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

Michael G. Acker, Albert A. Bowers, and Christopher T. Walsh*

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

Supporting Information

Contents:

1. Materials and General Methods

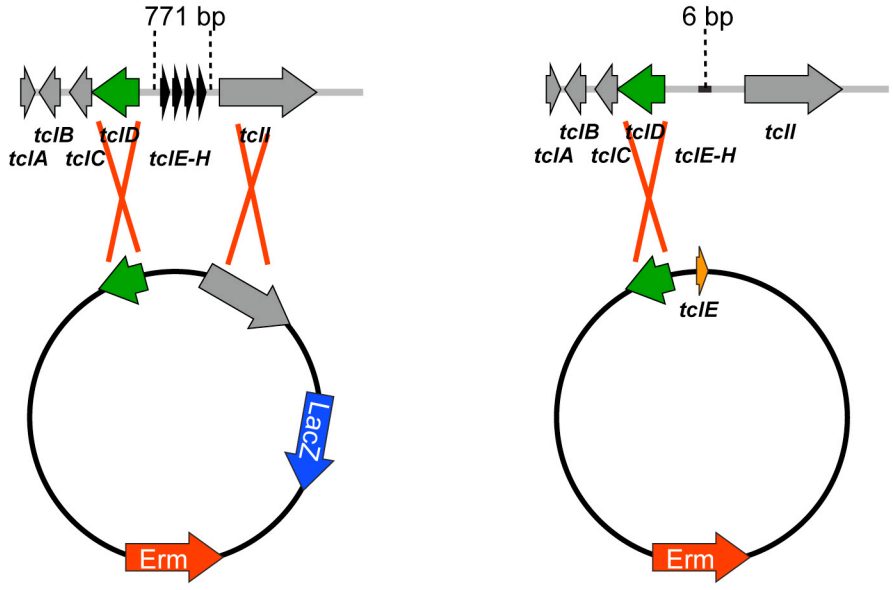
All molecular biology, recombinant DNA manipulation and microbiological assays were performed following the protocols of Sambrook *et al*.[1] Unless otherwise specified, all chemicals were purchased from Sigma-Aldrich. Restriction enzymes and Quick Ligase were purchased from New England Biolabs (Boston, MA). Pfu Turbo DNA Polymerase was purchased from Invitrogen (Carlsbad, CA) and Paq 5000 DNA polymerase from Stratagene (La Jolla, CA).DNA oligonucleotide primers were synthesized by Integrated DNA technologies (Coralville, IA). PCR was performed on a Biorad MyCycler thermal cycler. DNA sequencing was performed by the Molecular Biology Core Facilities at the Dana Farber Cancer Institute (Boston, MA). Top10 chemically competent *E. coli* cells were purchased from Invitrogen. Restriction endonuclease cleanup and gel extraction of DNA fragments were performed with QiaQuick PCR cleanup kit from Qiagen. Recombinant plasmids were isolated using the QiaPrep Spin Miniprep Kit from Qiagen. *B. cereus* ATCC 14579 genomic DNA was isolated from cultures using the DNeasy Kit from Qiagen. Extraction of thiocillins from cell-free media was performed on Sep-Pak C18 cartidges from Waters Corp. (Milford, MA). Analytical RP-HPLC was performed on a Beckman System Gold (Beckman Coulter) instrument using a Phenomenex Luna 5 μm C18(2) 100 Å 250 x 4.6 mm column, monitoring eluent absorption at 220 and 350 nm. Preparative RP-HPLC was

performed on a Beckman System Gold (Beckman Coulter) instrument using a Phenomenex Luna 10 µm C18(2) 100 Å 250 x 21.20 mm column. Purification of derivatives from crude extracts was also performed on a Biotage Isolera flash purification system (Biotage) using silica gel columns. ^{1}H NMR spectra were recorded on a Varian 600 MHz spectrometer. Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance resulting from incomplete deuteration as the internal standard (CDCl3 δ 7.26, D2O δ 4.79, CD3OD δ 3.31). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constants (Hz), and integration.

2. Generation of tclΔE-H Knockout Strain

Plasmid pMGA-tclΔE-H was generated from plasmid pKM082[2], containing ampicillin and erythromycin resistance cassettes. To enable blue-white selection for identification of tclE-H knockout strains, a complete *LacZ* gene containing promoter and coding region was cloned from pMAD[3] with PstI restriction endonuclease sites at both 5' and 3' sites (**SI Table 2.1**) and ligated into the PstI site of pKM082. Transformants containing LacZ were isolated based on their blue color when plated on LB plates containing 50 µg/mL XGAL. Into this new plasmid, two DNA fragments of ~1 kb homology to the tclE gene cluster surrounding tclE-H were individually cloned (**SI Figure 2.1**). The left fragment (LF) contained homology from *tclD* up to but not including the start codon of tclE and was inserted using 5' BamHI and 3' SphI restriction endonuclease sites. Finally, the right fragment (RF) contained homology beginning immediately after the *tclH* stop codon and continuing into *tclI*. The RF was cloned with both 5' and 3' SphI restriction sites. After ligation into the plasmid SphI site restriction site, transformants were sequenced to identify those containing RF in the correct directional orientation. The resulting plasmid contained two ~1 kb regions of homology to the tcl gene cluster, replacing the sequence from the *tclE* start codon to the *tclH* stop codon (771 bp) with the sequence GCATGC corresponding to the SphI restriction sequence.

SI Figure 2.1. Strategies for *tclE* knockout and *in vivo* replacement



Because *B. cereus* are less efficient at double-crossover homologous recombination than *B. subtilis*, removal of tclE-H from the genome of *B. cereus* ATCC 14579 was accomplished through two individual rounds of homologous recombination. In the first round, the

entire plasmid is integrated into the chromosome by Campbell integration. Plasmid pMGA-tclE-H was transformed into *B. cereus* ATCC 14579 following the previously published protocol[4]. Positive transformants (containing a chromosomally-integrated plasmid) were selected for on MLS LB-agar plates (containing 1µg/ml erythromycin and 25µg/mL lincomycin) for 36 hours at 30 °C. The second round of homologous recombination involves the removal of the plasmid through a second homologous recombination event. This event removes both the erythromycin resistance cassette and the LacZ cassette, allowing for identification of revertants by their white color when grown on plates containing Xgal. Individual colonies were transferred to LB liquid cultures without antibiotic and incubated for 24 hours at 30 °C. The liquid cultures were diluted 1:1000 in antibiotic free LB and again incubated for 24 hours at 30 °C. After 5-7 rounds of dilution and growth, the cultures were diluted 10^{-4} – 10^{-7} and 100 µl were plated on LB agar supplemented with 100µg/mL Xgal. After 20-24 hours at 37 °C, plates were analyzed for the presence of white colonies, indicating removal of the plasmid by homologous recombination. Because the original plasmid contained two areas of homology to the thiocllin gene cluster, the recombination event to remove the plasmid from the genome can result in two possible products with equal probability: 1) a reversion to the WT *B. cereus* or 2) the removal of *tclE-H*, generating a knockout of these genes. To differentiate between the two possibilities, colony PCR reactions were carried out using oligonucleotide primers to *tclD* and *tclI*. Knockout strains were confirmed by the loss of 771 bp in the PCR product due to the removal of *tclE-H* and verified by DNA sequencing.

SI Table 2.1. Oligonucleotides used for cloning and *tclE* mutagenesis

Oligo	Sequence	Role
Primer 72	5'-GGAATTCCCTGCAGCGAGGCC TTTAGCATATTATGTTGCC-3'	PCR of LacZ –PstI ends
Primer 89	5'-GGAATTCCCTGCAGAGATCTGTCTAGTTAATGTGTAACGTAAC-3'	PCR of LacZ –PstI ends
LCW001	5'-GTACGGATCCCATACTCCCAGATTATTTGCAGTCGTAAC-3'	PCR of LF-BamHI/SphI ends
Primer 133	5'-GATCGCATGCACTCATCCCACCTACAAGAAATTTTTGG-3'	PCR of LF-BamHI/SphI ends
Primer 87	5'-GGAATTCCGCATGCTTTTTCAAGAAGCTTAATTGTTCTCCCCC-3'	PCR of RF-SphI ends
Primer 88	5'-GGAATTCCGCATGCGCGGAATTTGTTTCCTAGAACCTCTACC-3'	PCR of RF-SphI ends
LCW019	5'-GTACACTAGTCTCCTCTCTAATTGATTTAATTACAG-3'	Clone tclE-EcoRI/SpeI ends
LCW021	5'-GTACGAATTCTCAAGTTGTACAACAACTGCATGTAC-3'	Clone tclE-EcoRI/SpeI ends
Primer 125	5'-CAGTAAGCATATGTATACTCCTCTCTAATTGATTTAATTACAG-3'	Make pLW111 tclE 5'UTR WT
Primer 126	5'-CTGTAATTAAATCAATTAGAGAGGAGTATACATATGCTTACTG-3'	Make pLW111 tclE 5'UTR WT
Primer 129a	5'-GATCGCATGCTTTTTCAAGAAGCTTAATTGTTCTCC-3'	Clone tclH 3'UTR-SphI ends
Primer 129b	5'-GATCGCATGCTCAAATATTTACTGATTTCACTAAAAAACG-3'	Clone tclH 3'UTR-SphI ends
Primer 131	5'-GTACATGCAGTTGTTGTACAACTTAATTTTTCAAGAAGCTTAATTGTTC TCC-3'	Remove EcoRI-SphI sequence
Primer 132	5'-GGAGAACAATTAAGCTTCTTGAAAAATTAAGTTGTACAACAACTGCAT GTAC-3'	Remove EcoRI-SphI sequence
Primer 55	5'-ATTATGGGAGCGTCATGTAAAACATGCGTATGTACATGC-3'	T3K mutagenesis
Primer 56	5'-GCATGTACATACGCATGTTTTACATGACGCTCCCATAAT-3'	T3K mutagenesis
Primer 53	5'-ATTATGGGAGCGTCATGTGATACATGCGTATGTACATGC-3'	T3D mutagenesis
Primer 54	5'-GCATGTACATACGCATGTATCACATGACGCTCCCATAAT-3'	T3D mutagenesis

Primer 209	5’-GAAATTATGGGAGCGTCATGTGCGACATGCGTATGTACATGCAG-3’	T3A mutagenesis
Primer 210	5’-CTGCATGTACATACGCATGTCGCACATGACGCTCCCATAATTTC-3’	T3A mutagenesis
Primer 213	5’-GGAGCGTCATGTACGAAATGCGTATGTACATGC-3’	T4K mutagenesis
Primer 214	5’-GCATGTACATACGCATTTCGTACATGACGCTCC-3’	T4K mutagenesis
Primer 67	5'-ATTATGGGAGCGTCATGTACGGTTTGCGTATGTACATGCAGTTG-3'	T4V mutagenesis
Primer 68	5'-CAACTGCATGTACATACGCAAACCGTACATGACGCTCCCATAAT-3'	T4V mutagenesis
Primer 61	5’-GCGTCATGTACGACATGCGATTGTACATGCAGTTGTTGTAC-3’	V6D mutagenesis
Primer 62	5’-GTACAACAACTGCATGTACAATCGCATGTCGTACATGACGC-3’	V6D mutagenesis
Primer 63	5’-GCGTCATGTACGACATGCAAATGTACATGCAGTTGTTGTAC-3’	V6K mutagenesis
Primer 64	5’-GTACAACAACTGCATGTACATTTGCATGTCGTACATGACGC-3’	V6K mutagenesis
Primer 59	5’-GCGTCATGTACGACATGCGCTTGTACATGCAGTTGTTGTAC-3’	V6A mutagenesis
Primer 60	5’-GTACAACAACTGCATGTACAAGCGCATGTCGTACATGACGC-3’	V6A mutagenesis
Primer 150	5’-CATGTACGACATGCGTATGTACATGCAGTTGTTGTACAACTTGA-3’	T8C mutagenesis
Primer 151	5’-TCAAGTTGTACAACAACTGCAACAACATACGCATGTCGTACATG-3’	T8C mutagenesis
Primer 211	5’-CATGTACGACATGCGTATGTGCATGCAGTTGTTGTACAACTTG-3’	T8A mutagenesis
Primer 212	5’-CAAGTTGTACAACAACTGCATGCACATACGCATGTCGTACATG-3’	T8A mutagenesis
Primer 215	5’-CATGTACGACATGCGTATGTAAATGCAGTTGTTGTACAACTTG-3’	T8K mutagenesis
Primer 216	5’-CAAGTTGTACAACAACTGCATTTACATACGCATGTCGTACATG-3’	T8K mutagenesis
Primer 189	5’-GTATGTACATGCAGTTGTTGTGCAACTTGATTTTTCAAGAAGC-3’	T13A mutagenesis
Primer 190	5’-GCTTCTTGAAAAATCAAGTTGCACAACAACTGCATGTACATAC-3’	T13A mutagenesis

3. Rescue of Thiocillin Production by *tclE* Integration

In order to rescue production of thiocillin in the *B. cereus tcl∆E-H* strain, we inserted a single copy of *tclE* into the knock-out genome by Campbell integration of a plasmid containing *tclE*. To minimize any problems due to potential promoter or terminator requirement for tclE production, the 5’ UTR of *tclE* and the 3’ UTR of *tclH* (the fourth and final copy of tclE in the tcl gene cluster), which could contain these elements, were included. Plasmid pMGA-tclE-KI was generated using pLW111[2] which already contains ~1 kb of homology to *tclD*. Briefly, *tclE* along with its 5’ UTR was cloned from *B. cereus* ATCC 14579 genomic DNA with SpeI and EcoRI restriction endonuclease sites at the 5’ and 3’ ends respectively, and cloned into pLW111. This resulted in a duplication of a small section of the 5’UTR (which already existed in pLW111) and this was removed by site directed mutagenesis. The 3’ UTR of *tclH* was cloned from genomic DNA with SphI sites at both the 5’ and 3’ ends, and inserted into the corresponding site in the plasmid. DNA sequencing identified those clones containing *tclH* 3’UTR in the correct directional orientation. Finally, site directed mutagenesis was performed to remove extra sequence between the EcoRI and SphI sites, resulting in a plasmid containing the unaltered sequence *tclD*-5’UTR-*tcE*-3’UTR.

pMGA-tclE-KI was transformed into *B. cereus tcl∆E-H* and positive transformants were selected for on MLS-LB agar for 36 hours at 30 °C. *tclE* knock-in clones were confirmed by the appearance of a product from PCR reactions using oligos specific to *tclE*.

4. Mutagenesis of *tclE* in pMGA-tclE-KI

Mutants of *tclE* were generated by site-directed mutagenesis of plasmid pMGA-tclE-KI using overlapping primer extension. Briefly, homologous primers were designed each containing the mutation of interest flanked by 15-20 bps of homologous plasmid DNA sequence. PCR was performed with Pfu Turbo to extend the primers, generating entire circular plasmid strands, each containing the mutation of interest. Restriction endonuclease DpnI was then added to the reaction. DpnI selectively cleaves the methylated template plasmid, having been purified from bacterial cultures, while leaving intact the unmethylated mutant plasmid generated by PCR. The resulting mixture was transformed into chemically competent *E. coli* TOP10 cells and positive transformants were selected for on LB agar supplemented with 100 µg/mL ampicillin. Plasmid

DNA was purified and mutants were confirmed by DNA sequencing. pMGA-tclE mutant plamids were transformed into B. cereus and selected as described above for pMGA-tclE-KI.

5. Extraction of Thiocillin Compounds

WT or *tclE* mutant *B. cereus* starter cultures (5 mL) were grown in LB for 20 hours at 30 °C. Larger cultures (0.5 L LB in 2 L culture baffles culture flasks) were inoculated with 300 µL of starter culture and grown for 68 hours at 30 °C with shaking at 200 rpm. (*tclE* mutant strains were grown in media supplemented with 1µg/mL erythromycin and 25µg/mL lincomycin.) Cultures were harvested and both the cell pellet and spent media were saved. To the pellet, 50 mL methanol was added along with 15 g sodium sulfate. The mixture was vortexed vigorously and allowed to sit for at least 10 minutes. The mixture was then filtered through Whatman filter paper (no. 1) and the methanol was removed by vacuum. Solid was solubilized in 10 mL 33% acetonitrile in water for HPLC analysis. *tclE* mutants that produced compound at low levels were grown in a 5L fermenter in ECPM1 media lacking glycerol (20 g N-Z amine; 3 g Yeast Extract; 1 g KH_2PO_4; 4 g K_2HPO_4; 1 g NH_4Cl; 2.4g K_2SO_4 in 1 L supplemented with 10 mL 100X Trace Elements (5 g EDTA; 0.5 g $FeCl_3$•$6H_2O$; 0.05 g ZnO; 0.01 g $CuCl_2$•$2H_20$; 0.01 g $Co(NO_3)_2$•6H2O; 0.01 g $(NH_4)_6$ Mo_7O_{24} in 1 L) and 2 mL of 500X Mg/Ca solution (203 g $MgCl_2$; 66.2 g $CaCl_2$ in 1 L). Cells and media were harvested after 24 hours and extraction was performed as detailed above, scaled accordingly.

Further purification was accomplished by ethyl acetate extraction. Solvents were removed from the crude compound extracts on a rotary evaporator. The crude residue was then dissolved in 40 mL of 1:1 EtOAc: water. The biphasic solution was transferred to a 60mL separatory funnel, shaken and the organic layer removed. The aqueous layer was washed with a further 20 mL of EtOAc and the combined organics were dried over Na_2SO_4, filtered through a 60 mL coarse fritted glass funnel, and evaporated to dryness. For purposes of assessing the thiocillin content of the individual layers, the residue from the organic layer was redissolved in 10 mL of acetonitrile. 180 µL of the acetonitrile solution was combined with 180 µL of water and 300 µL of this solution was injected onto the analytical HPLC. 300 µL of the aqueous layer was also injected, being careful to avoid the surface organics retained from the extraction.

Additional compound was extracted from the cell free media. The cell free media was passed over a Sep-Pak C18 column (Waters) and material was sequentially eluted with 10 mL of 20%, 50% and 100% acetonitrile in water. Derivatives commonly eluted in 50% acetonitrile. All compounds were finally purified by silica gel chromatography (Biotage, eluate: 95:5% CH_2Cl_2:MeOH) for use in disk diffusion and liquid culture assays. Compounds were eluted as mixtures of the tailored states and used as such. Compounds characterized by NMR were further separated by RP-HPLC.

6. LC-MS and MS/MS Analysis.

a. Procedure. High-resolution LC-MS data was collected in positive ion mode, on an Agilent 6520 Accurate-Mass Q-TOF Mass Spectrometer fitted with an electrospray ionization (ESI) source. The capillary voltage was set to 3500 kV, and the fragmentor voltage at 250 V. The drying gas temperature was maintained at 350°C with a flow rate of 12 L/min and a nebulizer pressure of 45 psi. Separation was effected on a Gemini-NX C18 reverse phase column (5µm, 110A, 2.0 x 50 mm, Phenomonex). Compounds were eluted in a gradient of solvents A (0.1% TFA in water) and B (0.1% TFA in acetonitrile): 2 min. isocratic 2%B, then increasing to 100%B over 10 min., and finally isocratic at 100%B for 2 min. before returning to 2%B and reequilibrating over 4 min. The order of elution relative to tailored states of the final products was conserved across variants, except where the short gradient created elution overlap. In order to better search for the presence of trace quantities of all tailored states, the

cellular extract was loaded “as-is” without further dilution. Under these conditions, the large quantities of major products essentially overloaded the short column in places, causing double peaking and ghost peaks. In order to demonstrate that these second peaks were in fact due to overloading and not regioisomers, the same samples were repeated under dilute conditions and double peaks were observed to coalesce.

b. Summary

SI Table 6.1. Summary of tclE mutants and the thiocillin compounds produced

			RED[a]	PURPLE	NAVY	BLACK	GREEN	LIGHT BLUE	ORANGE	PINK
	Mutant	Tailoring (R^1, R^2, R^3)	H, OH, red	CH_3,OH, red	H, OH, ox	CH_3, OH, ox	H, H, red	CH_3, H, red	H, H, ox	CH_3, H, ox
1a	**T3K**	Expected	1186.25224	1200.26789	1184.23659	1198.25224	1170.25733	1184.27298	1168.24168	1182.25733
		Observed						1184.2744		1182.256
		ppm error	--	--	--	--	--	-1.20	--	1.12
1b	**T3K-succ**	Expected	1286.26824	1300.28389	1284.25259	1298.26824	1270.27333	1284.28898	1268.25768	1282.27333
		Observed						1284.2882		1282.2754
		ppm error	--	--	--	--	--	0.61	--	-1.61
2	**T3D**	Expected	1173.18422	1187.19987	1171.16857	1185.18422	1157.18931	1171.20496	1155.17366	1169.18931
		Observed		1187.2011		1185.1864		1171.207		1169.1918
		ppm error	--	-1.04	--	-1.84	--	-1.74	--	-2.13
3	**T3A**	Expected	1129.19439	1143.21004	1127.17874	1141.19439	1113.19948	1127.21513	1111.18383	1125.19948
		Observed	1129.1934	1143.2091	1127.1763	1141.1948	1113.1982		1111.1853	
		ppm error	0.88	0.82	2.16	-0.36	1.15	--	-1.32	--
4a	**T4K**	Expected	1204.26281	1218.27846	1202.24716	1216.26281	1188.26789	1202.28354	1186.25224	1200.26789
		Observed		1218.2753		1216.2586		1202.2807		1200.2674
		ppm error	--	2.59	--	3.46	--	2.36	--	0.41
4b	**T4K-succ**	Expected	1304.27886	1318.29451	1302.26321	1316.27886	1288.28394	1302.29959	1286.26829	1300.28394
		Observed								
		ppm error	--	--	--	--	--	--	--	--
5a	**T4V**	Expected	1175.23626	1189.25191	1173.22061	1187.23626	1159.24134	1173.25699	1157.22569	1171.24134
		Observed	1175.2375	1189.2536	1173.2222	1187.2383				
		ppm error	-1.06	-1.42	-1.36	-1.72	--	--	--	--
5b	**T4V-2xOH**	Expected	1191.23117	1205.24682	1189.21552	1203.23117	xx	xx	xx	xx
		Observed		1205.2486		1203.2331				
		ppm error	--	-1.48	--	-1.60				
6a	**V6D**	Expected	1175.16797	1189.18362	1173.15232	1187.16797	1159.16857	1173.18422	1157.15292	1171.16857
		Observed								
		ppm error	--	--	--	--	--	--	--	--
7a	**V6K**	Expected	1188.23151	1202.24716	1186.21586	1200.23151	1172.23659	1186.25224	1170.22094	1184.23659
		Observed								
		ppm error	--	--	--	--	--	--	--	--
7b	**V6K-succ**	Expected	1288.24756	1302.26321	1286.23191	1300.24756	1272.25264	1286.26829	1270.23699	1284.25264
		Observed								
		ppm error	--	--	--	--	--	--	--	--
8	**V6A**	Expected	1115.17874	1129.19439	1113.16309	1127.17874	1131.17366	1145.18931	1129.15801	1143.17366
		Observed	1115.1791	1129.1942	1113.1634	1127.1792	1131.1738	1145.1897		
		ppm error	-0.32	0.17	-0.28	-0.41	-0.12	-0.34	--	--
9a	**T8C**	Expected	1161.16647	1175.18212	1159.15081	1173.16647	1145.17155	1159.1872	1143.1559	1157.17155
		Observed		1175.1824		1173.1664		1159.1864	1143.1543	
		ppm error	--	-0.24	--	0.06	--	0.69	1.40	--

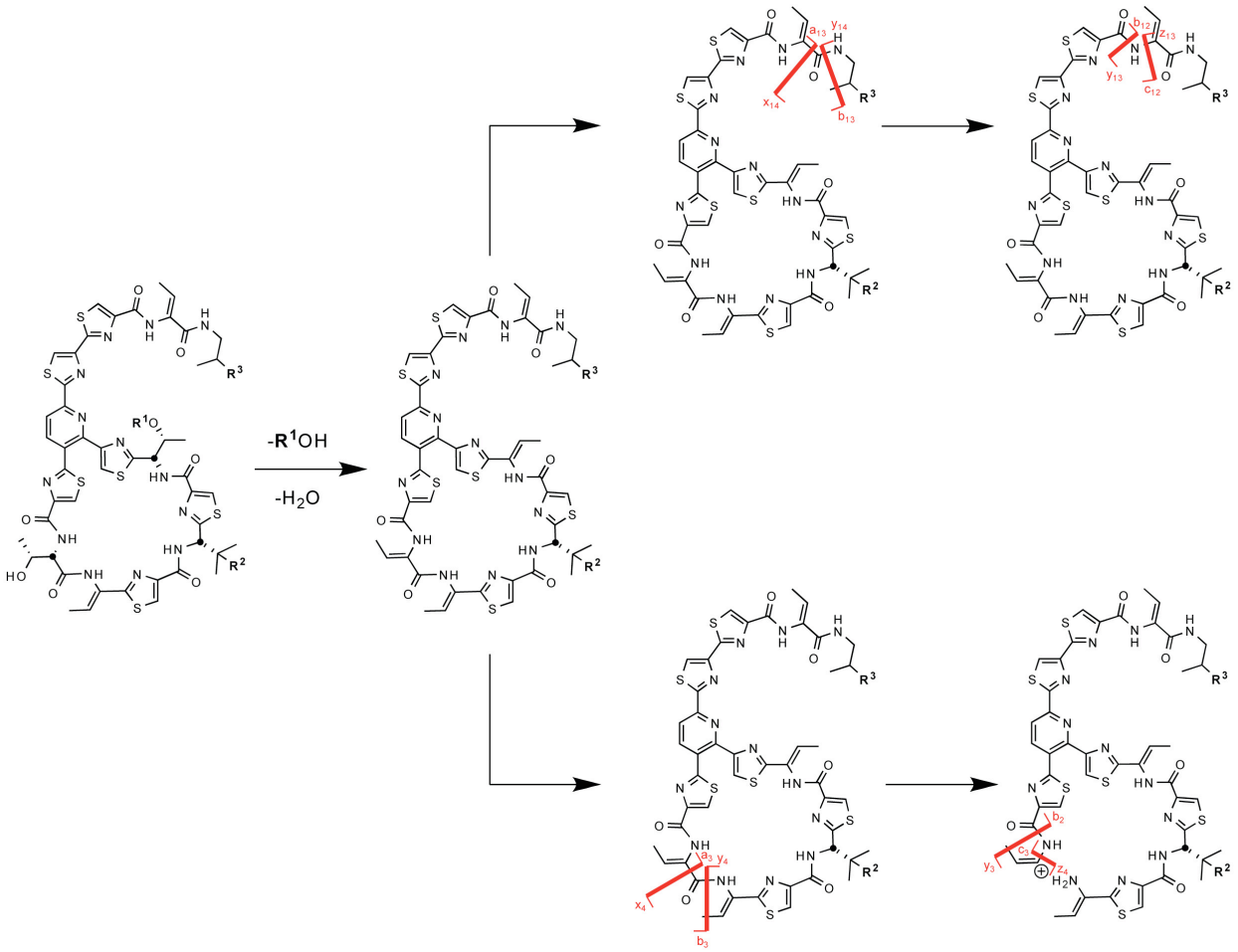
9b	**T8C -** thiazole	Expected	1141.14025	xx	1139.1246	xx	1125.14534	xx	1123.12969	xx
		Observed								
		ppm error	--	--	--	--	--	--	--	--
9c	**T8C -** thiazoline	Expected	1143.1559	xx	1141.14025	xx	1127.16099	xx	1125.14534	xx
		Observed			1141.1389		1127.1603		1125.1445	
		ppm error	--		1.18		0.61		0.75	
10	**T8A**	Expected	1129.19439	xx	1127.17874	xx	1113.19948	xx	1111.18383	xx
		Observed	1129.1912		1127.18		1113.1996		1111.1841	
		ppm error	2.83	--	-1.12	--	-0.11	--	-0.24	--
11a	**T8K**	Expected	1186.25224	xx	1184.23659	xx	1170.25733	xx	1168.24168	xx
		Observed	1186.2512		1184.236		1170.2576		1168.2417	
		ppm error	0.88	--	0.50	--	-0.23	--	-0.02	--
11b	**T8K-succ**	Expected	1286.26829	xx	1284.25264	xx	1270.27338	xx	1268.25773	xx
		Observed					1270.274		1268.2592	
		ppm error	--	--	--	--	-0.49	--	-1.16	--
12	**T13A**	Expected	1147.205	1161.2206	1145.1893	1159.205	1131.21	1145.2257	1129.1944	1143.21
		Observed	1147.205	1161.2211		1159.2043	1131.2096	1145.2265	1129.1936	1143.2105
		ppm error	0.00	-0.43	--	0.60	0.35	-0.70	0.71	-0.44
13	**WT**[b]	Expected	1159.20496	1173.22061	1157.18931	1171.20496	1143.21004	1157.22569	1141.19439	1155.21004
		Observed		1173.22	1157.1895	1171.2047	1143.2087	1157.2231	1141.1922	
		ppm error	--	0.52	-0.16	0.22	1.17	2.24	1.92	--
14	**V6I**	Expected	1173.22061	1187.23626	1171.20496	1185.22061	1157.22569	1171.24134	1155.21004	1169.22569
		Observed		1187.2392	1171.2079	1185.2234	1157.2287	1171.2424	1155.2116	
		ppm error	--	-2.48	-2.51	-2.35	-2.60	-0.91	-1.35	--
15	**V6L**	Expected	1173.2206	1187.2363	1171.205	1185.2206	1157.2257	1171.2413	1155.21	1169.2257
		Observed		1187.2365		1185.2227	1157.2271	1171.239		1169.2278
		ppm error	--	-0.17	--	-1.77	-1.21	1.96	--	-1.80

[a] Colors correspond to peaks on the traces in SI Figures 6.1-6.13. [b] WT thiocillins were extracted from 0.5 L cultures and 6 total compounds were identified. In previous work, WT cultures were grown in 50 x 3 mL samples, resulting in identification of 8 compounds[2].

c. MS Traces of Individual Compounds

d. MS/MS data. To confirm structural anomalies (e.g., T3K succinylation and T8C cyclodehydration), samples were submitted to targeted CID-MS/MS. For all samples examined, the collision energy was varied between 40 and 65 eV, with optimum fragmentation generally being observed at 45 eV. Representative spectra are illustrated below. Essential diagnostic peaks have been labeled. Two key points of fragmentation proved useful in structural confirmation (**SI Figure 6.14**): 1) cleavage of the C-terminal peptidic residues, and 2) loss of CO from threonine-3 and subsequent fragmentation of the remainder of the residue. These fragmentations were observed in all compounds examined, including the two wild type compounds, Micrococcin P1 and Micrococcin P2. Succinylated T3K was purified by preparative HPLC prior to analysis. All other compounds were submitted as crude mixtures from the methanol extracts.

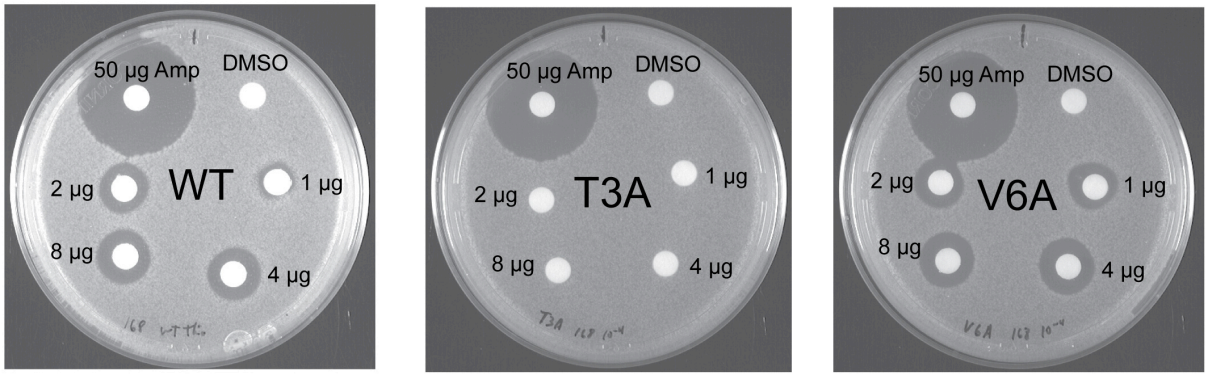
SI Figure 6.14. Scheme of MS/MS fragmentation of thiocillin variants



8. Disk Diffusion Antibiotic Activity Assays

Thiocillin variants were diluted to 1 µg/µL - 0.125 µg/µL by serial dilution of stock solutions into dimethylsulfoxide. A 5 mL culture of *B. subtilis* strain 168 was grown for 20 hours at 37 °C. The bacterial culture was diluted 1:10,000 in LB-top agar (Luria-Bertani (LB) broth supplemented with 0.7% agar), 4 mL were added to the surface of LB-agar plates and allowed to cool. Paper disks (6 mm diameter, BD Biosciences) were placed on the surface of the plate and 8 µL of thiocillin variant solutions were added to each paper disk. Included on each plate was a negative control containing DMSO only and a positive control containing 0.5 µL of 100 mg/mL ampicillin. Plates were incubated at 37 °C overnight and thiocillin variants that displayed antibiotic activity were identified by zones of inhibition of bacterial growth surrounding the paper disks (**SI Figure 8.1**).

SI Figure 8.1. *B. subtilis* growth inhibition by Thiocillin variants determined by disk diffusion assay



9. Determination of Minimum Inhibitory Concentrations (MICs)

Over night cultures of the individual strains (MRSA strains COL and MW2 grown in TSB media and *B. subtilis* strain 168 in LB at 37 °C) were diluted 1000-fold and used to fill 96-well plates (150 µL per well). Serial dilutions of the variant mixtures (1.5 µL of 800-6.25 µg/µL solutions in DMSO) were transferred from library plates to the culture plates. For each variant mixture one adjacent well was treated with 1.5 µL of unadulterated DMSO and one with 1.5 µL of erythromycin (10 µg/µL in 95% ethanol). The plates were incubated at 30 °C for 20 hours. The OD_{600} was read on a Perkin Elmer Envision plate reader. MICs were designated as the lowest concentration that produced an increase of less than 10% in OD over that of the adjacent erythromycin well.

SI Table 9.1. MICs for individual WT Thiocillin compounds against *B. subtilis* and Methicillin-resistant *S. aureus* (MRSA)

Strain	MIC (µg/mL)			
	Micro P1 (H, H, red)	Micro P2 (H, H, ox)	YM-266183 (H, OH, ox)	Thiocillin I (H, OH, red)
B. subtilis	0.2	0.5	0.9	0.9
S. aureus MW2	< 0.03	0.06	0.06	0.1
S. aureus COL	0.06	0.1	0.1	0.1

Four individual WT thiocillin compounds isolated from *B. cereus* ATCC 14579 were subjected to MIC analysis against *B. subtilis* and two different strains of methicillin-resistant *Staphylococcus aureus* (MRSA). For each strain, all four compounds inhibited growth with similar MICs (**SI Table 3**), validating our method of pooling all compounds produced by each *tclE* mutant strain for analysis of antibiotic activity by both disk diffusion assays and MIC of liquid culture growth inhibition.

(1) Sambrook, J.; Fitsch, E. F.; Maniatis, T., Molecular Cloning. A Laboratory Manual. 3rd ed. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, **2001**.
(2) Brown, L. C.; Acker, M. G.; Clardy, J.; Walsh, C. T.; Fischbach, M. A. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, 106, 2549.
(3) Arnaud, M.; Chastanet, A.; Debarbouille, M. *Appl. Environ. Microbiol.* **2004**, 70, 6887.
(4) Turgeon, N.; Laflamme, C.; Ho, J.; Duchaine, C. *J. Microbiol. Methods.* **2006**, 67, 543.