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Discovery of a New Family of Dieckmann Cyclases Essential to Tetramic Acid and Pyridone-Based Natural Products Biosynthesis

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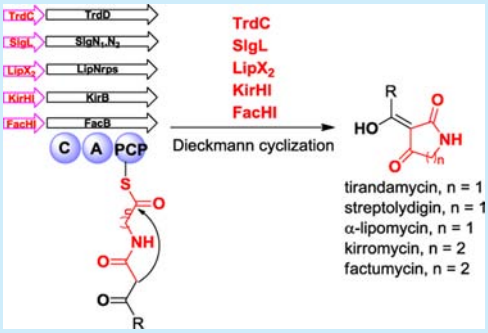
Supporting Information

ABSTRACT: Bioinformatic analyses indicate that TrdC, SlgL, LipX$_2$, KirHI, and FacHI belong to a group of highly homologous proteins involved in biosynthesis of actinomycete-derived tirandamycin B, streptolydigin, α-lipomycin, kirromycin, and factumycin, respectively. However, assignment of their biosynthetic roles has remained elusive. Gene inactivation and complementation, *in vitro* biochemical assays with synthetic analogues, point mutations, and phylogenetic tree analyses reveal that these proteins represent a new family of Dieckmann cyclases that drive tetramic acid and pyridone scaffold biosynthesis.



Natural products containing tetramic acid (pyrrolidine-2,4-dione) and pyridone core scaffolds have been isolated from fungi, bacteria, and sponges and display a wide array of antimicrobial, antitumor, and antiviral activities.[1,2] Of the more than 250 tetramic acid/pyridone-based natural products discovered to date, fewer than 20 have had their biosynthetic machineries rigorously characterized. These agents are typically assembled by hybrid type-I polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) biosynthetic machineries.[1−3] Three paradigms have been used to explain the generation of substituted tetramic acids. These include the following: (i) the action of a reductive domain (R) within the hybrid PKS−NRPS megasynthase terminal module as reflected by the fungal metabolites fusaridione A, cyclopiazonic acid, and preaspridone A, in which the dissected R domains from FsdS[4] (formerly EqiS),[5] didomain TR* from CpaS,[6] and full length ApdA[7] have been shown *in vitro* to catalyze Dieckmann cyclizations en route to the corresponding tetramates (Supporting Information, Figure S1, A); (ii) the action of a hybrid iterative PKS−NRPS megasynthase TE bearing module as highlighted by the bacterial polycyclic tetramic macrolactams (PTM) HSAF,[8] frontalamides,[9] and ikarugamycins,[10] for which the terminal TE domain of Orf6 in the HSAF pathway (possessing both protease and peptide ligase activities) orchestrates tetramic acid formation (Supporting Information, Figure S1, B);[11] and (iii) chemistry performed by products of a conserved but phylogenetically distinct two gene cassette (PyrD$_3$, homologous to various acyltransferase E2 subunits 2-oxoacid acid dehydrogenase, and PyrD$_4$, belonging to α/β-hydrolase superfamily) located within the PKS gene region, catalyzing a Dieckmann cyclization *in vitro* in the bacteria-derived spiro-tetramates pyrroindomycins (Supporting Information, Figure S1, C).[12] Conversely, only one strategy thus far seems to explain pyridone natural products biosynthesis; oxidative ring expansion from the tetramic acid precursor catalyzed by a cytochrome P450 enzyme. Examples of this chemistry include (i) generation of 2-pyridone pretennellin B from pretennellin A as catalyzed by the CYP450 oxidase TenA[13] and (ii) ApdE-catalyzed generation of aspyridone A from preaspyridone (Supporting Information, Figure S1, B).[14]

We report here a new paradigm for biosynthesis of both tetramic acid and pyridone scaffolds. Specifically, we report the discovery and characterization of a family of novel Dieckmann cyclase enzymes, representatives of which generate actinomycete-derived tetramic acid and pyridone natural products in the tirandamycin B (**1**),[15] streptolydigin (**2**),[16] α-lipomycin (**3**),[17] kirromycin (**4**),[18] and factumycin (**5**)[19] biosynthetic pathways (Scheme 1), using a uniform strategy.

The tirandamycin gene cluster has been identified from marine-derived *Streptomyces* sp. SCSIO 1666 and also from *Streptomyces* sp. 307-9.[15] Early investigations revealed that TrdH and TrdK serve as positive and negative regulators of tirandamycin biosynthesis, respectively.[15a] Additionally, TrdE is an atypical glycoside hydrolase responsible for dehydrative installation of the C-11/C-12 double bond,[15c] TrdL is a novel covalently bound FAD-dependent oxidase responsible for

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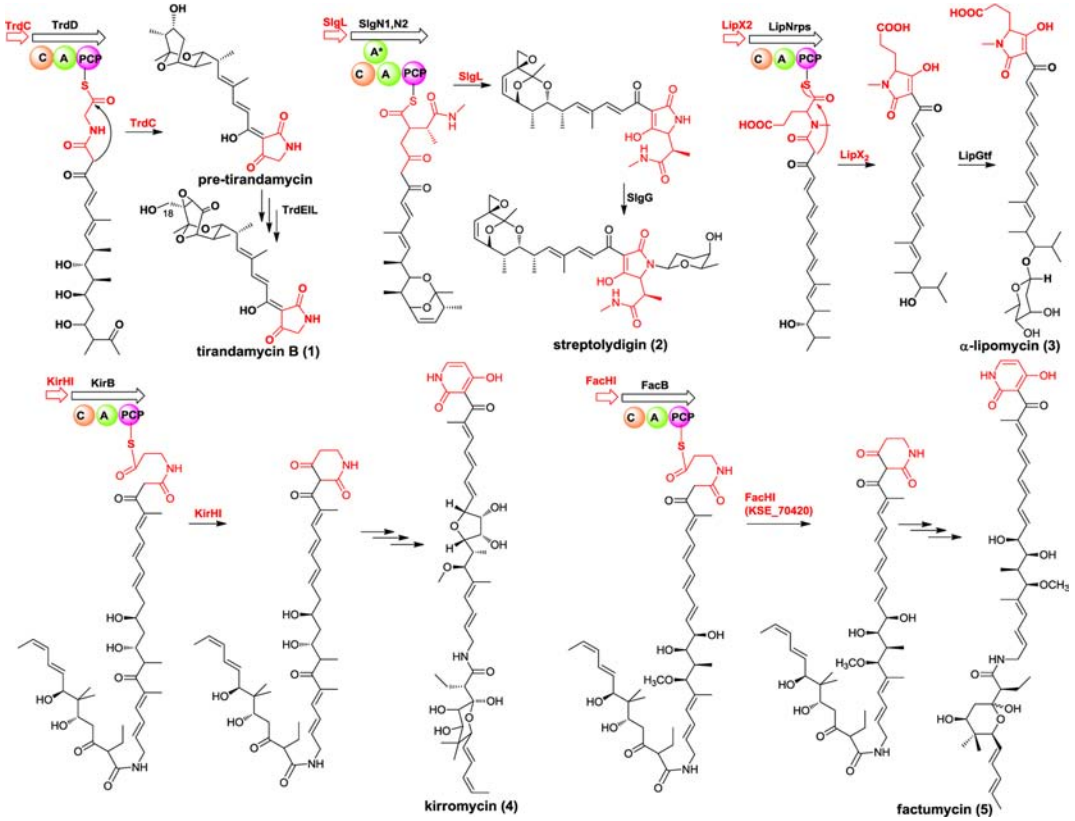
Scheme 1. Conserved Gene Cassettes and Their Putative Roles in the Biosynthetic Pathways for Tirandamycin, Streptolydigin, α-Lipomycin, Kirromycin, and Factumycin



oxidation of the C-10 hydroxy group to its keto form,[15b,e] and TrdI is responsible for C-10 hydroxylation, C-11/C-12 epoxidation, and C-18 hydroxylation.[15a,e]

The absence of both TE and R domains within the tirandamycin PKS–NRPS assembly line has stymied our understanding of tetramic acid generation during tirandamycin biosynthesis. Immediately upstream of the NRPS gene (*trdD*) resides *trdC*; both are transcribed in the same direction. We performed bioinformatics analysis of TrdC and its homologues in the GenBank database; BLAST results revealed that TrdC (276 aa) shows homology to SlgL (similarity 53%/identity 37%) residing immediately upstream of the NRPS gene ($slgN_2$) in the streptolydigin pathway,[16] to $LipX_2$ (similarity 55%/identity 39%) residing immediately upstream of the NRPS gene *lipNrps* in the α-lipomycin pathway,[17] to KirHI (similarity 58%/identity 45%) residing immediately upstream of the NRPS gene *kirB* in the kirromycin pathway,[18] and to KSE_70420 (similarity 55%/identity 41%) residing immediately upstream of the NRPS gene *facHI* in the factumycin pathway.[19] Intriguingly, streptolydigin, α-lipomycin, kirromycin, and factumycin all belong to a group of 3-acyltetramic acid/pyridone natural products isolated from four other actinomycetes; their biosynthetic assembly lines have been identified, but none have been found to possess TE or R domains. Consequently, we reason that this group of genes, highly conserved both in terms of sequence homology and localization within the gene cluster, are responsible for tetramic acid/pyridone core structure release from the assembly line (Scheme 1).

To determine whether TrdC plays a role in tirandamycin biosynthesis, we inactivated *trdC* by gene replacement with the apramycin gene cassette using λ-RED recombination technology to yield the Δ*trdC* mutant. The mutant was identified and confirmed on the basis of its kanamycin sensitive and apramycin resistant phenotype and further validated by PCR (Figure S2, Supporting Information). The Δ*trdC* mutant was fermented, and the resulting broth extracted with butanone; HPLC analysis of the extract revealed that *trdC* inactivation ablated tirandamycin B (**1**) production. Complementation of the Δ*trdC* mutant *in trans* was undertaken by cloning *trdC* into our modified pSET152AKE vector.[20] Introduction of the modified vector into the Δ*trdC* mutant by conjugation yielded the Δ*trdC:trdC* mutant. Fermentation of and subsequent metabolite analysis for Δ*trdC:trdC* revealed restoration of tirandamycin B production (Figure 1), thus validating the indispensability of TrdC activity for tirandamycin B biosynthesis. These findings parallel previous studies involving *slgL* in the streptolydigin pathway; an engineered Δ*slgL* mutant unable to produce streptolydigin was amenable to *in trans slgL* complementation that restored streptolydigin production to the

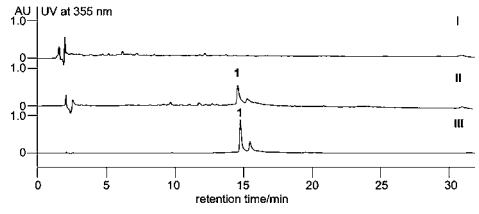


Figure 1. HPLC analysis of fermentation broth of (I) Δ*trdC* mutant strain; (II) complementary Δ*trdC:trdC* strain; (III) *Streptomyces* sp. SCSIO1666. **1** represents tirandamycin B.

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Δ*slgL* mutant strain.[16b] These results have also been verified independently in our laboratory using a different inactivation/complementation system (Figure S4, Supporting Information). These data collectively demonstrate that *trdC* and *slgL* are absolutely required for tirandamycin and streptolydigin biosyntheses, respectively.

We subsequently synthesized biosynthetic intermediate mimics to evaluate the *in vitro* biochemical activities of representatives of this family of enzymes. Acetoacetyl Gly-, acetoacetyl Ala-,[5] and acetoacetyl *β*-Ala- were each attached as thioesters to *N*-acetylcysteamine (NAC); the identities of the resultant enzyme substrate mimics *N*-acetoacetyl glycyl-SNAC (**8a**), *N*-acetoacetyl L-alanyl-SNAC (**9a**), and *N*-acetoacetyl *β*-alanyl-SNAC (**10a**) were confirmed by MS and ^{1}H and ^{13}C NMR data. Correspondingly, *trdC*, *slgL*, and *kirHI* were each cloned into the pET28a(+) vector, overexpressed in *Escherichia coli* BL21 (DE3), and their products purified to homogeneity to yield soluble *N*-terminus His_6-tagged proteins.

Enzymatic reactions with substrate mimics were performed in the presence of 50 mM Tris−HCl (pH 7.0), 1 mM substrate (**8a**, **9a**, or **10a**), 5 *μ*M enzyme (TrdC, SlgL, or KirHI), and revealed that TrdC, SlgL, and KirHI rapidly converted **8a** and **9a** to tetramic acid compounds **8b** and **9b**, respectively (Figure 2). Conversely, although the conversion of **10a** to **10b**$_1$/**10b**$_2$

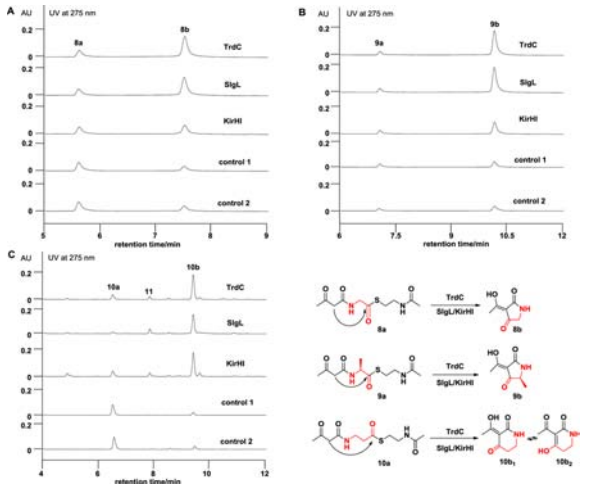

Figure 2. *In vitro* TrdC-, SlgL-, and KirHI-catalyzed transformations of **8a**, **9a**, and **10a** to **8b**, **9b** and **10b**$_1$/**10b**$_2$, respectively. A and B, at 28 °C for 1 h; C, at 37 °C for 12 h. Control 1, no enzyme added; control 2, trypsin-digested enzymes. **11**, dimer of *N*-acetylcysteamine.

was observed, the efficiency of this enzymatic transformation was apparently quite low relative to those involving substrate mimics **8a** and **9a** (Figure 2). Despite this, these data clearly demonstrate the ability of TrdC, SlgL, and KirHI to serve as Dieckmann cyclases. Moreover, these findings imply that these enzymes drive formation of tetramic acid or pyridone scaffolds in the corresponding antibiotic biosynthetic pathways.

We next phylogenetically analyzed the TrdC family of proteins with the NRPS R or R* domains from fungal natural product biosynthetic pathways.[5−7] Similar analyses were carried out for terminally embedded TE domains in hybrid PKS−NRPSs for PTM natural product biosynthetic pathways[21,22] and for PyrD3 and PyrD4 within the pyrroindomycin biosynthetic pathway;[12] the ability of these proteins to catalyze Dieckmann cyclization chemistry has been validated. Notably, TrdC and its homologues are phylogenetically distinct from these previously reported proteins (Figure 3). Additionally, *in vitro* biochemical assays (Figure 2) clearly indicate that KirHI represents a new

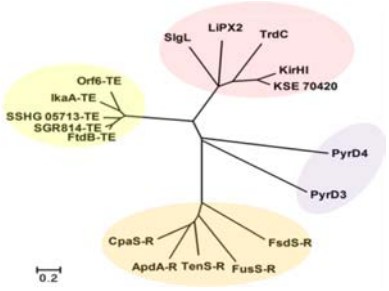

Figure 3. Unrooted phylogenetical tree of TrdC-type protein, TEs involved in PTM antibiotics biosynthesis, PyrD4 and PyrD3 involved in spiro-tetramate and pyrroindomycin synthesis, and R domain involved in biosynthesis of pyridone compounds originated from fungi.

and unique catalyst for generating 2-pyridone core scaffolds in bacterially derived pyridone-based natural products. Notably, the KirHI system contrasts those involved in the biosynthesis of the 2-pyridone scaffold found in the aspyridones,[14] tenellin,[13] and demethylbassianin.[23]

To better understand the reaction mechanism catalyzed by TrdC and its homologous proteins, we analyzed the secondary structure of TrdC using HHblit methods.[24] To our surprise, TrdC showed the greatest similarity to a variety of TE domains (Figure S6, Supporting Information) involved in fatty acid synthase-,[25] PKS-, and NRPS-derived natural products including fengycin,[26] erythromycin,[27] and tautomycetin.[28] For the TE-catalyzed reaction, the full-length acyl/peptidyl moieties are required for transfer to a conserved serine residue affording the transient oxoester. Additionally, the typical catalytic triad of Ser-Asp-His characteristic of these TEs has been validated by X-ray crystallography.[26−28] Within the TrdC family of proteins, and in the same corresponding positions, this conserved motif is replaced with a Cys_{88}-Asp_{115}-His_{253} triad. To explore how this Cys for Ser substitution in the TrdC may affect TrdC function, we converted Cys_{88} to Ser_{88} using site-directed mutagenesis. The C88S mutant TrdC was overexpressed in *E. coli* BL21 (DE3), purified, and employed in *in vitro* enzymatic assays using **8a** as a substrate. HPLC analyses of these reactions revealed that, contrary to its anticipated hydrolytic activity, the C88S TrdC retained Dieckmann cyclase activity as ascertained by comparison to reactions of **8a** with native TrdC (Figure S7, Supporting Information).

Finally, we investigated the biochemical roles of the Cys_{88}-Asp_{115}-His_{253} residues composing the postulated catalytic triad of TrdC by making point mutations and carrying out subsequent in trans complementations with the Δ*trdC* mutant. In addition, the roles of 13 other conserved (mostly polar) amino acid residues in TrdC-family homologous proteins were investigated by mutation to nonpolar and nonreactive residues (Figure S8, Supporting Information). Consequently, 16 *trdC* point mutants were generated and each introduced into the Δ*trdC* mutant strain. Subsequent HPLC-based metabolite analyses revealed that mutant strains harboring W50F, W70F, Q105I, E143L, R182L, D211A, W216F, S224A, and D225A mutations restored tirandamycin B (**1**) production capabilities to the Δ*trdC* strain; tirandamycin B was absent in similar analyses of fermentations with strains possessing R29L, D23A, F34A, C88A, D115A, E192L, and H253F mutations (Figure

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S10, Supporting Information). Thus, the three N-terminal residues R29, D23, and F34, the three residues C88, D115, and H253 corresponding to the catalytic triad in the TE domain, as well as the E192 residue within the TrdC protein are essential to TrdC activity.

In summary, we have found that the hypothetical protein TrdC is essential to tirandamycin biosynthesis. *In vitro* biochemical assays with purified proteins demonstrated that TrdC and its homologues SlgL and KirHI function as Dieckmann cyclases capable of catalyzing formation of both tetramic acid and pyridone scaffolds. This is the first report that the highly conserved new family of proteins represented by TrdC, SlgL, $LipX_2$, KirHI, and FacHI catalyze formation of tetramic acid and pyridone moieties during secondary metabolism by means of Dieckmann cyclization chemistry. Importantly, the TrdC-type motif is becoming increasingly prevalent among actinobacteria as reflected by review of the GenBank database. Many gene clusters displaying this Dieckmann cyclase motif remain silent in their host strains (Figure S11, Supporting Information). Consequently, these homologues might serve as effective genetic markers enabling the identification of new tetramic acid and/or 2-pyridone-based natural products using genome mining approaches.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures, NMR data, and spectra for synthetic and enzymatic products. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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