Supplemental materials for

Competence-stimulating peptide-dependent localized cell death and extracellular DNA production in *Streptococcus mutans* biofilms

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Figure S1-S9
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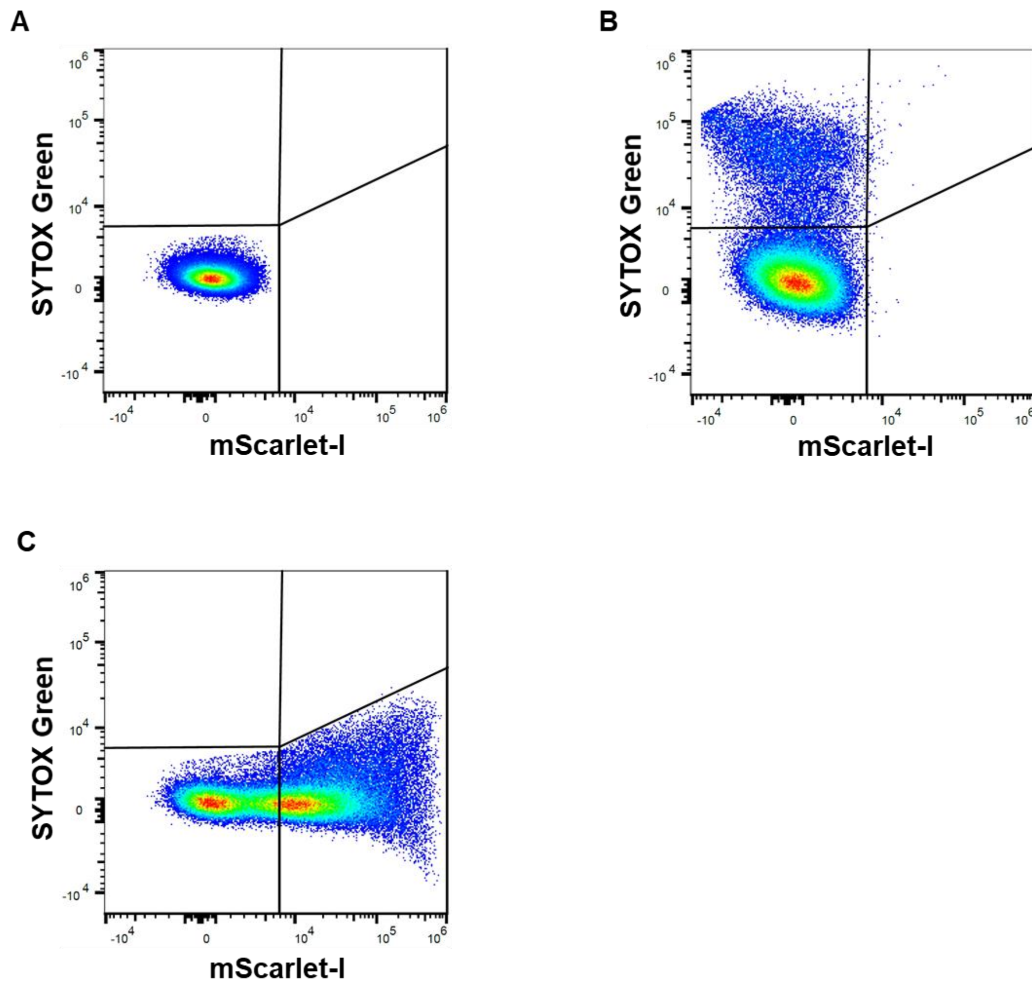

Fig. S1 Gating for determining the negative or positive fluorescence was set with a nonfluorescent sample and single fluorescent samples. *S. mutans* UA159 WT cells cultured in BHI and BHI with sCSP were used for the nonfluorescent sample (A) and the SYTOX Green fluorescent sample (B), respectively. The P_{lytF} reporter strain cultured in BHI with sCSP was used for the mScarlet-I fluorescent sample (C). The cells were grown in an aerobic atmosphere containing 5% CO_2 at 37°C for 6 h. SYTOX Green fluorescent samples were stained with 500 nM SYTOX Green for 15 min and then subjected to flow cytometry.

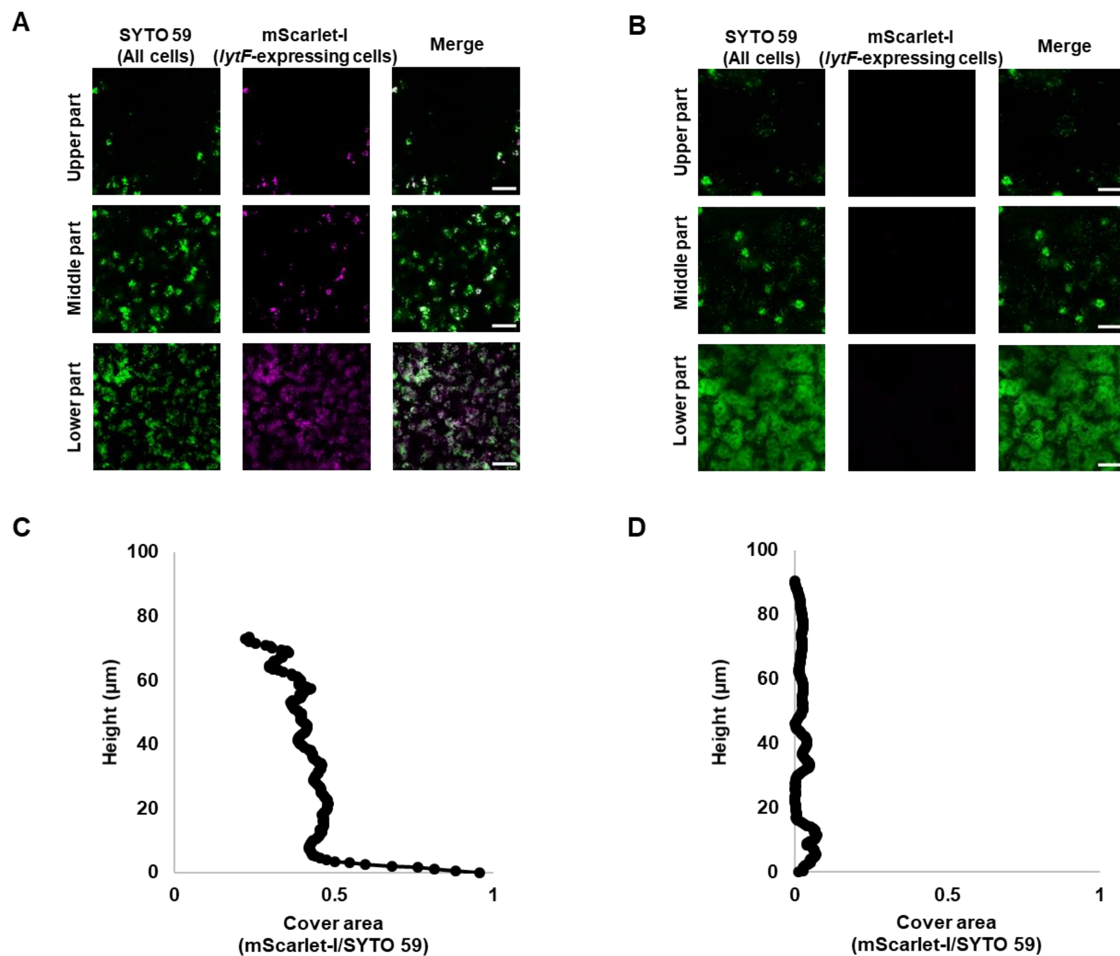

Fig. S2 *lytF*-expressing cells are abundant near the bottom of the biofilm. The P_{lytF} reporter strain was grown in BHIs with sCSP (A, C) or BHIs (B, D) in an aerobic atmosphere containing 5% CO_2 at 37°C for 6 h in a 6-well plate. Nonadherent cells were removed by washing twice with PBS. The biofilm cells were stained with 5 µM SYTO 59 for 30 min and observed with upright CLSM. Z-stacks were acquired at 0.5 µm intervals. (A, B) Distances of 2, 30, and 60 µm from the polystyrene surface were defined as the lower, middle, and upper parts of the biofilm, respectively. SYTO 59 (all cells) and mScarlet-I (*lytF*-expressing cells) are shown in green and magenta, respectively. The scale bars indicate 20 µm. (C, D) In each image of the *xy* sections, the cover areas where the fluorescence of mScarlet-I and SYTO 59 were detected were calculated by ImageJ and Fiji. The x-axis shows the mScarlet-I/SYTO 59 ratio of the cover area, and the y-axis shows the height of the biofilm. Representative data from independent experiments are presented.

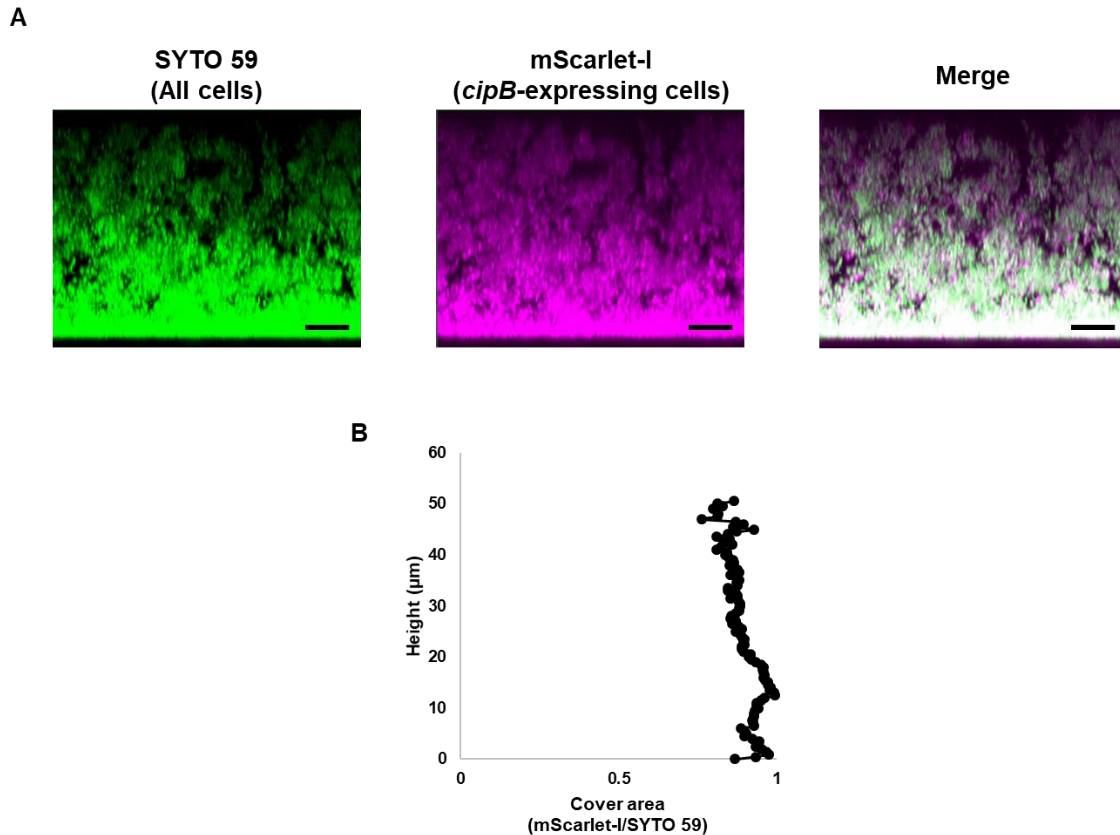

Fig. S3 Almost all cells in the biofilm captured the sCSP signal. We used the *cipB* promoter as a reporter for CSP capture because *cipB* is one of the genes directly controlled by the two-component regulatory system ComDE that recognizes CSP signals. The P_{cipB} reporter strain was grown in BHIs with sCSP in an aerobic atmosphere containing 5% CO_2 at 37°C for 6 h in a glass-bottom dish. Nonadherent cells were removed by washing twice with PBS. The biofilm cells were stained with 5 µM SYTO 59 for 30 min and observed with inverted CLSM. Z-stacks were acquired at 0.5 µm intervals. SYTO 59 (all cells) and mScarlet-I (*lytF*-expressing cells) are shown in green and magenta, respectively. The scale bars indicate 10 µm. Representative images from independent experiments are presented.

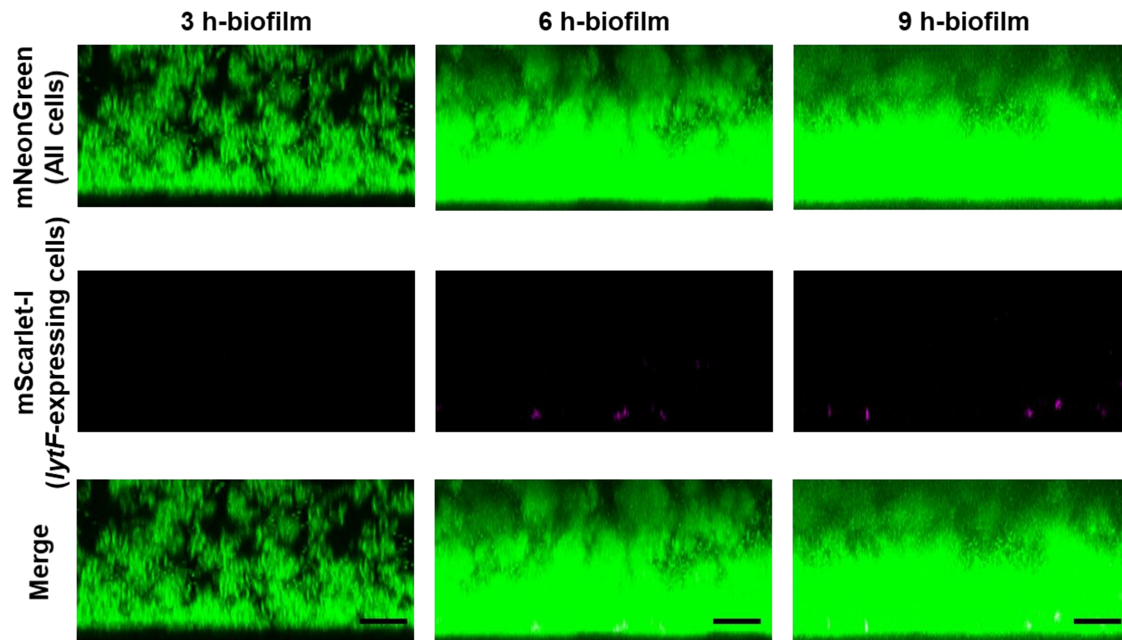

Fig. S4 *lytF*-expressing cells were observed near the bottom of the 6 h and 9 h biofilms when cultured in the absence of sCSP. The P_{ldh} P_{lytF} dual-reporter strain was grown in an aerobic atmosphere containing 5% CO_2 at 37°C in BHIs for 3, 6, or 9 h in glass-bottom dishes. Nonadherent cells were removed by washing twice with PBS. An inverted CLSM was used to make observations at a distance of approximately 40 µm from the surface was observed using an inverted CLSM. Z-stacks were acquired at 0.5 µm intervals. These images show side views of the biofilms. Since the samples for each culture time were individually prepared, these images were not in the same field of view. mNeonGreen (all cells) and mScarlet-I (*lytF*-expressing cells) are shown in green and magenta, respectively. The scale bars indicate 10 µm. Representative images from independent experiments are presented.

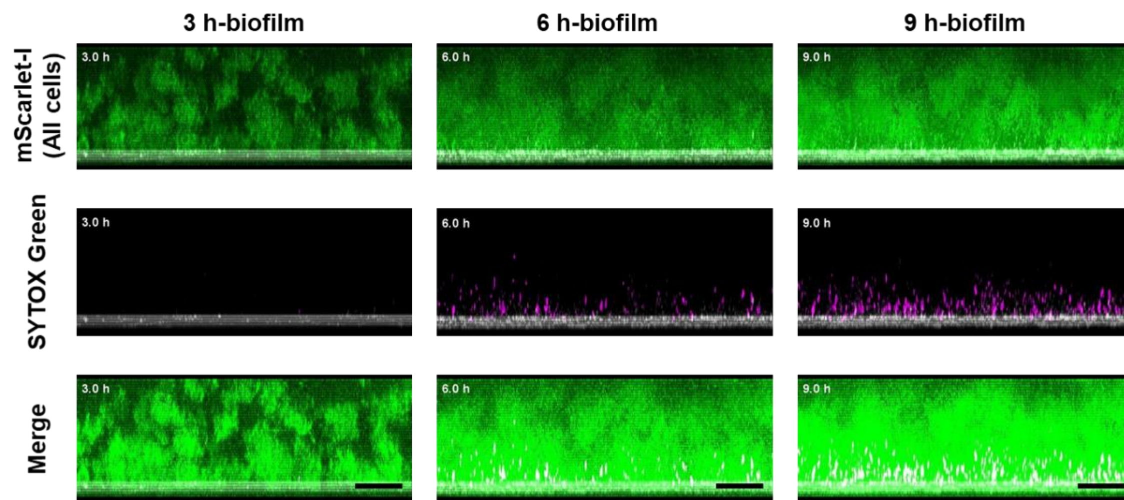

Fig. S5 Dead cells and eDNA are abundant near the bottom of the biofilm formed in BHIs without sCSP. The P_{ldh} reporter, a constitutive promoter reporter, was grown in a glass-bottom dish in BHIs at 37°C. The medium was supplemented with 1.25 µM SYTOX Green to stain dead cells and extracellular nucleic acids. The same field of view within a range of approximately 40 µm from the glass surface was observed at 30 min intervals while culturing at 37°C. Z-stacks were acquired at 0.55 µm intervals. These images show side views of the biofilms, and the white line below each image shows the glass surface obtained by reflection. mScarlet-I (all cells) and SYTOX Green (dead cells and extracellular nucleic acids) are shown in green and magenta, respectively. The scale bars indicate 10 µm. Representative images from independent experiments are presented.

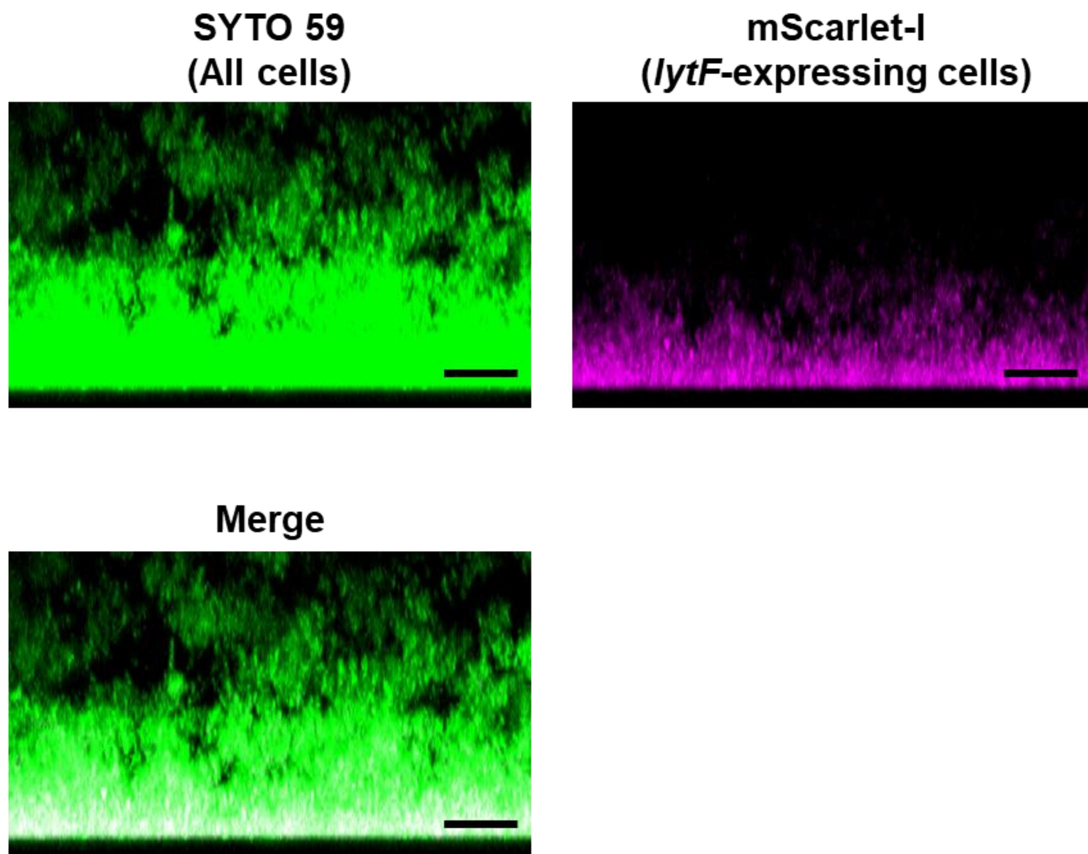

Fig. S6 *lytF*-expressing cells are abundant near the bottom of the biofilm formed on the saliva-coated glass surface. To examine whether the nature of the biofilm-forming surface affects the distribution of *lytF*-expressing cells within the biofilm, the biofilm formed on the saliva-coated glass surface was observed. A filter-sterilized whole saliva sample was added to a glass-bottom dish and allowed to stand overnight at 4°C. The dish was used after washing twice with sterile PBS. The P_{lytF} reporter strain was grown in an aerobic atmosphere containing 5% CO_2 at 37°C in BHIs with sCSP for 6 h in a saliva-coated glass-bottom dish. Nonadherent cells were removed by washing twice with PBS. The biofilm cells were stained with 5 µM SYTO 59 for 30 min and observed with inverted CLSM. Z-stacks were acquired at 0.5 µm intervals. These images show side views of the biofilm. SYTO 59 (all cells) and mScarlet-I (*lytF*-expressing cells) are shown in green and magenta, respectively. The scale bars indicate 10 µm. Representative data from independent experiments are presented.

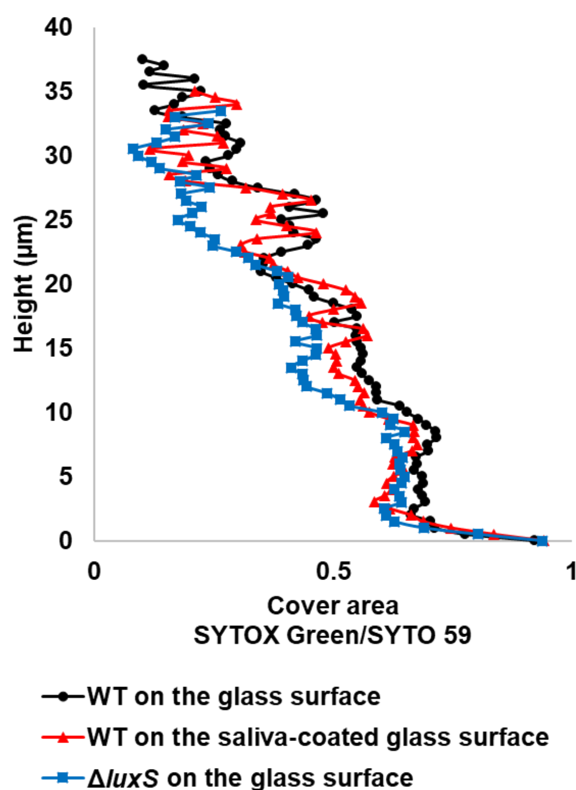

Fig. S7 Saliva and AI-2-mediated quorum sensing has no effect on the localization of *lytF*-expressing cells in the biofilm. The distributions of *lytF*-expressing cells in the biofilm formed by WT on the glass surface and the saliva-coated glass surface (Fig. S7) and the biofilm formed by Δ*luxS* on the glass surface (Fig. S8) were examined by image analysis. In each two-dimensional image, the cover areas where the fluorescence of mScarlet-I (*lytF*-expressing cells) and SYTO 59 (all cells) were detected were calculated by ImageJ and Fiji.

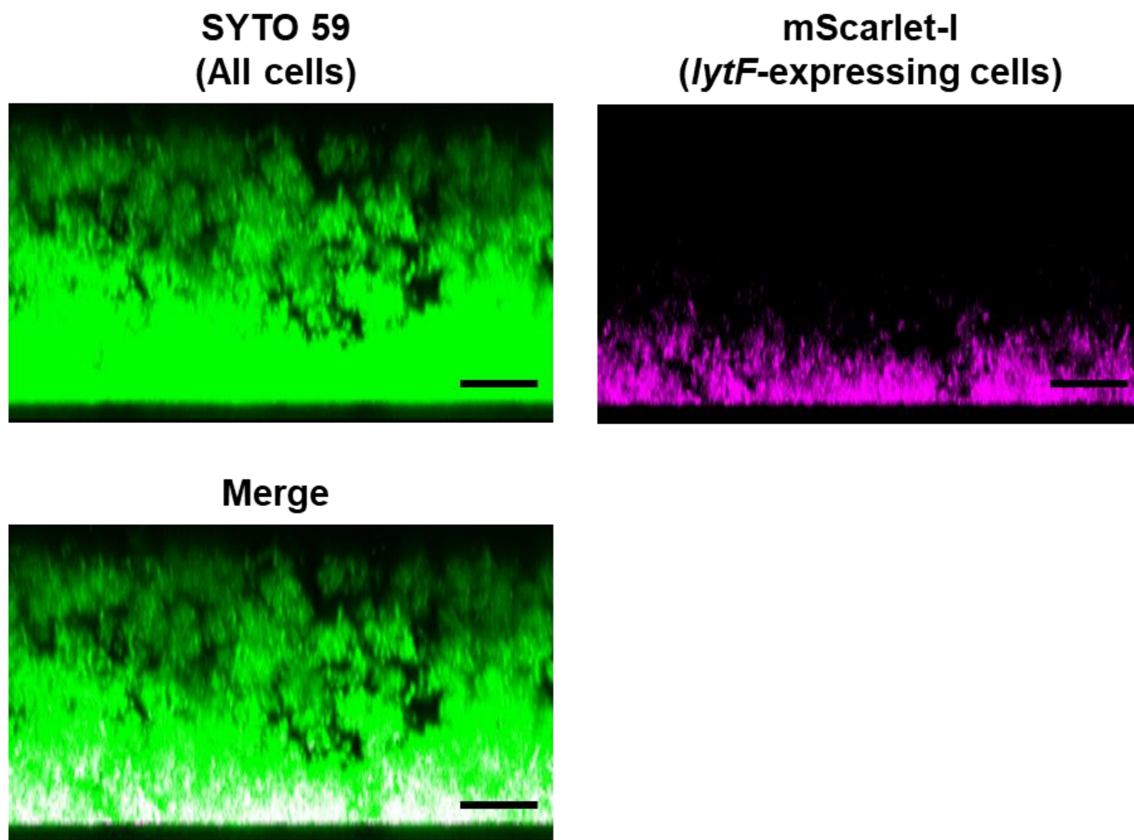

Fig. S8 *lytF*-expressing cells are abundant near the bottom of the biofilm formed by Δ*luxS*. To investigate whether quorum sensing via AI-2 is involved in the heterogeneous distribution of *lytF*-expressing cells in the biofilm, we analyzed the biofilm of the Δ*luxS* strain that cannot synthesize AI-2. The Δ*luxS* P_{lytF} reporter strain was grown in an aerobic atmosphere containing 5% CO_2 at 37°C in BHIs with sCSP for 6 h. Nonadherent cells were removed by washing twice with PBS. The biofilm cells were stained with 5 μM SYTO 59 for 30 min and observed with inverted CLSM. Z-stacks were acquired at 0.5 μm intervals. These images show side views of the biofilm. SYTO 59 (all cells) and mScarlet-I (*lytF*-expressing cells) are shown in green and magenta, respectively. The scale bars indicate 10 μm. Representative data from independent experiments are presented.

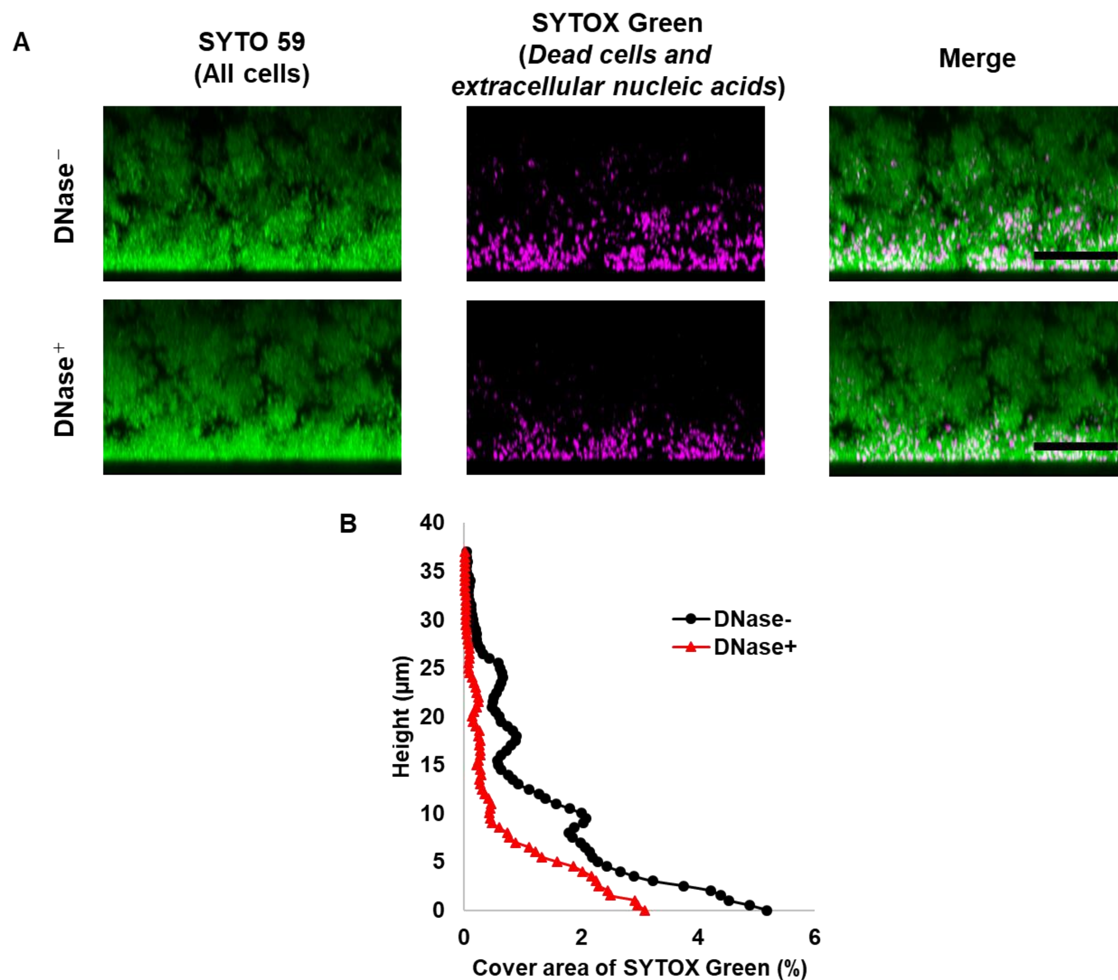

Fig. S9 The addition of DNase I to the culture medium reduces eDNA in the biofilm. CLSM observations and image analysis were performed to determine whether the addition of DNase I to the culture medium prior to culture initiation reduced the eDNA in the biofilm. The WT strain was cultured in a glass-bottom dish under the same conditions as in Fig. 8B (DNase I^- and DNase I^+). The biofilm cells were stained with 5 µM SYTO 59 and 500 µM SYTOX Green for 30 min and observed with inverted CLSM. Z-stacks were acquired at 0.5 µm intervals. (A) SYTO 59 (all cells) and SYTOX Green (eDNA and extracellular nucleic acids) are shown in green and magenta, respectively. The scale bars indicate 20 µm. (B) In each two-dimensional image, the cover area where the fluorescence of SYTOX Green were detected was calculated by ImageJ and Fiji.

Table S1 Primers for mutant construction

Primer	Sequence
comDE up Fw	CTCAAGGCTTTGATAAATTGATGCC
comDE up Rv fuse to ermBP	CTTTTACGTTTCCGGGTACAATTCGGTGGTTTCAAGACGTCCTTG
comDE down Fw fuse to ermBP	GAAATAATTCTATGAGTCGCCAATCATCATCATTTATTCAAGCAACTCC
comDE down Rv	GGCAGTAAGGACAACTTGACTAG
cipB up Fw	GGTTCAAAATGAGATAATGGCAC
cipB up Rv fuse to ermBP	CTTTTACGTTTCCGGGTACAATTCCGTTAAATTGTTCAAATGCTTGTG
cipB down Fw fuse to ermBP	GAAATAATTCTATGAGTCGCGGAGGAGCTCTTAATTCCTGTG
cipB down Rv	CCATAGGTCACCATATGATTAGATC
comR 5	GCAGATCTGCCTCGTCTCA
comR N5	CCCAGTAGTATCGTTGATGCAAC
comR N3	CAATTCGTAATCATGTCATAGCTGTTTCCTAAAACCTTTTCCTATAATCTCTGTCTAAAC
comR C5	GGGAGTGCAGTCGAAGTGGGCAAGTTGAAAGCCTATTGTTAATCTTCTGACATGG
comR C3	AAGGTGTGTATTTATTAGAAATCACACC
comR 3	CCTGGAAACGGAACCCCTCCA
comS 5	CAACCTATGGCGACCAACAAAG
comS N5	CCTGAAGAAGAACAATTGATTATTGATGGC
comS N3	CAATTCGTAATCATGTCATAGCTGTTTCCTCCTGTTATTCTCCTTTCTTTTTGATATCA
comS C5	GGGAGTGCAGTCGAAGTGGGCAAGTTGAAATAATAGACAGCCCTTATGTCAGATG
comS C3	GCAGATGTAGACTTTGACCATGTTG
comS 3	AAACAAGCTTATATTGCTGCGATTG
comX 5	TTCCGGCATAGCTCAGTTGG
comX N5	AATGAAGCATCTTTACCTAGGTGC
comX N3	CAATTCGTAATCATGTCATAGCTGTTTCCTCTTCTTCCATCTATTACGATGACCTCC
comX C5	GGGAGTGCAGTCGAAGTGGGCAAGTTGAAAGGCAAAGTATGCTGAAAGATTTACG
comX C3	CACTTCCAATTTCAAAACCAACATCAATTAG
comX 3	AGGTTCTACAATTTCACCTTTACCTGA
lytF up Fw	GTGTAGAAGAAGAGGGTTATTATCATG
lytF up Rv fuse to ermBP	CTTTTACGTTTCCGGGTACAATTCCTCAATCGAAATCTCCTTTATTCTTTTTTAC
lytF up Rv fuse to spc	GCCAGTCACGTTACGTTATTAGCTCAATCGAAATCTCCTTTATTCTTTTTTAC
lytF down Fw fuse to ermBP	GAAATAATTCTATGAGTCGCTTATATTTATCCTGACTAAGAGAACAAAAC
lytF down Fw fuse to spc	GATAAAATCCGATTAAGATACTGCCTACTTATATTTATCCTGACTAAGAGAACAAAAC
lytF down Rv	CTGAAAAAAACAGAGCAAGCTAAC
gtfB up Fw	CATGAAACCAGTTGGTCTGGAATAC

gtfB up Rv fuse to ermBP	CTTTTACGTTTCCGGGTACAATTCCACTTTCTTGTCCATTAGGAACCTC
gtfB down Fw fuse to ermBP	GAAATAATTCTATGAGTCGCGGAGAACGAGTTCGGATTAACTAATTG
gtfB down Rv	CGAAGTTTGCAGCATCTGTACTATAG
ermBP Fw	AGGAAACAGCTATGACATG
ermBP Rv	TTTCAACTTGCCCACTTCGAC
ermBP Fw 2	GAATTGTACCCGGAAACGTAAAAG
ermBP Rv 2	GCGACTCATAGAATTATTTCCTCC
spec Fw	CTAATAACGTAACGTGACTGGCAAG
spec Rv	GTAGGCAGTATCTTAATCGGATTTTATCG

Table S2 Primers for promoter reporter construction

Primer	Sequence
SMU438c 5	CAAAACGCTTGGCTAAACTAGG
SMU438c C3-TadhE	CGAGAGGCAAAAAAAGCCCGCATAAGCGGGCCTGACTGCTAAACCCTCAATCGGAGTTCTCT
SMU436c C5	TGAATTGTTTTAGTACCTAGTTAAGCTCGCTCGTTCAACC
SMU436c 3	CCAATGCCATTATGATGGCAAG
Pldh-TadhE Fw	CCGCTTATGCGGGCTTTTTTTGCCTCTCGAAGAGCCCGAGCAACAATAAC
Pldh-mScarlet Rv	CTCTTTAATAACAGCTTCACCTTTAGATACCATGTTCTAAACATCTCCTTA
mScarlet-Cp Fw	ATGGTATCTAAAGGTGAAGCTGTTA
mScarlet-Cp-Tldh Rv	AAGCATTACACAATGCTTGGTATTTTTATGGATTTTTATTGTTTACTTATAAAGTTCATCCATCCCTCCA
KanR-Tldh Fw	TCCATAAAAATACCAAGCATTGTGTAATGCTTGGTATTTTTATTAAACGTTGAGGAGGCAGATTGCCTTG
kanR- SMU436c Rv	AACGAGCGAGCTTAACTAGGTACTAAAACAATTCATCCAG
SMU_438c N5	AACCTCCTTGAAAAGCCAATGA
SMU436c C3	GGTATAGATGAAGTTTGCGGTC
SMU438c N5-2	AAGCACTTGAAAAATCGGAACG
SMU436c C3-2	AAATAAAAGAAGGACACCTAGCAG
PlytF-TadhE Fw	CCCGCTTATGCGGGCTTTTTTTGCCTCTCGGTGCATGGGAGGAATAGTAC
PlytF-mScarlet Rv	CTCTTTAATAACAGCTTCACCTTTAGATACCATTCTCAATCGAAATCTCCT
1405 up Fw	CAGAAGAAATGGGCAAGGTAGATG
1405 up Rv fuse to PcomS	CAAACCACTAACAGTTAAGCAGGATAAATAGTGTTGGCCAGCAAGAG
PcomS Fw fuse to 1405 up	CCTCTTGCTGGCCAACACTATTTATCCTGCTTAACTGTTAGTGGTTTG
PcomS Rv fuse to mNeon	CATATTATCCTCTTCACCTTTTGAAACCATCCTGTTATTCTCCTTTCTTTTTGATATC
mNeon Fw fuse to PcomS	CAAAAAGAAAGGAGAATAACAGGATGGTTTCAAAAGGTGAAGAGGATAATATGG
1405 down Rv	CCGATTGCTATTGCCATCATCAATG
1405_rspD-term 1	GAGCGTGAAGATTTTCTAAGTAATCGTTTTAAAAACCCCT
1405_rpsD-term 2	AGGGGTTTTTAAAACGATTACTTAGAAAATCTTCACGCTC

spoVG-term_erm 1	TTTCAAACTTAGTTGCACTCCAGGAAACAGCTATGACATG
spoVG-term_erm 2	CATGTCATAGCTGTTTCCTGGAGTGCAACTAAGTTTGAAA
erm_1405 1	TCGAAGTGGGCAAGTTGAAAAAAGCAACGTACCTTTGACA
erm_1405 2	TGTCAAAGGTACGTTGCTTTTTTCAACTTGCCCACTTCGA
smu_1405 F 1	AAAACCTTACTCTATTGGACTTGATATTGG
smu_1405 F 2	TGTTGTGACAGATGACTACAAAGTTCCTGC
smu_1405 F 3	CTGGGAAATACAGATAAAAGTCATATCGAG
smu_1405 R 1	AACCGGATGTTCTTTAAGAATTTGACTTCC
smu_1405 R 2	AACCTTTCAAACGTTGCTGTGAATTTCGTC
smu_1405 R 3	ATTTTCAGGTTGATGTCCCATAATTTTGAC
rspDterm_Pldh_1	CTCTTGCTGGCCAACACTATTTAAAGAGCCCGAGCAACAATAA
rspDterm_Pldh_2	TTATTGTTGCTCGGGCTCTTTAAATAGTGTTGGCCAGCAAGAG

Table S3 Primers for construction of complemented strain

Primer	Sequence
437 up Fw	GTGAAGCTGTTGTTCCAGATTTG
437 up Rv fuse to PlytF	GTACTATTCCTCCCATGCACCGAGAGGCAAAAAAAGCCC
lytF Fw fuse to 437 up	GCGGGCTTTTTTTGCCTCTCGGTGCATGGGAGGAATAGTACTTTTTG
lytF Rv fuse to Km	ATGGATTTTTATTGTTTATTAGTCAGGATAAATATAAGTTAATCTTCCC
Km-437 Fw fuse to lytF	GATTAACTTATATTTATCCTGACTAATAAACAATAAAAATCCATAAAAATACCAAGC
437 down Rv	GGTATAGATGAAGTTTGCGGTC

Movie S1 A subpopulation of cells releases nucleic acids into the extracellular environment in response to sCSP. We inoculated WT cells in the exponential phase into a microfluidic device and grew them two-dimensionally at 37°C in BHI (A) or BHI with sCSP (B) in flow cell conditions. SYTOX Green (1.25 μM) was added to the media to visualize dead cells and extracellular nucleic acids. The media flowed from left to right in these movies at a constant rate (150 μL/h). We observed the cell morphology by bright-field microscopy and acquired images every 10 min for a total of 18 h. Green indicates dead cells and extracellular nucleic acids.

Movie S2 Dead cells and eDNA are also abundant near the bottom of the biofilm. The P*ldh* reporter strain was grown in glass-bottom dishes in BHIs with sCSP (A) or BHIs (B). The media was supplemented with 1.25 μM SYTOX Green to stain dead cells and extracellular nucleic acids. The same field of view within a range of approximately 40 μm from the glass surface was observed at 30 min intervals while culturing at 37°C. Z-stacks were acquired at 0.55 μm intervals. These images show side views of the biofilms, and the white line below each image shows the glass surface obtained by reflection. mScarlet-I (all cells) and SYTOX Green (dead cells and extracellular nucleic acids) are shown in green and magenta, respectively. The scale bars indicate 10 μm. Representative images from independent experiments are presented.