Nine Enzymes Are Required for Assembly of the Pacidamycin Group of Peptidyl Nucleoside Antibiotics

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S *Supporting Information*

ABSTRACT: Pacidamycins are a family of uridyl peptide antibiotics that inhibit the translocase MraY, an essential enzyme in bacterial cell wall biosynthesis that to date has not been clinically targeted. The pacidamycin structural skeleton contains a doubly inverted peptidyl chain with a β-peptide and a ureido linkage as well as a $3'$-deoxyuridine nucleoside attached to $DABA_3$ of the peptidyl chain via an enamide linkage. Although the biosynthetic gene cluster for pacidamycins was identified recently, the assembly line of this group of peptidyl nucleoside antibiotics remained poorly understood because of the highly dissociated nature of the encoded nonribosomal peptide synthetase (NRPS) domains and modules. This work has identified a minimum set of enzymes needed for generation of the pacidamycin scaffold from amino acid and nucleoside monomers, highlighting a freestanding thiolation (T) domain (PacH) as a key carrier component in the peptidyl chain assembly as well as a freestanding condensation (C) domain (PacI) catalyzing the release of the assembled peptide by a nucleoside moiety. On the basis of the substrate promiscuity of this enzymatic assembly line, several pacidamycin analogues were produced using in vitro total biosynthesis.

Pacidamycins are members of a large class of uridyl peptide antibiotics that also includes mureidomycins, napsamycins, and sansanmycins.[1,2] They are sufficient structural mimics of the UDP-*N*-acetylmuramoyl pentapeptide intermediate in the bacterial cell wall assembly to inhibit the translocase MraY, a clinically unexploited target in the development of new antibacterial drugs.[3] Elucidation of the enzymatic mechanism for the pacidamycin scaffold assembly will therefore facilitate the generation of new MraY-targeted peptidyl nucleoside antibiotics through combinatorial biosynthesis.

More than 10 related pacidamycin family compounds have been isolated from *Streptomyces coeruleorubidus*,[1] all of which share a common structural skeleton having a (2*S*,3*S*)-diaminobutyric acid (DABA) residue serving as a connection point for the $3'$-deoxyuridine moiety via a $4',5'$-enamide linkage. A ureido dipeptide (Ala₄-Phe/Trp/*m*-Tyr₅) is attached to the α-amino group of DABA at the C-terminus, and a single amino acid

(Ala/*m*-Tyr₂), a bicyclic heterocycle, or a dipeptide (Ala/Gly₁-*m*-Tyr₂) is linked to the β-amino group of DABA at the N-terminus. Thus, in the pacidamycin framework, the tetra/pentapeptidyl chain reverses direction twice, at Ala/*m*-Tyr₂-DABA₃ and at Ala₄-Phe/Trp/*m*-Tyr₅ via a β-peptide and a ureido linkage, respectively (Figure 1). All of these nonstandard connectivities suggest novel chemical logic and enzymatic machinery for assembly of the pacidamycin scaffold.

The biosynthetic gene cluster for pacidamycins was reported recently,[4,5] allowing for the heterologous production of pacidamycin D/S, the uridyl tetrapeptides containing a single N-terminal Ala tethered to the β-amino group of DABA (Figure 1). Highly dissociated nonribosomal peptide synthetase (NRPS) modules were encoded, presenting a challenge in dissecting the peptidyl core assembly (Figure S1 in the Supporting Information). We characterized the substrate specificities of several encoded adenylation (A) domains (PacLOP) and reconstituted the formation of the C-terminal ureido dipeptide (Ala₄-Phe/Trp/*m*-Tyr₅) using PacJLNO.[5] In this work, the functions of six additional proteins (PacDHIUVW) have been defined by in vitro characterization, and a picture of the complete assembly line for generation of the unusual uridyl tetrapeptide scaffold has been provided.

PacP has previously demonstrated a strong preference for the activation of L-2,3-diaminopropionate (DAP), indicating that it would be the DABA activation enzyme.[5] Therefore, (2*S*,3*S*)- and (2*R*,3*R*)-DABA were chemically synthesized using the published methods[6] (Figure S2) and tested in the ATP−[³²P]PP$_i$ exchange assay. As expected, PacP exhibited a preference for reversible formation of (2*S*,3*S*)-DABA-AMP; no substantial activation of (2*R*,3*R*)-DABA was detected, validating the stereoselectivity of PacP (Figure 2a). PacV, a standalone methyltransferase encoded by the gene cluster, was predicted to catalyze the N-methylation of DABA. The purified PacV indeed transferred the methyl group from *S*-adenosylmethionine (SAM) to holo-PacP (∼90 kD), as demonstrated by SDS-PAGE autoradiography, only after DABA had been activated by the A domain of PacP and presumably loaded onto the in cis thiolation (T) domain (Figure 2b). No methylation of free DABA/DAP catalyzed by PacV could be
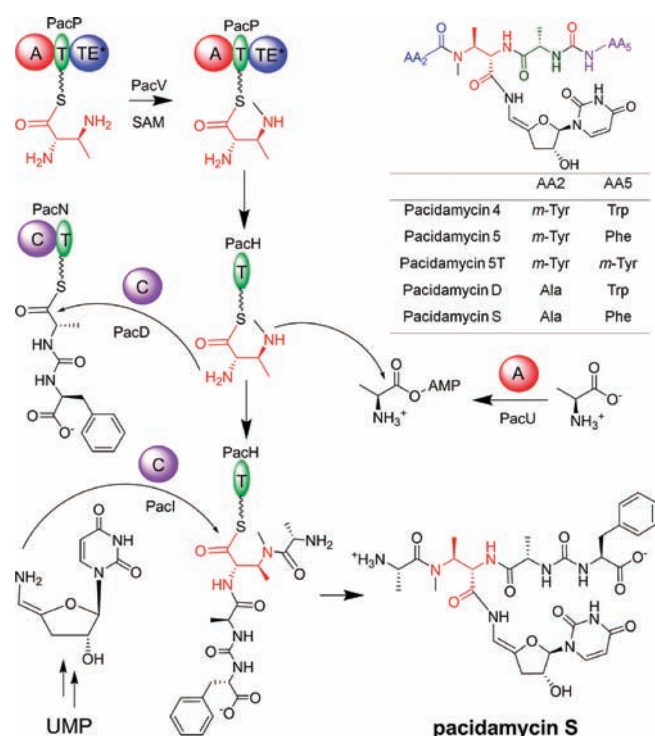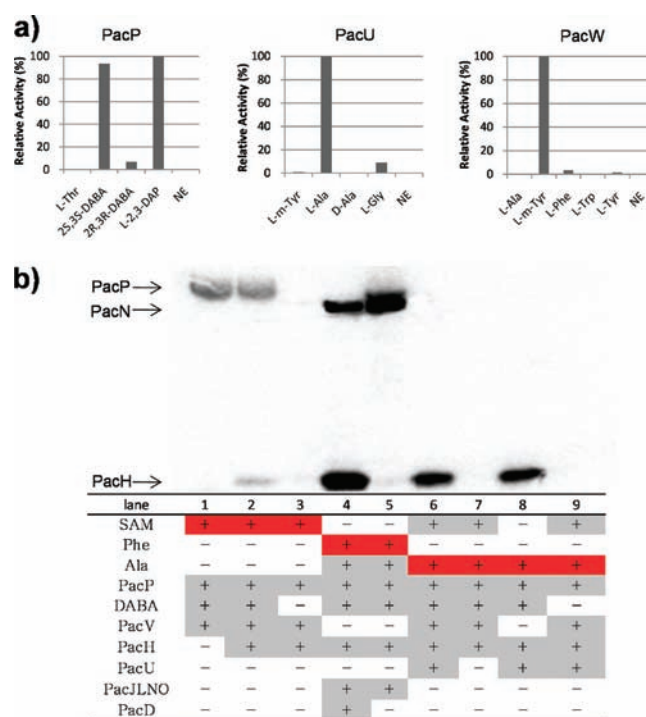
Figure 1. Structures of selected pacidamycins and proposed biosynthetic scheme for pacidamycin S. Domain notation: T, thiolation; A, adenylation; C, condensation; TE, thioesterase.

Figure 2. Characterization of NRPSs. (a) A-domain activities of PacP, PacU, and PacW. Abbreviations: NE, no enzyme; DABA, diaminobutyric acid; DAP, diaminopropionate; m-Tyr, m-Tyrosine (DL). A relative activity of 100% for L-2,3-DAP-, L-Ala-, and m-Tyr-dependent exchange corresponds to 282k, 70k, and 141k cpm, respectively. (b) Autoradiograph of one SDS-PAGE gel illustrating the covalent loading of ^{14}C-labeled substrate (highlighted in red). Enzymes and substrates used in each lane are indicated in the table (highlighted in gray).

detected by liquid chromatography–mass spectrometry (LC–MS), and the preincubation of DABA/DAP with PacP significantly increased the initial rate of methylation (Figure S3), indicating that PacP-tethered DABA could be the natural substrate for the SAM-dependent methyltransferase PacV to install a methyl moiety on the β-amino group of DABA.

Upon the addition of a freestanding holo-T protein (PacH, ~10 kD), the radioactive $[^{14}C]CH_3$ derived from SAM was transferred from PacP to PacH (Figure 2b). The PacH homologue from *Streptomyces roseosporus* (88% similarity), which was also purified in holo form, exhibited higher loading efficiency and was used throughout subsequent studies. Transfer of aminoacyl groups between T domains to facilitate subsequent tailoring has been observed previously, promoted by an acyltransferase of the α,β-hydrolase family, such as CmaE in coronamic acid biosynthesis.[7] However, the thioesterase (TE) domain of PacP lacks the typical catalytic triad of this family, and no obvious in trans acyltransferase candidate could be found encoded in the gene cluster; the aminoacyl moiety may be shuttled between the *HS*-pantetheinyl prosthetic groups of PacP and PacH by spontaneous transthiolation. In addition, transfer of the DABA moiety was not dependent on the N-methylation, as DABA-*S*-PacH could be formed and detected by Fourier transform MS (FTMS) (Figure 3a).

We then probed the mechanism for attachment of the N-terminal Ala to the β-amino group of thioester-tethered DABA in pacidamycin D/S biosynthesis. PacU, a standalone A-domain protein, was indicated to be specifically related to the activation of N-terminal Ala by targeted gene disruption, although it failed to activate L-Ala in vitro in our initial trials.[5] PacU was later repurified from *Escherichia coli* and exhibited a strong preference toward reversible formation of L-Ala-AMP (Figure 2a), correlating

well with the in vivo PacU disruption result. L-$[^{14}C]$Ala was further used in the loading assays to trace the covalent aminoacyl-*S*-thiolation intermediate. Radioactivity migrated with PacH but not PacP and was completely dependent on the presence of PacU and DABA (Figure 2b). These results suggested that Ala was condensed to DABA-*S*-PacH but not DABA-*S*-PacP after activation as Ala-AMP by PacU. The radioactivity signal remained the same in the absence of methyltransferase PacV and SAM, indicating that N-methylation of DABA is not a prerequisite for the aminoacylation of DABA-*S*-PacH by Ala. Indeed, the formation of Ala$_2$-DABA$_3$-*S*-PacH was confirmed by FTMS (Figure 3b). PacV and SAM were then excluded in the following assays to simplify the in vitro reconstitution system. It is notable that we also identified a digene cassette (designated *pacWX*) on the genome of *S. coeruleorubidus* (GenBank accession no. HQ874646) encoding a standalone A-domain protein and a phenylalanine hydroxylase. PacW shows high sequence similarity to PacU (87% similarity) and upon purification demonstrated a strong preference for the activation of m-Tyr over other aromatic amino acids (Figure 2a). PacW is therefore hypothesized to be involved in the activation of N-terminal m-Tyr found in pacidamycin 4/5/5T (Figure 1).
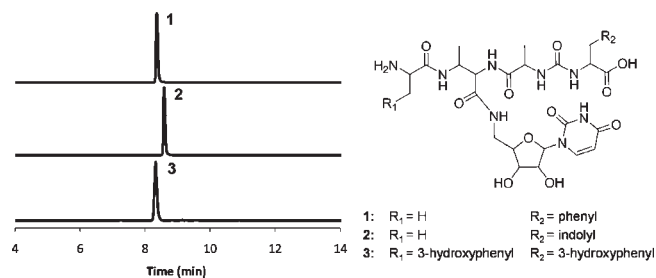
The enzymatic mechanism and timing of the incorporation of the ureido dipeptide onto the α-amino group of DABA was also investigated using SDS-PAGE autoradiography and FTMS. Our prior work showed that the ureido dipeptide (Ala$_4$-Phe/Trp/m-Tyr$_5$) could be formed and tethered on the pantetheinyl arm of PacN through the activity of PacJLNO.[5] When PacN-*S*-Ala$_4$-CO-$[^{14}C]$Phe$_5$ and DABA-*S*-PacH were combined, ^{14}C-labeled
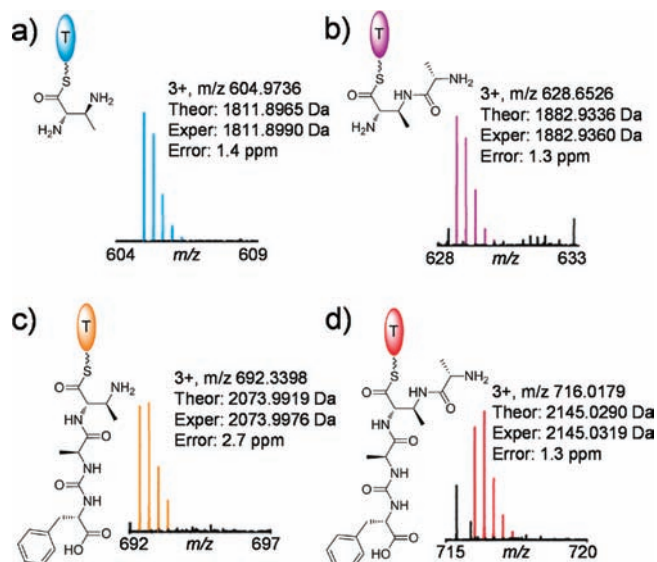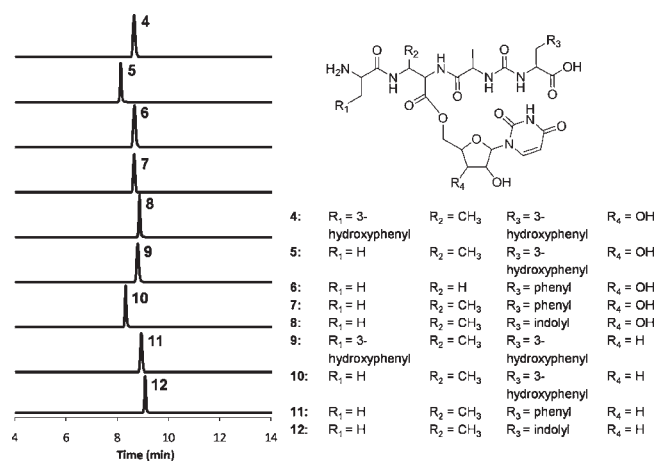
Figure 3. Detection of PacH-bound biosynthetic intermediates by FTMS after trypsin digestion. ATP, L-Phe, L-Ala, DABA, and PacPH were present in all assays. PacU was present in assays (b) and (d), while PacDJLNO were present in assays (c) and (d). Theoretical mass calculations are shown in Figure S4.

Figure 4. Extracted ion chromatograms showing production of pacidamycin analogues using 5′-aminouridine as an offloading substrate. The calculated mass with 10 ppm mass error tolerance was used. The assay components are shown in Table S2; the high-resolution MS (HR-MS) and HR-MS/MS analyses of each compound are shown in Figures S6−S8. The MS/MS fragments confirmed the attachment of the uridine moiety to DABA$_3$ carbonyl through an amide linkage as well as the regiospecific modifications of the DABA$_3$ amino group (Nα vs Nβ).

Figure 5. Extracted ion chromatograms showing the production of pacidamycin ester analogues. The calculated mass with a 10 ppm mass error tolerance was used. The assay components are shown in Table S2; the HR-MS and HR-MS/MS analyses of each compound are shown in Figures S9−S18.

L-Phe was transferred from PacN ($\sim$63 kD) to PacH only in the presence of a standalone condensation (C)-domain protein PacD (Figure 2b). The PacH-S-DABA$_3$-Ala$_4$-CO-Phe$_5$ species was further detected by FTMS (Figure 3c), confirming that PacD catalyzes the nucleophilic attack of the α-amino group of DABA on the PacN-tethered thioester of the C-terminal ureido dipeptide. No radioactive L-Phe was transferred to DABA-S-PacP, suggesting that PacH, not PacP, was one of the cognate loading domains for PacD recognition. It could also be deduced that the aminoacylation of the α- and β-amino groups of DABA could take place independently.

The tetrapeptidyl thioester of PacH (Figure 3d) was stable under incubation conditions, presumably awaiting offloading to the 5′-amino group of a deoxyuridine species to generate pacidamycins. PacI, the remaining standalone C-domain protein ($\sim$47 kD), was anticipated to release the tetrapeptidyl chain from the thioester linkage on PacH by a uridine derivative. Because 3′-deoxy-4′,5′-enaminouridine was not available, 5′-aminouridine was chemically synthesized[8] and tested as a surrogate substrate in the in vitro assays to probe the function of PacI. In fact, PacI catalyzed the offloading of the tetrapeptidyl chain from PacH by 5′-aminouridine via an amide linkage (Figure 4 and Figures S6−S8 and S19). PacI shows low sequence similarity to any other known proteins and is the first identified C-domain protein shown to condense peptide and nucleoside substrates. In addition, PacI could also utilize uridine and 3′-deoxyuridine as alternative substrates, forming an oxoester rather than the natural amide linkage (Figure 5 and Figures S9−S18). The relaxed substrate specificities of PacI together with other catalytic domains in the assembly line could facilitate the enzymatic synthesis of pacidamycin analogues. For example, nine analogues (**1**−**2**, **5**−**8**, and **10**−**12**) varied at the C-terminal aromatic amino acid, central diamino acid, and uridine moieties were generated in vitro using the nine proteins PacDHIJLNO-PU (Figures 4 and 5). In addition, when PacU was replaced by PacW, only products with N-terminal m-Tyr (**3**−**4**, **9**) were

formed (Figure 4 and 5), confirming the orthogonal functions of PacU and PacW in the activation of N-terminal Ala and m-Tyr, respectively.

In summary, we have dissected the distributed 10-protein assembly line that builds the scaffold of tetrapeptidyl nucleoside pacidamycin antibiotics. The trifunctional (2S,3S)-DABA is the central building block, and it is activated by and loaded onto PacP, methylated by PacV, and transferred to PacH. The tetrapeptide framework is then assembled on PacH, perhaps reflecting the participation of that 10 kD T-domain protein scaffold in recognition by the downstream enzymes. The assembly includes attachment of the N-terminal Ala (activated by PacU) or m-Tyr (activated by PacW) to the β-amino group of DABA and attachment of the C-terminal ureido dipeptide (formed by PacJLNO) to the α-amino group of DABA promoted by PacD. Finally, PacI catalyzes the release of the tetrapeptidyl intermediate from PacH by uridines (Figure 1 and Figure S20). Our work provides the basis for rational reprogramming of various uridyl peptide antibiotic assembly lines, including pacidamycins, mureidomycins, and napsamycins, as the recently identified biosynthetic gene

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cluster for napsamycins encodes the highly homologous discrete NRPSs.[9] We will next scale up the in vitro total biosynthesis of various pacidamycin analogues to obtain material to test for antibiotic activities and evaluate structure—activity relationships.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Experimental procedures, SDS-PAGE analysis of purified proteins, synthesis of DABA and amino-uridine, methylation time course, MS calculation of PacH-bound biosynthetic intermediates, compound characterizations, and biosynthetic pathway schemes. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

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