Supplement

Influence of *yjiL* and upstream genes on the antibacterial activity of proline-rich antimicrobial peptides overcoming *Escherichia coli* resistance induced by the missing SbmA transporter system

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Materials used in this study and suppliers: AppliChem GmbH (Darmstadt, Germany): Tetracycline hydrochloride (≥95%), tris(hydroxymethyl)aminomethane (Tris); Biosolve BV (Valkenswaard, Netherlands): dimethylformamide (DMF, peptide synthesis grade), acetonitrile (HPLC-S gradient grade); Bruker Daltonics GmbH (Bremen, Germany): alpha-cyano-4-hydroxycinnamic acid (CHCA); Carl Roth GmbH, Karlsruhe, Germany): Agar-Agar Kobe I, ampicillin sodium salt (≥99%), chloramphenicol (≥98,5%), potassium chloride (≥99%), potassium dihydrogen phosphate (≥99%), magnesium chloride (≥99%), dithiothreitol (≥99%), dichloromethane (DCM), tryptone, yeast extract; Gibco® (Paisley, UK): phosphate buffered saline (PBS, pH 7.4); Greiner Bio-One GmbH (Frickenhausen, Germany): 96- and 384-well microtiter plates; Iris Biotech (Marktredwitz, Germany): leucin-Wang resin; Merck KGAa (Darmstadt, Germany): diethyl ether (puriss); MultiSynTech GmbH: Rink amide 4-methylbenzhydrylamine (MBHA) resin, 4-benzyloxybenzyl alcohol (Wang) resin, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronoium hexafluorophosphate (HBTU); Orpegen Pharma GmbH (Heidelberg, Germany) or MultiSynTech GmbH (Witten, Germany) or Iris Biotech: all 9-fluorenylmethoxycarbonyl- (Fmoc-) protected amino acids; Phenomenex Inc. (Torrance, CA, USA): Jupiter C_{18}-columns (internal diameter (ID): 21.2 mm, length: 250 mm, particle size: 15 µm, pore size: 30 nm; ID: 10 mm, length: 250 mm, particle size: 5 µm, pore size: 30 nm; ID: 2 mm, length: 150 mm, particle size: 5 µm, pore size: 30 nm); Sigma-Aldrich GmbH (Taufkirchen, Germany): 1,2-ethandithiole (≥98%), m-cresole (99%), thioanisole (≥99%), *N*,*N*-diisopropylcarbodiimide (DIC, >98% by GC), *N*,*N*-diisopropylethylamine (DIPEA), 5(6)-carboxyfluorescein (Cf, for fluorescence); 1-hydroxy-benzotriazole (HOBt, >98%), trifluoroacetic acid (TFA, UV-grade for HPLC), TFA (purum) for peptide synthesis; N-methylmorpholine (NMM, >95% GC), , paraformaldehyde (95%), triisopropylsilane (TIS), sodium azide (≥99,5%), sodium chloride (≥99,5%), disodium hydrogen phosphate × 12 H_2O (≥99%), Mueller Hinton broth (MHB), Tryptic Soy broth (TSB); Thermo Scientific GmbH (Schwerte, Germany): Phusion High-Fidelity DNA Polymerase (2U/µl), dNTP Mix (2 mmol/L each).

Phage lysate λNK1323 containing transposon Tn*10* Tet^R carrying a tetracycline resistance was obtained from Prof. Dr. Garys Sawers (Universität Halle/Saale).

Table S1. Knock-out (KO) and control (K) primer for *yjiL*, *yjiM*, *yjiN* and *mdtM* and primer using for the arbitrary-primed PCR.

Primer	**Sequence**	**Source**
yjiL 3'KO	5'-ATG ACG CCG ACC GTG GAG TGA AAT AAA AAT AAA AAC TCT TGC GAT TGT GTA GGC TGG AGC T-3'	This study
yjiL 5'KO	5'-CCG TGT CGC GGC CTT TAT TGA GAT GCT GTA AGG AGT GGC ACC ATG GTC CAT ATG AAT ATC CTC C-3'	
yjiL 3'K	5'-CCG TGG AGT GAA ATA AAA AT-3'	
yjiL 5'K	5'-GCC TTT ATT GAG ATG CTG TA-3'	
yjiM 3'KO	5'-AGA CGG AAT CAA TGC CAA TCG AAT ATG CCA CTG CCA CTC CGC GAT TGT GTA GGC TGG AGC T-3'	
yjiM 5'KO	5'-CCT CTT TTC ATT ATC TCC CGT GGT ACG GGG AAG GAA AAT CCC ATG GTC CAT ATG AAT ATC CTC C-3'	
yjiM 3'K	5'-AAT GCC AAT CGA ATA TGC CA-3'	
yjiM 5'K	5'-TTA TCT CCC GTG GTA CGG GG -3'	
yjiN 3'KO	5'-GAT CTC TCT CGC GGT TAG CCA CTT AGT TTT TCA TGG ATT TGC GAT TGT GTA GGC TGG AGC T-3'	
yjiN 5'KO	5'-TTT TTG CGC GAT CCG GCC GTC AGG CTC TAT TCT TAA CGT TCC ATG GTC CAT ATG AAT ATC CTC C-3'	
yjiN 3'K	5'-GCG GTT AGC CAC TTA GTT TT-3'	
yjiN 5'K	5'-ATC CGG CCG TCA GGC TCT AT-3'	
mdtM 3'KO	5'-AAC GTT AAG AAT AGA GCC TGA CGG CCG GAT CGC GCA AAA AGC GAT TGT GTA GGC TGG AGC T-3'	
mdtM 5'KO	5'-TTT TCC CCG TTG GGG TTC TCC GGA CAA GGA GTT GTT TGT TCC ATG GTC CAT ATG AAT ATC CTC C-3'	
mdtM 3'K	5'-ATA GAG CCT GAC GGC CGG AT-3'	
mdtM 5'K	5'-TGG GGT TCT CCG GAC AAG GA-3'	
IS10-R	5'CAAGATGTGTATCCACCTTAACTTAATG-3'	
ARB2	5'-GGCCACGCGTCGACTTAGTTAC-3'	(1)
ARB6	5'-GGCCACGCGTCGACTAGTACNNN NNNNNNNACGCC-3'	
Tn10-2R	5'-ACCTTTGGTCACCAACGCTTTTCC-3'	

Table S2. Antimicrobial peptides used in this study.

Peptide[a]	**Sequences**	**Reference or source**
Api88	gu-ONNRPVYIPRPRPPHPRL-NH_2	(2)
Api137	gu-ONNRPVYIPRPRPPHPRL-OH	(3)
Onc18	VDKPPYLPRPRPPRRIYNR-NH_2	(4)
Onc72	VDKPPYLPRPRPPROIYNO-NH_2	(5)
Onc112	VDKPPYLPRPRPPRrIYNr-NH_2	
Bac7(1-35)	RRIRPRPPRLPRPRPRPLPFPRPGPRPIPRPLPFP-NH_2	(6)
Bac7(1-60)	RRIRPRPPRLPRPRPRPLPFPRPGPRPIPRP LPFPRPGPRPIPRPLPFPRPGPRPIPRP-NH_2	
Chex1-20Arg	Chex-RPDKPRPYLPRPRPPRPVR-NH_2	(7)
A3-APO	(Chex-RPDKPRPYLPRPRPPRPVR)$_2$Dab-NH_2	
Drosocin	H-GKPRPYSPRPTSHPRPIRV-OH	(8)
Pyrrhocoricin	H-VDKGSYLPRPTPPRPIYNRN-OH	(9)
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2	(10)

a Chex, Dab, O, r, and gu denote 1-amino cyclohexyl carboxylic acid, 2,4-diaminobutyric acid, L-ornithin, D-arginine, and *N*,*N*,*N*',*N*'-tetramethylguanidino, respectively

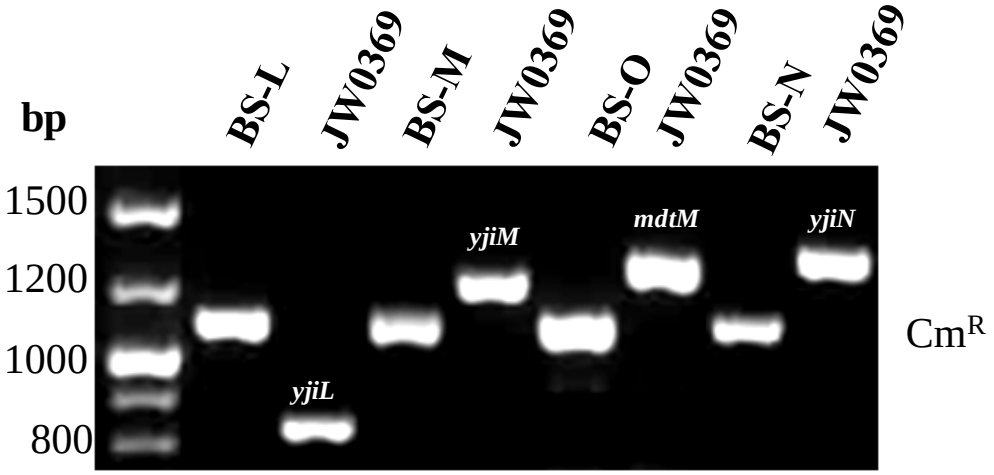

Figure S1. Agarose gel of the PCR products after the amplifications of the *yjiL*, *yjiM*, *mdtM*, and *yjiN* genes with the genomic DNA of JW0369, BS-L, BS-M, BS-O, and BS-N using the control primers of each gene. In case of the Δ*sbmA* mutant JW0369, the PCR products showed the expected bands for *yjiL* (≈ 768 bp), *yjiM* (≈ 1152 bp), *mdtM* (≈ 1233 bp), and *yjiN* (≈ 1281 bp). The knock-out mutants BS-L, BS-M, BS-N, and BS-O yielded PCR products of similar sizes of around 1100 bp belonging to the chloramphenicol resistance cassette (Cm^R) of pkD3, which confirmed the knock-out of the favored genes *yjiL*, *yjiM*, *mdtM*, and *yjiN*.

Figure S2. Alignment of the reverse complement sequence of *sbmA* and the sequence of the arbitrary-primed PCR product of the genomic DNA of the MC4100 Tn*10* insertion mutant M22. The remaining sequence of the IS10 element of the Tn*10* transposon next to the IS10-R primer binding site is shown in red, followed by the identified *sbmA* sequence. The Tn*10* transposon was inserted in *sbmA* 761 bp after the start codon, which resulted in the *sbmA* (1-761 bp) – Tn*10* – *sbmA* (761-1221 bp) gene in the genomic DNA of M22. The same result was obtained for the other Api88-resistant MC4100 Tn*10* insertion mutants. The Tn*10* insertion in the *sbmA* gene leads to a truncated, 254 amino acid residues long, and most likely non-functional SbmA protein (full length SbmA contains 406 residues).

A

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yjiL        ACAGTGTTTGCTGAATCAGAAGCGATCAGCCTGCGCTCAGCGGGCGTCGCGCCAGAAGCG 540
BS2         ------------------------------------------------------------

yjiL        ATTCTCGCAGGAGTGATTAACGCGATGGCGCGGAGGAGTGCCAATTTCATTGCTCGTCTC 600
BS2         -----------------------------------AAATTTTATCATTAGGGGATTCAT  24

yjiL        TCCTGTGAAGCGCCGATTCTGTTTACTGGTGGCGTTAGTCATTGCCAGAAGTTTGCCCGG 660
BS2         CAGTGTGAAGCGCCGATTCTGTTTACTGGTGGCGTTAGTCATTGCCAGAAGTTTGCCCGG  84
               ************************************************************

yjiL        ATGCTGGAATCTCACCTGCGAATGCCGGTAAATACCCATCCTGATGCGCAATTTGCTGGC 720
BS2         ATGCTGGAATCTCACCTGCGAATGCCGGTAAATACCCATCCTGATGCGCAATTTGCTGGC 144
            ************************************************************

yjiL        GCAATTGGCGCGGCGGTAATTGGTCAACGAGTGAGGACACGCCGATGA------------ 768
BS2         GCAATTGGCGCGGCGGTAATTGGTCAACGAGTGAGGACACGCCGATGAAAGAGTTTTTAT 204
            ********************************************

yjiL        ------------------------------------------------------------
BS2         TTTTATTTCACTCCACGGTCGGCGTCCCCCCCCCCGTACTAAATCCGCCCGTGGCCCAAT 264
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B

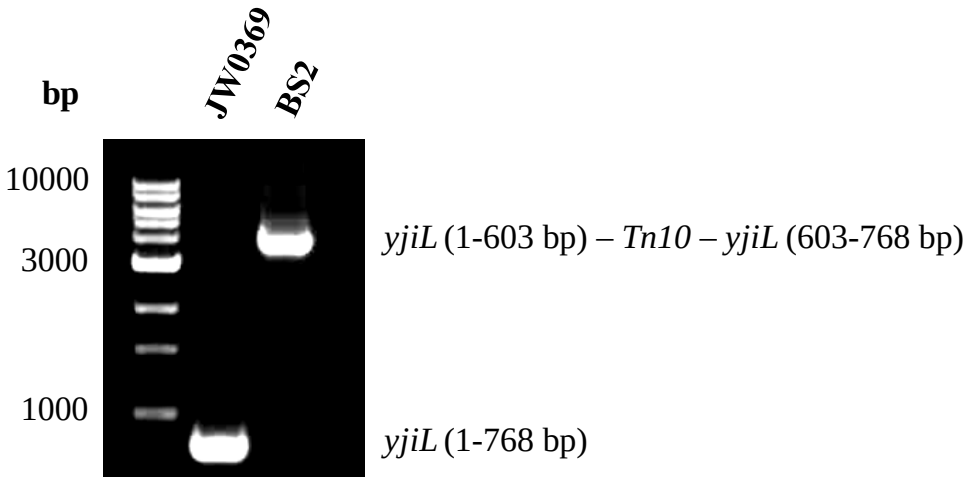

Figure S3. (A) Sequence alignment for *yjiL* and the arbitrary-primed PCR product of the genomic DNA of the JW0369 Tn*10* insertion mutant BS2. The remaining sequence of the IS10 element of the Tn*10* transposon next to the IS10-R primer binding site is shown in red, followed by the identified *yjiL* sequence. **(B)** Agarose gel of the PCR products after amplification of the *yjiL* gene with the genomic DNA of JW0369 and BS2 using the control primers yjiL3'K and yjiL5'K. The Tn*10* transposon was inserted in *yjiL* 603 bp after the start codon, which resulted in the *yjiL* (1-603 bp) – Tn*10* – *yjiL* (603-768 bp) gene in the genomic DNA of BS2. The same result was obtained for the other Onc112-resistant JW0369 Tn*10* insertion mutants. The Tn*10* insertion in the *yjiL* gene leading to a truncated, 201 amino acid residues long, most likely non-functional yjiL protein (predicted protein size is 255 residues).

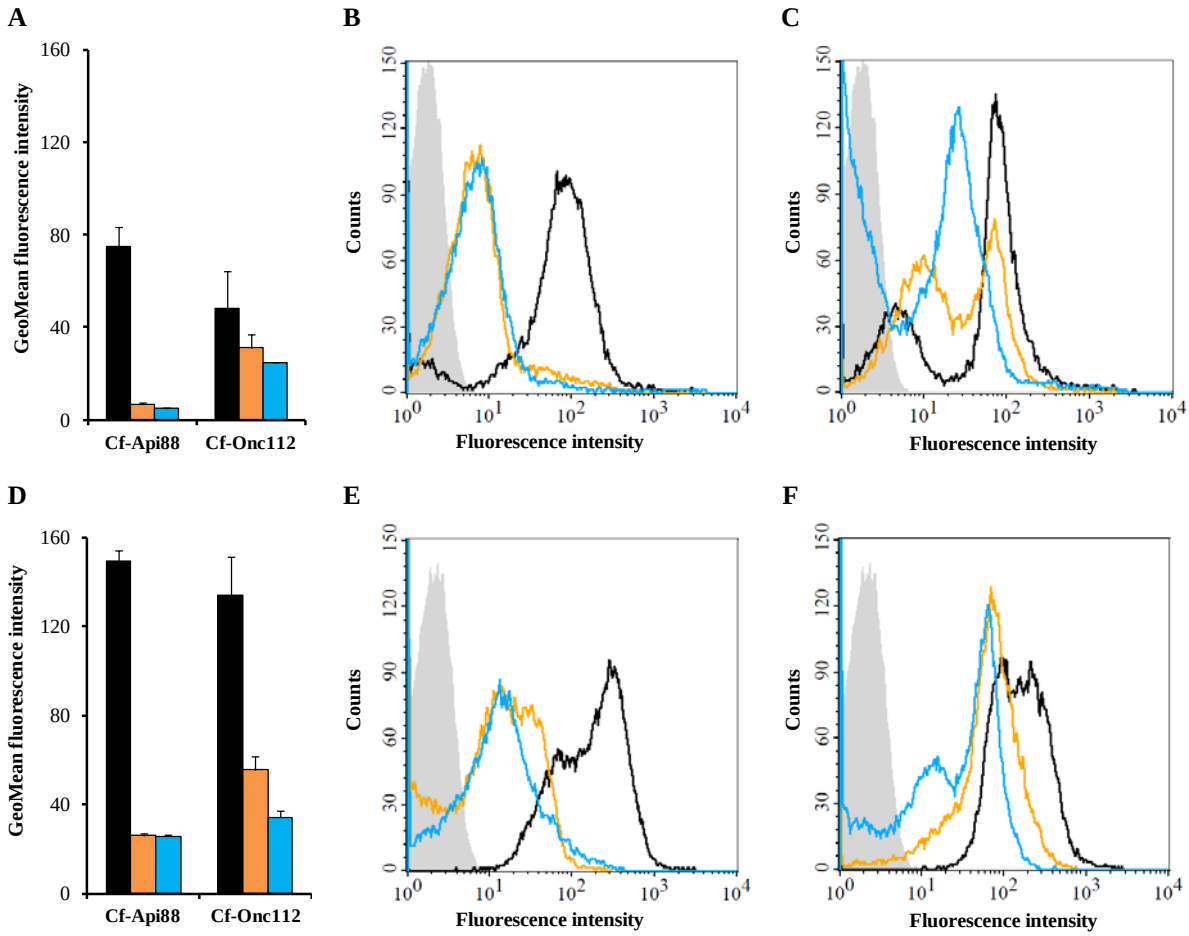

Figure S4: Flow cytometry analysis of *E. coli* strains BW25113 (black bars and lines), JW0368 (orange bars and lines), and BS2 (blue bars and lines) incubated with Cf-labelled peptides (6 µM) for 30 min **(A, B, C)** and 90 min **(D, E, F)** in 33% TSB-medium. GeoMean fluorescence intensities and histogram plots for cells treated with Cf-Api88 **(A, B, C, D)** and Cf-Onc112 **(A, C, D, F)**. BW25113 treated without peptide as a control is shown as light grey area **(B, C, E, F)**. Uptake studies showed that Cf-Api88 and Cf-Onc112 is transported in lower quantities into the Δ*sbmA* mutant JW0368 and into the Δ*sbmA yjiL*::Tn*10* mutant BS2 compared to BW25113 at both time points. The uptake of Cf-Onc112 into JW0368 is more efficient than Cf-Api88 after 30 min and 90 min. BS2 showed a similar uptake of Cf-Api88 and a slightly reduced uptake of Cf-Onc112 than JW0368. Shown are the results of two independent experiments.

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