Supporting online material for

Ring A of nukacin ISK-1: a lipid II-binding motif for type-A(II) lantibiotic

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Supporting online material

Experimental procedures:

Bacterial strains and growth condition:

Lactococcus lactis was grown in M17 medium supplemented with 0.5% glucose (GM17) at 30°C. *Lactobacillus sakei* subsp. *sakei* JCM 1157^T and *Enterococcus faecalis* JCM 5803^T were grown in MRS medium at 30°C. *Bacillus coagulans* JCM 2257^T was cultured in Luria-Bertani (LB) broth at 37°C. *Staphylococcus aureus* SG511 and *Staphylococcus simulans* 22 were grown in half-concentrated Mueller-Hinton (M-H) broth (Oxoid, Basingstoke, United Kingdom) at 37ºC. *Micrococcus luteus* DSM1790 and the nukacin ISK-1-producer strain *Staphylococcus warneri* ISK-1 were grown in trypticase soy broth (TSB) medium (Difco Laboratories, MI, USA) at 37°C.

Purification of lantibiotics:

Nukacin ISK-1 and derivative peptides were purified from the respective culture supernatants as described by Aso *et al.*[1] The nukacin ISK-1-producer strain *S. warneri* ISK-1 was grown in TSB for the purification of nukacin ISK-1, and nukacin derivatives were purified from *L. lactis* NZ9000 recombinant strains constructed in a previous study by Islam *et al.*[2] Commercial nisin A (Sigma-Aldrich, MO, USA) was purified by reverse-phase high-performance liquid chromatography (RP-HPLC). Nisin Z was purified from the culture supernatant of *L. lactis* NIZO22186 as described previously.[3]

Purification of peptidoglycan precursor:

Synthesis and purification were performed according to the protocol described by Brötz *et al*.[4] with modifications by Schneider T. *et al.*[5]. Briefly, lipid II was synthesized *in vitro* using membrane preparations of *M. luteus* DSM1790. The membranes were isolated from lysozyme-treated cells by centrifugation (35,000 *g*), washed twice in 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5, and stored at -80^{0}C until use. Substrate UDP-*N*-acetylmuramic acid pentapeptide (UDP-MurNAc-pp) was purified from *S. simulans* 22 as previously described by Kohlrausch and Höltje.[6] An analytical assay was performed to optimize the suitable conditions by using different

volumes of the membrane fraction and substrate purified as mentioned above. A total volume of 50 µL containing different volumes of the membrane protein, 5 nmol of C_{55}-P, different volumes of UDP-MurNAc-pp, 10 mM of UDP-GlcNAc in 1 M Tris-HCl, 1 M $MgCl_2$, pH 8, and 0.5% (w/v) Triton X-100 was used. For purifying higher quantities of lipid II, the analytical procedure was scaled up by a factor of 200. The reaction mixtures were incubated for 2 h at 30°C, and the lipids were extracted with the same volume of *n*-butanol/6 M pyridine-acetate, pH 4.2. Lipid I was synthesized by the methods described for lipid II with the omission of UDP-GlcNAc from the synthesis reaction. Lipid I/lipid II was purified using Sephadex G-25 column (HiTrap Desalting; GE Healthcare, Uppsala, Sweden) and eluted in a linear gradient from chloroform: methanol: water (2:3:1) to chloroform: methanol: ammonium bicarbonate (300 mM) (2:3:1). Lipid I- and lipid II-containing fractions were identified by TLC (silica gel 60 A, Whatman) using chloroform: methanol: water: ammonia (88:48:10:1) as the solvent.[7] Spots were visualized by PMA staining reagent. The concentration of purified lipids was determined as the amount of inorganic phosphates present after treatment with perchloric acid.[8]

MIC and antagonization tests:

MIC values were determined in microtiter plates as described by Wiedemann *et al.*[9] Respective indicator strains were grown in the appropriate culture broth. Serial 2-fold dilutions of the peptides were prepared in the appropriate growth medium with 0.1% Tween 80. Bacteria were added to a final inoculum of 10^5 CFU/ml in a volume of 0.2 mL. The microtiter plates were incubated at the temperature appropriate for each indicator strain. The MIC was considered as the lowest peptide concentration causing inhibition of visible growth; determinations were carried out at least twice.

For the antagonization test, cell wall precursors were mixed with 4×MIC of lantibiotics at a molar ratio of 2:1 (precursor-lantibiotic). After overnight incubation at 30°C, the antagonization potency was determined by observing the growth of *L. lactis* HP and comparing it to the control.

Intracellular accumulation of UDP-MurNAc-pp:

For the analysis of the cytoplasmic nucleotide pool, we followed the method of Sass *et al.*[10] In short *S. aureus* SG511 was grown in M-H broth to an OD_{600} of 0.5 and supplemented with 130 µg/mL of chloramphenicol. Chloramphenicol is necessary to prevent the induction of autolytic

processes as well as the *de novo* synthesis of the enzymes hydrolyzing the nucleotide-activated sugars interfering with the determination of the soluble precursor. After 15 min, peptides were added at 10×MIC, and the mixture was incubated for 30 min. Cells were harvested and extracted with boiling water. The suspension was then centrifuged (48,000×g, 30 min), and the supernatant was lyophilized. Nucleotide-linked cell wall precursors were analyzed by HPLC.

ITC measurements of peptide binding:

Isothermal titration calorimetry (ITC) experiments were carried out with a VP-ITC from MicroCal (Northampton, MA, USA). The device was electrically calibrated. Purified lipid II was solubilized in 10 mM Tris-Cl and 150 mM NaCl (pH 7.5) and subjected to repeated extrusion for 15 times through 0.1 µm polycarbonate filters. Peptides were also dissolved in the same buffer after complete lyophilization of HPLC purified samples. Prior to use, all the samples were degassed and equilibrated to the appropriate temperature before measurement. We selected 25°C as the experimental temperature. The reference power was set to 10 µal/s by using a syringe-stirring speed of 300 rpm. Titrations were performed by 25 injections of 10 µL each of lipid II solution (150 µM) into the sample cell containing 1.443 mL of the peptide at concentrations varying from 3.4 µM to 7.1 µM. The change in the heat rate during the titration steps was registered in real time. Raw data were processed using the Origin® 7 software provided with the instrument. In control experiments, the corresponding peptide solution (or lipid II solution) was injected into the buffer without lipid II (or without peptide). Heats of dilution were significantly lower and were subtracted from the actual measurements. Under the assumption of the one-site binding model with the legend in the sample cell, the equilibrium binding constant (K_D), and the thermodynamic parameters of enthalpy (ΔH), free energy binding (ΔG), and entropy change ($T\Delta S$) were calculated.

Supporting figure legends:

Figure S1:

Alignment of the unmodified propeptide sequence of different lantibiotics possessing mersacidin like lipid II-binding motif. Ring A of type-A(II) and additional lantibiotics showing similar sequence to lipid II-binding of mersacindin are shaded in gray. Underlined residues are the ones

modified during their maturation. A consensus sequence is drawn, in which ‘x' denotes undefined residues.

Figure S2:

Reactions in membrane-associated cell wall biosynthesis in *Staphylococcus aureus*. UDP-MurNAc-pp is synthesized on the cytoplasmic side and translocates to the inner face of the cytoplasmic membrane. MraY, MurG, and FemXAB enzymes catalyze the reactions to produce the membrane-bound precursor molecule lipid II with a pentaglycine chain. Lipid II translocates across the membrane by an unknown mechanism, and PBP converts lipid II into peptidoglycan. When this cycle is inhibited by an antibiotic at any stage, the soluble precursor UDP-MurNAc-pp accumulates in the cytoplasm.

Table S1: Thermodynamic parameters of peptides and lipid II interaction as determined by Origin® 7 software

Peptide/ Parameters[a]	$C_p°$ (µM)	$C_L°$ (µM)	ΔH (kcal/mol)	-TΔS (kcal/mol)	ΔG (kcal/mol)
Nukacin ISK-1	3.4	150	-6.6	2.6	-9.2
Nukacin D13E	3.4	150	-7.1	2.1	-9.2
Nukacin D13A	3.4	150	-	-	-
Nukacin D13A	7.1	150	-18.1	-10.7	-8.6
Nukacin C14S	5.6	150	-	-	-

[a] $C_p°$, initial peptide concentration; $C_L°$, initial lipid II concentration; ΔH, enthalpy change; -TΔS, entropy change; ΔG, free energy change

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Figure S1: Sequence alignment of unmodified propeptide of different lantibiotics containing mersacidin like lipid II-binding motif.

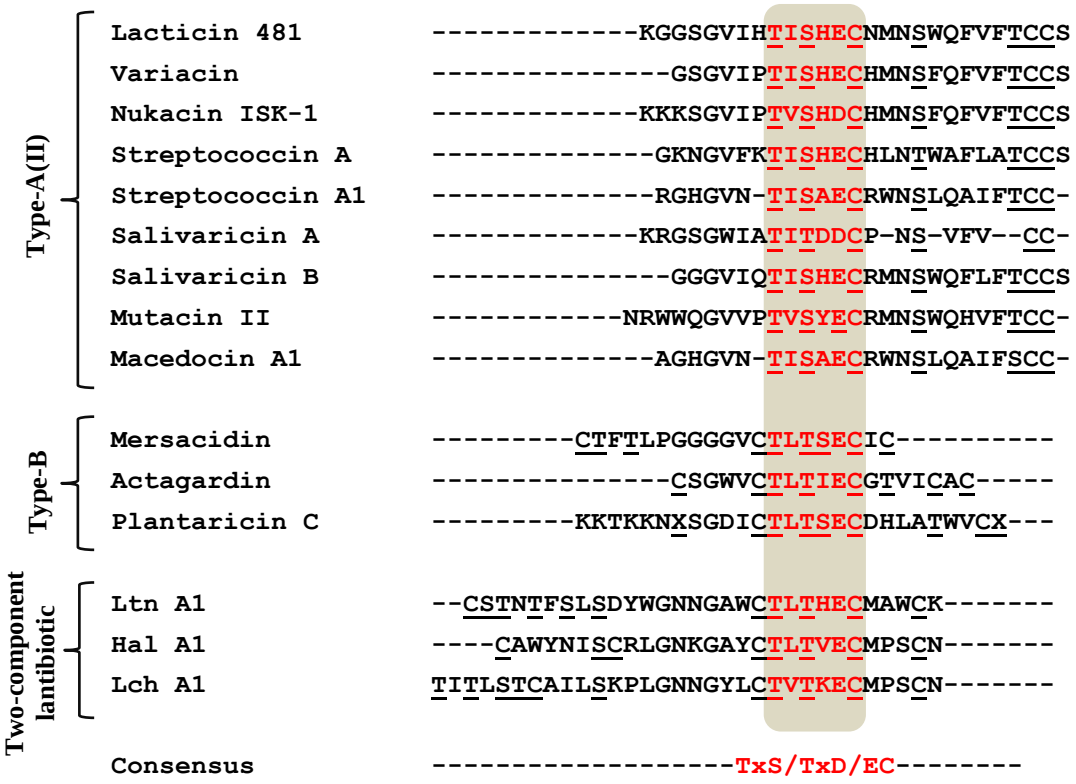

Figure S2: Reactions in membrane-associated cell wall biosynthesis in *Staphylococcus aureus*