

# Cloning, Sequencing, and Functional Analysis of the Biosynthetic Gene Cluster of Macrolactam Antibiotic Vicenistatin in *Streptomyces halstedii*

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## Summary

Vicenistatin, an antitumor antibiotic isolated from *Streptomyces halstedii*, is a unique 20-membered macrocyclic lactam with a novel aminosugar vicenisamine. The vicenistatin biosynthetic gene cluster (*vin*) spanning ~64 kbp was cloned and sequenced. The cluster contains putative genes for the aglycon biosynthesis including four modular polyketide synthases (PKSs), glutamate mutase, acyl CoA-ligase, and AMP-ligase. Also found in the cluster are genes of NDP-hexose 4,6-dehydratase and aminotransferase for vicenisamine biosynthesis. For the functional confirmation of the cluster, a putative glycosyltransferase gene product, VinC, was heterologously expressed, and the vicenisamine transfer reaction to the aglycon was chemically proved. A unique feature of the vicenistatin PKS is that the loading module contains only an acyl carrier protein domain, in contrast to other known PKS-loading modules containing certain activation domains. Activation of the starter acyl group by separate polyketides is postulated as well.

## Introduction

An antitumor antibiotic vicenistatin (Figure 1), produced by *Streptomyces halstedii* HC34, is unique in its structure of a 20-membered lactam core having an attachment of aminosugar vicenisamine [1, 2]. Of significance is that its antitumor activities were demonstrated against xenografted models of certain human colon cancers [1]. The characteristic structure and biological activities prompted us to launch its biosynthetic studies. We were particularly interested in the biosynthesis of the macrolactam aglycon because, while its major part was anticipated to be derived from a standard polyketide pathway, the starter portion appeared to be irrelevant to the acetate-propionate rule. In contrast to the polyketide extender units mostly including acetate and propionate, various kinds of starter units, such as branched fatty acids, aromatic carboxylic acids, and amino acids, are utilized depending upon PKSs, which turn out to provide important structural and biological diversities to polyketide metabolites [3]. Among those examples are ansa-

mycins [4], cytochalasins [5], hitachimycin [6], fluvirucins [7], epothilone [8], and antibiotic TA [9].

Concerning the biosynthesis of vicenistatin, we reported previously that the extender units of the aglycon were derived from acetate and propionate in a standard polyketide biosynthetic pathway, whereas the starter unit was derived from glutamate [10, 11]. Furthermore, (2*S*,3*S*)-3-methylaspartate but not (2*S*,3*R*)-3-methylaspartate was shown to be incorporated into vicenistatin [12]. We thus proposed that glutamate mutase should be involved in the rearrangement of L-glutamate into (2*S*,3*S*)-3-methylaspartate, which must subsequently be converted, after decarboxylation and epimerization, to a putative starter 3-amino-2-methylpropionyl unit.

In order to get closer insight into the unique and intriguing features of the vicenistatin biosynthesis, we undertook genetic and enzymatic studies. In the present paper, we describe the cloning, sequencing, and functional analysis of vicenistatin biosynthetic gene cluster.

## Results

### Cloning and Sequencing of the Vicenistatin Biosynthetic Gene Cluster

Since vicenistatin is structurally composed of a lactam polyketide aglycon and an aminosugar, the biosynthesis of each moiety is conceptually envisioned as homologous to those of well-known polyketides and deoxyhexoses. The early stages of the biosynthetic pathways for 2,6-deoxysugar are rather common in most organisms. Usually, NDP-glucose 4,6-dehydratase and NDP-4-keto-6-deoxyglucose 2,3-dehydratase are involved in most cases, and the genes for these two enzymes generally locate closely in a cluster. It has been well established that NDP-hexose 4,6-dehydratase genes are highly conserved in actinomycetes and can be used as a cloning probe [13]. The same is true for the 2,3-dehydratase genes. Consequently, we envisioned that the vicenistatin biosynthetic gene cluster should contain both of these 4,6-dehydratase and 2,3-dehydratase genes for the biosynthesis of vicenisamine. A cosmid library of the *S. halstedii* genome was thus constructed in pOJ446 according to a standard protocol and was screened by PCR methods using appropriate primers for these two genes. Restriction mapping of the resulting positive cosmids revealed the presence of at least three distinct 4,6-dehydratase genes and two distinct 2,3-dehydratase genes in the genome. Each 2,3-dehydratase gene was located in proximity to its respective 4,6-dehydratase gene. The last 4,6-dehydratase gene appeared to stand independently. Accordingly, these results suggested the presence of two possible gene clusters, each of which should contain a distinct gene set of 4,6-dehydratase and 2,3-dehydratase for the biosynthesis of 2,6-deoxysugar. Spot sequencing of each cluster was undertaken, and the presence of putative genes encoding a glutamate mutase and PKSs was successfully identified in the cosmid designated as K1B10.

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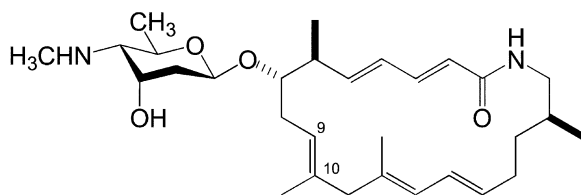


Figure 1. Structure of Vicenistatin

Since the feeding experiments had already shown that a glutamate mutase must be involved in the biosynthesis of the vicenistatin aglycon, we selected K1B10 as a core target for further genetic analysis.

An approximately 64 kbp region from four overlapping cosmids starting from K1B10 was ultimately sequenced

on both strands. The nucleotide sequence was deposited in the DNA Data Base of Japan (DDBJ) under the accession number AB086653. The sequence was then analyzed for putative open reading frames (ORFs) with the FRAME program [14], which makes use of a strong bias toward a G or C in the third position of *Streptomyces* codons, and the resulting ORFs were aligned with homologous sequences by BLAST programs available through the National Center for Biotechnology Information. Nineteen complete ORFs and one truncated ORF were identified and designated as *vin* genes (Figure 2A). Corresponding homologs and the putative function of each *vin* gene product are summarized in Table 1. Spot sequencing of the peripheral regions showed ORFs homologous to RNA polymerase, regulator, and geranylgeranyl diphosphate synthase, all of which are irrelevant to the putative biosynthetic genes for vicenistatin. Fur-

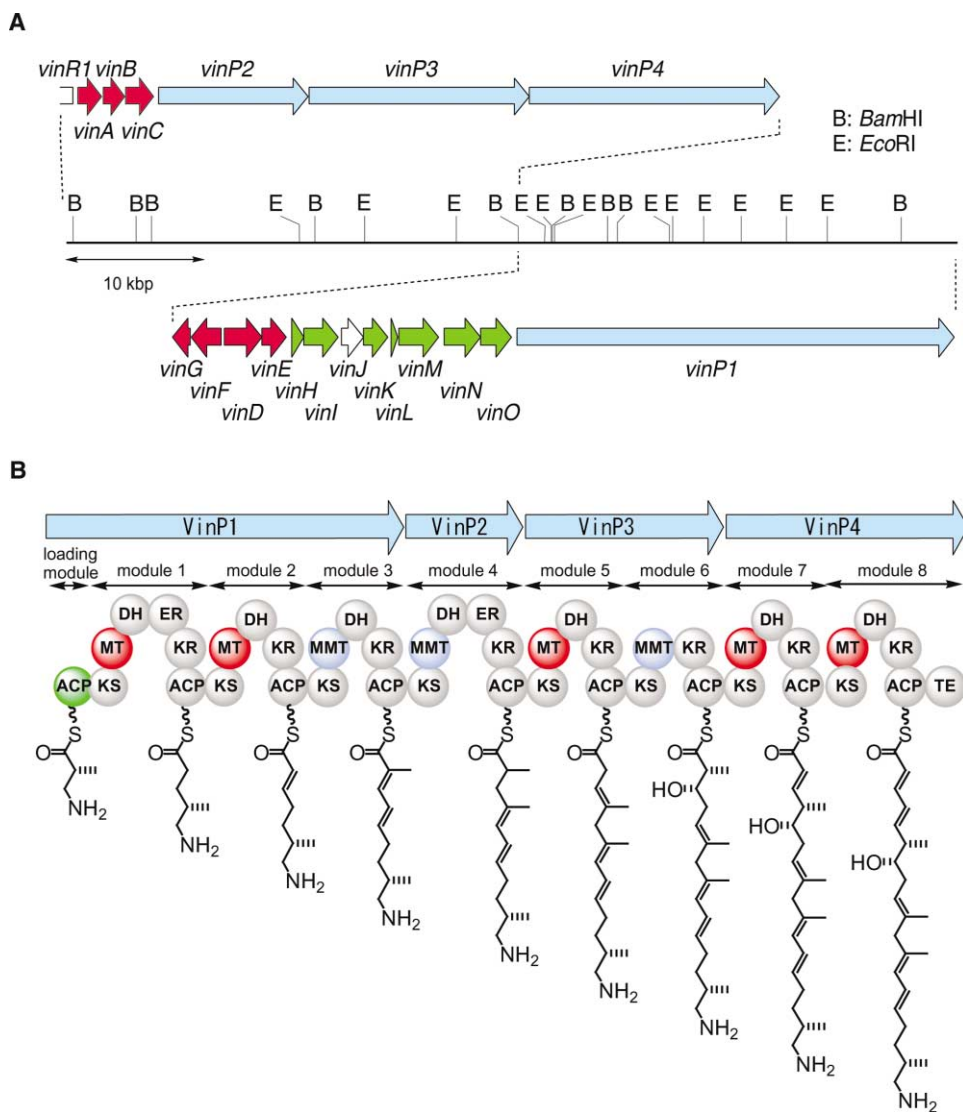


Figure 2. Organization of the Vicenistatin Biosynthetic Gene Cluster from *S. halstedii* HC34

(A) Genetic map of the *vin* gene cluster. Each arrow represents the direction of transcription of ORF.

(B) The modular architecture of polyketide synthases for vicenistatin aglycon.

Table 1. Summary of the *vin* Genes and Comparison with Database

| Protein | Amino Acids | Proposed Function               | Sequence Similarity (Protein, Origin)   | Identity/Similarity | Accession No. |
|---------|-------------|---------------------------------|---|---------------------|---------------|
| VinA    | 355         | dTDP-glucose synthase           | MtmD, <i>Streptomyces argillaceus</i>   | 69%/82%             | AJ007932      |
| VinB    | 323         | dTDP-glucose 4,6-dehydratase    | MtmE, <i>Streptomyces argillaceus</i>   | 72%/79%             | AJ007932      |
| VinC    | 419         | glycosyltransferase             | AknK, <i>Streptomyces galilaeus</i>     | 43%/59%             | AF257324      |
| VinD    | 489         | dTDP-glucose 2,3-dehydratase    | SnogH, <i>Streptomyces nogalater</i>    | 62%/76%             | AJ224512      |
| VinE    | 328         | dTDP-hexose 2,3-reductase       | TylCII, <i>Streptomyces fradiae</i>     | 60%/74%             | AF147704      |
| VinF    | 385         | aminotransferase                | DesI, <i>Streptomyces venezuelae</i>    | 52%/66%             | AF079762      |
| VinG    | 236         | N-methyltransferase             | AknX2, <i>Streptomyces galilaeus</i>    | 45%/63%             | AF264025      |
| VinH    | 165         | glutamate mutase S subunit      | NikU, <i>Streptomyces tendae</i>        | 55%/70%             | AJ250581      |
| VinI    | 469         | glutamate mutase E subunit      | NikV, <i>Streptomyces tendae</i>        | 58%/70%             | AJ250581      |
| VinJ    | 299         | proline iminopeptidase          | Pip2, <i>Sinorhizobium meliloti</i>     | 53%/68%             | AL591791      |
| VinK    | 327         | acyl CoA-ACP transacylase       | FabD, <i>Escherichia coli</i> K12       | 27%/39%             | AE000210      |
| VinL    | 82          | acyl carrier protein            | LnmP, <i>Streptomyces atroolivaceus</i> | 45%/61%             | AF484556      |
| VinM    | 524         | nonribosomal peptide synthetase | LnmQ, <i>Streptomyces atroolivaceus</i> | 42%/54%             | AF484556      |
| VinN    | 478         | CoA ligase                      | SMB20650, <i>Sinorhizobium meliloti</i> | 33%/49%             | AL603646      |
| VinO    | 414         | decarboxylase                   | BtrK, <i>Bacillus circulans</i>         | 31%/51%             | AB033991      |
| VinP1   | 5826        | PKS modules 1–3                 |   |                     |               |
| VinP2   | 2260        | PKS module 4                    |   |                     |               |
| VinP3   | 3362        | PKS modules 5 and 6             |   |                     |               |
| VinP4   | 3808        | PKS modules 7 and 8             |   |                     |               |
| VinR1   | —           | regulator                       | PikD, <i>Streptomyces venezuelae</i>    | 29%/46%             | AF079139      |

thermore, flanking the *vinR1* gene are ORFs with homology to regulators, thus apparently delineating the boundaries of the *vin* gene cluster.

#### The Genes Involved in Aglycon Biosynthesis

Identified in the *vin* cluster were four genes for PKSs comprised of 43 domains that were categorized into a loading module and eight extender modules as shown in Figure 2B. As mentioned above, the most intriguing issue for the biosynthesis of vicenistatin is the formation of the macrolactam starter unit. A PKS ORF (*vinP1*) appeared to encode a loading module and three extender modules. The loading module of *VinP1* is unusual because it contains only an acyl carrier protein (ACP) domain for loading. It is important to point out here that in most PKSs the loading module contains an acyltransferase (AT) and a  $\beta$ -ketoacyl ACP synthase (KS)-like domain, the latter usually having only decarboxylase activity, in addition to an ACP domain [3]. Therefore, the loading module of *VinP1* appears to be minimal so far.

All of the putative functions of the domains in this polyketide assembly line deduced from the homology research were consistent with the structure of the vicenistatin aglycon including the differentiation of malonyl and methylmalonyl extenders [15] and the oxidation state of each unit. Malonyl transferase (MT) domains appeared to be involved in modules 1, 2, 5, 7, and 8, and methylmalonyl transferase (MMT) domains were identified in modules 3, 4, and 6. The predicted ketoreductase (KR) domains all contain the consensus sequence GXGXXGXXA, characteristic of an NADP(H) binding site. All the dehydratase (DH) domains contain the conserved amino acid sequence HXXGXXXXP, often found in the functional DHs [16]. The enoylreductase (ER) domains in modules 1 and 4 both contain sequences that closely resemble a conserved sequence, LXXHXXGXXGXXAAXXXA, found in known functional ERs. Lastly, a thioesterase (TE) domain is found at the

C terminus of *VinP4*, which may be responsible for the release of the mature seco-acid and the macrolactam formation.

The amino acid sequences of *VinH* and *VinI* showed significant homology to S and E subunits of a coenzyme-B<sub>12</sub>-dependent mutase from *Streptomyces tendae*, respectively, which was shown to be involved in the isomerization of 2-oxoglutarate to 3-methylxaloacetate [17]. *VinH* and *VinI* are also similar to S and E subunits, respectively, of a glutamate mutase found in *Actinoplanes friuliensis* [18]. Glutamate mutase is a coenzyme-B<sub>12</sub>-dependent enzyme that catalyzes the reversible interconversion of (S)-glutamate to (2S,3S)-3-methylaspartate, and this type of catalysis was shown to be necessary for the biosynthesis of the starter unit as described above. Therefore, *VinH* and *VinI* were assigned as glutamate mutase. This may be the first glutamate mutase ever cloned from *Streptomyces*. The smaller subunit of glutamate mutase is known to contain a conserved sequence, DXHXXG, for the formation of the proposed catalytic triad with a conserved serine residue [19]; apparently, the first two conserved residues, Asp-39 and His-41, were found in *VinH*, and the third conserved glycine at 44 was replaced by asparagine.

Database search further suggested the putative roles of two ORFs, *VinM* and *VinN*, that may function in the activation of a carboxylic acid. *VinN* showed highest homology to ATP-dependent long-chain fatty acid-CoA ligase from *Sinorhizobium meliloti* (gene SMB20650, accession number AL603646) and *Mesorhizobium loti* (gene mll6742, accession number AP003010). *VinN* was further homologous to adenylation domains of nonribosomal peptide synthetases (NRPSs). An NRPS is a multifunctional modular enzyme containing an adenylation domain, a condensation domain, and a peptidyl carrier protein domain and functions in the generation of peptide-based secondary metabolites [20]. An adenylation domain selects a cognate amino acid and generates a

corresponding aminoacyl adenylate mixed anhydride at the expense of ATP. VinM was also shown to be homologous to such adenylation domains of various NRPSs and AMP ligases, such as LnmQ from *Streptomyces atroolivaceus* (accession number AF484556) [21], ACMSII from *Streptomyces chrysomallus* (accession number AF047717) [22], SypA from *Pseudomonas syringae* (accession number AF286216), and EntF from *E. coli* (accession number M60177) [23]. Apparently, both VinN and VinM have a putative AMP binding site. Previous isotope-tracer experiments showed that (2S,3S)-3-methylaspartate, generated by glutamate mutase, could be activated prior to epimerization and decarboxylation because (2S,3R)-3-methylaspartate and 3-amino-2-methylpropionate were not incorporated in the feeding experiments [12]. Thus, 3-methylaspartate may be activated either by VinM or VinN in the starter biosynthesis; however, the precise mechanism has yet to be clarified.

VinL showed highest homology to LnmP, a D-alanyl carrier from *Streptomyces atroolivaceus* (accession number AF484556) [21], and a putative 4'-phosphopantetheine binding serine residue was found in a highly conserved region of VinL. VinL is also homologous to other peptidyl carrier proteins and acyl carrier proteins. Therefore, VinL was assigned as an aminoacyl carrier protein involved in starter unit biosynthesis. VinK appeared to be homologous to malonyl transferase proteins of type II fatty acid synthases (FASs). VinK may thus be responsible for the transfer of the starter acyl unit to an ACP.

VinO was shown to be highly homologous to BtrK from *Bacillus circulans*, which was suggested to be a PLP-dependent decarboxylase involved in the biosynthesis of butirosin [24]. Good homology of VinO was also observed to LysA from *Pectobacterium chrysanthemi* (accession number AF416740), which is a PLP-dependent diaminopimelate decarboxylase involved in the primary metabolic pathway. Thus, VinO appeared to catalyze the decarboxylation of an amino acid intermediate involved in starter biosynthesis.

### Genes Involved in Sugar Biosynthesis

Sequence analysis suggested that the products of seven genes, *vinA*, *vinB*, *vinC*, *vinD*, *vinE*, *vinF*, and *vinG*, are involved in the vicenisamine biosynthesis. A high degree of similarity was observed for VinA and VinB with dTDP-glucose synthase MtmD [25] (accession number AJ007932) and dTDP-glucose 4,6-dehydratase MtmE [25] (accession number AJ007932), respectively, both of which were shown to be involved in the first two steps from glucose-1-phosphate to dTDP-4-keto-6-deoxyglucose in deoxysugar biosynthesis. Further, VinD was similar to SnogH (accession number AJ224512) [26], encoding an NDP-4-keto-6-deoxyglucose 2,3-dehydratase in the 2,6-deoxyhexose pathway. VinE was homologous to TylCII from *Streptomyces fradiae* (accession number AF147704) [27], which was shown to encode a dTDP-4-keto-6-deoxyhexose 2,3-reductase.

VinF showed good homology to DesI (accession number AF079762) [28] and RfbE (accession number X59554), which are aminotransferases involved in desosamine biosynthesis and in perosamine biosynthesis, respec-

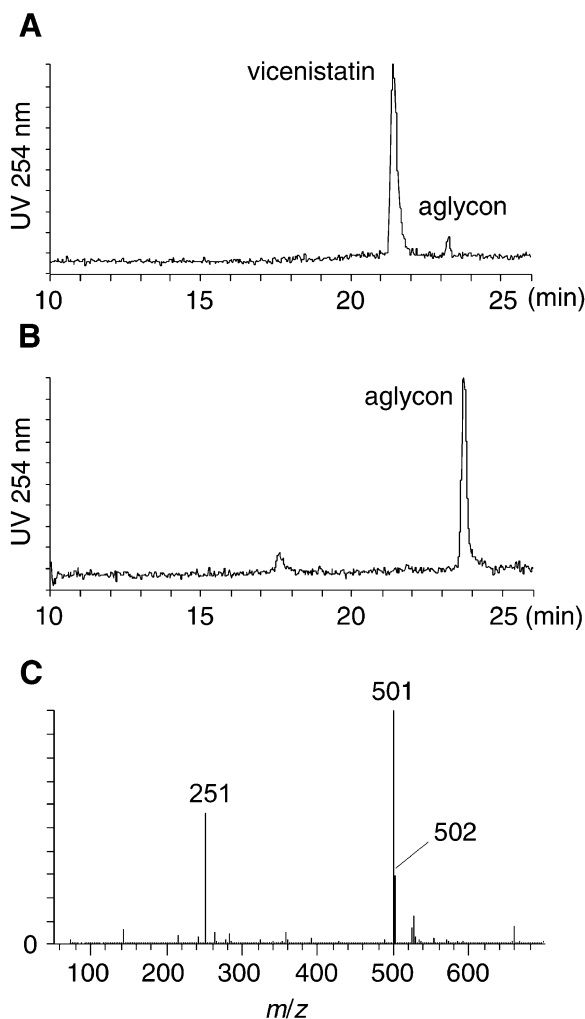


Figure 3. LC-MS Analysis of VinC-Catalyzed Reactions  
HPLC analysis of enzyme reactions using a cell-free extract from (A) *E. coli*/pET-vinC or (B) *E. coli*/pET30b(+) (as control). A peak at about 21.5 min is the VinC enzymatic reaction product, and (C) shows a corresponding mass spectrum of this product.

tively. VinG displayed sequence similarity to EryCVI from *Saccharopolyspora erythraea* [29] and to AknX2 from *Streptomyces galilaeus* [30], which were proposed to function as *N,N*-dimethyltransferase involved in aminohexose biosynthesis. We therefore propose that VinG is an *N*-methyltransferase involved in the last step of the dTDP-vicenisamine biosynthesis.

VinC was shown to have sequence homology to various glycosyltransferases involved in secondary metabolite biosynthesis, such as SpnP from spinosyn producer *Saccharopolyspora spinosa* (accession number AY007564) [31] and EryCIII from erythromycin producer *Saccharopolyspora erythraea*. It appears therefore that VinC may catalyze the glycosyl transfer from dTDP-vicenisamine to the aglycon, which has been successfully confirmed *vide infra*.

### Regulatory Genes

Although VinR1 has not yet been fully sequenced, it apparently contains conserved N-terminal triphosphate

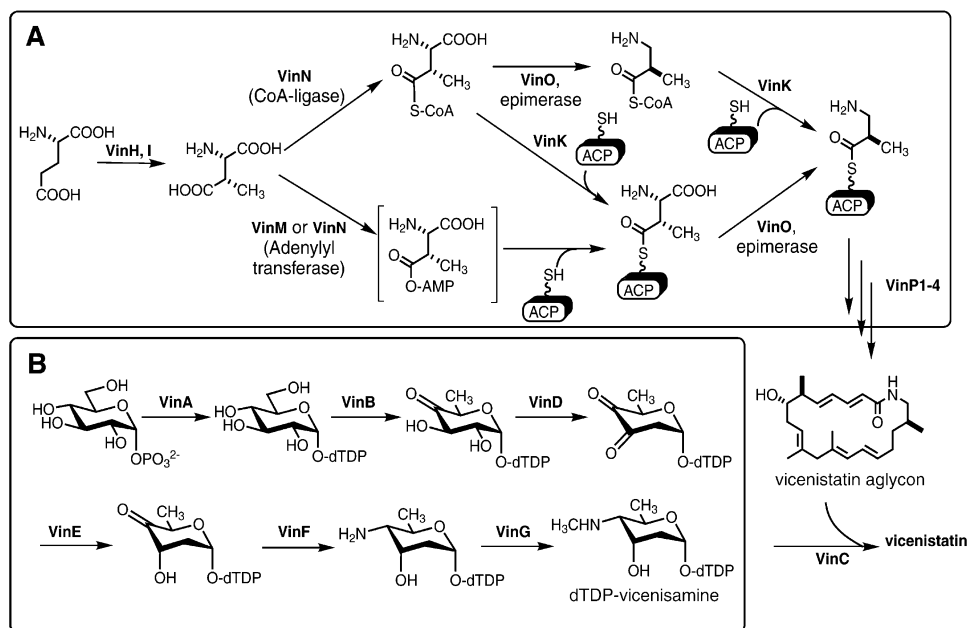


Figure 4. Proposed Biosynthesis of Vicenistatin  
(A) Several plausible pathways for the starter biosynthesis.  
(B) Biosynthetic pathway for vicenisamine.

binding domain of SARP (Streptomyces antibiotic regulatory proteins) [32]. In addition, the sequence of the *vinR1* product showed homology to NbmM (accession number AF521878) from *Streptomyces narbonensis* and PikD (accession number AF079139) from *Streptomyces venezuelae* [33], which are regulatory factors in desosamine biosynthesis and in pikromycin biosynthesis, respectively. Thus, it is plausible that the *vinR1* product is involved in regulation of vicenistatin biosynthesis.

#### Genes of Unknown Function

VinJ showed certain homology to a proline iminopeptidase PipIP from *Lactobacillus delbrueckii* [34], the function of which is to selectively hydrolyze peptidyl substrates containing proline at an N terminus to release proline. Proline iminopeptidase is rarely involved in polyketide biosynthetic gene clusters. Thus, a possible function of VinJ for vicenistatin biosynthesis, if any, is not clear at the moment.

#### Heterologous Expression and Functional Analysis of VinC

As described above, the sequence of VinC suggested its role as a glycosyltransferase. In order to confirm its putative glycosyltransferase function, *vinC* was cloned into an expression vector, pET30b(+), with which *E. coli* BL21(DE3) was transformed. SDS-PAGE of the transformant lysates clearly showed the production of soluble VinC protein. The vicenistatin aglycon and dTDP-vicenisamine were incubated with a cell-free extract of the transformant at 37°C for 24 hr. The reaction product was extracted with ethyl acetate and was compared with authentic vicenistatin by LC/ESI-MS, as shown in Figure 3. In an HPLC trace, a new peak was clearly observed

at about 21.5 min in the incubation with a cell-free extract of *E. coli*/pET-*vinC*, and no such peak was observed in a control incubation with a cell-free extract of *E. coli*/pET30b(+). A less mobile peak at about 24 min was the unreacted aglycon. The retention time, the molecular weight of the product, and the MS/MS fragmentation pattern were identical to those of authentic vicenistatin. These results clearly showed that VinC is the glycosyltransferase required for the final step of vicenistatin biosynthesis.

#### Discussion

In this paper, the sequence of the vicenistatin biosynthetic gene cluster that spans 64 kbp including 19 complete ORFs has been elucidated. The functional confirmation of VinC as a glycosyltransferase has proved that this *vin* cluster is indeed involved in the vicenistatin biosynthesis. Interesting organization of the *vin* genes and biochemical features are discussed below.

The first novel feature of the *vin* PKSs is its unique domain organization of the putative loading module, which contains only an ACP domain. Usually, the initial acyl transfer reaction to an ACP domain of a loading module is catalyzed by an AT domain or an A (adenylation) domain residing in the same loading module [3]. Apparently, this organization of the *vin* loading module is unprecedented in type I PKSs. In the vicenistatin PKS (VinP1), the starter acyl group may be loaded to the ACP domain by a certain "trans" enzyme encoded outside of VinP1. Several distinct scenarios are plausible for this unique acyl transfer reaction, as illustrated in Figure 4A, since several putative polypeptides have been deduced in the *vin* cluster as potential activation catalysts.

Based on the sequence homology to AMP ligase, both VinM and VinN appear to be capable of activating 3-methylaspartate to an aminoacyl adenylate, which may subsequently serve as an acyl donor onto the loading ACP domain. Alternatively, VinK as a discrete AT may transfer the starter acyl group to the ACP domain, and in this case, the requisite acyl-CoA substrate is generated by VinN, since VinN is homologous to CoA ligase as well.

Instead of direct acylation of the ACP domain of the loading module, VinL can first be acylated as an independent ACP, and the resulting acyl-VinL ultimately transfers the acyl group to the loading module. This putative trans-thioesterification of acyl chain is analogous to so-called skipping, which is known to be direct trans-thioesterification of an acyl group from an ACP to an ACP of the next-most module [35]. For this type of trans-thioesterification, the peptide-peptide interaction of the intermediary acylated VinL with the loading ACP domain of VinP1 should be important.

The second feature is the absence of an epimerase-encoding gene in the *vin* cluster sequenced so far. Previous feeding experiments suggested that epimerization of (2S,3S)-3-methylaspartate at C-3 is involved in the biosynthesis of aglycon. Although the possibility that a putative epimerase is encoded on a different part of the chromosome cannot be ruled out, alternatively, an epimerization may pleiotropically take place, for example, during the decarboxylation reaction.

The third feature of vicenistatin biosynthesis is the formation of a double bond between C-9 and C-10. The double bond in this position locates within the same extender unit, which is rather unusual. Normally, double bonds are formed by PKSs between the  $\alpha$  carbon of an extender unit and a carboxyl carbon of the acceptor unit. Similar unusual double-bond formation may be found in the biosynthesis of albocycline [36] and soraphen [37]; however, in the latter case nascent soraphen macrolactone synthesized by PKS appears to be fully reduced to a saturated state due to the presence of apparently functional KR, DH, and ER domains, and the double bond should therefore be generated by other modification enzymes [37]. On the contrary, in vicenistatin the C9-C10 double-bond formation seems to be through the catalysis of a DH domain in module 5, since the amino acid sequences of the DH domain and the neighboring region in module 5 are not so much different from those of other PKS modules. Thus, the double bond may be generated by direct dehydration of a  $\beta$ -hydroxyacyl intermediate due to stereochemical biases in the enzyme-substrate ternary complex. These interesting issues will be the subjects of future studies.

The biosynthesis of vicenisamine appears to be rather straightforward, as shown in Figure 4B. Glucose-1-phosphate is converted to dTDP-glucose by dTDP-glucose synthase VinA. Then, dTDP-glucose 4,6-dehydratase VinB is responsible for the conversion of dTDP-glucose to dTDP-4-keto-6-deoxyglucose, which is subsequently deoxygenated at C-2 by VinD to yield dTDP-3,4-diketo-2,6-dideoxyglucose. Further, the 3-keto group may be reduced in the next step by VinE, and VinF catalyzes transamination to the 4-keto group in the penultimate step. Last, VinG is responsible for *N*-methylation to yield

dTDP-vicenisamine, which is the substrate for the ultimate glycosyl-transfer reaction catalyzed by VinC to afford vicenistatin.

## Significance

The biosynthetic gene cluster (*vin*) of the antitumor antibiotic vicenistatin has been cloned from *Streptomyces halstedii* HC34 and sequenced. Precise information about the cluster should allow the understanding of biosynthesis of this unique macrolactam antibiotic. An important feature is the PKS loading module comprising only an ACP domain, which is unprecedented. The ACP domain should thus be loaded with a starter unit by the catalysis of enzymes other than PKS. The significance of this finding is to suggest that a loading module of PKSs may be segregated in certain cases. This rather simple loading system may thus provide the opportunity of a new approach to novel modified polyketides by gene manipulations. Furthermore, heterologous expression and functional confirmation of VinC as a glycosyltransferase involved in the transfer of vicenisamine to aglycon for the final step of vicenistatin biosynthesis proves that the elucidated *vin* cluster encodes the vicenistatin biosynthetic enzyme and provides another opportunity for modifying the carbohydrate portion.

## Experimental Procedures

### General Procedures

*Streptomyces halstedii* HC34 was a gift from Kirin Brewery Co., Ltd. *E. coli* DH5 $\alpha$  (TaKaRa) and plasmids pUC119 (TaKaRa) and LITMUS28 (New England Biolabs) were used for routine subcloning of DNA fragments. The cosmid vector pOJ446 was used for cosmid library construction [38]. Restriction enzymes and modification enzymes were purchased from TaKaRa. *E. coli* was grown at 37°C in Luria-Bertani (LB) broth or on LB agar supplemented, when necessary, with appropriate antibiotics. Ampicillin was added to 100  $\mu$ g/ml or kanamycin was added to 30  $\mu$ g/ml for selection of plasmid-containing *E. coli* cells. Purification of plasmids was carried out with GFX micro Plasmid Prep Kit (Amersham Biosciences). General DNA manipulations in *E. coli* were performed according to the standard protocols [39]. PCR was performed with a GeneAmp PCR System 9700 (Amersham Biosciences) using Ex-Taq DNA polymerase (TaKaRa). Oligo DNAs for PCR primer were synthesized in Amersham Biosciences.

### Construction of the *S. halstedii* Genomic Library

For the construction of the genomic library, the chromosomal DNA of *S. halstedii* HC34 was partially digested with Sau3AI. The resulting DNA fragments were then treated with shrimp alkaline phosphatase (Boehringer Mannheim) and ligated with a cosmid vector, pOJ446, that had previously been digested with HpaI, treated similarly with shrimp alkaline phosphatase, and digested with BamHI. The resulting ligation mixture was packaged into  $\lambda$  phage followed by phage transfection to *E. coli* XL1 Blue MRF' strain by using protocols described in the Gigapack III XL Packaging Extract (Stratagene).

### Screening of the *S. halstedii* Genomic Library

PCR primers were designed based on the reported multiple sequence alignments of several dTDP-glucose 4,6-dehydratases and dTDP-4-keto-6-deoxyglucose 2,3-dehydratases. For 4,6-dehydratase, primer 46DH-1, ACSGGYCSBGCCGCHTTCATCGG; primer 46DH-2, GRWRCTGRTRSGGCCGTAGTTGTT (S = C, G; Y = C, T; B = C, G, T; H = A, C, T; R = A, G; W = A, T); PCR conditions: 1 cycle at 94°C for 7 min followed by 30 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min. For 2,3-dehydratase, primer 23DH-1,

AGGCCACCCGSAGCAACTACAC; primer 23DH-2, GAASCGSCCG CCCTCCTCSGA; PCR conditions: 1 cycle at 94°C for 7 min followed by 30 cycles of 94°C for 50 s, 50°C for 50 s, and 72°C for 50 s.

#### Sequencing and Sequence Analysis

DNA sequencing was performed on double-stranded DNA templates by the dideoxynucleotide chain-termination method with a Model4000-1 (LI-COR) and a Thermo Sequenase Cycle Sequencing Kit. DNA sequences were analyzed with the Align-IR (LI-COR) and the DNASIS (Hitachi Software Engineering) programs. Homology search for protein sequences was carried out using BLAST and FASTA programs.

#### Expression of VinC in *E. coli*

The postulated glycosyltransferase gene (*vinC*) was amplified by PCR from cosmid K1B10 with primers VinC-Nterm (5'-AAGGTACCA TATGCGCGTCTGATGA-3') and VinC-Cterm (5'-CCCGGATCCGC TGGGCGGAGTGCTAC-3'). PCR conditions: 1 cycle at 98°C for 7 min followed by 30 cycles of 98°C for 30 s, 54°C for 30 s, and 72°C for 1 min using Ex taq DNA polymerase (TaKaRa). The introduced NdeI and BamHI restriction sites are underlined, respectively. The amplified product was cloned by using NdeI and BamHI sites into pET30b(+) to give pET-vinC. The pET-vinC was transformed into *E. coli* BL21(DE3) for overexpression.

The *E. coli* cells having pET-vinC and pET30b(+) (as a control) were separately grown in LB medium supplemented with 30 µg/ml kanamycin. Culture was grown at 37°C until OD<sub>600</sub> reached 0.6, 0.2 mM isopropyl β-D-thiogalactoside was added, and culture was continued at 37°C for 3 additional hr. The harvested wet cells (400 mg) were suspended in a 4 ml buffer (50 mM Tris-HCl [pH 7.5], 1 mM MnCl<sub>2</sub>, and 1 mM MgCl<sub>2</sub>) and disrupted by sonication (2 min, 10 times). The debris was removed by centrifugation (12000 × g, 30 min), and the supernatant was used as a cell-free extract for the following glycosylation reaction.

#### Enzyme Assay of VinC

Vicenistatin aglycon and dTDP-vicenisamine were prepared by adopting literature procedures [40]. A saturated solution (3.6 µl, 1.9 mg/ml) of vicenistatin aglycon in DMSO and 6.6 µg of dTDP-vicenisamine in water (14 µl) were incubated with a cell-free extract (100 µl) at 37°C for 24 hr. The reaction mixture was adjusted to pH 10 with NaOH and was extracted with ethyl acetate. The organic extract was evaporated and redissolved in MeOH, and the MeOH solution was analyzed with a LCQ mass spectrometer (Finnigan) coupled with a NANOSPAC HPLC (SHISEIDO) having a RP-18 GP column (KANTO). HPLC conditions were as follows: first washed with 10% MeOH and 0.1% TFA in water for 10 min, followed by elution with 90% MeOH and 0.1% TFA in water over 20 min, flow rate 50 µl/min. Elution was monitored with a NANOSPAC SI-1 UV detector (SHISEIDO) at a wavelength of 254 nm. Reference standard of vicenistatin was dissolved in methanol.

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#### References

- Shindo, K., Kamishohara, M., Odagawa, A., Matsuoka, M., and Kawai, H. (1993). Vicenistatin, a novel 20-membered macrocyclic lactam antitumor antibiotic. *J. Antibiot. (Tokyo)* **46**, 1076–1081.
- Arai, H., Matsushima, Y., Eguchi, T., Shindo, K., and Kakinuma, K. (1998). Absolute stereochemistry of vicenistatin, a novel 20-membered macrocyclic lactam antitumor antibiotic. *Tetrahedron Lett.* **39**, 3181–3184.
- Moore, B.S., and Hertweck, C. (2002). Biosynthesis and attachment of novel bacterial polyketide synthase starter units. *Nat. Prod. Rep.* **19**, 70–99.
- Hunziker, D., Tin-Wein, Yu., Hutchinson, C.R., Floss, H.G., and Khosla, C. (1998). Primer unit specificity in rifamycin biosynthesis principally resides in the later stages of the biosynthetic pathway. *J. Am. Chem. Soc.* **120**, 1090–1091.
- Vederas, J.C., and Tamm, C. (1976). Biosynthesis of cytochalasins. Part 6. The mode of incorporation of phenylalanine into cytochalasin D. *Helv. Chim. Acta* **59**, 558–566.
- Omura, S., Nakagawa, A., Shibata, K., and Sano, H. (1982). The structure of hitatimycin, a novel macrocyclic lactam involving β-phenylalanine. *Tetrahedron Lett.* **23**, 4713–4716.
- Puar, M.S., Gullo, V., Gunnarsson, I., Hegde, V., Patel, M., and Schwartz, J. (1992). Biosynthesis of macrolactam antifungal agents. *Bioorg. Med. Chem. Lett.* **2**, 575–578.
- Molnar, I., Schupp, T., Ono, M., Zirkle, R., Milnamow, M., Nowak-Thompson, B., Engel, N., Toupet, C., Stratmann, A., Cyr, D.D., et al. (2000). The biosynthetic gene cluster for the microtubule-stabilizing agents epothilones A and B from *Sorangium cellulosum* So ce90. *Chem. Biol.* **7**, 97–109.
- Paitan, Y., Alon, G., Orr, E., Ron, E.Z., and Rosenberg, E. (1999). The first gene in the biosynthesis of the polyketide antibiotic TA of *Myxococcus xanthus* codes for a unique PKS module coupled to a peptidyl synthetase. *J. Mol. Biol.* **286**, 465–474.
- Otsuka, M., Eguchi, T., Shindo, K., and Kakinuma, K. (1998). Non-fatty acyl polyketide starter in the biosynthesis of vicenistatin, an antitumor macrolactam antibiotic. *Tetrahedron Lett.* **39**, 3185–3188.
- Otsuka, M., Fujita, M., Matsushima, Y., Eguchi, T., Shindo, K., and Kakinuma, K. (2000). Biosynthetic pathway of macrolactam polyketide glycoside antitumor antibiotic vicenistatins. *Tetrahedron* **56**, 8281–8286.
- Nishida, H., Eguchi, T., and Kakinuma, K. (2001). Amino acid starter unit in the biosynthesis of macrolactam polyketide antitumor antibiotic vicenistatin. *Tetrahedron* **57**, 8237–8242.
- Decker, H., Gaisser, S., Pelzer, S., Schneider, P., Westrich, L., Wohleben, W., and Bechthold, A. (1996). A general approach for cloning and characterizing dNDP-glucose dehydratase genes from actinomycetes. *FEMS Microbiol. Lett.* **141**, 195–201.
- Ishikawa, J., and Hotta, K. (1999). FramePlot: a new implementation of the Frame analysis for predicting protein-coding regions in bacterial DNA with a high G+C content. *FEMS Microbiol. Lett.* **174**, 251–253.
- Ikeda, H., Nonomiya, T., Usami, M., Ohta, T., and Omura, S. (1999). Organization of the biosynthetic gene cluster for the polyketide anthelmintic macrolide avermectin in *Streptomyces avermitilis*. *Proc. Natl. Acad. Sci. USA* **96**, 9509–9514.
- Bevitt, D.J., Cortes, J., Haydock, S.F., and Leadlay, P.F. (1992). 6-Deoxyerythronolide-B synthase 2 from *Saccharopolyspora erythraea*. Cloning of the structural gene, sequence analysis and inferred domain structure of the multifunctional enzyme. *Eur. J. Biochem.* **204**, 39–49.
- Lauer, B., Russwurm, R., Schwarz, W., Kalmanczhelyi, A., Bruntner, C., Rosemeier, A., and Bormann, C. (2001). Molecular characterization of co-transcribed genes from *Streptomyces tendae* Tu901 involved in the biosynthesis of the peptidyl moiety and assembly of the peptidyl nucleoside antibiotic nikkomycin. *Mol. Gen. Genet.* **264**, 662–673.
- Heinzelmann, E., Berger, S., Puk, O., Reichenstein, B., Wohleben, W., and Schwartz, D. (2003). A glutamate mutase is involved in the biosynthesis of the lipopeptide antibiotic friulimicin in *Actinoplanes friuliensis*. *Antimicrob. Agents Chemother.* **47**, 447–457.
- Hoffmann, B., Konrat, R., Bothe, H., Buckel, W., and Krautler, B. (1999). Structure and dynamics of the B12-binding subunit of glutamate mutase from *Clostridium cochlearium*. *Eur. J. Biochem.* **263**, 178–188.
- Marahiel, M.A., Stachelhaus, T., and Mootz, H.D. (1997). Modular peptide synthetases involved in nonribosomal peptide synthesis. *Chem. Rev.* **97**, 2651–2673.
- Cheng, Y.Q., Tang, G.L., and Shen, B. (2003). Type I polyketide synthase requiring a discrete acyltransferase for polyketide biosynthesis. *Proc. Natl. Acad. Sci. USA* **100**, 3149–3154.
- Schauwecker, F., Pfennig, F., Schroder, W., and Keller, U. (1998).

- Molecular cloning of the actinomycin synthetase gene cluster from *Streptomyces chrysomallus* and functional heterologous expression of the gene encoding actinomycin synthetase II. *J. Bacteriol.* **180**, 2468–2474.
23. Gehring, A.M., Mori, I., and Walsh, C.T. (1998). Reconstitution and characterization of the *Escherichia coli* enterobactin synthetase from EntB, EntE, and EntF. *Biochemistry* **37**, 2648–2659.
  24. Ota, Y., Tamegai, H., Kudo, F., Kuriki, H., Koike-Takeshita, A., Eguchi, T., and Kakinuma, K. (2000). Butirosin-biosynthetic gene cluster from *Bacillus circulans*. *J. Antibiot. (Tokyo)* **53**, 1158–1167.
  25. Lombo, F., Siems, K., Brana, A.F., Mendez, C., Bindseil, K., and Salas, J.A. (1997). Cloning and insertional inactivation of *Streptomyces argillaceus* genes involved in the earliest steps of biosynthesis of the sugar moieties of the antitumor polyketide mithramycin. *J. Bacteriol.* **179**, 3354–3357.
  26. Ylihonko, K., Tuikkanen, J., Jussila, S., Cong, L., and Mantsala, P. (1996). A gene cluster involved in nogalamycin biosynthesis from *Streptomyces nogalater*: sequence analysis and complementation of early-block mutations in the anthracycline pathway. *Mol. Gen. Genet.* **251**, 113–120.
  27. Bate, N., Butler, A.R., Smith, I.P., and Cundliffe, E. (2000). The mycarose-biosynthetic genes of *Streptomyces fradiae*, producer of tylosin. *Microbiol.* **146**, 139–146.
  28. Zhao, L., Borisova, S., Yeung, S., and Liu, H.W. (2001). Study of C-4 deoxygenation in the biosynthesis of desosamine: evidence implicating a novel mechanism. *J. Am. Chem. Soc.* **123**, 7909–7910.
  29. Gaisser, S., Bohm, G.A., Cortes, J., and Leadlay, P.F. (1997). Analysis of seven genes from the eryAI-eryK region of the erythromycin biosynthetic gene cluster in *Saccharopolyspora erythraea*. *Mol. Gen. Genet.* **256**, 239–251.
  30. Raty, K., Kunnari, T., Hakala, J., Mantsala, P., and Ylihonko, K. (2000). A gene cluster from *Streptomyces galilaeus* involved in glycosylation of aclarubicin. *Mol. Gen. Genet.* **264**, 164–172.
  31. Waldron, C., Matsushima, P., Rosteck, P.R., Jr., Broughton, M.C., Turner, J., Madduri, K., Crawford, K.P., Merlo, D.J., and Baltz, R.H. (2001). Cloning and analysis of the spinosad biosynthetic gene cluster of *Saccharopolyspora spinosa*. *Chem. Biol.* **8**, 487–499.
  32. Arias, P., Fernandez-Moreno, M.A., and Malpartida, F. (1999). Characterization of the pathway-specific positive transcriptional regulator for actinorhodin biosynthesis in *Streptomyces coelicolor* A3(2) as a DNA-binding protein. *J. Bacteriol.* **181**, 6958–6968.
  33. Wilson, D.J., Xue, Y., Reynolds, K.A., and Sherman, D.H. (2001). Characterization and analysis of the PikD regulatory factor in the pikromycin biosynthetic pathway of *Streptomyces venezuelae*. *J. Bacteriol.* **183**, 3468–3475.
  34. Atlan, D., Gilbert, C., Blanc, B., and Portalier, R. (1994). Cloning, sequencing and characterization of the pepIP gene encoding a proline iminopeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397. *Microbiol.* **140**, 527–535.
  35. Thomas, I., Martin, C.J., Wilkinson, C.J., Staunton, J., and Leadlay, P.F. (2002). Skipping in a hybrid polyketide synthase. evidence for ACP-to-ACP chain transfer. *Chem. Biol.* **9**, 781–787.
  36. Taddei, A., and Zeeck, A. (1997). Biosynthesis of albocycline: origin of the carbon skeleton. *J. Antibiot. (Tokyo)* **50**, 526–528.
  37. Ligon, J., Hill, S., Beck, J., Zirkle, R., Molnar, I., Zawodny, J., Money, S., and Schupp, T. (2002). Characterization of the biosynthetic gene cluster for the antifungal polyketide soraphen A from *Sorangium cellulosum* So ce26. *Gene* **285**, 257–267.
  38. Bierman, M., Logan, R., O'Brien, K., Seno, E.T., Rao, R.N., and Schonher, B.E. (1992). Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene* **116**, 43–49.
  39. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, Second Edition (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).
  40. Matsushima, Y., Nakayama, T., Fujita, M., Bhandari, R., Eguchi, T., Shindo, K., and Kakinuma, K. (2001). Isolation and structure elucidation of vicenistatin M, and importance of the vicenistamine aminosugar for exerting cytotoxicity of vicenistatin. *J. Antibiot. (Tokyo)* **54**, 211–219.

#### Accession Numbers

The DNA Data Base of Japan (DDBJ) accession number for the *vin* cluster is AB086653.