Native and engineered clifednamide biosynthesis in multiple *Streptomyces* spp.

Yunci Qi[1], Edward Ding[1], Joshua A.V. Blodgett[1]*

[1]Department of Biology, Washington University in St Louis, St Louis, Missouri 63130, United States

*Corresponding author: jblodgett@wustl.edu

ABSTRACT

Polycyclic tetramate macrolactam (PTM) natural products are produced by actinomycetes and other bacteria. PTMs are often bioactive, and the simplicity of their biosynthetic clusters make them attractive for bioengineering. Clifednamide-type PTMs from *Streptomyces* sp. JV178 contain a distinctive ketone group, suggesting the existence of a novel PTM oxidizing enzyme. Here, we report the new cytochrome P450 enzyme (CftA) is required for clifednamide production. Genome mining was used to identify several new clifednamide producers, some having improved clifednamide yields. Using a parallel synthetic biology approach, CftA isozymes were used to engineer the ikarugamycin pathway of *Streptomyces* sp. NRRL F-2890 to yield clifednamides. Further, we observed that strong CftA expression leads to the production of a new PTM, clifednamide C. We demonstrate the utility of both genome mining and synthetic biology to rapidly increase clifednamide production and identify a PTM tailoring enzyme for rational molecule design.

KEYWORDS

Streptomyces, natural products, cytochrome P450, polycyclic tetramate macrolactams, genome mining, metabolic engineering

Actinomycete bacteria are widely studied for their ability to produce diverse bioactive secondary metabolites. Already the source of nearly two-thirds of clinical antibiotics[1], actinomycete genome sequencing has revealed a wealth of previously unrecognized biosynthetic gene clusters[2–4]. With many of these clusters apparently encoding drug-like molecules, these organisms remain as promising sources of much-needed future antibiotics and other therapeutics[5,6].

An unusually high proportion of sequenced actinomycete genomes contain polycyclic tetramate macrolactam (PTM) biosynthetic clusters[7]. PTMs are of therapeutic interest, with family members having documented activity against bacteria, protozoa, fungi, plants, and cancer cell lines[8–11]. In addition to their bioactivity, the relative simplicity and commonality of PTM biosynthetic loci has made them attractive targets for genomics-based discovery and engineering via synthetic biology approaches[12–14]. Despite containing only 3-6 genes, these small clusters encode diverse structures[15] (Figure 1).

The clifednamides are a family of PTMs discovered from *Streptomyces* sp. JV178, an environmental isolate from Connecticut garden soil[16]. The clifednamides have therapeutic potential due to their structural similarity with ikarugamycin, which is active against a wide range of organisms[8,10,11]. The clifednamides are distinguished from ikarugamycin by a ketone group on carbon 29 (Figure 1A). Despite growing research interest in PTMs, no structure-function studies have been carried out thus far. Comparing the bioactivities of the long-studied ikarugamycin and

43 its clifednamide analogs could provide valuable insight. However, efforts to extensively profile
44 clifednamide bioactivities have been limited by low yields from JV178 (Table 1).

45 To understand clifednamide biosynthesis towards engineering its production, JV178 was genome-
46 sequenced (see Methods). Due to the structural similarity of ikarugamycin and clifednamides, we
47 expected JV178 would encode an ikarugamycin-like PTM cluster. Using BLAST, we identified a
48 5-gene PTM locus that likely encodes the clifednamides. Homology analysis of these genes
49 allowed us to propose a plausible clifednamide biosynthetic pathway (Figure S1).

50 Three of the genes, *ikaA*, *ikaB* and *ikaC,* recapitulate the ikarugamycin cluster[12]. As such,
51 clifednamide biosynthesis likely begins with IkaA, an iterative polyketide synthase/non-ribosomal
52 peptide synthase fusion protein. Biochemical studies of its ortholog in HSAF biosynthesis[17]
53 indicate the protein initiates PTM biosynthesis by ligating two polyketide chains, built from six
54 malonyl-CoA precursor units each, to the non-proteinogenic amino acid L-ornithine (Figure S1)[18].
55 The resulting tetramate-polyene product is reductively cyclized by IkaB and IkaC to produce the
56 5-6-5 carbon ring system shared by ikarugamycin and clifednamides[19]. A *ftdA* homolog was also
57 found in the cluster. A PTM hydroxylase common to a number of PTM pathways[7,20,21], *ftdA* is
58 likely responsible for the C25 hydroxyl group of clifednamide B. The remaining open reading
59 frame is encoded between *ikaA* and *ikaB* (Figure 1A). Predicted to encode a novel cytochrome
60 P450, we reasoned its cognate enzyme (designated CftA, for clifednamide tailoring A) may install
61 the C29 ketone of the clifednamides. No additional PTM genes were detected in the JV178
62 genome, further suggesting the *cftA*-containing cluster encodes the clifednamides.

63 JV178 was found to be a poor host for genetic analysis (unpublished), preventing experimental
64 verification of the above model. To further study clifednamide biosynthesis, a genome mining
65 approach was used to identify additional producers. Several actinomycetes having publicly

66 available genome sequences were found to harbor PTM clusters syntenic with the JV178 locus
67 (Figure S2). Four such strains were obtained from the USDA NRRL strain collection. Each strain
68 was grown on a panel of solid media and extracted with ethyl acetate for LC-MS/MS analysis.
69 Clifednamide production was determined by comparison with extracts containing clifednamides
70 A and B from JV178. All four strains appear to produce both compounds based on product
71 retention times, UV absorbance spectra, and mass fragmentation patterns (Figure 2). Notably, *S.*
72 *negayawaensis* produced approximately 26 μM of clifednamides A and B combined, about 10-
73 fold greater than JV178 (Figure 2E, Table 1). The other three strains, *S. purpeofuscus*, *S.* sp. F-
74 6131, and *S. torulosus* produced considerably lower amounts (Figure 2B-D). Interestingly, a local
75 PTM-producing soil isolate (*Streptomyces* sp. KL33) also produced clifednamide A titers
76 comparable to *S. negayawaensis*, but no clifednamide B was detected (Figure 2F).

77 Of the newly obtained clifednamide producers, *Streptomyces*. *sp.* NRRL F-6131 proved to be the
78 most amenable to intergeneric conjugation. This strain was thus used to interrogate clifednamide
79 biosynthesis. A *rpsL*(K43N) mutant was isolated for streptomycin counterselection[22]. As noted for
80 other *Streptomyces* metabolites, this lesion also increased PTM production[23] (Figure 3A&B).
81 Markerless *cftA* deletion resulted in the loss of clifednamide production. However, the strain
82 produced increased amounts of a previously minor compound (*m/z* 479) (Figure 3C). This peak
83 was confirmed to be ikarugamycin by comparison with an authentic standard, and its apparent
84 accumulation is consistent with it being a clifednamide biosynthetic precursor. An additional peak
85 of interest (*m/z* 495) was detected in the Δ*cftA* mutant. This was tentatively identified as
86 butremycin, a known C25-hydroxyl derivative of ikarugamycin[24]. The experimental mass matches
87 the compound and its UV profile is consistent with other PTMs. MS/MS analysis revealed a
88 daughter ion with a *m/z* of 154, consistent with the C25-hydroxylated PTMs such as clifednamide

89 B. Because butremycin is structurally equivalent to clifednamide B lacking the C29 ketone, it is
90 an expected biosynthetic precursor.

91 Our biosynthetic analyses suggested an ikarugamycin-producing microbe could be engineered to
92 produce clifednamide via *cftA* expression. Using genome mining, *Streptomyces* sp. NRRL F-2890
93 was identified as a candidate host. Characterization of the strain revealed it has robust flux,
94 producing up to 28 μM (13.6 mg/L) of ikarugamycin (Figure 4A). A genetic system was
95 established in the strain to systematically evaluate the biosynthetic effects of a panel of CftA
96 isozymes. To do this, four *cftA* orthologs were cloned under two versions of the strong constitutive
97 P*ermE** promoter. While the original *ermE* promoter in *Saccharopolyspora erythraea* begins
98 transcription at the start codon[25], a short 5'-UTR containing a ribosome binding site is often added
99 to P*ermE** expression plasmids. The two plasmids used in this study have P*ermE** promoters with
100 variant 5'-UTR sequences that differentially express a *xylE* reporter (Figure S3)[26].

101 The resulting panel of P*ermE**-*cftA* constructs were chromosomally integrated in F-2890 at the
102 ΦC31 *attB* site[27] following intergeneric conjugation. As expected, the resulting strains all produced
103 clifednamide A (Figures 4, S4). Interestingly, CftA homologs from different strains produced
104 varying amounts of clifednamide A, with the JV178 homolog producing the most (20 μM, Table
105 1). No clifednamide B or butremycin was observed in any of these strains due to *ftdA* being absent
106 in the host. In general, constructs driven by the stronger pHM11a[28]-derived promoter converted
107 more ikarugamycin precursor to clifednamide A than the pDA1652-derived promoter[29].

108 Additionally, all four pHM11a constructs and two pDA1652 constructs resulted in a new peak
109 having a characteristic PTM UV profile and *m/z* 509 (Figures 4, S4). MS/MS fragmentation
110 produced a daughter ion with *m/z* of 139, a diagnostic daughter ion of PTM molecules lacking C25
111 hydroxylation such as clifednamide A, ikarugamycin, and capsimycin[30]. Furthermore, metabolic

112 labeling with $^{2}H_{7}$-L-ornithine results in a correspondingly heavier mass (*m/z* 516) and daughter
113 ion (*m/z* 146). These results parallel our results from clifednamide A (Figure S5). Based on these
114 results, we designated this peak clifednamide C. Its mass difference (*m/z* +30) from ikarugamycin
115 (*m/z* 479) and shorter C18 retention time suggest it has been oxidized twice. The first oxidation
116 presumably corresponds to the C29 keto oxidation by CftA to produce clifednamide A (*m/z* 493).
117 The subsequent oxidation of clifednamide A to clifednamide C is consistent with a hydroxylation
118 (*m/z* +16), perhaps resulting from above-optimal CftA activity. A clifednamide C peak was also
119 detected in *S. negayawaensis*, confirming its production in un-engineered clifednamide producers.

120 The tandem oxidation of clifednamide A by CftA is analogous to the activity of the recently
121 characterized IkaD[30]. A cytochrome P450 associated with some ikarugamycin biosynthetic
122 clusters, IkaD primarily installs an epoxide across carbons 7 and 8. However, it can also
123 hydroxylate C29, the same position targeted for keto insertion by CftA (Figure S1). We noted that
124 *Streptomyces* sp. NRRL F-2890, the ikarugamycin-producing host strain, also contains an IkaD
125 homolog. While F-2890 produces a small peak that is consistent with the mass of ikarugamycin
126 epoxide, this peak remains minor in CftA expression strains (unpublished data). Therefore, CftA
127 apparently lacks the epoxidase activity of IkaD.

128 Presumed CftA orthologs mined from public sequence repositories all share at least 80% amino
129 acid sequence identity, but have no more than 57% residue identity with the IkaD orthologs. Thus,
130 under the cytochrome P450 naming convention[31], CftA and IkaD are members of distinct
131 subfamilies within the CYP107 clade. Aside from IkaD and CftA, additional PTM-locus P450
132 enzymes also fall within the CYP107 group (Figure S6, S7). Beyond CYP107 family P450's, our
133 data also indicates the existence of three other distinct CYP families (<40% identity with CYP107)
134 associated with genome mined PTM-loci. With the largest group being comprised of FtdF

135 homologs (as found in the frontalamide cluster), no role has been established yet for these
136 enzymes. Together, our analyses suggest further investigations into PTM-associated CYPs will
137 continue to reveal enzymes with varied PTM scaffold specificity, oxidative activities and
138 regioselectivity.

139 In conclusion, we used a combination of comparative genomics, genetics, and genome mining to
140 reveal the novel cytochrome P450 CftA is responsible for clifednamide production. We engaged
141 in a synthetic biological approach to engineer clifednamide production by utilizing *cftA* orthologs
142 sourced from newly identified clifednamide producing strains. In addition to increased
143 clifednamide production over *Streptomyces* sp. JV178, we noted substantial differences from *cftA*
144 homologs. As such, this work demonstrates the value of leveraging genome-mined panels of
145 isozymes in a plug-and-play fashion to rapidly identify efficient biocatalysts. Further, we show
146 CftA belongs to an expanding family of PTM cytochrome P450 enzymes with the apparent
147 capability to carry out multiple oxidation events. These enzymes may be useful to engineer
148 diversity-oriented panels of PTMs for future bioactivity and structure-function analyses.

149 **Table 1.** PTM titers from key strains in this study

150

Strain	**Ikarugamycin**	**Clifednamide A**	**Clifednamide B**	**Clifednamide C**
S. sp. JV178	Undetected	0.94 μM	1.87 μM	Undetected
S. sp. NRRL F-2890	28.2 ± 5.27 μM	Undetected	Undetected	Undetected
S. sp. NRRL F-2890 P*ermE**$_{pDA1652}$-*cftA*$_{JV178}$	1.10 ± 0.68 μM	13.73 ± 1.04 μM	Undetected	2.62 ± 0.70 μM
S. sp. NRRL F-2890 P*ermE**$_{pHM11a}$-*cftA*$_{JV178}$	Undetected	19.84 ± 4.86 μM	Undetected	16.56 ± 0.01 μM
S. negayawaensis	Undetected	19.53 μM	6.46 μM	7.20 μM

151 **METHODS**

152 **Strains, Plasmids, Primers, Enzymes, Chemicals and General Methods**

153 Strains, plasmids, and primers are described in Tables S1-3. Several strains were obtained from
154 the Agricultural Research Service Culture Collection (NRRL). All primers were purchased from
155 Integrated DNA Technologies. All restriction enzymes and Taq polymerase were purchased from
156 New England BioLabs. T4 ligase was purchased from New England BioLabs and ThermoFisher.
157 KOD Hot Start DNA Polymerase (EMD Millipore) in FailSafe PCR 2X PreMix (Epicentre) was
158 used to amplify DNA sequences for cloning from *Streptomyces* genomic DNA. Taq polymerase
159 was used for colony PCR. Ikarugamycin (>99%) was purchased from Santa Cruz Biotechnology.
160 *L*-Ornithine-2,3,3,4,4,5,5-d_7 HCl (>98% atom D) was purchased from CDN Isotopes. All other
161 chemicals were obtained from Sigma Aldrich or Fisher Scientific. *Streptomyces* genomic DNA
162 was prepared for PCR by grinding a colony in 100 µL DMSO as described by Van Dessel *et al*[32].
163 Standard protocols for manipulating *E. coli* were based on those of Sambrook *et al*[33].
164 Streptomycetes were routinely propagated on ISP2 agar[34] and Trypticase Soy Broth (Difco) at
165 28˚C. Glass beads were added to liquid cultures to disrupt mycelial clumps.

166 **Genome sequencing of JV178** Genomic DNA was extracted from TSB-grown mycelia as
167 previously described[35]. Illumina 250-bp paired-end sequencing libraries were prepared using the
168 Nextera sample prep kit (Illumina Inc., San Diego, CA, USA) and were sequenced on an Illumina
169 MiSeq platform using V2 chemistry (Illumina, Inc., San Diego, CA, USA) by the Washington
170 University in St Louis Genome Technology Access Center. Sequencing reads were trimmed and
171 *de novo* assembled using the CLC Genomics Workbench (CLC Bio-Qiagen, Aarhus, Denmark).
172 An annotated sequence for the *Streptomyces* sp. JV178 clifednamide cluster was deposited to
173 GenBank (accession no. MF89327).

174 **Streptomycete conjugations.** *S.* sp. F-6131 spores were collected from ISP4 agar[34], while *S.* sp.
175 F-2890 spores were collected from ISP2 agar. Spores were harvested using TX Buffer[36].
176 Conjugations were performed using JV156 as the general *E. coli* donor as previously described[7].
177 Exconjugants were selected with 50 µg/mL colistin and 25 or 50 µg/mL apramycin. Successful
178 conjugations were verified by colony PCR.

179 ***cftA* markerless gene disruption** The *cftA* coding sequence of *S.* sp. F-6131 was replaced with a
180 truncated gene containing the first nine codons and the last ten codons of the wild-type coding
181 sequence with homologous recombination as previously described[37]. Streptomycin-resistant (Str^R)
182 mutants of *S.* sp. F-6131 were isolated on ISP2 + Str^{25} agar. The *rpsL* genes were amplified and
183 sequenced. JV739 bearing the *rpsL* (K43N) mutation was chosen for subsequent experiments as
184 the mutation did not disrupt clifednamide production. The 990 bp upstream flanking region of *cftA*
185 was amplified using primers YQ273 and YQ274 (introduced a *Xba*I site and homology to pUC19).
186 The 1079 bp downstream flanking region of *cftA* was amplified using primers YQ275 (introduced
187 homology to the upstream flanking region) and YQ276 (introduced a *Hind*III site and homology
188 to pUC19). The 2668 bp fragment of pUC19 was amplified with primers YQ268 and YQ269. PCR
189 amplicons were assembled using the NEBuilder HiFi Assembly kit (New England BioLabs). The
190 resulting pUC19-Δ*cftA* was digested with *Xba*I and *Hind*III and the 1956 bp fragment was ligated
191 into pJVD52.1 digested with *Xba*I/*Hind*III. The resulting pJVD52.1-Δ*cftA* was introduced into
192 JV739 by intergeneric conjugation, and apramycin-resistant (Apr^R) exconjugants were selected.
193 Exconjugants were grown in TSB non-selectively at 37˚C and double-recombinants were selected
194 for on ISP2 + Str^{100}. The Δ*cftA* mutants were confirmed by PCR.

195 ***cftA* heterologous expression** The *cftA* homologs were amplified using primers ED9-16
196 (introduced *Nde*I and *Xba*I sites). The PCR products were digested with *Nde*I and *Xba*I and ligated

197 into pJMD2 or pJMD3 to generate plasmids pED1-8. After confirming the inserts by Sanger
198 sequencing (Genewiz), the constructs were introduced into *S.* sp. F-2890 by intergeneric
199 conjugation.

200 **PTM detection by HPLC-MS/MS** Strains were cultivated in 15 mL of TSB liquid medium in
201 125 mL Erlenmeyer flasks shaken in 1-inch orbitals at 250rpm at 28˚C. 6 mm glass beads were
202 added to disrupt mycelial clumps. After 2 days of growth, 200 µL of cultures were plated on HT[38],
203 ISP4, ATCC172, or JBFM1 (adapted from Medium 2[39]: 2% D-fructose; 5% D(+)Mannose;
204 0.167% Na-L-aspartate; 0.06% L-arginine HCl; 0.05% L-histidine HCl; 0.2% K_2HPO_4; 0.2%
205 KH_2PO_4; 0.5% NaCl; 0.006% $ZnSO_4$-$7H_2O$; 0.0256% $MgSO_4$-$7H_2O$; 0.051% $MgCl_2$-$6H_2O$;
206 0.001% $CoCl_2$-$6H_2O$; 0.036% $NaSO_4$; 2.13% MES free acid; 1.5% agar; 2% R2 Trace elements;
207 1.5% Agar; pH 6.0) and incubated at 28˚C. After 6 days, the agar was diced and immersed in ethyl
208 acetate overnight. The ethyl acetate was evaporated at low pressure and the extract was suspended
209 in 400 µL of HPLC-grade methanol and syringe filtered.

210 Analysis was performed using a Phenomenex Luna C18 column (75 x 3 mm, 3 µm pore size)
211 installed on an Agilent 1260 Infinity HPLC connected to an Agilent 6420 Triple-Quad mass
212 spectrometer using the following method: T = 0, 5% B; T = 3, 40% B; T = 13, 60% B; T = 17,
213 100% B, T = 20, 100% B; A: water + 0.1% formic acid, B: acetonitrile + 0.1% formic acid; 0.9
214 mL/min. 10 µL of the methanol-dissolved extract was injected per run. The precursor ion scan
215 mode was used to identify molecules that fragmented (collision energy, 30 V) into daughter ions
216 with m/z of 139.2 or 154.2. The resulting data was analyzed offline with Agilent MassHunter
217 Qualitative Analysis software. PTMs were quantified using integrated peak areas of absorbance at
218 320nm detected with an in-line diode array detector (DAD). A standard curve was generated using
219 an authentic ikarugamycin standard.

220 **ASSOCIATED CONTENT**

221 Supplementary methods, tables, figures, and references

222

223 **AUTHOR INFORMATION**

224 **Author contributions**

225 Y.Q., E.D., and J.A.V.B. designed the experiments and wrote the manuscript. Y.Q. and E.D.
226 performed experiments.

227 **Notes**

228 The authors declare no competing interest

229

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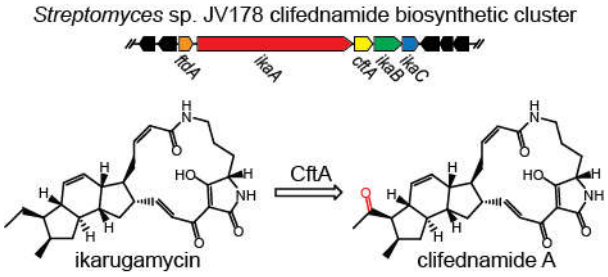
300

301 FIGURES



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303 Graphical abstract

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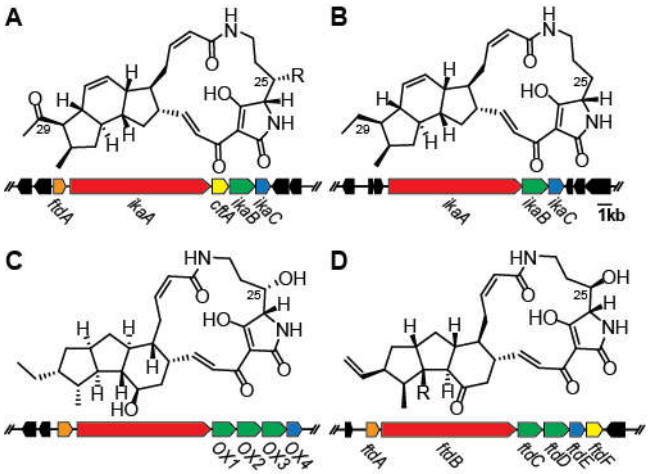

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306 Figure 1. Representative PTM biosynthetic clusters and their products. (A) the *Streptomyces* sp.
307 JV178 clifednamide cluster (A: R=H, B: R=OH); (B) the *Streptomyces* sp. ZJ306 ikarugamycin
308 cluster; (C) The *Lysobacter enzymogenes* C3 HSAF cluster; (D) the *Streptomyces* sp. SPB78
309 frontalamide cluster (A: R=OH, B: R=H). Orange ORFs encode for sterol desaturases, red for
310 iterative polyketide synthase non-ribosomal peptide synthase fusion proteins, green for FAD-
311 dependent oxidoreductases, blue for zinc-dependent alcohol dehydrogenases, and yellow for
312 cytochrome P450s. ORFs in black are not conserved between PTM biosynthetic clusters.

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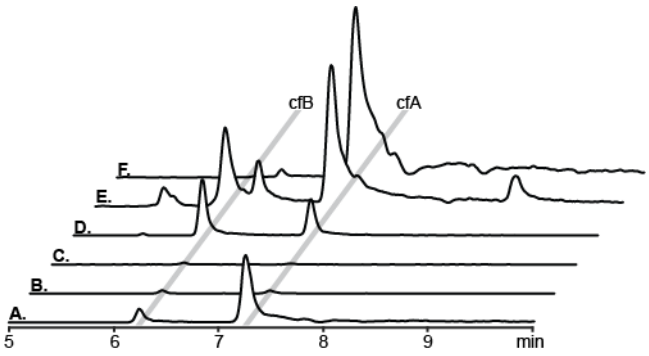

315 Figure 2. Clifednamide production by genome-mined strains. LC-MS/MS chromatograms of
316 extracts from (A) JV178, (B) *S. purpeofuscus*, (C) *S.* sp. F-6131, (D) *S. torulosus*, (E) *S.*
317 *negayawaensis*, and (F) KL33. The retention times for clifednamides A and B (cfA and cfB) are
318 indicated by grey bars.

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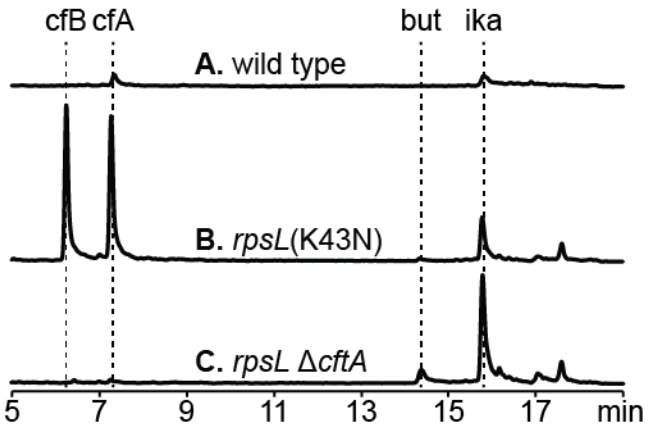
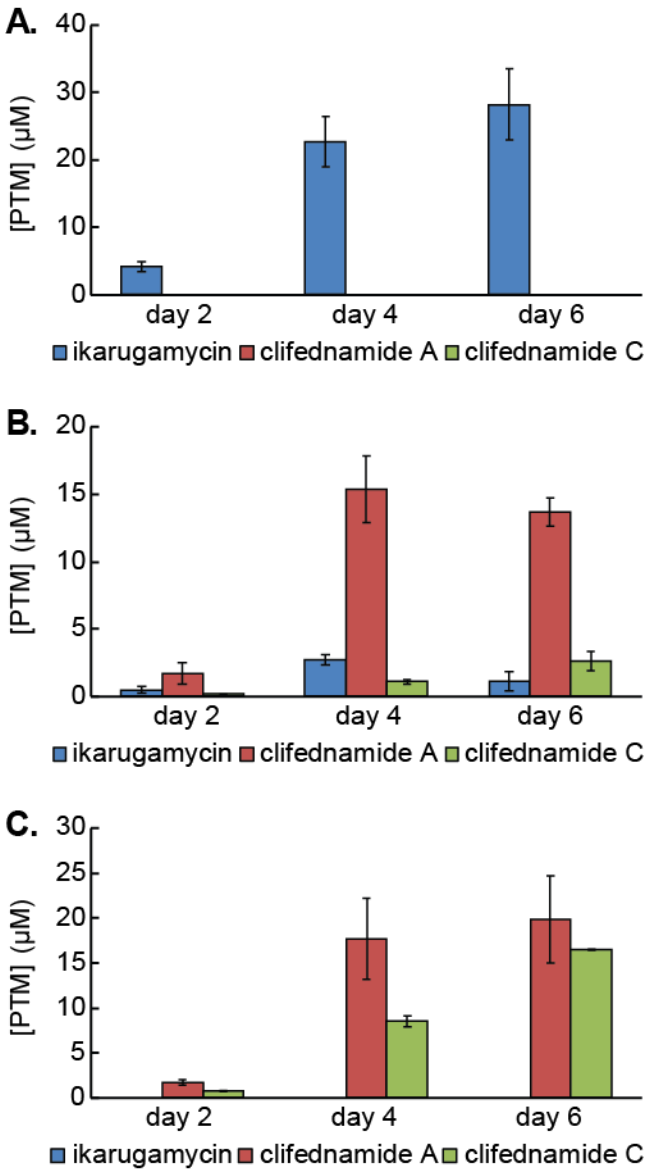


Figure 3. Deletion of *cftA* in *S.* sp. F-6131. LC-MS/MS chromatograms of extracts from (A) wild-type *S.* sp. NRRL F-6131, (B) the *rpsL* (K43N) mutant, and (C) the *rpsL* Δ*cftA* double mutant. The peaks for ikarugamycin (ika), butremycin (but), clifednamide A (cfA), and clifednamide B (cfB) are indicated with dotted lines.



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327 Figure 4. Production of PTMs in *Streptomyces* sp. F-2890 expressing the JV178 CftA. (A) Wild-
328 type F-2890, (B) F-2890 *attB*ΦC31::P*ermE**$_{pDA1652}$-*cftA*$_{JV178}$, and (C) F-2890
329 *attB*ΦC31::P*ermE**$_{pHM11a}$-*cftA*$_{JV178}$. Strains were grown in triplicate and extracted at days 2, 4, and
330 6.

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