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Supporting Information

Supporting Figures (S1-S8)

Supporting Table (S1 and S2)

Supporting References

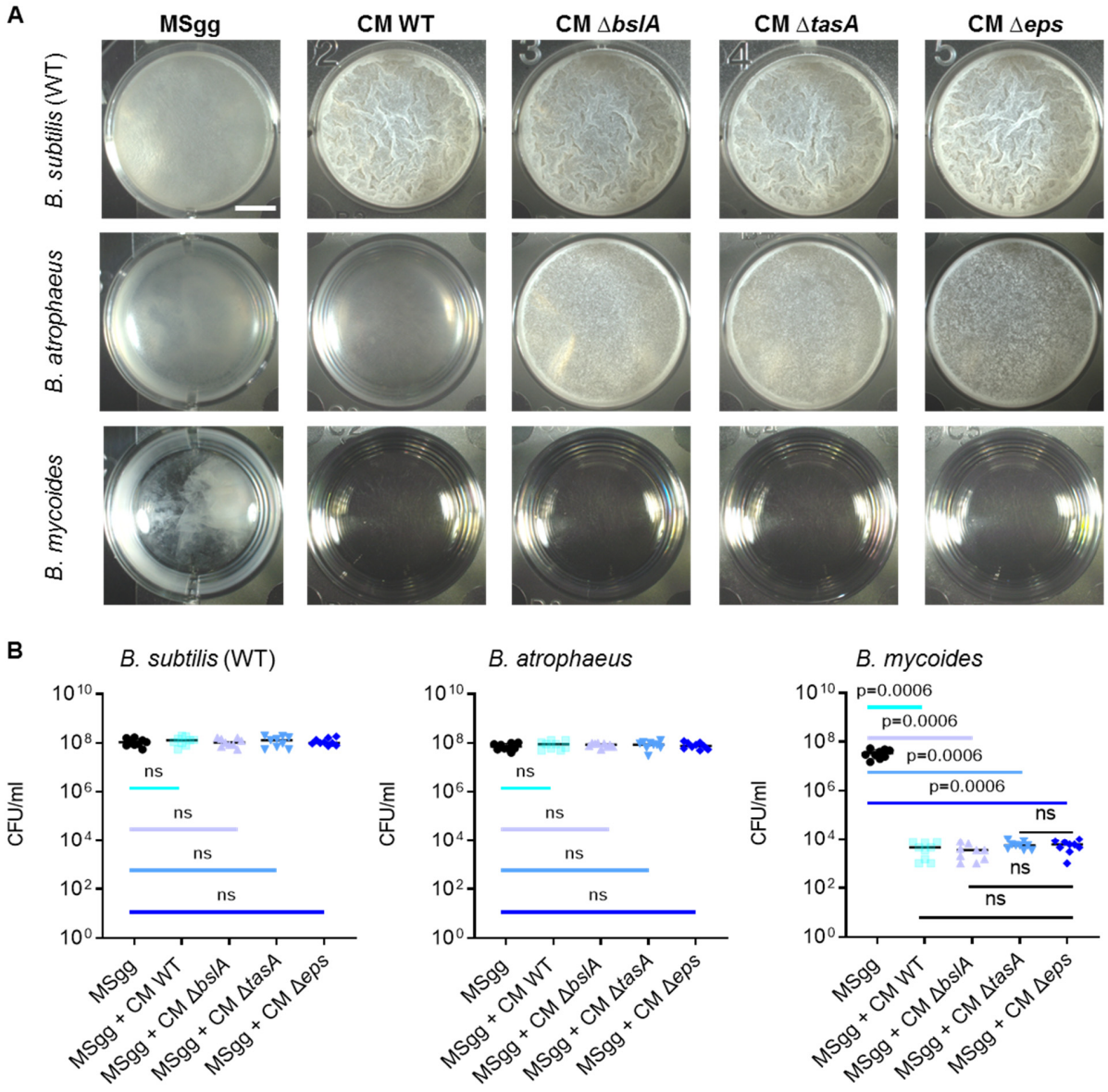
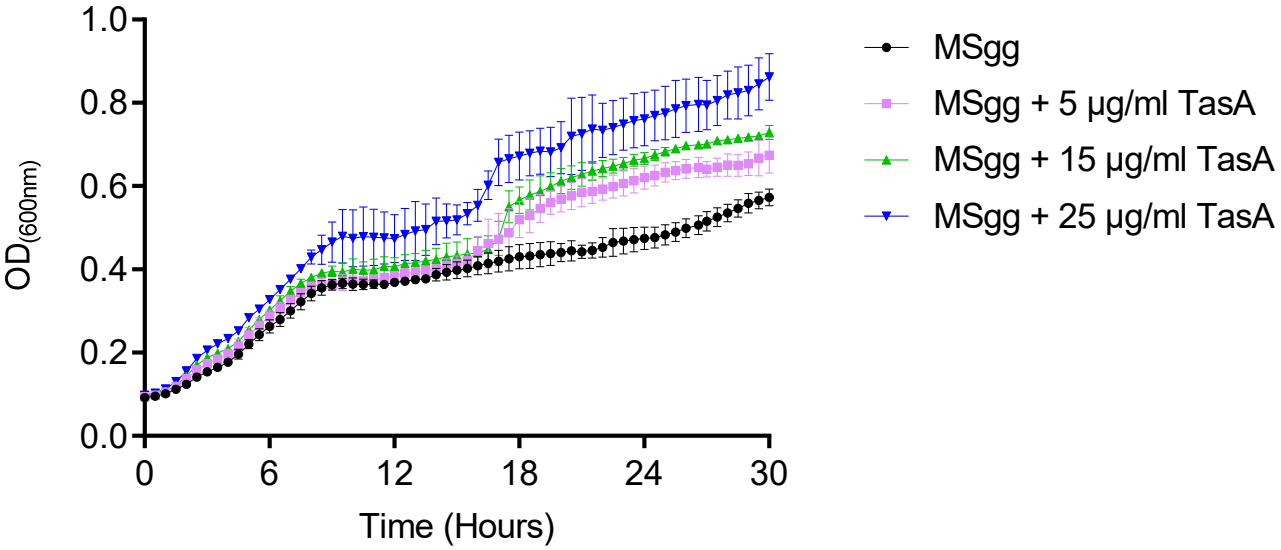
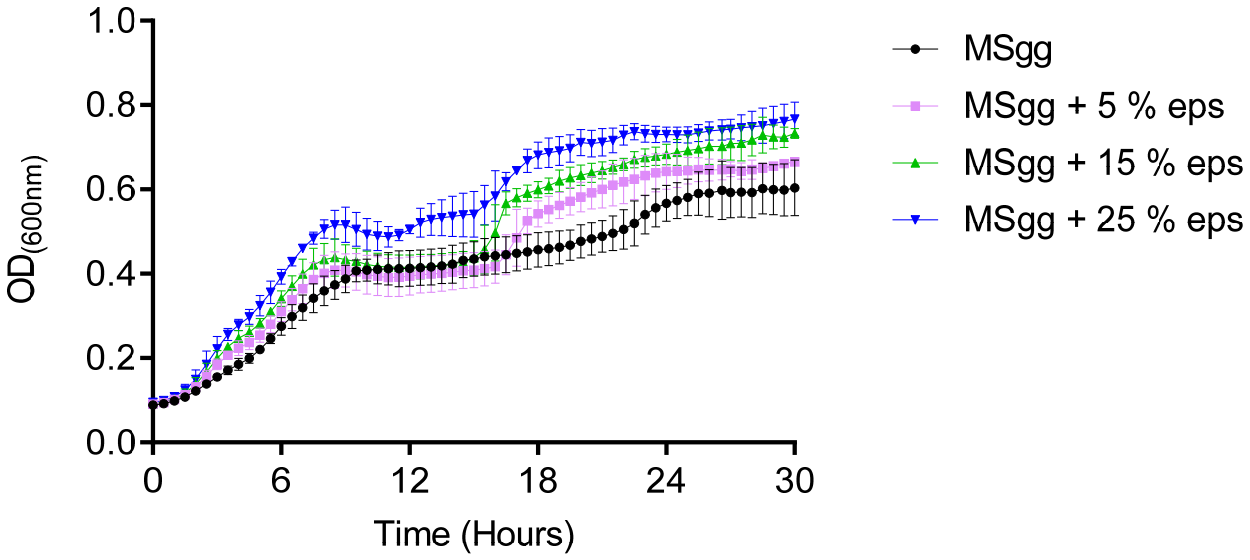


Figure S1. A) Monitoring pellicle formation and growth of the indicated strains when grown in MSgg medium and MSgg medium supplemented with conditioned medium (CM) from WT *B. subtilis* and its ECM mutants (10%, v/v). Cells were grown at 30°C and images were obtained at 24 h post inoculation and are representative of data presented in B. **B)** The number of CFU obtained when indicated strains were either grown in MSgg medium or MSgg medium supplemented with conditioned medium (CM) from WT *B. subtilis* and its ECM mutants (10%, v/v). Cells were grown at 30°C, and were harvested at 24 h post inoculation. Graphs represent mean ± SD from three independent experiments (n = 9). Statistical analysis was performed using Brown-Forsthye and Welch's ANOVA, with Dunnett's T3 multiple comparisons test. P < 0.05 was considered statistically significant. Scale bar = 5mm



	5h	10h	15h	20h	25h	30h
MSgg vs. MSgg + 5 µg/ml TasA	0.7508	0.9914	0.8785	<0.0001	<0.0001	0.0004
MSgg vs. MSgg + 15 µg/ml TasA	0.4099	0.387	0.4022	<0.0001	<0.0001	<0.0001
MSgg vs. MSgg + 25 µg/ml TasA	0.0417	0.0001	<0.0001	<0.0001	<0.0001	<0.0001

Figure S2. Monitoring growth of *B. mycoides* when supplemented with purified TasA protein Growth of *B. mycoides* was monitored in MSgg medium and MSgg medium supplemented with the purified TasA protein in increasing concentrations. An increase in the growth was observed in the presence of purified tasA protein. Graph represent the mean ± SD from three independent experiments (n = 9). Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison post hoc testing. P < 0.05 was considered



	5h	10h	15h	20h	25h	30h
MSgg vs. MSgg + 5 % eps	0.4661	0.9664	0.6626	0.0005	0.0609	0.0666
MSgg vs. MSgg + 15 % eps	0.0679	0.9292	0.9994	<0.0001	0.0002	<0.0001
MSgg vs. MSgg + 25 % eps	0.0005	0.0061	0.0004	<0.0001	<0.0001	<0.0001

Figure S3: Monitoring growth of B. mycoides when supplemented with purified eps. Growth of *B. mycoides* was monitored in MSgg medium and MSgg medium supplemented with the purified eps in increasing concentrations. An increase in the growth was observed in the presence of purified eps. Graph represent the mean ± SD from three independent experiments (n = 9). Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison post hoc testing. P < 0.05 was considered statistically significant. P values at

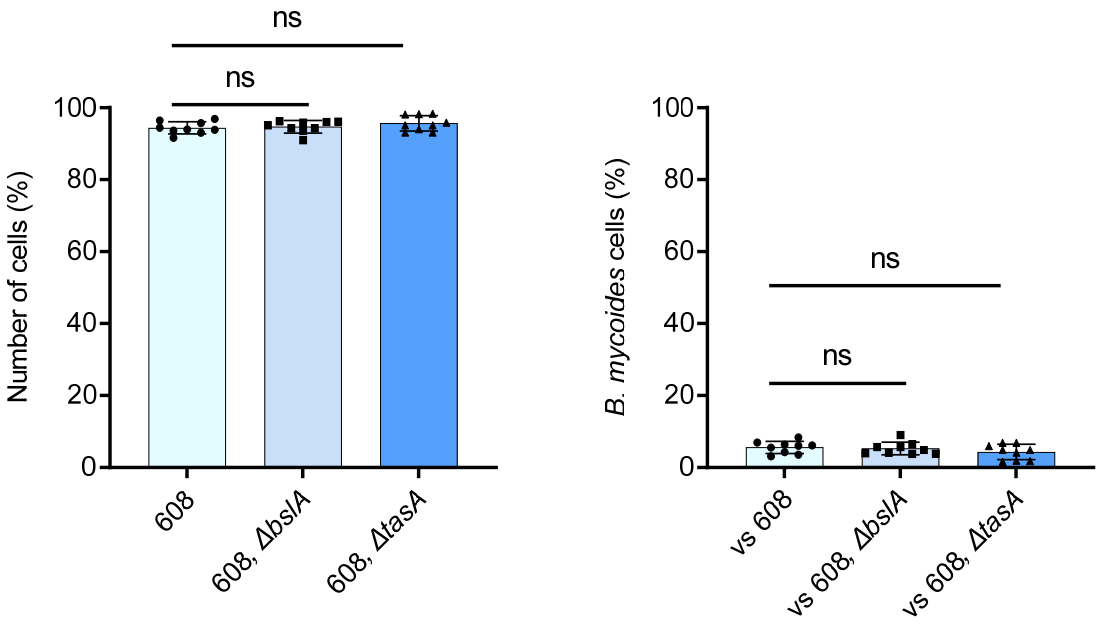
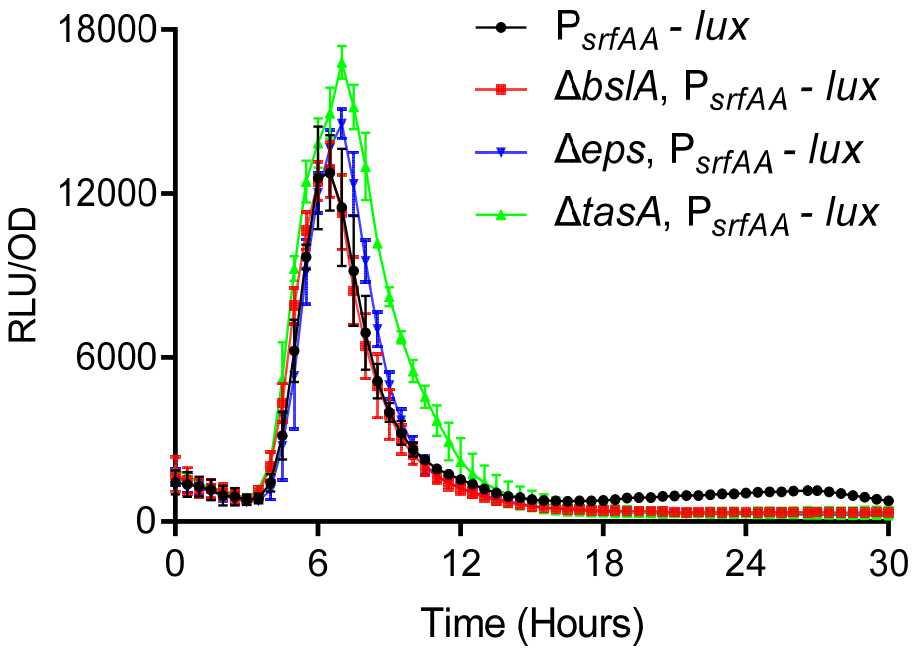


Figure S4: Quantifying the number of cells present in the pellicles of each competition assay using imaging flow cytometry. A competition assay was set up between WT *B. subtilis* stains harboring P $_{hyerspank}$-*gfp* (608), 608, Δ*tasA* and 608, Δ*bslA* mutants vs *B. mycoides,* in MSgg medium. As Δ*eps* mutant cannot form pellicles, hence it was not considered for this competition assay. **A)** Quantifying the number of 608 and its deletions mutants cells, in competition against *B. mycoides* cells, using imaging flow cytometry. **B)** Quantifying the number of *B. mycoides* cells in competition against 608 and its deletions mutants using imaging flow cytometry. Data were collected 72 h post inoculation, and 100,000 cells were counted. Graphs represent mean ± SD from three independent experiments (n=9). The data were analyzed using IDEAS 6.3. Statistics analysis was performed using Brown-Forsthye and Welch's ANOVA , with Dunnett's T3 multiple comparisons test. $P < 0.05$ was considered statistically significant.



	5h	8h	10h	20h	30h
P_{srfAA} - lux vs. *ΔbslA*, P_{srfAA} - lux	<0.0001	0.4753	0.9369	0.4375	0.5921
P_{srfAA} - lux vs. *ΔtasA*, P_{srfAA} - lux	<0.0001	<0.0001	<0.0001	0.3748	0.5439
P_{srfAA} - lux vs. *Δeps*, P_{srfAA} - lux	0.0498	<0.0001	0.9815	0.3428	0.3981

Figure S5: **Analysis of the luciferase activity in a WT *B. subtilis* strain harboring P_{srfAA}-*lux* (surfactin) reporter, and its indicated ECM deletion mutants**. Luminescence was monitored in MSgg medium, at 30°C for 30 h. Graphs represent mean ± SD from three independent experiments (n = 9). Statistical analysis was performed using two-way ANOVA followed by Dunnett's multiple comparison test. P < 0.05 was considered statistically significant. P values at different time points are shown in the table. Luciferase activity was normalized to avoid artifacts related to differential cell numbers as RLU/OD.

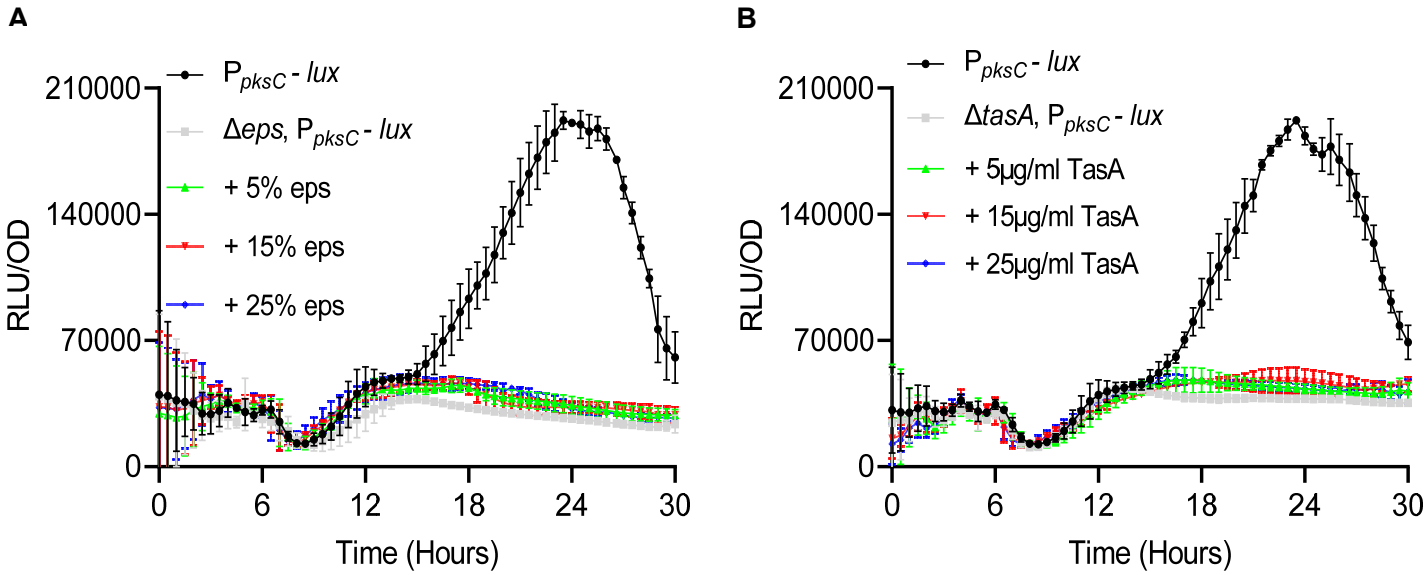


Figure S6: **Analysis of the luciferase activity in a WT *B. subtilis* strain harboring P_{pksC}-*lux* (bacillaene) reporter, and its indicated Δ*eps* and Δ*tasA* deletion mutants**. The mutants were supplemented with **A)** purified eps in increasing concentrations. **B)** Purified TasA in increasing concentrations. Luminescence was monitored in MSgg medium, at 30°C for 30 h. Graphs represent mean ± SD from three independent experiments (n = 9). Luciferase activity was normalized to avoid artifacts related to differential cell numbers as RLU/OD.

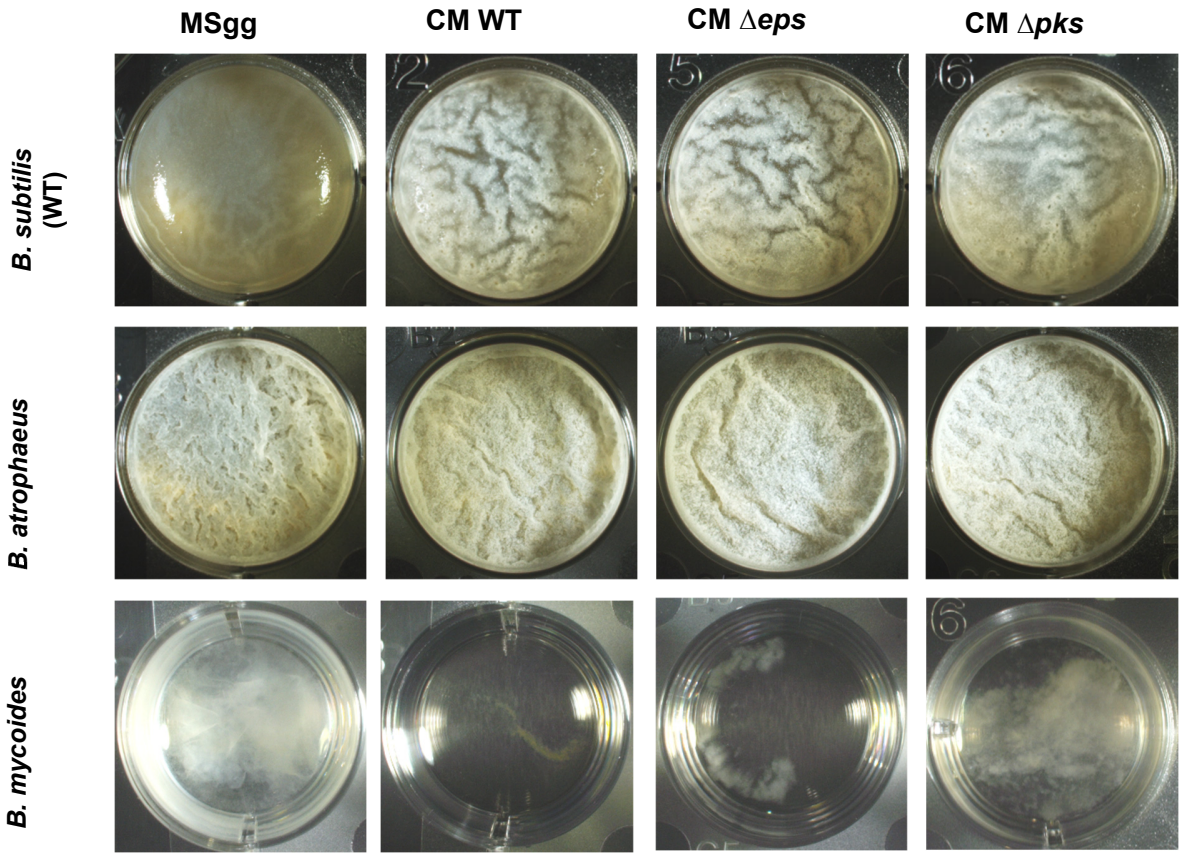


Figure S7. The bioactivity of the CM on pellicle formation. Monitoring pellicle formation and growth of the indicated strains in MSgg medium supplemented with conditioned medium (CM) of WT *B. subtilis* and its Δ*eps* and Δ*pks* mutants (10%, v/v). Cells were grown at 30°C and images were obtained at 72 h post inoculation, and are representative of data shown in Figure 4B.

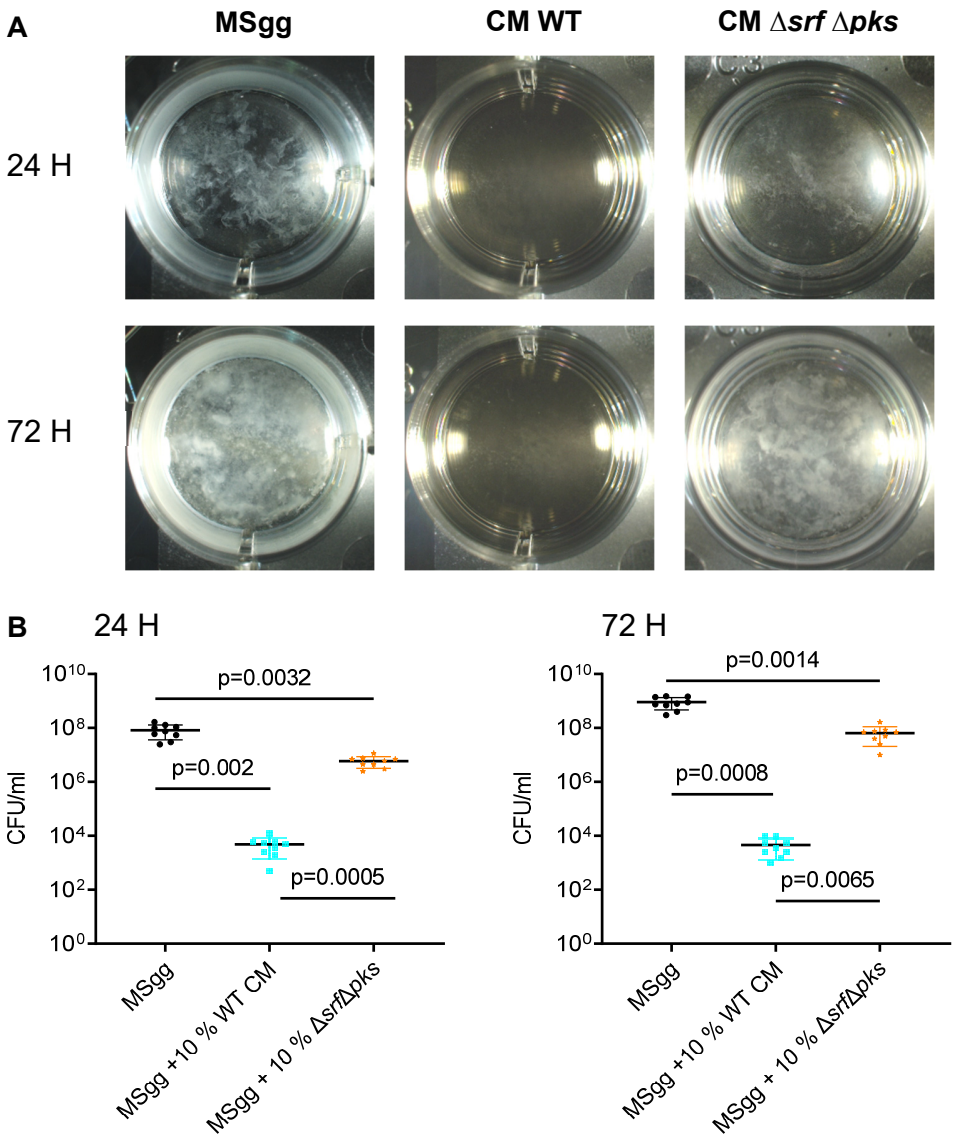


Figure S8. The bioactivity of the CM on B. *mycoides* growth. A) Monitoring growth of *B. mycoides* in MSgg medium supplemented with conditioned medium (CM) of WT *B. subtilis* and its Δ*srf* Δ*pks* mutants (10%, v/v). Cells were grown at 30°C and images were obtained at 24 h and 72 h post inoculation, and are representative of data shown in Figure S8B. **B)** The number of CFU obtained when indicated strains were either grown in MSgg medium or MSgg medium supplemented with conditioned medium (CM) from WT *B. subtilis* Δ*srf* Δ*pks* mutants (10%, v/v). Cells were grown at 30°C, and were harvested at 24 h and 72 h post inoculation. Graphs represent mean ± SD from three independent experiments (n = 9). Statistical analysis was performed using Brown-Forsthye and Welch's ANOVA, with Dunnett's T3 multiple comparisons test. $P < 0.05$ was considered statistically significant.

Supplementary Table 1

Expected m/z	Observed m/z	Mass error (ppm)	Observed RT (min)	Observed drift (ms)	Formula	Observed CCS (Å²)	Feature
234.1125	234.1131	2.49	26.17	6.67	C13H16NO3		
251.143	251.1434	1.27	26.17	6.38	C18H19O		
380.222	380.2224	1.02	26.17	6.26	C24H30NO3		
382.2377	382.2384	1.9	26.18	6.37	C24H32NO3		
432.2533	432.2547	3.09	26.17	6.48	C28H34NO3		
449.2799	449.2806	1.72	26.17	6.41	C28H37N2O3		
563.348	563.3492	2.22	26.17	6.53	C34H47N2O5		-OH
603.3405	603.3409	0.66	26.17	6.46	C34H48N2O6Na	245.1	+Na
581.3585	581.3594	1.55	26.17	6.43	C34H49N2O6	244.26	+H

Table S1. Bacillaene was putatively identified using adducts, fragmentation, and mass accuracy. Identified mass signals associated with bacillaene are presented in the table above.

Supplementary Table 2

List of strains used in the study

Strain	Description	Source or reference
B. subtilis NCIB 3610	Wild type	[1]
B. atrophaeus 1942	Wild type	Rotem Sorek lab, WIS, Israel
B. mycoides AH621	Wild type	Rotem Sorek lab, WIS, Israel
$P_{hyperspank}$-*gfp* (608)	*B. subtilis* amyE::$_{Phyperpsank}$-*gfp* (Cm ^{r}), constitutively expressing hyperspank promoter tagged to the gfp reporter integrated in the neutral *amyE* locus	[2]
Δ*bslA*, $P_{hyperspank}$-*gfp*	DNA was extracted from *B. subtilis* Δ*bslA* (lab stock), and transferred into *B. subtilis* $P_{hyperspank}$-*gfp* as described in materials and methods. Cm ^{r}, SP ^{r}	This study
Δ*epsA-O*, $P_{hyperspank}$-*gfp*	DNA was extracted from *B. subtilis* Δ*epsA-0* (lab stock), and transferred into *B. subtilis* $P_{hyperspank}$-*gfp* as described in materials and methods. Cm ^{r}, Lnc ^{r} , Ery ^{r}	This study
Δ*tasA*, $P_{hyperspank}$-*gfp*	DNA was extracted from *B. subtilis* Δ*tasA* (lab stock), and transferred into *B. subtilis* $P_{hyperspank}$-*gfp* as described in materials and methods. Cm ^{r}, Kan ^{r}	This study
P_{pksC}-*lux*	*B. subtilis* sacA::P_{pksC}-*lux* (Cm ^{r}), promoter of bacillaene operon tagged to the luciferase reporter integrated in the neutral *SacA* locus	[3]

P_{srfAA}-*lux*	*B. subtilis* sacA::P_{srfAA}-*lux* (Cm ^{r}), promoter of surfactin operon tagged to the luciferase reporter integrated in the neutral *sacA* locus	[4]
Δ*bslA,* P_{srfAA}-*lux*	DNA was extracted from *B. subtilis* Δ*bslA* (lab stock), and transferred into *B. subtilis* P_{srfAA}-*lux* as described in materials and methods. Cm ^{r}, SP ^{r}	This study
Δ*espA-O,* P_{srfAA}-*lux*	DNA was extracted from *B. subtilis* Δ*epsA-O* (lab stock), and transferred into *B. subtilis* P_{srfAA}-*lux* as described in materials and methods. Cm ^{r}, Lnc ^{r} , Ery ^{r}	This study
∆*tasA* (Tn 10), P_{srfAA}-*lux*	DNA was extracted from *B. subtilis* ∆*tasA* (Tn 10), (lab stock), and transferred into *B. subtilis* P_{srfAA}-*lux* as described in materials and methods. Cm ^{r}, Sp ^{r}	This study
Δ*bslA,* P_{pksC}-*lux*	DNA was extracted from *B. subtilis* Δ*bslA* (lab stock), and transferred into *B. subtilis* P_{pksC}-*lux* as described in materials and methods. Cm ^{r}, SP ^{r}	This study
Δ*espA-O,* P_{pksC}-*lux*	DNA was extracted from *B. subtilis* Δ*epsA-O* (lab stock), and transferred into *B. subtilis* P_{pksC}-*lux* as described in materials and methods. Cm ^{r}, Lnc ^{r} , Ery ^{r}	This study
∆*tasA* (Tn 10), P_{pksC}-*lux*	DNA was extracted from *B. subtilis* ∆*tasA* (Tn 10), (lab stock), and transferred into *B. subtilis* P_{pksC}-*lux* as described in materials and methods. Cm ^{r}, Sp ^{r}	This study
P_{pksC}-*gfp*	*B. subtilis* amyE::P_{pksC}-*gfp* (Cm r, Sp ^{r}), promoter of bacillaene operon tagged to the luciferase reporter integrated in the neutral *amyE* locus	[3]
Δ*espA-O,* P_{pksC}-*gfp*	DNA was extracted from *B. subtilis* Δ*epsA-O* (lab stock), and transferred into *B. subtilis* P_{pksC}-*gfp* as described in materials and methods. Cm ^{r}, Sp ^{r}, Lnc ^{r} , Ery ^{r}	This study)

Cm ^{r} – chloramaphenicol resistance, Kan ^{r} – kanamycin resistance, Sp ^{r} – spectinomycin resistance , Lnc ^{r} – lincomycin resistance , Ery ^{r} – erythomycin resistance

References

1 Branda, S.S. *et al.* (2001) Fruiting body formation by Bacillus subtilis. *Proc. Natl. Acad. Sci. U. S. A.* 98, 11621–6

2 Rosenberg, G. *et al.* (2016) Not so simple, not so subtle: the interspecies competition between Bacillus simplex and Bacillus subtilis and its impact on the evolution of biofilms. *npj Biofilms Microbiomes* 2, 15027

3 Ogran, A. *et al.* (2019) The plant host induces antibiotic production to select the most-beneficial colonizers. *Appl. Environ. Microbiol.* 85,

4 Maan, H. *et al.* (2021) Resolving the conflict between antibiotic production and rapid growth by recognition of peptidoglycan of susceptible competitors. *bioRxiv* DOI: 10.1101/2021.02.07.430110