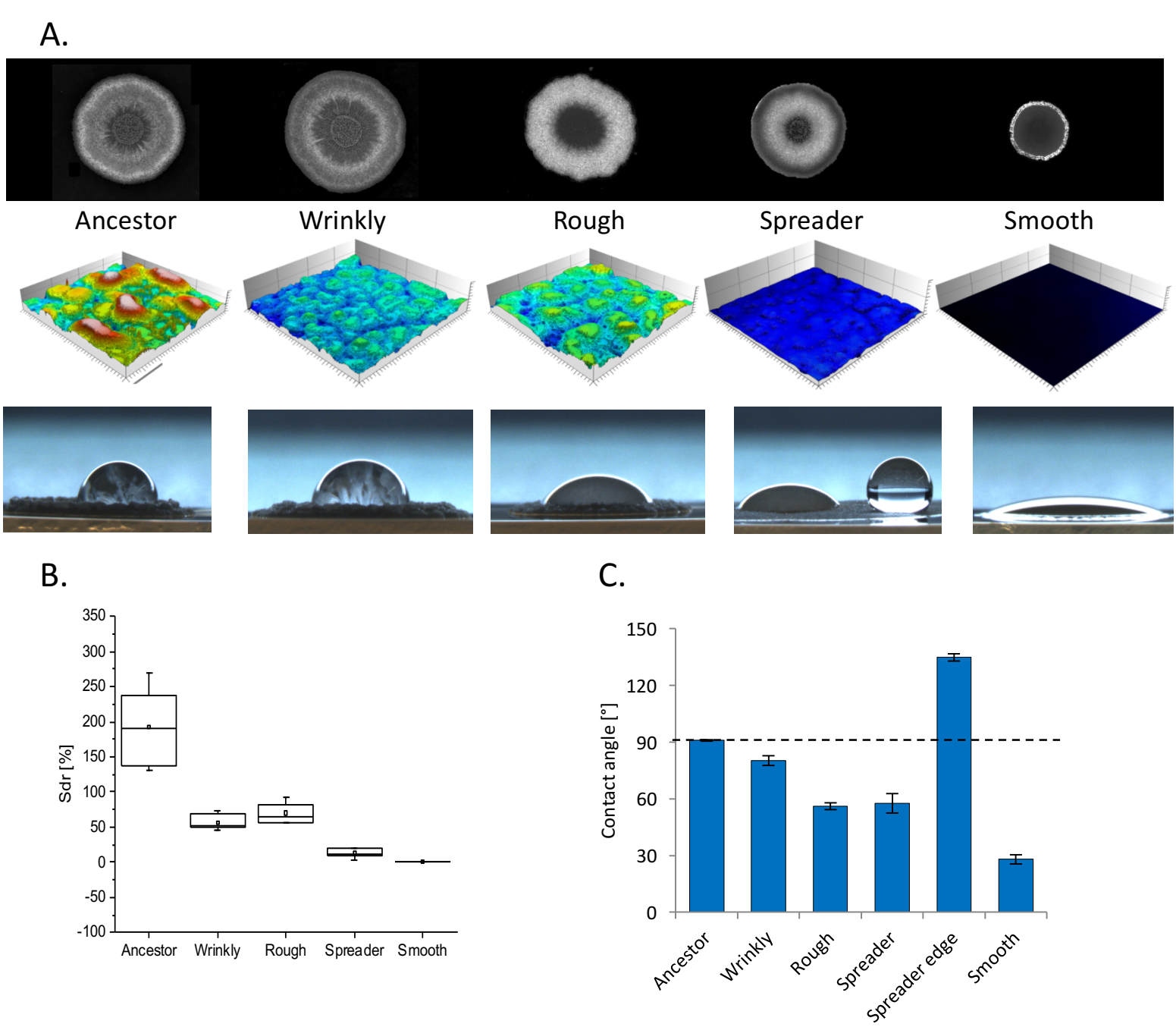
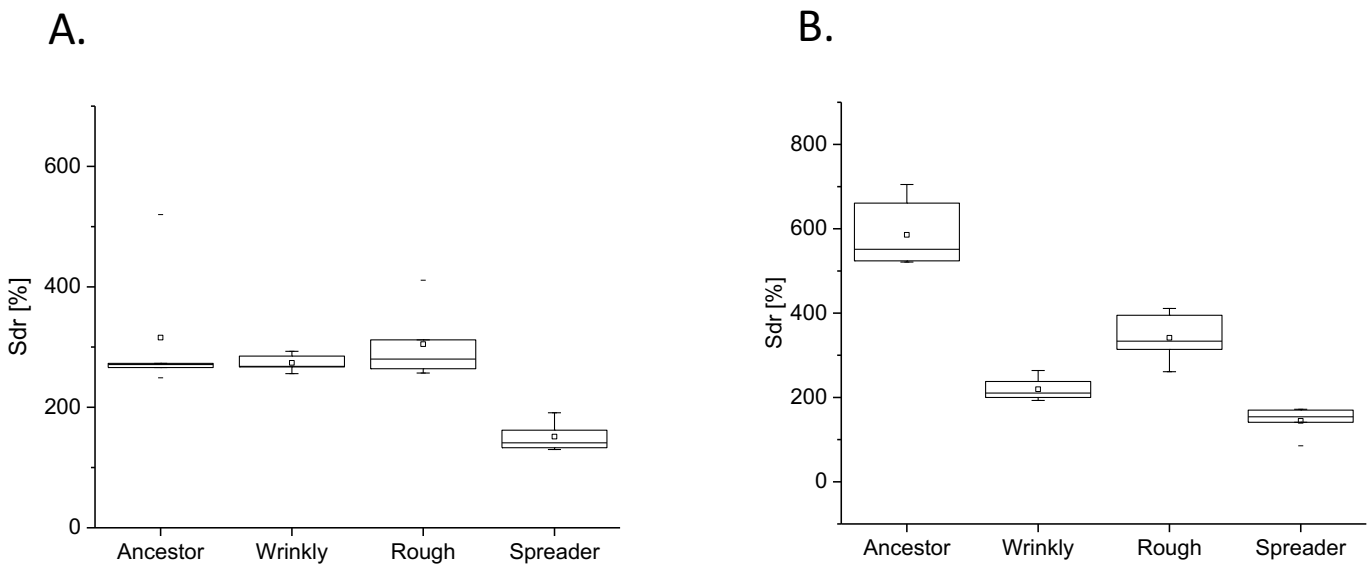
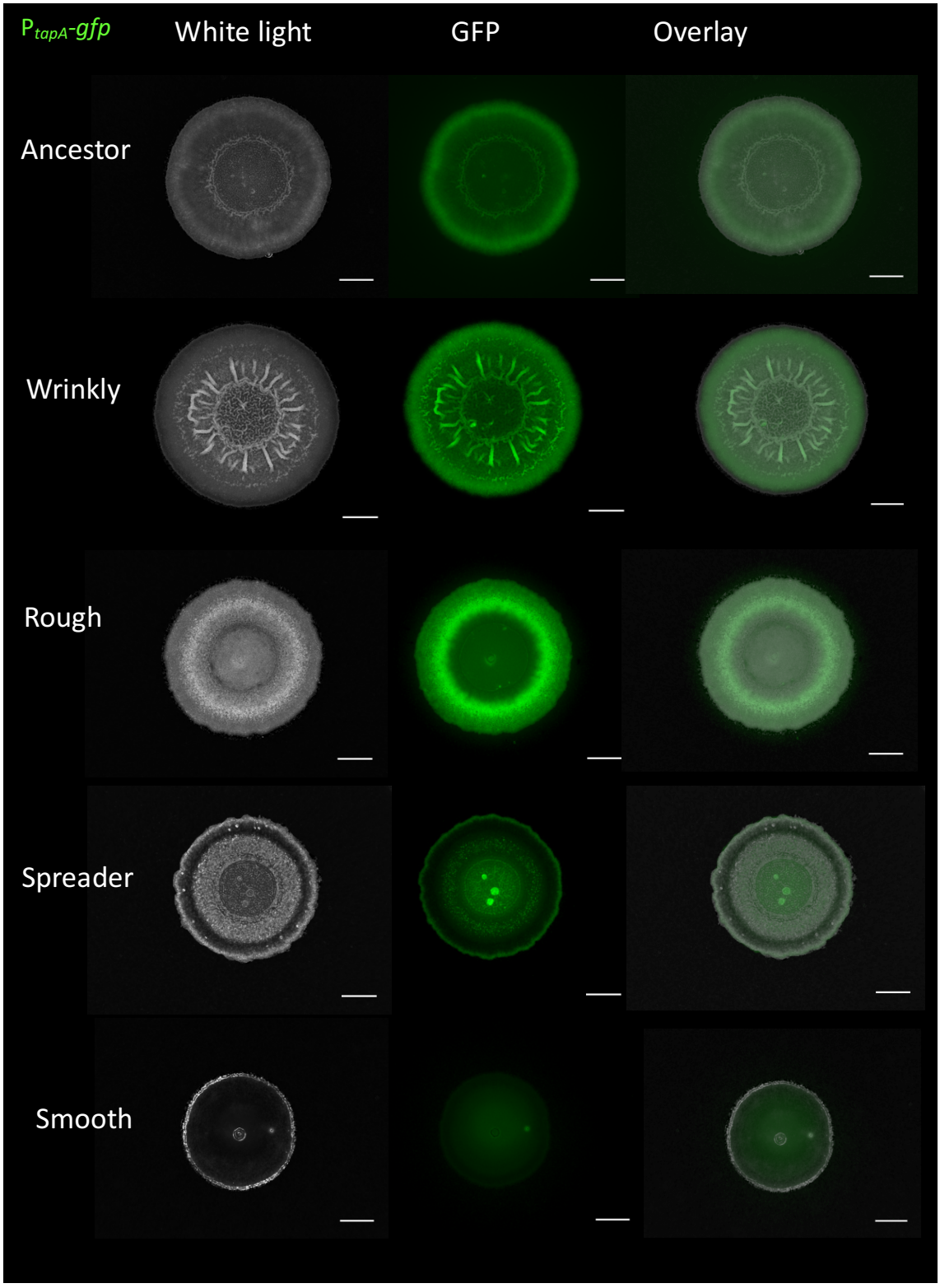


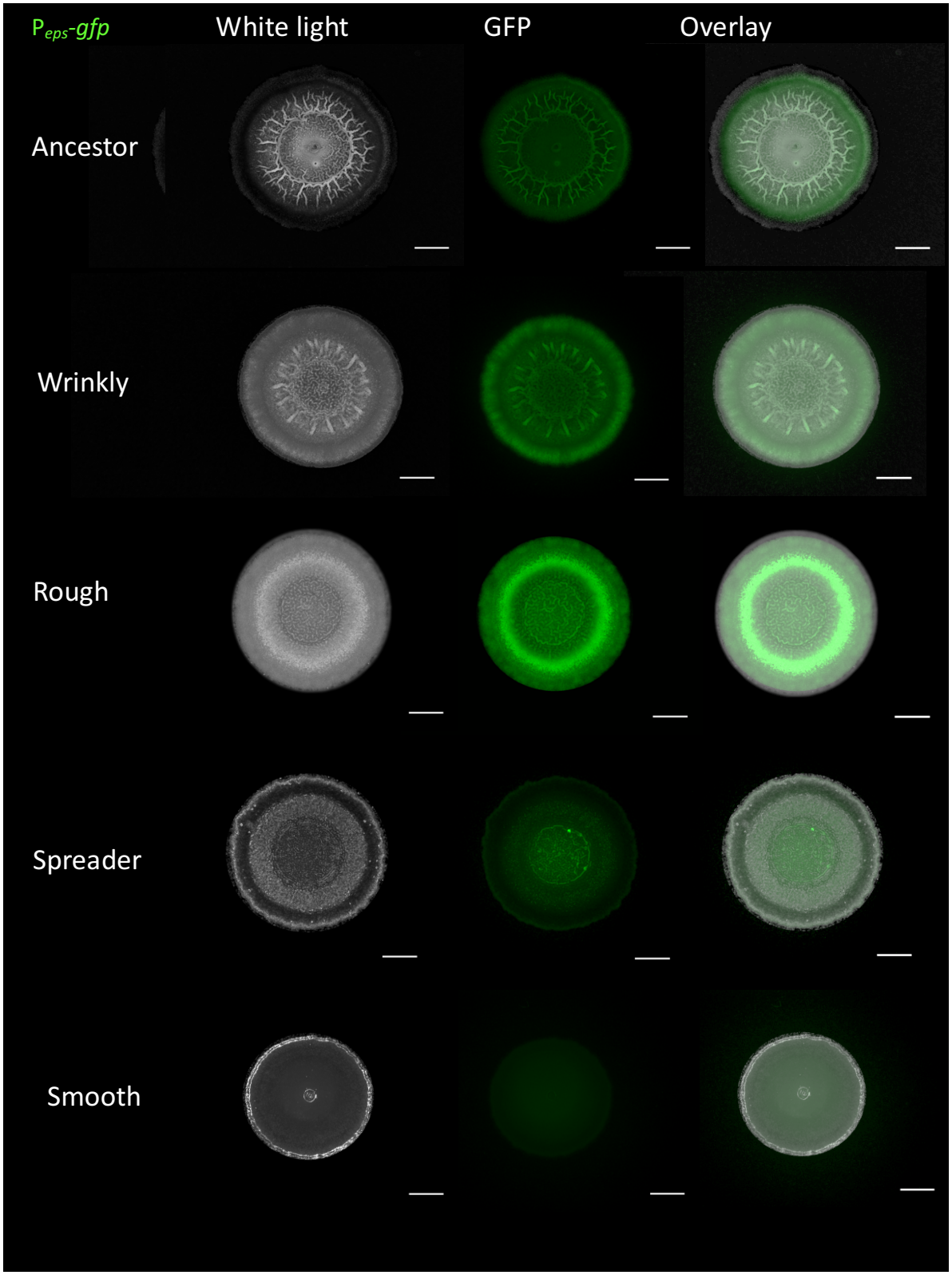
Supplementary Figure S1. Morphotypes morphology on MSgg and quantitative characterization of their colony features displayed on rich LB medium. **(A)** Colony morphologies of the ancestor and four distinct morphotypes spotted on MSgg medium (1.5% agar) are shown above. Middle, the surface topologies of the colonies developed on LB medium. Scale bar represents 200µm. Below, image of 10µl water droplet spotted in the center, or colony periphery in case of the Spreader morphotype. **(B)** Developed interfacial area *Sdr* calculated for colony center of the ancestor and four morphotypes (n=6 or n=5) for colonies grown on LB medium. Boxes represent Q1–Q3, lines represent the median, small squares represent the mean and bars span from max to min. **(C)** Contact angles of water spotted in the colony center, or colony periphery in case of the Spreader (n=3) for colonies grown on LB medium. Dashed line represents contractual hydrophobicity cut-off, separating the surfaces on hydrophilic (below the line) and hydrophobic (above). Data points represent mean and error bars represent standard error.



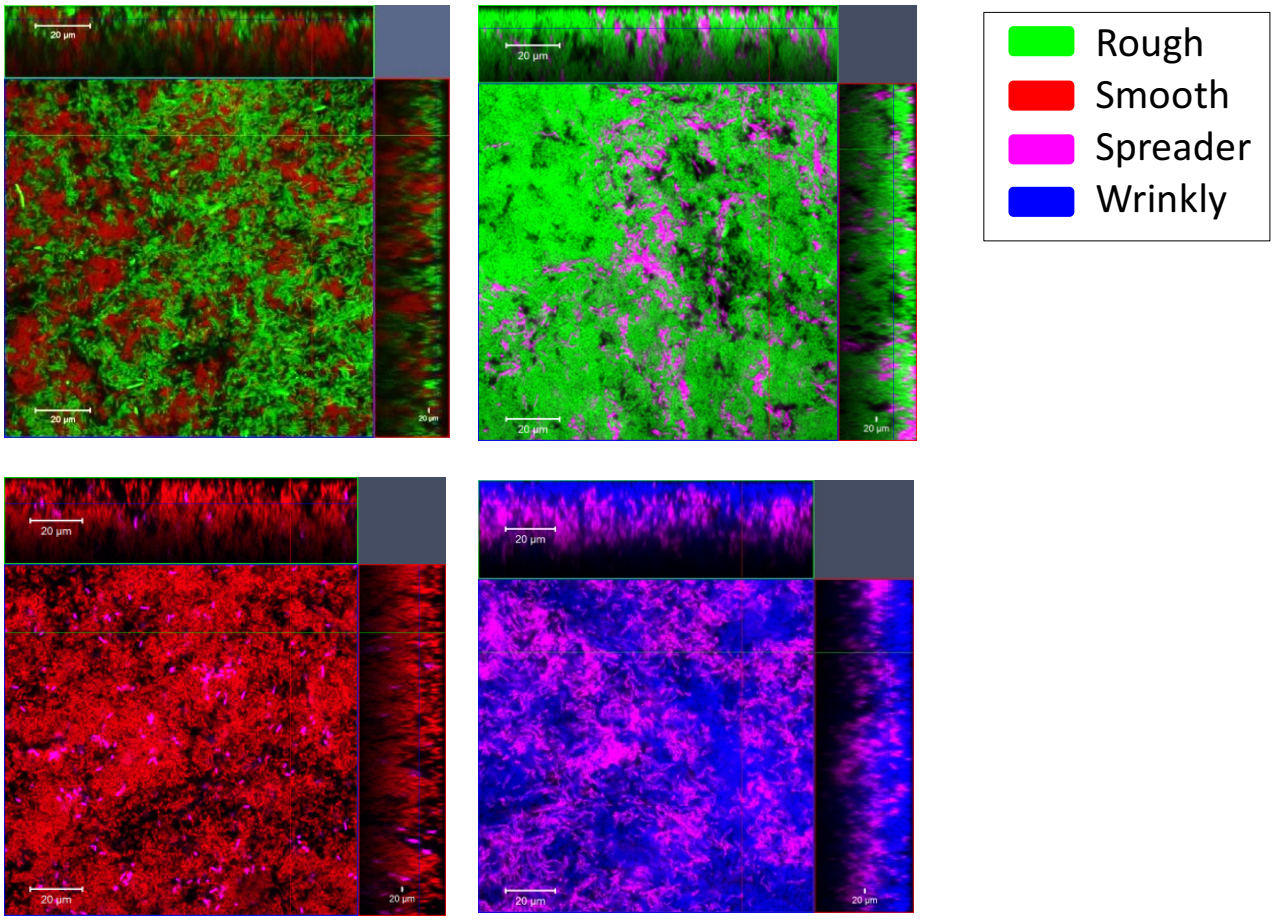
Supplementary Figure S2. Characterization of colony surface profiles at colony periphery. **(A)** Developed interfacial area *Sdr* calculated for colony periphery for colonies developed on MSgg medium (n=6 or n=5). **(B)** Developed interfacial area *Sdr* calculated for colony periphery for colonies developed on LB medium (n=6). Boxes represent Q1–Q3, lines represent the median, small squares represent the mean and bars span from max to min.



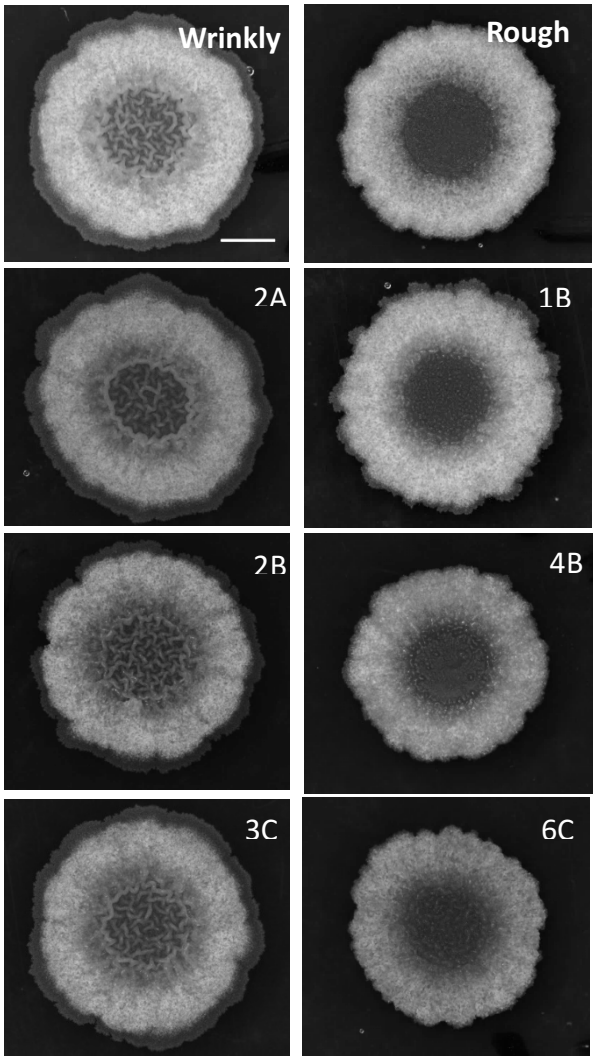
Supplementary Figure S3. Qualitative comparison of matrix-genes expression by different morphotypes. Expression of *tapA* was monitored in the ancestor and all morphotypes in colonies developed on MSgg using P_{tapA}-*gfp* reporter fusion. Scale bar represents 2mm.

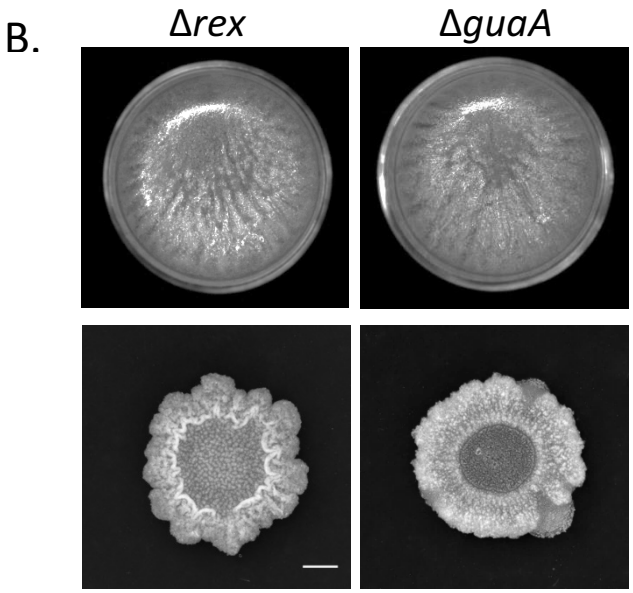


Supplementary Figure S4. Qualitative comparison of matrix-genes expression by different morphotypes. Expression of *eps* operon was monitored in the ancestor and all morphotypes in colonies developed on MSgg using P_{eps}-*gfp* reporter fusion. Scale bar represents 2mm.



Supplementary Figure S5. Control experiments with swapped fluorescent markers for estimating the spatial assortment of morphotypes in the pellicles. Confocal microscopy images of pellicle biofilms formed by the mix of four morphotypes were taken. Each time only 2 selected morphotypes were labelled with constitutive fluorescent reporters and could be visualized in the pellicle. Morphotypes were mixed in ratios 0.9: 5.3:0.6:3.2 ratios and pellicle were allowed to form for 48h at 30°C. To simplify image comparison, each morphotype was artificially labelled with the same assigned color (regardless on actual fluorescent marker used) across all images: Rough – green, Smooth – red, Spreader – purple, Wrinkly - blue. The actual mixed cultures were: $Rough_{GFP}$ + $Smooth_{mKate}$ (+Wrinkly and Spreader unlabeled), $Rough_{mKate}$ + $Spreader_{GFP}$ (+Wrinkly and Smooth unlabeled), $Smooth_{mKate}$ + $Spreader_{GFP}$ (+Rough and Wrinkly unlabeled), $Spreader_{mKate}$ + $Wrinkly_{GFP}$ (+Rough and Smooth unlabeled).





Supplementary Figure S6. Exploring molecular evolution pattern during diversification. **(A)** Morphologies of randomly picked colonies from populations 2-6 were evaluated and assigned to Wrinkly or Rough category based on similarity to those morphotypes from population 1. Isolates were subjected to whole- genome sequencing together with all morphotypes from population 1. Scale bar represents 2 mm. **(B)** Pellicle and colony morphologies of the ancestor strain carrying *rex* and *guaA* knockout strains. Pellicles and colonies were grown for 48h at 30°C. Well diameter is 1.5 cm. Scale bar represents 1 mm.

Strain name	Genotype	Reference
DK1042	NCIB 3610 *comI*^{Q12I}	Konkol, Blair and Kearns 2013
NRS2243	3610 *sacI::*P$_{eps}$-*gfp* (Km^{R})	Murray, Strauch and Stanley-Wall 2009
NRS2394	3610 *sacI::*P$_{tapA}$-*gfp* (Km^{R})	Murray, Strauch and Stanley-Wall 2009
BKE12280	168 *rex::mls*	Koo *et al.* 2017
BKE06360	168 *guaA::mls*	Koo *et al.* 2017
168 hyGFP	168 *amyE*::P$_{hyperspank}$-GFP (Cm^{R})	van Gestel *et al.* 2014
168 hymKate	168 *amyE*::P$_{hyperspank}$-mKATE2 (Cm^{R})	van Gestel *et al.* 2014
DTUB18	comI Rough *sacI::*P$_{eps}$-*gfp* (Km^{R})	This work
DTUB19	3610 *comI*^{Q12I} Wrinkly *sacI::*P$_{eps}$-*gfp* (Km^{R})	This work
DTUB20	3610 *comI*^{Q12I} Spreader *sacI::*P$_{eps}$-*gfp* (Km^{R})	This work
DTUB21	3610 *comI*^{Q12I} Smooth *sacI::*P$_{eps}$-*gfp* (Km^{R})	This work
DTUB22	3610 *comI*^{Q12I} Rough *sacI::*P$_{tapA}$-*gfp* (Km^{R})	This work
DTUB23	3610 *comI*^{Q12I} Wrinkly *sacI::*P$_{tapA}$-*gfp* (Km^{R})	This work
DTUB24	3610 *comI*^{Q12I} Spreader *sacI::*P$_{tapA}$-*gfp* (Km^{R})	This work
DTUB25	3610 *comI*^{Q12I} Smooth *sacI::*P$_{tapA}$-*gfp* (Km^{R})	This work
DTUB10	3610 *comI*^{Q12I} Rough *amyE*::P$_{hyperspank}$-*gfp* (Cm^{R})	This work
DTUB11	3610 *comI*^{Q12I} Wrinkly *amyE*::P$_{hyperspank}$-*gfp* (Cm^{R})	This work
DTUB12	3610 *comI*^{Q12I} Spreader *amyE*::P$_{hyperspank}$-*gfp* (Cm^{R})	This work
DTUB13	3610 *comI*^{Q12I} Smooth *amyE*::P$_{hyperspank}$-*gfp* (Cm^{R})	This work
DTUB14	3610 *comI*^{Q12I} Rough *amyE*::P$_{hyperspank}$-*mKATE2* (Cm^{R})	This work
DTUB15	3610 *comI*^{Q12I} Wrinkly *amyE*::P$_{hyperspank}$-*mKATE2* (Cm^{R})	This work
DTUB16	3610 *comI*^{Q12I} Spreader *amyE*::P$_{hyperspank}$-*mKATE2* (Cm^{R})	This work
DTUB17	3610 *comI*^{Q12I} Smooth *amyE*::P$_{hyperspank}$-*mKATE2* (Cm^{R})	This work
TB963	3610 *comI*^{Q12I} *rex::mls*	This work
TB964	3610 *comI*^{Q12I} *guaA::mls*	This work

Supplementary Table S1. Strains that were used in this study. NCIB 3610 *comI*^{Q12I} served as an ancestral strain in evolution experiment. Strains carrying fluorescent fusions were used as a source of genomic DNA to transform the evolved colony types. Strains lacking *rex* and *guaA* were used as a source of gDNA to transform the ancestral strain in order to test for phenotypic effects of those mutations.

REFERENCES

van Gestel J, Weissing FJ, Kuipers OP *et al.* Density of founder cells affects spatial pattern formation and cooperation in *Bacillus subtilis* biofilms. *ISME J* 2014;**8**:2069–2079.

Konkol MA, Blair KM, Kearns DB. Plasmid-Encoded ComI Inhibits Competence in the Ancestral 3610 Strain of *Bacillus subtilis*. *J Bacteriol* 2013;**195**:4085–4093.

Koo B-M, Kritikos G, Farelli JD *et al.* Construction and analysis of two genome-scale deletion libraries for *Bacillus subtilis*. *Cell Syst* 2017;**4**:291–305.

Murray EJ, Strauch MA, Stanley-Wall NR. SigmaX is involved in controlling *Bacillus subtilis* biofilm architecture through the AbrB homologue Abh. *J Bacteriol* 2009;**191**:6822-6832.