Supplementary information

Redundant and Distinct Roles of Secreted Protein Eap and Cell Wall-Anchored Protein SasG in Biofilm Formation and Pathogenicity of *Staphylococcus aureus*

Running Title: Roles of Eap and SasG in Biofilm and Pathogenesis

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Supplementary Methods

Purification of recombinant Eap. Recombinant Eap from MR23 was expressed and purified using a *Brevibacillus* expression system as previously described (1). Purified Eap was subjected to SDS-PAGE and quantified by measuring the band intensity using LAS-4000 Image Analyzer (GE Healthcare). Bovine serum albumin was used as a standard. Then, purified Eap was supplemented into biofilm cultures to address whether it promotes biofilm formation.

Atmospheric scanning electron microscopy (ASEM). *S. aureus* MR23 wild-type and Δ*eap* strains were grown in BHIG for 37°C for 4 h in ASEM dishes, as previously described (2). Biofilms formed on the dishes were fixed with 1% (v/v) glutaraldehyde/1% (v/v) paraformaldehyde and labeled with positively charged Nanogold reagent (Nanoprobes, Stony Brook, NY). After gold enhancement using GoldEnhance-EM (Nanoprobes) for 10 min at 25°C, biofilms were washed three times with doubly distilled water. Subsequently, biofilms were stained overnight with 2% (w/v) phosphate tungstic acid at 4°C. After washing three times with doubly distilled water, biofilms were immersed in radical scavenger 1% (w/v) ascorbic acid solution and imaged by ASEM (3) using the ClairScope ASEM system (JASM-6200, JEOL, Ltd, Tokyo, Japan). The acceleration voltage of the inverted SEM was 30 kV, and the applied electron dose was less than 0.8 $e^{-}/Å^{2}$.

Supplementary Figure Legends

FIG S1 Enzymatic susceptibility of preformed *S. aureus* biofilms. For the experiments, 24-h biofilms formed by WT (A), Δ*eap* (B), Δ*eap* Δ*srtA* (C), Δ*sasG* (D), and Δ*eap* Δ*sasG* (E) strains were treated with the indicated enzymes or left untreated. Biomasses of the residual biofilms were quantified as described in Fig. 1. Means and standard deviations from three independent experiments are shown. **, $P < 0.01$; NS, not significant.

FIG S2 Characterization of SasG in various *S. aureus* strains. Expression levels of SasG were analyzed by immunoblotting for clinical strains of methicillin-resistant (MRSA) and -sensitive *S. aureus* (MSSA) that produce protein-dependent biofilms. Molecular sizes are also indicated at the left of the panels.

FIG S3 Biofilm formation by *S. aureus* RN4220 and its isogenic mutant strains. (A) Biofilms of the indicated RN4220-derived strains were formed and quantified as indicated in Figure 2A. (B) Biofilm formation by RN4220 Δ*eap* Δ*sasG*-expressing Eap, SasG, and both was also analyzed. The optimal concentrations of anhydrotetracycline (aTc) were added to induce the expression of each protein. (C) SasG in the cell wall fraction and Eap in the ECM fraction from the strains grown under the indicated condition in B were detected by SDS-PAGE with CBB-staining. Red arrows indicate SasG. (D) Biofilm formation of RN4220 Δ*eap* Δ*sasG* harboring pLC1 or pEap-SasG was analyzed in the presence of 3.13 ng/ml aTc. The indicated concentrations of purified Eap was added to biofilm cultures from the onset of biofilm formation. The means and standard deviations of biofilm biomasses from three independent experiments are shown. *, $P < 0.05$; **, $P < 0.01$; NS, not significant.

FIG S4 ASEM analysis of biofilms. ASEM images of 4-h biofilms formed by MR23 WT (A) and Δ*eap* Δ*sasG* (B) strains labeled with positively charged Nanogold and phosphate tungstic acid are shown. Scale bars, 10 μm (×1,000 magnification) and 1 μm (×10,000 magnification).

FIG S5 Proliferation of *S. aureus* in silkworm larvae. Silkworm larvae were sacrificed and

body fluids collected 24 h after injection of the indicated *S. aureus* strains. The body fluids were diluted and spread on mannitol salt agar plates. The plates were incubated at 37°C overnight and the obtained colonies counted. Means and standard deviations from three independent experiments are shown. NS, not significant.

TABLE S1 Bacterial strains and plasmids used in the current study.

Strains and plasmids	Description	Source or reference
S. aureus strains		
RN4220	NCTC8325-4 derivative, restriction deficient mutant	4
RN4220 Δ*eap*	The *eap* gene was deleted from RN4220	This study
RN4220 Δ*sasG*	The *sasG* gene was deleted from RN4220	This study
RN4220 Δ*eap* Δ*sasG*	The *eap* and *sasG* genes were deleted from RN4220	This study
MR23	A clinical isolate of MRSA from the Jikei hospital	1
MR23 Δ*eap*	The *eap* gene was deleted from MR23	This study
MR23 Δ*srtA*	The *srtA* gene was deleted from MR23	5
MR23 Δ*eap* Δ*srtA*	The *eap* and *srtA* genes were deleted from MR23	This study
MR23 Δ*sasG*	The *sasG* gene was deleted from MR23	This study
MR23 Δ*eap* Δ*sasG*	The *eap* and *sasG* genes were deleted from MR23	This study
MR23 Δ*eap* Δ*fnbA* Δ*fnbB*	The *eap*, *fnbA* and *fnbB* genes were deleted from MR23	This study
MR23 Δ*eap* Δ*clfA* Δ*clfB*	The *eap*, *clfA* and *clfB* genes were deleted from MR23	This study
MR23 Δ*eap* Δ*spa*	The *eap* and *spa* genes were deleted from MR23	This study
MR23 Δ*eap* Δ*isdAB*	The *eap* and *isdAB* genes were deleted from MR23	This study
E. coli strain		
DH5α	*fhuA2* Δ(*argF-lacZ*)*U169 phoA glnV44 φ80* Δ(*lacZ*)*M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Toyobo, Osaka, Japan
BLR (DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal lac ile dcm Δ(srl-recA)306::Tn10 (*tet^R*)(DE3)*	Novagen
Plasmids		
pKOR1	*E. coli-S. aureus* shuttle vector plasmid for knockout of genes by allelic exchange, Amp^R, Cm^R	6
pSrtA-ko	pKOR1-derivative plasmid for knockout of the MR23 *srtA* gene, Amp^R, Cm^R	5
pEap-ko	pKOR1-derivative plasmid for knockout of the MR23 *eap* gene, Amp^R, Cm^R	This study
pSasG-ko	pKOR1-derivative plasmid for knockout of the MR23 *sasG* gene, Amp^R, Cm^R	This study
pFnbA-ko	pKOR1-derivative plasmid for knockout of the MR23 *fnbA* gene, Amp^R, Cm^R	This study
pFnbB-ko	pKOR1-derivative plasmid for knockout of the MR23 *fnbB* gene, Amp^R, Cm^R	This study
pClfA-ko	pKOR1-derivative plasmid for knockout of the MR23 *clfA* gene, Amp^R, Cm^R	This study
pClfB-ko	pKOR1-derivative plasmid for knock out of the MR23 *clfB* gene, Amp^R, Cm^R	This study
pSpa-ko	pKOR1-derivative plasmid for knock out of the MR23 *spa* gene, Amp^R, Cm^R	This study
pIsdAB-ko	pKOR1-derivative plasmid for knock out of the MR23 *isdAB* gene, Amp^R, Cm^R	This study
pLC1	*E. coli-S. aureus* shuttle vector plasmid with *tetO/xyl* promoter, Amp^R, Cm^R	7
pEap	pLC1-derivative plasmid for expression of Eap, Amp^R, Cm^R	This study
pSrtA	pLC1-derivative plasmid for expression of Sortase A, Amp^R, Cm^R	This study
$pSasG^{WT}$	pLC1-derivative plasmid for expression of $SasG^{WT}$, Amp^R, Cm^R	This study
$pSasG^{\Delta L}$	pLC1-derivative plasmid for expression of $SasG^{\Delta L}$, Amp^R, Cm^R	This study
pEap-$SasG^{WT}$	pLC1-derivative plasmid for co-expression of Eap and $SasG^{WT}$, Amp^R, Cm^R	This study

pCold I	Cold shock promoter, His_6-tag gene, ColE1*ori*, Ap^R	Takara
pCold-SasG	pCold I-derivative plasmid for expression of SasG(51-954)	This study

Amp^R, ampicillin-resistant; Cm^R, chloramphenicol-resistant; tet^R, tetracycline-resistant .

TABLE S2 Oligonucleotide primers used in the current study.

Primers	Sequences (5’ to 3’)	Descriptions
attB1-*eap*-F	ggggacaagtttgtacaaaaaagcaggctataaaagttcatgatgttgaccc	Disruption of *eap* in MR23
eap-R	gaattacacaaaaaaggagagataattttctttaattttataataaggcatctcac	Disruption of *eap* in MR23
eap-F	gtgagatgccttattataaaattaaagaaaattatctctccttttttgtgtaattc	Disruption of *eap* in MR23
attB2-*eap*-R	ggggaccactttgtacaagaaagctgggttcagtaagtgcaccaactaataataaat	Disruption of *eap* in MR23
attB1-*srtA*-F	ggggacaagtttgtacaaaaaagcaggctttaataatcttattttcactcgttatctta	Disruption of *srtA* in MR23
srtA-R	attcatccattagcgtaatagaacgttaaggctccttttataca	Disruption of *srtA* in MR23
srtA-F	tgtataaaaggagccttaacgttctattacgctaatggatgaat	Disruption of *srtA* in MR23
attB2-*srtA*-R	ggggaccactttgtacaagaaagctgggttaaccatctattaaatttaaaacctacatt	Disruption of *srtA* in MR23
attB1-*sasG*-F	ggggacaagtttgtacaaaaaagcaggctagtattagcatatttagatgtatttaaaaa	Disruption of *sasG* in MR23
sasG-R	aatcaactattatttaaattatgaattattttgcatactccttttcca	Disruption of *sasG* in MR23
sasG-F	tggaaaaaggagtatgcaaaataattcataatttaaataatagttgatt	Disruption of *sasG* in MR23
attB2-*sasG*-R	ggggaccactttgtacaagaaagctgggtcatcattgataatttaaagatgat	Disruption of *sasG* in MR23
attB1-*fnbA*-F	ggggacaagtttgtacaaaaaagcaggctcgtttcaggtatcatcttcattttattc	Disruption of *fnbA* in MR23
fnbA-R	gaaataaacccgtcaatttttgtttaatataatatctccctttaaatgcaaaattca	Disruption of *fnbA* in MR23
fnbA-F	tgaattttgcatttaaagggagatattatattaaacaaaaattgacgggtttatttc	Disruption of *fnbA* in MR23
attB2-*fnbA*-R	ggggaccactttgtacaagaaagctgggtcaataaatgcaattctatattgttcgg	Disruption of *fnbA* in MR23
attB1-*fnbB*-F	ggggacaagtttgtacaaaaaagcaggctcaaagcataattaaacaaaaattgacg	Disruption of *fnbB* in MR23
fnbB-R	aaataaacctgtcaattttggattgatataatattctcccttaaatgcaaaattca	Disruption of *fnbB* in MR23
fnbB-F	tgaattttgcatttaagggagaatattatatcaatccaaaattgacaggtttattt	Disruption of *fnbB* in MR23
attB2-*fnbB*-R	ggggaccactttgtacaagaaagctgggtgataaattgacgcgcagaactaat	Disruption of *fnbB* in MR23
attB1-*clfA*-F	ggggacaagtttgtacaaaaaagcaggctttgatattcatcatcaaaataaa	Disruption of *clfA* in MR23
clfA-R	atcatatgattaatttaatatcattattactttattccctctttttaaaaag	Disruption of *clfA* in MR23
clfA-F	ctttttaaaaagagggaataaagtaataatgatattaaattaatcatatgat	Disruption of *clfA* in MR23
attB2-*clfA*-R	ggggaccactttgtacaagaaagctgggttatttttttctcttcactaaaaaat	Disruption of *clfA* in MR23
attB1-*clfB*-F	ggggacaagtttgtacaaaaaagcaggcttgactgtcaaattttgatgcata	Disruption of *clfB* in MR23
clfB-R	taatctagaaattgaaatggagtaatatttagactcggatagcgactcag	Disruption of *clfB* in MR23
clfB-F	ctgagtcgctatccgagtctaaatattactccatttcaatttctagatta	Disruption of *clfB* in MR23
attB2-*clfB*-R	ggggaccactttgtacaagaaagctgggtatgaattttatgaaatcaaacagttttt	Disruption of *clfB* in MR23
attB1-*isdAB*-F	ggggacaagtttgtacaaaaaagcaggctacagttatttgaacatacaatttacc	Disruption of *isdAB* in MR23
isdAB-R	aatttaataattaaatataaagacgatttagttgttttcctcctaaggata	Disruption of *isdAB* in MR23
isdAB-F	tatccttaggaggaaaacaactaaatcgtctttatatttaattattaaatt	Disruption of *isdAB* in MR23
attB2-*isdAB*-R	ggggaccactttgtacaagaaagctgggtgaagtaatcgatgaggatcaaT	Disruption of *isdAB* in MR23
attB1-*spa*-F	ggggacaagtttgtacaaaaaagcaggctgaacgctcaactgaagatgaaag	Disruption of *spa* in MR23
spa-R	gatatctatcgttgtgtattgtttgtttaatataacgaattatgtattgcaatac	Disruption of *spa* in MR23
spa-F	gtattgcaatacataattcgttatattaaacaaacaatacacaacgatagatatc	Disruption of *spa* in MR23
attB2-*spa*-R	ggggaccactttgtacaagaaagctgggtcattacttgtggcagctaacac	Disruption of *spa* in MR23
pLC1-F	ctgcagccaagctagcttggc	Linearization of pLC1 vector
pLC1-R	cccgggagatctgatatcaagcttatttta	Linearization of pLC1 vector

pEap-F	atcagatctcccggggcttggtataaacaaaaccaaaca	Construction of Eap-expressing plasmid
pEap-R	ctagcttggctgcagatcattaattgtaattttgcttgcattata	Construction of Eap-expressing plasmid
pSrtA-F	atcagatctcccgggtgtataaaaggagccttaacgtatga	Construction of SrtA-expressing plasmid
pSrtA-R	ctagcttggctgcagttatttgacttctgtagctacaaagatttt	Construction of SrtA-expressing plasmid
pSasG^{WT}-F	atcagatctcccgggtggaaaaaggagtatgcaaatg	Construction of SasG^{WT}-expressing plasmid
pSasG^{WT}-R	ctagcttggctgcagttaattctttcttctacgagccaata	Construction of SasG^{WT}-expressing plasmid
pSasG$^{\Delta L}$-F	taactgcagccaagctagcttggc	Construction of SasG$^{\Delta L}$-expressing plasmid
pSasG$^{\Delta L}$-R	gcttggctgcagttattctgctcgtttttttctcttgat	Construction of SasG$^{\Delta L}$-expressing plasmid
pEap-SasG^{WT}-F1	ctgcagccaagctagcttggc	Construction of Eap-SasG^{WT}-co-expressing plasmid
pEap-SasG^{WT}-R1	atcattaattgtaattttgcttgcattatagaacacaa	Construction of Eap-SasG^{WT}-co-expressing plasmid
pEap-SasG^{WT}-F2	attacaattaatgattggaaaaaggagtatgcaaatg	Construction of Eap-SasG^{WT}-co-expressing plasmid
pEap-SasG^{WT}-R2	ctagcttggctgcagttaattctttcttctacgagccaata	Construction of Eap-SasG^{WT}-co-expressing plasmid
pCold-SasG-F	gaaggtaggcatatggctgaaaacaatattgagaatccaactacattaaaagataatgtcc	Construction of SasG(51-954)-expressing plasmid
pCold-SasG-R	agagattacctatctttattctgctcgtttttttctcttgattagctactgattctttagc	Construction of SasG(51-954)-expressing plasmid
SasG-s1	agtattagcatatttagatgtatttaaaaa	For DNA sequencing of *sasG* in MR23
SasG-s2	tagttgaatctaccccaattacaattcaag	For DNA sequencing of *sasG* in MR23
SasG-s3	tatgcggacaattcaactaatacatcagat	For DNA sequencing of *sasG* in MR23
SasG-s4	ccaaaattacaacctggtgaagagcgagtg	For DNA sequencing of *sasG* in MR23
SasG-s5	gtaaattcactgtaagtaaagtggaaaata	For DNA sequencing of *sasG* in MR23
SasG-s6	tcgaaagaagaaatcacaaaagatccgatt	For DNA sequencing of *sasG* in MR23
SasG-s7	accaaaagaagagattacaaaagatccgat	For DNA sequencing of *sasG* in MR23

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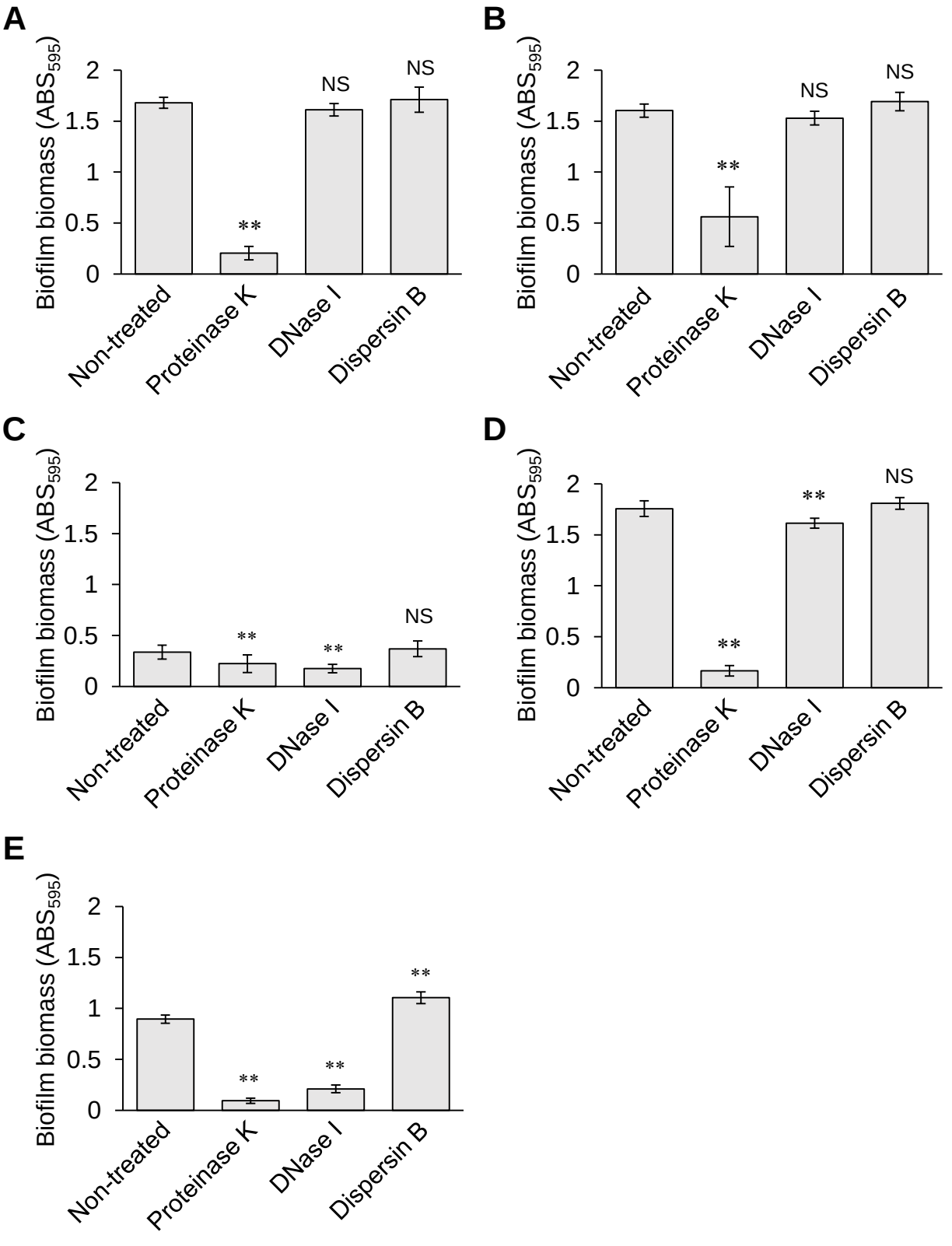

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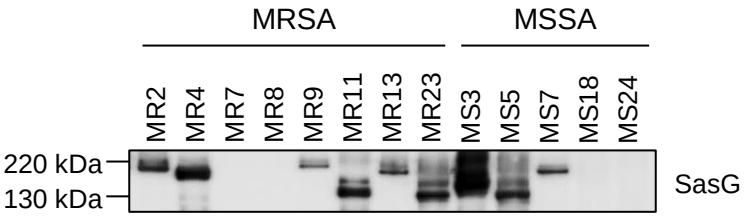

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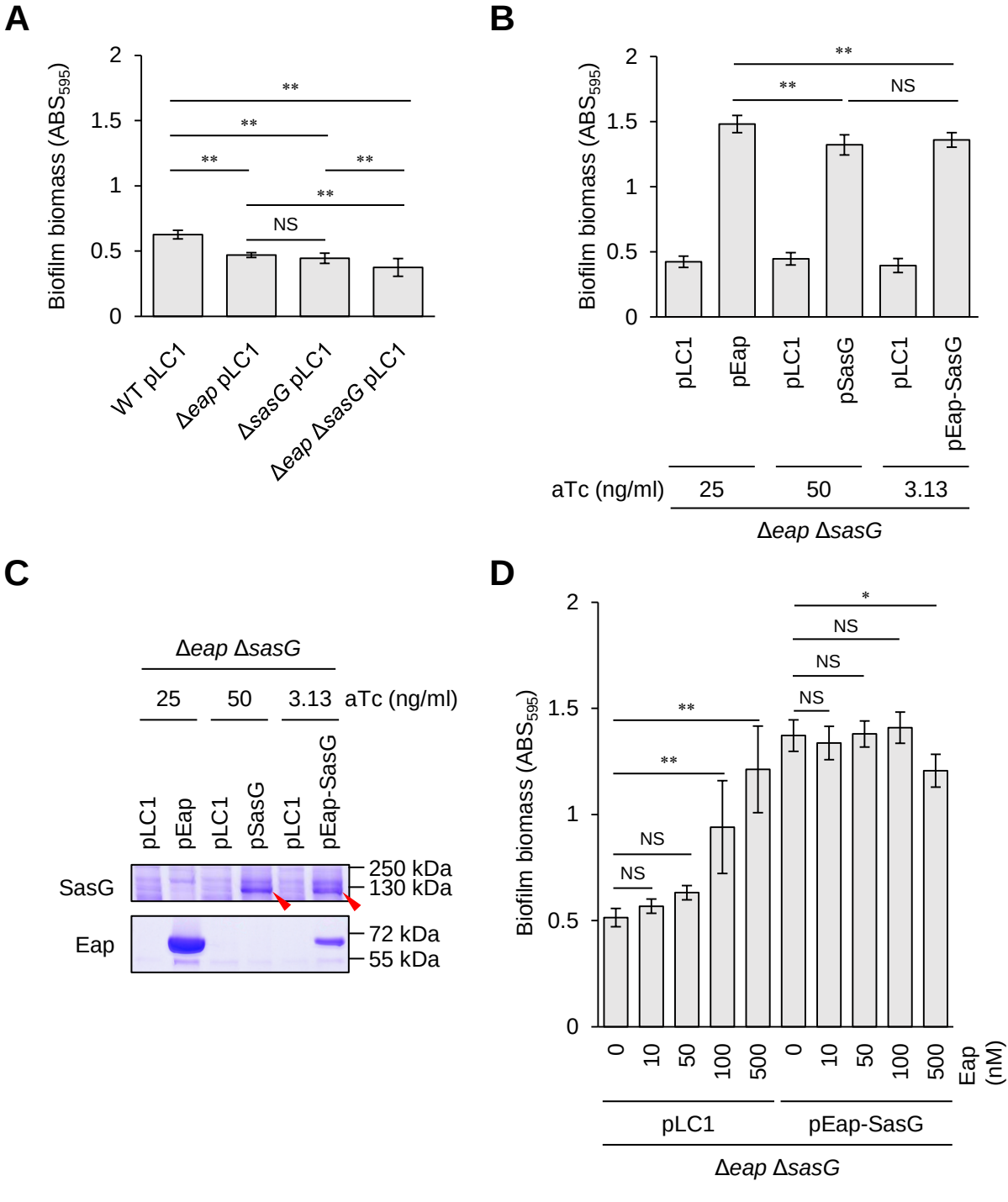

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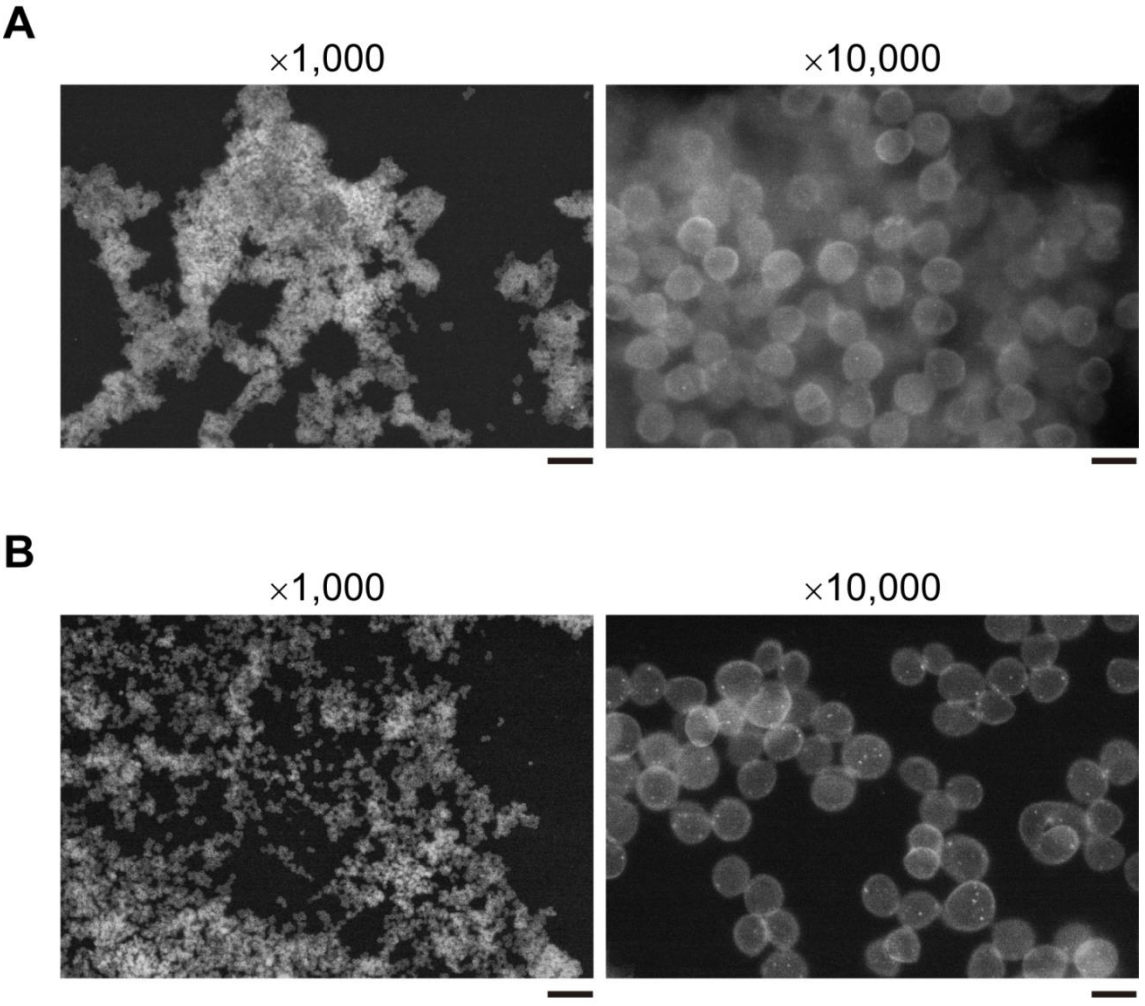

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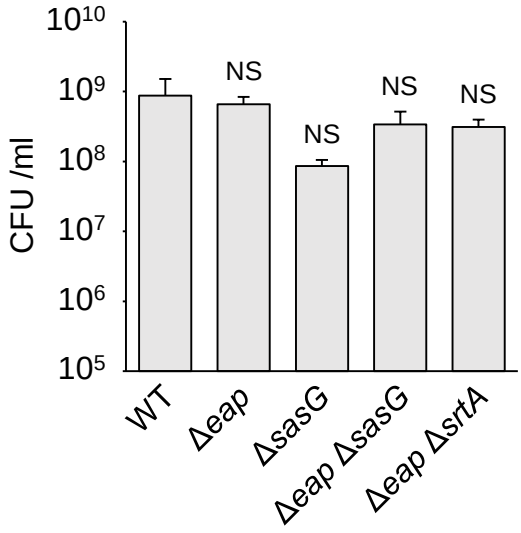

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