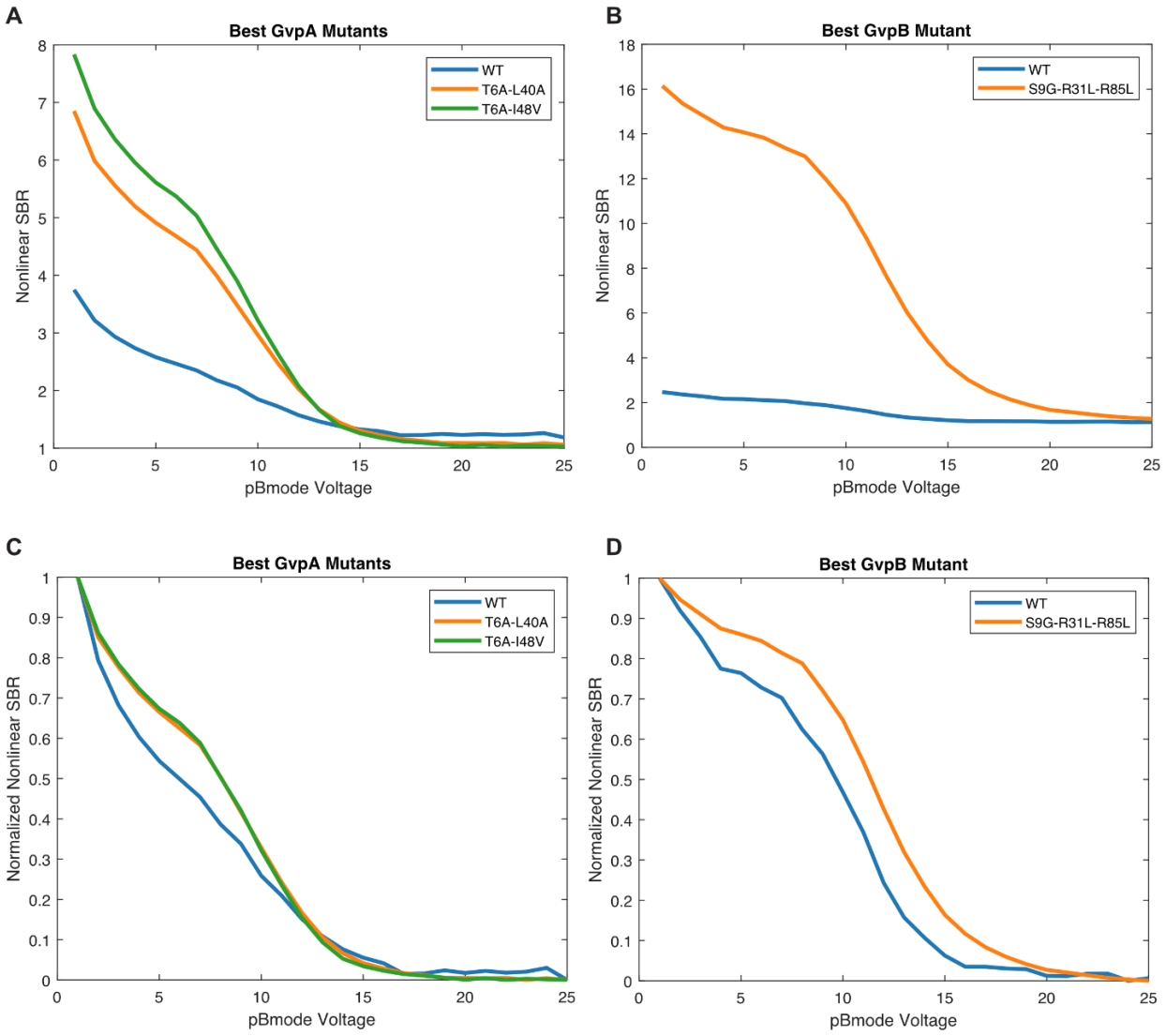

Figure S5. Acoustic collapse pressure curves for the best mutants identified in this study. Acoustic pressure was applied using a parabolic Bmode pulse sequence with increasing voltages, and nonlinear signals were acquired using xAM at 3.1 MPa after each voltage step. (A-B) xAM acoustic collapse pressure curves for the top-performing mutants identified in this study. (C-D) Data from A-B normalized to the same min and max. N=4 biological samples.

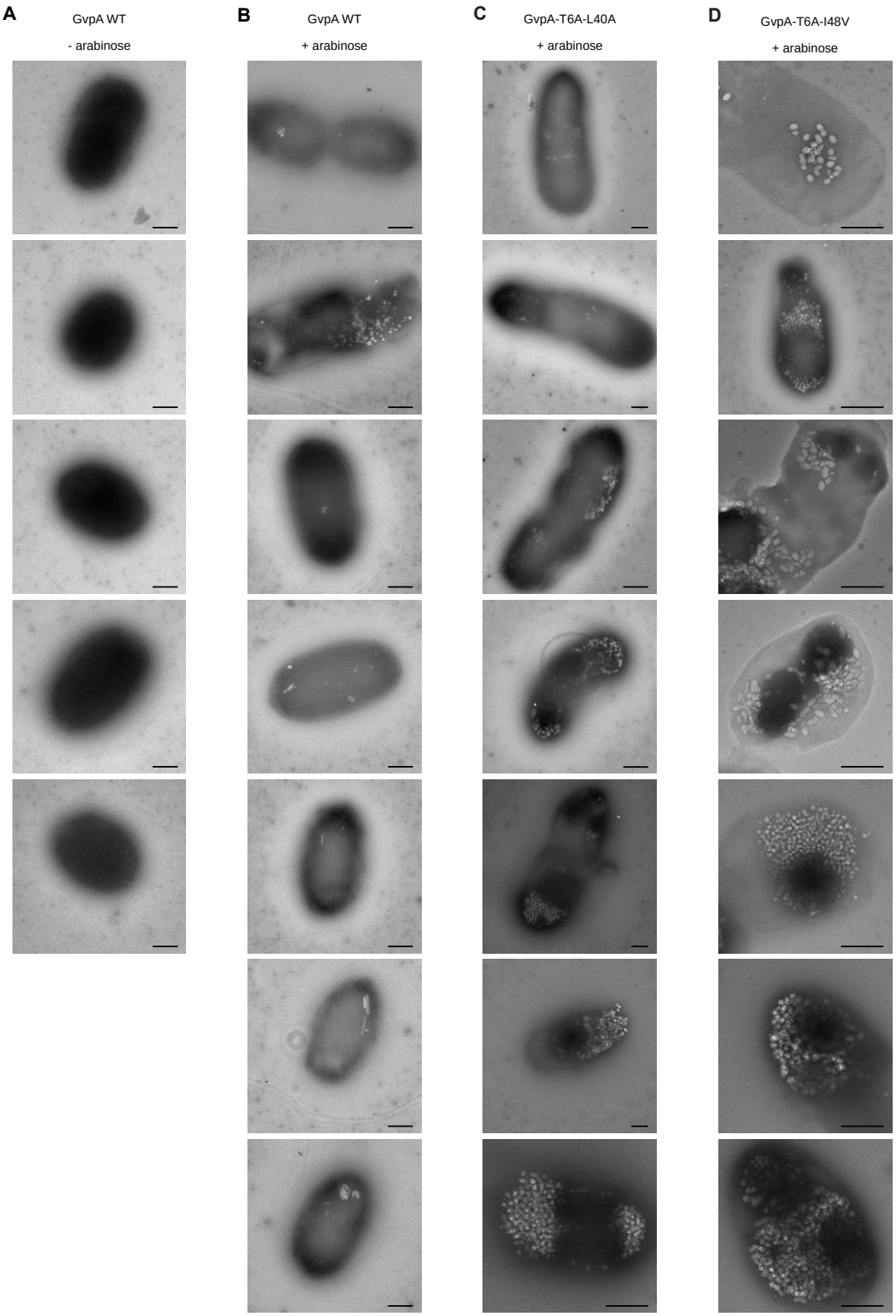

Figure S6. TEM images of *E. coli* cells expressing WT or mutant *A. flos-aquae* GVs. For each sample, 5-25 images were collected; a representative set is shown, ordered from least to most GVs produced per cell. Scale bars 500 nm.

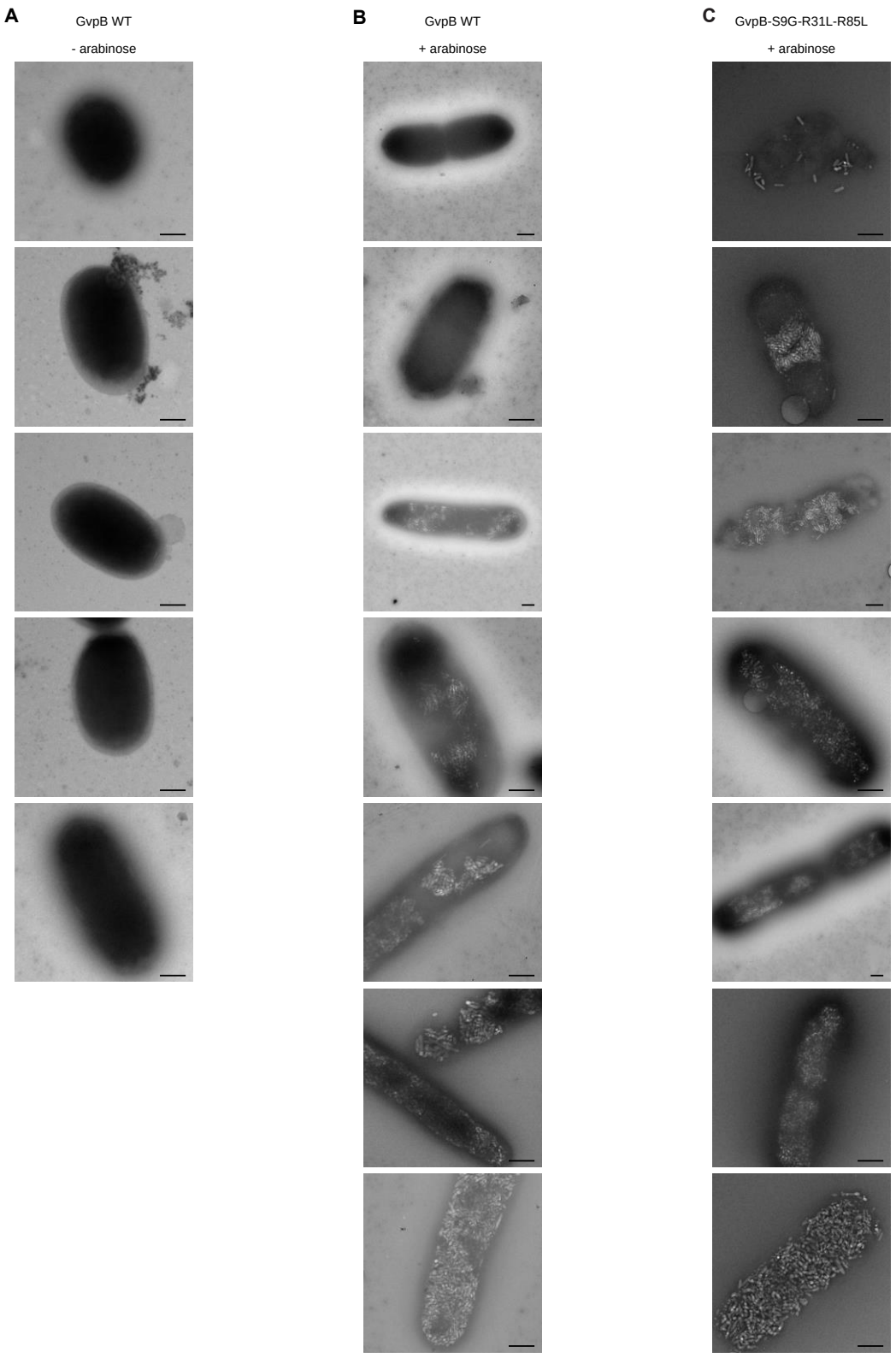

Figure S7. TEM images of *E. coli* cells expressing WT or mutant *B. megaterium* GVs. For each sample, 5-25 images were collected; a representative set is shown, ordered from least to most GVs produced per cell. Scale bars 500 nm.

Supplementary Note 1: Golden Gate reactions

Master mix recipes

Component	Amount per reaction	Stock concentration (NEB)	For 66 reactions
T4 Ligase Buffer	1 uL	10X	66 uL
Hi-T4 DNA Ligase	500 U	400 U/uL	83 uL
BsmBI-v2	15 U	10 U/uL	100 uL
Water	to 5 uL		81 uL

Component	Amount per reaction	Stock concentration (NEB)	For 66 reactions
T4 Ligase Buffer	1 uL	10X	66 uL
Hi-T4 DNA Ligase	500 U	400 U/uL	83 uL
BsaI-HF-v2 or BbsI-HF	15 U	20 U/uL	50 uL
Water	to 5 uL		131 uL

To set up reactions, combine 75 ng of the backbone part with 150 ng of each insert part in a PCR tube with 5 uL of the appropriate master mix and fill to 10 uL with water. Miniprepped parts give higher assembly efficiencies than linear PCR products.

Golden Gate thermocycler protocol
20 min 37/42°C
3 minutes 37/42°C
4 minutes 16°C

Cycle 2-3 x30
10 minutes 50°C
10 minutes 80°C
Hold 4°C