Supporting Information

Discovery and characterization of bicereucin, an unusual D-amino acid-containing mixed two-component lantibiotic

Liujie Huo and Wilfred A. van der Donk*

Department of Chemistry and Howard Hughes Medical Institute, University of Illinois at Urbana−Champaign, 600 South Mathews Avenue, Urbana, Illinois 61801, United States

*To whom correspondence should be addressed: E-mail: vddonk@illinois.edu;

phone: (217) 244-5360, fax:(217) 244-8533.

Full reference 1: Arnison, P. G.; Bibb, M. J.; Bierbaum, G.; Bowers, A. A.; Bugni, T. S.; Bulaj, G.; Camarero, J. A.; Campopiano, D. J.; Challis, G. L.; Clardy, J.; Cotter, P. D.; Craik, D. J.; Dawson, M.; Dittmann, E.; Donadio, S.; Dorrestein, P. C.; Entian, K. D.; Fischbach, M. A.; Garavelli, J. S.; Goransson, U.; Gruber, C. W.; Haft, D. H.; Hemscheidt, T. K.; Hertweck, C.; Hill, C.; Horswill, A. R.; Jaspars, M.; Kelly, W. L.; Klinman, J. P.; Kuipers, O. P.; Link, A. J.; Liu, W.; Marahiel, M. A.; Mitchell, D. A.; Moll, G. N.; Moore, B. S.; Muller, R.; Nair, S. K.; Nes, I. F.; Norris, G. E.; Olivera, B. M.; Onaka, H.; Patchett, M. L.; Piel, J.; Reaney, M. J.; Rebuffat, S.; Ross, R. P.; Sahl, H. G.; Schmidt, E. W.; Selsted, M. E.; Severinov, K.; Shen, B.; Sivonen, K.; Smith, L.; Stein, T.; Süssmuth, R. D.; Tagg, J. R.; Tang, G. L.; Truman, A. W.; Vederas, J. C.; Walsh, C. T.; Walton, J. D.; Wenzel, S. C.; Willey, J. M.; van der Donk, W. A. *Nat. Prod. Rep.* **2013**, *30*, 108.

Materials

All Chemicals were purchased from Sigma-Aldrich, Roche Biosciences or Fisher Scientific unless noted otherwise. All oligonucleotides were purchased from Integrated DNA Technologies and shown in Table S1. Restriction endonucleases, DNA polymerases, dNTP solutions and Gibson Assembly® Cloning Kit were purchased from New England Biolabs. Endoproteinases (Glu-C, Lys-C and trypsin) were purchased from Roche Applied Science. Gel extraction, plasmid miniprep, and PCR purification kits were purchased from QIAGEN. Genomic DNA isolation kit (UltraClean® Microbial DNA isolation kit) was purchased from MO BIO Laboratories, Inc. Media components for bacterial cultures were purchased from Fisher Scientific. Protein Calibration Standard I and Peptide Calibration Standard II for MALDI-TOF MS were purchased from Bruker. Defibrinated rabbit blood was ordered from Hemostat Laboratories.

General methods

For peptide residue numbering, positive residue numbers are used for amino acids in the core peptide counting forwards from the leader peptide cleavage site. Negative numbers are used for amino acids in the leader peptide counting backwards from the cleavage site. All polymerase chain reactions (PCR) were carried out on an automated thermocycler (C1000™, Bio-Rad). Gibson assembly reaction solutions were made based on a published protocol.[1] DNA sequencing

was performed by the Biotechnology Center at the University of Illinois at Urbana-Champaign (UIUC) and ACGT, Inc. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses were carried out at the Mass Spectrometry Facility of UIUC on an UltrafleXtreme mass spectrometer (Bruker Daltonics). For MALDI-TOF MS analysis, samples were desalted using ZipTipC18 (Millipore) and spotted onto a MALDI target plate with a matrix solution containing 35 mg/mL 2,5-dihydroxybenzoic acid (DHB) in 3:2 MeCN/H_2O with 0.1% trifluoroacetic acid (TFA) or 15 mg/mL sinapinic acid in 3:2 MeCN/H_2O with 0.1% TFA. Peptides were desalted by C4 solid-phase extraction (SPE) column and further purified by preparative reversed-phase high performance liquid chromatography (RP-HPLC) on a Delta 600 instrument (Waters) equipped with a Phenomenex C18 column at a flow rate of 8 mL/min. For RP-HPLC, solvent A was 0.1% TFA in H_2O and solvent B was 4:1 MeCN/H_2O containing 0.086% TFA. An elution gradient from 0% solvent B to 100% solvent B over 45 min was used unless specified otherwise. Liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-Q/TOF-MS/MS) was carried out and processed using a Synapt ESI quadrupole ToF Mass Spectrometry System (Waters) equipped with an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters). Absorbance of rabbit hemoglobin in 96-well plates was measured with a Synergy H4 Microplate Reader (BioTek).

Strains and plasmids

Bacillus cereus SJ1 (NRRL B-59452) was obtained from the culture collection of the Agricultural Research Service (ARS). *E. coli* DH5α and *E. coli* NEB® Turbo was used as host for cloning and plasmid propagation and *E. coli* BL21 (DE3) was used as a host for expression of proteins and peptides. Co-expression vector pRSFDuet-1 and pETDuet-1 were obtained from Novagen. The sources of all the indicator strains used in the antimicrobial activity assays of bicereucin and their corresponding cultivation conditions are summarized in Table S3.

Bioinformatic searches for precursor peptides lacking Cys

The nucleotide and amino acid sequences of LanJ-like enzymes, their LanA substrates and the synthetase LanMs were obtained from the National Center for Biotechnology Information (NCBI) sequence database. To search for biosynthetic gene clusters putatively producing Cys-lacking and D-amino acid containing RiPPs, BlastP searches were performed using $NpnJ_A$ and NpnM or $BsjJ_B$ and BsjM sequences as query. The individual adjacent open reading frames (ORFs) were analyzed with the Conserved Domain Database in order to identify putative LanMs or other enzymes involved in lanthipeptide biosynthesis (e.g.: LanT for cross-membrane transportation, LanP for leader peptide removal, etc.). To identify putative LanAs lacking Cys, the short ORFs near genes encoding LanM and LanJ were inspected manually.

Construction of pRSFDuet-1 derivatives for co-expression of BsjM and His_6-BsjA1/BsjA2

The gene encoding BsjM harboring flanking sequences homologous to pRSFDuet-1 multiple cloning site 2 was first amplified via PCR using genomic DNA of *Bacillus cereus* SJ1 as template and primers BsjM-mcsII-up and BsjM-mcsII-dn (Table S1). Subsequently the PCR fragment was cloned into the multiple cloning site 2 of the pRSFDuet-1 vector (without His tag) linearized by *Nde*I using Gibson assembly to generate the pRSFDuet-1/BsjM-2 vector. The genes encoding BsjA1 and BsjA2 harboring flanking sequences homologous to pRSFDuet-1 multiple cloning site 1 were then amplified using primers BsjA1-mcsI-up and BsjA1-mcsI-dn for BsjA1 and BsjA2-mcsI-up and BsjA2-mcsI-dn for BsjA2 (Table S1). Then the corresponding fragments were cloned into the multiple cloning site 1 (MCS1) of the pRSFDuet-1/BsjM-2 vector (with His tag) linearized by *Eco*RI using Gibson assembly to generate pRSFDuet-1/His-BsjA1/BsjM and pRSFDuet-1/His-BsjA2/BsjM.

Construction of pETDuet-1 derivatives for expression of $BsjJ_B$

The gene encoding $BsjJ_B$ harboring flanking sequences homologous to pETDuet-1 multiple cloning site 2 was first amplified using genomic DNA of *Bacillus cereus* SJ1 as template and primers $BsjJ_B$-mcsII-up and $BsjJ_B$-mcsII-dn (Table S1). Subsequently the PCR fragment was cloned into the multiple cloning site 2 of the pETDuet-1 vector (without His tag) linearized by *Nde*I using Gibson assembly to generate pETDuet-1/$BsjJ_B$-2.

Construction of pRSFDuet-1 derivatives for co-expression of BsjM and BsjA1

The gene encoding BsjA1 harboring flanking sequences homologous to pRSFDuet-1 multiple cloning site 1 was amplified using primers BsjA1-mcsI-nohis-up and BsjA1-mcsI-nohis-dn (Table S1). Then the fragment was cloned into the multiple cloning site 1 (MCS1) of the pRSFDuet-1/BsjM-2 vector (without His tag) linearized by *Nco*I using Gibson assembly to generate pRSFDuet-1/BsjA1/BsjM.

Construction of pRSFDuet-1 derivatives for co-expression of BsjM, His_6-$BsjA2^{C40A}$

Fragment $BsjA2^{C40A}$ harboring flanking sequences homologous to pRSFDuet-1 multiple cloning site 1 was amplified using primers BsjA2-mcsI-up and $BsjA2^{C40A}$-mcsI-dn (Table S1). Then the fragment was cloned into the multiple cloning site 1 (MCS1) of the pRSFDuet-1/BsjM-2 vector (with His tag) linearized by *Eco*RI using Gibson assembly to generate pRSFDuet-1/His-$BsjA2^{C40A}$/BsjM.

Construction of pETDuet-1 derivatives for expression and purification of His_6-$BsjJ_B$

The gene encoding $BsjJ_B$ harboring flanking sequences homologous to pETDuet-1 multiple cloning site 1 was first amplified using genomic DNA of *Bacillus cereus* SJ1 as template and primers $BsjJ_B$-mcsI-up and $BsjJ_B$-mcsI-dn (Table S1). Then the PCR fragment was cloned into the multiple cloning site 1 of the pETDuet-1 vector (with His tag) linearized by *Eco*RI using Gibson assembly to generate pETDuet-1/$BsjJ_B$-1.

Construction of pETDuet-1 derivatives for expression and purification of BsjT-150

The N-terminal 450 nucleotides of gene encoding BsjT harboring flanking sequences homologous to pETDuet-1 multiple cloning site 1 was first amplified using genomic DNA of *Bacillus cereus* SJ1 as template and primers BsjT150-up and BsjT150-dn (Table S1). Subsequently the PCR fragment was cloned into the multiple cloning site 1 of the pETDuet-1 vector (with His tag) linearized by *Eco*RI using Gibson assembly to generate pETDuet-1/BsjT150.

Construction of pET28a derivatives for expression and purification of His_6-MBP-BsjP

The gene encoding BsjP excluding the first N-terminal 75 nucleotides harboring flanking sequences homologous to pET28a-MBP[2] multiple cloning site 1 was first amplified using genomic DNA of *Bacillus cereus* SJ1 as template and primers BsjP-MBP-up and BsjP-MBP-dn (Table S1) and cloned into the multiple cloning site of the pET28a-MBP vector linearized by *Bam*HI using Gibson assembly to generate pETMBP/BsjP.

Overexpression of modified His_6-BsjA1 and His_6-BsjA2/$BsjA2^{C40A}$

E. coli BL21 (DE3) cells were transformed with pRSFDuet-1/His-BsjA1/BsjM and pETDuet-1/$BsjJ_B$-2, with pRSFDuet-1/His-BsjA2/BsjM and pETDuet-1/$BsjJ_B$-2, or with pRSFDuet-1/His-$BsjA2^{C-40A}$/BsjM and pETDuet-1/$BsjJ_B$-2, and plated on a Luria Broth (LB) agar plate containing 50 mg/L kanamycin and 100 mg/L ampicillin. A single colony was picked and grown in 50 mL of LB with 50 mg/L kanamycin and 100 mg/L ampicillin at 37 °C for 12 h, and the resulting culture was used to inoculate 4 L of LB. Cells were cultured at 37 °C until the OD_{600}

reached 0.5 and cooled on ice for 30 min. Subsequently IPTG was added to a final concentration of 0.6 mM. The cells were cultured at 18 °C for another 18 h before harvesting.

Overexpression of His_6-$BsjJ_B$

E. coli BL21 (DE3) cells were transformed with pRSFDuet-1/BsjA1/BsjM and pETDuet-1/$BsjJ_B$-1 and plated on a Luria Broth (LB) agar plate containing 50 mg/L kanamycin and 100 mg/L ampicillin. A single colony was picked and grown in 50 mL of LB with 50 mg/L kanamycin and 100 mg/L ampicillin at 37 °C for 12 h, and the resulting culture was used to inoculate 4 L of LB. Cells were cultured at 37 °C until the OD_{600} reached 0.5 and cooled on ice for 30 min. Subsequently IPTG was added to a final concentration of 0.6 mM. The cells were cultured at 18 °C for another 18 h before harvesting.

Overexpression of His_6-BsjT150 and His_6-MBP-BsjP

E. coli BL21 (DE3) cells were transformed with pETDuet-1/BsjT150 and plated on Luria Broth (LB) agar plate containing 100 mg/L ampicillin, or with pETMBP/BsjP and plated on a Luria Broth (LB) agar plate containing 50 mg/L kanamycin. A single colony was picked and grown in 50 mL of LB with corresponding antibiotics at 37 °C for 12 h, and the resulting culture was used to inoculate 4 L of LB. Cells were cultured at 37 °C until the OD_{600} reached 0.5 and cooled on ice for 30 min. Subsequently IPTG was added to a final concentration of 0.6 mM. The cells were cultured at 18 °C for another 18 h before harvesting.

Purification of modified His_6-BsjA1 and His_6-LanA2/BsjA2^{C40A}

The cell pellets were resuspended at room temperature in LanA start buffer (20 mM NaH_2PO_4, pH 7.5 at 25 °C, 500 mM NaCl, 0.5 mM imidazole, 20% glycerol) and lysed using a high pressure homogenizer (Avestin, Inc.). The sample was centrifuged at 23,700g for 30 min, and the supernatant was kept. The pellets were then resuspended in LanA buffer 1 (6 M guanidine hydrochloride, 20 mM NaH_2PO_4, pH 7.5 at 25 °C, 500 mM NaCl, 0.5 mM imidazole) and lysed again. The insoluble portion was removed by centrifugation at 23,700 g for 30 min, and the soluble portion was kept. Both soluble portions were passed through 0.45-μm syringe filters (Fisherbrand®), and the His_6-tagged modified peptides were purified by immobilized metal affinity chromatography (IMAC) as previously described[3]. The eluted fractions were desalted by preparative reversed phase (RP) HPLC using a Waters Delta-pak C4 column (15 μm; 300 Å; 25 mm × 100 mm). The desalted peptides were lyophilized and stored at -20 °C.

Purification of His_6-$BsjJ_B$ and His_6-BsjT150

All steps were performed at 4 °C in a cold room or on ice. The cell pellets were resuspended in buffer A (20 mM HEPES, 500 mM NaCl, 20% glycerol, pH 7.5 at 25 °C) and lysed using a high pressure homogenizer (Avestin, Inc.). The sample was centrifuged at 23,700g for 30 min. The supernatant was passed through 0.45-μm syringe filters (Fisherbrand®) and loaded onto a 5 mL HisTrap IMAC column pre-charged with Ni^{2+} and equilibrated with buffer A. The column was attached to an ÄKTA fast protein liquid chromatography (FPLC) system (GE Healthcare) and further washed with up to 25% buffer B (20 mM HEPES, 500 mM NaCl, 500 mM imidazole, 20% glycerol, pH 7.5 at 25 °C) in buffer A at a flow rate of 1.5 mL/min. Then the protein was eluted using a gradient of 25-100% buffer B. UV absorbance at 280 nm was monitored and fractions were collected and analyzed by SDS-PAGE (Bio-Rad). The fractions containing the desired proteins were combined and exchanged back to buffer A using a PD-10 desalting column (GE Healthcare) and subsequently concentrated using an Amicon Ultra-15 Centrifugal Filter Unit (Millipore). Protein aliquots were frozen in liquid nitrogen and stored at -80 °C.

His_6-$BsjJ_B$ *in vitro* assay
The 8-fold dehydrated His_6-BsjA1 (50 μM) was incubated with 1 μM His_6-$BsjJ_B$ enzyme in the presence of 0.5 mM NADH and 0.5 mM FMN in 50 mM HEPES buffer at pH 7.5 overnight at room temperature. The reaction was quenched by addition of 1% trichloroacetic acid (TCA). The conversion from Dha to D-Ala or Dhb to D-Abu was monitored by MALDI-TOF MS.

Proteolytic cleavage and purification of BsjA core peptide
The modified BsjA1 and/or BsjA2 precursor peptides were dissolved in 50 mM HEPES buffer to a concentration of 3 mg/mL at pH 7.5. To 5 mL of peptide solution, 500 μL of 1 mg/mL BsjT150/BsjP or 100 μL of 1 mg/mL GluC protease were added and the assay was incubated for 12 h, then quenched with 0.1% trifluoroacrtic acid (TFA). The cleavage of leader peptide was monitored by MALDI-TOF MS. Purification of digested core peptide was carried out using RP-HPLC as described in the general method section.

Iodoacetamide (IAA) assay to detect (non)cyclized Cys residue of Bsjβ
An aliquot of Bsjβ was diluted to a final concentration of 1 mM in buffer containing 250 mM Tris-HCl, 25 mM IAA and 13 mM TCEP, pH 9.0. The reaction was incubated at room temperature for 60 min and analyzed by MALDI-TOF MS.

Marfey assay of Ala and Abu residues
A sample of around 0.1 mg of purified peptide was hydrolyzed by treatment with 2 mL of 6 N HCl and heating to 120 °C for 24 h in a sealed tube. The hydrolysate was dried under N_2. To a total of 1 μmol of amino acids in an eppendorf tube, 2 μL of a 1% acetone solution of Marfey's reagent (FDAA, N-(5-fluoro-2,4-dinitrophenyl)-D-alaninamide) and 8 μmol of a 1 M solution of $NaHCO_3$ were added. The reaction mixture was heated with frequent shaking over a hot plate at 40 °C for 1 h and then cooled to room temperature. The reaction was quenched by adding 5 μL of 2 M HCl and diluted with 0.2 mL of MeOH. Standards (L or D-Ala and L or D-Abu) were treated with identical procedures.

LC-MS analysis of derivatized Ala and Abu residues
The FDAA-derivatized samples (with around 2 nmol of derivatized Ala residues) were injected onto a Synapt LC-MS (Waters) equipped with a C18 UPLC column. The molecular weight and retention times were compared with those of standard FDAA-derivatized Ala or Abu residues. Acetonitrile/water containing 0.1% formic acid was used as the mobile phase with a linear gradient elution mode (0-40% MeCN, 20 min) at a flow rate of 0.18 mL/min. A mass range of m/z 100 - 3000 was covered with a scan time of 1 s, and data were collected in positive ion mode. Glu-1-Fibrinopeptide B (Glu-Fib) was used as external calibration standard. Extracted ion chromatograms were generated with an m/z window of 342.1 ± 0.2 Da for FDAA-Ala and 356.1 ± 0.2 Da for FDAA-Abu.

LC-ESI-Q/TOF MS and MS/MS analyses.
A 5 μL volume of 100 μg/mL peptide was injected on a Waters Acquity UPLC system equipped with a BEH C8 column (1.7 μm, 100 mm × 1.0 mm; Waters). The column was pre-equilibrated in aqueous solvent. The solvents used for LC were: solvent A = 0.1% formic acid in water and solvent B = 0.1% formic acid in methanol. A solvent gradient of 3–97% B over 15 min was used, and the fractionated sample was directly subjected to ESI-Q/TOF MS analysis using a Waters Synapt mass spectrometer. The mass spectrometer was calibrated before any sample was injected. Data was acquired in ESI-positive mode with the capillary voltage set to 3.0–3.5 kV. The ionization source and desolvation gas were heated to 120 °C and 300 °C, respectively. Cone gas was set to 0 L/h, and desolvation gas was set to 600 L/h. The transfer collision energy was set to 4 V for both MS and MS/MS analyses. The trap collision energy was set to 6 V for MS analysis.

For MS/MS analysis, a trap collision energy ramp ranging from 20–40 V was applied on multiply charged parent ions to achieve fragmentation. Suitable trap collision energy was determined by choosing the spectra where both fragment peaks and parent peak could be observed. [Glu[1]]-Fibrinopeptide B (Sigma) was directly infused as lock mass with lock spray sampling if desired. The acquired spectra were processed using MaxEnt3 software and analyzed by Protein/Peptide Editor in BioLynx 4.1 (Waters). The MS/MS data of AVE-Bsjα and AVE-Bsjβ shown in Figure 2a and 2b were uploaded to the Global Natural Product Social Molecular Network (GNPS) under submission number MSV000079622.

GC/MS analysis.

The Bsjβ peptide was hydrolyzed, and the resulting solution was dried and directly used for derivatization.[4,5] The derivatized samples were analyzed by GC/MS using an Agilent 7890 gas chromatograph equipped with a Varian CP-Chirasil-L-Val–fused silica column (25 m × 0.25 mm × 0.15 μm). Samples were dissolved in methanol and introduced to the instrument via a split (1:5) injection at a flow rate of 1.7 mL/min or 2.0 mL/min helium gas. The temperature method used was 160 °C for 5 min, 160 °C to 180 °C at 3 °C/min and 180 °C for 6 min. The mass spectrometer was operated in simultaneous scan/selected-ion monitoring (SIM) mode, monitoring at the characteristic fragment masses of 365 Da for derivatized Lan.

Agar diffusion growth inhibition assay of Bsjα and Bsjβ

Bsjα and Bsjβ or Bsjβ^{C40A} were obtained as described above by GluC cleavage followed by HPLC purification. Peptides were dissolved in H_2O to achieve a concentration of 100 μM. The peptide solutions were diluted to prepare a 10 μM solution. Agar plates were prepared by combining 20 mL of melted agar (cooled to 42 °C for 5 min) with 200 μL of dense overnight cell culture. The seeded agar was poured into a sterile 100-mm round dish (VWR) and allowed to solidify at 25 °C for 30 min. A sample of 10 μL of 10 μM Bsjα, Bsjβ, Bsjβ^{C40A} separately or combined Bsjα with Bsjβ or Bsjα with Bsjβ^{C40A} were directly spotted on the solidified agar. Plates were incubated at various temperatures shown in Table S3 for 16 h, and the antimicrobial activity was determined by the presence or absence of zones of growth inhibition. A negative control was conducted using sterile H_2O.

Liquid growth inhibition assay of Bsjα and Bsjβ

Modified Bsjα and Bsjβ or Bsjβ^{C40A} were obtained as described above by GluC cleavage followed by HPLC purification. Peptides were dissolved in H_2O to achieve a stock concentration of 500 μM. Bacterial culture was prepared by combining 100 μL of dense overnight culture with 10 mL of fresh liquid media and distributed into a 96-well plate to give a final volume of 50 μL in each well after addition of serial dilutions of Bsjα and/or Bsjβ. Subsequently the 96-well plate was incubated at various temperatures shown in Table S3 for 16 h, and the growth inhibition was determined by the measurement of OD_{600} at different time points and the minimal inhibitory concentration (MIC) was determined by plotting an isobologram. A negative control was conducted using sterile H_2O.

Hemolytic assay of Bsjα and Bsjβ

The approach described previously[6] was used. A sample of 1 mL of defibrinated rabbit blood was diluted with 20 mL of PBS in a 50-mL conical tube on ice and mixed well by gently inverting the tube. The PBS-diluted blood sample was centrifuged at 1,000 g for 5 min at 4 °C, and the supernatant containing released hemoglobin was discarded. The process was repeated two to four times until the supernatant was clear. The blood cells were then diluted with PBS to make a 5% solution, which was immediately used to test the hemolytic activity of the peptides. Rabbit blood

was used within 10 d after receipt to minimize autolysis. Bsjα and Bsjβ peptide solutions made as described above were diluted with PBS to prepare a 10-μM solution. To a 96-well plate, 100 μL of 5% washed rabbit blood sample was added to each well, followed by the addition of the desired peptide samples or controls. PBS was used to adjust the final volume to 150 μL. The 96-well plate was kept in a 37 °C incubator to allow the lytic reaction to proceed. At each time point, 20 μL of each reaction mixture was taken out, diluted with 200 μL of fresh PBS and centrifuged at 1,000 g for 5 min. The supernatant was transferred to a new well, and the absorbance was measured at 415 nm (wavelength was optimized for rabbit hemoglobin). The absorbance of each sample at each time point was analyzed in triplicate, and the maximum absorbance was determined by adding 50 μL of 0.1% Triton in PBS to 100 μL of a 5% blood sample and using the same analysis procedure.

Supporting Tables and Figures

Table S1. Primers used in the study

Primer name	Primer sequence (5'-3')
BsjM-mcsII-up	ATTAGTTAAGTATAAGAAGGAGATATACATATGATTAAGAATGTGAACTTGAAAGAAGC
BsjM-mcsII-dn	TGGCCGGCCGATATCCAATTGAGATCTGCCATTTAATCTAACGTTGCTATCGAAGC
BsjA1-mcsI-up	ATCATCACCACAGCCAGGATCCGAATTCGACAAATGAAGAAATTATTGTAGCATGGAA
BsjA1-mcsI-up	AAGCTTGTCGACCTGCAGGCGCGCCGAGCTTTAATTAACTGTGATATAACTTGCAACAA
BsjA2-mcsI-up	ATCATCACCACAGCCAGGATCCGAATTCGACAAATGAAGAAATCATTGTAGCATGGAA
BsjA2-mcsI-dn	CGCAAGCTTGTCGACCTGCAGGCGCGCCGAGCTTTAACCTAGGCATGTTTTATTGTT
$BsjJ_B$-mcsII-up	TATTAGTTAAGTATAAGAAGGAGATATACATATGAAACATGTCCTCGCATACGTTGGATC
$BsjJ_B$-mcsII-dn	CGCGTGGCCGGCCGATATCCAATTGAGATCTGCTTAACAATGAGAATATTCGTTAATCTT
BsjA1-mcsI-nohis-up	TTAACTTTAATAAGGAGATATACCATGACAAATGAAGAAATTATTGTAGCATGGAA
BsjA1-mcsI-nohis-dn	TGGCTGTGGTGATGATGGTGATGGCTGCTGCCTTAATTAACTGTGATATAACTTGCA
$BsjA2^{C\text{-}40A}$-mcsI-dn	ATTAGCGGTATTTTTTCAAACAATAAAACAGCACTAGGTTAAAGCTCGGCGCGCCTGCAG GTCGACAAGCT
$BsjJ_B$-mcsI-up	ATCACCATCATCACCACAGCCAGGATCCGAATTCGATGAAACATGTCCTCGCATACGTTG
$BsjJ_B$-mcsI-dn	CCGCAAGCTTGTCGACCTGCAGGCGCGCCGAGCTTTAACAATGAGAATATTCGTTAATCT
BsjT150-up	ACCATCATCACCACAGCCAGGATCCGAATTCGATGAAAAAAATAAGATATGTTGAACAAA
BsjT150-dn	GCTTGTCGACCTGCAGGCGCGCCGAGCTTTATAATAAATGCTTGAGAAAAATATTGTTTTT
BsjP-MBP-up	TCTGAGAACCTGTACTTCCAATCCGGATCCCTCGAGGAAGAGGAAGAAAACTATTATACA
BsjP-MBP-dn	GTGGTGGTGCTCGAGTGCGGCCGCAAGCTTGTCGATTAGTGGGTTACTGCCTTATATACA
BsjM-mid1	AGTTTAAAATTAGCAATATTTCTAT
BsjM-mid2	ATTCAATATAGAGATGAATTATTACATAG
BsjAleader-der-up	ACAAATGAAGAAATTATTGTAGCAT
BsjAleader-der-up	CGACTTAAGCATTATGCGGCCGCTTATGATGGATATCCGAATGAATATCTTTGTTCAACTGC TCCACCTGTTACTTGCGCCATTTCAT

Table S2. Proteins involved in bicereucin biosynthesis

Proteins	Residues (aa)	Putative annotation	Proposed function in bicereucin biosynthesis
BsjA1	81	Structural gene	Precursor peptide
BsjA2	88	Structural gene	Precursor peptide
BsjA3	81	Structural gene	Precursor peptide
BsjM	1018	Type-II Lanthipeptide synthetase	Dehydration of Ser/Thr to form Dha/Dhb
$BsjJ_B$	251	NAD(P)H-dependent FMN reductase	Reduction of Dha/Dhb to form D-Ala/D-Abu
BsjT	708	Lanthipeptide transporter LanT-like	Initial proteolytic cleavage of leader peptide
BsjP	462	Serine Protease S8 family	Final proteolytic cleavage of leader peptide
BsjR	151	AraC family transcription regulator	Pathway regulation
ORF1	351	Cytolysin immunity protein	Self-resistance

Table S3. Summary of strains used in the antimicrobial assay of Bsjα and Bsjβ[7]

Strain	Source	Media	Temprature (°C)
Lactococcus lactis 481	CNRZ[a] 481	M17 + 0.5% lactose, BD	30
Bacillus subtilis	ATCC[b] 6633	Luria Broth (LB), Fisher	30
Staphylococcus epidermidis 15×	Ekkelenkamp *et al.*	Nutrient Broth, BD	37
Micrococcus luteus	ATCC 4698	Tryptic Soy Broth (TSB), Sigma	30
Streptococcus mutans	ATCC 25175	Brain Heart Infusion (BHI) Broth, BD	37
Staphylococcus aureus Rosenbach, (Methicillin-resistant)	ATCC BAA-1717	Nutrient Broth, BD	37
Enterococcus faecium, (Vancomycin-resistant)	ATCC 700221	Brain Heart Infusion (BHI) Broth, BD	37
Escherichia coli DH5α	UIUC-CMF[c]	Luria Broth (LB), Fisher	37
Enterobacter cloacae 10-19C	ATCC 29893	Nutrient Broth, BD	30
Pseudomonas aeruginosa strain K	UIUC-CMF[c]	Tryptic Soy Broth (TSB), Sigma	37

[a]CNRZ: National Centre for Zootechnical Research. [b]ATCC: American type culture collection. [c]UIUC-CMF: University of Illinois at Urbana-Champaign Cell and Media Facility

Table S4. Summary of activity assessed by agar diffusion growth inhibition assay

Strain	Source	Bsjα	Bsjβ	Bsjα+Bsjβ
Lactococcus lactis 481	CNRZ 481	-	-	+
Bacillus subtilis	ATCC 6633	+	+	++
Staphylococcus epidermidis 15×	Ekkelenkamp *et al.*	+	+	++
Micrococcus luteus	ATCC 4698	+	+	++
Streptococcus mutans	ATCC 25175	-	-	+
Staphylococcus aureus Rosenbach, (Methicillin-resistant)	ATCC BAA-1717	-	-	+
Enterococcus faecium, (Vancomycin-resistant)	ATCC 700221	-	-	++

-: no inhibition zone observed. +: medium inhibition zone observed. ++: strong inhibition zone observed.

Table S5. Specific activity (minimal inhibitory concentration, MIC) of bicereucin

Strain	Source	Bsjα	Bsjβ	Bsjα+Bsjβ
		MIC (μM)	MIC (μM)	MIC (μM)
Gram-positive bacteria:				
Bacillus subtilis	ATCC[b] 6633	25.0	25.0	1.0 + 0.5, in ratio 1:2
Micrococcus luteus	ATCC 4698	12.5	12.5	0.5 + 0.25, in ratio 1:2
Lactococcus lactis 481	CNRZ[a] 481	n.d.	n.d.	5.0 + 2.5, in ratio 1:2
Staphylococcus epidermidis 15×	Ekkelenkamp *et al.*	n.d.	n.d.	12.5 + 6.25, in ratio 1:2
Streptococcus mutans	ATCC 25175	n.d.	n.d.	20.0 + 10.0, in ratio 1:2
Staphylococcus aureus Rosenbach, (Methicillin-resistant)	ATCC BAA-1717	n.d.	n.d.	12.5 + 6.25, in ratio 1:2
Enterococcus faecium, (Vancomycin-resistant)	ATCC 700221	n.d.	n.d.	2.5 + 1.25. in ratio 1:2
Gram-negative bacteria:				
Escherichia coli DH5α	UIUC-CMF[c]	n.d.	n.d.	> 500 + 250, in ratio 1:2
Enterobacter cloacae 10-19C	ATCC 29893	n.d.	n.d.	> 500 + 250, in ratio 1:2
Pseudomonas aeruginosa strain K	UIUC-CMF[c]	n.d.	n.d.	> 500 + 250, in ratio 1:2

[a]CNRZ: National Centre for Zootechnical Research. [b]ATCC: American type culture collection. [c]UIUC-CMF: University of Illinois at Urbana-Champaign Cell and Media Facility

Table S6. Specific activity (minimal inhibitory concentration, MIC) of Bsjα and Bsjβ^{C40A}

Strain	Source	Bsjβ-C40A	Bsjβ	Bsjα+Bsjβ-C40A	Bsjα+Bsjβ
		MIC (μM)	MIC (μM)	MIC (μM)	MIC (μM)
Bacillus subtilis	ATCC 6633	**>500**	25.0	1.0 + **>500**	1.0 + 0.5
Micrococcus luteus	ATCC 4698	**75**	12.5	0.5 + **25**	0.5 + 0.25
Lactococcus lactis 481	CNRZ 481	n.d.	n.d.	5 + **75**	5 + 2.5

Table S7. Summary of LanAs lacking Cys from searches using the $NpnJ_A$ and NpnM sequence as query.

Strain	Accession number	LanA sequence
Scytonema hofmanni PCC 7110		MTTNSEALNPQDLQERIIAKAIEDPAYKQRLLSNPKALLSEELGAELPEDLTVQVLQQSPKHLYLLLPIDIDELIRDGVLS ESELEAVAGGVLFTVVTIYTVKQAPKISKAIKGWFS
	2551959285	MTTNSEALNSQDLQERIIAKAIENPAYKQRLLSDAKAVLEEELDIELPADLSVQVLQQSPKQLYLLLPFDIDELVREGILS ESELEAVAGGGVLSLVANTAAQVSIFTSIQYSIEYSLRKTAQLKRKK
	2551959286	MTTNFEEFNSQTLEERIIVKAIEDPNYKQRLLSDAKAVVEEELGDKLGEDVTIQVLQQSAKHLYLLLPVDIDDMIREGLI TQEELEAVAGGSARLIKVTKALHNSVNAKSKSDVSQGVSGVVATVTSLASALYSAVRSRKG
	2551959287	MTKTSEELNPQTLEERIITKAMEDPAYKQRLISNAKAVVEEELGDKLGENITIEVLQQSAKNLYLLLPADIDEMIRDGLIS QEELEVVAGGRSIIRSTSIDFKQTSRNLKNIEAGLKSAVAIVSTVVLSRRATTPPK
Nostoc punctiforme PCC 73102		
	YP_001866608	MSEQQAQTRKDIESRIIAKAWKNEAFKQELLTNPKPIIEQEFGVELPAELNVSVYEENSTSLYFVLPILPQIEGRELSEE ELESVAGGFIGGLITIAVGVTPFTGDIVKATKKLTKK
	YP_001866607	MSEQEQAQTRKNIEARIVAKAWKDEGYKQELLTNPKAIIEREFGVEFPAEVSVQVLEENSTSLYFVLPISPVAIAQELSE EQLEAIAGGYMTTLASANASAKINPILPIRHSLVKTLR
	YP_001866606	MSEQEQAQTRQDIEARIIAKAWKDEVYKQELLTNPKAVIEREFGVEFPADVNVQVLEENPTSLHFVLPISPVTIAQELS EEELLAIAAGGQIKELTKISANLVKNYKTTRAAVSATALISGASIGASSVHL
	YP_001866605	MSEQEQAQTRQDIEARIIAKAWKDEAYKQELVTNPKAVIEREFGVEFPADVNVQVLEENPTSLHFVLPISPVAIAQELS EEQLEAIAGGKDWRVELAKGALSVGLAITLVDTLKRLTK
	YP_001866604	MTQQEQAQTRQDIEARIIAKAWKDEAYKQELLTNPKAVIEREFGVEFPADVNVQVLEENPTSLHFVLPISPVAIAQELS EEELLALAAGVNYSAVTVAIVKNTVKQNTNIITRAAVSVTALVTGASIGASSVHL
	YP_001866603	MSEQEQAQTRQDIEARIIAKAWKDESYKQELLTNSKAVIEREFGVEFPADVTVQVLQENPTSLYFVLPLSPTAIMQELS EEQLQAIAGGGISAVIGNPKLISSALSGLLVSVSYLASHQIRR
Azospirillum sp. B510 plasmid pAB510a		
	YP_003451044	MIEIDGVEGEGGGQVLRTALALLLADGPSRLVLDGGTDNPHAPPFDFLDRSLLPLLGRMGVPAEQVGGRAATRMAG YLASGAFAGP
Cyanobacterium sp. PCC 10605	2503745297	MSKEAVEEFFAKIPEDDNLQQKLVSILQADIDHRQETAKLAQEYGFDITAEELSEEVKKRQEEFAQRQESGELSEEELE SVAGGLSFPGTFSFPSPIPGTFPFPTPRPGPIPFPKPKW
	2503745298	MSKEAVEQFFARIPEDNELQQKLVSILQEDIDHRQETAKLAQEYGFDVTAEELSEEAKQRQEEFAQNPESEELSEEEL ESVAGGSIPSITVGIFQPLTINTYNMPGSISNPFPKPKW
	2503745299	MSQEAVISFLDAVPQNEELQRKLATILESSENDREDAAQLANEYGYDITPDELWAEIQKRQQETKFRQEAGELTDEEL EAVAGGELLVATIASTVAMTVSVGGSIAVGILAPKIKW
	2503745300	MSQEAAIQFLEAVPENEELQQKLVAILESSENDREDAAQLANEYGYDITPDELWAEIQKRQQDLEARQEAGELTDEEL EAVAGGEFVATAVVATVVGAAGLGWTIGGGIAPKIKW
	2503745301	MSIAAVEAFLDKVQTDEELQKELATALEAENDRQAVTDLANSKNYEFTPDELWAEIQKRQNDALARQQSGELSDEEL EAVAGGEFVVGSVLITAASFAASVTAGVAIPNIKW

Table S8. Summary of LanAs lacking Cys from searches using $BsjJ_B$ and BsjM sequences as query.

Strain	Accession number	LanA sequence
Bacillus cereus AH621	ZP_04298129	MTVEEQEFIVRCHQEDLHTKHIYHMRYVFKAKTVTQVKQQLNAWFKRYQVHMYGSVSKITKTIILRGQAILNTIISPL
	ZP_04298127	MKLFLKKLQNAGFIQLLEDKHNRRKTYLATQKGKIILMSEIERRSKMVDHGKSALKSSVTKFLNR
Clostridium beijerinckii NCIMB 8052	YP_001311651	MLNEKALKKVGPSYEEVGAKKDGGVTPTSDISVLTAATLISATYTVTITATND
Bacillus cereus F65185	ZP_04206900	MSPSLTQTGKIVFDVPKDAKGFILKTSGDMMGKEIKS
Caldicellulosiruptor sp. F32	2554121233	MNKVAYSLDNFLLNIYIKTLIYEYYLWRENNSENIKNKKMYFSYSFIIYSIIKEYTSL
Lachnospiraceae bacterium C6A11	2562060207	MEVNMTRNDKELIAVVEGRLDTITSPELEDKLMPVLAETEKLVFDFANLEYVSSAGLRVLMATFKVMKGKGGSMVIRNVT PKVKEVFMVTGLINGFDFE
	2562060204	MNERIKDLFTEIEKNPELKKKLQALPDEDASKDKAIEIAKEYGFVLTREDLEGDINGEISNEELMMVAGGLRMEDYARDAS GSGFVLTAIRKALM
	2562060203	MNENLKLFLQKVAADKEIQAKMQSFDDIDEAYKYAVTVQDGFTKEEFEELMTKLSKATSESDEISDADLSRVAGGMTTTD EAGLIGSLTTVASVAALSLSLSAM
	2562060201	MNENLKMFLQKVAADEEKQAKMQSFTDMDEAYEYAATVQEGFTKEEFTELMTKIKDFASQNNDEINDDDLANVAGGTS TSACIARAATAATAAVTMSLLAV
	2562060200	MNDSLKEFLKKLSEDEELCAKLESAGDSDKAYEIAKTIQEGFTKEEFVDAMTRINNSISADGEINDDDLISVAGGLTTDEE TLLMDGTFLGSAIVAASSAGAV
	2562060199	MNENLKMFLQKVAADEKIQAKMKSFTDIDEAYEYAVSIQDGYSKEEFEAVMAKSAKGSTINDEISDTDLESVAGGTITEGA KGFRASAGDEVDYSLFAATAI
	2562060198	MNENLEKFFQKVSADEKIQAKMKSFTDIDEAYDYAVSIQDGYSKEEFEAVMVEITKGSAENDEISDDDLENVAGGIISEAR GLKAVNGNSDNLTNFAAGAI
Clostridium beijerinckii SA-1 ATCC 35702		
	2574385795	MLNEKALKKVGPSYEEVGAKKDGGVTPTSDISVLTAATLISATYTVTITATND
Clostridium botulinum CDC54075	2569227337	MNKYKDLFLCLSLFILGIFIWIYKTIITSDIPINISLNEFLKVFFIIFIYDLLQYIYIKKLKSHLYLLNLIFLTILLLIWLGNLITSLIY

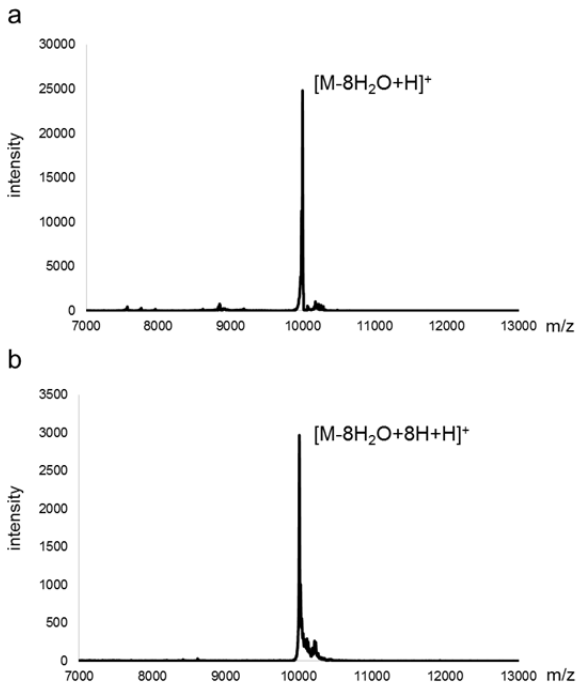

Figure S1. MALDI/TOF mass spectra for (a) His_6-BsjA1 modified by BsjM (calculated m/z = 9991.02 Da, observed m/z = 9990.89 Da) and (b) His_6-BsjA1 modified by BsjM and $BsjJ_B$ (calculated m/z = 9999.03 Da, observed m/z = 9998.75 Da) in *E. coli*.

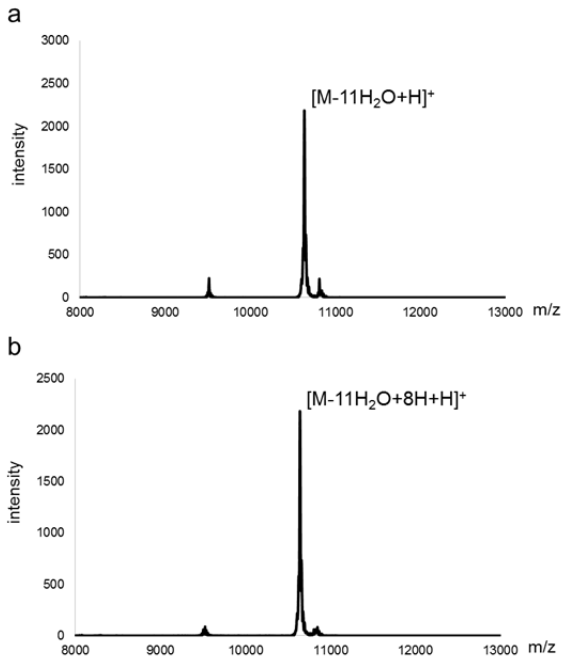

Figure S2. MALDI/TOF mass spectra for (a) His_6-BsjA2 modified by BsjM (calculated m/z = 10604.33 Da, observed m/z = 10605.02 Da) and (b) His_6-BsjA2 modified by BsjM and $BsjJ_B$ (calculated m/z = 10612.32 Da, observed m/z = 10612.02 Da) in *E. coli*.

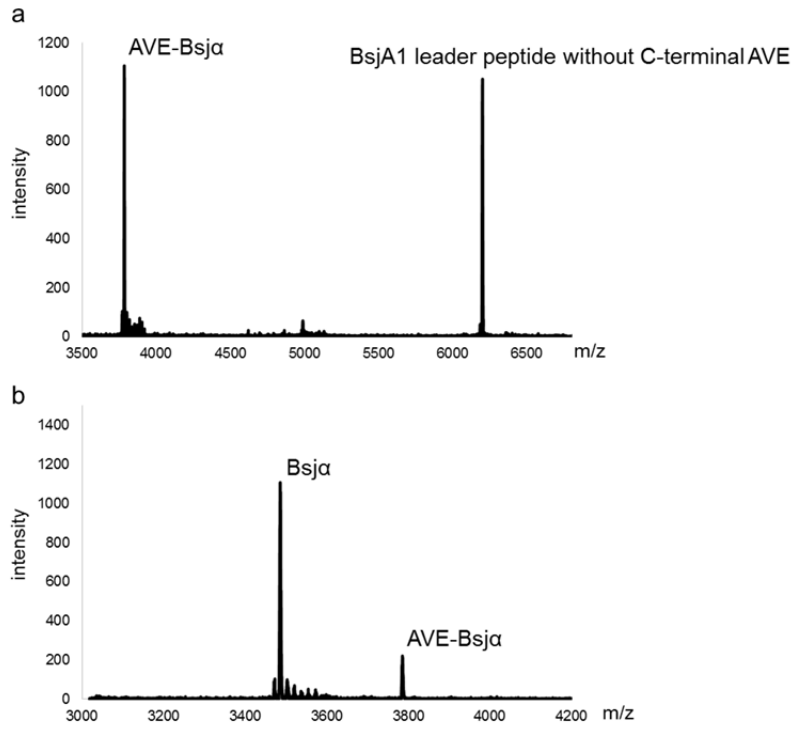

Figure S3. *In vitro* activity of (a) BsjT150 and (b) BsjP . (a) MALDI-TOF mass spectra of 3 mg/mL His_6-BsjA1 peptide incubated with 0.1 mg/mL His_6-BsjT-150 at 20 °C for 12 h. (b) MALDI-TOF mass spectra of 1 mg/mL AVE-BsjAα peptide incubated with 0.5 mg/mL His_6-MBP-BsjP at 20 °C for 12 h.

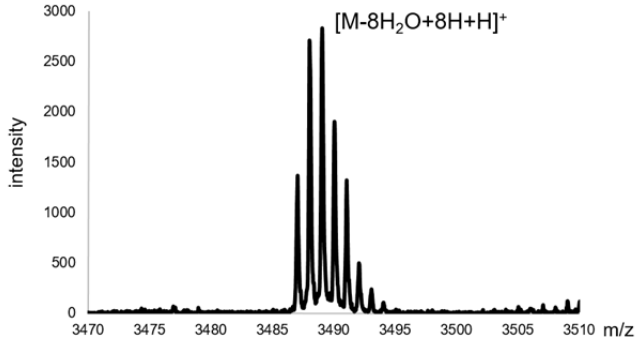

Figure S4. MALDI-TOF mass spectrum of Bsjα core peptide after modification of BsjA1 by BsjM and $BsjJ_B$ in *E. coli* and proteolysis by Glu-C (calculated m/z = 3485.92 Da, observed m/z = 3486.02 Da).

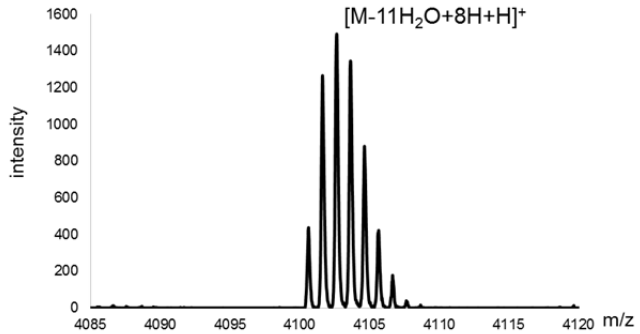

Figure S5. MALDI-TOF mass spectrum of Bsjβ core peptide after modification of BsjA2 by BsjM and $BsjJ_B$ in *E. coli* and proteolysis by Glu-C (calculated m/z = 4100.23 Da, observed m/z = 4099.98 Da).

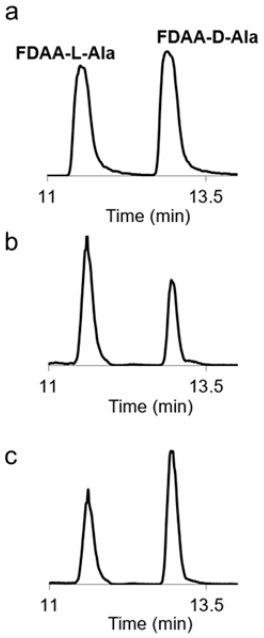

Figure S6. Elucidation of the stereochemistry of the introduced Ala of Bsjβ using Marfey's method. a) Extracted ion chromatograph (EIC) of [M+H] = 342.1 ± 0.2 Da corresponding to FDAA-L- and D-Ala standards. II) EIC of hydrolysate of AVE-Bsjβ derivatized with FDAA. III) EIC of hydrolysate of AVE-Bsjα derivatized with FDAA co-injected with authentic D-Ala derivatized with FDAA.

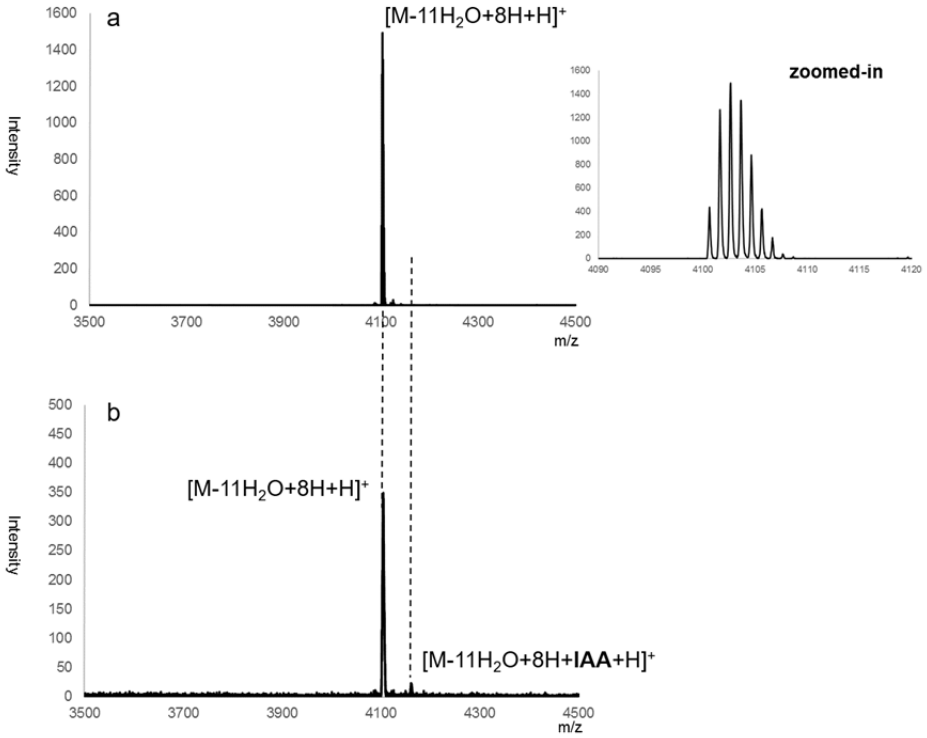

Figure S7. MALDI-TOF mass spectrum of iodoacetamide (IAA) assay with the Bsjβ core peptide after modification of BsjA2 by BsjM and $BsjJ_B$ in *E. coli* and proteolysis by Glu-C. (a) Bsjβ as negative control (calculated m/z = 4100.23 Da, observed m/z = 4099.98 Da). (b) Bsjβ reacted with IAA (calculated m/z = 4158.24 Da, observed m/z = 4158.74 Da).

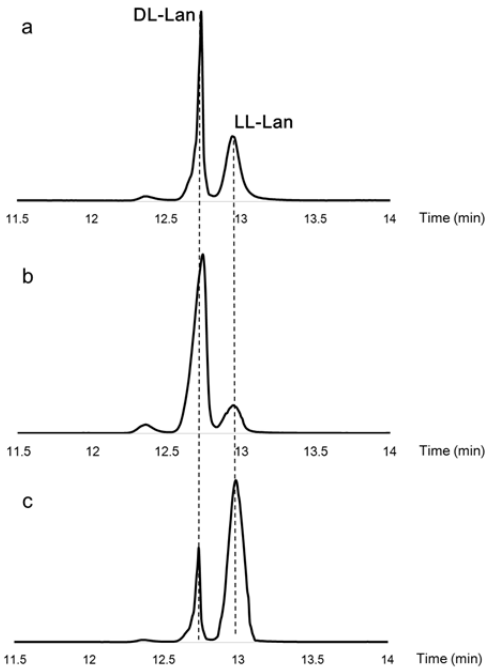

Figure S8. GC/MS traces for co-injections of synthetic, derivatized Lan standards and hydrolyzed/derivatized Lan residues obtained from *in vivo* modified Bsjβ (selected ion monitoring, SIM, at 365 Da for Lan). (a) Hydrolyzed and derivatized Lan residues from modified Bsjβ. b) Co-injected with derivatized DL-Lan standard. c) co-injected with derivatized LL-Lan standard.

	725	781	836
LctM	SYA**H**GNSGI. . .	SQW**C**HGASGQ. . .	CL**CH**GILGNL
CylM	GFG**H**GIYSY. . .	NSW**C**KGTVGE. . .	CL**CH**GNAGTL
GeoM	GLA**H**GSSGF. . .	SMW**C**HGAAGI. . .	CL**CH**GDLGNL
HalM1	GFS**H**GVSGV. . .	VAW**C**HGAPGI . . .	SL**CH**GDFGQL
HalM2	GLS**H**GAAGF. . .	TFW**C**HGAPGI . . .	SL**CH**GDFGNL
LtnM1	GLA**H**GVSQI. . .	TSW**C**NGTSGI . . .	IM**CH**GSLGVY
LtnM2	GIA**H**GELGY. . .	VGW**C**NGLSGI . . .	SV**CH**GASGVL
CinM	GFS**H**GSGGI. . .	NAW**C**NGAAGI . . .	TL**CH**GTSGNA
ProcM	GFS**H**GTAGY. . .	ASW**C**HGAPGI . . .	HL**CC**GSLGLM
BsjM	GFS**Q**GISSL. . .	SNW**Q**NGLVGI . . .	LS**C**GNSGTGE

Figure S9. Sequence alignment showing the conserved His and Cys residues among class II lanthipeptide synthetases (LanMs). Highly conserved residues are shown in bold. The conserved His residue believed to be the acid that protonates the enolate formed during cyclization is shown in red and the counterpart in BsjM is shown in blue. Conserved active site residues critical for zinc ion binding are shown in green and their counterparts in BsjM are shown in yellow. The residue numbers on the top are based on LctM.

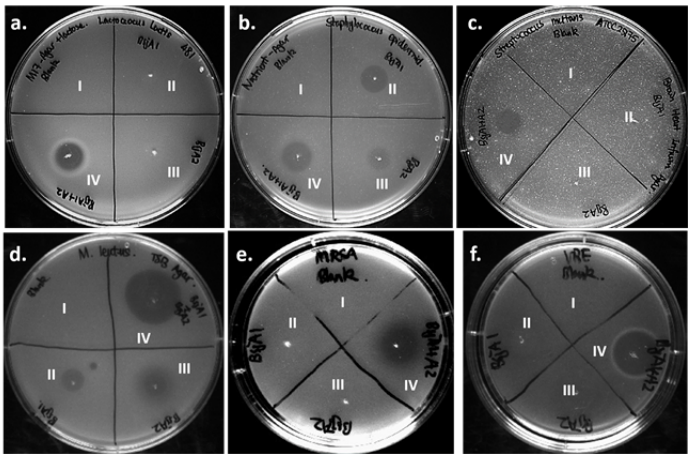

Figure S10. Antimicrobial activity assay of Bsjα and Bsjβ against a) *Lactococcus lactis* CNRZ 481, b) *Staphylococcus epidermidis* 15x, c) *Streptococcus mutans* ATCC 25175, d) *Micrococcus luteus* ATCC 4698 e) Methicillin-resistant *Staphylococcus aureus* Rosenbach ATCC BAA-1717, f) Vancomycin-resistant *Enterococcus faecium* ATCC 700221. I: Blank, II: Bsjα, III: Bsjβ, IV: Bsjα combined with Bsjβ, spotted peptide amount: 1 µmol.

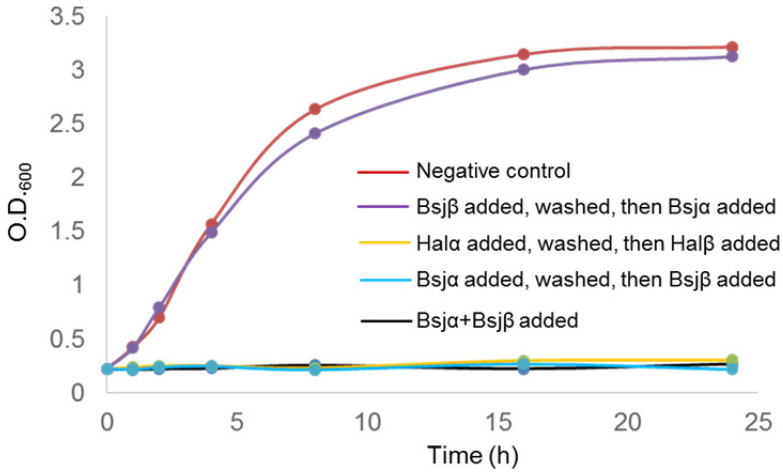

Figure S11. Sequential activity of bicereucin peptides against *Lactococcus lactis* 485. Growth curves are shown for (blue) Bsjα treatment, washing, and Bsjβ addition, (purple) Bsjβ treatment, washing, and Bsjα addition, (yellow) positive control using Haloduracin: Halα treatment, washing, and Halβ addition, (black) both Bsjα and Bsjβ were added at the same time, (red) negative control using sterile water.

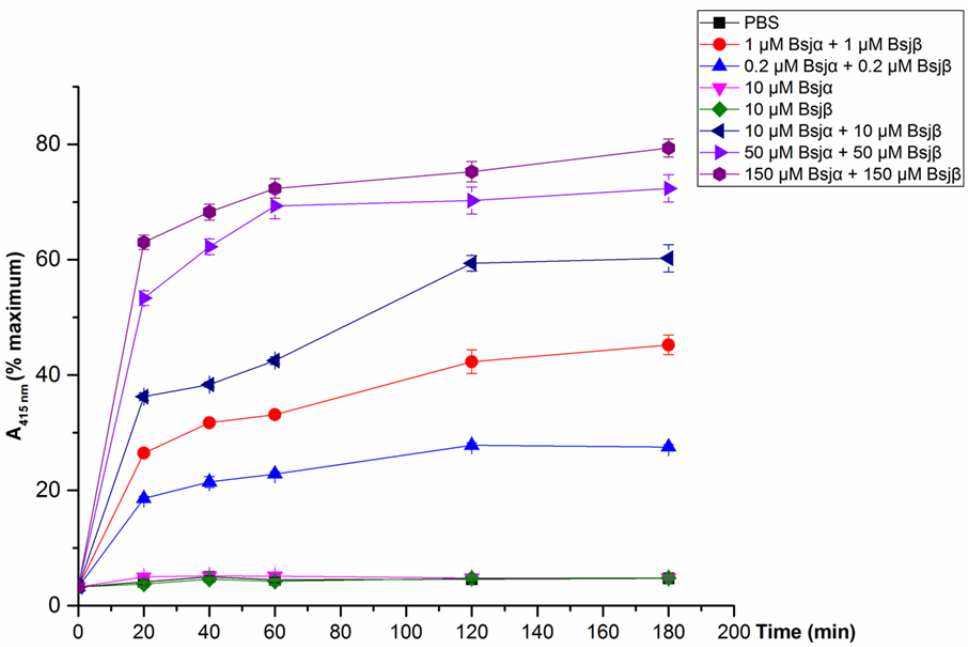

Figure S12. Hemolytic assay of Bsjα and Bsjβ with rabbit red blood cells. At each time point, the hemoglobin released from the lysed cells was quantified in triplicate by measuring the absorbance of the supernatant at 415 nm. Error bars indicate the standard deviation of the three independent experiments. Maximum absorbance was determined by measuring the blood sample treated with 0.1% Triton in PBS using same analysis procedure.

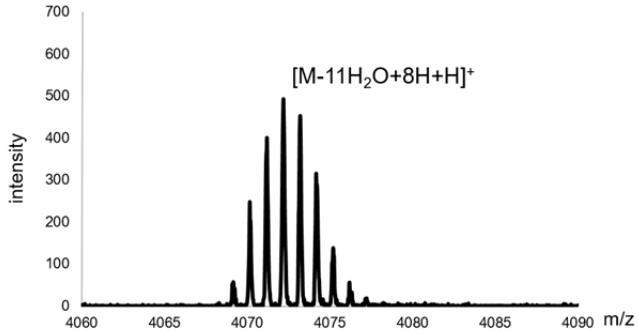

Figure S13. MALDI-TOF mass spectrum of Bsjβ^{C40A} core peptide modified by BsjM and BsjJ$_B$ (calculated m/z = 4068.25 Da, observed m/z = 4068.35 Da) in *E. coli* and digested by Glu-C.

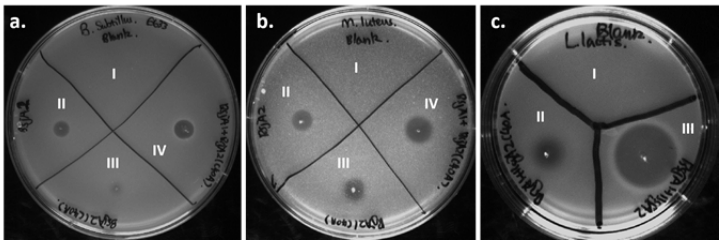

Figure S14. Antimicrobial activity assay of Bsjα and/or Bsjβ compared with Bsjβ^{C40A} against a) *Bacillus subtilis* ATCC6633, I: Blank, II: Bsjβ, III: Bsjβ^{C40A}, IV: Bsjα combined with Bsjβ^{C40A}. b) *Micrococcus luteus* 4698, I: Blank, II: Bsjβ, III: Bsjβ^{C40A}, IV: Bsjα combined with Bsjβ^{C40A}. c) *Lactococcus lactis* CNRZ 481, I: Blank, II: Bsjα combined with Bsjβ^{C40A}, III: Bsjα combined with Bsjβ. Spotted peptide amount: 1 μmol.

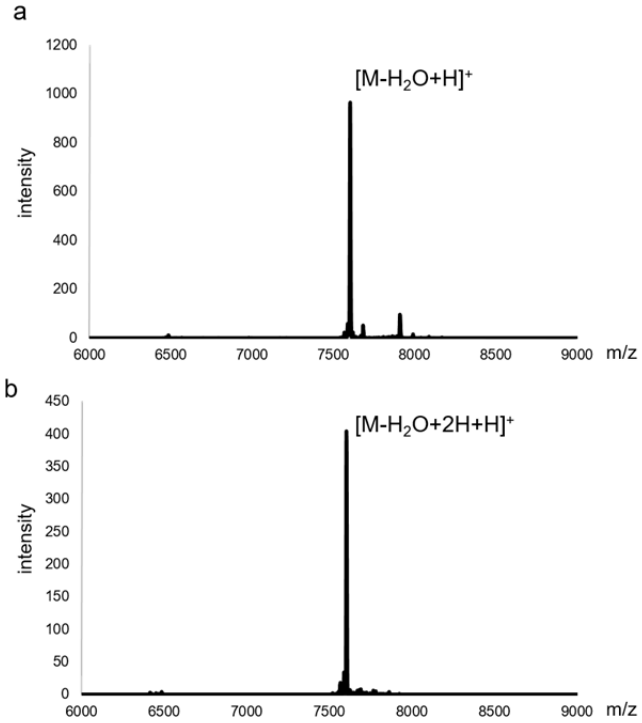

Figure S15. MALDI-TOF mass spectra of His_6-BsjA1-leader-dermorphin modified by (a) BsjM (calculated m/z = 7729.65 Da, observed m/z = 7730.02 Da) and (b) BsjM and $NpnJ_A$ (calculated m/z = 7731.64 Da, observed m/z = 7731.89 Da) in *E. coli*.

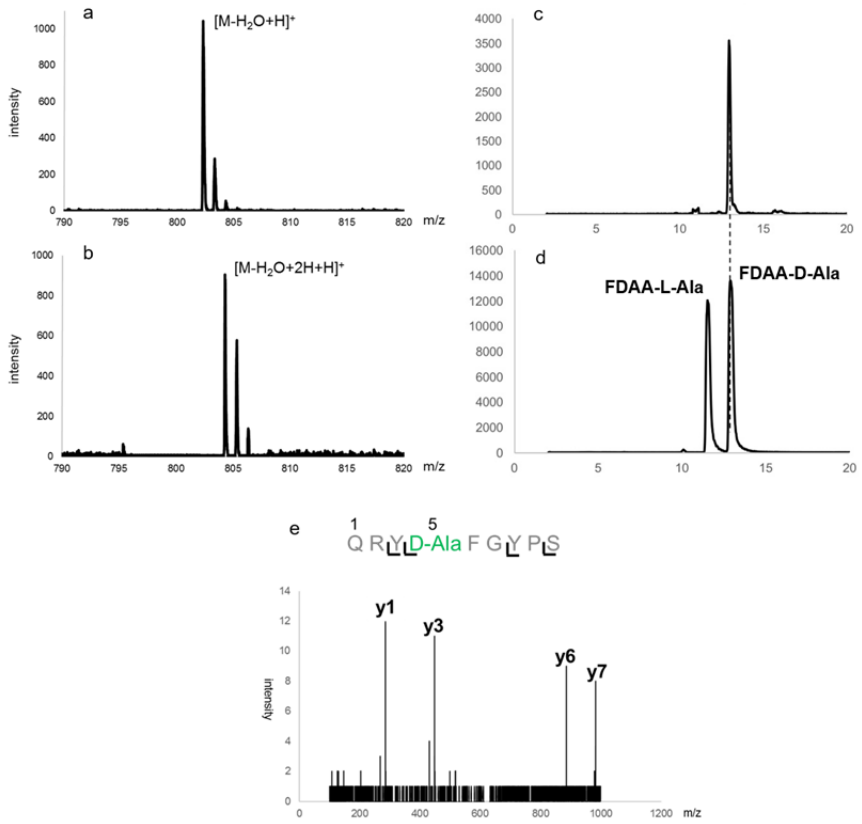

Figure S16. Dermorphin analogue (without C-terminal amidation) produced from His_6-BsjA1-leader-dermorphin modified by (a) BsjM (calculated m/z = 802.33 Da, observed m/z = 802.32 Da), (b) BsjM and $NpnJ_A$ (calculated m/z = 804.32 Da, observed m/z = 804.29 Da) in *E. coli* and digested by trypsin. (c) Extracted ion chromatography (EIC) of [M+H] = 342.1 ± 0.2 Da of hydrolysate of the dermorphin analogue derivatized with FDAA Marfey reagent. (d) EIC of [M+H] = 342.1 ± 0.2 Da corresponding to standard L- and D-alanine derivatized with FDAA Marfey reagent. (e) Fragmentation pattern of the dermorphin analogue produced from His_6-BsjA1-leader-dermorphin modified by BsjM and $NpnJ_A$ in *E. coli* and treated with trypsin.

References

(1) Gibson, D. G.; Young, L.; Chuang, R. Y.; Venter, J. C.; Hutchison, C. A., 3rd; Smith, H. O. *Nat. Methods* **2009**, *6*, 343.

(2) Yang, X.; van der Donk, W. A. *J. Am. Chem. Soc.* **2015**, *137*, 12426.

(3) Li, B.; Cooper, L. E.; van der Donk, W. A. *Methods Enzymol.* **2009**, *458*, 533.

(4) Liu, W.; Chan, A. S.; Liu, H.; Cochrane, S. A.; Vederas, J. C. *J. Am. Chem. Soc.* **2011**, *133*, 14216.

(5) Tang, W.; van der Donk, W. A. *Biochemistry* **2012**, *51*, 4271.

(6) Tang, W.; van der Donk, W. A. *Nat. Chem. Biol.* **2013**, *9*, 157.

(7) Ekkelenkamp, M. B.; Hanssen, M.; Danny Hsu, S. T.; de Jong, A.; Milatovic, D.; Verhoef, J.; van Nuland, N. A. *FEBS Lett.* **2005**, *579*, 1917.