Identification of the biosynthetic gene cluster for the pacidamycin group of peptidyl nucleoside antibiotics

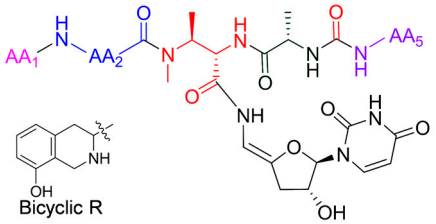
Wenjun Zhang[a,b], Bohdan Ostash[c,d], and Christopher T. Walsh[a,1]

[a]Department of Biological Chemistry and Molecular Pharmacology and [c]Department of Microbiology and Moledular Genetics, Harvard Medical School, Boston, MA 02115; [b]Department of Chemical and Biomolecular Engineering, University of California, Berkeley, CA 94720; and [d]Department of Genetics and Biotechnology, Ivan Franko National University of L'viv, L'viv 79005, Ukraine

Contributed by Christopher T. Walsh, August 3, 2010 (sent for review July 28, 2010)

Pacidamycins are a family of uridyl tetra/pentapeptide antibiotics that act on the translocase MraY to block bacterial cell wall assembly. To elucidate the biosynthetic logic of pacidamcyins, a putative gene cluster was identified by 454 shotgun genome sequencing of the producer *Streptomyces coeruleorubidus* NRRL 18370. The 31-kb gene cluster encodes 22 proteins (PacA-V), including highly dissociated nonribosomal peptide synthetase (NRPS) modules and a variety of tailoring enzymes. Gene deletions confirmed that two NRPSs, PacP and PacO, are required for the biosynthesis of pacidamycins. Heterologous expression and in vitro assays of PacL, PacO, and PacP established reversible formation of *m*-Tyr-AMP, L-Ala-AMP, and diaminopropionyl-AMP, respectively, consistent with the amino acids found in pacidamycin scaffolds. The unusual Ala_4-Phe_5 dipeptidyl ureido linkage was formed during in vitro assays containing purified PacL, PacJ, PacN, and PacO. Both the genetic and enzymatic studies validate identification of the biosynthetic genes for this subclass of uridyl peptide antibiotics and provide the basis for future mechanistic study of their biosynthesis.

adenylation | ureido-bond | uridine



	AA1	AA2	AA5
Pacidamycin 1	Ala	*m*-Tyr	Trp
Pacidamycin 2	Ala	*m*-Tyr	Phe
Pacidamycin 3	Ala	*m*-Tyr	*m*-Tyr
Pacidamycin 4	-	*m*-Tyr	Trp
Pacidamycin 5	-	*m*-Tyr	Phe
Pacidamycin 5T	-	*m*-Tyr	*m*-Tyr
Pacidamycin 6	Gly	*m*-Tyr	Trp
Pacidamycin 7	Gly	*m*-Tyr	Phe
Pacidamycin D	-	Ala	Trp
Pacidamycin 4N	-	Bicyclic R	Trp

Fig. 1. Structures of pacidamycins.

Pacidamycins are a family of uridyl tetra/pentapeptide antibiotics isolated from *Streptomyces coeruleorubidus*. Since their discovery in 1989, at least 10 related compounds have been reported (1, 2), which share a common structural skeleton with a 3′-deoxyuridine nucleoside attached to an *N*-methyl 2,3-diaminobutyric acid (DABA) residue via a 4′,5′-enamide linkage. Attached to the α-amino of DABA is L-Ala, which is linked to the *C*-terminal aromatic amino acid via a ureido linkage. An *N*-terminal amino acid or dipeptide is attached to the β-amino of DABA to give the tetra/pentapeptide framework (Fig. 1). Similar to many other uridyl peptides and uridyl lipopeptides, including liposidomycins (3), tunicamycins (4), capuramycins (5, 6), muraymycins (7), and more closely related mureidomycins (8) and napsamycins (9), pacidamycins exhibited antimicrobial activity targeting the translocase MraY to block formation of lipid I from UDP-*N*-acetylmuramoyl-pentapeptide and undecaprenyl phosphate during bacterial cell wall assembly (Fig. S1) (10, 11). The uracil-ribose moiety is a key determinant of binding to the MraY target (10).

We have begun to focus on the biosynthesis of pacidamycin family uridyl peptide antibiotics due to their three unusual structural features (Fig. 1). The first one is the presence of the nonproteinogenic amino acids L-*meta*-tyrosine (L-*m*-Tyr) and DABA, which suggests that nonribosomal peptide synthetase (NRPS) modules might be involved in the tetra/pentapeptide framework formation. The second feature is that the peptide chain direction reverses twice during assembly. As exemplified in pacidamycin 1, the peptide bond between *m*-Tyr_2 and $DABA_3$ is a β- rather than an α-peptide linkage. Consequently, the $DABA_3$-Ala_4 peptide bond uses the α-amino of DABA moiety and the chain is reversed at Ala_4. Next, the attachment of that Ala_4 to Trp_5 involves a ureido linkage, which constitutes a second chain direction reversal such that Tyr_5 has a free *C*-terminal carboxylate. Our laboratory has recently established that an NRPS module in syringolin biosynthesis catalyzes such a ureido-bond formation (12). The third remarkable feature of the pacidamycin scaffold is the nature and attachment mode of the uracil-based nucleoside to the peptide chain. The carboxyl moiety of DABA, rather than being utilized for a normal peptide linkage in chain extension (for example, linked to the amino group of Ala_4), is tethered as an amide but a special one: the *cis*-enamide of a 3′-deoxy-4′,5′-enaminouridine. Thus the DABA residue serves both as a branching element and a connection point for the unusual uridine moiety.

The biosynthesis of peptidyl nucleoside antibiotics has been actively pursued recently for the possible development of previously undescribed classes of MraY-targeted antibacterial drugs. In the past 2 years the biosynthetic gene clusters were identified for polyoxins (13), caprazamycins (14) and related liposidomycins (15), and A500359s (a member of the capuramycin family) (16) via PCR screening of the cosmid libraries. However, little is known about the biosynthesis of the subfamily of pacidamycins, mureidomycins, and napsamycins uridyl peptide antibiotics, and none of the above PCR probes would be useful for identifying the gene cluster for this subfamily. A β-replacement reaction has been reported in the conversion of threonine to DABA in mureidomycin A, albeit the dedicated enzyme was not identified (17).

Author contributions: W.Z. and C.T.W. designed research; W.Z. and B.O. performed research; W.Z. and C.T.W. analyzed data; and W.Z. and C.T.W. wrote the paper.

The authors declare no conflict of interest.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. HM855229).

[1]To whom correspondence should be addressed. E-mail: christopher_walsh@hms.harvard.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1011557107/-/DCSupplemental.

To understand what types of reactions and enzyme catalysts are involved in both the construction of the nonproteinogenic amino acids and uridine building blocks and the assembly of these branched deoxyuridine-ureido-peptide scaffolds, we have undertaken the identification of the pacidamycin biosynthetic gene cluster from the producer *S. coeruleorubidus* NRRL 18370 through genome scanning. The identity of the cluster has been confirmed by both genetic and biochemical characterizations. After completion of this study, a parallel effort in *S. coeruleorubidus* genome sequencing has independently identified the same 22 gene cluster (18).

Results and Discussion

Identification of Pacidamycin Gene Cluster from *S. coeruleorubidus*. The 454 shotgun sequencing of *S. coeruleorubidus* NRRL 18370 with the GS FLX Titanium series at University of California at Los Angeles (UCLA) generated a total of ~208 million bases. Assembly of the unpaired sequence reads resulted in 10,850,001 nonredundant bases distributed over 212 contigs. Using a local BLASTP program queried against a database consisting of all the contigs, more than 20 putative NRPS modules were found. To spot the sequence region potentially responsible for pacidamycin synthesis, sequences of a cysteine synthase and an argininosuccinate lyase were further used as probes for BLASTP. These two enzymes have been demonstrated to be essential for DABA synthesis in the lipopeptide antibiotic friulimicin by gene inactivation and subsequent DABA feeding experiments (19). The bioinformatic search identified one putative gene cluster for pacidamycins, which spans ~31 kb of genomic DNA on a single contig and consists of 22 open reading frames (ORFs), here designated *pacA-V* (Fig. 2 and Table 1). Of these ORFs 17 can be assigned roles in pacidamycin biosynthesis: 8 encode dissociated NRPS modules including a total of 4 adenylation (A) domains, 4 thiolation (T) domains, 3 condensation (C) domains, and 1 thioesterase (TE) domain. None of these NRPSs have more than three domains, analogous to the previously described fragmented assembly line of andrimid (20). Thus there are one freestanding T domain and two freestanding A and C domains, raising questions about how functional modules assemble and provide specific flux during peptidyl-chain growth. One MbtH-like protein (PacJ) is encoded in the cluster that might interact with A domains as indicated previously (21, 22). One stand-alone *S*-adenosylmethionine (SAM)-dependent *N*-methyltransferase (PacV) was also detected, and putatively assigned to *N*-methylation of DABA. We propose that all of these NRPS-related ORFs function in the assembly of tetra/pentapeptide framework, with

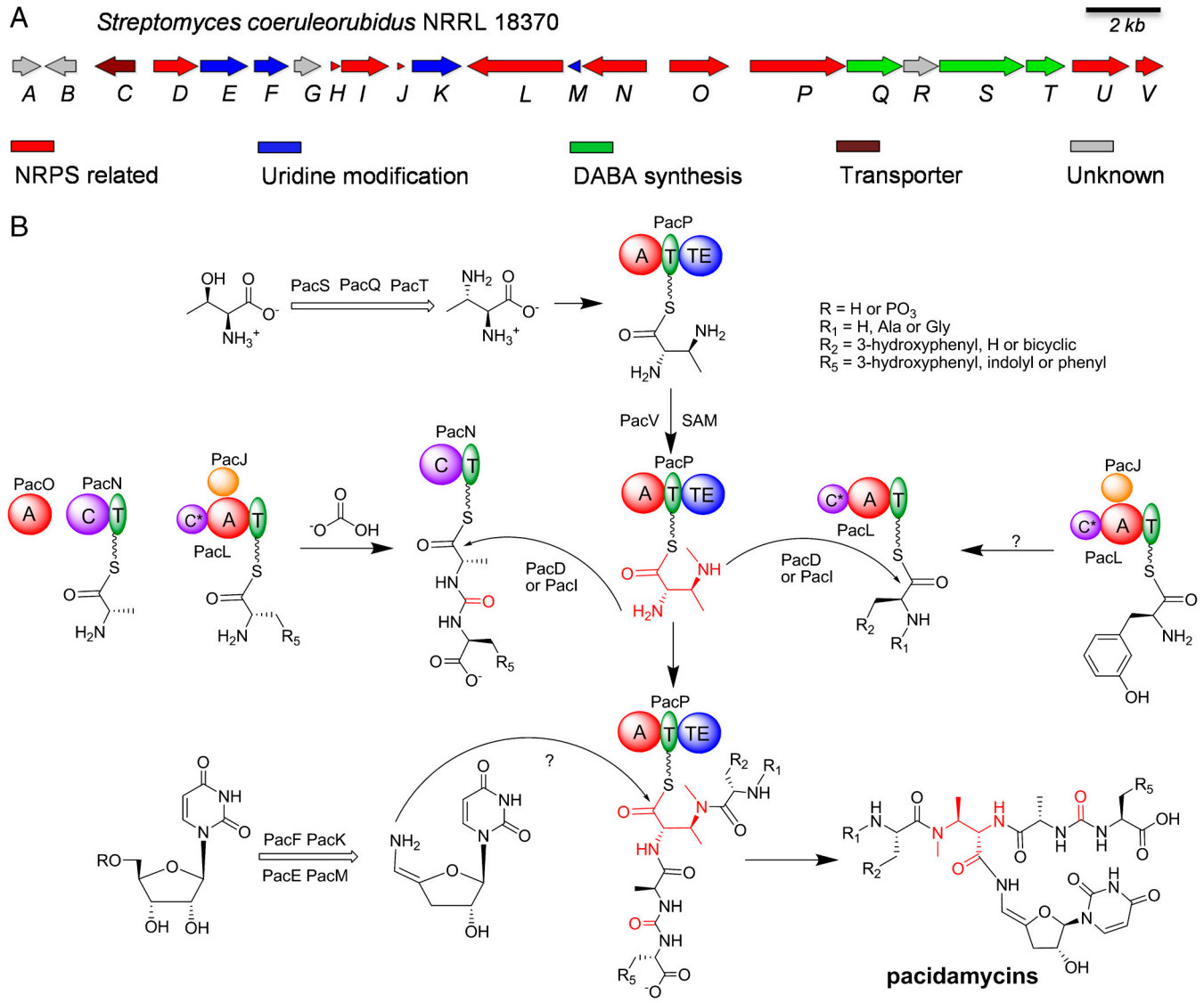


Fig. 2. Map of pacidamycin gene cluster and proposed biosynthetic pathway. (*A*) Organization of *pac* genes. (*B*) Proposed pathway for pacidamycin biosynthesis. Domain notation: T, thiolation; A, adenylation; C, condensation; TE, thioesterase.

Table 1. Genes in the pacidamycin biosynthetic cluster from *S. coeruleorubidus* NRRL 18370 contig00048 and deduced roles based on sequence homology

Gene	Size, aa	Deduced role	Protein homolog*	Accession number	Protein similarity/ identity, %/%
pacA	258	Hypothetical protein	Caci_2672 [*Catenulispora acidiphila* DSM44928]	YP_003113429	39/52
pacB	285	Hypothetical protein	SSEG_09055 [*Streptomyces sviceus* ATCC 29083]	ZP_05017494	43/60
pacC	364	Major facilitator transporter	Caci_7785 [*Catenulispora acidiphila* DSM 44928]	YP_003118450	54/71
pacD	398	NRPS (C)	Pden_3015 [*Paracoccusdenitrificans* PD1222]	YP_916794	28/40
pacE	426	PLP-dependent aminotransferase	CetH [*Actinomyces sp.* Lu 9419]	ACH85568	51/65
pacF	311	Fe(II)/2-oxoglutarate oxygenase	RegB [*Actinoplanes friuliensis*]	CAD32906	37/51
pacG	244	Hypothetical protein	SSCG_02980 [*Streptomyces clavuligerus* ATCC 27064]	ZP_06771352	29/48
pacH	93	NRPS (T)	SghaA1_010100031768 [*Streptomyces ghanaensis* ATCC 14672]	ZP_04689798	40/66
pacI	421	NRPS (C)	NrpA [*Ralstonia eutropha* H16]	YP_841202	28/42
pacJ	72	MbtH domain-containing protein	Bcep1808_1578 [*Burkholderia vietnamiensis* G4]	YP_001119420	58/74
pacK	443	FAD-dependent oxidoreductase	SSEG_08816 [*Streptomyces sviceus* ATCC 29083]	ZP_05016850	54/65
pacL	849	NRPS (C*-A-T)	PstC [*Actinoplanes friuliensis*]	CAM56770	46/60
pacM	121	Cupin-2 domain-containing isomerase	Mbar_A1689 [*Methanosarcina barkeri* str. Fusaro]	YP_305211	32/56
pacN	574	NRPS (C-T)	AptA2 [*Anabaena sp.* 90]	ACZ55942	31/50
pacO	527	NRPS (A)	bcere0026_56870 [*Bacillus cereus* AH603]	ZP_04200912	40/57
pacP	848	NRPS (A-T-TE)	SghaA1_010100001257 [*Streptomyces ghanaensis* ATCC 14672]	ZP_04683778	48/61
pacQ	498	Argininosuccinate lyase	orf_R2 [*Streptomyces collinus* Tu 365]	CAN89657	51/62
pacR	318	Kinase	PduX [*Streptomyces kanamyceticus* NBRC 13414]	BAE95583	54/65
pacS	755	Fusion protein containing a PLP-dependent cysteine synthase and an argininosuccinate lyase	orf_R4 [*Streptomyces collinus* Tu 365]	CAN89659	58/68
pacT	350	PLP-dependent threonine aldolase	bcere0007_32170 [*Bacillus cereus* AH621]	ZP_04295986	61/76
pacU	507	NRPS (A)	Strop_2774 [*Salinispora tropica* CNB-440]	YP_001159594	58/69
pacV	250	SAM-dependent *N*-methyltransferase	sce7770 [*Sorangium cellulosum* "So ce 56"]	YP_001618419	37/51

*Results generated by BLAST analysis, *S. roseosporus* homologs (best homologs for all ORFs) are excluded.

some domains perhaps used iteratively. The highly dissociated nature of these NRPSs, together with nonstringent domain–domain interactions and relaxed amino acid substrate specificity, may account for the production of up to 10 pacidamycins varied in the peptide scaffold.

Three genes predicted to be on the same operon, *pacQST*, are postulated to be involved in the biosynthesis of the DABA moiety. PacQ displayed significant homology to an argininosuccinate lyase, and PacS was predicted to be a fusion protein containing both a pyridoxal phosphate (PLP)-dependent cysteine synthase and an argininosuccinate lyase domain. Consistent with DABA synthesis in friulimicin (19), PacS is proposed to catalyze the β-replacement of threonine hydroxyl by the α-amino of aspartate, and the intermediate then breaks down to give DABA with the release of fumarate promoted by argininosuccinate lyase PacQ or PacS. The configuration of DABA in pacidamycins was determined to be *2S,3S*, different from the *2S,3R* configuration of DABA in friulimicin and natural L-Thr (11). PacT, a PLP-dependent threonine aldolase homolog, not encoded in friulimicin gene cluster, is thus proposed to be responsible for the *3S* configuration of DABA (Fig. S2). The remaining four tailoring enzymes encoded in the gene cluster are putatively assigned for uridine nucleoside modification, including a Fe(II)/α-ketoglutarate-dependent oxygenase (PacF), a flavin adenine dinucleotide (FAD)-dependent oxidoreductase (PacK), a PLP-dependent aminotransferase (PacE), and a cupin-2 domain-containing isomerase (PacM). Although uridine-5′-aldehyde might be a common intermediate in the nucleoside modification of pacidamycins, caprazamycins, and liposidomycins, the proposed dedicated alcohol dehydrogenase (LpmW/Cpz25) encoded in caprazamycin and liposidomycin gene clusters was not found in the pacidamycin cluster (14, 15). Instead, either PacF or PacK might work on uridine or uridine-5′-monophosphate to give uridine-5′-aldehyde, which is then subjected to 3′,4′-dehydration, 5′-transamination possibly catalyzed by PacE, and isomerization presumably catalyzed by PacM to yield the unique building block 3′-deoxy-4′,5′-enamino-uridine (Fig. S3).

PacC showed strong sequence similarity to major facilitator transporters, and probably functions to export pacidamycins out of the cell. There are other ORFs in the gene cluster encoding proteins with no obvious function, some of which might be related to regulation and antibiotic resistance. Interestingly, the whole biosynthetic gene cluster was also found in the published genome of *Streptomyces roseosporus* NRRL 15998, a known daptomycin producer, although no pacidamycin production has been reported from *S. roseosporus*. By comparing the sequences around the two clusters, the boundary of the *pac* cluster was putatively identified (Fig. S4). Notably there is a phenylalanine hydroxylase encoded in the cluster from *S. roseosporus*, which presumably catalyzes the formation of *m*-Tyr from Phe. The absence of the corresponding gene in the *pac* cluster from *S. coeruleorubidus* indicates that the identified cluster might be incomplete, and the putative gene encoding a phenylalanine (meta)-hydroxylase is located elsewhere on the genome revealed by BLASTP analysis.

In Vivo Gene Disruption Analysis. A set of gene disruption experiments was carried out to test the necessity of various genes in the biosynthesis of pacidamycins. The knockout targets include PacO, a single A domain, PacP, an A-T-TE tridomain NRPS, PacU, another single A domain and PacQ, the argininosuccinate lyase homolog. All of the genes were deleted in-frame through double crossover according to standard methods (23), and the resulting mutants were confirmed by PCR (Fig. S5). Following the reported growth condition and extraction technique (24), all 10 known pacidamycins could be detected from the culture of wild-type strain as major products by LC-HRMS analysis (Fig. 3 and Fig. S6). The production of pacidamycins was completely abolished in Δ*pacO* and Δ*pacP* mutants, demonstrating that these two genes are essential for the biosynthesis of pacidamycins (Fig. 3 and Figs. S7 and S8). In addition, when *pacO* was introduced back into the Δ*pacO* mutant, the transconjugant was found

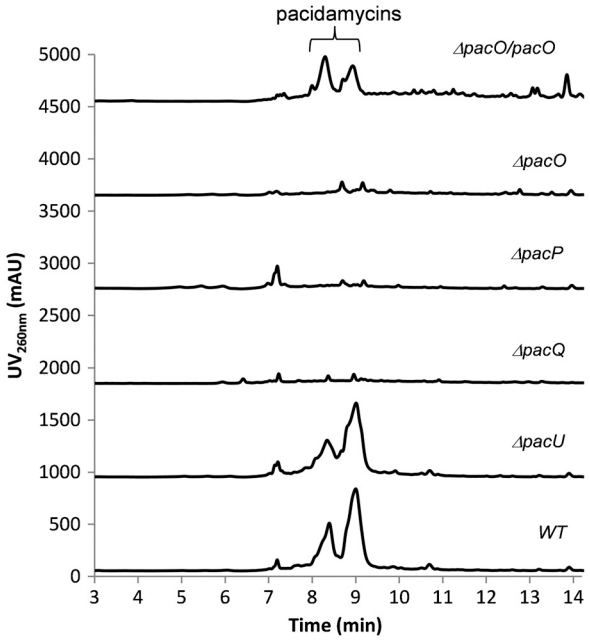


Fig. 3. UV traces (260 nm) during LC-HRMS analysis of metabolites produced by wild-type and mutant *S. coeruleorubidus* strains. See SI Figs. S6–S11 for detailed HRMS analysis.

to have resumed pacidamycin production (Fig. 3 and Fig. S9). The disruption of *pacQ* significantly reduced the yield of all pacidamycins in comparison with that of the wild type (reduced to <1%), with only trace amount of pacidamycins produced indicated by mass ion extraction (Fig. S10). This result suggested that PacQ plays an important role in the biosynthesis; however, its function might be complemented by other enzymes, such as PacS, the fusion protein with an argininosuccinate lyase domain. Surprisingly, the deletion of *pacU* had no impact on the production of nine pacidamycin compounds, but did abolish pacidamycin D production in the extract of Δ*pacU* mutant shown by mass ion extraction (Fig. 3 and Fig. S11). Pacidamycin D is a uridyl tetrapeptide with a single Ala_1 attached to the β-amino group of DABA, whereas all other pacidamycins have a *m*-Tyr_2 or its derivative at the corresponding position. The abolishment of pacidamycin D to make the uridyltetrapeptide framework indicated that PacU may be specifically related to activation of the alternative *N*-terminal Ala. In summary, the in vivo gene disruption experiments unambiguously verified that the identified gene cluster from *S. coeruleorubidus* genome is directly involved in the biosynthesis of pacidamycins. Independently, Rackham et al. have shown that heterologous expression of this gene cluster in *Streptomyces lividans* yielded pacidamycin D and a related variant, providing a parallel in vivo identification of the cluster, but they did not report any in vitro studies (18). In contrast, we studied the activity of seven encoded enzymes that allowed the assignment of their functions in the biosynthesis of pacidamycins.

Adenylation Domain Activity Assays. Adenylation domains are gatekeeping enzymes in NRPSs that are responsible for the building block selection in the peptide scaffold assembly. Based on pacidamycin structures, at least three different A domain activities are needed: for L-Ala, DABA, and aromatic amino acids (L-m-Tyr, L-Phe, and L-Trp) activation, respectively. All four A domain-containing proteins, PacL, PacO, PacP, and PacU were overexpressed and purified from *Escherichia coli* (Fig. S12), and their ability to reversibly adenylate various amino acids was tested using the classical ATP-[^{32}P]PP_i exchange assay. PacL is a C*-A-T tridomain protein (C* indicates a truncated and presumably nonfunctional C domain) that was predicted to activate L-Val/L-Leu-like hydrophobic branched amino acids based on the 10-residue specificity sequence (10AA code) (Table S1). As the prediction was inconsistent with amino acid components in pacidamycins, a broad range of substrates was tested. Although PacL alone showed no obvious activity toward all of the amino acids tested, it preferentially activated aromatic amino acids in the presence of PacJ, the small MbtH-like protein (Fig. 4*A*). MbtH domain-containing proteins have been found in many NRPS-encoding gene clusters and have been shown to be important for metabolite assembly in vivo (22). The PacJ activation of PacL will be explored in detail elsewhere. The preferential activation of *m*-Tyr, L-Phe, and L-Trp but not L-Tyr executed by PacL/PacJ is in good agreement with the aromatic amino acids found at the *C* terminus of pacidamycins. PacP is a A-T-TE tridomain protein predicted to activate L-Ser/L-Arg by bioinformatic analysis. It is encoded in the same operon with *pacQST* (genes related to DABA synthesis) and is therefore postulated to be involved in DABA activation. Because *2S,3S*-DABA was not readily available, its analog L-2,3-diaminopropionate (DAP) was used in the ATP-PP_i exchange assay. PacP exhibited a strong preference for activation of DAP over all other amino acids, strongly suggesting that it is a DABA activation enzyme (Fig. 4*A*). The remaining two stand-alone A domain proteins, PacO and PacU, were predicted to activate L-Ala and L-Pro/L-p-hydroxyphenylglycine, respectively, based on the 10AA code. Indeed, PacO demonstrated a preference toward reversible formation of L-Ala-AMP, with modest activation of L-Gly (Fig. 4*A*). However, no substantial activation of any amino acids was detected in PacU assays, with or without the presence of PacJ. The amino acid specificities of PacP, PacO, and PacU correlate well with the in vivo gene disruption results, in which *pacP* and *pacO* were essential for pacidamycins synthesis, whereas *pacU* was not needed for the synthesis of 9 out of 10 pacidamycins (Fig. 3). It is unclear, given the in vivo deletion result noted above on loss of pacidamycin D, why PacU did not activate L-Ala in the in vitro assay; it is possible that PacU was purified from *E. coli* in an inactive form. It can also be deduced that Ala_4 in pacidamycins is selected by PacO.

Covalent Loading of Holo Forms of Thiolation Domains. The loading of the adenylated amino acids to the adjacent or dissociated T domain holo forms could then be investigated using ^{14}C-labeled substrate. Particularly significant is the loading of L-Ala activated by the freestanding adenylation domain PacO to identify its *in trans* loading partner. All four T domain-containing proteins, PacH, PacL, PacN, and PacP, were overexpressed and purified from *E. coli* BAP1 strain that contains a chromosomal copy of the phosphopantetheinyl transferase Sfp to ensure their posttranslational modification to the pantetheinylated forms (Fig. S12) (25). The ^{14}C-labeled covalent aminoacyl-*S*-thiolation intermediate was then detected by SDS-PAGE autoradiography. Using [^{14}C]L-Ala, PacN (a C-T didomain protein) was determined to be the cognate loading partner of PacO (Fig. 4*B*, lane 3); no Ala loading was detected on any of the other three holo T domains. In addition, as expected, PacL was covalently loaded with aromatic amino acid when using [^{14}C]L-Phe and [^{14}C]L-Trp as the substrate, respectively (Fig. 4*B*, lanes 1–2).

Ureido-Bond Formation Between Covalently Tethered Ala and Phe/Trp/*m*-Tyr. When [^{14}C]Phe/Trp-loaded PacL and Ala-*S*-PacN were combined, ^{14}C-labeled L-Phe or L-Trp was transferred to PacN, whereas [^{14}C]L-Ala remained on PacN (Fig. 4*B*, lanes 4–8). These results indicated the formation of covalent linkage between Ala tethered on PacN and aromatic amino acyl moiety undergoing transfer, presumably catalyzed by the C domain of PacN. To test if this represents ureido-linked Phe/Trp_5-Ala_4-*S*-PacN, because this 4–5 ureido link is observed in all the pacidamycins, [^{14}C]$NaHCO_3$ was fed as the only radioactive substrate

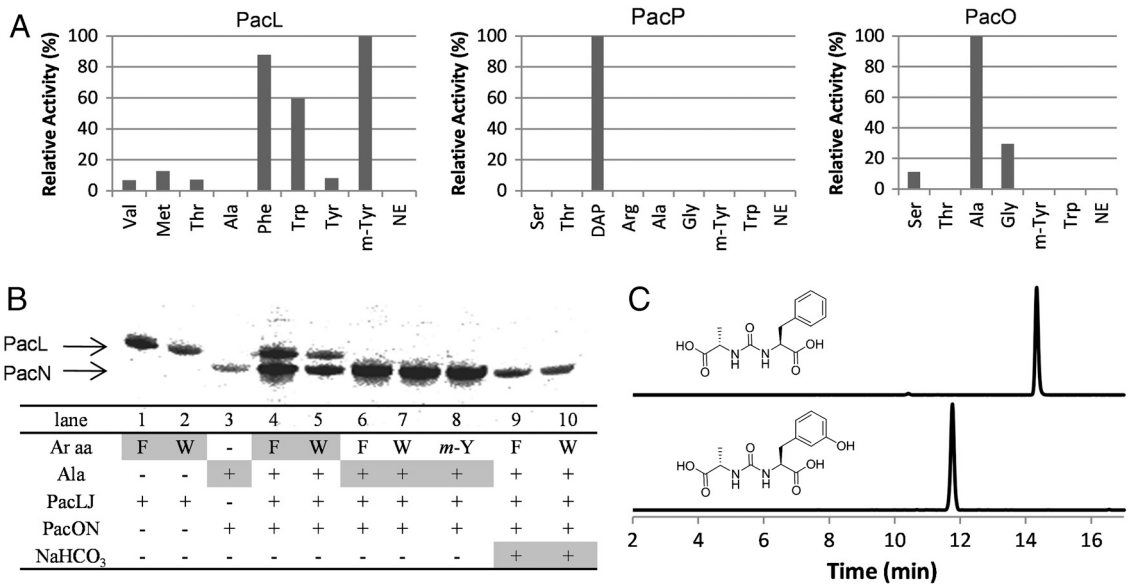


Fig. 4. Characterization of NRPSs. (*A*) A domain activity of PacL, PacP, and PacO. NE, no enzyme; *m*-Tyr, *meta*-tyrosine; DAP, L-2,3-diaminopropionate. All acids are L-configuration except *m*-Tyr (DL). One hundred percent relative activity for Ala, DAP, and *m*-Tyr-dependent exchange corresponds to 253 k, 251 k, and 139 k cpm, respectively. (*B*) Autoradiograph of SDS-PAGE gel illustrating the covalent loading of ^{14}C-labeled substrate (shaded). Enzymes and amino acids used in each lane are indicated in Table 1. (*C*) Extracted ion chromatograms showing production of ureido dipeptides Ala-CO-Phe and Ala-CO-*m*-Tyr. The calculated mass with 10-ppm mass error tolerance was used.

to the PacL, PacN assays. Our prior work on a comparable ureido link in syringolin formation has shown that bicarbonate is the carbon source of the carbonyl moiety of that ureido linkage (12). Radioactivity migrated with PacN but not PacL (Fig. 4*B*, lanes 9–10), suggesting that indeed a Phe/Trp$_5$-Ala$_4$ ureido dipeptyl thioester was formed and tethered on the pantetheinyl arm of PacN. The identification of the ureido dipeptide was confirmed by liquid chromatography–high resolution mass spectrometry after hydrolytic release of the peptidyl-*S*-T domain intermediate with thioesterase TycF (26). In vitro assay with ATP, L-Ala, L-Phe, PacO, PacN, PacL, and PacJ yielded the ureido dipeptide Ala-CO-Phe ($m/z = 303.0951$ $[M + Na]^+$, $\Delta = 0.8$ mmu), and its mass was shifted by +1 using [^{13}C]NaHCO$_3$, or by +2 using [2,3-^{13}C2]L-Ala as an alternative substrate (Fig. 4*C* and Fig. S13). The dipeptide Ala-CO-*m*-Tyr ($m/z = 319.0901[M + Na]^+$, $\Delta = -0.2$ mmu) was also formed when Phe was replaced by *m*-Tyr in vitro (Fig. 4*C* and Fig. S14). Therefore, we have successfully reconstituted the assembly of Ala$_4$ and *C*-terminal aromatic amino acid residue (Phe/Trp/*m*-Tyr) with the chain-reversing ureido linkage. Ala$_4$ is selected and activated as the Ala-AMP by PacO and loaded on *HS*-pantetheinyl-PacN; the *C*-terminal aromatic amino acid (Phe, Trp, or *m*-Tyr) is activated by PacL with the help of PacJ and loaded onto *HS*-pantetheinyl-PacL. PacN may then catalyze the ureido-bond formation between Ala-*S*-PacN and Phe/Trp/*m*-Tyr-*S*-PacL, yielding a ureido dipeptide tethered on PacN (Fig. 2*B*).

In summary, we have identified the gene cluster for pacidamycins that sets the stage for further deciphering of the chemical logic for assembly of this class of peptidyl nucleoside antibiotics. Our in vivo and in vitro experiments allow a first proposal of the pacidamycin biosynthetic pathway (Fig. 2*B*). The key bifunctional amino acid building block DABA is most probably synthesized from L-Thr catalyzed by PacQST, activated by and loaded onto PacP, and *N*-methylated by PacV. Promoted by one of the condensation domains (PacD or PacI), the α-amino of DABA nucleophilically attacks the PacN tethered thioester of the *C*-terminal ureido dipeptide, which is assembled by PacLJON as described above. The remaining condensation domain presumably directs the nucleophilic attack of β-amino of DABA (before or after its *N*-methylation) on the carbonyl of a thioester tethered *N*-terminal amino acid or dipeptide. Whether one or more of the above PacLJON also assembles Ala/Gly-*m*-Tyr as a normal dipeptidyl-*S*-T intermediate (rather than the ureido-linked dipeptide) for Ala/Gly$_1$-*m*-Tyr$_2$ is yet to be demonstrated. Finally, the TE domain of PacP is presumed to catalyze the release of tetra/pentapeptidyl-*S*-PacP by 3′-deoxy-4′,5′-enamino-uridine, a modified nucleoside possibly synthesized by PacEFKM from uridine, to yield uridyl tetra/pentapeptide pacidamycins via a 4′,5′-enamide linkage. Further biochemical study will provide more insight in the timing and mechanisms of the biosynthesis of this intriguing class of antibiotics.

Materials and Methods

454 Sequencing and Bioinformatic Analysis. *Streptomyces coeruleorubidus* NRRL 18370 was obtained from U.S. Department of Agriculture Agricultural Research Service Culture Collection. The genomic DNA used for sequencing was prepared using the phenol/chloroform extraction method. The shotgun sequencing was performed at the GenoSeq (UCLA Genotyping and Sequencing Core) with the GS FLX Titanium system (Roche). The 454 sequencing reads were assembled into contigs with the GS De Novo Assembler software (Roche). The assembled data were converted into a local BLAST database for search using stand-alone BLAST software (ver. 2.2.18) downloaded from the National Center for Biotechnology Information Web site. ORFs were detected and analyzed using online program FGENESB (Softberry), and the putative roles of the proteins were assigned using protein–protein BLAST and Pfam analysis. The NRPS A domain specificity was predicted using online program NRPSpredictor (27). The nucleotide sequence of the gene cluster was deposited at GenBank.

Gene Disruptions in *S. coeruleorubidus* and Mutants Analysis. In vivo generation of targeted mutations in *S. coeruleorubidus* was achieved by conjugative transfer of disruption plasmids from *E. coli* WM6026 and *Streptomyces* according to general protocols (23). The knockout cassettes were constructed using the ReDirect technology (28). An example for *pacP* is detailed here. A 6.5-kb fragment containing *pacP* flanked by 2-kb arms was PCR amplified from genomic DNA and inserted into PCRBlunt vector. This plasmid was introduced into *E. coli* BW25113/pIJ790 by electroporation. The *acc(3)IV-oriT* cassette amplified by PCR from pIJ773 was then introduced to replace the entire *pacP* using PCR targeting and λ-Red-mediated recombination. The resulting knockout cassette was transformed into *E. coli* WM6026 (a diaminopimelic acid auxotroph) for conjugation with *S. coeruleorubidus*. The double-crossover strain was obtained from antibiotic selection (Apra^{R}Kan^S) and confirmed by PCR (Fig. S5). The *pacO* complementation experiment was

performed using plasmid pIJ6902 and confirmed by PCR. Mutant analysis was carried out following the reported procedure (24). See *SI Appendix* for details. LC-HRMS analysis was performed on an Agilent Technologies 6520 Accurate-Mass QTOF LC-MS instrument with a Luna 3u C18 column (4.6×75 mm) from Phenomenex. A linear gradient of 2 to 95% CH_3CN (vol/vol) over 15 min in H_2O supplemented with 0.1% (vol/vol) formic acid at a flow rate of 0.5 mL/min was used.

ATP-PP$_i$ Exchange Assays. All proteins were expressed and purified with His_6 tag following the general protocol (see *SI Appendix*, Fig. S12). The assays were preformed in 100 μL of reaction buffer (50 mM Tris-HCl/2 mM $MgCl_2$, pH 7.8) containing 5 mM ATP, 1 mM $Na_4[^{32}P]PP_i$ ($\sim 4 \times 10^6$ cpm/mL), 1 mM (*tris*(2-carboxyethyl)phosphine) (TCEP), 5 mM substrate, and 1 μM enzyme. Reactions were incubated at 25 °C for 1 h, then quenched by the addition of charcoal suspension (1.6% wt/vol activated charcoal, 0.1 M Na_4PP_i, 3.5% $HClO_4$). Free $[^{32}P]PP_i$ was removed by centrifugation of the sample followed by washing twice with wash solution (0.1 M Na_4PP_i and 3.5% $HClO_4$). Charcoal-bound radioactivity was measured on a Beckman LS 6500 scintillation counter.

Loading Assays with ^{14}C-Labeled Substrates. A typical assay contained, in a total volume of 20 μL, 5 mM ATP, 2 mM $MgCl_2$, 1 mM TCEP, 50 μM amino acids, 10 μM enzymes, and 50 mM Hepes, pH 8.0. ^{14}C-labeled substrate was added to each reaction accordingly (L-Ala [0.13 μCi], L-Phe [0.4 μCi], L-Trp [0.052 μCi], or $NaHCO_3$ [0.5 μCi]. After 1-h incubation at 25 °C, samples were quenched by adding 1× SDS sample buffer. Following SDS-PAGE, radiolabeled protein was detected using a BAS-III imaging plate (Fuji Film, 48- to 96-h exposure) and a Typhoon 9400 phosphorimager (GE Healthcare).

LC-HRMS Product Assays. Assays were performed in 50 μL of 50 mM Hepes (pH 8.0) containing 5 mM ATP, 2 mM $MgCl_2$, 1 mM TCEP, 5 mM L-Ala, 5 mM L-Phe or m-Tyr, 10 μM PacO, 20 μM PacN, 20 μM PacL, and 40 μM PacJ. After 2-h incubation at 25 °C, TycF (5 μM) was added and further incubated for 30 min. The proteins were then removed by 5-kDa MWCO (Molecular Weight Cutoff) filter tubes, and the filtered reaction mixture was subjected to LC-HRMS analysis. A linear gradient of 2 to 40% CH_3CN (vol/vol) over 15 min in H_2O supplemented with 0.1% (vol/vol) formic acid at a flow rate of 0.5 mL/min was used.

ACKNOWLEDGMENTS. We thank Dr. Chris Neumann for providing the purified TycF and Dr. Heidi Imker for helpful discussion and proofreading of the manuscript. This work was supported by NIH Grant GM49338 (C.T.W.).

1. Fronko RM, et al. (2000) New pacidamycins produced by Streptomyces coeruleorubidus, NRRL 18370. *J Antibiot (Tokyo)* 53:1405–1410.
2. Chen RH, Buko AM, Whittern DN, McAlpine JB (1989) Pacidamycins, a novel series of antibiotics with anti-Pseudomonas aeruginosa activity. II. Isolation and structural elucidation. *J Antibiot (Tokyo)* 42:512–520.
3. Muroi M, Kimura K, Osada H, Inukai M, Takatsuki A (1997) Liposidomycin B inhibits in vitro formation of polyprenyl (pyro)phosphate N-acetylglucosamine, an intermediate in glycoconjugate biosynthesis. *J Antibiot (Tokyo)* 50:103–104.
4. Tamura G, Sasaki T, Matsuhashi M, Takatsuki A, Yamasaki M (1976) Tunicamycin inhibits the formation of lipid intermediate in cell-free peptidoglycan synthesis of bacteria. *Agric Biol Chem* 40:447–449.
5. Hotoda H, et al. (2003) Synthesis and antimycobacterial activity of capuramycin analogues. Part 1: Substitution of the azepan-2-one moiety of capuramycin. *Bioorg Med Chem Lett* 13:2829–2832.
6. Hotoda H, et al. (2003) Synthesis and antimycobacterial activity of capuramycin analogues. Part 2: Acylated derivatives of capuramycin-related compounds. *Bioorg Med Chem Lett* 13:2833–2836.
7. McDonald LA, et al. (2002) Structures of the muraymycins, novel peptidoglycan biosynthesis inhibitors. *J Am Chem Soc* 124:10260–10261.
8. Isono F, Inukai M (1991) Mureidomycin A, a new inhibitor of bacterial peptidoglycan synthesis. *Antimicrob Agents Chemother* 35:234–236.
9. Chatterjee S, et al. (1994) Napsamycins, new Pseudomonas active antibiotics of the mureidomycin family from Streptomyces sp. HIL Y-82,11372. *J Antibiot (Tokyo)* 47:595–598.
10. Winn M, Goss RJ, Kimura K, Bugg TD (2010) Antimicrobial nucleoside antibiotics targeting cell wall assembly: Recent advances in structure-function studies and nucleoside biosynthesis. *Nat Prod Rep* 27:279–304.
11. Boojamra CG, et al. (2001) Stereochemical elucidation and total synthesis of dihydropacidamycin D, a semisynthetic pacidamycin. *J Am Chem Soc* 123:870–874.
12. Imker HJ, Walsh CT, Wuest WM (2009) SylC catalyzes ureido-bond formation during biosynthesis of the proteasome inhibitor syringolin A. *J Am Chem Soc* 131:18263–18265.
13. Chen W, et al. (2009) Characterization of the polyoxin biosynthetic gene cluster from Streptomyces cacaoi and engineered production of polyoxin H. *J Biol Chem* 284:10627–10638.
14. Kaysser L, et al. (2009) Identification and manipulation of the caprazamycin gene cluster lead to new simplified liponucleoside antibiotics and give insights into the biosynthetic pathway. *J Biol Chem* 284:14987–14996.
15. Kaysser L, Siebenberg S, Kammerer B, Gust B (2010) Analysis of the liposidomycin gene cluster leads to the identification of new caprazamycin derivatives. *ChemBioChem* 11:191–196.
16. Funabashi M, et al. (2009) Identification of the biosynthetic gene cluster of A-500359s in Streptomyces griseus SANK60196. *J Antibiot (Tokyo)* 62:325–332.
17. Lam WH, Rychli K, Bugg TD (2008) Identification of a novel beta-replacement reaction in the biosynthesis of 2,3-diaminobutyric acid in peptidylnucleoside mureidomycin A. *Org Biomol Chem* 6:1912–1917.
18. Rackham EJ, Gruschow S, Ragab AE, Dickens S, Goss RJ (2010) Pacidamycin biosynthesis: Identification and heterologous expression of the first uridyl peptide antibiotic gene cluster. *ChemBioChem* 11:1700–1709.
19. Muller C, et al. (2007) Sequencing and analysis of the biosynthetic gene cluster of the lipopeptide antibiotic Friulimicin in Actinoplanes friuliensis. *Antimicrob Agents Chemother* 51:1028–1037.
20. Jin M, Fischbach MA, Clardy J (2006) A biosynthetic gene cluster for the acetyl-CoA carboxylase inhibitor andrimid. *J Am Chem Soc* 128:10660–10661.
21. Heemstra JR, Jr, Walsh CT, Sattely ES (2009) Enzymatic tailoring of ornithine in the biosynthesis of the Rhizobium cyclic trihydroxamate siderophore vicibactin. *J Am Chem Soc* 131:15317–15329.
22. Lautru S, Oves-Costales D, Pernodet JL, Challis GL (2007) MbtH-like protein-mediated cross-talk between non-ribosomal peptide antibiotic and siderophore biosynthetic pathways in Streptomyces coelicolor M145. *Microbiology* 153:1405–1412.
23. Kieser T, Bibb MJ, Buttner MJ, Chater KF, Hopwood DA (2000) *Practical Streptomyces Genetics* (John Innes Foundation, Norwich, UK).
24. Gruschow S, et al. (2009) New pacidamycin antibiotics through precursor-directed biosynthesis. *ChemBioChem* 10:355–360.
25. Pfeifer BA, Admiraal SJ, Gramajo H, Cane DE, Khosla C (2001) Biosynthesis of complex polyketides in a metabolically engineered strain of *E. coli*. *Science* 291:1790–1792.
26. Yeh E, Kohli RM, Bruner SD, Walsh CT (2004) Type II thioesterase restores activity of a NRPS module stalled with an aminoacyl-S-enzyme that cannot be elongated. *ChemBioChem* 5:1290–1293.
27. Rausch C, Weber T, Kohlbacher O, Wohlleben W, Huson DH (2005) Specificity prediction of adenylation domains in nonribosomal peptide synthetases (NRPS) using transductive support vector machines (TSVMs). *Nucleic Acids Res* 33:5799–5808.
28. Gust B, Challis GL, Fowler K, Kieser T, Chater KF (2003) PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. *Proc Natl Acad Sci USA* 100:1541–1546.