Electronic Supplementary Information

Atomic Force Microscopy and Surface Plasmon Resonance for Real-time Single-cell Monitoring of Bacteriophage-mediated Lysis of Bacteria

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1 Materials and Methods

1.1 Composition of the Cultivation Media
Tryptic soy broth (TSB CM0129, Oxoid, UK, 30 g of TSB dissolved in distilled water to 1 L, pH 7.4), Tryptic soy agar 1.5% (30 g of TSB CM0129, 15 g of Agar no. 1 LP0011, all Oxoid, UK, dissolved in distilled water to 1 L, pH 7.4), Tryptic soy agar 0.7% (30 g of TSB CM0129, 7 g of Agar no. 1 LP0011, all Oxoid, UK, dissolved in distilled water to 1 L, pH 7.4).

1.2 Adsorption Kinetics of Phage P68
Adsorption efficiency of the phage P68 on S. aureus strains RN4220 and RN4220 ΔtarM under different growth conditions (in Tris buffer and TSB at 37 °C and 25 °C) was determined as described previously. The adsorption was analyzed using the MOI of 0.1, and the adsorption rate was determined according to Mašlaňová et al.

1.3 RNA Extraction
Bacterial strains S. aureus RN4220 and RN4220 ΔtarM in an early exponential phase (OD600 of 0.35) were infected with phage P68 (MOI of 0.01) and incubated at 37 °C with shaking. Aliquots of 2 mL were taken periodically at 0, 10, 20, 30, 40, 50, 60, and 120 min and
centrifuged at 11,000 g for 1 min. The supernatant was discarded, and pellets were snap-frozen in liquid nitrogen and stored at −70 °C.

The pellets were resuspended in 1 mL of TRIzol Reagent (Invitrogen, USA), and the cells were lysed using Lysing Matrix B (MP Biomedicals, USA) at FastPrep-24 Classic bead beating grinder and lysis system (MP Biomedicals, USA) for 6 × 20 s. The total RNA was extracted according to the TRIzol Reagent manufacturer’s instructions. The concentration of isolated RNA was measured at 260 nm on NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, USA). Genomic DNA was removed using a Turbo DNA-free kit (Invitrogen, USA). The RNA integrity was established using a 2100 Bioanalyzer instrument (Agilent, USA); the used extraction method provides RIN values between 7.5 and 9. The pure RNA was transcribed into cDNA using EvoScript Reverse Transcriptase (Roche, USA). To exclude DNA contamination after RNA extraction, the no-reverse transcriptase control (NRT) was performed. The cDNA was stored at −20 °C for subsequent qPCR analyses. The performance of all RT-qPCR experiments followed the MIQE guidelines for RT-qPCR.4

1.4 qPCR
The qPCR was used to determine the expression of the major capsid protein gene (mcp) of phage P68. The LightCycler 480 Instrument II (Roche, USA) was used, and the reactions were carried out in triplicates in a white LightCycler 480 Multiwell Plate 96. Each reaction mixture (20 μL) contained 10 μL of 2× LightCycler 480 SYBR Green I Master (Roche, USA), forward and reverse primers (each 300 µM; mcp_P68_F: TACATCGTGCATCAGGTGGT; mcp_P68_R: AAACACGCCACCTAAGTCGT), and template cDNA (5 µL). An initial denaturation of DNA at 95 °C for 10 min was followed by 30 cycles of amplification (95 °C for 15 s, 55 °C for 20 s, 72 °C for 15 s), and a dissociation phase at 95 °C for 15 s, 60 °C for 60 s, 95 °C for 5 s, and 60 °C for 15 s. The plasmid DNA (pCG248) with inserted P68 mcp gene (pCG248-mcp) ranging from 30 ng to 0.30 pg in ten-fold dilutions was used as a standard for absolute quantification. The amplification efficiency of qPCR was calculated from C_t values of a standard curve prepared from the plasmid DNA with the cloned mcp gene; a linear regression curve through the data points was generated. The efficiency of reaction (E) was calculated using the equation: $E = 10^{-\frac{1}{slope}}$, yielding $E$ of 1.79, the slope of −3.97, and the error of the standard curve of 0.20.

1.5 Lysis Evaluated using Turbidimetry
The lysis experiments in solution were performed in non-binding 96-well polystyrene microtiter plates (Greiner, Austria), and the lysis was evaluated using Synergy 2 reader (BioTek, USA) as the decrease of OD600. First, the bacterial culture of S. aureus was transferred to the TSB, TSB without Ca^{2+}, and Tris buffer. Varying concentrations of lysis agents – either lysostaphin or phage P68 in the corresponding buffers were added in a ratio of 1:4 (v/v). The lysis was monitored for 6 h at 25 °C and 37 °C. The lysostaphin-mediated lysis was tested using concentrations of 0.1, 0.3, 1, 3, 10, 30, and 100 µg/mL and the S. aureus concentration of 1.1×10^9 CFU/mL. In the case of phage-mediated lysis, the number of phages was expressed as MOI, defined as the ratio of concentrations of phage P68 to S. aureus. The MOI values of 0.0001, 0.001, 0.01, 0.1, 1, and 10 were tested.
1.6 Lysis Evaluated using Optical Microscopy
Bright-field optical microscopy without staining the sample was performed using an Olympus BX41 microscope equipped with a 100× immersion objective and an Olympus E-510 camera (Olympus, Japan). For the immobilization of bacteria, the surface of the polystyrene Petri dish was coated with 10 µg/mL of PLL for 1 h and washed with distilled water. *S. aureus* bacterial culture in PBS (1.4×10⁹ CFU/mL) was applied for 1 h, followed by rinsing with distilled water and drying with compressed air. The bacteria were immediately rehydrated by either the Tris buffer or TSB. The initial state of the immobilized bacteria was imaged, followed by the addition of the enzyme or phage and incubation at 25 °C or 37 °C. The lysis progress was evaluated under the microscope after 1.5 h and then every 30 min. Samples with different lysostaphin concentrations (0, 0.1, 1, 10, and 100 µg/mL) were examined. The control sample (either in the absence of the lysing agent or using the phage-resistant culture of *S. aureus* RN4220) was treated by the same procedure. The phage concentration was 8×10⁹ PFU/mL; however, non-specific adsorption of phages on the Petri dish surface must be considered. Therefore, the exact MOI value cannot be reliably determined.

1.7 AFM Imaging in Air
Visualization of the specific binding of phage P68 to *S. aureus* was performed in air with mica as the substrate. *S. aureus* (1.1×10⁹ CFU/mL) in PBS buffer was adsorbed on a freshly cleaved the mica surface (SPI Supplies, USA) at 25 °C for 30 min, followed by the addition of phage P68 in phage buffer (4.6×10¹⁰ PFU/mL), and incubation for 45 min. Afterward, the surface was rinsed with distilled water and dried with compressed air to prevent undesired drying of the buffer salts. The same procedure was repeated in the case of visualization of the lysis of bacteria; the only difference was the use of TSB medium for the incubation.

The AFM scanning was performed using Dimension FastScan Bio (Bruker, USA) in PeakForce Tapping mode. A ScanAsyst-Air probe (Bruker, USA) with a spring constant $k$ of 0.4 N/m was used. The 10 µm × 10 µm images were recorded at the resolution of 512 × 512 pixels. The AFM scanning of SPR chips after the SPR measurements was done using the same procedure.
2 Results and Discussion

Figure S1: Turbidimetric measurement of the bacterial lysis by lysostaphin at 25 °C in (A) Tris buffer and (B) TSB, comparing S. aureus RN4220 ΔtarM with lysostaphin-resistant Kocuria kristinae. (ATCC 27570)

Figure S2: Adsorption kinetics of phage P68 evaluated by double agar overlay plaque assay. The comparison of adsorption efficiency of phage P68 on S. aureus RN4220 and RN4220 ΔtarM in TSB growth medium and Tris buffer at 25 °C and 37 °C. The data points were connected with the B-spline function.
Figure S3: Turbidimetric measurement of *S. aureus* RN4220 ΔtarM lysis by bacteriophage P68 in (A) TSB, (B) TSB without Ca\(^{2+}\), (C) resistant strain *S. aureus* RN4220 in TSB, and (D) *S. aureus* RN4220 ΔtarM mutant in Tris buffer, all tested at 25 °C and 37 °C.
**Figure S4:** Expression of P68 major capsid protein gene (*mcp*) during phage infection of *S. aureus* RN4220 Δ*tarM* in TSB and Tris buffer at (A) 25 °C and (B) 37 °C determined by qPCR. The data points were connected with the B-spline function.

**Figure S5:** Dependence of the lysis time on MOI evaluated from the turbidimetric measurement of the phage-mediated lysis in TSB at 37 °C (Figure 2B).
Figure S6: Bright-field optical microscopy images of lysostaphin-mediated lysis of *S. aureus* RN4220 ΔtarM on the PLL-modified polystyrene surface at 25 °C.

Figure S7: Bright-field optical microscopy images of phage-mediated lysis of *S. aureus* RN4220 ΔtarM (and resistant strain RN4220) on the PLL-modified polystyrene surface at 37 °C.
Figure S8: Bright-field optical microscopy images of phage-mediated lysis of *S. aureus* RN4220 Δ*tarM* on the PLL-modified polystyrene surface at 25 °C and 37 °C.
Figure S9: (A) SPR sensorgrams of *S. aureus* RN4220 ΔtarM lysis by lysostaphin in Tris buffer at 25 °C. (B) Adsorption of lysostaphin in Tris buffer on the gold sensor chip coated with PLL monolayer and blocked with 1% BSA. The orange triangles indicate the beginning and end of the lysostaphin injection.
Figure S10: (A) AFM scans of *S. aureus* RN4220 ΔtarM on the SPR chip surface after lysostaphin-mediated lysis in Tris buffer at 25 °C, including height profiles of marked bacteria. (B) AUC corresponding to the integrated bacteria cross-sections. The level of significance is marked according to the *P*-values: * 0.05–0.01; ** < 0.01.
Figure S11: AFM scans of *S. aureus* RN4220 ΔtarM on the SPR chip surface after phage-mediated lysis in TSB at 25 °C, including height profiles of marked bacteria. The phage concentrations are expressed in PFU/mL.
Figure S12: Optical microscopy images of *S. aureus* RN4220 ΔtarM on the SPR chip surface after phage-mediated lysis experiments. The phage concentrations are expressed in PFU/mL.
**Figure S13:** (A) Examination of the effect of AFM scanning on *S. aureus* RN4220 ΔtarM during 150 min in Tris buffer at 25 °C, including height profiles of marked bacteria. The time dependency of (B) AUC corresponding to the integrated bacteria cross-sections and (C) relative stiffness (normalized to the average stiffness of the native bacteria). The level of significance is marked according to the *P*-values: * 0.05–0.01; ** < 0.01. The gray box represents YM of PLL-modified Petri dish.
Figure S14: (A) Height image of phages P68 adsorbed on mica surface with a detail of one virion (bottom-right corner, size 110 nm × 110 nm), and (B) cross-sections of marked P68 virions. PeakForce Error images of (C) phage P68 bound on *S. aureus* RN4220 ΔtarM in Tris buffer with (D) zoomed section and a detail (bottom-left corner, size 205 nm × 205 nm) of two phages with a hole after DNA was released from the capsid, and (E) lysed bacteria after 45 min of incubation with P68 in the TSB.
Figure S15: (A) AFM visualization with height profiles of the lysis of *S. aureus* RN4220 ΔtarM by $1.2 \times 10^8$ PFU of phage P68 at 25 °C in Tris buffer. The time dependency of (B) AUC corresponding to the integrated bacteria cross-sections and (C) relative stiffness (normalized to the average stiffness of the native bacteria). The level of significance is marked according to the $P$-values: * $0.05-0.01$; ** $<0.01$. The gray boxes represent YM with completely lysed bacteria and PLL-modified Petri dish.
Figure S16: (A) Examination of the effect of AFM scanning on *S. aureus* RN4220 ΔtarM during 150 min in TSB at 37 °C, including height profiles of marked bacteria. The time dependency of (B) AUC corresponding to the integrated bacteria cross-sections and (C) relative stiffness (normalized to the average stiffness of the native bacteria). The level of significance is marked according to the *P*-values: * 0.05–0.01; ** < 0.01. The gray box represents YM of PLL-modified Petri dish.
3 References