Pyridomycin is a structurally unique antitumor antibiotic produced by *Streptomyces pyridomyceticus* NRRL B-2517 (Fig. 1) (8). Pyridomycin is an unusual 12-membered ring depsipeptide composed of four moieties in the following order: N-3-hydroxypicolinyl-L-threonine, 3-(3-pyridyl)-L-alanine, propionic acid, and 2-hydroxy-3-methylpent-2-enoic acid, which is probably epimerized from α-keto-β-methylvaleric acid (9, 10). To the best of our knowledge, it is the only known depsipeptide that contains an enolic acid. Isotope labeling studies (11) indicated that the biosynthesis of pyridomycin might involve the assembly of the backbone by a hybrid NRPS/PKS system using 3-hydroxypicolinic acid (3-HPA) as the starter unit. Feeding experiments confirmed that 3-HPA is a precursor of the 3-hydroxypicolinyl moiety, whereas lysine was not incorporated into the compound, indicating that the formation of the 3-hydroxypicolinyl moiety follows a different pathway from that involved in the biosynthesis of streptogramin B antibiotics, such as pristinamycin I, virginiamycin S (Fig. 1), and etamycin, which also use 3-HPA as the starter unit (12–14). Chemical synthesis schemes have been devised for pyridomycin (15), but little was known about the pathway of pyridomycin biosynthesis.

Here we report the cloning and sequencing of the pyridomycin biosynthetic gene cluster within a 42.5-kb DNA region containing 26 open reading frames (ORFs) (Fig. 2A). At the center of this region is a hybrid NRPS/PKS system (NRPS/PKS/NRPS). The last NRPS contains an essential KR domain in an unusual position. Based on targeted mutagenesis, *in vitro* enzyme studies, and sequence analysis, we propose a putative pathway for pyridomycin biosynthesis. *In vivo* and *in vitro* characterization...
of PyrA and PyrU demonstrated that PyrA and PyrU constituted the loading module that initiated the assembly of the pyridomycin backbone. Together with the observed broad substrate specificity of PyrA, these findings provide opportunities to generate pyridomycin derivatives with novel or enhanced bioactivities by rational engineering of the biosynthetic pathway or combinatorial biosynthesis. Given its special molecular architecture (9, 16), pyridomycin also offers an opportunity to discover new chemistry for natural product biosynthesis.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Culture Conditions**—Strains and plasmids used in the study are summarized in Supplemental Tables S1 and S2. *S. pyridomyceticus* NRRL B-2517 and its derivatives were cultivated at 30 °C in YEME liquid medium for growth of mycelia, on COM medium (1% corn starch, 1% oat flour, 0.1% malt extract, 0.1% yeast extract, 0.1% tryptone, 0.1% yeast extract, 0.1% tryptone, 1.2% agar, pH 7.2) for sporulation, and on 2CM medium (17% soluble starch, 0.2% tryptone, 0.1% NaCl, 0.2% (NH₄)₂SO₄, 0.1% K₂HPO₄, 0.1% MgSO₄, 0.2% CaCO₃, 1.2% agar with 1 ml of inorganic salt solution/liter and adjusted to pH 7.2) for conjugation. Luria-Bertani (LB) broth and agar were used for cultivation of *E. coli* strains. All plasmid subcloning experiments were performed in *E. coli* DH10B using standard protocols (18). The final antibiotic concentrations used for selection of *E. coli* were as follows: 5 μg/ml apramycin and 15 μg/ml streptomycin for *S. pyridomyceticus*; 30 μg/ml apramycin, 50 μg/ml kanamycin, 100 μg/ml ampicillin, and 12.5 μg/ml chloramphenicol for *E. coli*.

**DNA Isolation and General Manipulations**—DNA isolation and manipulation in *E. coli* and *Streptomyces* were as follows: 5 μg/ml streptomycin and 15 μg/ml apramycin for *S. pyridomyceticus*; 30 μg/ml apramycin, 50 μg/ml kanamycin, 100 μg/ml ampicillin, and 12.5 μg/ml chloramphenicol for *E. coli*.

**DNA Sequence Analysis**—ORFs were analyzed and identified using the Frame Plot 3.0 beta online program (21), and the deduced proteins were compared with other known proteins in the databases using the NCBI BLAST server (22). Multiple nucleotide sequence alignments and analysis were performed using the BioEdit Sequence Alignment Editor (available on the World Wide Web) or Vector NTI Advance 11.0 (Invitrogen). The NRPS-PKS architecture was predicted by NRPS-PKS using the BioEdit Sequence Alignment Editor (available on the World Wide Web) (23).

**Inactivating PyrG-KR in S. pyridomyceticus NRRL B-2517 by Point Mutation**—The mutated sequence on pJTU4676PSY (Thio⁵) was introduced into *S. pyridomyceticus* by interspecific conjugation. Thiostrepton-sensitive (Thio⁵) clones were selected and confirmed to contain the desired chromosomal point mutation (HTT19PSY) using PCR and sequencing.

**Genetic Manipulation of pyrA and pyrU in S. pyridomyceticus**—See the supplemental material.

**Pyridomycin Fermentation, Isolation, Feeding Experiments, and HPLC-MS Analysis**—See the supplemental material.

**Cloning of pyrU and pyrA**—pyrU and pyrA were amplified using KOD-plus high fidelity PCR polymerase (Toyobo) and cosmid 9A3 as template (primers listed in supplemental Table S3). The purified PCR products pyrU (255-bp Ndel/BamI fragment) and pyrA (1632-bp Ndel/EcoRI fragment) were ligated into pET28a digested with the same restriction enzymes to generate plasmids pJTU4655 and pJTU4637. The correct sequences of pyrU in pJTU4655 and pyrA in pJTU4637 were confirmed by DNA sequencing.

**Construction of pJTU4655(S47A) and pJTU4652(S47A)**—To achieve the mutation of serine to alanine at position 47 in PyrU, pJTU4655(S47A) and pJTU4652(S47A) were constructed by site-directed mutagenesis using the primers PyrUS47AF and PyrUS47AR (supplemental Table S3); pJTU4655 and pJTU4652 served as templates. The mutations were verified by DNA sequencing. pJTU4655(S47A) was used for PyrU(S47A) overproduction, whereas pJTU4652(S47A) was used to complement the ΔpyrU mutant.

**Overproduction and Purification of Recombinant Proteins**—See the supplemental material.

**Phosphopantetheinylation of PyrU and PyrU(S47A)**—The in vitro assays for phosphopantetheinylation of PyrU or PyrU(S47A) were carried out in a 100-μl reaction containing 30 μM apo-PyrU, 0.5 mM CoA, 3 μM Sfp, 10 mM MgCl₂, 2 mM DTT, and 20 mM Tris·HCl, pH 8.0, at 37 °C for 45 min. Reactions were started by adding Sfp (24) and quenched by flash freezing at −80 °C. The in vivo assays were performed by coexpression of PyrU and PyrU(S47A) with Sfp in *E. coli* BL21 (DE3), followed by purification of the recombinant proteins.

The assays were analyzed by HPLC on an Agilent HPLC series 1100 with a ZORBAX 300SB-C18 column (4.6 × 250 mm, 5 μm, 300 Å, Agilent), using a 10–90% (v/v) gradient of acetonitrile/water containing 0.1% (v/v) trifluoroacetic acid (TFA) for 30 min at a flow rate of 0.5 ml/min and UV detection at 280 nm.

The LC-MS analyses were performed on a 6530 Accurate-Mass QTOF spectrometer coupled to an Agilent HPLC 1200 series (Agilent Technologies) that was developed for 30 min...
using a 5–95% linear gradient of acetonitrile/water containing 0.1% TFA at a flow rate of 0.2 ml/min. Data were obtained in the positive mode, and the mass scan range was set between 600 and 2500 m/z. The resulting spectra were analyzed using the software Mass Hunter, which calculated the masses of the intact proteins.

**Determination of Substrate Specificity of PyrA**—PyrA activity was measured by monitoring PPi release at 360 nm continuously for 30 min, using the EnzChek pyrophosphate assay kit according to the manufacturer’s instructions (MicroProbes) on a Synergy 2 multimode microplate reader (BioTek). PyrA (1.9 μg and 0.3 μM) was used to determine the initial velocity with 3-HPA or its analogs (4 mM) as the substrates. The standard curve was created using the pyrophosphate standard from the kit. Reactions were carried out in triplicate with boiled PyrA as control. Kinetic analysis of PyrA for 3-HPA, 2,3-dihydroxybenzoic acid (2,3-DHBA), and 4-amino-2-hydroxybenzoic acid (4A2HBA) were performed by varying each substrate concentration (0.01–0.32 mM 3-HPA, 0.4–4.8 mM 2,3-DHBA, and 0.4–4.0 mM 4A2HBA) in the presence of 2 mM ATP. The reactions were initiated, adding 1.2, 0.3, and 0.6 μM PyrA, respectively. The velocity was calculated based on the increase in absorbance at 360 nm. The Michaelis-Menten equation was fitted to plots of velocity of PPi release versus substrate concentration to extract values for $k_m$ and $k_{cat}$ using the program GraphPad Prism 5.

**Acylation of Holo-PyrU**—Loading of 3-HPA and its analogs onto holo-PyrU catalyzed by PyrA was performed in standard reactions containing 50 mM Tris-HCl (pH 8.0), 10 mM MgCl$_2$, 2 mM DTT, 5 mM ATP, 2 mM substrate, 5 μM PyrA, and 30 μM holo-PyrU. Reactions were initiated by the addition of PyrA, incubated at 37 °C for 30 min, and quenched by flash freezing at −80 °C. After centrifugation, the clarified supernatant was separated by HPLC, and the new peak for candidate enzymatic products was collected and concentrated using a Savant SPD111V SpeedVac concentrator (Thermo Scientific). The identities of products were determined by HPLC and QTOF MS analysis as for the analysis of PyrU.

**RESULTS**

**Cloning, Sequencing, and Analysis of the pyr Gene Cluster**—To identify the pyridomycin biosynthetic gene cluster, a genomic library of *S. pyridomyceticus* NRRL B-2517 was constructed in pJ446 (about 2,000 cosmid clones). Degenerate PCR primers (supplemental Table S3) designed according to the conserved core regions A3 and A7 of NRPS adenylation (A) domains (23, 25) were used for the initial screening of the cosmid library, and more than 100 A domain positive cosmids were isolated. These were then tested using degenerate primers designed according to the conserved regions that are unique to KS domains of hybrid NRPS/PKS (7). More than 20 cosmids hybridizing to both the A domain and the KS domain probes were isolated. Restriction mapping produced two separate contigs. In order to identify the contig containing the pyridomycin biosynthesis gene cluster, additional degenerate primers specific for conserved motifs in 3-HPA:AMP ligases (responsible for activation of 3-HPA in the biosynthesis of pristinamycin, virginiamycin, and etamycin) (13, 14, 26) were used. The deduced protein sequence of the amplified fragment from contig 2 resembled the 3-HPA:AMP ligase from *Streptomyces pristinaeaparalis* (supplemental Fig. S2). From this, we concluded that contig 2 may be involved in pyridomycin biosynthesis. To confirm this hypothesis, a 20-kb sequence in cosmid 9A3 of contig 2 was replaced by a kan cassette. This mutation was then introduced into the *S. pyridomyceticus* chromosome. The resulting strain, HT77, no longer produced pyridomycin (supplemental Fig. S3). Thus, cosmid 9A3 was sequenced to yield a continuous 42.5-kb DNA region, the GC content of which is 73.56%, typical for *Streptomyces* (19), and 26 ORFs were predicted (Fig. 2 and Table 1).

**Assembly of the Pyridomycin Core**—Among the 26 ORFs, two typical NRPS genes, *pyrE* and *pyrG*, and a PKS gene, *pyrF*, were identified, and their functional domains matched the chemical structure of the pyridomycin core (Fig. 2A).

PyrE consists of two minimal NRPS modules and is most similar (45% identity) to DhbF, involved in the biosynthesis of the catecholic siderophore bacillibactin from *Bacillus subtilis* (27). The two A domains of PyrE are very similar to known A domains, and they feature all 10 highly conserved motifs. Module 1 of PyrE is similar to the pristinamycin 1 synthetase 2, which forms a 3-hydroxypicolinic acid-threonine moiety as was predicted for pyridomycin biosynthesis. Also, the 10 residues in the aa binding pocket predict incorporation of threonine by module 1 (28), consistent with the chemical structure and the feeding experiments (11). PyrE module 2 probably incorporates 3-(3-pyridyl)-L-alanine, which is similar to the weakly predicted phenylalanine.

PyrF is a minimal PKS module. The KS domain features the highly conserved catalytic Cys-His-His triad (29). The KS domain is most similar to typical NRPS/PKS hybrid KSs from *Bacillus epollid* (36% identity) or *epoD* (34% identity) (30, 31). The acyltransferase domain of PyrF contains the highly conserved active site GHSXG and is similar to methylmalonyl-CoA acyltransferases.

Special features of the PyrG (2451 aa) architecture are the location of a PKS KR domain and two A domains. PyrG is therefore an NRPS/PKS hybrid protein. The C domain in PyrG probably forms a C–O (ester) link as has been found in fumonisin (32) and antibiotic C-1027 (33).

The KR domain in PyrG was predicted to be functional because it contains a Rossmann fold for NAD(P)H binding and conserved Lys, Ser, and Tyr residues (34) (supplemental Fig. S4). Mutation of conserved active sites of the KR domain S163A/Y176F resulted in complete loss of pyridomycin production, and no intermediate product was detected in the supernatant or in the mycelium of three independent mutant clones. Analysis of the aa binding pockets of the two NRPS A domains gave no firm prediction for the type of extender unit. The PyrG-A1 domain lost the conserved A3 motif, which is critical for adenylate formation, whereas the A6 and A8 motifs in PyrG-A2 are not conserved (35) (supplemental Fig. S5). A TE domain at the C-terminal end of PyrG is probably responsible for lactone formation, as shown in Fig. 2A.

**Bioisynthesis of the Pyridyl Molecules**—PyrB is an L-lysine 2-aminotransferase similar to VisA (61% identity and 70% similarity) involved in 3-HPA formation for virginiamycin biosyn-
thesis in Streptomyces virginiae (14, 36) and to NikC (56% identity and 69% similarity) that catalyzes the initial reaction for converting lysine to the pyridyl residue in nikkomycin D in Streptomyces tendae (37). However, L-aspartic acid instead of L-lysine (along with glycerol or pyruvic acid) was incorporated into the two pyridyl residues in pyridomycin. This suggested that pyrB should not be involved in the pyridomycin biosynthesis and that the formation of the pyridyl residues may follow either the NAD biosynthetic pathway (path a in Fig. 2B) (38, 39) or the aspartate family of amino acids biosynthetic pathway (path b in Fig. 2B) (40).

Five genes (pyrP to -T) are transcribed in the same orientation and may constitute an operon (Table 1 and Fig. 2A). They initially seemed to be involved in the biosynthesis of pyridyl moieties. PyrQ is a putative aspartate kinase (40). PyrP resembles 3-dehydroquinate synthase (41). The other genes encode oxidoreductases. However, the inactivation of all of these genes did not affect pyridomycin production (supplemental Tables S2 and S3). These findings indicated that the biosyntheses of the pyridyl moieties would follow path a in Fig. 2B, using some genes from the primary metabolism.

Unknown or Tentative Role in Pyridomycin Biosynthesis—Immediately downstream of the NRPS/PKS genes, pyrH to -N may form an operon (Fig. 2A and Table 1). PyrH (71 aa) is 74% identical to the MbtH-like protein from Streptomyces fungicidicus, and it contains the three conserved Trp residues that may be important for moderating protein-protein interactions (42, 43). Similar proteins are integral parts of several NRPSs that stimulate specific A domains (44). PyrI, -K, -L, and -M are putative oxidoreductases (cytochrome P450 or dehydrogenases), and PyrN is similar to an esterase. The roles of these enzymes in pyridomycin biosynthesis remain unclear.

pyrC and pyrD, located upstream of NRPS/PKS genes, encode a flavin-dependent oxidoreductase and a sarcosine oxidase, respectively. Similar gene pairs of unknown functions (snaO (67% aa identity) and snaN (58% aa identity), and virN (65% aa identity) and virM (58% aa identity)) occur in the prisatinamycin II (45) and in the virginamycin M biosynthesis gene clusters (14).
**Pyridomycin Biosynthesis**

**TABLE 1**

<table>
<thead>
<tr>
<th>ORF</th>
<th>Size*</th>
<th>Homologous protein, species</th>
<th>Identity/Similarity</th>
<th>Accession number</th>
<th>Proposed function*</th>
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<tr>
<td>Pyr1</td>
<td>49</td>
<td><em>Ccel_0989</em>, Clostridium cellulolyticum H10</td>
<td>44/68</td>
<td>YP_002505331</td>
<td>FkbH-like protein</td>
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<td>246</td>
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<td>NP_651322</td>
<td>Ketoreductase</td>
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<td>Pyr3</td>
<td>334</td>
<td>Bthr0003, 63860, Bacillus thuringiensis serovar thuringiensis str. T01001</td>
<td>29/53</td>
<td>ZP_04137149</td>
<td>Transport/self-resistance</td>
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<td>Pyr4</td>
<td>210</td>
<td>Shewm77, 2680, Shewanella sp. MR-7</td>
<td>33/47</td>
<td>YP_737822</td>
<td>Unknown</td>
</tr>
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<td>Pyr5</td>
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<td>Ssgd_07479, S. pristinae spiralis ATCC 25486</td>
<td>71/87</td>
<td>EFH32214</td>
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</tr>
<tr>
<td>PyrB</td>
<td>418</td>
<td>Ssgd_07479, S. pristinae spiralis ATCC 25486</td>
<td>66/75</td>
<td>EFH32215</td>
<td>Aminotransferase</td>
</tr>
<tr>
<td>PyrA</td>
<td>543</td>
<td>Ssgd_07480, S. pristinae spiralis ATCC 25486</td>
<td>68/76</td>
<td>EFH32216</td>
<td>3-Hydroxyproline acid:AMP ligase</td>
</tr>
<tr>
<td>PyrU</td>
<td>84</td>
<td>Ssgd_05103, S. pristinae spiralis ATCC 25486</td>
<td>36/59</td>
<td>ZP_05014213</td>
<td>Phosphopantetheine binding</td>
</tr>
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<td>PyrC</td>
<td>384</td>
<td>Ssgd_05104, S. pristinae spiralis ATCC 25486</td>
<td>67/76</td>
<td>ZP_05014214</td>
<td>Oxidoreductase</td>
</tr>
<tr>
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<td>Ssgd_05106, S. pristinae spiralis ATCC 25486</td>
<td>60/71</td>
<td>ZP_05014216</td>
<td>Oxidase</td>
</tr>
<tr>
<td>PyrE</td>
<td>2147</td>
<td>SgrIT_01000025868, Streptomyces griseoflavus Tu4000</td>
<td>49/61</td>
<td>ZP_05541578</td>
<td>Dimodular NRPS (C-A-T-C-A-T)</td>
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<td>1411</td>
<td>Ava_1612, Anabaena variabilis ATCC 29413</td>
<td>38/55</td>
<td>YP_322130</td>
<td>PKS (KS-AT-ACP)</td>
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<tr>
<td>PyrG</td>
<td>2451</td>
<td>SAML0567, S. ambiaciens ATCC 23877</td>
<td>45/55</td>
<td>CAJ89348</td>
<td>NRPS (C-A-A2-KR-TE)</td>
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<tr>
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<td>71</td>
<td>Sros_3469, S. fungicidicus</td>
<td>63/74</td>
<td>YP_003339149</td>
<td>MbtH-like protein</td>
</tr>
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<td>Pyr1</td>
<td>401</td>
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<td>65/76</td>
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<td>Aminotransferase</td>
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<td>PyrK</td>
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<td>53/65</td>
<td>YP_00278538</td>
<td>Oxidoreductase</td>
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<td>PyrL</td>
<td>402</td>
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<td>ZP_0501560</td>
<td>Dehydrogenase</td>
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<td>PyrM</td>
<td>488</td>
<td>nfa46290, Nocardia farcinica IFM 10152</td>
<td>53/64</td>
<td>YP_120844</td>
<td>Dehydrogenase</td>
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<td>PyrN</td>
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<td>SBI_07222, S. binghengensis BCW-1</td>
<td>49/60</td>
<td>ADI10342</td>
<td>Esterase</td>
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<td>PyrP</td>
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<td>SAV_2270, Streptomyces avermitilis MA-4680</td>
<td>45/64</td>
<td>NP_823446</td>
<td>TetR family regulator</td>
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<tr>
<td>PyrP</td>
<td>370</td>
<td>Franki3_4206, Frankia sp. C013</td>
<td>47/66</td>
<td>YP_482832</td>
<td>3-Deoxyquinate synthase</td>
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<tr>
<td>PyrQ</td>
<td>454</td>
<td>SCAB_41651, Streptomyces sp. T01001</td>
<td>39/56</td>
<td>YP_003489785</td>
<td>Aspartate kinase</td>
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<td>PyrR</td>
<td>408</td>
<td>MXAN_4919, Myxococcus xanthus DK 1622</td>
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<td>PyrS</td>
<td>513</td>
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<td>YP_440349</td>
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<td>PyrT</td>
<td>497</td>
<td>SAML0550, Nocardia farcinica IFM 10152</td>
<td>68/82</td>
<td>YP_119476</td>
<td>Alddehyde dehydrogenase</td>
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---

* Number of amino acids in the ORF predicted by Frame Plot 3.0
* Functions of the most similar proteins from the NCBI database and from the predicted conserved motifs. NRPS and PKS domains are abbreviated as follows: A, adenyla-
* tion; C, condensation; T, thiolation; KS, ketoacyl synthase; AT, acyltransferase; ACP, acyl carrier protein; KR, ketoreductase; TE, thioesterase.

**Genes Involved in Regulation and Self-resistance**—Pyr3, a putative membrane protein, could be involved in a transport system for pyridomycin export and self-resistance. PyrO resembles TetR regulators containing a conserved helix-turn-helix DNA-binding domain (Pfam00440) (46). The inactivation of pyr2 (HTT12; supplemental Table S1) had no effect on pyridomycin production (confirmed by LC-MS analysis; data not shown), and it may be outside the biosynthetic gene cluster.

**3-HPA Activation by PyrA and PyrU**—The above analysis of PyrE to -G found no loading module. PyrA (543 aa), upstream of the NRPS/PKS/NRPS locus, is a probable 3-HPA:AMP ligase because it is similar to SnpA involved in pristinamycin I biosynthesis by *S. pristinae spiralis* (68% identity) and VisB involved in virginamycin S biosynthesis by *S. virginiae* (65% identity) (14, 26). Presumably, 3-HPA is activated by PyrA and incorporated into the pyridomycin assembly line (Fig. 2A). To confirm the role of *pyrA* in pyridomycin biosynthesis, an internal 1101-bp DNA fragment (encoding the entire catalytic triad) was replaced in the *S. pyridomyceticus* genome using the PCR targeting method (Fig. 3, A and B). HPLC and bioassay showed that pyridomycin production was completely abolished in the *pyrA* deletion mutant HTT6, but it was restored to near wild-type level by in trans complementation by a full-length *pyrA* gene expressed from *Perme* promoter (promoter of the erythromycin resistance gene) in the integrating plasmid pJTU4637b (Fig. 3, C and D).

Next to *pyrA* but reading in the opposite direction is *pyrUL*, which encodes a putative carrier protein. PSI-BLAST of its deduced amino acid sequence showed 42% identity to isochloromatase from *Vibrio harveyi* HY01. 37% to enterobactin synthetase component B in *Azotobacter vinelandii* DJ, and 36% to a conserved hypothetical protein from *S. pristinae spiralis* ATCC 25486. Sequence alignment with homologous proteins revealed an LGXXS motif in PyrU, which is a conserved motif in carrier proteins (47) (supplemental Fig. S6). Thus, PyrU is proposed to function as a PCP for tethering 3-HPA. No similar protein has been found among the pristinamycin I and virginamycin S biosynthetic genes.

To confirm that *pyrUL* is critical to the biosynthesis of pyridomycin, the ApyrU mutant HTT5 was constructed (Fig. 4, A and B) and lost the production of pyridomycin, as analyzed by HPLC. Complementation by introducing pJTU4655 containing *pyrUL* constitutively expressed from the ermE' promoter partially restored pyridomycin production of HTT5 (Fig. 4C). As expected, the mutant gene *pyrUL* failed to restore normal pyridomycin production. The bioassay using *Mycobacterium smegmatis* mc^2^155 indicated that a trace amount of pyridomycin was produced by HTT5, and this was confirmed by LC-MS analysis ([M + H]^+^ 541.3 m/z, identical to the pyridomycin standard; Fig. 4, C and D). All of these findings clearly demonstrated that the putative PCP PyrU was essential for pyridomycin biosynthesis and that the serine 47 residue is the active site for the proposed phosphopantetheinylation.

**PyrUL Is a Peptidyl Carrier Protein for Loading 3-HPA in Vivo and in Vitro**—To test whether PyrU functions as a PCP for loading 3-HPA, Hisg-apo-PyrU, Hisg-holo-PyrU, and, as a control, the presumably inactive mutant Hisg-PyrUS47A were overproduced separately in *E. coli* (supplemental Fig. S7).

Phosphopantetheinylation *in vivo* was tested by coexpression of PyrU and Sfp (*B. subtilis* phosphopantetheinylation transferase expressed from pSV20) (48). Expression of PyrU without Sfp was used as a control. Protein purification and HPLC analysis showed that coexpression produced a new peak at 27.8 min
compared with the peak eluting at 28.3 min from PyrU alone (Fig. 5A, a and b). The identity of the two peaks was confirmed by ESI-QTOF-MS analysis, giving 11,294.90 Da (calcd: 11,294.77 Da for apo-PyrU) and 11,635.21 Da (calcd: 11,635.86 Da for holo-PyrU), which is a 340 mass shift, consistent with a 4'-phosphopantetheine cofactor covalently attached to the Ser residue of apo-PyrU (Fig. 5B) (49). However, PyrU with the S47A mutation coexpressed with pSV20 only gave one peak at 28.8 min on HPLC analysis, showing that it was inactive (Fig. 5A, d and e).

Subsequently, we monitored the incorporation of the 4'-phosphopantetheine cofactor in vitro. When incubated with CoA and Sfp, apo-PyrU was quantitatively converted to holo-PyrU (Fig. 5A, c), as demonstrated by HPLC analysis, whereas no change was observed for PyrUS47A. Together, the data provide direct evidence that PyrU is a PCP with an active site Ser47."
Substrate Specificity of PyrA—To determine the substrate specificity in vitro, the 58.9-kDa N-terminally His6-tagged PyrA was produced in E. coli (supplemental Fig. S8). The reaction velocity of PPi release from ATP, catalyzed by PyrA with different aromatic acids and amino acids at 4 mM concentrations, was determined using a continuous spectrophotometric assay (see “Experimental Procedures”). The results depicted in Fig. 6A showed that 2,3-DHBA and 4A2HBA were activated with even higher reaction velocity than 3-HPA. The $K_m$ and $k_{cat}$ values for these three substrates were determined and are shown in Fig. 6C. Although higher turnover numbers ($k_{cat}$) were indeed found for 2,3-DHBA and 4A2HBA, the catalytic efficiency ($k_{cat}/K_m$) of PyrA for 3-HPA was 22- and 40-fold higher than that for 2,3-DHBA and 4A2HBA, respectively. Taken together, the results are consistent with the assignment of 3-HPA as the native substrate of PyrA. However, the capability of PyrA to accept a range of different substrates if supplied at sufficient concentration offers the prospect to generate pyridomycin derivatives by feeding alternative starter units to a strain that has lost by mutation the ability to synthesize 3-HPA.

In Vitro Reconstitution of the Loading Module for the Pyridomycin Biosynthesis—The functions and the location of PyrA and PyrU led to the expectation that PyrA may activate 3-HPA and tether it to PyrU, followed by transfer to the NRPS/PKS assembly line. To confirm this hypothesis, His6-holo-PyrU, His6-PyrA, and the substrate 3-HPA were incubated in a buffer containing Mg$_2^+$, DTT, and ATP. HPLC analysis and high resolution ESI-QTOF-MS showed a new peak at $m/z$ 11,756.32 Da, consistent with the expected 3-HPA-holo-PyrU (calcld: 11,756.87 Da) (Fig. 7, a and b).

The 3-HPA analogs 4A2HBA and 2-fluorobenzoic acid (2-FBA) also yielded the expected products at $m/z$ 11,770.89 and 11,757.87 Da, respectively (Fig. 7, c and d). Therefore, PyrA tethers 3-HPA and alternative aromatic molecules to PyrU, which acts as the loading module for pyridomycin assembly.

Production of New Pyridomycin Analogues—Inspired by the broad substrate specificity of PyrA and PyrU, we created new pyridomycin analogs by feeding picolinic acid, 2,3-DHBA, 4A2HBA, 2-FBA, and 2-chlorobenzoic acid into the wild-type strain. LC-QTOF-MS revealed three analogs derived from picolinic acid, 2,3-DHBA, and 4A2HBA (Table 2 and supplemental Fig. S9). No new product was generated from 2-FBA or 2-chlorobenzoic acid.

DISCUSSION

In this work, we report the cloning and characterization of a 42.5-kb DNA fragment of S. pyridomyceticus NRRL B-2517, which contains a gene cluster that encodes the enzymes for the assembly of the core structure of the antimycobacterial antibiotic pyridomycin. The gene cluster represents an unusual NRPS/PKS hybrid system. PyrE, an NRPS elongation module containing two minimal modules (C-A-PCP), activates and tethers threonine and 3-(3-pyridyl)-L-alanine to the PCPs of NRPS1 and NRPS2, respectively, and forms an amide bond. Next, PyrF, a typical PKS elongation module, probably activates and transfers a methylmalonyl CoA to the PKS3 acyl carrier protein and elongates the chain.

Surprisingly, the PKS3, PyrF, lacks a KR domain that was thought to be responsible for the reduction of the $\beta$-keto group (*) to the hydroxyl group (*) of pyridomycin (Fig. 2A). A probably functional KR domain is embedded in the subsequent NRPS4 (PyrG). Based on the structural analysis of pyridomycin, the KR in PyrG was initially predicted to catalyze the reduction of the $\omega$-keto group (*) of the biosynthetic intermediate transferred from PKS3. Disruption by nonpolar point mutations should thus have produced a keto group at the posi-
tion in pyridomycin (indicated by asterisk in Fig. 2A). However, no product was detected, indicating that chain assembly was prematurely aborted.

Similar NRPS modules have been described for cereulide, valinomycin, cryptophycin, and hectochlorin biosynthesis (50–53). They all catalyze the reduction of the α-keto carboxyl acid that is tethered directly to the PCP by a cognate A domain. As predicted from the in vitro biochemical investigation (54), the KR point mutant HTT19PSY (S163A/Y176F) did not produce any pyridomycin intermediate. The conservative point
mutations were unlikely to change the overall structure of the NRPS4. It therefore seemed likely that the KR domain needs to be functional and is essential for pyridomycin production. Probably, α-keto-β-methylvaleric acid (derived from isoleucine) is activated by the tandem A domains of NRPS4 and tethered to PCP. Then the KR domain may reduce the α-keto group of α-keto-β-methylvaleric acid to hydroxy, ready for ester bond formation catalyzed by the C domain (Fig. 2A). The TE domain then detaches and circularizes the chain. This would generate a hypothetical precursor that needs to be reduced at the position indicated by the asterisk in Fig. 2A and oxidized to form a double bond (#) in the structure of pyridomycin. The enzymes catalyzing these steps remain to be identified.

The tandem A domains of PyrG may act together to activate the substrate and tether it to PCP because PyrG-A1 lacks the conserved A3 motif for adenylate formation, and the A6 and A8 motifs in PyrG-A2 are not conserved. The TE domain at the C terminus of PyrG was proposed to catalyze the cyclization of the mature pyridomycin linear chain to form the lactone.

PyrE, PyrF, and PyrG constitute the hybrid NRPS/PKS that synthesizes the pyridomycin ring structure. However, an enterobactin EntB-like loading module containing an ArL-Arcidom was still missing for the pyridomycin assembly line (55).

PyrA, a predicted 3-HPA:AMP ligase, was shown to link ATP and 3-HPA, releasing PPi. 3-HPA:AMP ligases were proved to be involved in the biosynthesis of streptogramin B antibiotics, such as pristinamycin I, etamycin, and virginiamycin S (12–14). Therefore, PyrA was envisioned to activate 3-HPA using ATP. Indeed, the in vitro experiments proved that PyrA selected and activated 3-HPA in the presence of ATP, a function that is normally performed by NRPS A domains.

The search for a carrier protein identified PyrU, a small 84-aa protein that has not been observed in the streptogramin B antibiotic biosynthesis (14, 45). Although it shows low homology with known PCPs or aryl carrier proteins (ArCPs), it features the conserved LGXXS motif for phosphopantetheinylation (supplemental Fig. S6). Therefore, PyrU was proposed to function as a PCP or ArCP receiving the activated 3-HPA from PyrA (55–57). To confirm the function of PyrU, the ΔpyrU mutant HTT5 was constructed. It almost completely lost pyridomycin production, which was restored by trans complementation, demonstrating the involvement of PyrU in pyridomycin biosynthesis. To obtain clear evidence that PyrU functions as a PCP/ArCP for loading 3-HPA, PyrU and its mutant PyrUS47A were overproduced and purified for biochemical characterization. Phosphopantetheinylation of PyrU but not of PyrUS47A was observed in vivo and in vitro, clearly identifying PyrU as a PCP/ArCP. With the characterized PyrA and PyrU in hand, we successfully reconstituted the loading module for pyridomycin assembly in vitro.

Pyridomycin and pristinamycin I use the same two initial building blocks. A predicted PCP gene (pyrU orthologue; SSDG_07480; Table 1) was found in the S. pristinaeae spiralis ATCC 25486 genome sequence, outside the pristinamycin biosynthetic gene cluster (45). We thus predict that the separate PCP may participate in the biosynthesis of pristinamycin I and other streptogramin B compounds.

Streptogramin B and pyridomycin contain 3-HPA starter units. The streptogramin 3-HPA is derived from lysine (14, 36, 37), but the labeling experiment indicated that both pyridyl moieties of pyridomycin originate from 1-aspatic acid, glyceral, and/or pyruvate, but lysine is not incorporated (11). Pyridyl ring formation from aspartate is known from the primary metabolism NAD biosynthetic pathway (39) (path a in Fig. 2B). The three initial steps of path b in Fig. 2B (aspartate kinase, aspartate semialdehyde dehydrogenase, and dihydropicolinate synthase) are also used in the biosynthesis of lysine (40). For pyridyl ring formation, dehydrogenation produces pyridine 2,6-dicarboxylic acid (58), which can be envisioned to be converted to picolinic acid by decarboxylation. However, inactivation of the predicted aspartate kinase pyrQ did not reduce pyridomycin production. We therefore propose that both pyridyl moieties are synthesized by the reactions shown in Fig. 2, path a, similar to NAD biosynthesis in the primary metabolism and the shikimate pathway (38).

PyrA activated a series of aromatic acids, including two aromatic amic acids, and transferred them to PyrU. Precursor feeding of S. pyridomycetus yielded three pyridomycin analogs (Figs. 6 and 7 and supplemental Fig. S9), but no product was generated from 2-chlorobenzoic acid or 2-fluorobenzoic acid. A combination of rational engineering of the biosynthetic pathway and precursor feeding will provide opportunities to produce novel pyridomycin derivatives. This work sets the stage for ongoing in depth investigations of pyridomycin biosynthesis.

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Identification and Characterization of the Pyridomycin Biosynthetic Gene Cluster of *Streptomyces pyridomyceticus* NRRL B-2517

Tingting Huang, Yemin Wang, Jun Yin, Yanhua Du, Meifeng Tao, Jing Xu, Wenqing Chen, Shuangjun Lin and Zixin Deng

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