Applied and Environmental Microbiology, Apr. 2006, p. 2514–2519 Vol. 72, No. 4
0099-2240/06/$08.00+0 doi:10.1128/AEM.72.4.2514–2519.2006

Characterization of the P450 Monooxygenase NysL, Responsible for C-10 Hydroxylation during Biosynthesis of the Polyene Macrolide Antibiotic Nystatin in *Streptomyces noursei*

Olga Volokhan,[1] Håvard Sletta,[2] Trond E. Ellingsen,[2] and Sergey B. Zotchev[1]*

Department of Biotechnology, Norwegian University of Science and Technology, N-7491 Trondheim, Norway,[1] and Department of Industrial Biotechnology, SINTEF Materials and Chemistry, SINTEF, N-7034 Trondheim, Norway[2]

Received 10 December 2005/Accepted 23 January 2006

The *nysL* gene, encoding a putative P450 monooxygenase, was identified in the nystatin biosynthetic gene cluster of *Streptomyces noursei*. Although it has been proposed that NysL is responsible for hydroxylation of the nystatin precursor, experimental evidence for this activity was lacking. The *nysL* gene was inactivated in *S. noursei* by gene replacement, and the resulting mutant was shown to produce 10-deoxynystatin. Purification and an in vitro activity assay for 10-deoxynystatin demonstrated its antifungal activity being equal to that of nystatin. The NysL protein was expressed heterologously in *Escherichia coli* as a His-tagged protein and used in an enzyme assay with 10-deoxynystatin as a substrate. The results obtained clearly demonstrated that NysL is a hydroxylase responsible for the post-polyketide synthase modification of 10-deoxynystatin at position C-10. Kinetic studies with the purified recombinant enzyme allowed determination of K_m and k_{cat} and revealed no inhibition of recombinant NysL by either the substrate or the product. These studies open the possibility for in vitro evolution of NysL aimed at changing its specificity, thereby providing new opportunities for engineered biosynthesis of novel nystatin analogues hydroxylated at alternative positions of the macrolactone ring.

The genetics and biochemistry of antibiotic biosynthesis in bacteria attract much attention due to the possibility of using this information to design and produce new pharmaceuticals (26). Filamentous gram-positive bacteria of the genus *Streptomyces* are known to produce a wide range of chemically diverse secondary metabolites with different biological activities. Over 200 gene clusters governing antibiotic biosynthesis in *Streptomyces* have been isolated and analyzed, giving rise to a wealth of information regarding the enzymology of antibiotic formation. Biosynthetic pathways for many antibiotics produced by *Streptomyces* include steps catalyzed by P450 monooxygenase enzymes, which mostly perform hydroxylation of pathway intermediates (19).

P450 monooxygenases are widely distributed in nature and are mostly known to perform monooxygenation, although other catalytic functions, such as dehydrogenation, C—C and C═N bond cleavage, and dehydration, have also been reported for P450 enzymes (16). The "classical" reaction catalyzed by these enzymes is the transfer of one oxygen atom from O_2 to a variety of substrates, using electrons supplied by NAD(P)H through ferredoxin (Fdx) and ferredoxin oxidoreductase (Fdr). Although in some bacteria the genes for P450 monooxygenases are usually clustered with the Fdx and Fdr genes, such a gene arrangement is rare in *Streptomyces*, suggesting that in streptomycete bacteria these enzymes are rather nonselective in their choice of electron transfer partners (19).

A considerable number of streptomycete P450 monooxygenases involved in antibiotic biosynthesis have been unraveled, including those performing monooxygenation of intermediates in the biosynthetic pathways for macrolides (5, 15, 17), anthracyclines (27), glycopeptides (6), coumarins (12) etc. Interestingly, some bacterial P450 monooxygenases exhibit substantial flexibility regarding the structural features of their substrates (13, 28), suggesting the possibility for using these enzymes in combinatorial biosynthesis. Recently, characterizations of P450 monooxygenase genes involved in the biosynthesis of two polyene macrolide antibiotics, pimaricin and amphotericin B, have been published (10, 11, 17). Deletion of *pimD* in the pimaricin producer *Streptomyces natalensis* resulted in the accumulation of 4,5-deepoxy pimaricin, suggesting a role for PimD as a 4,5-epoxydase. The latter was recently confirmed via an enzymatic assay with heterologously expressed protein (18). Inactivation of the *amphL* gene in the amphotericin producer *S. nodosus* led to the production of 8-deoxy amphotericins A and B, implying that AmphL is a C-8 hydroxylase in amphotericin biosynthesis. Another P450 enzyme from the amphotericin biosynthetic pathway was shown to be responsible for the oxidation of the methyl group, resulting in the appearance of the exocyclic carboxyl moiety. Most interestingly, the amphotericin B analogue lacking the latter group was purified and shown to have considerably less hemolytic activity than amphotericin B (11). A similar result was obtained upon inactivation of RimG, the P450 monooxygenase responsible for oxidation of the exocyclic methyl group on the rimocidin/C-108 precursor in *S. diastaticus* (22). Considering the importance of polyene macrolides as antifungal agents used in human therapy, a better understanding of the reactions catalyzed by these P450 monooxygenases and their substrate range might help in combinatorial biosynthesis of novel polyene macrolides aimed at the development of new antibiotics.

Streptomyces noursei ATCC 11455 produces a complex mixture of polyene macrolides with antifungal activity which are

* Corresponding author. Mailing address: Department of Biotechnology, Norwegian University of Science and Technology, N-7491 Trondheim, Norway. Phone: 47 73 59 86 79. Fax: 47 73 59 12 83. E-mail: sergey.zotchev@nt.ntnu.no.

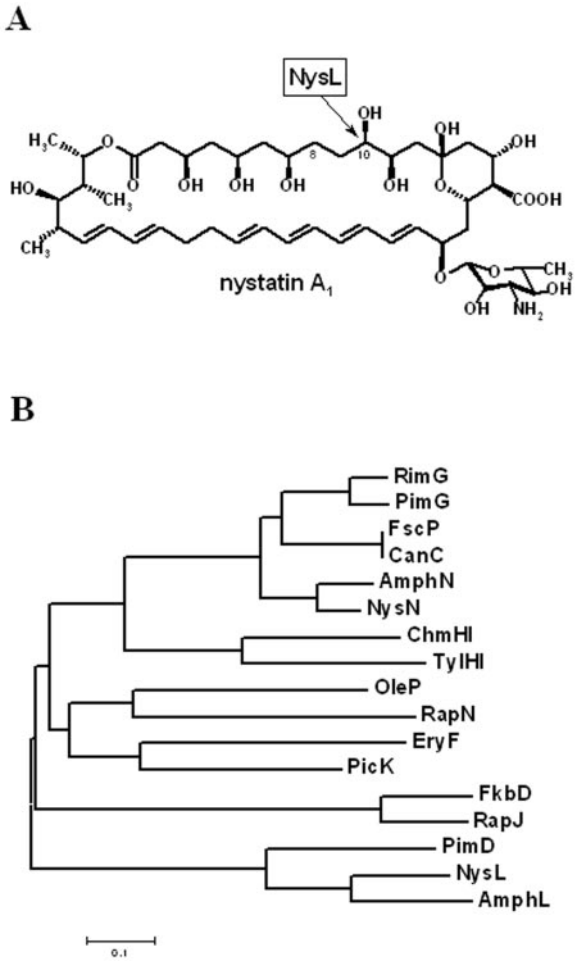

FIG. 1. (A) Chemical structure of nystatin A_1. The site of NysL-catalyzed hydroxylation is indicated by an arrow. (B) Phylogenetic tree of actinomycete P450 monooxygenases known to be responsible for post-PKS modification of macrolide antibiotic precursors in the biosynthetic pathways of amphotericin (AmphL and AmphN), chalcomycin (ChmHI), candicidin (CanC), erythromycin (EryF), FK520 (FkbD), FR-008 (FscP), nystatin (NysL and NysN), oleandomycin (OleP), picromycin (PicK), pimaricin (PimD and PimG), rapamycin (RapH and RapJ), rimocidin (RimG), and tylosin (TylHI).

generally referred to as nystatins (9). The major component of the nystatin complex, nystatin A_1, is composed of a 38-membered macrolactone ring synthesized by six large polyketide synthase (PKS) proteins (7). The macrolactone ring of nystatin A_1 is further modified in the course of biosynthesis via the addition of a mycosamine deoxysugar moiety, oxidation of the methyl group at C-16, and hydroxylation at C-10 (14). The last two modifications in the biosynthesis of nystatin were suggested to be performed by P450 monooxygenase enzymes encoded by the genes *nysL* and *nysN* in the nystatin biosynthetic gene cluster, although experimental evidence for the enzymatic activities of these monooxygenases was not available. In the present work, we have proven the role of NysL as a C-10 hydroxylase (Fig. 1A) involved in nystatin biosynthesis via gene inactivation in *S. noursei*, heterologous expression of NysL, and in vitro enzyme assays for the recombinant protein.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Some of the plasmids are described below. *S. noursei* strains were maintained on ISP2 agar medium (Difco) and grown in tryptic soy broth (Oxoid) for DNA isolation. *Escherichia coli* strains were handled using standard techniques (21). Conjugation from *E. coli* ET12567(pUZ8002) to *S. noursei* and the gene replacement procedure were performed as described previously (23). Assessments of nystatin and 10-deoxynystatin production and accurate mass determinations were done by liquid chromatography-mass spectroscopy (LC-MS) of dimethyl sulfoxide extracts of 5-day-old cultures from well plate cultivations (9). Well plate cultivations were performed in semidefined 0.5× SAO-23 medium according to a previously described protocol (25).

DNA manipulation. General techniques for DNA manipulation were used as described elsewhere (21). Isolation of the DNA fragments from agarose gel was done with a QIAEX kit (QIAGEN, Germany). Southern blot analysis was performed with a DIG High Prime labeling kit (Roche Biochemicals, Germany) according to the manufacturer's manual. Oligonucleotide primers were purchased from MWG-Biotech AG. The DNA sequence of the nystatin biosynthetic gene cluster has been reported previously (7).

Construction of *nysL* deletion mutant. A 2,148-kb DNA fragment designated Nldel12, encompassing part of *nysL* and the downstream region, was amplified from the pL95X template using primers NLD1 (5′-GACGAATTCAACTGGT GGCGGAGCTGA-3′) and NLD2 (5′-GACCTGCAGCTGCTTGAGTTCGGT G-3′). A 2,089-kb DNA fragment designated NLdel34, containing the upstream region and some of the coding region of *nysL*, was amplified from the plasmid pL95X template using primers NLD3 (5′-GACCTGCAGTCGAGGAACTGC GGGTGCT-3′) and NLD4 (5′-GCAAAGCTTTGCGGGCGATGGCGTTCA

TABLE 1. Bacterial strains, plasmids, and phage used for this study

Strain or plasmid	Relevant properties[a]	Source or reference
Escherichia coli strains		
DH5α	General cloning host	BRL
ET12567(pUZ8002)	Strain for intergeneric conjugation; Km^r Cm^r	23
Streptomyces noursei strains		
ATCC 11455	Wild type, nystatin producer	ATCC
NDA59	Mutant with in-frame *nysA* deletion, nystatin nonproducer	8
NLD101	NDA59 with in-frame deletion of the *nysL* gene	This work
Phage		
N95	Recombinant λ phage containing the *nysL* gene and flanking regions	7
Plasmids		
pGEM3Zf(−)	ColE1 replicon; Ap^r; 3.2 kb	Promega
pQE2	N-terminal six-His tag expression vector	QIAGEN
pQNL2	Vector for heterologous expression of the six-His–NysL protein	This work
pSOK20	pSG5 and ColE1 replicons; *oriT*; Am^r; *E. coli-Streptomyces* conjugative vector	29
pSOK804	*E. coli-Streptomyces* conjugative and integrative vector; VWB_{int}; ColE1 replicon; *oriT*; Am^r	24
pNLD1	*nysL* replacement vector	This work

[a] Abbreviations: Am, apramycin; Ap, ampicillin; Km, kanamycin; Tc, tetracycline; Cm, chloramphenicol.

C-3′). The NLdel12 and NLdel34 PCR products were digested with the EcoRI/PstI and PstI/HindIII endonucleases, respectively, and ligated together with the 3.0-kb EcoRI/HindIII fragment from pSOK201, yielding the *nysL* replacement vector pNLD1. Using this vector, we obtained the *nysL* in-frame deletion mutant NLD101 by double homologous recombination in the previously constructed non-nystatin-producing mutant NDA59, which can be complemented with the *nysA* gene to restore nystatin production (8).

Purification of 10-deoxynystatin and test for antifungal activity. The purification of 10-deoxynystatin was performed according to a previously described procedure (25). The antifungal activities of nystatin and 10-deoxynystatin were determined using *Candida albicans* as a test organism according to a method described previously (9).

Heterologous expression of NysL. The *nysL* gene was amplified for insertion into the N-terminal His tag expression vector pQE-2 (QIAGEN) by PCR. The forward primer NLQE21 (5′-GGACCATATGAGCACACCGACCGCAC-3′) introduced a unique NdeI site at the 5′ end of the gene, while the reverse primer NLQE22 (5′-CCAGAAGCTTCATCACGTCACCAGGTGAC-3′) carried a HindIII site downstream from translational stop codons. The amplified DNA fragment was digested with NdeI and HindIII and cloned into the same sites of pQE2 to generate pQNL2, the vector for heterologous expression of the N-terminal six-His–NysL protein. The cloned fragment was sequenced in order to check its integrity after PCR amplification. Expression and purification of six-His–NysL were performed essentially as described elsewhere (20), but after induction of the culture with 1 mM IPTG (isopropyl-ß-D-thiogalactopyranoside) and incubation for 4 h at 30°C.

NysL enzyme assay and studies of enzyme kinetics. NysL activity was measured in a mixture containing 0.3 to 105 μM 10-deoxynystatin, 16 to 48 nM six-His–NysL, 100 μg/ml spinach ferredoxin, 0.2 U/ml spinach ferredoxin-$NADP^+$ reductase, 1.4 mM NADPH, 10 mM glucose-6-phosphate, and 8 U/ml glucose-6-phosphate dehydrogenase in 50 mM Tris-HCl, pH 8.0 (16). The reaction mixture was incubated at 30°C in the temperature-controlled autoinjector of the Agilent 1100 LC-MS equipment used in the assay. The reaction was started by adding the enzymes to the preheated substrate mixture. Every fifth minute after the reaction was started, an aliquot from the reaction mixture was injected into the LC-MS instrument for determination of the nystatin and 10-deoxynystatin concentrations. The LC-MS analyses were performed on an Agilent 1100 high-performance liquid chromatography (HPLC) system connected to a diode array detector and an Agilent single-quadrupole mass spectrometer using electrospray ionization in negative mode. A Zorbax Bonus-RP (2.1 × 50 mm) chromatographic column operated at 0.3 ml/min was used for analyte separation. The mobile phase consisted of 10 mM ammonium acetate, pH 4.0, and 30% acetonitrile.

RESULTS

Inactivation of *nysL* gene in *S. noursei* leads to production of 10-deoxynystatin. The *nysL* gene in the *S. noursei* nystatin biosynthetic gene cluster is located downstream of *nysK*, the gene encoding the last module of the nystatin PKS and a terminal thioesterase (7). Amino acid sequence analysis of the deduced *nysL* product revealed a high degree of similarity of this polypeptide to bacterial P450 monooxygenases, including those involved in antibiotic biosynthesis (data not shown). Further phylogenetic analysis (Fig. 1B) revealed clustering of the NysL amino acid sequence with those of PimD and AmphL, proven to act as an epoxidase and a hydroxylase in the biosynthesis of the polyene macrolides pimaricin and amphotericin, respectively. Although we have suggested earlier that NysL is most probably a C-10 hydroxylase, the experimental evidence for this assumption was lacking. Repeated attempts to inactivate *nysL* by in-frame deletion via double homologous recombination in an *S. noursei* wild-type strain failed. We then used a previously constructed mutant that does not produce nystatin, NDA59, with an inactivated *nysA* gene encoding the nystatin PKS loading module, which can be complemented with the wild-type copy of *nysA* to restore nystatin production (8). The use of the latter mutant, along with the extension of flanking regions for the gene replacement construct up to 2 kb, allowed isolation of the Δ*nysL* mutant designated NLD101. The scheme for NDL101 mutant construction is presented in Fig. 2. The deletion within the *nysL* gene in NLD101 was confirmed by Southern blot analysis (data not shown).

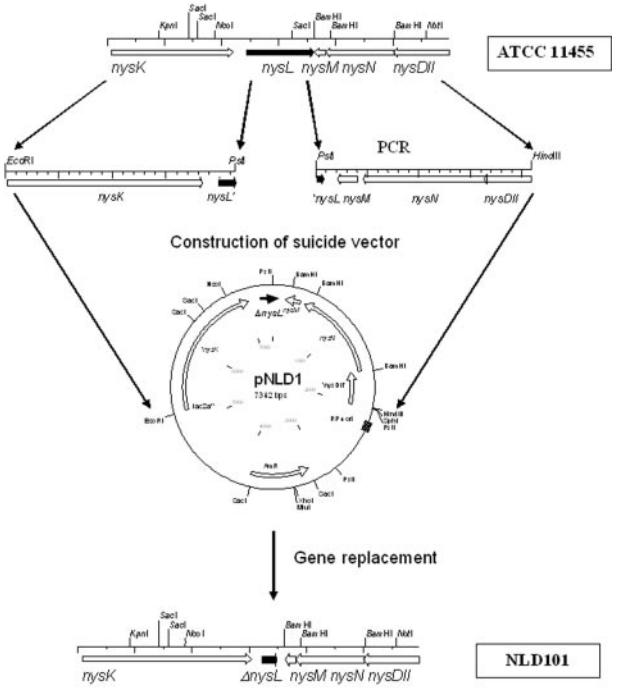

FIG. 2. Schematic representation of Δ*nysL* mutant construction in *S. noursei*.

To analyze polyene macrolide production by the Δ*nysL* mutant, the integrative plasmid pNA0 (8) carrying the *nysA* gene was introduced into the NLD101 mutant. The resulting recombinant strain, NLD101(pNA0), was incubated for 5 days in defined production medium, and culture extracts were analyzed for the presence of nystatin-related polyene macrolides by using HPLC and LC-MS. The latter analysis (Fig. 3) confirmed the production of a 910-Da nystatin analogue by the NLD101(pNA0) strain at a volumetric yield of 1.06 ± 0.04 g/liter, which was somewhat lower than the nystatin yield in the wild-type strain (1.51 ± 0.03 g/liter). The accurate atomic mass of this analogue, its retention time during HPLC, and the UV spectrum exactly matched those for 10-deoxynystatin, which was identified during studies of the NysH/NysG transporter system in *S. noursei* (25). The 10-deoxynystatin produced was then purified from the NLD101 culture extract for further testing.

To test whether the hydroxy group at C-10 has significance for the antifungal activity of nystatin, the MIC_{50} was determined for 10-deoxynystatin, using *Candida albicans* as a test organism and nystatin as a reference. The MIC_{50}s of 10-deoxynystatin and nystatin were found to be identical (0.45 μg/ml under the conditions tested), implying that C-10 hydroxylation has little significance for the activity of nystatin, at least against *C. albicans*.

Recombinant NysL protein efficiently converts 10-deoxynystatin to nystatin. To allow for enzymatic studies of the NysL

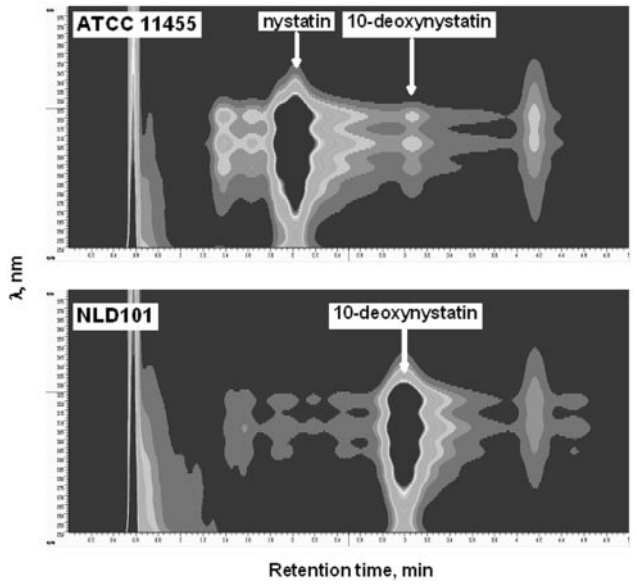

FIG. 3. Diode array detector-HPLC profiles of culture extracts from *S. noursei* ATCC 11455 and the NLD101 mutant.

monooxygenase, we heterologously expressed this protein in *Escherichia coli* with an N-terminal six-His affinity tag and purified the recombinant protein. The PCR-amplified product containing the coding region of the *nysL* gene was cloned into the pQE2 expression vector immediately after the six-His tag sequence (pQNL2) (see Materials and Methods). Induction of the T5 promoter in the culture of *E. coli* DH5α carrying the pQNL2 expression plasmid resulted in high-level production of the partially soluble 43-kDa protein, whose molecular mass corresponded to that of NysL. The recombinant six-His–NysL protein was purified on a Ni-nitrilotriacetic acid agarose column, and its purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 4A).

The ability of the purified six-His–NysL protein to convert its putative substrate 10-deoxynystatin to nystatin was analyzed in vitro. The reaction mixture (see Materials and Methods) contained an NADPH-regenerating system based on glucose-6-phosphate and glucose-6-phosphate dehydrogenase in addition to the buffer solution, spinach ferredoxin, spinach ferredoxin-NADP$^+$ reductase, NADPH, and 10-deoxynystatin. Only in the presence of such an electron-donating system was six-His–NysL able to convert 10-deoxynystatin to nystatin, and no conversion was observed without the participation of the NADPH-regenerating system. The progress of the reaction in terms of the conversion of 10-deoxynystatin to nystatin was monitored over 40 min (Fig. 4B). The conversion of >75% of the 10-deoxynystatin to nystatin was observed under these conditions.

Kinetic studies of recombinant NysL protein. The kinetics of the recombinant NysL-catalyzed reaction was studied next. With a substrate concentration of 45 μM, the initial rate of hydroxylation was found to increase linearly with the enzyme concentration in the range of 16 to 45 nM six-His–NysL. At 30°C, the phase of the reaction where the enzyme concentration and the rate of hydroxylation correlated linearly lasted ca. 15 to 20 min. An enzyme concentration of 16.1 nM and a reaction time of 5 min were chosen for kinetic studies. Enzymatic assays were performed with increasing concentrations of 10-deoxynystatin and nystatin. Data from the assays, monitored by LC-MS, did not reveal inhibition of the recombinant NysL enzyme by either the substrate or the product. At substrate concentrations in the range of 0.3 to 105 μM, the data could be fitted well to the Michaelis-Menten equation (Fig. 5).

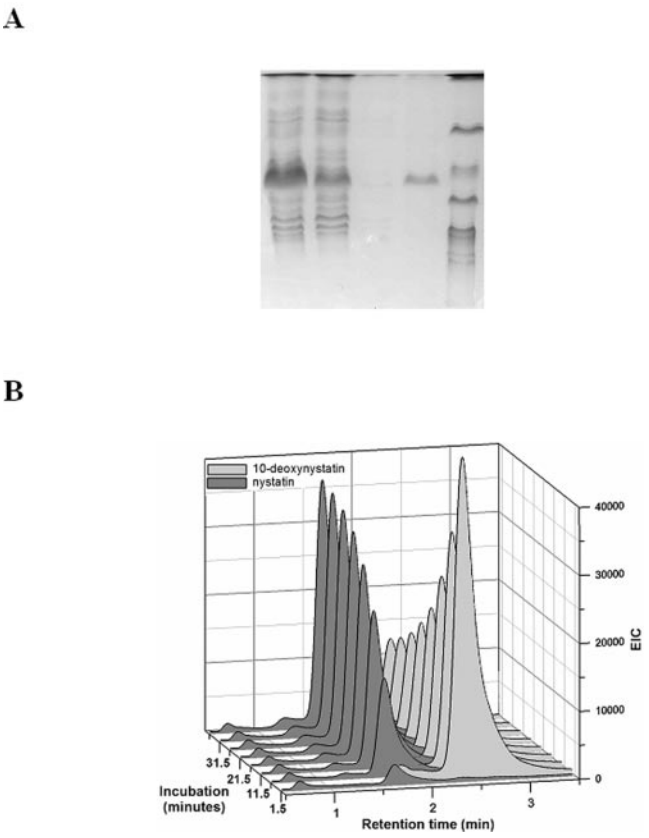

FIG. 4. (A) Heterologous expression of NysL in *Escherichia coli* as a His-tagged protein. Lanes: 1, crude cell extract; 2, flowthrough; 3, wash; 4, eluted His-NysL; 5, molecular weight marker. (B) Enzymatic assay monitored by HPLC and LC-MS showing conversion of 10-deoxynystatin to nystatin by His-NysL and the redox partners.

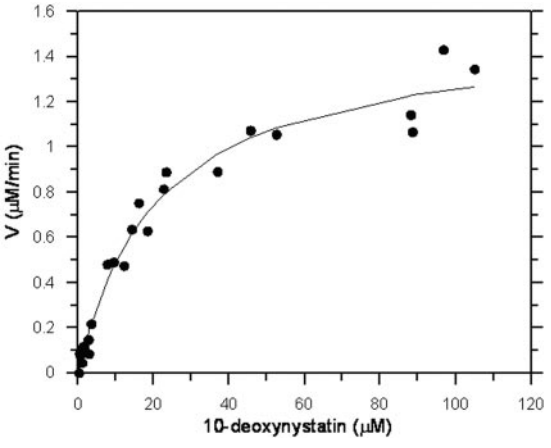

FIG. 5. Kinetics of six-His–NysL-catalyzed reaction. The presented data are based on three independent enzyme assays.

Further increases in the substrate concentration resulted in precipitation of 10-deoxynystatin and were therefore not considered. The K_m and V_{max} values were determined (based on three independent assays) to be 24 ± 3 μM and 1.5 ± 0.2 μM/min, respectively. Provided that all of the enzyme is active, this corresponds to a k_{cat} value of 1.6 ± 0.2 s^{-1} and a k_{cat}/K_m value of 0.07 ± 0.01 μM^{-1} s^{-1}.

DISCUSSION

P450 monooxygenases play an important role in the biosynthesis of many secondary metabolites produced by *Streptomyces* bacteria. Recent advances in engineered antibiotic biosynthesis in streptomycetes make P450 monooxygenases attractive targets for genetic manipulation that can lead to the production of novel antibiotic analogues (6, 10, 11, 17, 22). The biosynthesis of all polyene macrolide antibiotics known to possess potent antifungal activity involves oxidative steps apparently catalyzed by the P450 monooxygenases (1). Inactivation of the monooxygenase genes in the producers of the polyene macrolides pimaricin and amphotericin yielded new analogues of these antibiotics with properties different from those of the final products of the respective biosynthetic pathways (10, 11, 17).

In the present study, we have characterized the P450 monooxygenase NysL encoded by the nystatin biosynthetic gene cluster in *S. noursei* ATCC 11455. An alignment of the NysL amino acid sequence with those for P450 enzymes known to be involved in antibiotic biosynthesis, followed by phylogenetic analysis, clearly revealed that NysL is related to PimD and AmphL, an epoxidase and a hydroxylase in the pimaricin and amphotericin biosynthetic pathways, respectively. PimD, AmphL, and NysL represent a separate clade on the phylogenetic tree which is distant from that containing P450 monooxygenases from the polyene macrolide biosynthetic pathways known or assumed to catalyze the oxidation of an exocyclic methyl group (1, 11, 22).

Inactivation of the *nysL* gene in *S. noursei* yielded a recombinant strain producing 10-deoxynystatin as a major polyene macrolide (Fig. 3). This metabolite is present in trace amounts in wild-type strain extracts and was assumed to be accumulated due to the failure of NysL to hydroxylate this precursor at C-10 (9). Sletta et al. have demonstrated that the accumulation of 10-deoxynystatin by *S. noursei* also occurs upon inactivation of the presumed nystatin efflux system NysH-NysG (25). This observation suggested a link between the process of NysL-catalyzed hydroxylation of 10-deoxynystatin and active transport of the nystatin, and we hypothesized that the NysH-NysG transporter provides conditions favorable for C-10 hydroxylation by NysL.

Based on the current model for the polyene macrolide mode of action, which involves the formation of ion-permeable channels in fungal membranes (2), it could be assumed that removal of the C-10 hydroxyl group from the nystatin polyol region will decrease channel permeability. Indeed, according to the model, hydroxyl groups in the polyol region of the molecule are located on the inner part of the channel, creating a hydrophilic environment allowing ions and other small molecules to leak out of the cell. However, we have demonstrated that the antifungal activity of 10-deoxynystatin is equal to that of nystatin, at least for *C. albicans*. This observation was in contrast to the data for 4,5-deepoxypimaricin and 8-deoxyamphotericin B, which were obtained upon inactivation of the genes encoding P450 monooxygenases performing epoxidation and hydroxylation of pimaricin and amphotericin precursors, respectively, in polyol regions of the molecules. In both cases, a notable decrease in antifungal activity was observed (10, 17). One possible explanation for this phenomenon might be that the conformation of the polyol region of the nystatin molecule is different from those of pimaricin and amphotericin. This difference might result in limited participation of the nystatin C-10 hydroxyl group in channel formation and, thus, its low significance for antifungal activity.

Heterologous expression and purification of His-tagged NysL allowed for preliminary characterization of this enzyme in vitro. Recombinant NysL was shown to efficiently convert 10-deoxynystatin to nystatin A_1, and no inhibition was observed by either the substrate or the product. This is in contrast with the apparent substrate inhibition shown for the epoxidase PimD, the close homologue of NysL (18). The K_m and k_{cat} values determined for the recombinant NysL protein are similar to those reported for other macrolide-specific P450 monooxygenases and presented by Mendes et al. (18). According to the k_{cat}/K_m criterion, His-tagged NysL has a somewhat higher substrate specificity toward 10-deoxynystatin than recombinant PimD toward its substrate, 4,5-deepoxypimaricin. It could be interesting to test whether recombinant NysL can use other polyene macrolides as substrates, since this could open possibilities for combinatorial biosynthesis. The latter can be achieved through expression of the *nysL* gene in polyene macrolide-producing hosts in order to obtain novel analogues with different post-PKS hydroxylation patterns. For example, the expression of NysL in the amphotericin producer devoid of AmphL activity (10) could yield an amphotericin analogue hydroxylated at C-10 instead of C-8 (Fig. 1A). Alternatively, NysL can be engineered via either site-specific mutagenesis (4) or directed evolution (3) in order to change its specificity towards 10-deoxynystatin. This approach might provide novel nystatin analogues hydroxylated at alternative macrolactone ring positions in hope of obtaining antifungal compounds with improved pharmacologic properties.

ACKNOWLEDGMENTS

We are grateful to Per Bruheim and Randi Aune for help with analytical methods.

This work was supported by the Research Council of Norway.

REFERENCES

1. **Aparicio, J. F., P. Caffrey, J. A. Gil, and S. B. Zotchev.** 2003. Polyene antibiotic biosynthesis gene clusters. Appl. Microbiol. Biotechnol. **61:**179–188.
2. **Baginski, M., H. Resat, and E. Borowski.** 2002. Comparative molecular dynamics simulations of amphotericin B-cholesterol/ergosterol membrane channels. Biochim. Biophys. Acta **1567:**63–78.
3. **Bell, S. G., C. F. Harford-Cross, and L. L. Wong.** 2001. Engineering the CYP101 system for in vivo oxidation of unnatural substrates. Protein Eng. **14:**797–802.
4. **Bell, S. G., X. Chen, F. Xu, Z. Rao, and L. L. Wong.** 2003. Engineering substrate recognition in catalysis by cytochrome P450cam. Biochem. Soc. Trans. **31:**558–562.
5. **Betlach, M. C., J. T. Kealey, G. W. Ashley, and R. McDaniel.** 1998. Characterization of the macrolide P-450 hydroxylase from *Streptomyces venezuelae* which converts narbomycin to picromycin. Biochemistry **37:**14937–14942.
6. **Bischoff, D., S. Pelzer, A. Holtzel, G. J. Nicholson, S. Stockert, W. Wohlleben, G. Jung, and R. D. Sussmuth.** 2001. The biosynthesis of vancomycin-type glycopeptide antibiotics—new insights into the cyclization steps. Angew Chem. Int. Ed. Engl. **40:**1693–1696.

7. **Brautaset, T., O. N. Sekurova, H. Sletta, T. E. Ellingsen, A. R. Strøm, S. Valla, and S. B. Zotchev.** 2000. Biosynthesis of the polyene antifungal antibiotic nystatin in *Streptomyces noursei* ATCC 11455: analysis of the gene cluster and deduction of the biosynthetic pathway. Chem. Biol. **7:**395–403.
8. **Brautaset, T., S. E. Borgos, H. Sletta, T. E. Ellingsen, and S. B. Zotchev.** 2003. Site-specific mutagenesis and domain substitutions in the loading module of the nystatin polyketide synthase, and their effects on nystatin biosynthesis in *Streptomyces noursei*. J. Biol. Chem. **278:**14913–14919.
9. **Bruheim, P., S. E. Borgos, P. Tsan, H. Sletta, T. E. Ellingsen, J.-M. Lancelin, and S. B. Zotchev.** 2004. Chemical diversity of polyene macrolides produced by *Streptomyces noursei* ATCC 11455 and recombinant strain ERD44 with genetically altered polyketide synthase NysC. Antimicrob. Agents Chemother. **48:**4120–4129.
10. **Byrne, B., M. Carmody, E. Gibson, B. Rawlings, and P. Caffrey.** 2003. Biosynthesis of deoxyamphotericins and deoxyamphoteronolides by engineered strains of *Streptomyces nodosus*. Chem. Biol. **10:**1215–1224.
11. **Carmody, M., B. Murphy, B. Byrne, P. Power, D. Rai, B. Rawlings, and P. Caffrey.** 2005. Biosynthesis of amphotericin derivatives lacking exocyclic carboxyl groups. J. Biol. Chem. **280:**34420–34426.
12. **Chen, H., and C. T. Walsh.** 2001. Coumarin formation in novobiocin biosynthesis: beta-hydroxylation of the aminoacyl enzyme tyrosyl-*S*-NovH by a cytochrome P450 NovI. Chem. Biol. **8:**301–312.
13. **Cupp-Vickery, J. R., C. Garcia, A. Hofacre, and K. McGee-Estrada.** 2001. Ketoconazole-induced conformational changes in the active site of cytochrome P450eryF. J. Mol. Biol. **311:**101–110.
14. **Fjaervik, E., and S. B. Zotchev.** 2005. Biosynthesis of the polyene macrolide antibiotic nystatin in *Streptomyces noursei*. Appl. Microbiol. Biotechnol. **67:** 436–443.
15. **Gaisser, S., R. Lill, J. Staunton, C. Mendez, J. Salas, and P. F. Leadlay.** 2002. Parallel pathways for oxidation of 14-membered polyketide macrolactones in *Saccharopolyspora erythraea*. Mol. Microbiol. **44:**771–781.
16. **Mansuy, D.** 1998. The great diversity of reactions catalyzed by cytochromes P450. Comp. Biochem. Physiol. C **121:**5–14.
17. **Mendes, M. V., E. Recio, R. Fouces, R. Luiten, J. F. Martin, and J. F. Aparicio.** 2001. Engineered biosynthesis of novel polyenes: a pimaricin derivative produced by targeted gene disruption in *Streptomyces natalensis*. Chem. Biol. **8:**635–644.
18. **Mendes, M. V., N. Anton, J. F. Martin, and J. F. Aparicio.** 2005. Characterization of the polyene macrolide P450 epoxidase from *Streptomyces natalensis* that converts de-epoxypimaricin into pimaricin. Biochem. J. **386:**57–62.
19. **O'Keefe, D. P., and P. A. Harder.** 1991. Occurrence and biological function of cytochrome P450 monooxygenases in the actinomycetes. Mol. Microbiol. **5:**2099–2105.
20. **Ortiz de Orue Lucana, D., and H. Schrempf.** 2000. The DNA-binding characteristics of the *Streptomyces reticuli* regulator FurS depend on the redox state of its cysteine residues. Mol. Gen. Genet. **264:**341–353.
21. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Seco, E. M., S. Fotso, H. Laatsch, and F. Malpartida.** 2005. A tailoring activity is responsible for generating polyene amide derivatives in *Streptomyces diastaticus* var. 108. Chem. Biol. **12:**1093–1101.
23. **Sekurova, O., H. Sletta, T. E. Ellingsen, S. Valla, and S. Zotchev.** 1999. Molecular cloning and analysis of a pleiotropic regulatory gene locus from the nystatin producer *Streptomyces noursei* ATCC 11455. FEMS Microbiol. Lett. **177:**297–304.
24. **Sekurova, O. N., T. Brautaset, H. Sletta, S. E. Borgos, O. M. Jakobsen, T. E. Ellingsen, A. R. Strom, S. Valla, and S. B. Zotchev.** 2004. In vivo analysis of the regulatory genes in the nystatin biosynthetic gene cluster of *Streptomyces noursei* ATCC 11455 reveals their differential control over antibiotic biosynthesis. J. Bacteriol. **186:**1345–1354.
25. **Sletta, H., S. E. Borgos, P. Bruheim, O. N. Sekurova, H. Grasdalen, R. Aune, T. E. Ellingsen, and S. B. Zotchev.** 2005. Nystatin biosynthesis and transport: *nysH* and *nysG* genes encoding a putative ABC transporter system in *Streptomyces noursei* ATCC 11455 are required for efficient conversion of 10-deoxynystatin to nystatin. Antimicrob. Agents Chemother. **49:**4576–4583.
26. **Vosburg, D. A., and C. T. Walsh.** 2005. Natural product biosynthetic assembly lines: prospects and challenges for reprogramming. Ernst Schering Res. Found. Workshop **51:**261–284.
27. **Walczak, R. J., M. L. Dickens, N. D. Priestley, and W. R. Strohl.** 1999. Purification, properties, and characterization of recombinant *Streptomyces* sp. strain C5 DoxA, a cytochrome P-450 catalyzing multiple steps in doxorubicin biosynthesis. J. Bacteriol. **181:**298–304.
28. **Xue, Y., D. Wilson, L. Zhao, H. Liu, and D. H. Sherman.** 1998. Hydroxylation of macrolactones YC-17 and narbomycin is mediated by the *pikC*-encoded cytochrome P450 in *Streptomyces venezuelae*. Chem. Biol. **5:**661–667.
29. **Zotchev, S., K. Haugan, O. Sekurova, H. Sletta, T. E. Ellingsen, and S. Valla.** 2000. Identification of a gene cluster for antibacterial polyketide-derived antibiotic biosynthesis in the nystatin producer *Streptomyces noursei* ATCC 11455. Microbiology **146:**611–619.