The loading module for the nystatin polyketide synthase (PKS) in *Streptomyces noursei* is represented by the NysA protein composed of a ketosynthase (KS\(S\)), acyltransferase, dehydratase, and an acyl carrier protein. The absolute requirement of this protein for initiation of nystatin biosynthesis was demonstrated by the in-frame deletion of the *nysA* gene in *S. noursei*. The role of the NysA KS\(S\) domain, however, remained unclear, since no data on the significance of the “active site” serine (Ser-170) residue in the loading modules of type I PKSs were available. Site-specific mutagenesis of Ser-170 both in the wild-type NysA and in the hybrid loading module containing malonyl-specific acyltransferase domain from the extender module had no effect on nystatin biosynthesis. A second mutation (S413N) of the NysA KS\(S\) domain was discovered that completely abolished the ability of the hybrids to restore nystatin biosynthesis, presumably by affecting the ability of the resulting proteins to catalyze the required substrate decarboxylation. In contrast, NysA and its Ser-170 mutants bearing the same S413N mutation were able to restore nystatin production to significant levels, probably by using acetyl-CoA as a starter unit. Together, these data suggest that the KS\(S\) domain of NysA differs from the KS\(Q\) domains found in the loading modules of several PKS type I systems in that the active site residue is not significant for its activity.

Modular (type I) polyketide synthases (PKSs)\(^1\) are multifunctional proteins responsible for the biosynthesis of structurally diverse natural products, macrolides, with a wide range of pharmacological applications. PKSs catalyze decarboxylative condensations of simple carboxylic acids into the growing polyketide chain by a mechanism similar to the fatty-acid synthases. PKSs, however, are more diverse in their catalytic reactions, including the use of different primer and extender molecules. Each PKS module is a collection of domains with distinct catalytic functions during PKS catalysis, one module being responsible for one condensation step in the biosynthetic pathway. The minimal domains necessary for condensation are ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP). In addition, the domains ketoreductase, dehydratase (DH), and enoyl reductase responsible for different degrees of the reduction state of every \(\beta\)-carbon in the polyketide chain may be present in various combinations. It follows that the chemical structure of the final product is largely reflected in the modular organization of the PKS proteins (1).

A distinctive feature of modular PKS is the presence of a loading module for chain initiation, which is usually fused to the first extender module. The loading module of the 6-deoxyerythronolide B synthase catalyzing the biosynthesis of the erythromycin macrolactone ring in *Saccharopolyspora erythraea* is composed of the AT and ACP domains. Although several models for chain initiation have been proposed for this system (2, 3), evidence accumulated so far indicates that monocarboxylic acyl-CoA species are utilized as substrates. However, recent characterization of the new modular PKS proteins has shown that multidomain loading modules are more common. Such loading modules typically possess, in addition to AT and ACP, a domain designated KS\(Q\), which is similar to the chain length factor (CLF) of type II PKSs (4). Both CLF and KS\(Q\) are similar to the KS domains, while having a glutamine residue substituting cysteine in the active site. In the actinorhodin type II PKS CLF forms a heterodimer with KS, and by mutational studies it has been demonstrated that both domains are competent to catalyze substrate decarboxylation, as long as at least one of the domains has a glutamine residue positioned in its active site (4). A similar property has been suggested for the KS\(Q\) domains of some modular PKS, such as monensin PKS loading module, where a Q177A substitution in the KS\(Q\) domain resulted in a 10-fold reduction of the decarboxylation activity *in vitro* (4). In addition, KS\(Q\) is typically accompanied by a distinct AT domain, with proposed high specificity for dicarboxylic substrates compared with monocarboxylic substrates (3, 5). According to a model suggested by others (4), AT loads a dicarboxylic acid onto the ACP, whereas the KS\(Q\) domain governs decarboxylation in a way resembling the catalysis performed by extender modules. However, the residue in an “active site” position of the KS might be not the only one crucial for decarboxylation. Mutational analysis of the actinorhodin PKS, for example, demonstrated that additional KS residues might be critical, indicating that the precise mechanism for the KS-dependent decarboxylation still remains unclear (6).

We have reported previously (7) cloning and analysis of the complete gene cluster governing biosynthesis of the polyene antibiotic nystatin in *Streptomyces noursei*, and we showed that the deduced nys-PKS loading module (NysA) possesses...
Decarboxylation, presumably catalyzed by the KS S, takes place in situ by AT0 from its CoA ester to the phosphopantetheine arm of ACP. The elsewhere (14). Agar and liquid media, when appropriate, were supplied E. coli agar medium (Difco), and is recruited directly from acetyl-CoA, and no substrate decarboxylation before the resulting acetyl unit is transferred to NysB for condensation.

Some unusual features. NysA is a single module polypeptide composed of four catalytic domains (see Fig. 1) and thus resembles a typical extender module devoid of ketoreductase domain. It contains a KS-like domain with a serine residue (KS8) instead of a cysteine in the proposed active site position 170. The DH domain does not seem to have any function in NysA except probably for maintenance of a protein structural integrity, and most likely represents an evolutionary remnant. The primary sequence of the AT domain (AT0) displays an acetate-specific signature (8) and contains the conserved Arg-117 residue (Escherichia coli AT numbering) typical for such domains with high selectivity for CoA esters of dicarboxylic acids (3). It has been shown recently (9, 10) that two of these features, the KS8 domain and AT domain with the conserved Arg-117 residue, are shared by other polyene type I PKSs. However, in view of unclear significance of the KS8 domain for the NysA function, it remains unknown whether malonyl-CoA or acetyl-CoA is used as primer for nystatin synthesis (Fig. 1, A and B).

In the present study we have analyzed the in vivo role of NysA for the initiation of nystatin synthesis in S. noursei. In particular, the significance of the KS8 and the AT0 domains was investigated by site-directed mutagenesis and construction of the hybrid loading modules. Our data indicate that the KS8 domain is important, but not essential, for the initiation of nystatin biosynthesis.

**Experimental Procedures**

**Bacterial Strains, Media, and Growth Conditions**

Bacterial strains, plasmids, and recombinant phages used in this study are listed in Table I. S. noursei strains were maintained on ISP2 agar medium (Difco), and E. coli strains were grown in L broth or L agar (11). Conjugal plasmid transfer from E. coli ET 12567 (pUZ8002) and the gene replacement procedure were performed as described previously (12, 13). Transformation of E. coli was performed as described elsewhere (14). Agar and liquid media, when appropriate, were supplemented with antibiotics using the following concentrations: chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; ampicillin, 100 μg/ml; apramycin, 50 μg/ml. Analysis of the nystatin production by S. noursei strains was performed in SAO-23 liquid medium as reported previously (13, 15).

**DNA Manipulations, DNA Sequencing, and PCR**

General DNA manipulations were performed as described previously (11). DNA fragments from agarose gels were purified using the Qiaex II kit (Qiagen, Germany). Southern blot analysis was performed with the diogoxigenin-11-UTP High Prime labeling kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. Oligonucleotide primers were purchased from Amersham Biosciences, and DNA sequencing was performed using the ABI Prism Big Dye Terminator Cycle sequencing kit and the Genetic Analyzer 3100 (Applied Biosystems, Inc.). The PCRs were performed with the Expand High Fidelity PCR System (Roche Molecular Biochemicals) using the Eppendorf Mastercycler (Eppendorf, Germany), as described previously (15).

**Construction of Vectors for In-frame Deletion and Chromosomal Integration of nysA**

**nysA In-frame Deletion Vector**—The 11.0-kb BamHI fragment from plasmid pJ76B11 was cloned into pGEM3Zf(−), and from this construct (pL76B11) the 5.83-kb PstI-SacI DNA fragment encompassing the coding region of the nysA gene and surrounding sequences was ligated into the corresponding sites of plasmid pGEM3Zf(−). From the resulting plasmid (pL76PS5.8) the 1.88-kb MscI-PstI and the 2.79-kb SacI-SmaI DNA fragments were excised and ligated into the PstI/SacI-digested pGEM3Zf(−). From this construct, designated pNAD1, the 4.49-kb EcoRI-HindIII insert was ligated together with the 3.0-kb EcoRI-HindIII fragment from plasmid pSO2K01, resulting in the nysA replacement vector pNAD2.

**nysA Integration Vector**—The 4.63-kb EcoRI-SpeI fragment from plasmid pL76B11 (including the coding region of nysA and 157-bp upstream sequences) was ligated into the EcoRI/XhoI sites of pGEM3Zf(−), as well as into the EcoRI-SpeI sites of pLTRUMS28, resulting in plasmids pL76ES4.6 and pL77TE564.6, respectively. From pL77ES5.6 the 4.63-kb EcoRI-HindIII insert was excised and ligated into the EcoRI/HindIII sites of vector pSO2K04, resulting in the nysA integration vector pKS5AT0. Plasmid pSO2K04 is a mobilizable vector capable of site-specific integration into the S. noursei chromosome recently constructed in our laboratory (16).

**Site-directed Mutagenesis of the nysA KS8 Coding Region**

To prepare a suitable template for site-directed mutagenesis, the 2.95-kb ApaI fragment from plasmid pL76PS5.8 was ligated into the corresponding site of plasmid pGEM11Zf(−). From this construct, plL76A2.95, the 2.81-kb SpeI-SacI fragment including the nysA KS8 coding region was ligated into the XbaI/SacI sites of pLTRUMS28, resulting in plasmid pL77ES5.8. This plasmid was used as a template for mutagenesis using the QuickChange Site-directed mutagenesis kit (Stratagene). The putative active site serine residue in position 170 (Ser-170) of the nysA gene product was substituted with cysteine (S170C), glutamine (S170Q), and glycine (S170G) by using the following mutagenic primer pairs, respectively: S170C-F, 5'-CGTCACCGTCGACACCA- CGACGAGGA-TCC-3' (sense); S170C-R, 5'-GACGGCAGCAGCAAGCGACGAGCTGCACGCTGGTGTCGACGGTGA-ACGTCAGGCAG-3' (antisense); S170Q-F, 5'-CGTCCAGCGTCGACACCA-GCGACGAGGA-TCC-3' (sense), and S170Q-R, 5'-GACGGCAGCAGCAAGCGACGAGCTGCACGCTGGTGTCGACGGTGA-ACGTCAGGCAG-3' (antisense); S170G-F, 5'-CTTCCAGCGTCGACACCA-GCGACGAGGA-TCC-3' (sense), and S170G-R, 5'-GACGGCAGCAGCAAGCGACGAGCTGCACGCTGGTGTCGACGGTGA-ACGTCAGGCAG-3' (antisense). Mutated nucleotides are shown in boldface, and all mutations were verified by DNA sequencing. Underlined in all primers are new restriction sites (AfuI for mutations S170C and S170Q and BamHI for mutation S170G) introduced for simplified identification of positive clones. After mutagenesis, the 1.02-kb MluI-SpeI fragments including the newly introduced mutations were excised and ligated together with the 3.60-kb EcoRI-MluI fragment from plpL76T654.6 into the EcoRI/XhoI sites of vector pGEM3Zf(−). From the resulting constructs, the 4.63-kb EcoRI-HindIII inserts were ligated into the corresponding sites of vector pSO2K04, resulting in plasmids pK5S7AT0, pKS4AT0, and pKS4AT0, respectively. All three plasmids are analogous to the integration vector pK5S7AT0, except for the desired point mutations affecting Ser-170 in the NysA KS8 domain.

**PCR Amplification of DNA Regions Encoding Individual PKS Domains**

The nys-PKS regions encoding individual domains were PCR-amplified and labeled. Primers for the introduction of the termination sites were typically used to facilitate further assembly of the resulting fragments into hybrid genes. The new restriction sites introduced and used for the assembly procedure were in all cases positioned in the inter-domain linker regions to avoid any unwarranted structural effects for the resulting hybrid proteins. All the resulting constructs were verified by DNA sequencing.

**O. Sekurova, personal communication.**


**TABLE I**

**Bacterial strains, plasmids, and phages used in this study**

| Strain or plasmid | Relevant properties | Source or Ref. |
|-------------------|---------------------|---------------|
| *E. coli* DH5α   | General cloning host | BRL           |
| *E. coli* ET12567 (pUZ8002) | Strain for megacell conjugation, Km'Cm' | 13, 23 |
| *S. noursei* ATCC 11455 | Wild type, nystatin producer | ATCC          |
| *S. noursei* NDA59 | Wild type derivative with nysA in-frame deletion, nystatin non-producer | This work |

**Plasmids and phages**

| Strain or plasmid | Relevant properties | Source or Ref. |
|-------------------|---------------------|---------------|
| pUC18             | ColEl replicon, Ap', 2.7 kb | 24            |
| pSOK201           | pSG5 and ColEl replicons, ortT, Am' | 25            |
| pL76BI1           | 11-kb BamHI fragment from N76, including the nysA gene, cloned in pGEM3Zf(−) | This work |
| pL76PES5.8        | StiI-SpeI fragment from pL76BI1, including nysA, cloned in pGEM3Zf(−) | This work |
| pNAD1             | 1.68-kb MscI-PstI and 2.79 kb SacI-SmaI fragments from pL76PS5.8 ligated into the PstI/SacI sites of pGEM3Zf(−) | This work |
| pNAD2             | 4.49-kb EcoRI-HindIII insert of pNAD1, including nysA with a 1.3 kb in-frame deletion, cloned into the EcoRI/HindIII fragment of pSK0801 | This work |
| pL76ES4.6         | 4.63-kb EcoRI-SpeI fragment from pL76BI1 cloned in pGEM3Zf(−) | This work |
| pGEM-KSC          | 1.83-kb PCR fragment encompassing the nysA KSC coding region, cloned into XbaI/SacI sites of pLITMUS28 | This work |
| pGEM-KSN1         | 1.83-kb PCR fragment encompassing the mutant nysA KSN1 coding region cloned into the EcoRI/BamHI sites of pGEM3Zf(−) | This work |
| pGEM-KSN2         | 1.83-kb PCR fragment encompassing the mutant nysA KSN2 coding region cloned into the EcoRI/BamHI sites of pGEM3Zf(−) | This work |
| pGEM-DHACP        | 1.63-kb PCR fragment encompassing the nysA DH + ACP coding region cloned into the SphiIII sites of pGEM3Zf(−) | This work |
| pGEM-AT0          | 0.99-kb PCR fragment encompassing the nysA AT0 coding region cloned into the SphiI/BamHI sites of pGEM3Zf(−) | This work |
| pGEM-AT1          | 0.99-kb PCR fragment encompassing the nysB AT1 coding region cloned into the SphiI/BamHI sites of pGEM3Zf(−) | This work |
| pGEM-AT3          | 0.99-kb PCR fragment encompassing the nysC AT3 coding region cloned into the SphiI/BamHI sites of pGEM3Zf(−) | This work |
| pKSAT1 (AT0)      | Construct for integration of nysA hybrid module with substitution of AT0 with AT1 coding region | This work |
| pKSAT3 (AT0)      | Construct for integration of nysA hybrid module with substitution of AT0 with AT3 coding region | This work |
| pKSAT1 (AT1)      | Construct for integration of nysA hybrid module with substitution of AT1 with AT0 coding region | This work |
| pKSAT3 (AT1)      | Construct for integration of nysA hybrid module with substitution of AT1 with AT3 coding region | This work |

**PCR Amplification and Subcloning of DNA Regions Encoding Variant KS Domains**—The 1.75-kb DNA fragments covering the coding region of the variant NysA KS, KSC, and KSS domains, including the nysA promoter region, were PCR-amplified from the plasmid pLITMUS28.2.8 and its mutated derivatives (see above) DNA using the following primer pair: NAKS-F, 5′-TACGACTCACTATAGGCGCGC-3′ (sense), and NAKS-R, 5′-GGAGGATCCCTGGTGCGGC-GACCCG-3′ (antisense). The latter primer contains a BamHI recognition site (underlined) used for the hybrid assembly procedure (see below). The resulting PCR fragments were blunt-ligated into the SmaI site of pUC18, and from the resulting constructs the 1.75-kb EcoRI-BamHI inserts were excised and ligated into the corresponding sites of vector pGEM3Zf(−), resulting in the constructs pGEM-KSN1, pGEM-KSN2, and pGEM-KSN3, respectively (see Table I). In addition, a derivative of pGEM-KSN2 harboring a second point mutation (S413N) of the KS coding region was identified and denoted pGEM-KSN3. This mutation most probably resulted from an erroneous PCR amplification. The 1.0-kb MluI-SpeI fragment from plasmid pGEM-KSN3 (including the S413N but not the S170C mutation) was used to substitute the corresponding fragments in plasmids pGEM-KS3 and pGEM-KS4, respectively (see Table I).
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TABLE II

Complementation of S. noursei NDA59 with NysA mutants and hybrid-loading modules

| Vector for complementation | KS mutations | AT domain | Nystatin level, % |
|----------------------------|--------------|-----------|------------------|
| pKS\(^{AT0}\)               | None         | AT0       | 100 ± 3          |
| pKS\(^{AT0}\)               | S170C        | AT0       | 101 ± 8          |
| pKS\(^{AT0}\)               | S170Q        | AT0       | 90 ± 1           |
| pKS\(^{AT0}\)               | S170G        | AT0       | 98 ± 5           |
| pKS\(^{AT1}\)               | None         | AT1       | 0                |
| pKS\(^{AT1}\)               | S170C        | AT1       | 0                |
| pKS\(^{AT1}\)               | S170Q        | AT1       | 0                |
| pKS\(^{AT1}\)               | S170G        | AT1       | 0                |
| pKS\(^{AT2}\)               | None         | AT3       | 11 ± 2           |
| pKS\(^{AT2}\)               | S170C        | AT3       | 17 ± 1           |
| pKS\(^{AT2}\)               | S170Q        | AT3       | 12 ± 1           |
| pKS\(^{AT2}\)               | S170G        | AT3       | 0                |
| pKS\(^{AT3}\)               | S170C + S143N| AT3       | 0                |
| pKS\(^{AT3}\)               | S170Q + S143N| AT3      | 0                |
| pKS\(^{AT3}\)               | S170G + S143N| AT0      | 34 ± 1           |
| pKS\(^{AT0}\)               | S413N        | AT0       | 36 ± 2           |
| pKS\(^{AT0}\)               | S170Q + S143N| AT0      | 19 ± 2           |
| pKS\(^{AT0}\)               | S170G + S143N| AT0      | 0                |

a Description of the integration vectors used is given in Table I.
b KS domains harboring between zero and two amino acid substitutions were used, and the particular mutations are indicated.
c The variant AT domains used are as follows: AT0, NysA; AT1, NysB module 1; AT3, NysC module 3.
d Nystatin production was measured as described under “Experimental Procedures.” All experiments were performed in triplicate, and the mean values with the corresponding S.E. are given for all cases, 100% represents the production levels obtained in NDA59 (pKS\(^{SAT0}\)), and 0 nystatin is below 0.05%.

Sequence Alignment and Phylogenetic Analysis

Alignment of the amino acid sequences was done using the ClustalW 1.82 software provided on-line by the European Bioinformatics Institute (www.ebi.ac.uk/clustalw). The alignment was visualized using the Boxshade 3.21 on-line software from the Swiss node of EMBnet (www.ch.embnet.org/software/BOX_form.html).

The phylogenetic tree was drawn by the TreeView 1.6.6 software developed by R. D. M. Page at the University of Glasgow (taxonomy. zoology.gla.ac.uk/rod/treeview.html) using the distances calculated after the ClustalW alignment at the EBI server (see above).

RESULTS

In-frame Deletion and Complementation of nysA—In a previous report (7) we described the nysA gene disruption abolishing all nystatin synthesis in S. noursei. However, to rule out the possibility of any polar effects that might have been imposed by this mutation, we constructed a nysA in-frame deletion mutant NDA59 by double homologous recombination, using the gene replacement vector pNAD2. The 1.36-kb in-frame deletion affecting the nysA gene within this plasmid eliminated the coding sequence for the C-terminal part of KS\(^{S}\) and most of the AT0 domain (see Fig. 2). Analysis of NDA59 showed that it produces no nystatin (Fig. 2B), thus confirming that the NysA loading module is essential for initiation of nystatin synthesis in S. noursei. In an attempt to complement this mutant, plasmid pKS\(^{SAT0}\) was chromosomally integrated in the NDA59 strain. This plasmid harbors a DNA fragment encompassing the nysA gene and a 157-bp sequence upstream of its coding region, presumably containing the nysA promoter. Nystatin synthesis was restored to wild-type levels (Fig. 2C) in the NDA59 (pKS\(^{SAT0}\)) strain, demonstrating that the nysA gene expressed in trans from its endogenous promoter can efficiently complement the nysA deficiency in the NDA59 mutant. We next integrated plasmid pKS\(^{SAT0}\) into the wild-type strain in order to test whether expression of an additional copy of nysA may stimulate nystatin production. This manipulation had no detectable effect on the nystatin synthesis (data not shown) suggesting that the NysA expression level is not a limiting factor for the production of this antibiotic under the conditions tested.

Replacement of the Proposed Active Site Serine (Ser-170) in the NysA KS\(^{S}\) Domain Has No Effect on Nystatin Synthesis in Vivo—It is generally accepted that the initial condensation step
Initiation of Nystatin Biosynthesis in S. noursei

During the PKS-catalyzed polyketide synthesis takes place on the KS domain of the first extender module. Loading modules usually either lack the KS domains completely or have KSQ domains with a glutamine in place of a cysteine in the active site position. These KSQ domains in the loading modules are believed to be responsible for decarboxylation of the starter dicarboxylic acid CoA esters prior to condensation. The KSS domain of NysA has a serine residue in this position (Fig. 3A), and the role of this domain for initiation of nystatin synthesis was unknown. We proposed that if AT0 recruits malonyl-CoA, then KSS should possess a decarboxylase activity, whereas no such activity is needed if acetyl-CoA is used (Fig. 1). To test whether the Ser-170 in KSS is important for the NysA activity, this residue was substituted with glutamine (S170Q), cysteine (S170C), and the catalytically “silent” glycine (S170G). Integration vectors expressing the resulting mutant nysA genes (plasmids pKS^AT0, pKS^QAT0, and pKS^GAT0, respectively) were introduced into the S. noursei mutant NDA59, and the resulting recombinant strains were analyzed for nystatin production. Surprisingly, similar nystatin production by the NDA59 mutant NDA59 has an in-frame deletion in the nysA gene abolishing all the nystatin synthesis. C. chromosomal integration of vector pKS^AT0, expressing nysA from its endogenous promoters, restored nystatin production to almost wild-type levels in the mutant NDA59. All the production experiments were performed in triplicate, and the mean values with the corresponding S.E. are given in all cases.

**Fig. 2.** Deletion and complementation of nysA in S. noursei. A, the NysA protein in S. noursei ATCC 11455 (wild type (WT)) is composed of the KS^S, AT0, DH, and ACP domains. This organism produces 3.0 g/liter (equals to 100% nystatin under the conditions tested (see “Experimental Procedures”). B, S. noursei mutant NDA59 has an in-frame deletion in the nysA gene abolishing all the nystatin synthesis. C, chromosomal integration of vector pKS^AT0, expressing nysA from its endogenous promoters, restored nystatin production to almost wild-type levels in the mutant NDA59. All the production experiments were performed in triplicate, and the mean values with the corresponding S.E. are given in all cases.

**Fig. 3.** Comparative amino acid sequence analysis of the KS^S, KS^Q, and extender KS domains of modular PKS. A, alignment of the KS domain amino acid sequences encompassing the conserved active site and C-terminal regions. Active site residue and a serine conserved in the KS and extender KS domains are indicated with arrows. Nys, nystatin (GenBank^TM accession number AF263912); Amph, amphotericin (GenBank^TM AF357202); Fm, fumacin (GenBank^TM AF278573); Spn, spinosad (GenBank^TM AJ278573); Ome, olgomycin (GenBank^TM AB070940); Myc, mycinamicin (GenBank^TM AB017641); Pik, pikromycin (GenBank^TM AF079138); Tyl, tylosin (GenBank^TM U78289); Nid, niddamycin (GenBank^TM AF016585); and Ole, oleandomycin (GenBank^TM AF220851) PKSs. B, unrooted radial phylogenetic tree showing that KS^S are more closely related to the extender KS than to the KS^Q. The dendrogram was built as described under “Experimental Procedures.”

The KS^S, AT0, DH, and ACP domains were individually used to substitute for AT0 in NysA and its KS mutants. The purpose of these experiments was to unravel whether NysA can utilize dicarboxylic acid substrates in vivo, and whether this ability is dependent on the type of residue in position 170 of the loading module. Care was taken to keep the polypeptide length, the interdomain linkers, and the domain order of each hybrid similar to the native NysA protein. The C-terminal part of NysA encompassing DH and ACP domains was kept intact in all cases. The resulting vectors were introduced into S. noursei mutant NDA59, and the recombinant strains were analyzed for nystatin production.
None of the integration vectors expressing the AT1 hybrids (pKS\textsuperscript{S}AT1, pKS\textsuperscript{S}AT1, and pKS\textsuperscript{S}AT1) could restore synthesis of either nystatin or related polyenes when introduced into the NDA59 mutant (Table II). However, the integration vectors expressing the AT3 hybrids (pKS\textsuperscript{S}AT3, pKS\textsuperscript{S}AT3, and pKS\textsuperscript{S}AT3) were able to restore nystatin production in NDA59, although not as efficiently as the pKS\textsuperscript{Q}AT0 expressing the wild-type nysA (Table II). These results indicate that NysA is capable of using malonyl-CoA as a starter unit in a manner that seems to be independent of the type of residue in position 170. In this way our data contradict previous reports (3, 4) stating that the type of residue in this particular position is critical for decarboxylase activity and suggest that other residues within NysA may be essential. The lower production levels obtained with the AT3 hybrids (between 11 and 17%) compared with those obtained with the wild-type NysA may be due to several reasons, the improper folding of the hybrid proteins being most likely. This result may also in part be explained if the hybrids have narrower substrate specificity, i.e. if they are capable of accepting only malonyl-CoA, whereas both acetyl-CoA and malonyl-CoA may be used by the wild-type NysA.

A Novel KS Mutation (S413N) Abolishes the in Vivo Activity Exclusively in the AT3 Hybrids—During the hybrid construction procedure (see “Experimental Procedures”), an unwar- ranted mutation was discovered, resulting in substitution of the highly conserved serine residue in position 413 of the NysA KS domain with an asparagine (S413N). First, this mutation was introduced into the integration vectors expressing the AT3 hybrids, and the resulting plasmids (pKS\textsuperscript{S}AT3, pKS\textsuperscript{S}AT3, and pKS\textsuperscript{Q}AT3) were used to complement NDA59. Surpris- ingly, none of the resulting recombinant strains was able to produce nystatin (Table II), suggesting that Ser-413 was critical for the in vivo activity of the AT3 hybrids. Sequence com- parisons (Fig. 3A) revealed that this particular residue is highly conserved in the KSs of single polypeptide loading mod- ules as well as extender module KSs. We reasoned that if this mutation somehow affects the decarboxylase activity linked to KS\textsuperscript{S}, and wild-type NysA can use both acetyl- and malonyl-CoA as starter units, the effect of such a mutation would be less dramatic on the AT0 background. Therefore, the S413N muta- tion was introduced into the integration vectors expressing NysA and the Ser-170 mutants, and the resulting plasmids (pKS\textsuperscript{S}AT0, pKS\textsuperscript{S}AT0, and pKS\textsuperscript{Q}AT0) were introduced into the NDA59 mutant. Remarkably, the nystatin synthesis in the resulting recombinant strains was restored to 19–36% com- pared with the NDA59 (pKS\textsuperscript{AT}0) (Table II). Together, these results suggest that the observed effect of the S413N mutation on the ability of the loading modules to initiate nystatin bio- synthesis depends on the type of AT domain present (see “Discussion”).

DISCUSSION

Modular PKSs have proven to be exciting targets for the rational genetic engineering with the aim of producing new macrolide antibiotics. Several strategies such as single domain inactivation, domain swapping and insertion, and module re-arrangements have led to synthesis of novel polyketides (16). The process of initiation of polyketide synthesis governed by the distinct loading modules may also be a target for genetic manipulations aimed at the synthesis of new products (5, 17). The majority of the modular PKS loading modules can be divided into two classes, depending on their domain organiza- tion and, consequently, their mode of initiation of polyketide synthesis. One class is represented by the loading modules having, in addition to the AT and ACP domains, a KS\textsuperscript{S} domain with suggested decarboxylase activity. These modules, repre- sented by oleandomycin, niddamycin, and pikromycin PKSs, accept dicarboxylic acid-CoA esters such as malonyl-CoA and methylmalonyl-CoA as starters. Such starter molecules must be decarboxylated before they are loaded onto the first extender module, and the KS\textsuperscript{S} domain seems to be crucial for this process (4). Yet another class of loading modules found, for example, in the erythromycin and avermectin PKSs is charac- terized by having the AT and ACP domains only. These mod- ules apparently use monocarboxylic acid-CoA esters such as propionyl-CoA and isobutyryl-CoA to load them onto the first extender module of the corresponding PKS. Therefore, no decarboxylation is required during initiation of polyketide synthesis in the latter systems.

The deduced loading module for the nystatin PKS, NysA, is composed of the KS\textsuperscript{S}, AT0, DH, and ACP domains (see Fig. 1). By constructing a nysA in-frame deletion mutant, we have demonstrated that NysA is essential for in vivo production of nystatin in S. noursei. This result suggests that the first extension module of NysB cannot be loaded directly by a starter carboxylic acid, as was apparently the case for erythromycin PKSs, where inactivation of the 6-deoxyerythronolide B syn- thease loading module did not abolish erythromycin production completely (2, 18). In ery-PKS, the direct loading of the KS domain of the first extension module was proposed as an alter- native mechanism for chain initiation (3).

During the polyketide chain elongation the KS domains of each extender module supposedly play two roles: (i) they cata- lyze condensation of the extender unit onto the polyketide chain, and (ii) simultaneously promote the in situ decarboxy- lation of the recruited dicarboxylic acyl-CoA esters during the condensation process. These two functions seem to be insepara- ble in the extender KSs, since the condensation process strictly depends on decarboxylation (19, 20). The condensation irreversibly requires the conserved cysteine in the KS active site, whereas the mechanism and amino acid residue(s) in- volved in decarboxylation are still not completely unraveled (4, 6). It is suggested that there exists a cooperation between the KS and AT domains on each module for the discriminative recruitment and concomitant decarboxylation of dicarboxy- lated substrates (3, 5). In the complex loading modules contain- ing KS\textsuperscript{S} domains, the active site glutamine has been shown to be important for decarboxylase activity (4). In such loading modules the AT domains are found to be structurally and functionally similar to their counterparts found in the extender modules, with high specificity for the dicarboxylic acyl-CoAs (3). NysA has an extender-type AT domain suggesting that malonyl-CoA might be its preferred substrate. However, this assumption heavily relies on the decarboxylase activity of the KS\textsuperscript{S}, analogous to what has been demonstrated for the KS\textsuperscript{Q} in other systems. At least in the type II PKS system, the CLF with serine residue in the active site position retained most of its decarboxylase activity (6).

One obvious strategy to test whether the KS\textsuperscript{S} is at all re- quired for initiation of nystatin biosynthesis would be to make an in-frame deletion within the KS\textsuperscript{S}-coding region of nysA. However, previous attempts on using such strategies for inac- tivation of the nys-PKS reduction domains typically abolished all polyene production, presumably by disturbing the folding of the resulting mutant proteins.\textsuperscript{3} Our mutagenesis results clearly show that the type of residue in the active site position 170 of KS\textsuperscript{S} has no significance for the in vivo activity of NysA. However, the successful complementation of the nysA-deficient mutant NDA59 using the AT3 hybrids indicates that NysA is somehow capable of catalyzing the required decarboxylation of malonyl-CoA. Although the type of residue at position 170 had

\textsuperscript{3} T. Brautaset and S. B. Zotchev, unpublished results.
little effect on the ability of the hybrid proteins to initiate nystatin biosynthesis, the second S413N mutation completely aborted this function in such hybrids. The most probable explanation is that the latter mutation has abolished decarboxylase activity of these proteins preventing proper priming with the malonyl-CoA recruited by the AT3. Alternatively, this substitution might have caused unwarranted structural changes in the AT3 hybrids leading to loss of all in vivo activity. The latter explanation seems unlikely, since introduction of the S413N mutation in the wild-type NysA, as well as in the NysA KS\(^\text{C}\) and KS\(^\text{Q}\) mutants, did not abolish their ability to initiate nystatin synthesis, although it has been diminished by 64–81%. By assuming that the S413N mutation abolishes decarboxylase activity in NysA, the residual in vivo activity observed for NysA and its active site mutants bearing this mutation could be due to the utilization of acetyl-CoA as a starter. This notion is in agreement with a recent report showing that loading module AT of the ery-PKS has, in contrast to the extender AT, a relaxed specificity toward acyl-CoA substrates and can accept both propionyl-CoA, acetyl-CoA, and butyryl-CoA (21). No clues as to which amino acid residues might be responsible for the proposed broad substrate specificity of AT0 in NysA could be found by inspecting the alignment of the AT amino acid sequences (data not shown). Loading modules of the polypeptide PKSs seem to be rather unique, as they are represented by separate polypeptides, whereas in other modular PKS systems loading modules are fused to the first extender module. This feature may in principle provide a greater degree of freedom in folding of the polypeptide PKS loading modules that, in turn, might affect the choice of the starter unit by these proteins.

The inability of the AT1 hybrids to complement nystatin synthesis in NDA59 could be due to several reasons. Based on the results obtained with the AT3 hybrids, we suggest that these observations cannot be solely explained by the lack of the decarboxylase activity in the hybrid proteins. A possible explanation may be that the downstream nys-PKS module in NysB cannot use the propionate primer generated by these engineered proteins, and further elongation is aborted. Alternatively, the heterologous AT1 domain might be the cause of the misfolding of the hybrid modules and the subsequent loss of activity. Similar problems are documented by others (22), and in such cases site-directed mutagenesis has successfully been applied as an alternative strategy for changing the substrate specificity of AT domains.

Alignment of the amino acid sequences for the KS-like domains in the PKS type I loading modules clearly shows that all KS\(^\text{Q}\) domains contain a glycine at position 413 (NysA numbering), whereas the single polypeptide loading modules contain highly conserved serine (Fig. 3A). A threonine residue in the Pim50 loading module is structurally very similar to serine and thus might well serve as its functional substitute. Interestingly, the alignment of the KS domains from the extender modules of 13 PKS type I systems (encompassing together over 100 KS domains) shows that the Ser-413 residue is highly conserved among these domains, with only one exception; KS in the module 2 of rifamycin PKS (data not shown). Ser-413 was also found to be highly conserved in the extender KSs of the PKS type I systems discovered in such diverse bacterial genera as Mycobacterium, Nostoc, Stigmatella, Desulfoficrobium, and Planctothrix (data not shown). Finally, the phylogenetic tree based on the KS sequence alignments clearly shows that KS\(^\text{Q}\) cluster with the extender KS domains, whereas the KS\(^\text{Q}\) domains represent a separate branch (Fig. 3B). All these data further suggest that single polypeptide loading modules differ from their KS\(^\text{Q}\) counterparts, which could explain the discrepancy in the data obtained upon the active site residue mutagenesis reported here and by others (4).

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REFERENCES
1. Donadio, S., Staver, M., McAlpine, J. B., Swanson, S. J., and Katz, L. (1991) Science 252, 675–679
2. Pereda, A., Summers, R. G., Stassi, D. L., Ruan, X., and Katz, L. (1998) Microbiology 144, 543–553
3. Long, P. F., Wilkinson, C. J., Bisang, C. P., Cortés, J., Dunstaer, N., Oliynyk, M., McCormick, E., McArthur, H., Mendes, C., Salas, J. A., Staunton, J., and Leadlay, P. F. (2002) Mol. Microbiol. 43, 1215–1225
4. Bisang, C., Long, P. F., Cortés, J., Westcott, J., Crosby, J., Matharu, A. L., Cox, R. J., Simpson, T. J., Staunton, J., and Leadlay, P. F. (1999) Nature 401, 502–505
5. Wilkinson, C. J., Frost, E. J., Staunton, J., and Leadlay, P. F. (2001) Chem. Biol. 8, 1197–1208
6. Dreier, J., and Khosla, C. (2000) Biochemistry 39, 2088–2095
7. Brautaset, T., Sekurova, O. N., Sletta, H., Ellingsen, T. E., Strem, A. R., Valla, S., and Zotchev, S. B. (2000) Chem. Biol. 7, 385–403
8. Haydock, S. F., Aparicio, J. F., Molnar, I., Schwecke, T., Khaw, L. E., Konig, A., Marsden, A. F., Galloway, I. S., Staunton, J., and Leadlay, P. F. (1995) FEBS Lett. 374, 246–248
9. Aparicio, J. F., Foures, R., Mendes, M. V., Oliveira, N., and Martin, J. F. (2000) Chem. Biol. 7, 895–905
10. Caffrey, P., Lynch, S., Flood, E., Finnan, S., and Oliynyk, M. (2001) Chem. Biol. 8, 713–723
11. Sambroc, J., Fritsch, E. F., and Maniatis, T. (1999) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
12. Flett, F., Mersinias, V., and Smith, C. P. (1997) FEMS Microbiol. Lett. 155, 223–229
13. Sekurova, O., Sletta, H., Ellingsen, T. E., Valla, S., and Zotchev, S. B. (1999) FEMS Microbiol. Lett. 177, 297–304
14. Chung, C. T., Niemela, S. L., and Miller, R. H. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2172–2175
15. Brautaset, T., Bruheim, P., Sletta, H., Hagen, L., Ellingsen, T. E., Strem, A. R., Valla, S., and Zotchev, S. B. (2002) Chem. Biol. 9, 367–373
16. Staunton, J., and Weissman, K. J. (2001) Nat. Prod. Rep. 18, 380–416
17. Marsden, A. P. A., Wilkinson, B., Cortés, J., Dunstaer, N. J., Staunton, J., and Leadlay, P. F. (1998) Science 279, 199–202
18. Jacobsen, J. R., Cane, D. E., and Khosla, C. (1998) Biochemistry 37, 4298–4304
19. Witkowski, A., Joshi, A. K., Lindqvist, Y., and Smith, S. (1999) Biochemistry 38, 11643–11650
20. Jez, J. M., Ferrer, J. L., Bowman, M. E., Dixon, R. A., and Noel, J. P. (2000) Biochemistry 39, 890–902
21. Liu, G. F., Lau, J., Cane, D. E., and Khosla, C. (2003) Biochemistry 42, 200–207
22. Beeves, C. D., Murlu, S., Ashley, G. W., Piagentini, M., Hutchinson, C. R., and McDaniel, R. (2001) Biochemistry 40, 15464–15470
23. MacNeil, T. (1992) Gene 111, 61–68
24. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Methods Enzymol. 113, 83–89
25. Zotchev, S. B., Haugan, K., Sekurova, O., Sletta, H., Ellingsen, T. E., and Valla, S. (2000) Microbiology 146, 611–619
Site-specific Mutagenesis and Domain Substitutions in the Loading Module of the Nystatin Polyketide Synthase, and Their Effects on Nystatin Biosynthesis in *Streptomyces noursei*

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