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Discovery of a Novel Family of Gram-Negative-Active Cationic Cyclolipopeptides by Motif Search-Guided Chemical Synthesis

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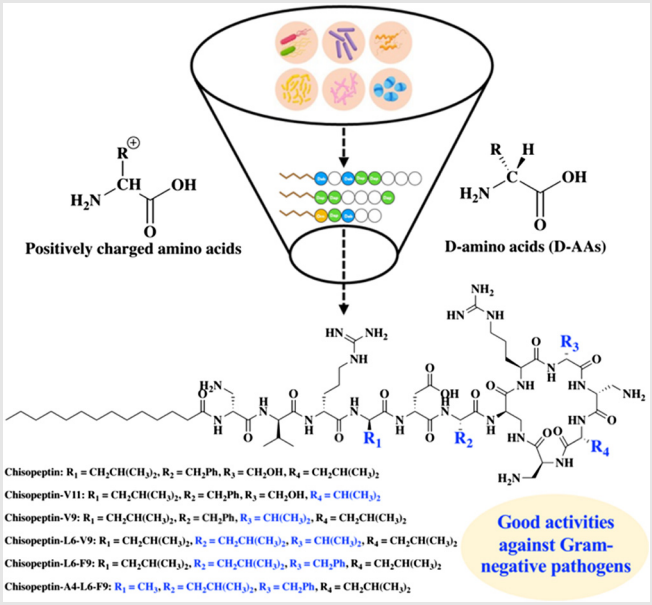
Emerging antimicrobial resistance is a global public health crisis, which necessitates the development of antibiotics with novel structural scaffolds and modes-of-action (MOAs). Cationic cyclolipopeptides have been increasingly recognized as types of promising therapeutics against Gram-negative pathogens. By two-round motif searching of 8982 sequenced genomes from 42 underexplored Gram-negative bacterial genera, here, we identified a novel family of cationic cyclolipopeptide biosynthetic gene clusters (BGCs). Then a synthetic-bioinformatic natural product (syn-BNP) approach that relies on structure prediction with high accuracy, followed by chemical synthesis, was used to rapidly access their predicted products with six positively charged amino acids and nine D-amino acids (D-AAs) out of 12 building blocks, thus generating chisopeptin and its five congeners. All of them showed good activities against a panel of Gram-negative pathogens with a minimum inhibition concentration (MIC) value of 2–8 μg/mL. Furthermore, chisopeptin was found to have a unique MOA to kill *Klebsiella pneumoniae* by possibly targeting the inner membrane protein YejM that regulates lipopolysaccharide biogenesis. The study of chisopeptin-type structures inspired by underexplored BGC families provides a productive approach to developing novel cyclolipopeptide-based therapeutics.



Keywords: antimicrobial resistance, underexplored sources, natural products, cationic cyclolipopeptides, chemical synthesis, YejM

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Introduction

The emergence of multidrug-resistant Gram-negative pathogens has become a major threat to global public health and necessitates the development of novel antibiotics with diverse modes of action (MOAs).[1–3] The chemical and mechanistic diversity of bacterial nonribosomal peptides (NRPs) encoded by giant NRP synthetases (NRPSs) have been known as promising natural products for novel antibiotic discovery.[4–7] Multiple chemical modifications such as cyclization, introduction of positively charged amino acids (PCAAs) or D-amino acids (D-AAs), and *N*-terminal fatty acid acylation, are usually used to optimize the physicochemical and proteolytic stability profiles of peptides, further improving their therapeutic potentials.[8] Particularly, cationic lipopeptides (CLPs) with at least two PCAAs, showed unique chemical properties for combating Gram-negative pathogens and have been developed for a series of clinically used antibiotics (i.e., colistin and polymyxin B).[9–11]

In the last 10 years, underexplored Gram-negative bacteria have been increasingly recognized as rich sources of NRPs for the discovery of novel CLP-type antibiotics.[12–15] In our previous study, 8982 Gram-negative bacterial genomes from 42 underexplored genera were screened for novel CLP biosynthetic gene clusters (BGCs).[16] A linear CLP chospeptin was rapidly identified by structure prediction, followed by solid-phase peptide synthesis (SPPS), which showed good activities against different colistin-resistant Gram-negative pathogens by directly targeting lipopolysaccharide (LPS).[16] However, although eight novel CLP BGCs were selected, we just synthesized their inspired linear products, possibly explaining why only chospeptin showed good antibacterial activity (Figure 1 and Supporting Information Table S1).[16] We proposed that the other seven CLP BGCs might encode cyclolipopeptides considering some hydrophilic AAs (i.e., Dap, Dab, Ser, and Thr) as nucleophiles could realize peptide cyclization through the *C*-terminal carboxyl. Actually, cyclization has emerged as the most widely used strategy to optimize the bioactivity of natural or synthetic peptides.[17] Considering more than 40 potential cyclolipopeptides inspired by the other seven CLP BGCs needed to be synthesized, another AA modification (introduction of D-AAs) was used for the second-round motif search (Supporting Information Table S1). Natural peptides usually comprise L-amino acids (L-AAs), which are vulnerable to enzyme degradation. By contrast, the introduction of D-AAs could often optimize the proteolytic stability of peptides, thus improving their bioactivity.[8,18,19] While multiple D-AAs in a single peptide were used for the second-round search, we found an interesting cationic lipopeptide 2 (CLP2) BGC from *Chitinophaga solisilvae* CGMCC 1.12462, whose predicted peptide contained up to nine D-AAs out of the 12 building blocks (Figures 1 and 2a).

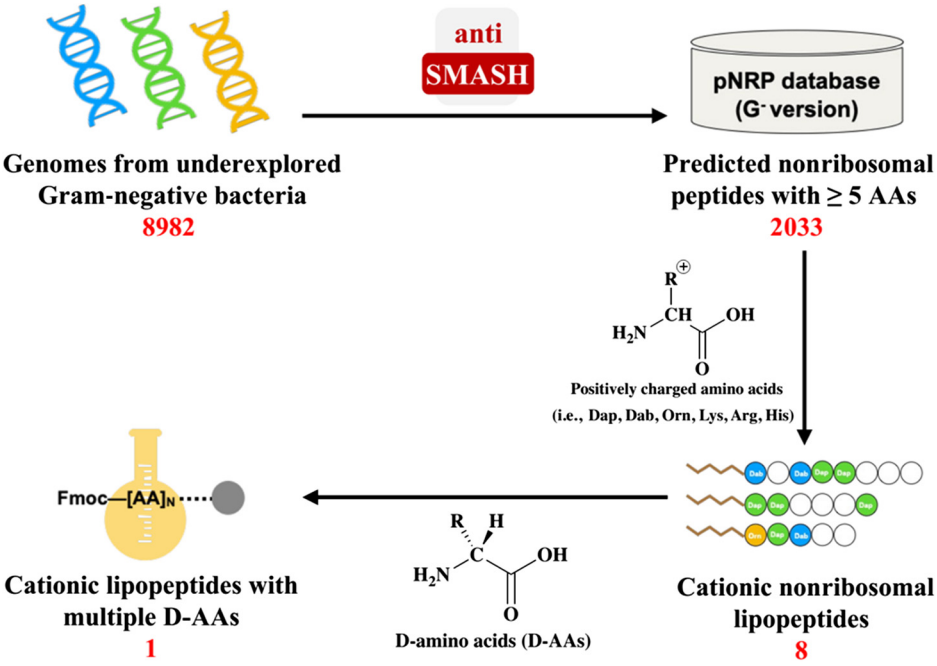

Figure 1 | *Two-round motif search for novel CLPs with multiple D-AAs from underexplored Gram-negative bacteria. One interesting CLP BGC whose encoded peptide contains nine D-AAs was selected for structure prediction followed by SPPS as either linear or cyclic structures.*

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Experimental Methods

Chemical reagents, consumables, and instruments

Standard *N*-Fmoc amino acids were purchased from GL Biochem (Shanghai, China). LPS from *Escherichia coli* O55:B5 was purchased from Beijing InnoChem (Beijing, China). GlcNAC, lipoteichoic acid, muramyl dipeptide, MurNAC, pentapeptide, and peptidoglycan were purchased from Bidepharm (Shanghai, China). Reagents for SPPS, including 2-chlorotrityl resin, dichloromethane (DCM), *N*,*N*-diisopropylethylamine (DIPEA), 4-dimethylaminopyridine, *N*,*N*-dimethylformamide (DMF), O-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*,*N*′-tetramethyluronium hexafluorophosphate (HATU), hexafluoroisopropanol (HFIP), (7-azabenzotriazol-1-yloxy) tripyrrolidinophosphonium hexafluorophosphate (PyAOP), and trifluoroacetic acid (TFA), were purchased from GL Biochem (Shanghai, China) or Shanghai Titan (Shanghai, China).

An Agilent 1260 Series high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection and equipped with an XBridge Prep C18 130 Å column (Santa Clara, California, USA) (10 × 150 mm, 5 μm) was used for peptide purification. For all liquid chromatography, solvent A = H_2O and solvent B = CH_3CN. An Agilent 1290 Series HPLC coupled to a 6546 Series QTOF mass spectrometer, equipped with a Phenomonex Kinetex PS C18 100 Å column (Torrance, California, USA) (2.1 × 50 mm, 2.6 μm) and controlled by Agilent MassHunter software (www.agilent.com.cn/zh-cn/product/software informatics/mass-spectrometry-software), was used to acquire the HPLC-high-resolution mass spectrometry (HRMS) data of synthetic peptides. A Bruker Avance DMX 700 MHz spectrometer (Billerica, Massachusetts, USA) equipped with cryogenic probes was used to acquire the ^{1}H, ^{13}C, distortionless enhancement by polarization transfer-135 degrees (DEPT-135), correlation spectroscopy (COSY), heteronuclear multiple-bond correlation (HMBC), and heteronuclear single quantum coherence (HSQC) NMR spectra of synthetic peptides. All spectra were recorded at 25 °C in dimethyl sulfoxide (DMSO)-d_6. Chemical shift values are reported in parts in million (ppm) and referenced to residual solvent signals: 2.50 ppm (^{1}H) and 39.52 ppm (^{13}C).

Solid phase peptide synthesis (SPPS)

Standard Fmoc chemistry-based SPPS methods were used to synthesize peptides on 2-chlorotrityl chloride resin. Peptide synthesis started from the last AA of each peptide, which was loaded on 2-cholorotrityl resin (0.3 g, 0.552 mmol/g) and was swollen in DCM for 20 min, drained as well as washed with DMF (3 mL, 3×). By using Fmoc-protected amino acids (2 equiv, relative to resin loading) mixed with HATU (2 equiv) and DIPEA (2 equiv) in DMF (5 mL), individual amino acids were coupled step-by-step. Finally, coupling reactions were performed for 1 h with occasional swirling and washed with DMF (3 mL, 3×).

For the synthesis of synCLP2-L, Fmoc-deprotection was done using 20% piperidine in DMF (3 mL) for 7 min and repeated twice. The resin was washed with DMF (3 mL, 5×) and then coupled with a subsequent *N*-Fmoc amino acid. Then tetradecanoic acid was coupled into the end of the linear peptide.

For the synthesis of synCLP2-C1, synCLP2-C7 (chisopeptin), and chisopeptin congeners, the alloc group of Dap was firstly removed by two rounds of palladium [$Pd(PPh_3)_4$]-catalyzed deprotection in DCM. In each round of deprotection, 0.25 equiv palladium and 12 equiv phenylsilane were added to resins suspended in DCM (3 mL) and reacted for 1 h. Then the 2-chlorotrityl resin was removed by treatment with 20% HFIP in DCM for 2 h to cleave the resin bead. Soluble peptides were further cyclized using PYAOP (8 equiv) and DIPEA (30 equiv) in DMF and extracted using DCM and H_2O with 1% formic acid. Air-dried cyclic peptides were dissolved in MeOH and completely dried under vacuum overnight.

For final cleavage, each Air-dried linear or cyclic peptides were dissolved in 3 mL of cleavage cocktail (95% v/v TFA, 2.5% v/v triisopropylsilane, and 2.5% v/v H_2O) for 1.5 h. Then to precipitate the peptide, a cold mixture of diethyl ether:hexane (1:1) was added and kept for 10 min at −20 °C. Peptide pellets were collected by centrifugation at 2500 g for 5 min at 4 °C, redissolved in 5 mL of methanol, and dried under vacuum overnight.

Peptide purification by HPLC

Crude synCLP2-L, synCLP2-C1, synCLP2-C7 (chisopeptin), and chisopeptin congeners were purified on an Xbridge Prep C18 HPLC column using a dual solvent system (A/B: H_2O/acetonitrile) (Supporting Information Figure S1). All synthetic peptides were eluted using a linear gradient from 20% to 45% gradient of B. The identity of each linear or cyclic peptide was confirmed by HRMS (Supporting Information Table S2 and Figures S2–S4). Furthermore, ^{1}H, ^{13}C, DEPT135, COSY, HSQC, and HMBC NMR spectra were recorded for chisopeptin and its five congeners (Supporting Information Figures S5–S40). Structural assignments were performed with additional information from NMR experiments (Supporting Information Tables S3–S8).

Antimicrobial assays against Gram-positive bacteria, Gram-negative bacteria and fungi

All antimicrobial assays were performed in 96-well microtiter plates using a broth microdilution method. For fungi strains, overnight cultures were diluted 2000-fold in yeast peptone dextrose (YPD) broth. Overnight cultures were diluted 1000- and 10000-fold in LB broth for

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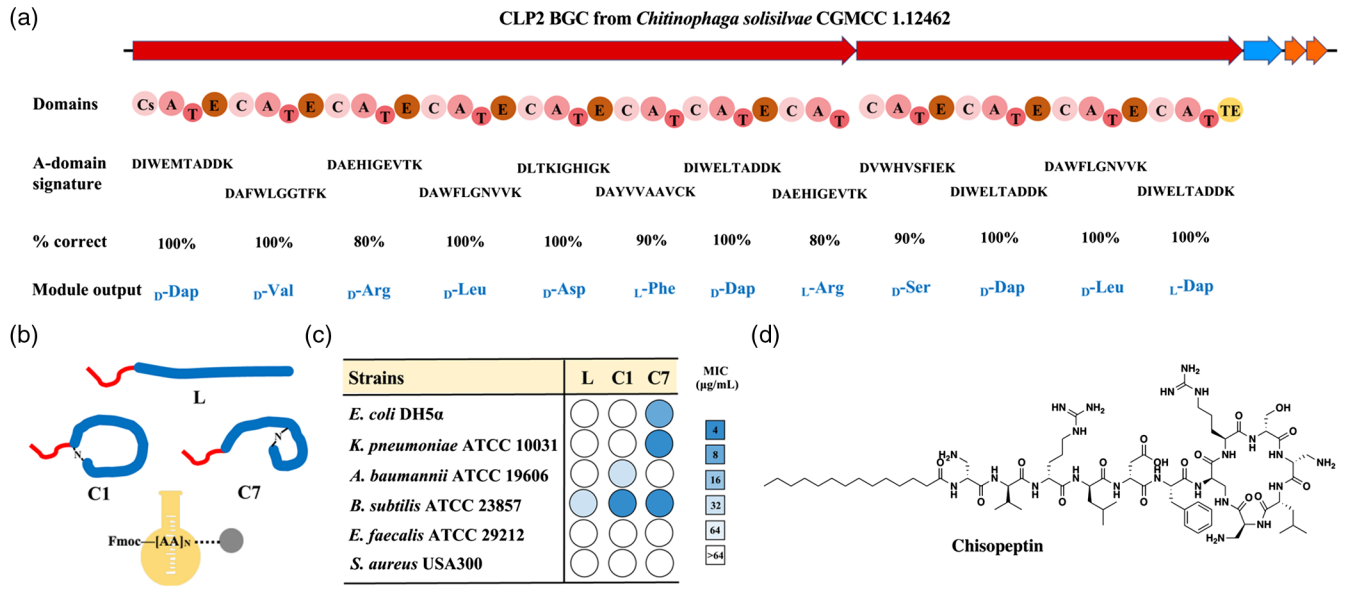

Figure 2 | *Discovery of chisopeptin. (a) The CLP2 BGC contains two NRPS genes. The thioesterase domain releases the mature peptide from the final T domain as either a linear or cyclic mode. (b) Diagrams of the three different peptide topologies that were made from the linear peptide predicted by the CLP2 BGC. (c) MIC data against three Gram-negative or -positive bacteria for the three predicted synthetic peptides depicted in (b) (n = 2). (d) Structure of chisopeptin, which corresponds to C7 in (b).*

Enterococcus faecium and *Staphylococcus aureus*, respectively. For other bacteria, overnight cultures were diluted 5000-fold in LB broth. 100 μL of each diluted culture was mixed with 100 μL of LB broth containing a synthetic peptide at 2-fold serial dilutions across a 96-well microtiter plate row. The final concentration of each synthetic peptide ranged from 64 to 0.125 μg/mL. Plates were incubated at 37 °C (bacteria) or 30 °C (fungi) for 16 h. The lowest concentration that inhibited visible bacterial or fungal growth was recorded as the minimum inhibition concentration (MIC). All MIC assays were performed in duplicate (n = 2).

Antibiotic-resistant mutant selection

A single *Klebsiella pneumoniae* American Type Culture Collection (ATCC) 10031 colony was grown overnight at 37 °C in a liquid Luria-Bertani (LB) medium. A portion of the overnight culture was diluted to ~10^8 cells per microliter, which were plated into solid LB with chisopeptin at 4× its MIC at 5 μL per well. The MICs of three individual colonies were then determined using the broth microdilution method described above. The genome was extracted from cultures of colonies that showed an elevated MIC relative to the wild-type, and the resulting genome was sequenced by the high-throughput next-generation sequencing (MGI's DNBSEQ-T7) platform developed by MGI Tech, China. Single-nucleotide polymorphisms for chisopeptin were identified using SNIPPY mapping software (https://snippy.en.softonic.com/) by mapping DNBSEQ-seq reads to the reference genome of *K. pneumoniae* ATCC 10031.

Cytotoxicity assessment

The human cervical cancer (HeLa) cell line was grown at 37 °C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM). To allow HeLa cells to adhere to 96-well flat bottom microtiter plates, they were incubated in DMEM at 37 °C for 24 h. Then the DMEM medium was replaced with 100 μL of fresh DMEM medium containing each synthetic peptide at 10 serially diluted concentrations ranging from 0.125 to 64 μg/mL. After 48 h at 37 °C, 10 μL of a cell counting kit-8 (CCK-8) solution was added to each well. After 2 h at 37 °C, the absorbance at 450 nm for each well was detected for measuring the cytotoxicity of each synthetic peptide using a microplate reader. Taxol and DMSO were used as the positive and negative controls, respectively, for the measurement of the half-maximal inhibitory concentration (IC_{50}) of each synthetic peptide against the HeLa cells.

Results and Discussion

Prediction and synthesis of the CLP2 BGC products

More than 90% of sequenced bacterial BGCs are not or lowly expressed under standard laboratory conditions, which could serve as rich sources for novel antibiotic discovery.[20–23] With the increasing accuracy of NP

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structure prediction algorithms and the advancement of modern synthetic organic chemistry, a bioactive compound could be rapidly generated by structure prediction followed by chemical synthesis.[24,25] The synthetic-bioinformatic natural product (syn-BNP) approach has been widely used for de novo and targeted discovery of diverse NRPs with novel structures or MOAs.[26–29] In this study, we used this syn-BNP approach to access linear or cyclic lipopeptide structures inspired by the CLP2 BGC for the test of antimicrobial activity. As shown in Figure 2a, all genes in the CLP2 BGC were predicted to form a single operon considering their expression were found to follow the same direction. The absence of any tailoring enzyme-encoding genes within 10 kb of the CLP2 NRPS genes suggested that the product of the CLP2 BGC could not be postmodified (Figure 2a and Supporting Information Figure S39). Meanwhile, A-domain substrate specificity analysis allowed us to predict the building block incorporated by each A-domain found in the CLP2 BGC with high accuracy (Figure 2a and Supporting Information Table S9). Therefore, the linear peptide encoded by the CLP2 BGC was predicted to be the direct precursor to the final peptide. Meanwhile, considering that the two amino acids, D-Dap-1 and D-Dap-7, in predicted peptides, could serve as nucleophiles for the cyclization of the predicted peptide through the *C*-terminal carboxyl, predicted CLP2 products could appear as either linear or cyclic structures. Meanwhile, the condensation starter (Cs) domain that usually couples a fatty acid onto the first amino acid of the peptide was encoded by the CLP2 BGC (Supporting Information Figure S41). Among the known bacterial CLPs, tetradecanoic acid is one of the most common lipids, so all predicted CLP2 peptides were *N*-terminal acylated with tetradecanoic acid.[30] Together, one linear predicted peptide (synCLP2-L) and two cyclic predicted peptides (synCLP2-C1 and synCLP2-C7) could have arisen from the CLP2 BGC and were rapidly generated based on standard Fmoc chemistry-based SPPS (Figure 2b and Supporting Information Figure S40).

Antimicrobial spectrum

All three synthetic structures were initially assayed for activity against three Gram-negative and three Gram-positive bacteria (Figure 2c). Although both synCLP2-C1 and synCLP2-C7 showed good activities against *Bacillus subtilis* ATCC 23857, only synCLP2-C7, cyclized through the hydroxyl of D-Dap-7 showed good activity against two Gram-negative bacteria, including *E. coli* DH5α and *K. pneumoniae* ATCC 10031, with MIC values from 4 to 8 μg/mL (Figure 2c). In broader bioactivity screening, synCLP2-C7 was also active against the two other Gram-negative pathogens, including

Table 1 | *Activities of synCLP2-L, synCLP2-C1, Chisopeptin and its Eight Congeners Against Microorganisms and Human HeLa Cell (MIC: μg/mL)*

Type	Pathogens	synCLP2 -L	synCLP2 -C1	Chisopeptin	Chisopeptin -NF	Chisopeptin -HA	Chisopeptin -DA	Chisopeptin -V11	Chisopeptin -V9	Chisopeptin -L6-V9	Chisopeptin -L6-F9	Chisopeptin -A4-L6-F9	Colistin	Amphotericin B
Gram-negative bacteria	*E. coli* K12	>64	>64	4	>64	>64	4	4	8	4	2	4	2	ND
	E. coli DH5α	>64	>64	8	>64	>64	8	4	8	8	4	8	<0.125	ND
	E. coli ATCC 25922	>64	>64	4	>64	>64	8	8	8	4	2	8	0.5	ND
	K. pneumoniae ATCC 10031	>64	>64	4	>64	>64	4	4	8	8	2	4	0.5	ND
	K. pneumoniae RJA166	>64	>64	8	>64	>64	8	8	8	8	4	8	<0.125	ND
	K. pneumoniae RJF293	>64	>64	8	>64	>64	8	8	8	8	4	8	<0.125	ND
	K. pneumoniae RJF999	>64	>64	8	>64	>64	8	8	4	8	4	4	<0.125	ND
	Erwinia amylovora LXX	>64	>64	4	>64	64	4	4	4	4	2	2	0.25	ND
	Vibrio cholerae ATCC 39315	>64	32	4	>64	>64	8	8	4	8	2	4	>64	ND
	Acinetobacter baumannii ATCC 19606	>64	32	>64	>64	>64	64	>64	>64	>64	>64	32	1	ND
	Pseudomonas aeruginosa ATCC 15692	>64	>64	32	>64	>64	16	>64	>64	>64	32	>64	0.5	ND
Gram-positive bacteria	*Bacillus subtilis* ATCC 23857	32	4	4	>64	16	4	2	2	4	2	4	>64	ND
	E. faecalis ATCC 29212	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	ND
	S. aureus USA300	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	ND
	Mycobacterium smegmatis mc2 155	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	ND
Fungi	*Candida albicans* ATCC 10231	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	0.5
	Saccharomyces cerevisiae BY4741	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64	0.25
Human cell line	HeLa	ND	ND	>64	ND	ND	ND	>64	>64	>64	>64	>64	>64	ND

Note: Colistin and amphotericin B were used as the positive controls. ND: not detected.

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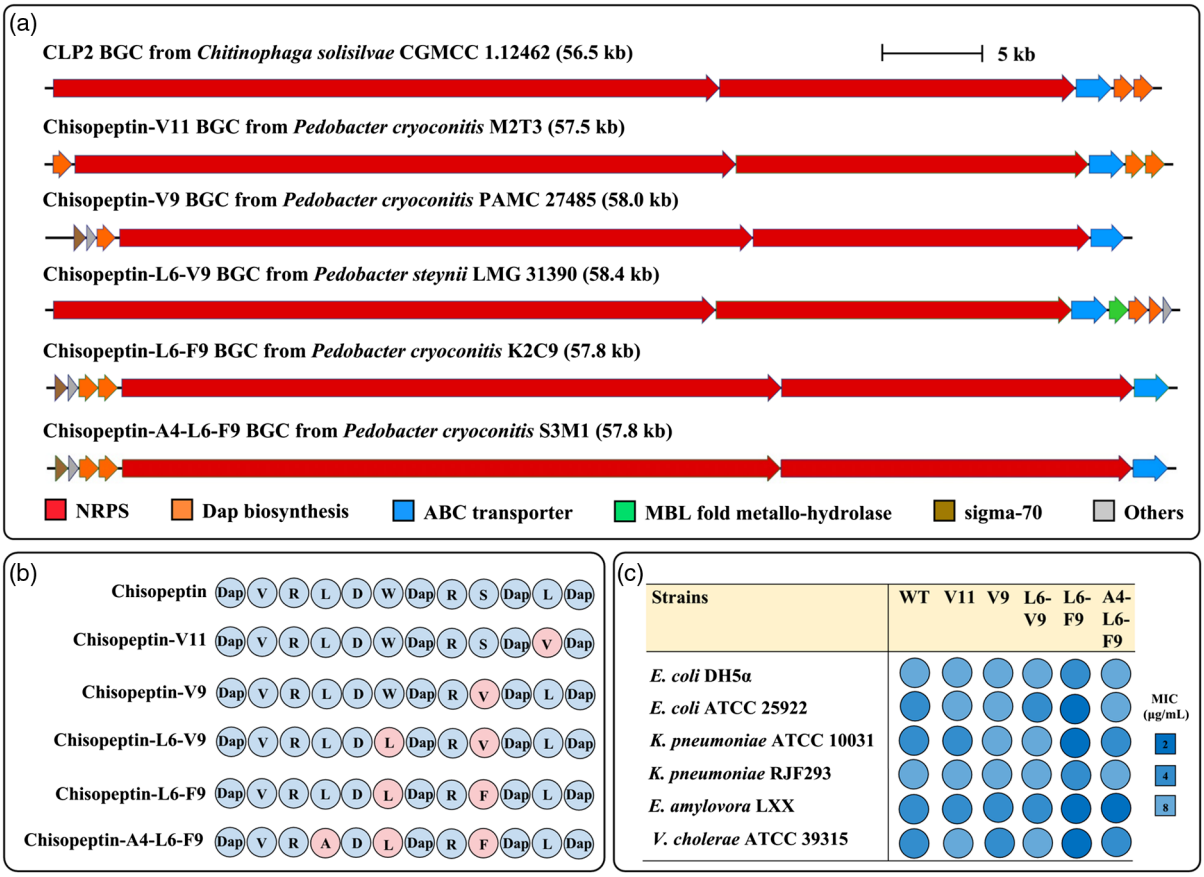

Figure 3 | *Discovery of five chisopeptin congeners. (a) The BGCs of CLP2 and five chisopeptin congeners. (b) Linear peptide sequences of chisopeptin and its five congeners. (c) MIC data against Gram-negative or Gram-positive pathogens for chisopeptin and its five congeners (n = 2).*

Erwinia amylovora LXX and *Vibrio cholerae* ATCC 39315 (Table 1). Even at the highest concentration tested (64 μg/mL), synCLP2-C7 did not show activity against two fungal strains or cytotoxicity to the human cell line HeLa (Table 1). These results indicated that synCLP2-C7, named chisopeptin, had a relatively narrow antimicrobial spectrum. Naturally occurring bacterial lipopeptides appeared as either linear or cyclic structures.[6,7,31] It is very rare that one nonribosomal lipopeptide BGC could encode both cyclic and linear peptides.[6,7,31,32] Considering only chisopeptin showed good anti-Gram-negative activity and either synCLP2-L or synCLP2-C1 was not active against tested pathogens, we speculated that chisopeptin was likely the closest to a natural product encoded by CLP2 BGC (Figure 2d). The structure of chisopeptin was further confirmed using HRMS and 1D/2D NMR data (Supporting Information Table S2, Figures S1–S2 and S5–S10). Then we synthesized three chisopeptin congeners without or by changing the length of the fatty acid side chain: chisopeptin-NF without the fatty acid side chain, chisopeptin-HA with the hexanoic acid side chain, and chisopeptin-DA with the decanoic acid side chain (Supporting Information Figures S1 and S3). As shown in Table 1, neither chisopeptin-NF nor chisopeptin-HA was active against tested Gram-negative bacteria and chisopeptin-DA showed comparable anti-Gram-negative activity to chisopeptin (with tetradecanoic acid as the fatty acid side chain). These results indicated that a long fatty acid side chain was necessary for the activity of chisopeptin against Gram-negative bacteria.

Prediction and synthesis of the five chisopeptin congener BGC products

In our first-round search pipeline to discover novel CLP BGCs using at least two PCAAs in a peptide as the motif, only one representative CLP BGC among highly similar BGCs was chosen for the next-step study. Therefore, we researched the predicted NRP database from 42 under-explored Gram-negative bacterial genera and found the five potential chisopeptin congener BGCs. Intriguingly, all five BGCs are from the genus *Pedobacter*, which has not traditionally been part of microbial NP discovery pipelines.[13] Similar to the CLP2 BGC, the expression of all genes in each BGC was also found to follow the same direction, which possibly formed a single operon

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(Figure 3a and Supporting Information Figures S42–S46). Except for an metallo-beta-lactamase (MBL) fold metallo-hydrolase encoded by the chisopeptin-L6-V9 BGC, there are no tailoring enzymes encoded by the five BGCs (Figure 3a and Supporting Information Figures S41–S45). Through A-domain substrate specificity analysis, we found that there were one to three different AAs in linear peptides predicted by the five BGCs from chisopeptin (Figure 3b and Supporting Information Tables S10–S14 and Figure S1). Then all five chisopeptin congeners, including chisopeptin-V11, chisopeptin-V9, chisopeptin-L6-V9, chisopeptin-L6-F9, and chisopeptin-A4-L6-F9, were designed to be cyclized through the hydroxyl of D-Dap-7 and rapidly generated by SPPS. Their structures were further confirmed using HRMS and 1D/2D NMR data (Supporting Information Tables S2 and S4–S8, Figures S4 and S11–S40). Similar to chisopeptin, all five congeners showed similar antimicrobial spectra to chisopeptin and were active against four Gram-negative pathogens with MIC from 2 to 8 μg/mL. In a subset of these congeners, the antibacterial activities of chisopeptin-L6-F9 in particular, were two-fold higher than those of chisopeptin (Figure 3c and Table 1). Collectively, a novel family of cationic cyclolipopeptides, including chisopeptin and its five congeners, were identified with good activities against a panel of Gram-negative pathogens.

Mode of action studies

Then, we tested whether *K. pneumoniae* ATCC 10031 would be lysed using 4× the MIC of chisopeptin, and found that chisopeptin could not result in rapid bacteriolysis (Figure 4a). Considering that chisopeptin as a cationic cyclolipopeptide might target LPS in the outer membrane of Gram-negative pathogens, we assayed for the ability of LPS to suppress chisopeptin to explore MOA of chisopeptin. However, when LPS was added to the LB medium, the MIC of chisopeptin did not increase in a dose-dependent manner for *K. pneumoniae*, which indicated that chisopeptin could not target LPS and its anti-Gram-negative activity was not dependent on LPS (Figure 4b). Next, we tried to raise mutants to chisopeptin and selected *K. pneumoniae* mutants that could grow on 4 × MIC of chisopeptin. At 4× chisopeptin's MIC

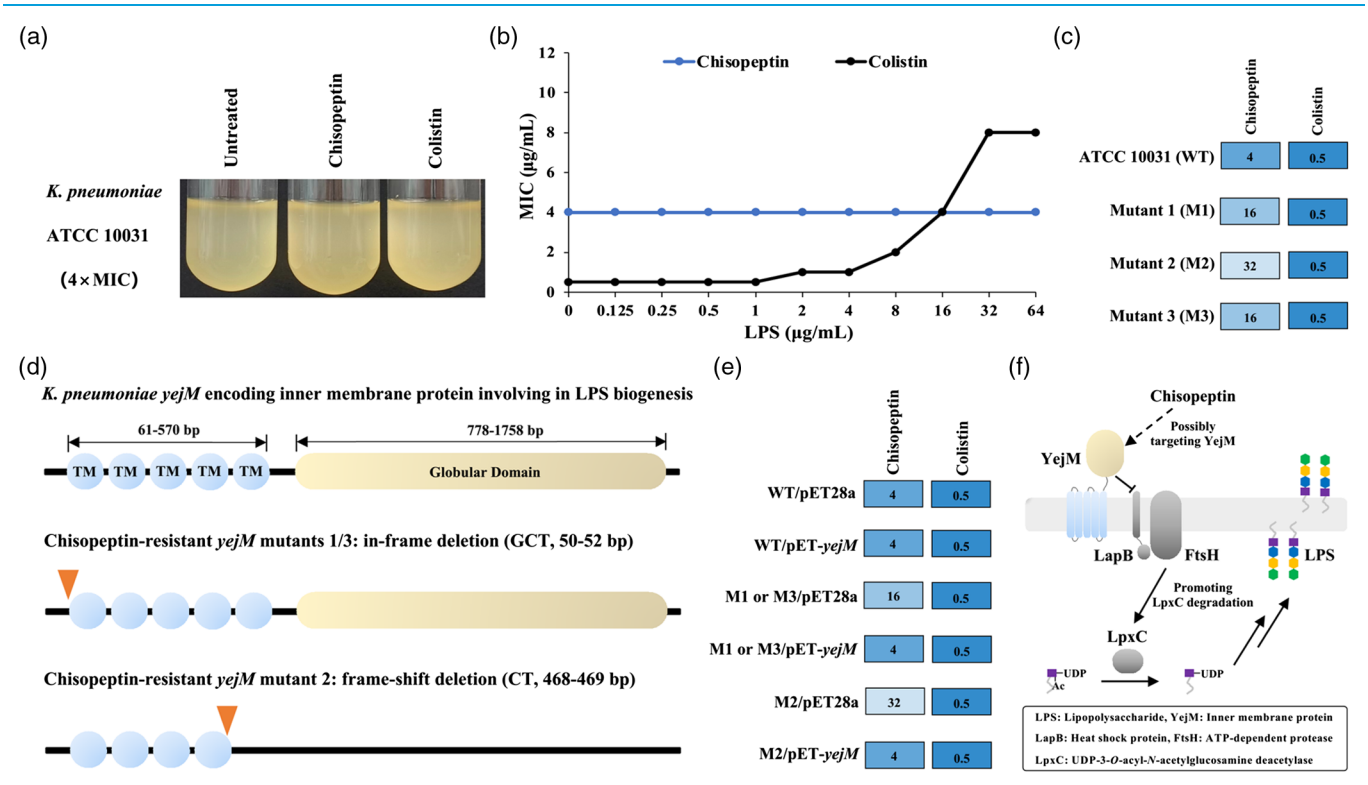

Figure 4 | *MOA of chisopeptin against the strain* K. pneumoniae *ATCC 10031. (a) Bactericidal activity of chisopeptin against* K. pneumoniae*. Cultures were incubated with each antibiotic at 4× its MIC. (b) The* K. pneumoniae *antibacterial activity of chisopeptin was determined in the presence of different concentrations of LPS (n = 2). (c) The MICs (μg/mL) of chisopeptin against* K. pneumoniae *mutants (n = 2). Colistin was used as the positive control. (d) The mutated results of the gene* yejM *in the three chisopeptin-resistant* K. pneumoniae *strains. (e) The MICs (μg/mL) of chisopeptin against* K. pneumoniae *mutants with pET28a or pET-*yejM *(n = 2). Colistin was used as the positive control. (f) A proposed model-of-action of chisopeptin against Gram-negative pathogens (i.e.,* K. pneumoniae*).*

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resistant mutants appeared at a frequency of 1×10^{-6}. We sequenced the genomes of three representative resistant strains (Figure 4c). In all cases, the resistant strains contained a mutation (in-frame or frame-shift deletion) in the gene *yejM* and no other mutations were detected in the genome (Figure 4d). When the gene *yejM* under the expression of the constitutive promoter *rpsLp* was complemented to the three *K. pneumoniae* mutants, all of them became sensitive to chisopeptin again (Figure 4e). YejM, as an inner membrane protein, is a key regulator of outer membrane integrity and LPS biogenesis by directly detecting LPS on the periplasmic leaflet of the inner membrane in Gram-negative bacteria.[33,34] In detail, YejM as an anti-adaptor protein could compete with FtsH for LapB to inhibit LpxC degradation (Figure 4f).[35] Deletion of the gene *yejM* could promote LpxC degradation, thus resulting in reduced LPS levels.[34,36] Previous studies indicated that the mutation of *yejM* also led to higher resistance to chloramphenicol in *E. coli*.[37] Considering that chisopeptin did not directly target LPS, we proposed that chisopeptin might target the inner membrane protein YejM, thereby interrupting normal LPS biogenesis and exhibiting anti-Gram-negative activity (Figure 4f). To explore whether chisopeptin could directly bind to YejM in vitro, we tried to express and purify YejM but failed, which might be because YejM is a membrane protein (data not shown). The more detailed MOA for chisopeptin could be further explored in the future.

Conclusion

Infections caused by multidrug-resistant Gram-negative pathogens are threatening to overwhelm healthcare systems worldwide.[2,3] In recent years, underexplored Gram-negative bacteria exhibit large biosynthetic potential for the discovery of novel antimicrobial peptides.[12–15] In this study, we used the two-round motif search pipelines, including at least two PCAAs and multiple D-AAs, to screen >8900 underexplored Gram-negative bacterial genomes and identified a novel family of cationic cyclolipopeptides (chisopeptin and its five congeners) with good activities against a panel of Gram-negative pathogens. All six synBNPs had very unique structural scaffolds, which contained six PCAAs and nine D-AAs out of 12 building blocks. Noteworthy, the kinds of cationic cyclolipopeptides identified had the potential MOA to kill *K. pneumoniae* by possibly targeting the inner membrane protein YejM that regulated LPS biogenesis. Considering that *K. pneumoniae* has a relatively high frequency of generating resistant mutants to chisopeptin, combinations of the chisopeptin-type antibiotics and of known Gram-negative-active drugs with different MOAs (i.e., penicillin-binding protein-targeting meropenem, ribosome-targeting amikacin, and DNA-targeting moxifloxacin), provided an alternative strategy for further preclinical development of this novel family of cationic cyclolipopeptides.[38] As seen with the characterization of bacterial CLPs, the study of chisopeptin-type structures inspired by underexplored BGC families provides an alternative approach for developing novel CLP-based therapeutics with diverse MOAs.[39,40]

Supporting Information

Supporting Information is available and includes chisopeptin and its congeners domain prediction tables, chisopeptin and its congeners BGC analysis, chisopeptin and its congeners HRMS and NMR spectra.

Conflict of Interest

There is no conflict of interest to report.

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