# Supplemental information

The roles of intracellular and extracellular

calcium in *Bacillus subtilis* biofilms

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The roles of intracellular and extracellular calcium in *Bacillus subtilis* biofilms
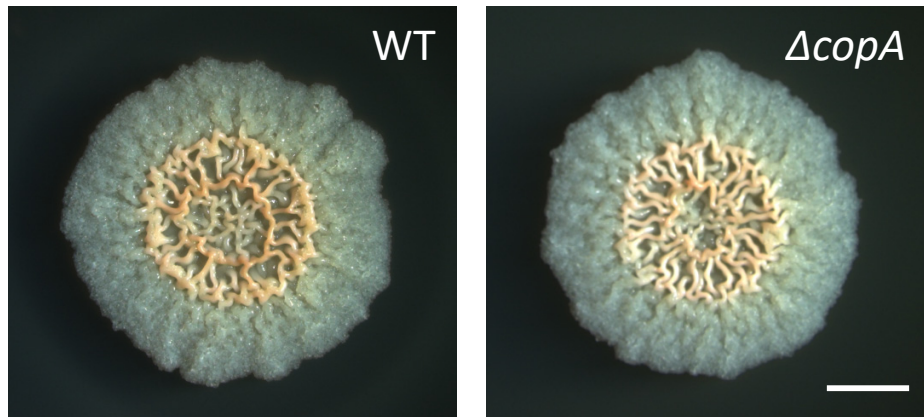
Supplementary Figures 1-8
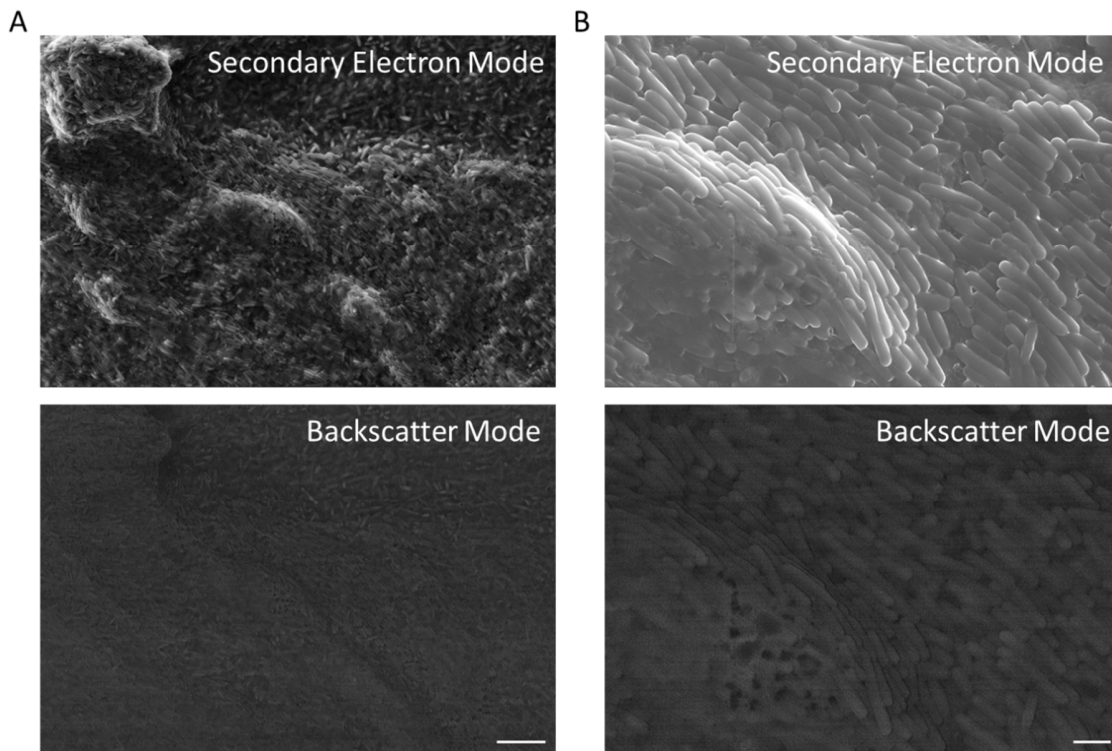
Supplementary Figure 1, Visualizing extracellular calcium carbonate in the absence of external calcium supplementation, related to Figure 2. Scanning Electron Microscopy (SEM) image of *B. subtilis* biofilm colony grown for 6 days on a B4 agar (without excess calcium). Shown are the surface of the biofilm in secondary electron and in backscattered mode of a representative field. **(A)** Magnification – X2500, scale bar – 10 μm. **(B).** Magnification – X10000, scale bar – 2 μm. Representative fields (out of n=4 fields, from 8 independent experiments) are shown.
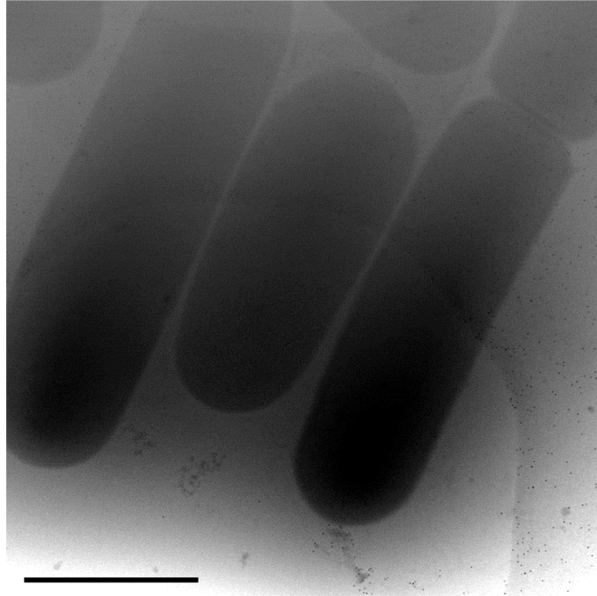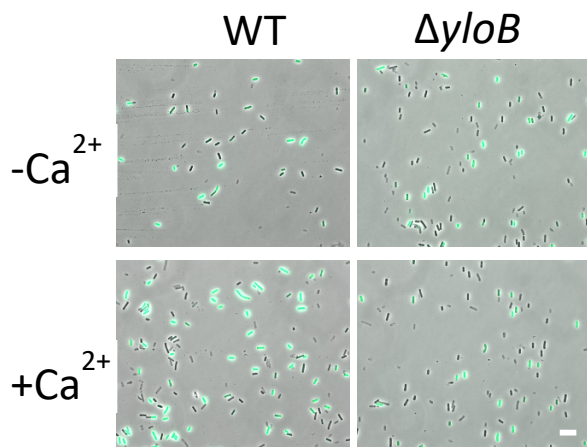
Supplementary Figure 2, Visualizing intracellular calcium in the absence of external calcium supplementation, related to Figure 3. Cryo-STEM bright-field images of representative biofilm cells from a *B. subtilis* colony grown for 10 days on B4 agar (without calcium). Scale bar 1 µm. A representative field imaged (out of n=30 cells, from 4 independent experiments is shown.

|	Dead	Spore	Mineral producer
Structured (n=510)	12.5%	12.7%	4%
Total colony (n=308)	7.5%	7.5%	1.6%

Supplementary Figure 3, Analysis of a subpopulation containing intracellular calcium deposits, related to Figure 3. Quantification of different subpopulations within a 6-day old *B. subtilis* biofilm colony, showing enrichment of cells with mineral foci in the structured areas (wrinkles) of the colony.

Supplementary Figure 5, Biofilm formation is impaired in *copA* mutant, deleted for the putative target of CueR, related to Figure 4. Light microscopy images of wild type *B. subtilis* (WT) and ΔcopA mutant. Biofilm colonies were grown on B4-Ca^{2+} agar for 3 days at 30°C. Scale bar – 2 mm. The experiment was repeated 3 times, in a technical quadruplicate – and representative images are shown.

Supplementary Figure 5. **Visualizing intracellular calcium in *yloB* mutant biofilm cells, related to Figure 5.**Intracellular calcium levels of biofilm cells (n=200) stained with calcein-AM, a calcium-specific fluorescent dye as done previously (1). Biofilm cells were washed with PBS and mildly sonicated as done by us previously (2) prior to staining. Scale bar corresponds to 2 μm.
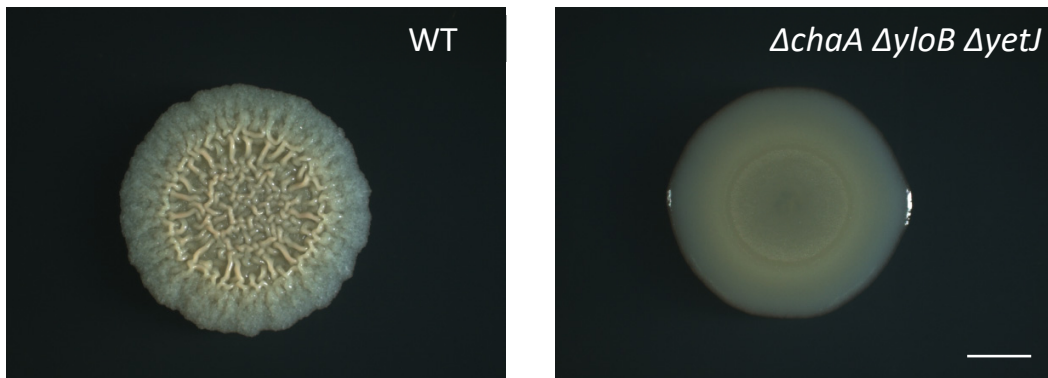
Supplementary Figure 6, Biofilm formation is impaired in *a triple* mutant in calcium importers, related to Figure 5. Light microscopy images of wild type (WT) and triple *ΔchaA ΔyloB ΔyetJ* mutant. Biofilm colonies grown on solid B4-Ca^{2+} agar for 3 days at 30°C. Scale bar – 2 mm. The experiment was repeated 3 times, in a technical quadruplicate – and representative images are shown.

Medium	pH					
	medium control	far from colony	close to colony	below the colony	colony center	Medium Langelier Saturation index (LSI)
B4Ca^{2+}	6	7.5	7.75	7.75	8	0.51-0.015

Supplementary Figure 7, the medium is not saturated by calcium carbonate promoting spontaneous sedimentation, related to Figure 6. pH of colonies and the agar medium they were grown on was assessed using pH-indicator strips (MColorpHast™). The experiment was conducted at least twice for each medium, with at least 4 technical repeats. LSI calculation refers to the indicated growth media. ND – undetermined. Solution is slightly scale forming and corrosive.

WT   **Δ*yloA***
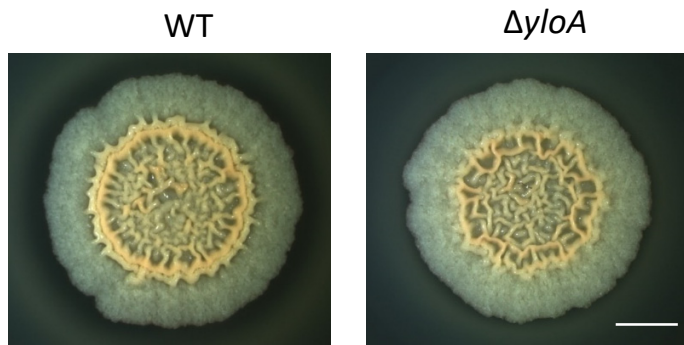
Supporting Figure 8, *yloA* mutant does not have a biofilm phenotype, related to Figure 5. Light microscopy images of wild type (WT) and *ΔyloA* mutant. Biofilm colonies grown on solid B4-Ca^{2+} agar for 3 days at 30°C. Scale bar – 2 mm. The experiment was repeated 3 times, in a technical quadruplicate – and representative images are shown.