Supporting Information

Decreasing the ring size of a cyclic nonribosomal peptide antibiotic by in-frame module deletion in the biosynthetic genes

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Construction of plasmids

To construct pΔA2, the DNA regions 5'- and 3'-flanking to *srfA-A2* were amplified by polymerase chain reaction (PCR; using the Expand Long Range PCR system from Boehringer Mannheim, Germany) from chromosomal DNA of *Bacillus subtilis* ATCC 21332 and cloned into pTZ18R (Genbank accession number L08956). The 1812 bp 5'-flanking fragment was amplified using oligonucleotides oHM53 and oHM54 (see Table 1), digested with *Eco* RI and *Bam* HI and ligated with similarly treated pTZ18R to give pTZ18-5'-srf. The 1341 bp 3'-flanking fragment was amplified using oligonucleotides oHM55 and oHM56, treated with *Bam* HI and *Pst* I and cloned into the corresponding sites of pTZ18R to give pTZ18-3'-srf. The 3'-flanking fragment was then excised from the latter plasmid using *Bam* HI and *Hin*d III and ligated into the corresponding sites of pTZ18-5'-srf resulting in pΔA2. Plasmid ptycC6 was constructed by cloning the *Bam* HI-*Bgl* II fragment of pProCAT-LeuCAT (Ref 1) into the *Bam* HI site of pΔA2. Correct orientation of the insert was verified by restricition mapping and DNA-sequencing. The fragment encoding module 2 of SrfA-A was PCR amplified from chromosomal DNA of *B. subtilis* ATCC 21332 using oligonucleotides oHM66 and oHM52, digested with *Bam* HI and *Bgl* II and ligated into the *Bam* HI site of pΔA2 to give psrfA-A2. Correct orientation of the insert was verified by restricition mapping and DNA-sequencing.

Genetic manipulation of *B. subtilis*

Gene exchange in *B. subtilis* strains was essentially performed as previously reported.[2,3] AS10 was transformed with one of the exchange vectors pΔA2, ptycC6 and psrfA-A2, as well as the congression plasmid pNEXT33A,[4] which confers neomycin resistance. Transformants were selected on agar plates of Difco Sporulation Medium (DSM) supplemented with 10 μg/mL neomycin. These were then tested for loss of the chloramphenicol resistance by restreaking on DSM plates supplemented with 10 μg/mL neomycin and 5 μg/mL

chloramphenicol. Colonies that exhibited the neomycin resistant and chloramphenicol sensitive phenotype were then examined for the desired genotype.

Preparation of lipopeptide extracts

200 mL SpIII growth medium (SpII medium[5] without calcium chloride) were inoculated with 2 mL of an overnight culture in the same medium. Cells were grown for 44h at 37°C with vigorous shaking and were then pelleted by centrifugation. 120 mL of the supernatant were extracted twice with 60 mL butanol. The mixture was vigorously shaken for 1h at room temperature. After centrifugation, the butanol phase was separated and evaporated under vacuum. The residual material was dissolved in 5 mL methanol (0.045% formic acid; v/v).

HPLC and MS analysis of the products

Reversed-phase HPLC was performed on an Agilent 1100 Series system using a 250/3 Nucleosil 120-3C8 column (Macherey & Nagel) at 25°C and a solvent system with 0.05% formic acid in water (buffer A) and 0.045% formic acid in methanol (buffer B). The gradient profile was as follows: 0 min, 70% B; 30 min, 95% B; 38 min, 95% B; 40 min, 70% B; 50 min, 70% B. Product yields relative to that of surfactin produced by *B. subtilis* ATCC 21332 and strain HM0542 were calculated from the UV-absorbance recorded at 210 nm and corrected for the optical density of the cells cultures at the point of cell harvesting.

Electro-spray ionization time-of-flight (ESI-TOF) mass spectrometry was carried out with a Qstar-Pulsar_i mass spectrometer (PESciex/Applied Biosystems). Figure 1 and 2 show mass spectra recorded for surfactin and Δ2-surfactin, respectively. The ESI-MSMS spectrum of Δ2-surfactin (see Figure 3) was obtained by selecting the most intensive ion (m/z 961 at low quadrupole resolution) and fragmenting it in the collison cell. 10,000 cycles were added in the multiple channel averaging (MCA) modus. During data acquisition, the collision energy was manually varied in the range between 50 and 100, while the collision gas Argon was kept fixed at 10 (instrument parameters). The mass section with an m/z ratio of 380 was enhanced to improve the fragmentation pattern.

Test for biological activity

Hemolytic activity of extracts prepared from *B. subtilis* ATCC 21332, HM0394 and AS10 was analyzed on blood agar plates as previously described.[3] Lysis of erythrozytes resulted in a plaque around the filter paper on which extracts were applied (see Figure 4).

Preparations of surfactin from *B. subtilis* ATCC 21332 and Δ2-surfactin from HM0394 were applied at the same concentrations as based on the quantification by HPLC. For this purpose, the Δ2-surfactin solution was concentrated under vacuum. The same volume of the extracts from AS10 was used for the analysis.

Table 1: Oligonucleotides used in this study

Oligonucleotide	Sequence 5'-…-3'; Restriciton site underlined
oHM52	ATA GGA TCC GGC AAT CAC CTG TGC CA
oHM53	ATA GAA TTC GAG AAA GAG AAG CTG CTT G
oHM54	ATA GGA TCC ATC AGC TTC CTC TGC AAG
oHM55	ATA GGA TCC GAA AAA GGG ACA GCC GC
oHM56	ATA CTG CAG CGC TTG CTT TTC TGT CTC G
oHM66	ATA GAA TTC AGA TCT GAA AGC AAA GCT TTT GCG

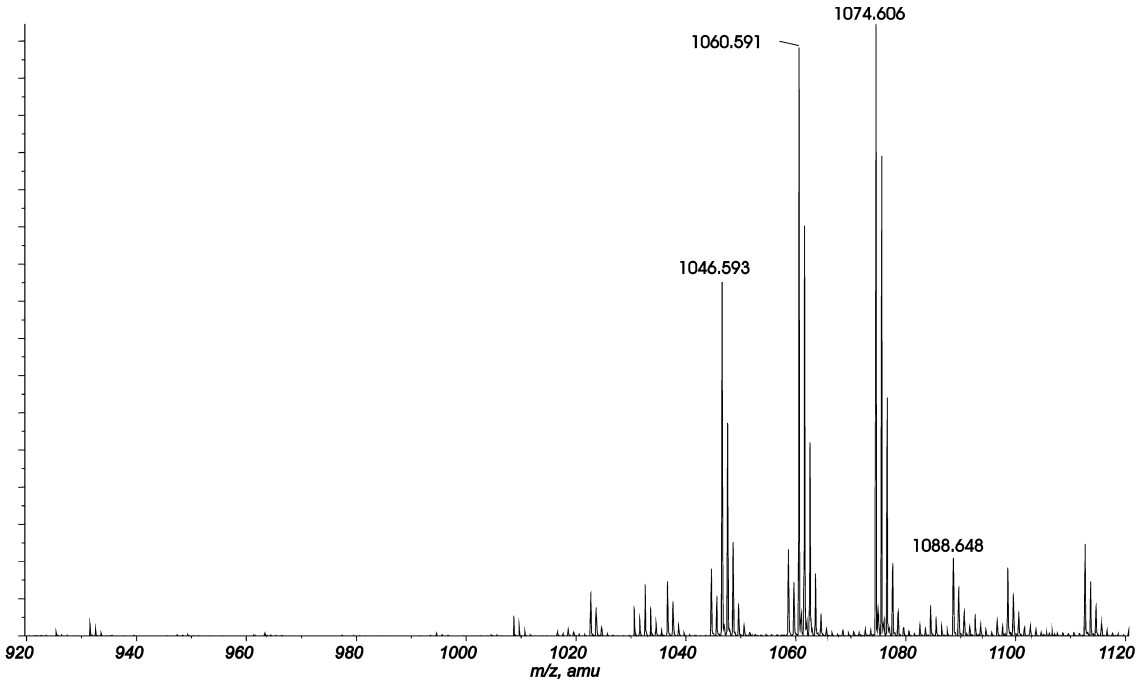

Figure 1. Mass spectra of surfactin prepared from *B. subtilis* ATCC 21332. The masses of the $[M+K]^+$ ions with variable chain length of the fatty acid were most prominent and are assigned in the figure (observed 1046.6, 1060.6, 1074.6, and 1088.6; calculated 1046.6

1060.6, 1074.7, and 1088.7), but also the Na^+ and H^+ adducts could be observed. Mass spectra obtained for the surfactin obtained from strains HM0519 and HM0542 were essentially identical.

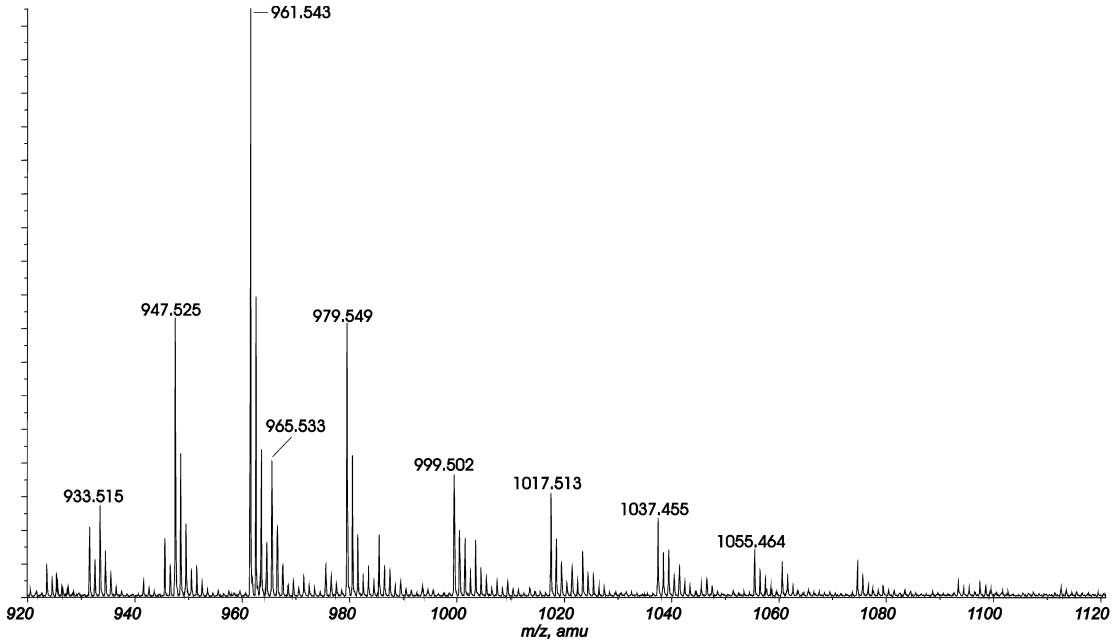

Figure 2. Mass spectra of Δ2-surfactin prepared from HM0394. The masses of the $[M+K]^+$ ions with variable chain length of the fatty acid were most prominent and are assigned in the figure, but also the Na^+ and H^+ adducts could be observed. Both cyclic Δ2-surfactin ($[M+K]^+$ = 933.515, 947.525 and 961.543) and linear linΔ2-surfactin ($[M+K]^+$ = 965.538 and 979.549) were detected. The distribution of the fatty acid moieties with respect to their variable chain were found to be essentially identical to that of wild-type surfactin. A slight variation dependent on the individual preparation was observed for all strains. This finding further supports the idea that fatty acid selection occurs during initiation of surfactin synthesis and is independent of further peptide backbone elongation. The signals at m/z=999.5, m/z=1037.5, m/z=1017.5, and m/z=1055.5 correspond to the calculated masses of the ions at m/z=961.5 and m/z=979.5 with one or two protons exchanged for potassium ions, respectively.

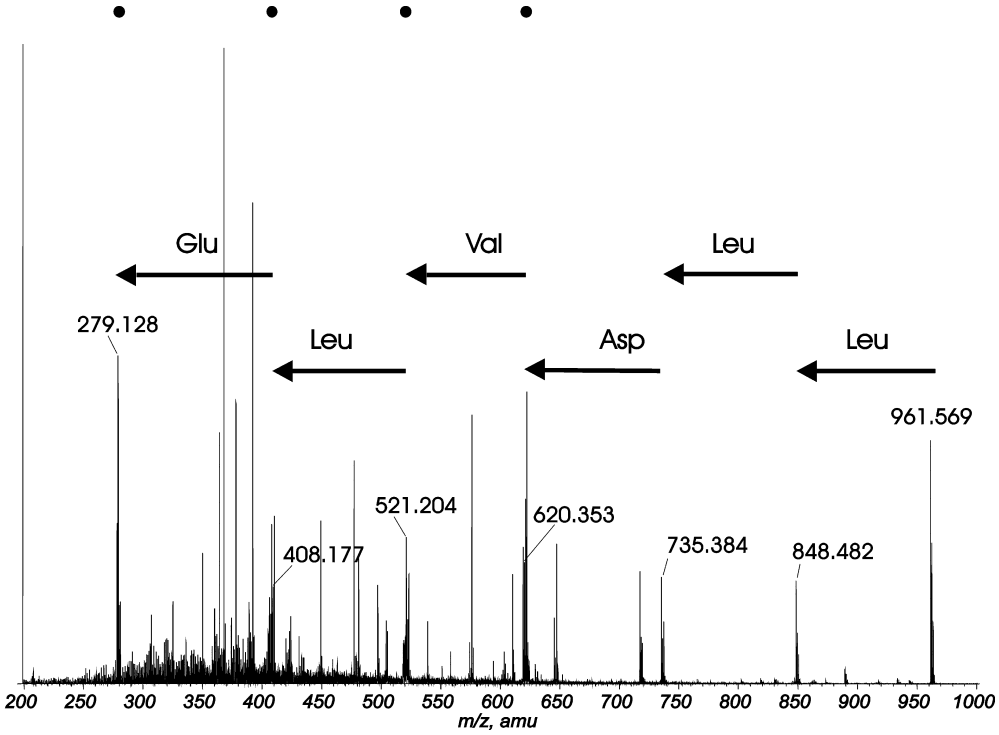

Figure 3. ESI-MSMS spectrum of Δ2-surfactin. The ion with m/z=961.569 was selected for fragmentation. Regions indicated with a dot are shown in a 5-fold enlarged scale.

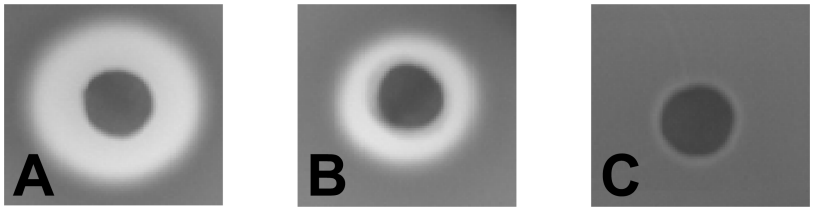

Figure 4. Analysis of hemolytic activity of lipopeptide preparations from *B. subtilis* ATCC 21332 (**A**), HM0394 (**B**) and AS10 (**C**) on blood-agar plates.

References

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5) Cutting, S. M.; Vander Horn, P. B. *Genetic Analysis - Chapter 2*; Harwood, C. R. and Cutting, S. M., Ed.; John Wiley & Sons: Chichester, U.K., 1990, pp 34.