SUPPLEMENTARY INFORMATION

Soft X-ray tomography reveals variations in *B.subtilis* biofilm structure upon *tasA* deletion

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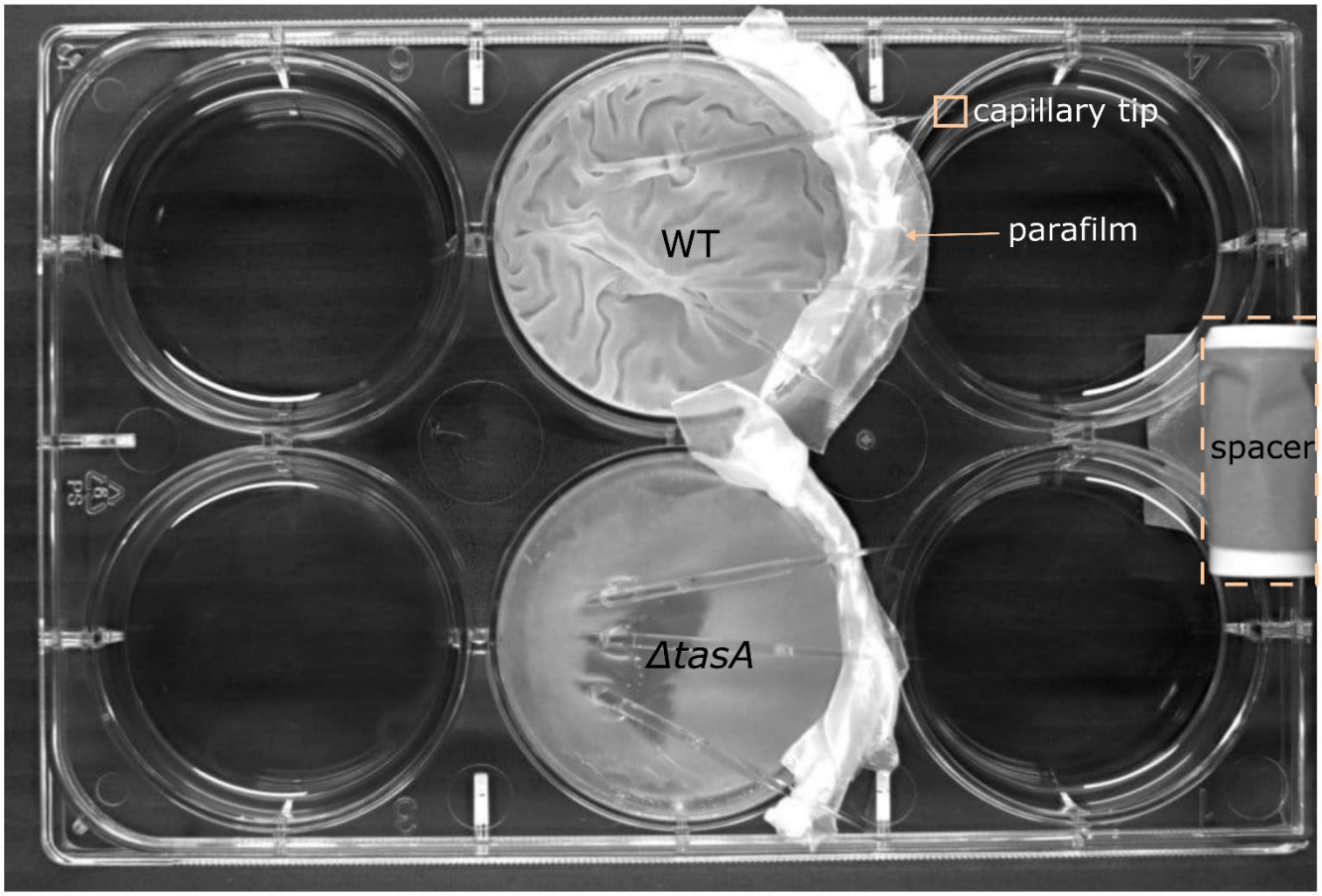
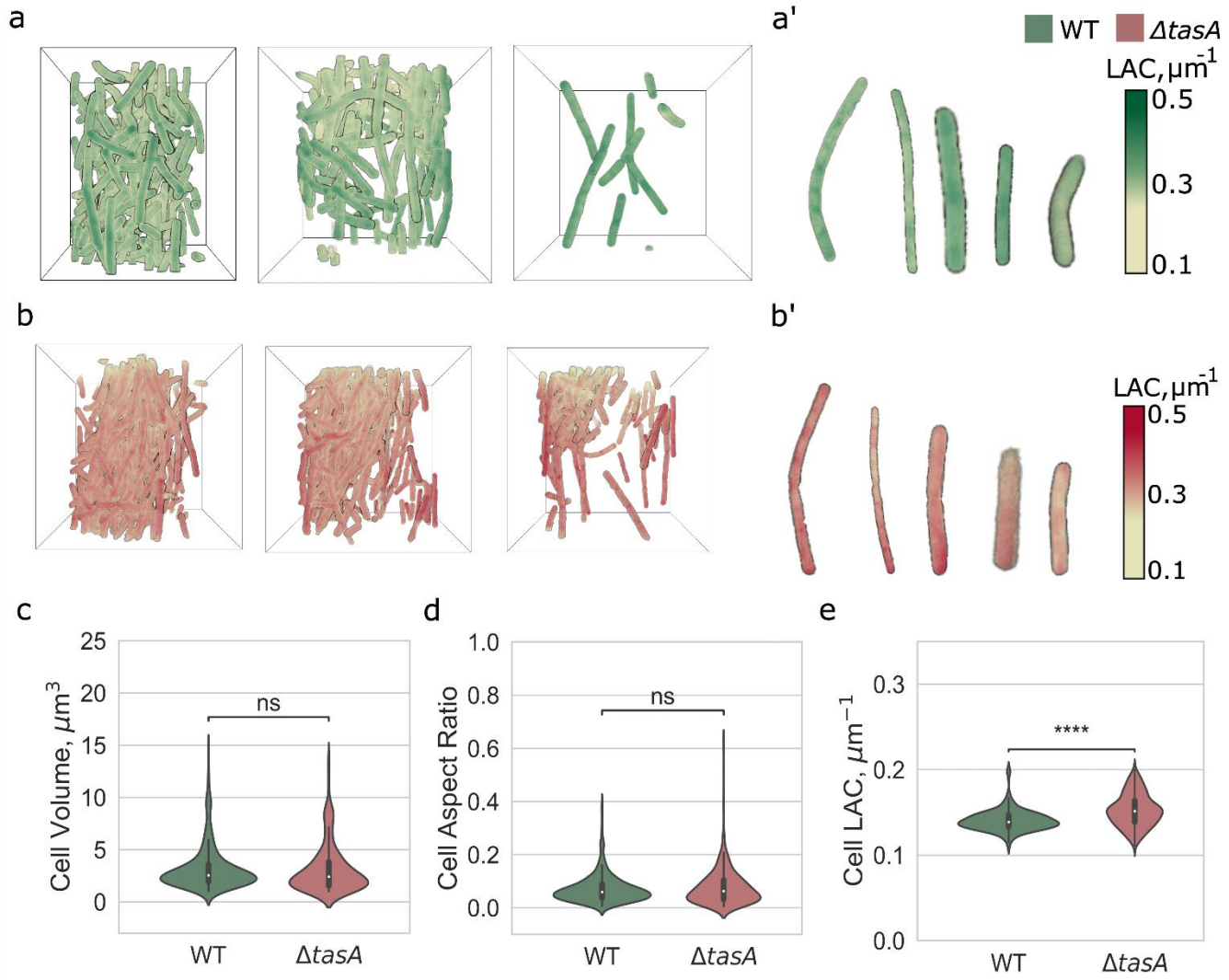
Supplementary Table 1

Genotype	WT			*ΔtasA*					*ΔtasA* + TasA	
Parameters	Bacteria t=0h	Bacteria t=22h	ECM fragments t=22h	Bacteria t=0h	Bacteria t=22h	ECM fragments t=22h	Bacteria t=44h	ECM fragments t=44h	Bacteria t=44h	ECM fragments t=44h
Sample size (n)	356	201	-	254	261	-	131	-	98	-
Volume (μm^3)	3.23 ± 2.14	2.86 ± 1.42	-	3.16 ± 2.33	3.24 ± 1.64	-	1.78 ± 0.48	-	1.56 ± 0.34	-
LAC (μm^{-1})	0.14 ± 0.01	0.16 ± 0.01	0.11 ± 0.02	0.15 ± 0.02	0.16 ± 0.01	0.14 ± 0.01	0.16 ± 0.01	0.10 ± 0.01	0.15 ± 0.00	0.11 ± 0.01
Aspect Ratio	0.07 ± 0.05	0.12 ± 0.07	-	0.08 ± 0.07	0.13 ± 0.11	-	0.19 ± 0.01	-	0.24 ± 0.08	-



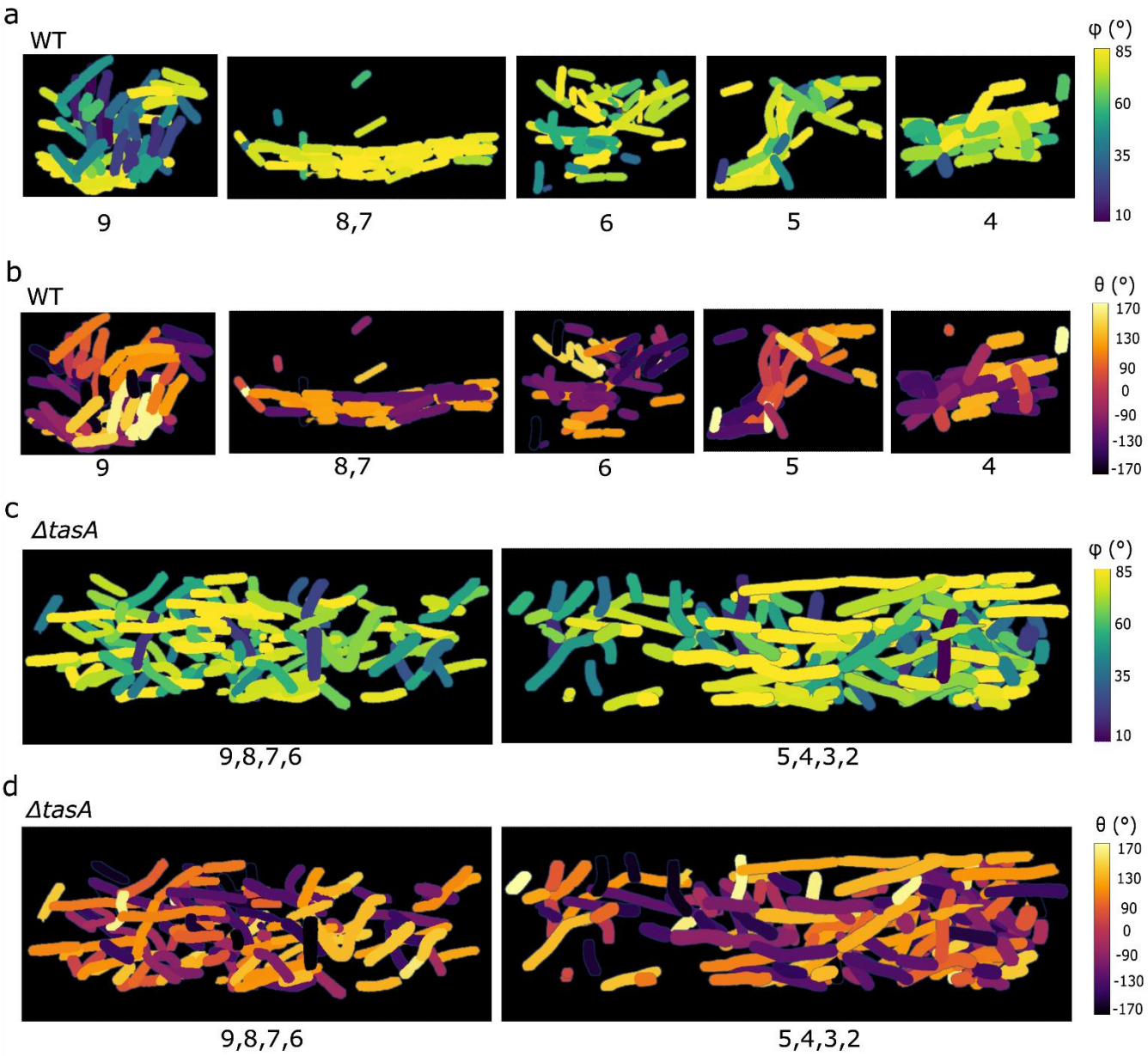
Supplementary Figure 1: 'Biofilm-in-capillary' set-up.

Biofilm from WT (top) and Δ*tasA* (down). The capillaries are positioned with their tip up, supported by wrapped parafilm on top of the 6-well plate cavities. A spacer is used to ensure tip integrity after covering it with the plate lid. Finally, the set-up is covered with a plastic bag and wet towels to ensure the required humidity for biofilm to grow for a day of incubation (30°C, t=22 h).



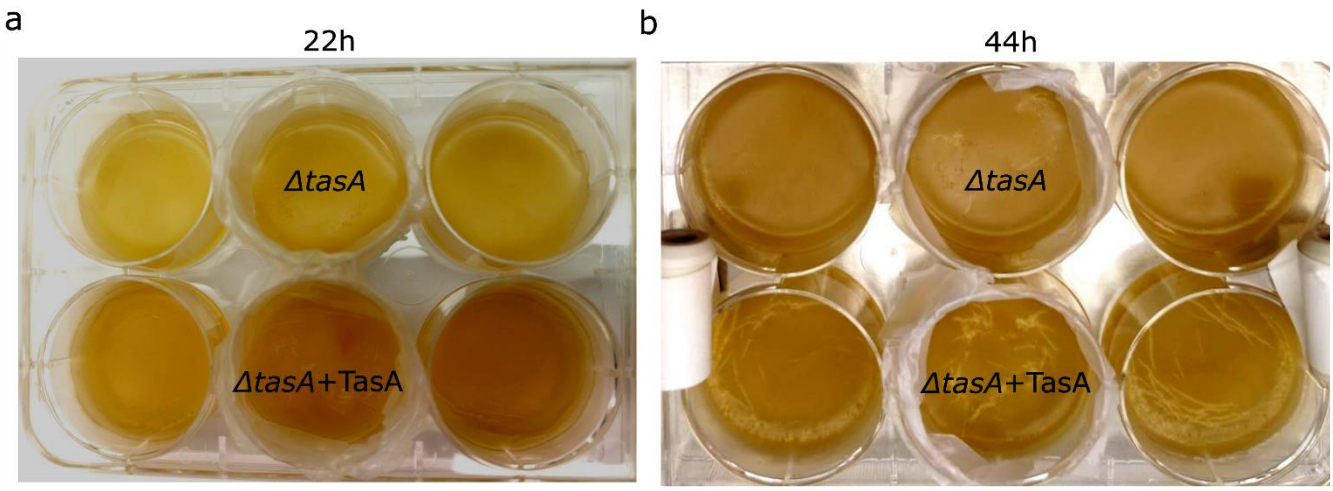
Supplementary Figure 2: Quantitative 3D analysis on single dispersed bacteria from shaking cultures on LB shows no major phenotypic differences between WT and Δ*tasA* populations.

(**a-b**) Three-dimensional renderings showing different *B.subtilis* culture densities without the formation of biofilm. Since the mean volume of *B.subtilis* is approximately 2-3 μm^3, within the field of view (FOV) of 15 μm in SXT, quantitative 3D analysis is performed on hundreds of WT (green) and Δ*tasA* (red) cells. (**a'-b'**) Three-dimensional renderings representing phenotypic variability of single bacteria, shown all together in panels (a-b). Linear absorption coefficient (LAC) scale is scaled from 0.1 μm^{-1} to 0.5 μm^{-1}. (**c**) Cell volume (μm^3) distribution range is increased from WT to Δ*tasA* group. (**d**) A subpopulation of Δ*tasA* bacteria tends towards a rounder shape compared to WT, even though overall both groups have a typical rod shape of bacilli. The single-bacteria renderings of (a'-b') show a similar picture. (**e**) Cell content density, characterized by LAC, is significantly increased in the Δ*tasA* group, which has higher variability, compared to the WT. Statistical analysis performed in t-test independent samples with Bonferroni correction, p-value (p) ns: 0.05 < p <= 1.00, (*): 0.01 < p <= 0.05, (**): 0.001 < p <= 0.01, (***): 0.0001 < p <= 0.001, (****): p <= 0.0001, with sample-size (n) for cell analysis, n=356 (WT) and n=254 (Δ*tasA*), respectively.



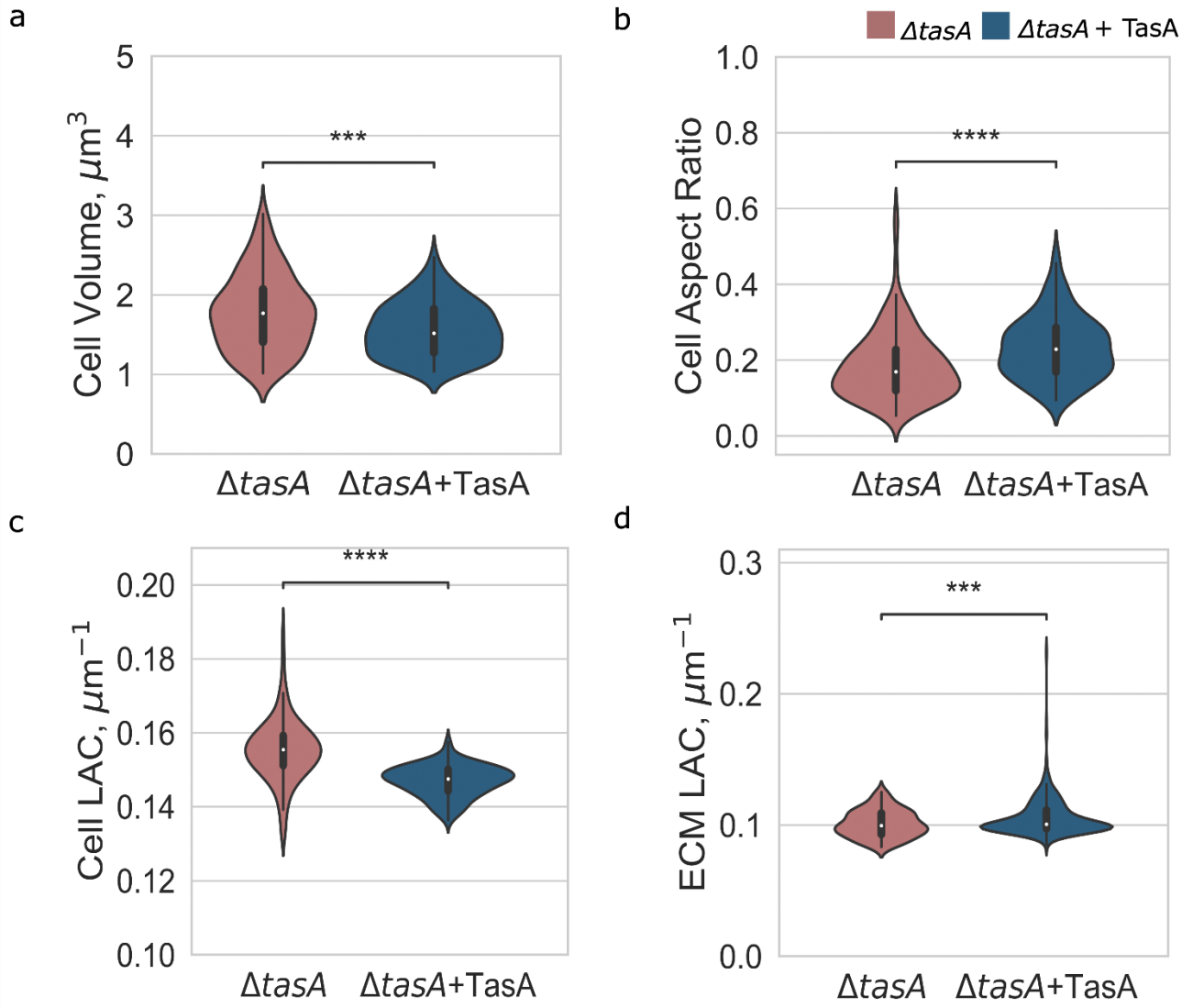
Supplementary Figure 3: Δ*tasA* bacteria lack the 3D organization that describes WT bacteria in the biofilm.

(**a-b**) WT bacteria in biofilm (t=22 h) show overall vertical alignment (yellow cells, φ=80-85 °) across the capillary length, as shown in FOVs 4-9. The bacteria that engage in the helix-loop (FOV 9) tend to be more horizontal (magenta, φ=10-20 °). A similar pattern is observed also for the θ ° alignment, with the best example shown in FOV 5. (**c-d**) 3D organization loss is visible in Δ*tasA* cultures (t=22 h), with a lot of vertical cells acquiring random θ ° angles (see FOV 2-5). Colors for φ ° are scaled from 10 ° to 85 ° and for θ ° from -170 ° to 170 °.



Supplementary Figure 4: The TasA addition partially rescues the *ΔtasA* biofilm phenotype.

(**a**) Macroscopic view of ΔtasA bacterial biofilm treated with TasA at 22h and (**b**) 44h. The aggregating fibrils on the surface are more prominent with the increased incubation time, partially restoring the biofilm phenotype.



Supplementary Figure 5: Cell and ECM analysis of Δ*tasA* and Δ*tasA* + TasA biofilm.
(**a-b**) Δ*tasA* Bacteria decrease in volume (μm^3), as they acquire a rounder shape, upon TasA addition (t=44 h). (**c**) Upon TasA treatment the Δ*tasA* cells decrease in their cell LAC (μm^{-1}). (**d**) TasA addition contributes to an increase in ECM LAC (μm^{-1}). Statistical analysis performed in t-test independent samples with Bonferroni correction, p-value (p) ns: 0.05 < p <= 1.00, (*): 0.01 < p <= 0.05, (**): 0.001 < p <= 0.01, (***): 0.0001 < p <= 0.001, (****): p <= 0.0001, with sample-size (n) for cell analysis, n=131 (Δ*tasA*, t=44 h) and n=98 (Δ*tasA*+TasA, t=44 h), respectively.

Supplementary video 1: Time-lapse epi-fluorescence imaging of individual bacterial cells migrating to the open tip of the capillary over 320s.

Supplementary video 2: 3D renderings of WT *B. subtilis* BKD211 biofilm (t=22 h) showing helix-like, orientation of the bacteria along the capillary. The ECM fragments (dark green) show an uneven distribution of foam-like substructures along the capillary.

Supplementary video 3: 3D renderings of *ΔtasA B. subtilis* BKD230 biofilm (t=22 h) revealing the loss of structured orientation of the *ΔtasA* bacteria along the capillary. The ECM fragments (dark red) show string-like structures.