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The antibacterial threaded-lasso peptide capistruin inhibits bacterial RNA polymerase

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SUMMARY

Capistruin, a ribosomally synthesized post-translationally modified peptide produced by *Burkholderia thailandensis* E264, efficiently inhibits growth of *Burkholderia* and closely related *Pseudomonas* strains. The functional target of capistruin is unknown. Capistruin is a threaded-lasso peptide (lariat peptide), comprising an N-terminal 9-amino-acid ring followed by a 10-amino-acid C-terminal tail that is threaded through the ring. The structure of capistruin is similar to that of microcin J25 (MccJ25), a threaded-lasso antibacterial peptide that is produced by some strains of *Escherichia coli* and targets DNA-dependent RNA polymerase (RNAP). Here, we show that capistruin, like MccJ25, inhibits wild-type *E. coli* RNAP but not mutant, MccJ25-resistant, *E. coli* RNAP. We show further that an *E. coli* strain resistant to MccJ25 due to a mutation in an RNAP subunit gene exhibits resistance to capistruin. The results indicate that the structural similarity of capistruin and MccJ25 reflects functional similarity and suggest that the functional target of capistruin, and possibly other threaded-lasso peptides, is bacterial RNAP.

Keywords

lariat peptides; capistruin; microcin J25 (MccJ25); RNA polymerase; RNA polymerase inhibitor

Introduction

Bacterial threaded-lasso peptides, also referred to as lariat peptides, are a unique class of ribosomally synthesized, biologically active natural products (Fig. 1).^{1–16} They are composed of an 8- or 9-residue N-terminal cycle, involving a backbone-sidechain lactam

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linkage between residue 1 and residue 8 or 9, and a C-terminal linear tail that is threaded through the cycle and held in place by steric constraints imposed by bulky amino acid sidechains above and below the cycle (Fig. 1b). The resulting rigid and compact threaded-lasso fold results in remarkable stability. For example, the structure and biological function of the *Escherichia coli* threaded-lasso peptide microcin J25 (MccJ25) are unperturbed by denaturants and by autoclaving.

To date, nine bacterial peptides have been experimentally demonstrated to assume a threaded-lasso fold: MccJ25 from *E. coli*;^{1–3,13–16} capistruin from *Burkholderia thailandensis*;⁴ lariatin A and lariatin B from *Rhodococcus* sp.;⁵ and RES-701-1, BI-32169, siamycin I (also known as MS-271, NP-06, and FR901724), siamycin II, and siamycin III (also known as RP71955 and aborycin) from *Streptomyces* spp.^{6–12} Depending on structural features including the presence or the absence of sets of cysteine residues, which can be involved in the formation of up to two intramolecular disulfide bonds, threaded-lasso peptides are divided into two classes: “class I” threaded-lasso peptides have disulfide bonds (siamycins I–III), and “class II” threaded-lasso peptides lacking disulfide bonds (MccJ25, capistruin, lariatin A, and lariatin B). BI-32169 unites structural features of class I and II and was recently proposed to form a third class of threaded lasso peptides.¹¹

The biosynthetic gene clusters for MccJ25 and capistruin have been identified and sequenced.^{4,17–20} Both biosynthetic gene clusters (*mcjABCD* for MccJ25 and *capABCD* for capistruin) encode precursor propeptides of mature threaded-lasso peptides (McjA and CapA), enzymes involved in processing precursor propeptides into mature threaded-lasso peptides (McjBC and CapBC), and export/immunity proteins (McjD and CapD). For both biosynthetic gene clusters, the export/immunity proteins belong to the ATP-binding cassette (ABC) transporter protein class and presumably secrete mature threaded-lasso peptide from the producing cell. In the case of McjD, it was shown that overproduction of this protein is sufficient to render cells resistant to external MccJ25.¹⁷

McjC and CapC exhibits strong sequence similarity to amidotransferases of the Asn-synthase/Gln-hydrolase class, which catalyze transfer of ammonia or an amine from an amide donor to an adenylyate-activated carboxyl acceptor.^{1,2,14,20} McjB/CapB exhibit weak sequence similarity to enzymes involved in activation of carboxyl groups and also to peptidases.^{14,20} It is likely that McjC/CapC mediates the condensation reaction between the backbone amino group and the side-chain carboxyl group that results in cyclization of the precursor, and that McjB/CapB mediates activation of the side-chain carboxyl group and/or cleavage of the precursor.

MccJ25 is the only threaded-lasso peptide for which the functional target is known. MccJ25 inhibits bacterial growth by inhibiting bacterial RNA polymerase (RNAP).^{21–24} MccJ25 inhibits RNAP by binding to, and obstructing, the RNAP secondary channel, also referred to as the RNAP NTP entrance channel, through which NTPs enter the RNAP catalytic center.^{23,24} Substitution of residues within the RNAP secondary channel result in resistance to MccJ25 *in vitro*.^{22,23} Mutations of RNAP subunit genes that alter the RNAP secondary channel confer resistance to MccJ25 *in vivo*, establishing that RNAP is the functional target of MccJ25.^{22,23} MccJ25 inhibits RNAP from *E. coli* and other enteric bacteria; from *P. aeruginosa* and *X. oryzae*, but has no effect on RNAP from Gram-positive bacteria.²²

We hypothesized that the similarity between the structures of the threaded-lasso peptides MccJ25 and capistruin might reflect a common mechanism of antibacterial function of the two peptides. In this work, this notion is experimentally confirmed.

Results

Capistruin inhibits transcription elongation by RNAP in vitro

To assess the effect of capistruin of transcript elongation by *E. coli* RNAP, elongation complexes (ECs) stalled at position +30 (EC³⁰) were obtained on a linear DNA fragment containing the *galP1* promoter (Fig. 2a, lane 1). Transcription past position +30 was prevented by omitting UTP from the reactions. The nascent transcripts in stalled EC³⁰ were radioactively labelled through incorporation of [α -³²P]-CMP. The addition of NTPs to stalled complexes allowed transcript elongation to proceed to the end of the template (“run-off” in Fig. 2a, lane 2); a transcript ending at a terminator located between the promoter and template end was also observed (“terminated transcript” in Fig. 2a, lane 2). Addition of MccJ25 together with NTPs led to formation of shorter transcripts (Fig. 2a; compare lanes 6, 7, and 8 with each other and with control lane 2). This is an expected effect, since MccJ25 is known to inhibit transcript elongation.^{23,24} The addition of capistruin together with NTPs had the same effect on transcript elongation (Fig. 2a; compare lanes 3 and 6, 4 and 7, and 5 and 8). We conclude that capistruin inhibits transcript elongation by *E. coli* RNAP and that the potency of inhibition is comparable to that of MccJ25.

The abilities of MccJ25 and capistruin to inhibit RNAP from other bacterial species were assessed. Both MccJ25 and capistruin inhibited RNAP from *Pseudomonas aeruginosa* (Fig. 2b, compare lane 4 with lanes 5 and 6). Each threaded-lasso peptide inhibited *P. aeruginosa* RNAP as efficiently as the *E. coli* enzyme (compare lane 3 with lane 6 and lane 4 with 7). RNAP from *Fransicella tularensis* was resistant to capistruin and MccJ25 (Fig. 2b, compare lane 10 with lanes 11 and 12). RNAPs from *Bacillus cereus* or *Thermus thermophilus* also were resistant to capistruin and MccJ25 (data not shown).

A mutant RNAP derivative that is resistant to MccJ25 is cross-resistant to capistruin in vitro

E. coli RNAP derivatives carrying substitutions in the RNAP secondary channel are resistant to inhibition of transcription elongation by MccJ25.^{21–23} To assess effects of a substitution within the RNAP secondary channel on inhibition of transcription elongation by capistruin, wild-type RNAP and a MccJ25-resistant mutant RNAP derivative containing a substitution within the RNAP secondary channel--[931Ile] β '-RNAP^{21–23}--were assayed using artificial ECs assembled on nucleic-acid scaffolds. Such ECs have been used extensively to study basic RNAP functions (see, for example, ref. 25). The addition of NTPs to ECs containing a radioactively labeled 8-nt RNA primer (EC⁸) led to extension of the RNA, (Fig. 3, lanes 2 and 5). In experiments with wild-type RNAP, the addition of MccJ25 or capistruin prior to addition of NTPs inhibited the reaction, resulting in the disappearance, or near-disappearance, of the band corresponding to the full-length run-off product and the appearance of bands corresponding to ECs stalled at various positions along the template (Fig. 3, lanes 3 and 4). The extent of inhibition was less than that observed in experiments with promoter templates (Fig. 2), an expected result since transcribed portion of scaffold templates is very short. Importantly, in experiments with [931Ile] β '-RNAP, the addition of MccJ25 or capistruin did not substantially inhibit the primer elongation reaction (Fig. 3, compare lane 5 with lanes 6 and 7, correspondingly). We conclude that [931Ile] β '-RNAP is resistant to both inhibition by MccJ25 and inhibition by capistruin. We conclude further that the RNAP secondary channel is required for both inhibition by MccJ25 and inhibition by capistruin. We infer that MccJ25 and capistruin function through overlapping--although not necessarily identical--binding sites within the RNAP secondary channel.

A mutation of an RNAP-subunit gene that confers resistance to MccJ25 *in vivo* confers cross-resistance to capistrui in vivo

To assess whether a mutation of an RNAP-subunit gene that confers resistance to MccJ25 *in vivo* also confers resistance to capistrui *in vivo*, we tested the antibacterial activities of capistrui against *E. coli* strains containing a plasmid carrying *rpoC*-931I, which encodes [931Ile]- β' which confers MccJ25 resistance *in vivo*,²¹⁻²³ or containing a plasmid carrying *rpoC*⁺, which encodes wild-type β' (Fig. 4). As expected,²¹⁻²³ the strain that contains the mutant RNAP β' -subunit gene *rpoC*-931I and produces [931Ile] β' -RNAP was observed to be resistant to the antibacterial effects of MccJ25 (Fig. 4; growth inhibition of ~50%, vs. 100% for wild-type *rpoC*). Strikingly, the strain carrying *rpoC*-931I also was observed to be resistant to the antibacterial effects of capistrui (Fig. 4; growth inhibition of ~30%, vs. 100% for wild-type *rpoC*). We conclude that a mutation of an RNAP subunit gene that confers resistance to MccJ25 *in vivo* also confers resistance to capistrui *in vivo*, and we infer that the RNAP secondary channel is the functional cellular target of capistrui.

Discussion

The results show that capistrui, an antibacterial threaded-lasso peptide produced by *Burkholderia thailandensis* E264, inhibits *E. coli* RNAP *in vitro* (Fig. 2). A mutant RNAP derivative that carries a substitution in the a RNAP secondary channel and that is resistant to the threaded-lasso peptide MccJ25 *in vitro*^{22,23} also is resistant to capistrui *in vitro* (Fig. 3). An *E. coli* strain carrying a mutation that alters the RNAP secondary channel and that is resistant to antibacterial effects of MccJ25²¹⁻²³ also is resistant to antibacterial effects of capistrui (Fig. 4). We conclude that MccJ25 and capistrui, two threaded-lasso peptides with minimally similar sequences (Fig. 1a) but similar three-dimensional structures (Fig. 1b), particularly in the cycle-threaded segment part that has been shown to be important for MccJ25 activity,²⁶ both inhibit RNAP and both function through binding sites--possibly identical binding sites, or possibly overlapping but nonidentical binding sites--within the RNAP secondary channel. We conclude further that RNAP is the functional target for the antibacterial effects of capistrui in *E. coli*.

Capistrui inhibits *E. coli* RNAP as potently as MccJ25 (Fig. 2), yet at least 10-fold higher concentrations of capistrui are required to inhibit *E. coli* growth.⁴ MccJ25 inhibits *P. aeruginosa* RNAP as potently as capistrui (Fig. 2b), yet unlike capistrui, MccJ25 has no effect on *P. aeruginosa* growth (ES and KS, unpublished data). The observed differences in species-specific rank-order antibacterial potencies are likely to be attributable to species-specific differences in cellular uptake and/or efflux. The availability of large numbers of single-amino-acid-substitution mutants of capistrui and MccJ25^{26,27} should allow identification of amino acids of capistrui and MccJ25 that determine species-specific efficiencies of cellular uptake and efflux.

Bioinformatic analysis reveals that clusters of genes homologous to MccJ25 and capistrui biosynthetic and export/immunity genes are widely distributed in bacteria.^{4,14,28} Analysis of neighboring DNA regions reveals short open reading frames likely to encode threaded-lasso peptide precursors^{4,14,28} (see also supplemental material). Our findings raise the possibility that many, possibly most, of these threaded-lasso peptides may be specific inhibitors of bacterial RNAP.²⁹

Methods

Capistrui

Burkholderia thailandensis E264 (DSM 13276) was incubated at 37 °C overnight in LB medium. Subsequently 1 liter M20 medium (20 g/L L-glutamic acid, 0.2 g/L L-alanine, 1.0

g/L sodium citrate, 20 g/L disodium hydrogen phosphate, 0.5 g/L potassium chloride, 0.5 g/L sodium sulfate, 0.2 g/L magnesium chloride, 0.0076 g/L calcium chloride, 0.01 g/L iron(II) sulfate and 0.0076 g/L manganese sulfate, pH 7.0) was inoculated to an OD₆₀₀ of 0.01 and incubated at 42 °C for 24 h. Cultures were harvested by centrifugation and the combined supernatants were applied to solid phase extraction using XAD16 resin (5 g XAD16 per liter supernatant). After 1 h incubation of the culture supernatant with the XAD16 resin, the supernatant was removed by filtration and the remaining resin was washed with water and eluted with methanol. The resulting methanol extract was evaporated to dryness, dissolved in 3 mL 10% acetonitrile and applied onto a RP-HPLC preparative Nucleodur C18ec column (250 mm x 21 mm). Elution was performed using the following gradient of water/0.1 % trifluoroacetic acid (solvent A) and acetonitrile/0.1 % trifluoroacetic acid (solvent B) at a flow rate of 18 mL/min: linear increase from 10% B to 40% B within 30 min followed by a linear increase to 95% B in 5 min. Capistruin showed a retention time of 26.2 min and could be purified with a yield of 0.7 mg/L culture.

MccJ25

MccJ25 was purified as in ref. 1.

RNAP derivatives

Wild-type *E. coli* RNAP and [931Ile]β'-RNAP were purified as in refs. 22 and 1. *P. aeruginosa* RNAP was prepared from PAO1 cell biomass using the standard procedure developed for *E. coli* RNAP (see ref. 22 and references therein). *Francisella tularensis* RNAP was prepared by heterologous co-overexpression of *Francisella tularensis* *rpo* genes in *E. coli* (KK, DM, and KS, in preparation).

In vitro transcription

A linear template containing the *galP1* promoter fused *S. typhimurium* *his* operon leader region was used as a template for *in vitro* transcription. The template was obtained by replacing the T7 A1 promoter region (bp -121 to +2 with respect to the transcription start point at +1) in DNA fragment amplified from transcription template plasmid pIA171³⁰ with the corresponding *galP1* sequence. To obtain stalled EC³⁰, 50 nM linear DNA template and 100 nM RNAP holoenzyme were combined in 10 µl of transcription buffer (10 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl₂, 1 mM DTT, pH 7.9). After a 20-min incubation at 30 °C, 2 µl of hot mix containing 1 µl of 10 µM [α -³²P]-CTP (3000 Ci/mmol), 0.5 µl of 0.6 mM ATP+GTP, and 0.5 µl of 2 mM CpApUpC were added and reactions were incubated for additional 5 min at 30 °C. Then 2.5 µl of halted EC was next combined with 6.5 µl of transcription buffer containing with or without capistruin or MccJ25 and incubated for 4 min at 30 °C. Reactions were supplemented with 0.025 mM NTPs, incubated for 5 min at 30 °C and terminated by the addition of equal volume of formamide-containing loading buffer. After heating at 100 °C for 1 min, reaction products were resolved by 10% PAGE with 7M urea, followed by PhosphorImager analysis.

EC⁸ was reconstituted by combining 100 nM *E. coli* RNAP core enzyme and 50 nM scaffold (assembled as in Ref. 25) in 10 µl of transcription buffer and incubation for 10 min at 37 °C. Following the addition of threaded-lasso peptides and 5-min incubation at 37 °C, transcription was initiated by the addition of 0.01 mM NTPs. After a 3-min incubation at 37 °C, reactions were terminated by the addition of 1 volume of formamide loading buffer. After heating at 100 °C for 1 min, samples were separated by 20% PAGE with 7M Urea, followed by PhosphorImager analysis.

In vivo antibacterial activity assays

An *E. coli* strain lacking the *tolC* gene was used as a host strain for *rpoC* expression plasmids. The disruption of *tolC* confers defect in efflux³¹ and $\Delta tolC$ strain was found to exhibit increased sensitivity to capistrin (E.S. and K.S., unpublished data). The strain originated from the Keio collection and was provided by Dr. K.A. Datsenko. Cells were grown in M63+YE broth (2 g (NH₄)₂SO₄, 13.6 g KH₂PO₄, 2 g yeast extract per 1L, adjusted to pH 7 by KOH) containing 100 µg/ml ampicillin and 0.1 mM IPTG for expression of the plasmid-borne *rpoC*-931I or *rpoC*⁺ gene until OD₆₀₀ ~ 0.5, cultures were diluted 10,000-fold into the same medium or into medium also containing 5 µM MccJ25 or 50 µM capistrin, and OD₆₀₀ was recorded after incubation for 16h at 37°C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Threaded-lasso peptides (lariat peptides) MccJ25 and capistrain

(a) Sequences of MccJ25 and capistrain.¹⁻⁴ The bracket in each sequence denotes the backbone-sidechain lactam linkage. Residues of MccJ25 and capistrain shown to be important for production and stability^{26,27} are colored red. Residues of MccJ25 important for inhibition of RNAP²⁸ are colored blue.

(b) Three-dimensional structures of MccJ25 (left; PDB 1PP5¹) and capistrain (right; BMR 20014⁴). Loop and terminal segments are shown schematically as dashed lines.

Figure 2. Capistrin inhibits transcript elongation by RNAP *in vitro*
(a) Stalled transcription elongation complexes containing *E. coli* RNAP and a radioactively labeled 30-nt long nascent RNA were prepared on a template containing the T7 A1 promoter. Reaction mixtures were supplemented with MccJ25 or capistrin at concentrations indicated, and transcription was re-started by the addition of NTPs to ??? μ M. Reaction products were resolved by denaturing PAGE and revealed by autoradiography
(b) Stalled transcription elongation complexes containing *E. coli* RNAP (*Ec*), *Pseudomonas aeruginosa* RNAP (*Pa*), or *Francisella tularensis* RNAP (*Ft*) were prepared and analyzed as in (a), in the absence or presence of 100 μ M MccJ25 or 100 μ M capistrin.

Figure 3. A mutant RNAP derivative that is resistant to MccJ25 is cross-resistant to capistruin Transcription elongation complexes containing a radioactively labeled 8-nt RNA primer were assembled on nucleic-acid scaffolds using wild-type RNAP or [931Ile]β'-RNAP. The structure of the scaffold used (Ref. 25) is schematically shown at the top of the figure. Reaction mixtures were supplemented with 0 or 50 μM MccJ25 or capistruin, and transcription was re-started by the addition of NTPs. Reaction products were analyzed as in Fig. 2. At the scaffold used, transcription (primer elongation) does not proceed to the end of the template but is halted when the transcript length reaches 16 nucleotides.

Figure 4. A mutation of an RNAP-subunit gene that confers resistance to MccJ25 *in vivo* confers cross-resistance to capistruin *in vivo*

An *E. coli* $\Delta tolC$ strain was transformed with plasmids expressing *rpoC*⁺ or mutant, MccJ25-resistant, *rpoC*-931I alleles^{22, 23} and induced cells were grown in the presence or in the absence of MccJ25 (5 μ M) or capistruin (50 μ M) for 16 hours at 37 °C as described in Methods. Ratios of OD₆₀₀ values reached by cultures containing MccJ25 or capistruin and control cultures (no threaded-lasso peptides added) are presented. Mean values obtained in three independent experiments are shown. The error bars indicate standard deviations.