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Generation of Thiocillin Variants by Prepeptide Gene Replacement and *In Vivo* Processing by *Bacillus cereus*

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The thiazolyl peptide antibiotics comprise a family of >80 members with the common characteristics of a central pyridine/piperidine ring typically decorated by three thiazole substituents and a macrocyclic peptide ring containing additional thiazoles[1] (Figure 1). Some members of this antibiotic group, such as thiostrepton and nosiheptide, have a second macrocyclic ring. The thiazolyl peptide natural products target one of two sequential steps in bacterial protein synthesis. Molecules such as GE2270 and thiomuracin bind tightly to EF-Tu and abrogate its aminoacyl-tRNA delivery function.[2a-c] In contrast, thiostrepton and the thiocillins bind directly to the 50S ribosomal subunit, interacting both with 23S rRNA loops and the amino acid side chains of protein L11, with the effect of disrupting EF-G activity and therefore preventing tRNA translocation on the ribosome.[2d-e] While thiazolyl peptides display potent antibiotic activity against gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA)[2f] poor aqueous solubility and pharmacokinetics have limited their clinical use. Furthermore, total syntheses of thiazolyl peptide compounds while representing remarkable achievements still present formidable challenges for structure-activity variations,[3a-f] limiting the production of novel compounds with improved pharmacokinetic properties.

Whether biosynthesis of the highly modified thiazolyl peptides occurs via nonribosomal or ribosomal assembly has long been debated;[4a] ribosomally-encoded natural products[4b] are known to contain dehydro-amino acids (the lantibiotics[4c-d]) and thiazoles (microcin B17, patellamide[4e-f]) like the thiocillins, but pyridine formation has not been seen in other ribosomal peptide scaffolds. Four recent reports have disclosed that GE2270, thiomuracin,[2b] nosiheptide,[5a] thiostrepton,[5b-c] and the thiocillins[5c-d] all arise by posttranslational modification of ribosomally generated prepeptides of 50-60 residues. The sequences that show up in the mature antibiotic scaffolds are derived from the C-terminus of these microbial prepeptides.

In the thiocillins from the producer *Bacillus cereus* ATCC 14579 at least 10, and up to 13, of the C-terminal 14 residues undergo posttranslational modification to generate a set of eight related antibiotics. Based on stable isotope feeding studies[6a-b], the ten core transformations are thought to involve dehydrations of Ser1[7] and Ser10 on the way to pyridine ring formation, dehydration of Thr4 and Thr13 to dehydrobutyrine residues, and cyclizations of Cys2, 5, 7, 9, 11, 12 to six thiazoles. Three additional posttranslational modifications appear to occur stochastically: hydroxylation at Val6, *O*-methylation at Thr8 and/or ketone/alcohol interconversion of the C-terminal residue arising from decarboxylation of Thr14, giving rise to $2 \times 2 \times 2 = 8$ possible thiocillins. We note that four of the eight thiocillins produced abundantly by *B. cereus* display similar efficacy against *Bacillus subtilis* and two MRSA

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Supporting Information Available. SI Figures (23), SI Tables (3), experimental procedures, and spectral data.

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strains, with minimum inhibitory concentrations (MIC) of 0.2 - 0.9 μg/mL and < 0.03 – 0.1 μg/mL, respectively (see SI).

With the recent discovery of the thiocillin gene cluster from *Bacillus cereus*[5d], we set out to genetically manipulate the biosynthetic machinery by site-directed mutagenesis to make modifications to the thiocillins, perhaps improving their pharmacokinetic properties without the need for new synthetic strategies. The thiocillin gene cluster contains four contiguous identical copies of a gene encoding a purported 52-residue precursor peptide (*tclE-H*), which is thought to be post-translationally modified to yield the mature antibiotic scaffold. To confirm that the tandem genes *tclE-H* are responsible for generating the thiocillin prepeptide, we generated a *B. cereustclE-H* knockout strain (*tcl*Δ*E-H*) by homologous recombination with a plasmid containing sequence homology to the *tcl* gene cluster but lacking *tclE-H* (see SI). Cultures of *Bacillus cereus* ATCC 14579 (WT) and *tcl*Δ*E-H* were extracted for compound and analyzed by reverse phase HPLC. WT *B. cereus* extracts contained thiocillins as observed by UV absorption at 350 nm and LC-MS (Figure 2). In contrast, extracts of *tcl*Δ*E-H* failed to yield any of the eight thiocillin compounds. To rescue production of the thiocillins and confirm tclE as the prepeptide responsible for thiocillin production, we inserted a single, plasmid-based copy of *tclE* into the chromosome of *tcl*Δ*E-H* by Campbell integration. Cultures of the knock-in strain (*tclE KI*) were extracted and analyzed by reverse phase HPLC and LC-MS to reveal that *tclE KI* rescued production of thiocillins to near WT levels (Figure 2).

The ability to rescue thiocillin production with *a single*, plasmid-based copy of *tclE* enables mutasynthesis of novel thiocillin compounds in *B. cereus* by use of variant *tclE* genes to initiate structure/activity relationship studies of the thiazolyl peptide antibiotics. Initially, we have focused on residues 3, 4, 6, 8, and 13 which are not involved in setting the trithiazolylpyridine core in the mature scaffold, reasoning that amino acid substitutions at these positions probe the promiscuity of the thiocillin tailoring enzymes while minimizing disruption to the core framework. In all, 14 single amino acid substitutions were made in the thiocillin prepeptide tclE at these 5 positions, with 12 resulting in production of one or more thiocillin variants (Table 1 and SI Table 6.1). The combination of possible post-translational modifications at positions 6, 8 and/or 14, gave rise to 65 thiocillin molecular variants detected by LC and high resolution MS (SI Table 6.1).

In a first set of site-directed mutants of the *tclE* gene, amino acids T3, V6, T8 and T13 were substituted with alanine, and T4 with valine, relatively conservative changes, minimizing the existing side chains to methyl or isopropyl groups. The *tclE* mutant plasmids were each transformed into *B. cereus tcl*Δ*E-H*. HPLC analysis of extracts from 0.5 L cultures confirmed the presence of a number of thiocillins. The T3A variant produced six of eight expected thiocillins, while four thiocillins were observed in T8A, where A8 cannot be methylated. T4V generated four of eight expected compounds plus two additional derivatives with masses corresponding to the addition of two hydroxyl groups, suggesting that tclD, the V6 hydroxylase is also able to hydroxylate a valine at position 4. Further evidence of the promiscuity of tclD is the identification of forms with hydroxylated Cβ of the alanine side chain among the six compounds isolated from the V6A variant.

To determine antimicrobial activity, the extracts from each compound were subjected to normal phase chromatography on silica gel and fractions containing compounds absorbing at 350 nm were collected. Because the individual thiocillin derivatives produced by WT *B. cereus* inhibited bacterial growth with similar MICs, the tclE variants from a given mutant were pooled for antibiotic activity assays by disk diffusion on LB plates containing *B. subtilis* strain 168. V6A, T8A and T13A variants maintain similar levels of antibiotic activity to the wild type thiocillin set (Table 1). In contrast, T3A and T4V derivatives failed to inhibit growth at amounts up to 8 μg suggesting that these particular variations disrupt binding or positioning of the

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compounds at the L11/23S rRNA binding interface on the large ribosomal subunit. To quantify antibiotic activity MICs were determined in serial dilution liquid culture assays with both *B. subtilis* and *S. aureus*. V6A and T13A variants showed 2-4 fold improved activity against *B. subtilis* and Methicillin-resistant *Staphylococcus aureus* (MRSA) strain COL and were equally active against MRSA strain MW2. In contrast, T8A was slightly decreased in antibiotic activity.

To introduce charge, T3D and V6D were then generated to determine if the side chain carboxylate anions, which could potentially improve the solubility of the thiocillin scaffold, can be accepted in these positions by the nine posttranslational modification ORFs *tclDJKLMNOPS*. Cultures of the V6D mutant failed to produce any thiocillin compounds as determined by LC and high res MS. In contrast, T3D produced four of eight expected compounds in sufficient quantity to be purified; however at amounts up to 8 μg, the T3D variants failed to inhibit growth of *B. subtilis*. Positions T3 and V6 were also substituted with lysine. No thiocillin compounds were identified from extracts of the V6K mutant. Although production levels were significantly reduced from those of WT thiocillin, requiring growth in a 5L fermenter, the T3K variant produced two thiocillin compounds. As with T3D, T3K was inactive against *B. subtilis* in disk diffusion assays containing up to 8 μg of compound.

LC-MS analysis of extracts of T3K identified multiple compounds with additional mass increases of 100.106 Da, suggesting $C_4H_4O_3$ as the added functional group. MS/MS analysis confirmed the extra mass in all fragments containing lysine 3 and *N*-succinylation was confirmed by MS/MS and NMR (see SI). *N*-succinylation of T3K and T8K, presumably by succinyl-CoA may be a self-protection strategy by the producer organism. *N*-succinylated T3K derivatives with the extended carboxylate side chain were as inactive as the unmodified T3K thiocillin scaffold in disk diffusion assays. The corresponding T4K and T8K mutants in the *tclE* gene were next explored. T8K generated all four expected compounds (no 8 *O*-methylation) as well as three *N*-succinylated derivatives. T4K produced four compounds, all of which were methylated at position T8. Interestingly, not a single *N*-succinylated derivative of T4K was observed. Production of T4K was insufficient for antibiotic activity analysis and T8K failed to inhibit growth of both *B. subtilis* and MRSA.

As a third test of the thiocillin biosynthetic processing machinery to accept tclE prepeptide modifications, we introduced a cysteine substitution as variant T8C. In the maturation of WT thiocillin, all 6 cysteine residues in the 14-amino acid C-terminus of tclE are converted to thiazoles. The addition of a seventh cysteine could give distinct outcomes: 1) complete conversion to a seventh thiazole like the six native cycteines, via an intermediate thiazoline; 2) *S*-methylation of C8 in analogy to *O*-methylation of T8 in the native tclE; or 3) no modification. Extracts of the T8C variant contained near WT levels of compounds, and LC-MS analysis identified seven thiocillins. No thiazole at residue 8 was detected, however three of four expected thiazoline derivatives were identified by MS/MS; the *S*-methyl Cys8 forms predominated. Disk diffusion assays confirmed the antibiotic activity of T8C compounds against *B. subtilis*, and MIC values against *B. subtilis*, MRSA COL and MRSA MW2 indicate retention of almost full antibiotic efficacy.

The results presented herein begin to decipher the functional requirements for processing of the 52-residue tclE prepeptide to the mature thiocillin scaffold and the stochastic variants at side chains 6, 8, and 14. This is also a beginning to map the antibiotic activity of thiocillin variants. In all, 65 novel thiazolyl peptide compounds were generated. While conservative variants at positions 6, 8 and 13 maintained considerable or equivalent antibiotic activity, those at positions 3 and 4 as well as more drastic charge insertion mutants were completely inactive in the concentration ranges tested. These results correlate well with the position of micrococcin modeled into the complex with the 50S ribosome by Harms and colleagues[2e]. Threonine-3 and dehydrobutyrine-4 appear in close proximity to ribosomal protein L11; disrupting these

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contacts could result in perturbation of micrococcin binding to the ribosome and loss of antibiotic activity.

The ability to express *tclE* gene mutants in a *B. cereus* strain deleted of its four tandem endogenous copies of *tclE-H* sets the stage for more extensive structure-activity evaluations. These include alterations that still allow processing to the mature trithiazolylpyridine core in this highly morphed ribosomal peptide antibiotic framework (e.g., the requirement for three thiazoles surrounding the pyridine core and the size and flexibility of the macrocyclic ring connecting thiazole 2 and thiazole 9). Fermentation of the variant thiocillins will also allow evaluation of the subset of scaffolds that retain antibiotic activity and show improvements in such parameters as aqueous solubility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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7. thiocillin numbering: Ser1-Thr14 where Ser1 is Ser39 of the prepeptide

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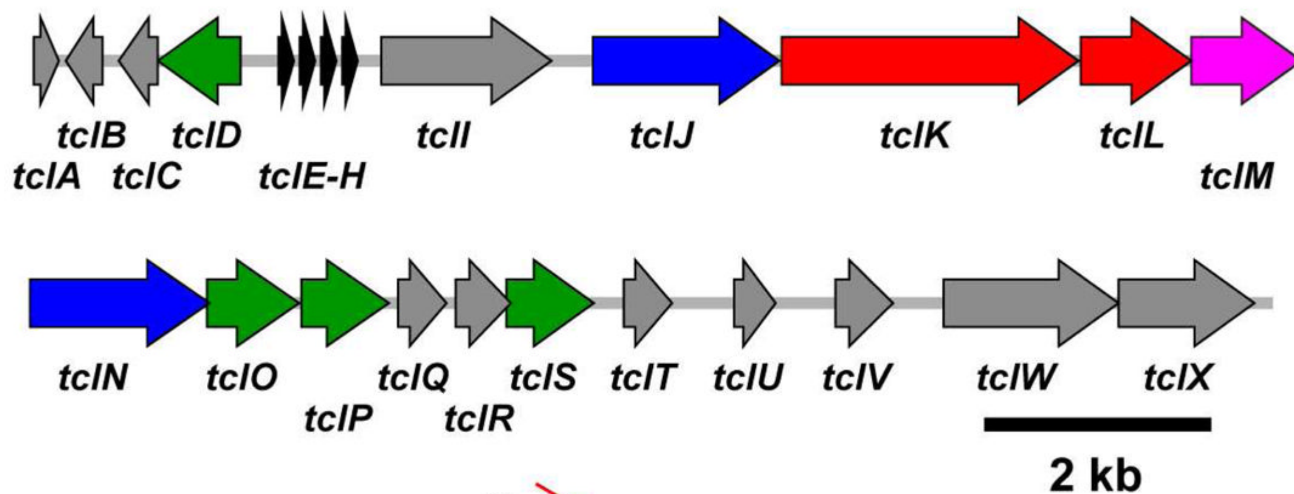

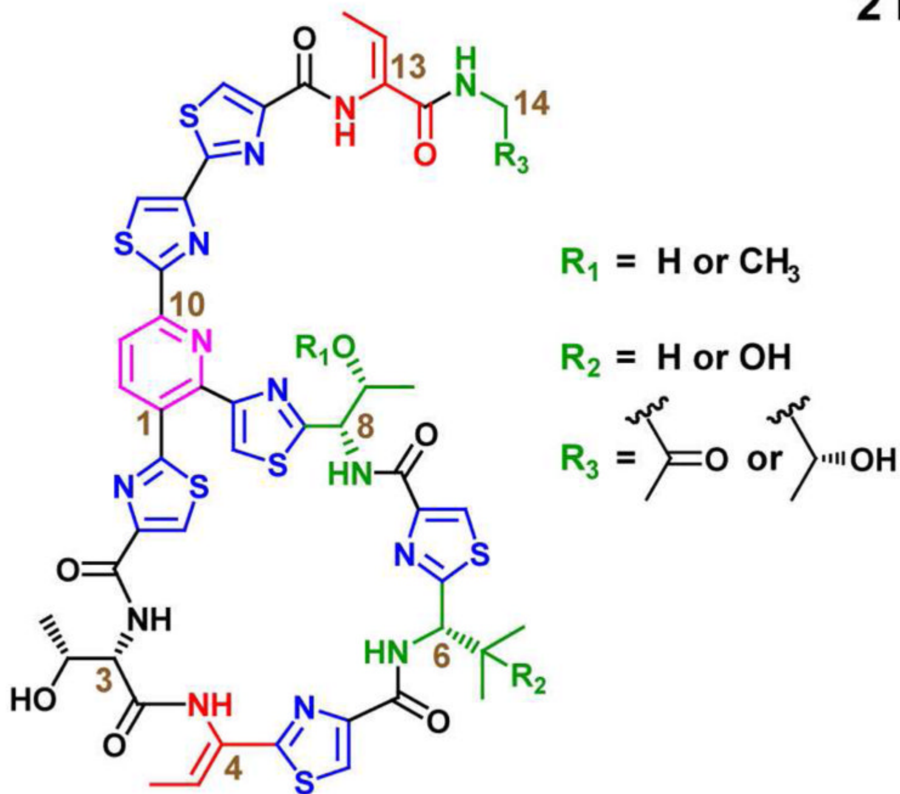

Figure 1.

Annotated gene cluster responsible for the production of the thiocillins. Structure of thiocillin depicts the three positions of stochastic modification (R_1, R_2, R_3) in green.

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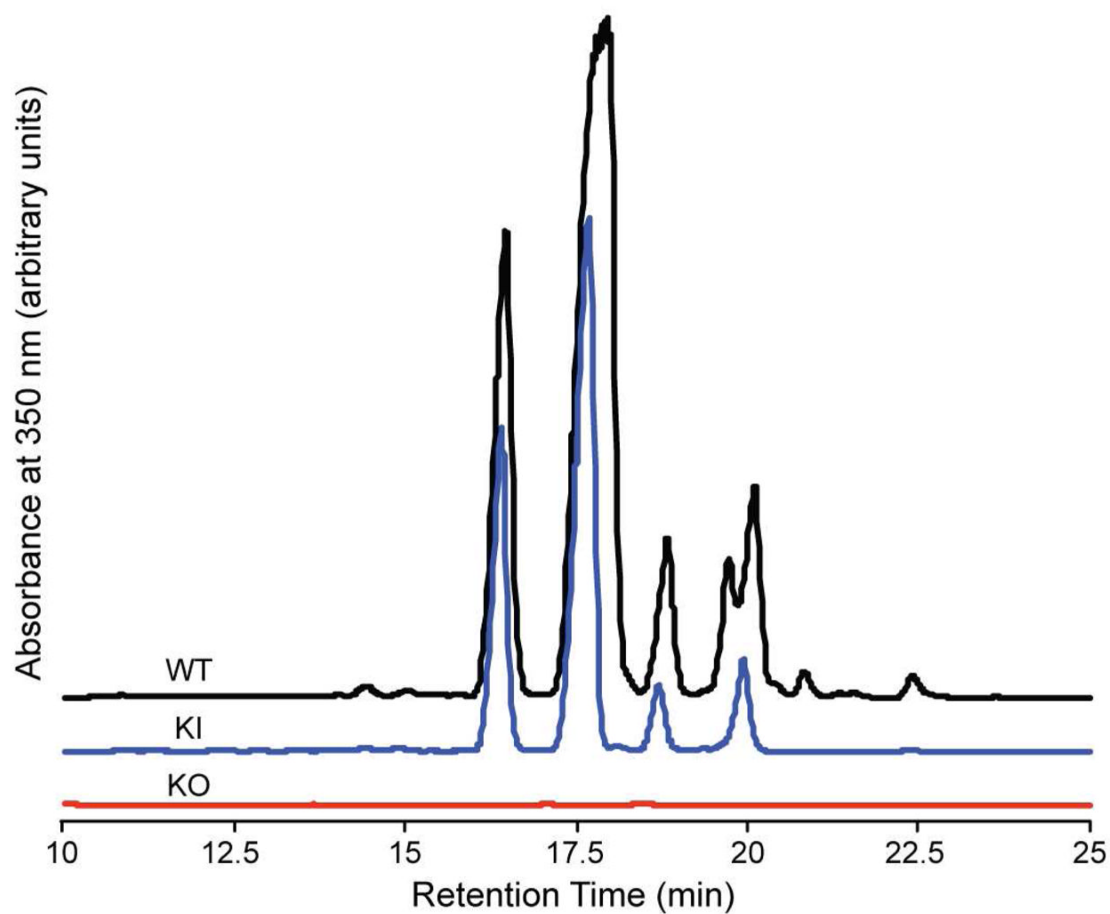

Figure 2.
HPLC trace of thiocillin extractions from *B. cereus* ATCC 14579 (WT), tclE-H knockout *tclE*Δ*E-H* (*KO*) and *tclE KI* (KI).

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Table 1

Summary of thiocillin variants produced in this study and their antibiotic efficacy against *B. subtilis* and methicillin-resistant *S. aureus*.

	Mutant	Compounds Observed[b]	Additional Modifications	MIC (μg/mL)[a]		
				168[c]	*COL*[d]	*MW2*[e]
1	T3K	4	*N*-succinylation	>8	>8	>8
2	T3D	4	--	>8	>8	>8
3	T3A	6	--	>100	>100	>100
4	T4K	4	--	nt[f]	nt	nt
5	T4V	6	--	>100	>100	>100
6	V6D	N/A	--	nt	nt	nt
7	V6K	N/A	--	nt	nt	nt
8	V6A	6	*Ala*-hydroxylation	0.13	0.06	0.06
9	T8C	7	*S*-methylation, *Cys*-cyclization	0.3	0.15	0.08
10	T8A	4	--	1	0.25	0.25
11	T8K	6	*N-succinylation*	>4	>4	>4
12	T13A	7	--	0.13	0.06	0.06
13	*WT*[g]	6	--	0.5	0.06	0.13

[a] Minimum inhibitory concentrations determined by overnight culture in 96-well plate format.

[b] details available in SI.

[c] *Bacillus subtilis* 168.

[d] Methicillin-resistant *Staphylococcus aureus* COL.

[e] Methicillin-resistant *Staphylococcus aureus* MW2.

[f] nt = not tested.

[g] WT = wild type.