Identification of a gene cluster for antibacterial polyketide-derived antibiotic biosynthesis in the nystatin producer *Streptomyces noursei* ATCC 11455

Sergey Zotchev,¹ Kåre Haugan,² Olga Sekurova,¹ Håvard Sletta,³ Trond E. Ellingsen³ and Svein Valla¹

Author for correspondence: Sergey Zotchev. Tel: +47 73 59 86 79. Fax: +47 73 59 87 05.
e-mail: sergey.zotchev@chembio.ntnu.no

¹ UNIGEN Center for Molecular Biology and Department of Biotechnology, NTNU, N-7489 Trondheim, Norway

² Nord-Trøndelag College, Dept of Engineering, N-7600 Levanger, Norway

³ SINTEF Applied Chemistry, SINTEF, N-7034 Trondheim, Norway

***Streptomyces noursei* ATCC 11455 produces the antifungal polyene antibiotic nystatin containing the deoxysugar moiety mycosamine. Part of the deoxythymidyl diphosphate (TDP)-glucose dehydratase gene (*gdhA*) known to be involved in deoxysugar biosynthesis was amplified by PCR from genomic DNA of *S. noursei* ATCC 11455. A gene library for *S. noursei* was made and screened with the *gdhA* probe. Several overlapping phage clones covering about 30 kb of the *S. noursei* genome were physically mapped. A partial DNA sequencing analysis of this region resulted in the identification of several putative genes typical of macrolide antibiotic biosynthetic gene clusters. A gene-transfer system for *S. noursei* has been established, and gene deletion or disruption experiments within the putative biosynthetic gene cluster were performed. All of the knock-out mutants retained the ability to produce nystatin, suggesting that the identified gene cluster is not involved in biosynthesis of this antibiotic. Culture extracts from the wild-type strain and three knock-out mutants were analysed by TLC followed by a bioassay against *Micrococcus luteus*. Two antibacterial compounds were found to be synthesized by the wild-type strain while only one was produced by the mutants. This provided evidence for the involvement of the identified gene cluster in the biosynthesis of a presumably novel antibacterial macrolide antibiotic in *S. noursei*.**

Keywords: *Streptomyces noursei*, gene cluster, macrolide antibiotic

INTRODUCTION

Filamentous soil bacteria belonging to the genus *Streptomyces* are widely recognized as industrially important micro-organisms because of their ability to produce antibiotics. In the past few years there has been rapid progress in genetic characterization of the biosynthetic pathways for various antibiotics produced by these

organisms (Schwecke *et al.*, 1995; Katz & Donadio, 1995). Knowledge acquired during these studies allows one to more or less precisely predict the order of biochemical reactions leading to the synthesis of a given antibiotic. Moreover, knowledge gained through the functional analysis of antibiotic biosynthesis genes gives an opportunity to manipulate the pathways in a rational manner in order to produce novel compounds with improved properties (Hopwood, 1997).

A large number of antibiotics used in medical and veterinary care, and agriculture are of a polyketide origin. The polyketide backbone of these antibiotics is synthesized by polyketide synthase (PKS)-assisted condensation of the building units represented usually by acetyl-, propionyl- and (methyl)malonyl-CoA (Hopwood, 1997). Both the sequential manner in which

Abbreviations: ABC, ATP-binding cassette; PKS, polyketide synthase; TDP, deoxythymidyl diphosphate; WT, wild-type.

The GenBank accession numbers for the sequences reported in this paper are AF071512 for ORF1, AF071513 for ORF2, AF071514 for ORF3, AF071515 for ORF4, AF071516 for ORF5, AF071517 for ORF6, AF071518 for ORF7, AF071519 for *gdhA*, AF071520 for ORF8, AF071521 for ORF9, AF071522 for ORF10 and AF071523 for ORF11.

Table 1. Bacterial strains, plasmids and phages used in this study

Ap^R, apramycin resistant; Km^R, kanamycin resistant; Thio^R, thiostrepton resistant; Am^R, ampicillin resistant; Neo^R, neomycin resistant.

Strain, plasmid or phage	Properties	Source/reference
Bacterial strains		
<i>E. coli</i> DH5 α	General cloning host	BRL
<i>E. coli</i> ET12567	<i>dam-13::Tn9 dcm-6 hsdM</i>	MacNeil <i>et al.</i> (1992)
<i>M. luteus</i> ATCC 10240	Wild-type	ATCC†
<i>S. lividans</i> 1326	Wild-type	H. Schrempf‡
<i>S. noursei</i> ATCC 11455	Wild-type, nystatin producer	ATCC†
Plasmids		
pGEM3Zf(–)	ColE1 replicon, Ap ^R , 3.2 kb	Promega
pUC4K	ColE1 replicon, Km ^R Ap ^R , 3.9 kb	Pharmacia
pUZ8002	RK2 derivative, Km ^R Tc ^R	D. H. Figurski§
pGM11	pSG5 replicon, Neo ^R , 5.3 kb	Wohlleben & Muth (1993)
pWHM3	ColE1 + pIJ101 replicons, Thio ^R , 7.2 kb	Vara <i>et al.</i> (1989)
pHZ1351	ColE1 + replicon from <i>Streptomyces</i> sp. FR-005, Thio ^R , 8.3 kb	Z. Deng
pKC1218	ColE1 + SCP2* replicon, <i>oriT</i> , Am ^R , 5.8 kb	Bierman <i>et al.</i> (1992)
pSET152	ColE1 replicon + ϕ C31 int, <i>oriT</i> , Am ^R , 5.5 kb	Bierman <i>et al.</i> (1992)
pSOK101	pWHM3 derivative with 3.1 kb <i>Bam</i> HI– <i>Sph</i> I fragment replaced with 3.0 kb <i>Bam</i> HI– <i>Sph</i> I fragment from pSET152 containing ColE1, <i>oriT</i> and Am ^R , 7.1 kb	This work
pSOK201	pGM11 derivative with 1.2 kb <i>Eco</i> RI– <i>Hind</i> III fragment replaced with 3.0 kb <i>Eco</i> RI– <i>Hind</i> III fragment from pSOK101 containing ColE1, <i>oriT</i> and Am ^R , 7.1 kb	This work
pCHZ101	pHZ1351 derivative with 4.0 kb <i>Bam</i> HI– <i>Hind</i> III fragment replaced with 3.0 kb <i>Bam</i> HI– <i>Hind</i> III fragment from pSOK101 containing ColE1, <i>oriT</i> and Am ^R , 7.3 kb	This work
pGEM-RN4	1.6 kb <i>Eco</i> RI– <i>Bam</i> HI fragment from KH11 and 1.35 kb <i>Bam</i> HI– <i>Sal</i> I fragment from KH12 cloned into pGEM3Zf(–), Ap ^R , 6.15 kb	This work
pRN4	2.95 kb <i>Eco</i> RI– <i>Hind</i> III fragment from pGEM-RN4 joined to the 3.0 kb <i>Eco</i> RI– <i>Hind</i> III fragment from pSOK101 containing ColE1, Am ^R and <i>oriT</i> , 5.95 kb	This work
pRN8	1.4 kb <i>Bam</i> HI– <i>Eco</i> RI fragment from KH12 joined to the 1.3 kb Km ^R <i>Eco</i> RI fragment from pUC4K, the 1.4 kb <i>Eco</i> RI– <i>Sph</i> I fragment from KH12 and the 3.0 kb <i>Bam</i> HI– <i>Sph</i> I fragment from pSET152 containing ColE1, Am ^R , <i>oriT</i> , 7.3 kb	This work

Table 1. (cont.)

Strain, plasmid or phage	Properties	Source/reference
pGEM-ND2	1.3 kb <i>Bam</i> HI– <i>Eco</i> RI fragment from KH16 cloned into pGEM3Zf(–), Ap ^R , 4.5 kb	This work
pND2	1.3 kb <i>Hind</i> III– <i>Eco</i> RI fragment from pGEM-ND2 joined to 3.0 kb <i>Hind</i> III– <i>Eco</i> RI fragment from pSOK101 containing ColE1, Am ^R , <i>oriT</i> , 4.3 kb	This work
Phages DASHII	Bacteriophage λ vector	Stratagene

† American Type Culture Collection, Manassas, VA, USA.

‡ FB, Biologie/Chimie, University of Osnabrück, D-49069 Osnabrück, Germany.

§ Dept of Microbiology, College of Physicians and Surgeons, Columbia University, NY 10032, USA

|| Huazhong Agricultural University, Wuhan, China.

the backbone is assembled and the fact that specific domains within a PKS are responsible for a given biosynthetic step provides an excellent opportunity for protein engineering. Indeed, several research groups have reported successful manipulations of PKS genes which resulted in biosynthesis of new compounds (for example, Kuhstoss *et al.*, 1996; Ruan *et al.*, 1997). However, the polyketide backbone by itself usually has little, if any, biological activity. Modifications of this backbone by means of hydroxylation, methylation, acylation and glycosylation are usually required for achieving full potency, and the genes providing these activities are often found in the proximity of the PKS genes (Katz & Donadio, 1995). This fact further expands the possibilities for manipulations of the biosynthetic pathways, potentially leading to overproducing strains or new antibiotics (Sezonov *et al.*, 1997; Solenberg *et al.*, 1997).

We have initiated molecular genetic studies on *Streptomyces noursei* ATCC 11455, the producer of the antifungal polyene antibiotic nystatin. The latter is currently being used for the treatment of many fungal infections in humans. The structure of nystatin predicts the involvement of type I (modular) PKS enzymes in biosynthesis of its polyketide backbone, while a mycosamine moiety attached to the aglycone implies involvement of deoxysugar biosynthesis genes. Using a PCR-derived probe for the deoxythymidyl diphosphate (TDP)-glucose dehydratase gene, we have identified part of the gene cluster apparently governing the biosynthesis of a macrolide antibiotic. Gene disruption and replacement experiments showed that this gene cluster is not required for nystatin biosynthesis, but that it governs the synthesis of an antibacterial agent in *S. noursei* ATCC 11455.

METHODS

Bacterial strains, plasmids and phages. Bacterial strains, plasmids and phages used in this study are listed in Table 1

except for plasmids pKH10, pKH11, pKH12, pKH14, pKH15, pKH16 and pKH17. These plasmids were constructed by cloning the corresponding *Eco*RI KH fragments depicted in Fig. 1(a) into the *Eco*RI site of pGEM3Zf(–).

Media, growth conditions and transformation procedures. *S. noursei* ATCC 11455 and its mutants were grown on solid ISP2 medium (Difco) and in liquid TSB medium (Sigma). For preparation of protoplasts, cultures were grown at 30 °C for 40 h in TSB medium supplemented with 1.5% glycine. *S. noursei* protoplasts were prepared and regenerated according to Hopwood *et al.* (1985) except that P medium contained 20% sucrose and regeneration medium R5 contained 15% sucrose. *Streptomyces lividans* 1326 protoplasts were obtained and transformed as described by Hopwood *et al.* (1985). Intergeneric conjugation from *Escherichia coli* ET12567(pUZ8002) into *Streptomyces* strains was done as described by Flett *et al.* (1997). *E. coli* strains were grown and transformed as described by Sambrook *et al.* (1989) except for *E. coli* ET12567(pUZ8002), which was maintained on media containing 20 µg chloramphenicol ml^{–1} and 50 µg kanamycin ml^{–1}. *Micrococcus luteus* ATCC 10240 was maintained essentially as *E. coli* strains except that the incubation temperature was 35 °C.

Molecular genetic techniques. Plasmid, phage and total DNA preparations, endonuclease digestions, ligations and fractionation were performed as described previously (Sambrook *et al.*, 1989; Hopwood *et al.*, 1985). Modification of plasmid DNA *in vitro* was done according to Matsushima & Baltz (1994). DNA fragments were isolated from agarose gels using the Qiagen QIAEX kit, labelled by the use of the HighPrime kit from Boehringer Mannheim, and used for Southern blot analysis. PCR amplification of the *gdh* fragment was done according to Decker *et al.* (1996). DNA sequencing reactions were performed with a DNA sequencing kit from Applied Biosystems and analysed with an automated DNA sequencer from the same supplier. DNA sequences were analysed using the GCG program package (Devereux *et al.*, 1984) and deposited in GenBank.

Analysis of secondary metabolites. For nystatin production, *S. noursei* strains were grown for 120 h in 50 ml SAO-23 medium (g per l: glucose, 45; NH₄NO₃, 2.5; corn meal, 3; MgSO₄·7H₂O, 0.4; KH₂PO₄, 0.2; CaCO₃, 5) in shake flasks at

Fig. 1. Putative macrolide antibiotic biosynthesis gene cluster in *S. noursei* ATCC 11455. (a) Partial physical and genetic map. Shaded boxes represent fragments used for subcloning and DNA sequencing; E = *EcoRI*, Ba = *BamHI*, S = *SphI*. White boxes indicate DNA fragments used in gene inactivation experiments (see text and Table 1 for details). (b) Genetic maps of the mutants constructed via gene replacement and disruption.

28 °C (220 r.p.m.). Cells were then pelleted and extracted with DMSO, and extracts analysed by HPLC. For production of antibacterial compounds, *S. noursei* was grown for 120 h in 50 ml MP1 medium (g per l: glucose, 40; yeast extract, 1.5; NH_4NO_3 , 2.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; KH_2PO_4 , 0.5; CaCO_3 , 3) in shake flasks at 28 °C (220 r.p.m.). Culture supernatants were then extracted with equal volumes of ethyl acetate, and extracts were evaporated under vacuum. The pellets were dissolved in small amounts of ethyl acetate and subjected to TLC analysis. TLC plates (Merck) were developed in ethyl acetate/methanol/water (100:2.5:1, by vol.). For antibacterial agent bioassay, TLC plates were placed for 30 min on the surface of LB agar plates inoculated with *M. luteus* ATCC 10240. The agar plates were then incubated for 16 h at 35 °C and screened visually for growth-inhibition zones.

RESULTS

Identification of a macrolide antibiotic biosynthesis gene cluster in *S. noursei* ATCC 11455

Several groups have reported the cloning of gene clusters for biosynthesis of antibiotics containing a deoxysugar moiety using a probe for the TDP-glucose dehydratase (*gdh*) gene, which governs one of the initial steps in deoxysugar formation (Liu & Thorson, 1994). Since the

nystatin molecule contains the deoxysugar mycosamine, we decided to use a similar approach to identify the nystatin biosynthesis gene cluster. Part of a presumptive *gdh* gene was amplified from the *S. noursei* chromosome by PCR with primers designed by Decker *et al.* (1996). DNA sequence analysis of the 550 bp PCR product designated *gdhA* showed that it encodes a peptide with 41.6% identity to the RfbB TDP-glucose dehydratase from *Xanthomonas campestris* (Table 2). Hybridization of the labelled *gdhA* probe under high stringency conditions with *S. noursei* chromosomal DNA digested with several restriction enzymes showed the presence of one copy of *gdhA* in the genome (data not shown). A *S. noursei* gene library was then prepared in the bacteriophage λ vector DASHII. Screening of this library with the *gdhA* probe allowed the isolation of several overlapping phage clones which were then aligned according to their physical maps and hybridization with *gdhA*. Next, an *EcoRI* and some *BamHI* DNA fragments from the identified region were subcloned in the pGEM3Zf(–) vector and subjected to partial DNA sequence analysis. This analysis revealed several putative genes typical of those governing the biosynthesis of polyketide-derived antibiotics (Table 2). In particular,

Table 2. Genes identified within the putative macrolide antibiotic biosynthesis gene cluster from *S. noursei* ATCC 11455 by partial DNA sequencing and comparative analysis

ORF	Partial sequence	Match in the databases	Identity (%)
1	133 aa	OleB ABC transporter (Olano <i>et al.</i> , 1995), aa 338–467	62.4
2	157 aa	Streptomycin 6-kinase (Vogtli & Hutter, 1987), aa 250–309	35.5
3	170 aa	EryBV glycosyltransferase (Gaisser <i>et al.</i> , 1997), aa 28–189	52.9
4	117 aa	Putative transposase (Kaluza <i>et al.</i> , 1985), aa 257–354	27.1
5	144 aa	EryF hydroxylase (Weber <i>et al.</i> , 1991), aa 306–407	57.1
6	174 aa	MycF O-methyltransferase (Inouye <i>et al.</i> , 1994), aa 70–223	71.2
7	115 aa	SnoY (Ylihanko <i>et al.</i> , 1996), aa 1–114	28.2
<i>gdbA</i>	186 aa	RfbB TDP-glucose dehydratase (Koplin <i>et al.</i> , 1993), aa 9–197	41.6
8	187 aa	Deoxyhexose reductase (Gaisser <i>et al.</i> , 1997), aa 9–162	48.7
9	181 aa	TDP-glucose dehydratase (Lombo <i>et al.</i> , 1997), aa 6–172	67.2
10	110 aa	TDP-glucose synthase (Tsukioka <i>et al.</i> , 1997), aa 1–110	60.9
11	181 aa	EryA2 PKS (Donadio <i>et al.</i> , 1991), aa 120–300	75.7

genes presumably encoding proteins homologous to an ABC (ATP-binding cassette) transporter (ORF1), glycosyltransferase (ORF3), putative transposase (ORF4), P450 hydroxylase (ORF5), O-methyltransferase (ORF6), deoxyhexose reductase (ORF8), a second TDP-glucose dehydratase (ORF9), TDP-glucose synthase (ORF10) and type I PKS (ORF11) have been identified (see Fig. 1a for their locations).

Development of a gene-transfer system for *S. noursei* ATCC 11455

To specifically inactivate the genes within the identified cluster, a gene transfer system for *S. noursei* had to be established. Initial studies showed that the organism is resistant to high concentrations of thiostrepton ($>100 \mu\text{g ml}^{-1}$), hygromycin ($>200 \mu\text{g ml}^{-1}$), gentamicin ($>50 \mu\text{g ml}^{-1}$) and erythromycin ($>100 \mu\text{g ml}^{-1}$). *S. noursei* plated on agar media supplemented with neomycin ($>50 \mu\text{g ml}^{-1}$), kanamycin ($>50 \mu\text{g ml}^{-1}$) or viomycin ($>50 \mu\text{g ml}^{-1}$) consistently gave some background growth. However, *S. noursei* was sensitive to apramycin at concentrations $>30 \mu\text{g ml}^{-1}$ so this antibiotic was chosen for selection of transformants.

Although a method for efficient protoplast formation and regeneration for *S. noursei* ATCC 11455 was established (see Methods), all our attempts to achieve *S. noursei* protoplast transformation with vectors pKC1218 and pSET152 using both standard and modified techniques failed. To extend the choice of vectors, the new plasmids pSOK101 (pIJ101 replicon), pSOK201 (pSG5 replicon) and pCHZ101 (pHZ1351 replicon) were constructed (see Fig. 2 and Table 1). However, transformation of *S. noursei* protoplasts with the above vectors failed to give any transformants. All five plasmids used in these transformation experiments contain the origin of transfer, *oriT*, from plasmid RP4 allowing mobilization of the construct during conjugation from an appropriate *E. coli* strain into *Streptomyces* sp. (Mazodier *et al.*, 1989). The plasmids could

easily be introduced into *S. lividans* 1326 via both protoplast transformation and conjugation from *E. coli* ET12567(pUZ8002). Conjugation experiments with *S. noursei* (Table 3) using pSOK101, pKC1218 and pCHZ101 were unsuccessful. For pSOK201, a relatively high number of small apramycin-resistant colonies appeared on the conjugation plates. However, these putative transconjugants stopped growing after 3–4 d incubation and did not develop into normal colonies. Attempts to transfer these small colonies to fresh selective media resulted in slow-growing and poorly sporulating colonies from which no plasmid DNA could be isolated. About 1–2 colonies per plate were isolated when plasmid pSET152 carrying the site-specific integration system from streptomycete phage ϕ C31 was used for conjugation. Southern blot analysis of two such *S. noursei* (pSET152) clones with labelled pSET152 DNA indicated integration of the plasmid into a specific site on the chromosome (data not shown).

Gene replacements and disruption within the identified gene cluster

The successful introduction of pSET152 into *S. noursei* ATCC 11455 provided evidence for intergeneric conjugation being the only tool for gene transfer into this organism. To test the possible involvement of the ORFs within the identified gene cluster in nystatin biosynthesis, gene-inactivation experiments were carried out. For this purpose, the suicide vector pRN4 was constructed by cloning, in the original orientation, a 1.6 kb *EcoRI*–*Bam*HI fragment from pKH11 and a 1.35 kb *Bam*HI–*Sal*I fragment from pKH12 (*Sal*I site not shown on the map) into a suicide vector carrying *oriT* and an apramycin-resistance gene (see Fig. 1a and Table 1). *S. noursei* ATCC 11455 transconjugants carrying pRN4 integrated into the chromosome via a single-crossover event were obtained at a frequency four times higher than that found for the site-specific integration of pSET152. One of the transconjugants was allowed three

Fig. 2. New *E. coli*-*Streptomyces* bifunctional cloning vectors capable of intergeneric conjugation. *Bam*HI, *Eco*RI and *Hind*III sites in all vectors are unique. For details on construction see Table 1.

rounds of sporulation on ISP2 agar without antibiotic selection and its progeny were screened for loss of the apramycin-resistance marker. Four such clones were

Table 3. Gene transfer to *S. noursei* ATCC 11455 via conjugation from *E. coli* ET12567(pUZ8002).

Plasmid	Replicon	Efficiency per recipient	Notes
pSOK101	pIJ101	$<3 \times 10^{-8}$	Colonies are sick and grow poorly when retransferred on selective medium
pKC1218	SCP2*	$<3 \times 10^{-8}$	
pSOK201	pSG5	6×10^{-5}	
pCHZ101	pHZ1351	$<3 \times 10^{-8}$	Site-specific integration
pSET152	ϕ C31 int	5×10^{-8}

Fig. 3. Southern blot analysis of the *Bam*HI-digested total DNA isolated from *S. noursei* ATCC 11455 (WT), RN412 and RN413 with labelled KH10 probe. Hybridizing fragments present in WT and absent from RN412 and RN413 are indicated with the arrows. Intact KH10 fragment was used as a positive control.

selected and two of them, RN412 and RN413, were shown by hybridization of the labelled KH10 DNA fragment with their total DNA to contain the desired deletion (Fig. 3). As shown in Fig. 1(b), the above mutants have deletions of approximately 10.6 kb, affecting ORF5, ORF6, ORF7, *gdhA* and ORF8.

ORF9, presumably encoding the second TDP-glucose dehydratase, was inactivated by gene replacement with pRN8. The latter was made by cloning the 1.4 kb *Bam*HI-*Eco*RI fragment from pKH12, a 1.3 kb *Eco*RI kanamycin-resistance fragment and a 1.4 kb *Eco*RI-*Sph*I fragment from pKH16 in a mobilizable suicide vector (see Fig. 1a and Table 1). The ORF9 gene replacement procedure was followed as above except that it was

Fig. 4. Analysis of the antibacterial compounds produced by *S. noursei* strains, by TLC and bioassay against *M. luteus* ATCC 10240. Erythromycin [1 mg (E1) or 5 mg (E5)] was applied as a control on the same TLC plate.

performed in *S. noursei* strain RN413. In three recombinant strains, designated RN803, RN805 and RN807, ORF9 replacement was confirmed by Southern blot analysis (data not shown).

Finally, inactivation of ORF11, presumably encoding a type I PKS enzyme in *S. noursei* ATCC 11455, was made by gene disruption with pND2. pND2 was constructed by cloning the internal 1.3 kb *Bam*HI–*Eco*RI fragment of ORF11 from KH16 into a mobilizable suicide vector (see Fig. 1a and Table 1). Integration of pND2 into the chromosome via a single crossover upon intergeneric conjugation resulted in strains ND21 and ND22. The integration of pND2 in these strains was also confirmed by Southern blot analysis of their total DNA (data not shown). The genotypes of all the recombinant strains constructed are presented in Fig. 1(b).

Analysis of secondary metabolites produced by the wild-type strain and knock-out mutants

S. noursei strains RN413, RN803 and ND21 were tested for nystatin production by shake flask experiments in the semi-defined medium SAO-23. All the mutants constructed produced nystatin in amounts similar to the wild-type (WT) strain ATCC 11455. Thus, it was concluded that the identified gene cluster is not involved in the biosynthesis of nystatin. From the information we had on the putative genes within the cluster, it was logical to assume that the cluster governs the biosynthesis of some macrolide antibiotic which is exported out of the cell (presence of gene for a putative ABC transporter). Thus, we focused our attempts on isolation of this unknown antibiotic. The shake flask fermentations were carried out with the mutant and the WT strains in the semi-defined medium MP1. Ethyl acetate extracts of the supernatants from 120 h cultures were analysed by TLC followed by a bioassay against *M. luteus* ATCC 10240. A large growth inhibition zone

corresponding to the TLC spot with R_F 0.44 (A) was observed in the case of the WT strain (Fig. 4), while none of the mutants displayed a similar inhibition zone. We thus concluded that this zone corresponds to the compound (A) synthesized by the products of the putative genes within the identified gene cluster. In addition, the extracts from all strains contained another compound with antibacterial activity, as small inhibition zones corresponding to TLC spots with R_F 0.12 (B) were also observed (Fig. 4). Thus, the WT strain is capable of producing at least two antibacterial compounds, A and B, while the mutants fail to produce A due to the specific mutations within its biosynthetic gene cluster.

DISCUSSION

Using a PCR-amplified DNA probe for the putative TDP-glucose dehydratase gene we have isolated a 30 kb genomic region from *S. noursei* ATCC 11455, the producer of the antifungal antibiotic nystatin. A partial DNA sequencing analysis within the cloned segment revealed the presence of several ORFs presumably encoding homologues of the proteins involved in biosynthesis of, and resistance to, antibiotics in different streptomycetes. Establishment of a gene-transfer system for *S. noursei* allowed gene-inactivation experiments, which demonstrated that the identified gene cluster is not responsible for nystatin biosynthesis. However, by comparing the biological activities in culture supernatants of the WT strain and its constructed mutants, we were able to demonstrate the involvement of this gene cluster in the biosynthesis of a presumably novel antibacterial compound.

We speculate that this new compound is a macrolide antibiotic on the basis of the observed absence of the specific antibacterial activity in the mutant ND21 with disrupted ORF11, presumably encoding a type I PKS.

Sequence comparisons also suggest that the polyketide backbone of this compound might be modified by the means of hydroxylation (ORF5) and O-methylation (ORF6) during antibiotic synthesis. Five of the ORFs found within the cluster appear to be responsible for synthesis (*gdhA*, ORF8, ORF9 and ORF10) and attachment (ORF3) of deoxysugar(s) to the macrolide antibiotic precursor(s). It thus seems likely that at least one deoxysugar is attached to the polyketide moiety. Interestingly, two putative genes, *gdhA* and ORF9, both presumably encoding Gdh, are present in this cluster. It is possible that the two putative *gdh* genes are responsible for synthesis of two different deoxysugars, even though to our knowledge there is no precedent for finding two *gdh* genes within one cluster. Alternatively, one of the identified *gdh* genes may not encode a true dehydratase, but another as yet undescribed enzyme involved in deoxysugar formation. The modifications of the polyketide moiety seem to be crucial for the antibiotic activity, as a deletion affecting ORFs 5–8 and *gdhA* abolishes the specific antibacterial activity in mutant RN413. However, due to the limited DNA sequence data, we cannot exclude the possibility of a regulatory gene being affected by the deletion.

The two ORFs found within the cluster might be responsible for the resistance of *S. noursei* to the macrolide antibiotic identified. ORF1 seems to encode an ABC transporter homologous to the *Streptomyces antibioticus* OleB ATP-binding protein involved in the efflux of oleandomycin from the producing organism (Olano *et al.*, 1995). Another putative resistance gene might be represented by ORF2, which displays similarity to the *Streptomyces glaucescens* streptomycin 6-kinase responsible for the inactivation of streptomycin by means of its phosphorylation (Vogtli & Hutter, 1987).

Finally, the putative ORF4 product resembles transposases from the *Rhizobium japonicum* insertion element (Kaluza *et al.*, 1985) and insertion elements from some other bacteria. There is at least one other reported case of an insertion element being present in the antibiotic biosynthesis gene cluster; IS1136 was found between the PKS-encoding genes in the erythromycin biosynthesis gene cluster of *Saccharopolyspora erythraea* (Donadio & Staver, 1993).

So far, the production of two antifungal antibiotics of polyketide origin by *S. noursei*, cycloheximide and nystatin, had been documented (Roszkowski *et al.*, 1972). Although Brown & Hazen (1953) reported isolation of the antibacterial antibiotic phalamycin from a spontaneously obtained morphological variant of *S. noursei*, this strain was apparently a nystatin non-producer and phalamycin was not produced by the wild-type *S. noursei*. Thus, it is not clear whether the phalamycin-producing strain was really *S. noursei*. We therefore suggest that the antibacterial compound identified in this study and the putative genes for its biosynthesis are novel. Further characterization of this antibiotic aimed at its purification, and elucidation of its chemical structure and potency, is under way.

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