Supplementary Figures

Targeting the Achilles' Heels of Multidrug-Resistant *Staphylococcus aureus* by The Endocannabinoid Anandamide

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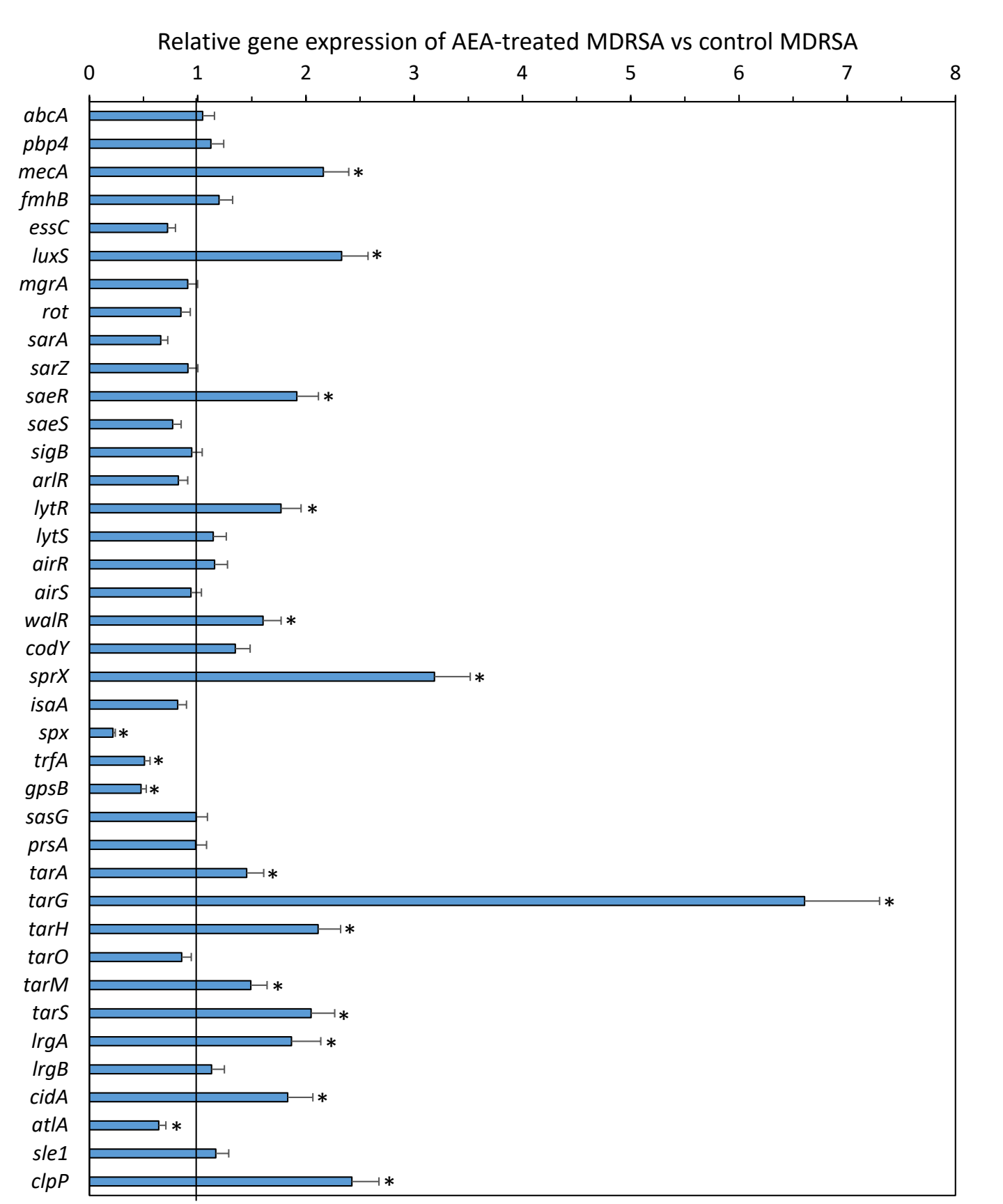
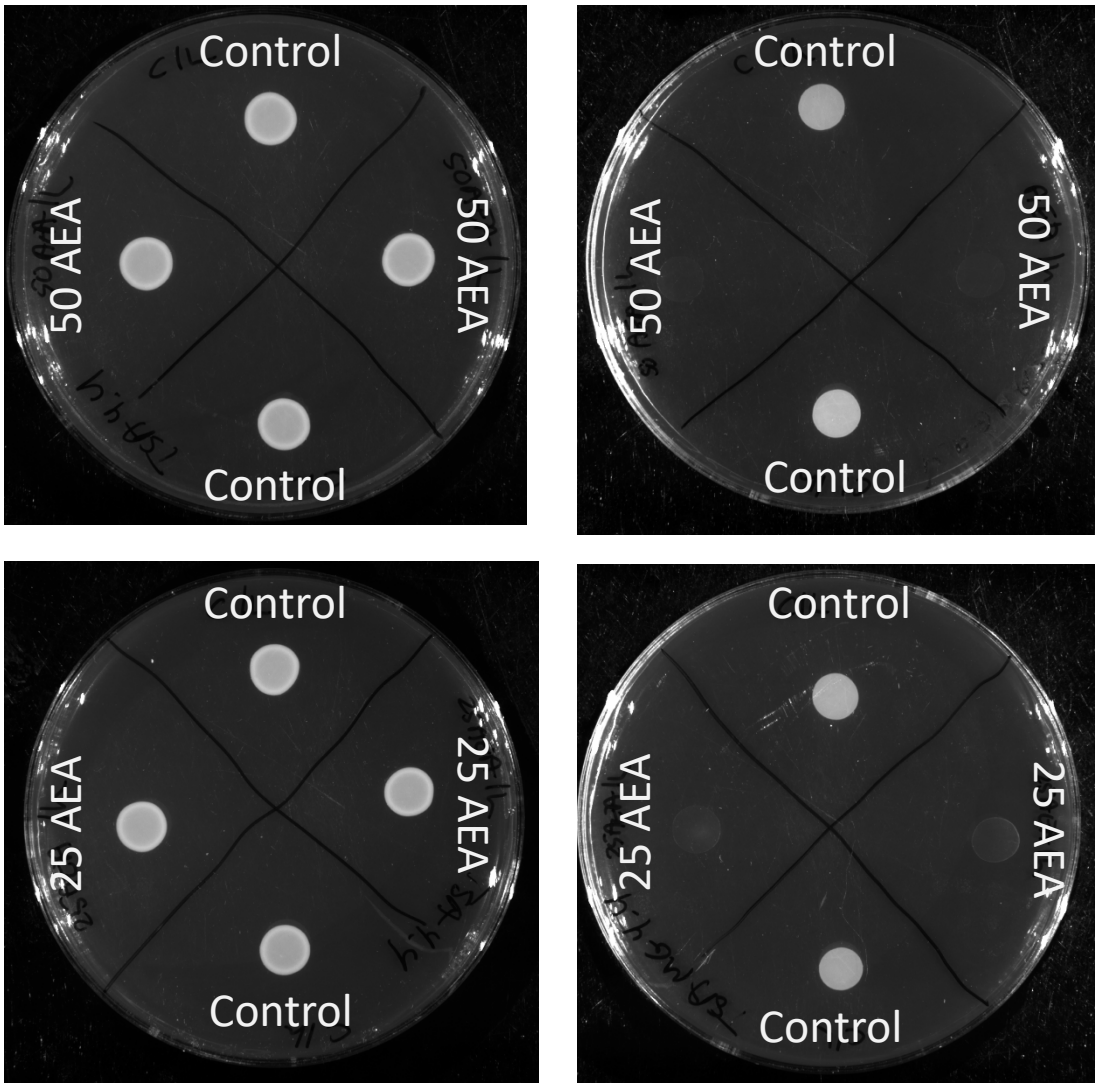


Supplementary Figure S1. Effect of AEA on gene expression relevant to antibiotic resistance and biofilm formation. MDRSA CI-M was incubated in the absence or presence of 50 µg/ml of AEA for 2 h, and then the relative gene expression was determined by quantitative RT-PCR. The data presents a representative experiment where two AEA-treated samples were calculated against two control samples, and using the following 9 housekeeping genes: *gmk*, *glyA*, *gyrA*, *gyrB*, *proC*, *recF*, *rho*, *rpoB*, and *asnC*. *p<0.05. The relevant function of these genes are described in *Supplementary Table S1*. Other genes are published in Banerjee *et al*. **Sci. Rep.** 11: 8690, 2021.

TSA

TSA + 0.02% methyl green



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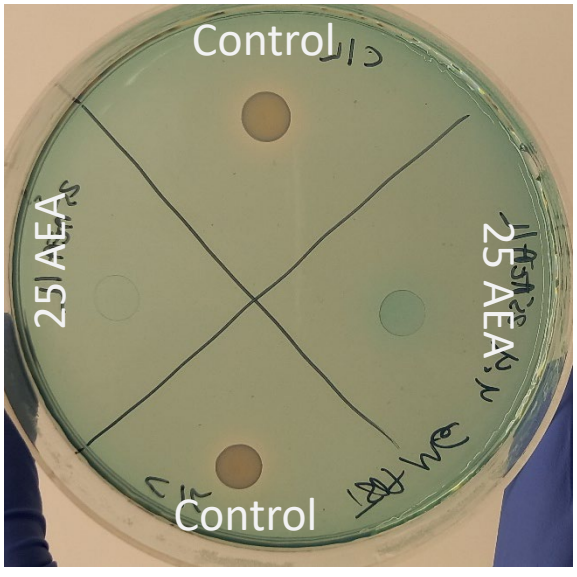
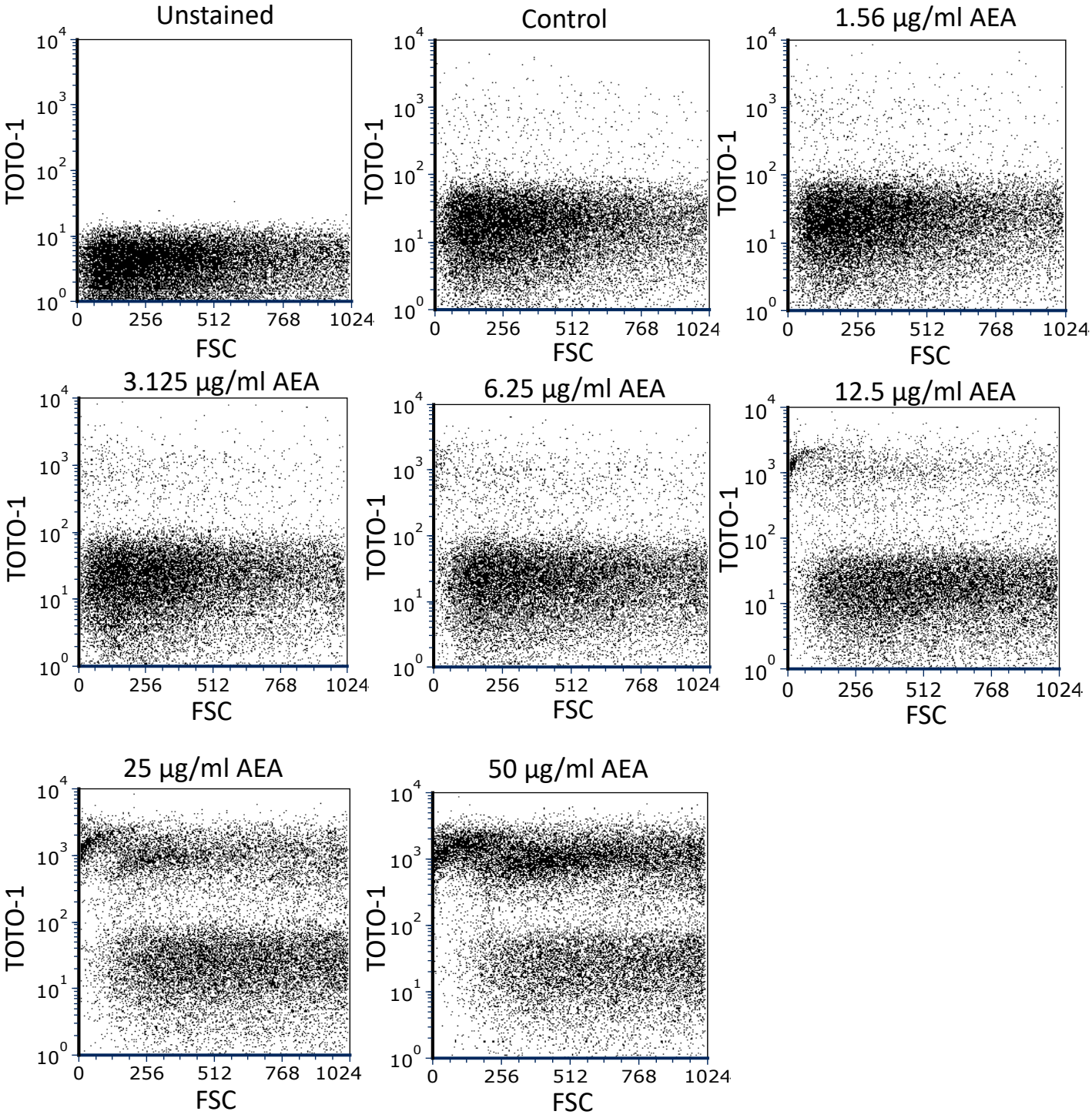
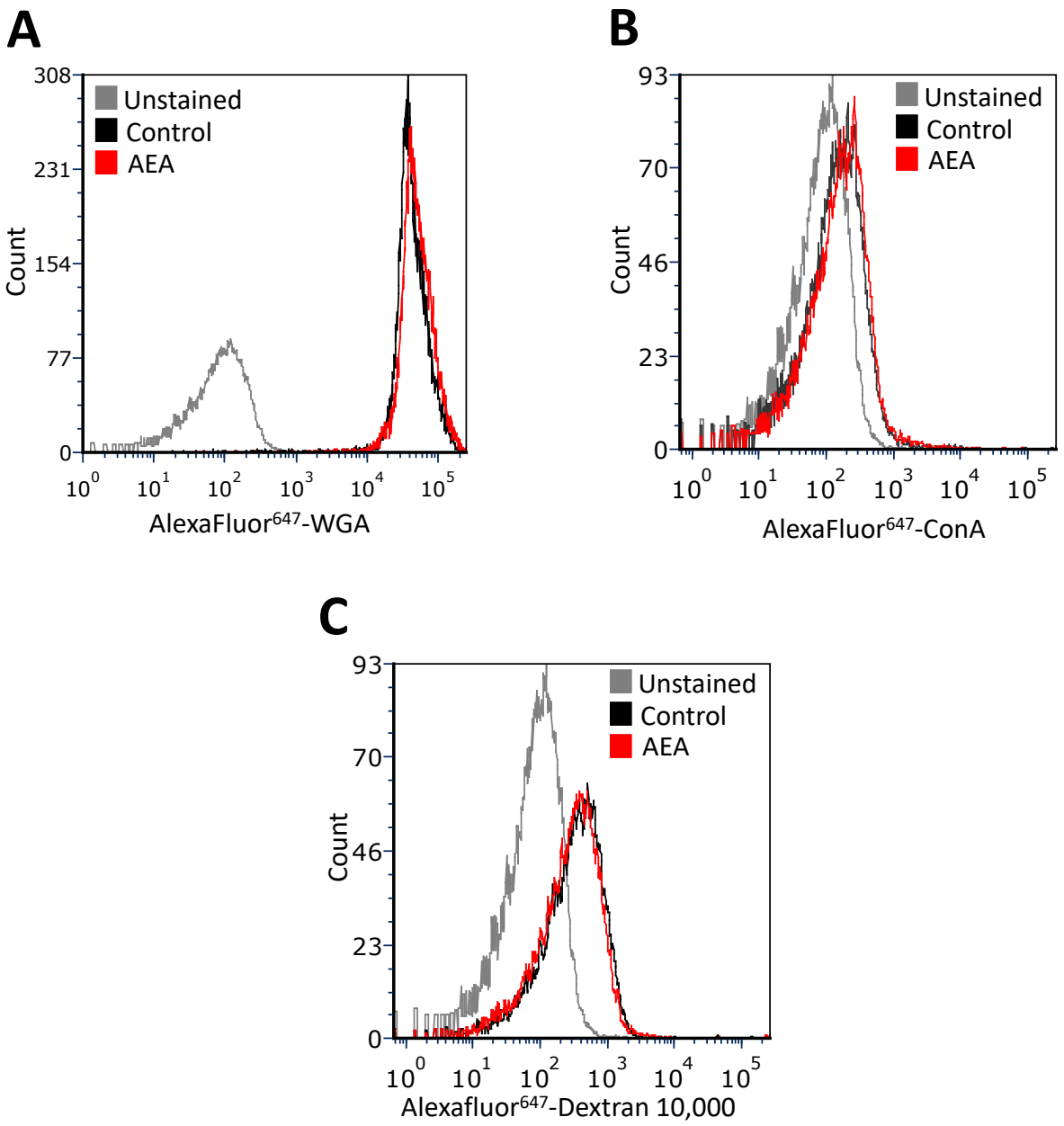


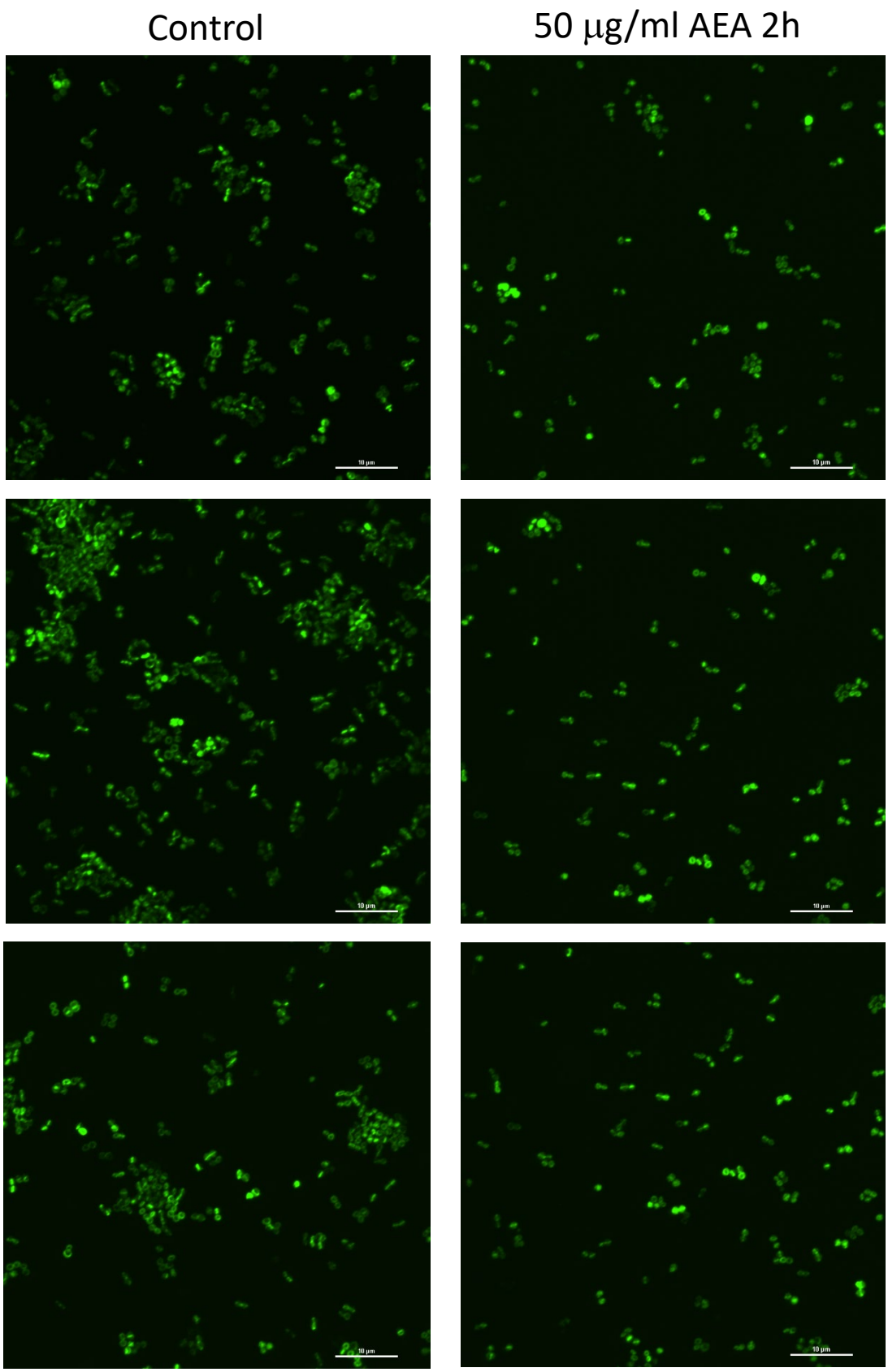
Supplementary Figure S2. AEA treatment increases the susceptibility to methyl green. MDRSA CI-M was exposed to 50 or 25 µg/ml AEA for 1 h in TSBG, and then 10 µl was spotted on TSA or TSA with 0.02% methyl green, and the plates were incubated overnight at 37°C.



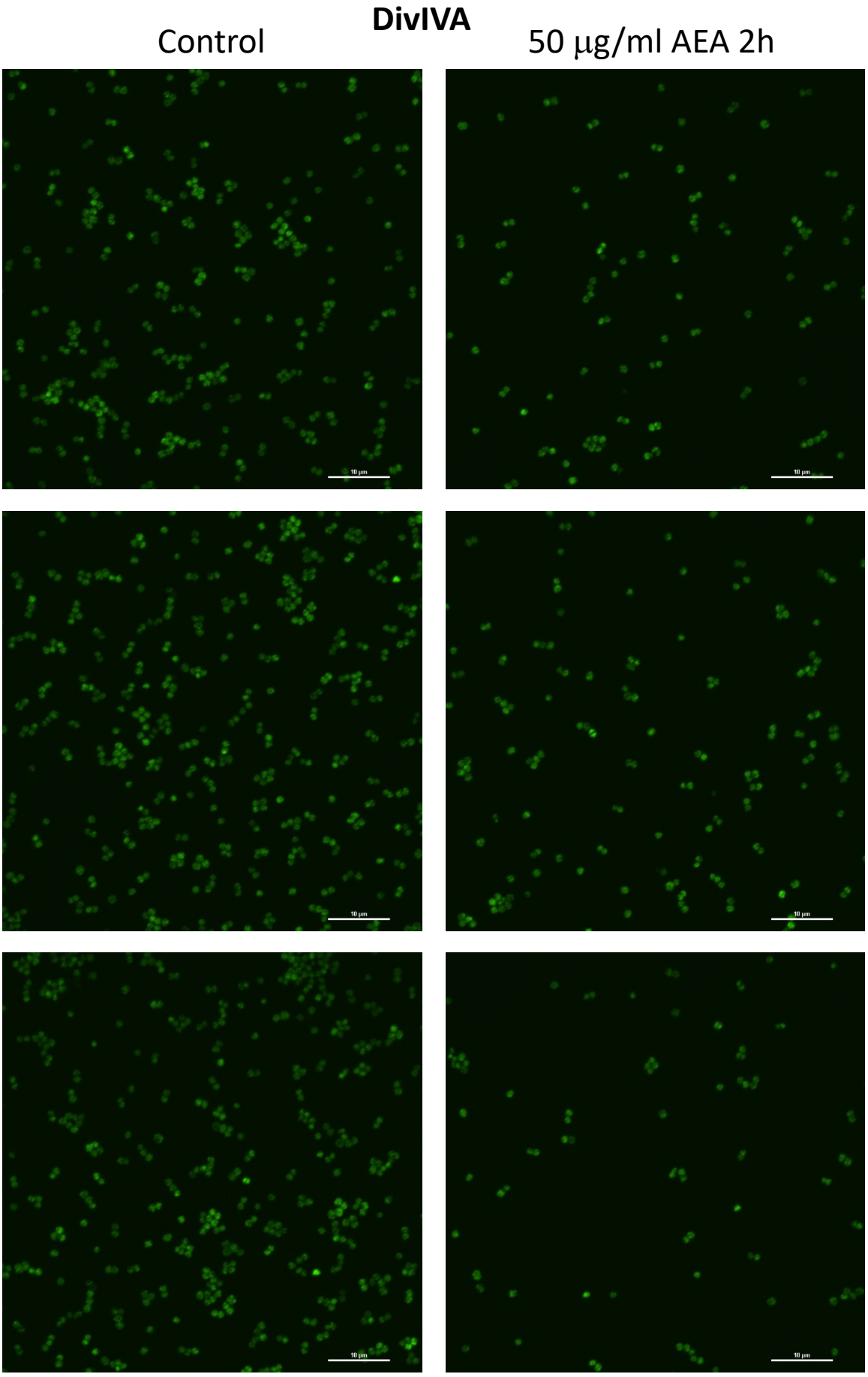
Supplementary Figure S3. AEA increases cell-bound extracellular DNA. MDRSA CI-M was exposed to 50 µg/ml AEA for 2 h in TSBG at 37°C, washed in PBS and then exposed to 2 µM TOTO-1 for 20 min at room temperature.



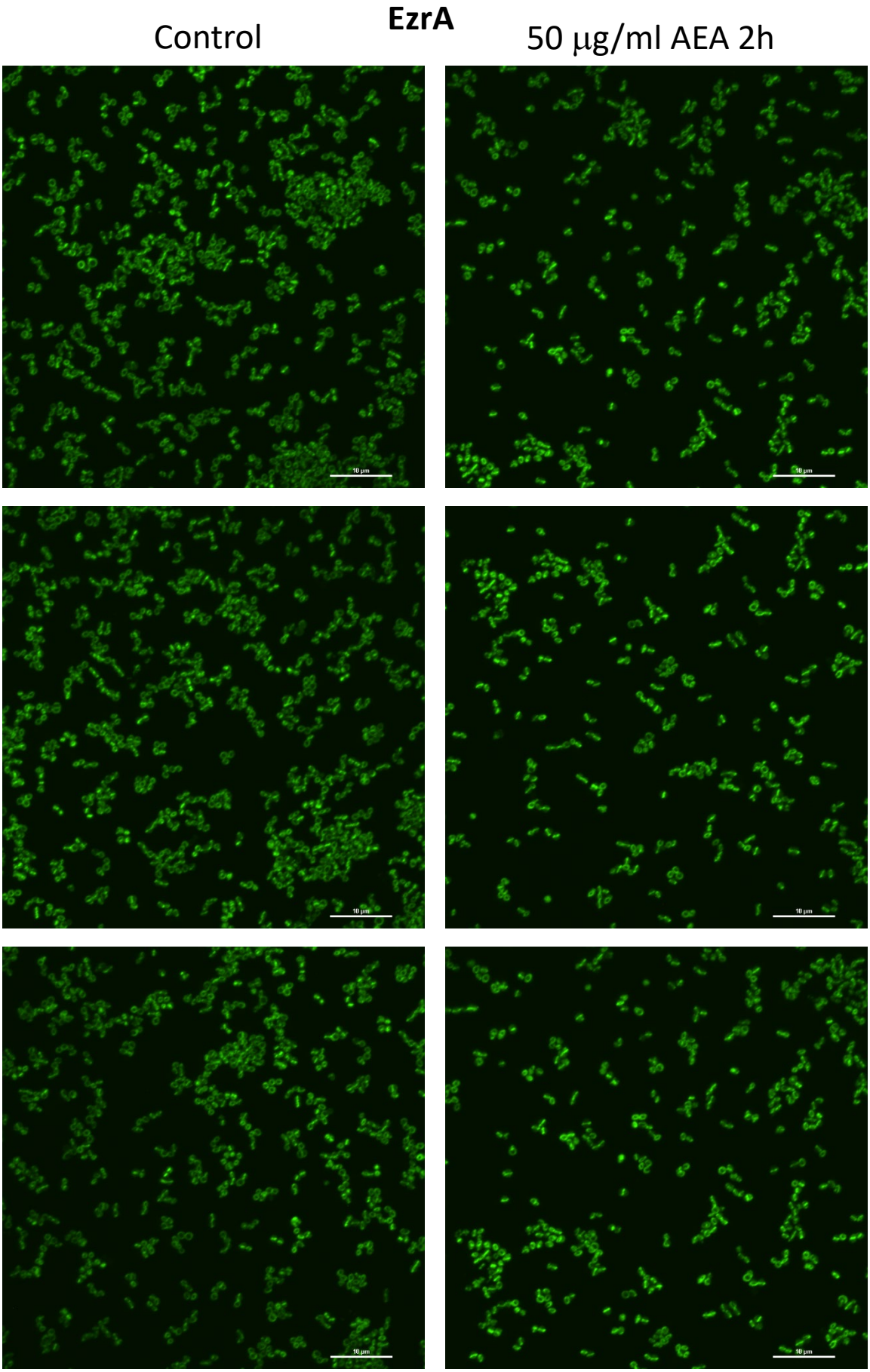
Supplementary Figure S4. AEA did not alter the affinity of wheat germ agglutinin (WGA), concanavalin (ConA) or Dextran (average MW 10,000 Dalton) to the bacterial surface. MDRSA CI-M was incubated in the absence or presence of 50 µg/ml of AEA for 2 h, and then the bacteria were incubated with 10 µg/ml of AlexaFluor^{647}-conjugated WGA. ConA or Dextran for 20 min prior to analysis by flow cytometry. Grey line: Unstained bacteria; Black line: Control; and Red line: AEA-treated bacteria.



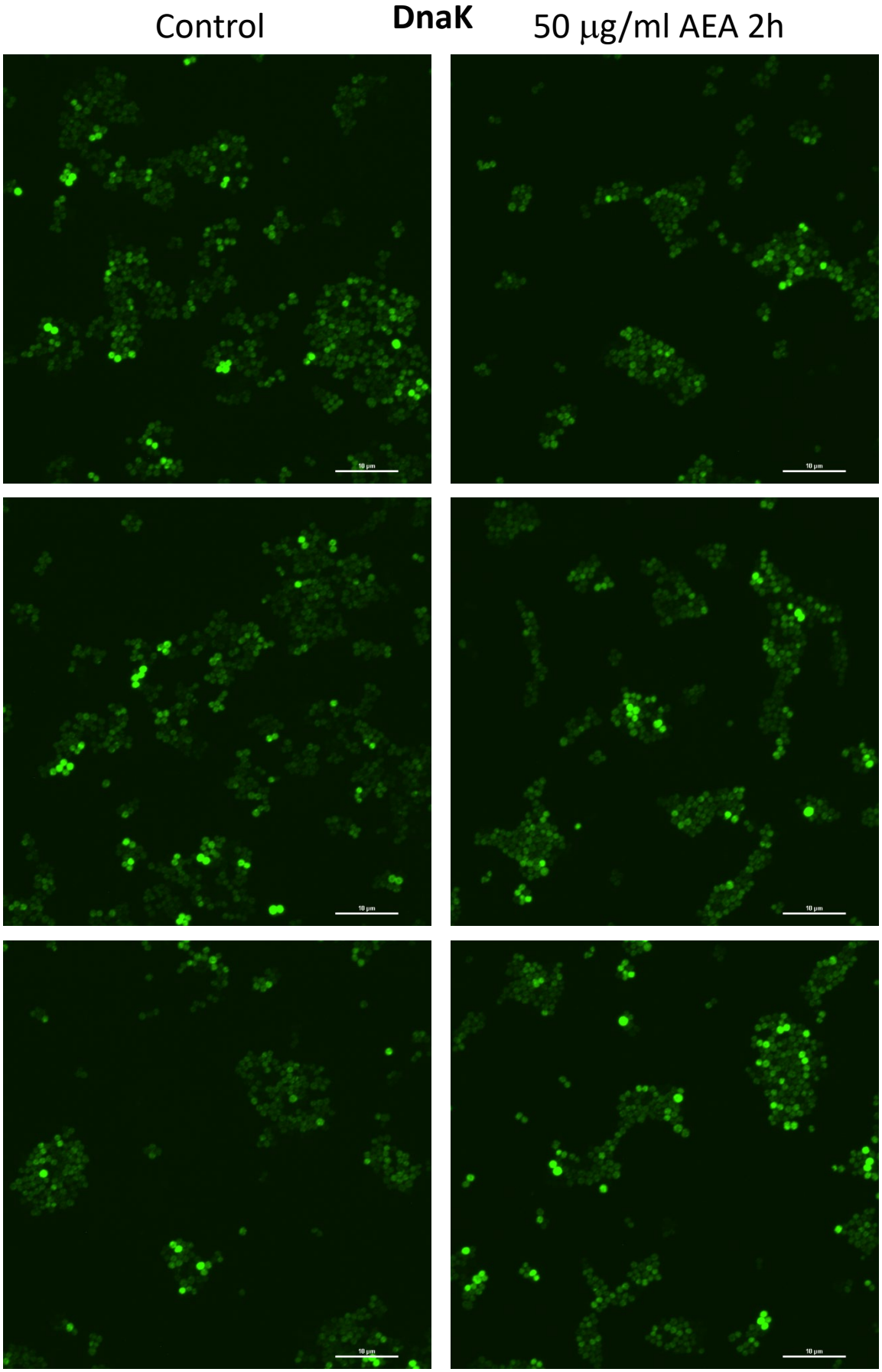
Supplementary Figure S5. LH607 expressing an inducible FtsZ-GFP (SA103; pLOW *ftsZ-gfp*, pGL485 (*erm*^{R}, *cat*^{R}, *tet*^{R})) was exposed to 50 μM IPTG for 2 h and then incubated in the absence or presence of 50 μg/ml AEA for 2 h and the green fluorescence visualized by spinning disk confocal microscopy.



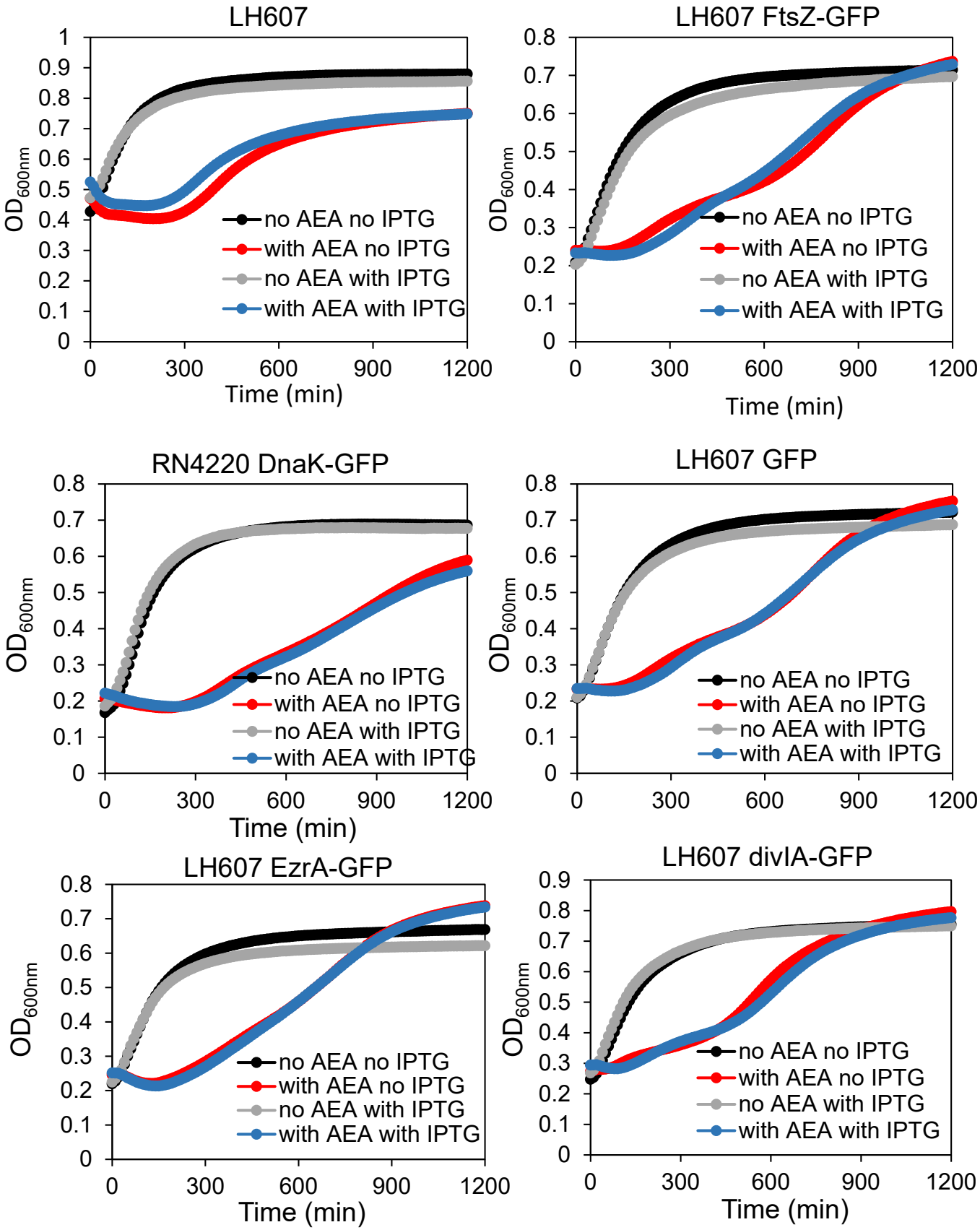
Supplementary Figure S6. LH607 expressing inducible DivIA-GFP (SA356; PdivIVA divIVA-gfp::Pspac divIVA, pGL485 (ermR catR tetR)) was exposed to 50 µM IPTG for 2 h and then incubated in the absence or presence of 50 µg/ml AEA for 2 h and the green fluorescence visualized by spinning disk microscopy.



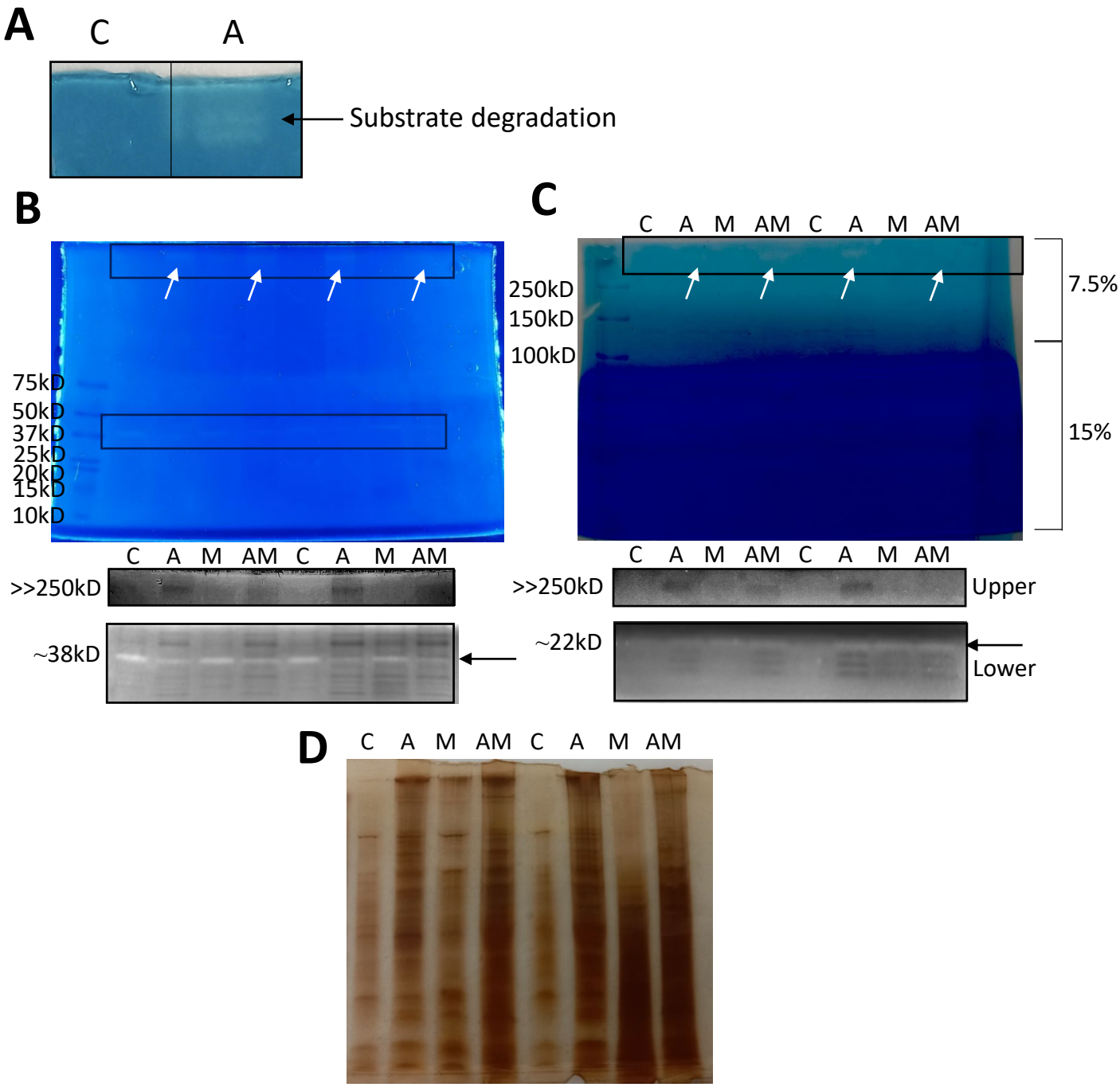
Supplementary Figure S7. LH607 expressing EzrA-GFP (SA353; *ezrA::ezrA-gfp*, pGL485 (*erm*^{R} *cat*^{R})) was exposed to 50 µM IPTG for 2 h and then incubated in the absence or presence of 50 µg/ml AEA for 2 h and the green fluorescence visualized by spinning disk microscopy.



Supplementary Figure S8. RN4220 expressing inducible DnaK-GFP (SA307; pLOW *dnaK-msgfp*, pGL485 (*erm*^{R} *cat*^{R})) was exposed to 50 µM IPTG for 2 h and then incubated in the absence or presence of 50 µg/ml AEA for 2 h and the green fluorescence visualized by spinning disk microscopy.

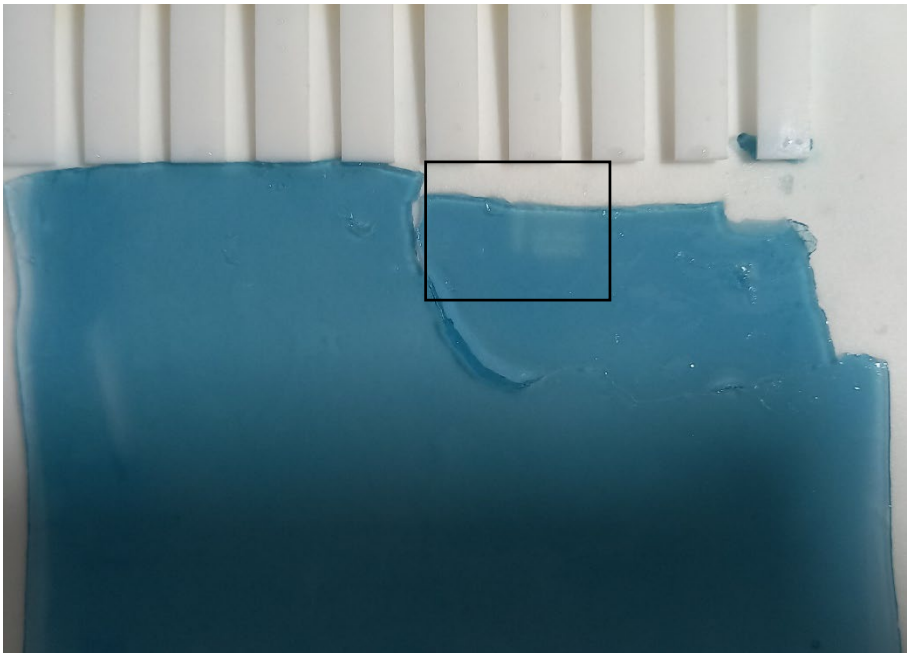


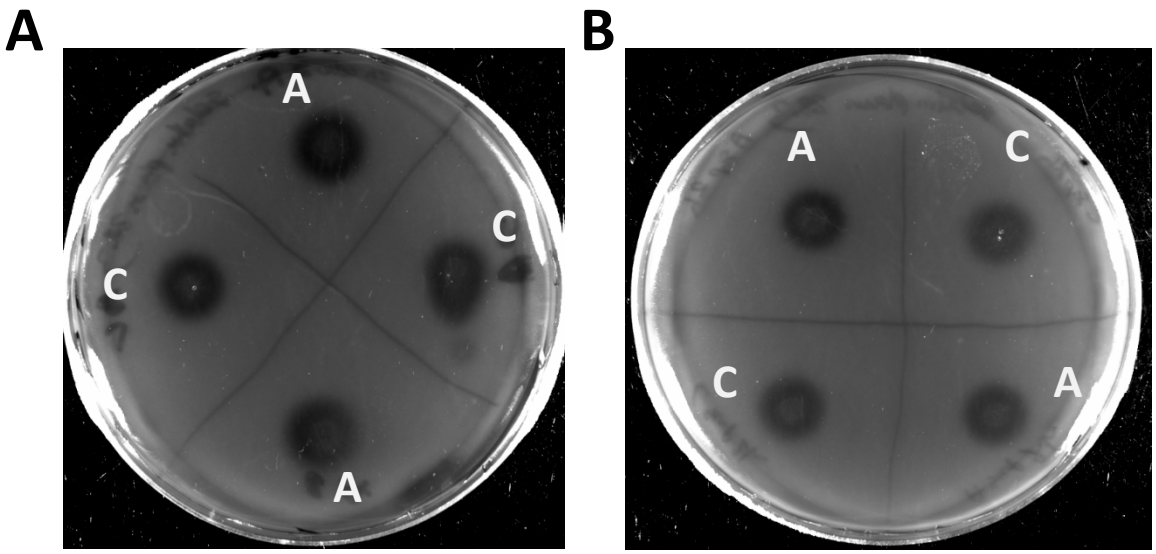
Supplementary Figure S9. Overnight cultures of the various *S. aureus* strains were resuspended to an OD_{600nm} of 0.1 in TSBG and incubated in TSBG for 2 hrs with 50 µM IPTG to induce gene expression. Then the bacteria were incubated in the absence or presence of 50 µg/ml AEA and the planktonic growth was measured each 10 min for 20 hrs.



Supplementary Figure S10. Appearance of a high molecular weight protease activity following AEA treatment. A. Gelatin zymogram: SDS-extracts of control or 50 µg/ml AEA (2h)-treated MDRSA CI-M were run in a 5% non-reduced SDS-PAGE containing 1.2 mg/ml gelatin, and following incubation in protease reaction buffer for 24 h at 37°C, the gel was stained with Coomassie blue. A double band clear region was observed in the AEA-treated bacteria in the upper part of the gel representing protein complexes >>250kD. **B-C.** Gelatin (**B**) and casein (**C**) Zymograms of MDRSA CI-M that have been exposed to 50 µg/ml AEA and/or 50 µg/m MET for 2h. The upper part of the gel was 7.5%, while the lower part was 15%. Some of the substrate have electrophoresed from the 7.5% gel into the 15% gel. The two lower panels of B and C are black and white images of the two separate parts of the gel. To better visualize the >>250kD proteolytic bands, the images were inverted. In the lower panel, the proteolytic bands appear as clear regions within the gel. **D.** Silver stain of the same samples run in **B** and **C** using a 4-15% gradient gel. C=Control; A=AEA; M=MET; AM= AEA+MET. SDS-extraction was done on the same OD of bacteria.

The image of the whole gel of Suppl. Fig. 10A.





Supplementary Figure S11. A. Anandamide treatment didn't interfere with secreted protease activity. A-B. MDRSA CI-M was incubated in the absence or presence of 50 µg/ml AEA for 2 h, and then either 10 µl of the bacterial culture (**A**) or 10 µl of the culture supernatant (**B**) were inoculated on TSA agar plates containing 1.5% gelatin. The plates were incubated for 24 h and stained with crystal violet. Clear areas are seen in both control and AEA-treated bacteria indicating the degradation of gelatin by secreted proteases.

Supplementary Table S1 –Relevant function of genes studied.

Gene	Relevant gene functions	Reference
abcA	An ATP-dependent transporter that confers resistance to β-lactam antibiotics. *abcA* is regulated by MgrA, NorG, Rot and sarZ.	[1,2]
airSR	AirSR two-component system is involved in resistance to reactive oxygen species by upregulating Staphyloxanthin production. An *airSR Staphylococcus aureus* mutant exhibited reduced autolysis rates and reduced viability in the presence of vancomycin.	[3,4]
arlR	The two-component system ArlRS confers oxacillin resistance by inducing the expression of *spx*. ArlR regulates the expression of more than hundred genes involved in different functions, including autolysis, cell division, growth, and pathogenesis. ArlRS activates MgrA-mediated transcription of genes including cell wall-anchored adhesins (*ebh*, *sdrD*), polysaccharide and capsule synthesis genes, cell wall remodeling genes (*lytN*, *ddh*), genes involved in metal transport (*feoA*, *mntH*, *sirA*), anaerobic metabolism genes (*adhE*, *pflA*, *nrdDG*) and a large number of virulence factors (*lukSF*, *lukAB*, *nuc*, *gehB*, *norB*, *chs*, *scn* and *esxA*).	[5-8]
atlA	The initial attachment of *S. aureus* to a surface depends on the autolysin AtlA. Autolysins promotes the release of extracellular DNA to the biofilm matrix. AtlA is also involved in cell division, cell wall turnover and bacterial lysis.	[9-11]
cidA	The CidA murein hydrolase regulator contributes to extracellular DNA release and biofilm formation in *Staphylococcus aureus*. CidA increases the activity of murein hydrolases and promotes the detachment of bacteria from the biofilm and their spread to new infection sites in the dispersion phase. CidA is a holin that is antagonized by the anti-holin Lrg system.	[12,13]
clpP	Inactivation of ClpXP protease led to increased β-lactam resistance in a MRSA USA300 strain. A truncating mutation in *clpP* leads to vancomycin resistance. ClpP degrades the autolysin Sle1 required for proper cell division. ClpXP degrades superoxide dismutase (SodA) making the bacteria more prone to cell death. ClpXP degrades the transcription factor Spx that confers antibiotic resistance.	[14-18]
codY	CodY is a nutrient-sensing regulator that affect the expression of over 200 genes. Among others it represses metabolic genes and virulence genes. CodY represses capsule production. A *codY* mutant is resistant to butyrate-induced growth inhibition. A strain lacking *codY* regulatory activity produces a PIA-dependent biofilm.	[19-24]
essC	*essC* encodes a ESAT-6 secretion system C component belonging to the Type VII protein secretion system (T7SS) involved in virulence, EssC possesses a membrane-bound multidomain ATPase and is involved in protein transport.	[25]
fmhB	FmhB is involved in the first step of peptidoglycan pentaglycine interpeptide formation. This interpeptide plays a role in the stability of the *S. aureus* cell wall, acts as an anchor for cell wall-associated proteins and is essential for methicillin resistance. Any shortening of the pentaglycine side chain reduces or even abolishes methicillin resistance.	[26]
gpsB	GpsB localizes to mid-cell during cell division and interacts with the core divisome component FtsZ. GpsB stimulates the GTPase activity of FtsZ and promotes bundling of FtsZ filaments, thus enabling cell division. Depletion of GpsB caused cell division arrest and cell lysis, whereas overproduction of GpsB led to too early activation of FtsZ, resulting in the formation of enlarged cells.	[27]

isaA	IsaA is a highly immunogenic, noncovalently cell wall-bound lytic transglycosylase that is co-regulated with the glycylglycine endopeptidase LytM. Deletion of *isaA* in a MRSA strain led to decreased biofilm formation and reduced β-lactam resistance.	[28,29]
lrgAB	LrgA is an anti-holin that antagonizes the activity of murein hydrolases. A *lrgAB* mutant showed increased extracellular murein hydrolase activity. The *lrgAB* mutation were more sensitive to penicillin when approaching the stationary phase of growth, the time at which the *lrgAB* operon is maximally expressed. However, the *lrgAB* mutation did not affect penicillin-induced killing of cells growing in early-exponential phase, a time in which *lrgAB* expression is minimal. Inactivation of *lrgB* increases cell lysis-dependent eDNA release and enhances biofilm formation.	[13,30,31]
luxS	LuxS mutants of *S. aureus* showed increased biofilm formation, reduced autolysis and increased expression of the vancomycin resistance-associated VraRS two-component regulatory system.	[32-35]
lytSR	LytSR senses changes in the membrane potential and confers resistance to antimicrobial peptides. LytSR is a two-component system that regulates the expression of the anti-holin *lrgA* and *lrgB*.	[30,36,37]
mecA	mecA encodes for the PBP2a variant that shows low affinity for β-lactam antibiotics, and thus confers β-lactam resistance.	[38]
pbp4	A penicillin-binding protein that can confer β-lactam resistance, which is thought to be due to its high transpeptidase activity, that results in the production of a highly cross-linked cell wall peptidoglycan.	[1,39,40]
prsA	The foldase PrsA is required for proper folding of PBP2a and thereby promotes β-lactam resistance. Deletion of *prsA* altered oxacillin resistance and caused a decrease in PBP2A membrane expression without affecting *mecA* mRNA levels.	[41,42]
saeRS	The SaeRS two component system controls the production of over 20 virulence factors including hemolysins, leukocidins, superantigens, surface proteins, and proteases. SaeRS negatively regulates the expression of genes involved in cytolysis (*lrgA*) and autolysis (*lytS, atlE and aae*). A *saeRS* mutant showed increase susceptibility to penicillin and oxacillin and was more prone to autolysis.	[43-45]
sasG	The SasG surface protein promotes biofilm formation, especially during the accumulation phase, which requires physiological levels of zinc ions.	[46,47]
sigB	SigB affects biofilm maturation by repressing the expression of RNAIII that has anti-biofilm activities. A *sigB* mutant showed increased RNAIII expression, elevated extracellular protease levels and altered murine hydrolase activity.	[48]
sle1	The autolysin Sle1 is important for the onset of daughter cell separation. Sle1 is a substrate of the ClpXP protease. High Sle1 levels in bacteria lacking ClpXP activity confer β-lactam hyper-resistance.	[18]
sprX	SprX is a small non-coding RNA that positively regulates the expression of the autolysin regulator WalR, resulting in increased induction of the autolysins *isaA* and *lytM*. SprX upregulates the expression of the virulence genes cell wall-associated clumping factor B (*clfB*) and delta hemolysin (*hld*). Down-regulation of *sprX* resulted in decreased biofilm formation and higher resistance to Triton X-100-induced lysis.	[49,50]
spx	Spx is a stress-induced transcriptional regulator that controls the expression of *trfA* implicated in antibiotic resistance. Spx expression is regulated by the ArlRS two-component system. Deletion of *arlRS* sensitized MRSA to oxacillin, while overexpression of Spx in the *ΔarlRS* strain restored oxacillin resistance.	[5,51-53]

	A *spx* mutant was hypersensitive to a wide range of stress conditions including high and low temperature, high osmolarity, and hydrogen peroxide due to lack of *trxB* thioredoxin reductase transcription. YjbH controls the degradation of Spx by ClpP.	
tarO	TarO is involved in the initial step of cell wall teichoic acid synthesis. TagO catalyzes the reversible transfer of GlcNAc-1-P from UDP–GlcNAc to the undecaprenyl phosphate scaffold to produce lipid-α (GlcNAcα–PP-Undecaprenyl). A *tagO* mutant showed increased cell surface hydrophobicity, enhanced autolytic activity, impaired biofilm formation, and reduced expression of *icaADBC* and *PIA* genes. Deletion of *tarO* in a MRSA strain restored their sensitivity to methicillin.	[54-56]
tarA	TagA is involved in the step after TagO in cell wall teichoic acid synthesis. TagA is a ManNAc transferase that adds ManNAc from a sugar nucleotide donor (UDP-ManNAc), producing a ManNAc (β1 → 4) GlcNAcα–PP- Undecaprenyl product, called lipid-β.	[54]
tarM/tarS	TarM and TarS add α-linked and β-linked N-acetylglucosamine, respectively, to the poly-ribitol chain of the growing wall teichoic acid. Eliminating *tarS* from a MRSA strain sensitized the bacteria to β-lactams. This suggests that β-O-GlcNAcylation of wall teichoic acids is required for MRSA resistance.	[54,57]
tarG/tarH	The TagGH transporter transfer the wall teichoic acid across the membrane. TagG is essential for bile-induced biofilm formation in *S. aureus* and its expression protects the bacteria from bile-induced cell lysis.	[54,58]
trfA	TrfA is required for the degradation of the MazE antitoxin and thus affects dormancy and tolerance to antibiotics. *trfA* transcription is regulated by the redox sensitive transcriptional factor Spx.	[51,53,59]
walKR	The WalKR two-component system controls cell wall metabolism by regulating autolysin production such as *sceD, ssaA, lytM* and *atlA*. A deletion mutation in *walRK* conferred vancomycin resistance.	[15,60]

Supplementary Table S2 – Primers used for quantitative real-time PCR for *Staphylococcus aureus*.

Gene	Forward Primer	Reverse Primer	Reference
16S rRNA	CCAGCAGCCGCGGTAAT	CGCGCTTTACGCCCAATA	[61]
abcA	CAAGAACCTATTGAACCGACAGAA	GTGGGATTTGGAACGACACA	[1]
airR	TGCTGATGGTTATGAAATGA	CATCTTGTGCCTTAGGATGT	[19]
airS	TTCCTAGCCAAAATGACAATA	TTCAGTATTTGGAGACGCTAC	[19]
arlR	TTCTTCAATATCAAACGGCTTA	GACAACAATCTACACCTAT	[5]
asnC	TCGGTGGATCTGAACGTGTGGA	GTGGCACACTACCATAACGACG	[62]
atlA	AACAGCACCAACGGATTAC	CATAGTCAGCATAGTTATTCATTG	[49]
cidA	CTACTACTACAACTAGGAATCATC	TTTAGCGTAATTTCGGAAGC	[63]
clpP	AACAACAAATCGCGGTGAAC	CATAAATCGCAAAACCAGCTGT	This paper
codY	ATCGCATCAAAAGTTGCAGA	CGTGATTCAATTACACCAGCA	[19]
essC	ACCATCGTTCGCCAAGGA	TGGCTGTGGCGGTCTTTC	[64]
fmhB	AAGCGAGGTACGACAGTAGAACG	CATCTCCATCTTCATGCAACGCA	[61]
glyA	CTACAAACTCACAGCCAC	GTATCGGAAGCGGTTATG	[61]
gmk2	CCATCTGGAGTAGGTAAAGG	CTACGCCATCAACTTCAC	[61]
gtf	TGGTGACGCCGAAGGACTC	GCAGCACGAGCAGGAACAC	[61]
gpsB	TCCTGAGGTCTTGATGTTGC	TGGCTCGTGGCTATAGAAGA	This paper
gyrA	TGGCCCAAGACTTTAGTTATCGTTATCC	TGGGGAGGAATATTTGTAGCCATACCTAC	[61]
gyrB	GGTGCTGGGCAAATACAAGT	TCCCACACTAAATGGTGCAA	[61]
isaA	GCAGGTGCTACTGGTTCATCAG	GATTCACGAGCGATGATTGC	[49]
lrgA	TGAAACAACAAAAAGACGCATCAAAACCAG	ACTTCGCCTAACTTAACAGCACCAG	[6]
lrgB	TATTTGGTGTGGCCTTCCTC	AAACAGATTGTTGCCGGTTC	[63]
luxS	CGGACTACATTCATTAGAACATT	TTACAAGCAGGCACTTCA	[65]
lytR	ATTAGGAGCTAAGATTCAAAAGATG	TTGACTGCTTGTTCAATACG	[63]
lytS	GCATGGTTCTATCGTCGGTACATTG	ACTTACTTTGCGTTTCGGCTTCAC	[6]
pbp4	CTAAAGGTGAGCAAAGGATAAATGG	TCTCTTGGATAGTCCGCGTGT	[1]
proC	GGCAGGTATTCCGATTGA	CCAGTAACAGAGTGTCCAAC	[61]
prsA	AGTTAATGATAAGAAGATTGACGAACAAA	GAAGGGCCTTTTCAAATTTATCTTT	[42]
recF	AGTTATAGACACGGCACG	GCGTCGTCTTATTTGAGG	[61]
rho	GGAAGATACGACGTTCAGAC	GAAGCGGGTGGAAGTTTA	[61]
RNAII	TATGAATAAATGCGCTGATGATATACCACG	TTTTAAAGTTGATAGACCTAAACCACGACC	[61]
rpoB	TCCTGTTGAACGCGCATGTAA	GCTGGTATGGCTCGTGATGGTA	[61]
saeR	AAGTGGCGACCATTACAT	CATTATTGCCTCAAATACGT	[66]
saeS	TGCCAATACCTTCATCGCTAA	CAATATCGAACGCCACTTGA	[67]
sasG	ATCGTCAGTCACTCATAAC	TATCAACACTTCCGTAACC	[65]
sigB	TCGATAACTATAACCAAAGCCT	AAGTGATTCGTAAGGACGTCT	[68]
sle1	TCAGGATCTGCAACAACGAC	CCTTTACCAATTTCAGCACGAC	[15]
sprX	ATAATCTTTCTAGACGTATTCAAA	CAGGCTATATAGTTCACTCCTACT	[6]
spx	GCTTATTACGTCGTCCAATTATTTTA	CGTACGAACTTTTCTAGGTAAGAA	[16]
tarA	GTTGCTGATGGGACAGGAGT	TGCATATTGTGCCGCTTCTA	[17]
tarG	ATCAGTATGTGGTTCTTCATC	TGCTGCACGGTATGATTCAG	[17]
tarH	ATCATTGGCGGTTCTTTGTC	TGCACGCATACCACTTGAAT	[17]
tarO	TTCCATCCTGCCAAAATA	GAATGGAACTGCTAAGATAACA	[17]
tarM	TAATGCTAATAATGGTGCTG	GGTCCATCACAAATCATAAT	[18]
tarS	CACGAAACAAGAAGCACA	TGATTACCAACACGCACT	[18]
trfA	ATCGAGGCCCGTGGATTTAG	TCGACACCTTTTTCAAAGGCA	[4]
walR	CAAATGGCTAGAAAAGTTGTTGTAG	CAGTAAGCATTATTATTGGCATTTCG	[6]

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