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Studies on the biosynthesis of antibiotic nystatin with emphasis on regulation

Thesis for the degree of Doctor Philosophiae

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I wish to thank my husband Sergey, the one who introduced me into this field, constantly inspiring and supporting me at work and at home, for being the best friend, colleague and farther for our two beloved daughters.

These days, finishing my writing, I often mentally turn myself back, to my childhood and family. My older sister was always a very good friend and an inspiring example in my life, and who knows, what field of knowledge I would select for study at the age of 18 (indeed, I was interested in so much different things!) if not her, with gripping stories about mysterious science of genetics, amazing marine creatures, the unusual journeys and her studies at the biological department!

I would like to dedicate this work to my dear parents, who are unfortunately not with us in this world any longer. They were always very proud of me and my small and big achievements. I think they would be very happy today.

Olga Sekurova Trondheim, February 2013

ABSTRACT

Nystatin is an antifungal polyene macrolide antibiotic produced by *Streptomyces noursei*, first described in 1950 (Hazen and Brown, 1950). Nystatin is currently being used for treatment of superficial fungal infections, and is considered a medically important drug. However, until recently, nothing was known about the biosynthesis of this antibiotic in *S. noursei*. This study is the part of the detailed investigation of the genetics and biochemistry of nystatin biosynthesis with emphasis on the regulation.

First, the pleiotropic regulatory gene locus from *S.noursei* capable of enhancing actinorhodin (Act) production in *S.lividans* was cloned and sequenced. Two genes, designated *ssmA* and *ssmB*, have been suggested to be responsible for the phenomenon. Putative product of *ssmA* showed limited homology to the peptide encoded by *afsR2,* known as a pleiotropic regulator from *S.lividans* and *S. coelicolor*. Recombinant *S.lividans* strains carrying deletion derivatives of the locus were tested for Act production. The results of these experiments showed that *ssmA* is required for Act overproduction, while *ssmB* is possibly involved in the negative regulation of antibiotic production. Further experiments suggested that *ssmA* is involved in the carbon source-dependent regulation of nystatin production in *S.noursei*.

Next, the entire nystatin biosynthetic gene cluster from *S.noursei* was cloned and sequenced, putative functions for the biosynthetic genes were implied, and a model for the nystatin biosynthesis was suggested. Six genes encoding PKS type I, genes for posttranslational modifications of nystatin aglycon, efflux of antibiotic out of the cell, and putative cluster-specific regulatory genes have been identified in the cluster. Inactivation of PKS-encoding genes in the cluster resulted in nystatin non-producing mutants, confirming their roles in the biosynthesis of this antibiotic. Genes presumably encoding nystatin efflux pump were studied via gene inactivation and analysis of resulting mutants. It was shown that the efflux is tightly linked to C-10 hydroxylation of the nystatin macrolactone ring. Several genes for post-PKS modifications have been found in the nystatin cluster, among them three genes for synthesis and attachment of mycosamine moiety to the nystatin aglycon. Effect of inactivation of these genes on nystatin biosynthesis was studied. Combined, these results have helped to refine the model of nystatin biosynthesis.

The regulatory locus of 6 genes has been found on a right flank of nystatin biosynthetic cluster. Four of them were shown by gene inactivation to be directly involved in the regulation of nystatin biosynthesis. Promoter-probe studies revealed the main targets of regulation in the

nystatin gene cluster, and cross-complementation experiments allowed establishing the hierarchy among the regulators. Finally, the model for regulatory cascade was suggested.

The results of studies described above provided important information needed for rational engineering of novel polyene macrolides by manipulation of the nystatin biosynthetic genes. Seven analogs of nystatin with altered polyol region and carboxylic group have been obtained and subjected to in vitro antifungal and hemolitic activities tests. It was shown that combinations of several mutations could be beneficial for the activity-toxicity properties of the new compounds. The two most active and less toxic analogs were chosen for in vivo tests in a mouse model, where they proved to be considerably less toxic and at least as active as amphotericin B, the antifungal antibiotic used for treatment of systemic fungal infections. These results indicate that two obtained nystatin analogs can be used for further development into antifungal drugs for human therapy, and that genetic engineering is an effective tool for obtaining new compounds with improved therapeutic properties.

ABBREVIATIONS

aa	Amino acid(s)
ABC	ATP-binding cassette
ACP	Acyl carrier protein
Act	Actinorhodin
AT	Acyltransferase
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
bp	Base pair(s)
BTAD	Bacterial transcription activation domain
CDA	Calcium dependent antibiotic
CREA	Catabolite repressor from *Aspergillus nidulans*
CSR	Cluster specific regulator
CoA	Coenzyme A
Da	Dalton
DEBS	Deoxyerythronolide B synthase
6dEB	6-deoxyerythronolide B
DH	Dehydratase
DIG	Digoxygenin
DMSO	Dimethyl sulfoxide
ER	Enoyl reductase
Ery	Erythromycin
HPLC	High performance liquid chromatography
HTH	Helix-turn-helix
kb	Kilo base pairs
kD	Kilo dalton
KR	Ketoreductase
KS	Ketosynthase
LAL	Large ATP-binding regulators of the LuxR type
LC-MS	Liquid chromatography-mass spectrometry
LD	Lethal dose
Mb	Mega base pairs
MIC50	Minimum inhibitory concentration (50 % inhibition of growth of test organism)

MTD	Maximal tolerated dose
NRPS	Non-ribosomal peptide synthase
nt	Nucleotide(s)
NTP	Nucleoside triphosphate
orf	Open reading frame
PCR	Polymerase chain reaction
PK	Polyketide
PKS	Polyketide synthase
Red	Undecylprodigiosin
RNAP	RNA polymerase
SARP	Streptomyces antibiotic regulatory proteins
TE	Thioesterase

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1. INTRODUCTION

Antibiotics belong to the large, chemically heterogeneous group of natural products classified as secondary metabolites. They are synthesized by many bacterial, fungal, plant and animal species and are not required for normal growth of the producing organisms. By definition, antibiotics are secondary metabolites that have the ability to kill or inhibit the growth of other microorganisms.

Discovery of penicillin by Alexander Fleming in 1928 opened the modern history of antibiotics. Penicillin is the first antibiotic found in microorganisms, it is synthesized by *Penicillum notatum, P. chrysogenum* and several other fungi. It showed activity against a wide range of bacteria, and was soon introduced into medical practice as a successful treatment against pneumonia, blood poisoning, strep throat, diphtheria, scarlet fever, syphilis, gonorrhea, meningitis, rheumatic fever and many other diseases (Chain et al., 1940). This "wonder" drug saved millions of lives during the World War II, and also today, together with its derivatives, it is one of the most commonly used antibiotics. Back then, however, nobody could make a prediction that the use of penicillin would become limited, because bacteria would develop resistance against penicillin. As early as in 1952, about three-fifths of all staphylococcus infections were caused by penicillin–resistant bacteria. It became clear that different approaches, as for example, changing the chemical structure of antibiotic molecules in order to get their new properties, must be considered. The discovery of penicillin also brought about the idea that if there is one antibiotic in nature it must be more, and since that time many more antibiotics have been found, though only few of them are fungal in origin. Most of the antibiotics known today are derived from bacteria belonging to the order *Actinomycetales* and particularly to the genus *Streptomyces.*

1.1 Antibiotics produced by *Streptomyces* bacteria

Actinomycete bacteria are the source of ca. 60% of all known antibiotics produced by microorganisms. As Berdy reports the number of antibiotics produced by soil-inhabiting actinomycetes discovered by 2002, estimated as 8700, compared with 4900 from fungi and 2900 from all other bacteria. Nearly 80% of actinobacteria-derived antibiotics were found in *Streptomyces* species (Berdy, 2005). It has been estimated that the streptomycetes alone may

produce up to 250,000 different biologically active molecules of different classes, including some important drugs as anti-cancer, anti-parasitic agents and immunosuppressants (Watbe et al., 2001).

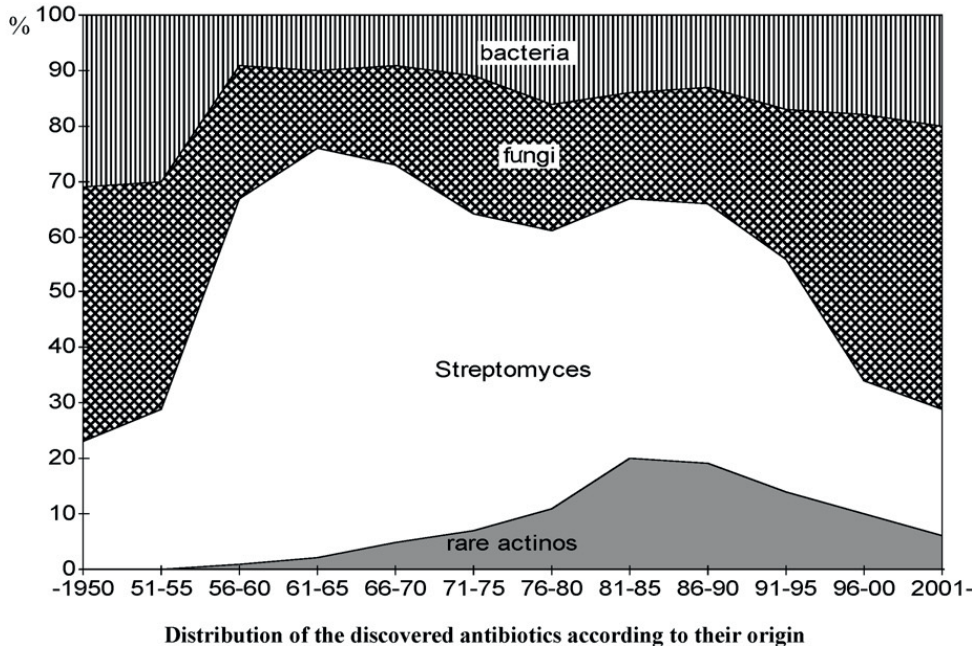

Figure 1.1. Origin of antibiotics discovered in 1950-2001. (Adapted from Berdy (2005).

The *Streptomyces* bacteria form complex colonies on solid media, which represent a mould-like system of interconnected hyphae that first grows as a so-called vegetative/substrate mycelium and then later, after the nutrient resources become limited, produces aerial sporulating hyphae. These features are not typical bacterial, that's why microbiologists were confused about their origin for a long time. From the discovery of the first actinomycetes in the 1870s and till as late as 1950s, they were considered as an intermediate between bacteria and fungi. Nevertheless, in early 1950s there were the first pointers on bacterial origin of streptomycetes as, for example, the cell-wall composition resembling that of typical Gram-positive bacteria, and also their sensitivity to anti-bacterial antibiotics. Later, with a help of electron microscope, it was shown that *S. coelicolor* lacks a nuclear membrane, defining streptomycetes as bacteria (Hopwood, 1999). *Streptomyces* can also be distinguished from other bacteria by a much higher G+C content in their DNA, often up to 75% compared with about 50% for low G+C Gram-positive bacteria like *Bacillus* or *Streptococcus.*

Interest in *Streptomyces* as a possible source of antibiotics was sparked after the discovery of streptomycin, the first known antibiotic from a bacterium, by Selman Waksman in 1943 (Jones et al., 1944). This antibiotic was first isolated in the laboratory of Waksman from the strain of *Streptomyces griseus*. It is difficult to overestimate the significance of this bactericidal drug as the first effective chemotherapeutic treatment for pulmonary tuberculosis and bubonic plague (Singh and Mitchison, 1954). It appeared also to be effective against causative agents of other diseases: typhoid fever, cholera, urinary tract infections, etc. In 1952, S.Waksman was awarded a Nobel prize for this outstanding discovery.

Since then, many more useful antibiotics have been discovered in *Streptomyces* bacteria. Some examples of these antibiotics are given in Table 1.1

Table 1.1. Useful antibiotics from *Streptomyces* spp. and their application (Kieser et al.,2000).

Antibiotic	**Producer**	**Chemical class**	**Target**	**Application**
Actinomycin	*Streptomyces* spp.	Peptide	Transcription	Antitumor
Avermectin	*S. avemitilis*	Macrolide(PK)	Chloride ion channels	Antiparasitic
Amphotericin	*S. nodosus*	Polyene macrolide	Membrane	Antifungal
Bleomycin	*S. verticillus*	Glycopeptide	DNA strand breakage	Antitumor
Candicidin	*S. griseus*	Polyene macrolide	Membrane	Antifungal
Daunorubicine	*S. peucitius*	Anthracycline(PK)	DNA	Antitumor

			intercalation	
Kanamycin	*S. kanamyceticus*	Aminoglycoside	Protein synthesis	Antibacterial
Tetracycline	*S. aureofaciens*	Tetracycline(PK)	Protein synthesis	Antibacterial
Thiostrepton	*S. azureus*	Thiopeptide	Protein synthesis	Growth promotant
Validamycin	*S. hygroscopicus*	Aminoglycoside	Protein synthesis	Plant protectant
Virginiamycin	*S. virginiae*	Macrocyclic lactone+macrocyclic peptidolactone	Protein synthesis	Growth promotant

1.2 Genetics and biochemistry of antibiotic biosynthesis in *Streptomyces*

1.2.1 The short historical overview of the development of knowledge on the genetics and biochemistry of Streptomyces

After the potential of *Streptomyces* as antibiotic producers was understood, many laboratories in different countries began intensive research on genetics and biochemistry of these unique organisms. To the most important discoveries of the 1960s belongs the creation of the first genetic map of *Streptomyces coelicolor* chromosome by Hopwood (Hopwood, 1967). After this publication, *S. coelicolor* became a model organism for genetics and molecular biology studies.

The identification of A-factor as a diffusible molecule required for activating streptomycin production and sporulation in *S. griseus* by Khokhlov and his collaborators is another important finding in *Streptomyces* physiology (Khokhlov et al., 1967). This hormone-like

molecule was characterized as a novel γ-butyrolactone by the same research group in 1976 (Kleiner et al., 1976). In later works the discovery of this small signaling molecule was significantly developed in *S. griseus, S. coelicolor* and *S. virginiae,* and has been among the most important features of the modern understanding of regulatory network in biosynthesis of antibiotics in *Streptomyces* (Yamada et al., 1987; Onaka et al., 1998).

The discovery of the ΦC31 temperate phage by the group of N. Lomovskaya was another very important event in studying *Streptomyces* biology (Lomovskaya et al., 1970). Approximately at the same time, the first *Streptomyces* plasmid SCP1 was postulated, but all active attempts to physically isolate it failed (Hopwood and Wright, 1973). Only in 1987 the group of H. Kinashi could identify the reason for that failure. He and his colleagues with a help of newly developed pulsed-field gel electrophoresis technology showed that SCP1 is large (350kb) and linear DNA molecule (Kinashi and Shimaji, 1987). The significance of this discovery was understood when it appeared that linear replicons, including the chromosome, are widely spread among *Streptomyces*.

The first circular *Streptomyces* plasmid SCP2 was isolated by Schrempf et al. in 1975. (Schrempf et al.,1975). This discovery was an important step toward introducing DNA into *Streptomyces* by transformation and finally cloning genes in these organisms. Since then, several methods of the gene transfer into *Streptomyces* have been developed, including transformation of protoplasts (Bibb et al., 1978), phage-mediated transduction (Stuttard, 1979), and plasmid-mediated conjugation (Mazodier et al., 1989).

Early finding that genes for biosynthesis of antibiotic methylenomycin are localized on a plasmid led to the speculations that plasmid determination of antibiotic production genes might be typical for *Streptomyces* (Kirby et al., 1975). However, in the subsequent studies on antibiotic actinorhodin in *S. coelicolor* it was shown that genes for its biosynthesis are chromosomally localized and clustered (Wright and Hopwood, 1976). Such organization of antibiotic biosynthetic genes appears to be a main rule, even some more examples of plasmid-located gene clusters have been found **(**Kinashi et al., 1987).

Cloning of the first genes in *S. coelicolor* and *S. lividans* marked the beginning of intensive studies on the genetics and biochemistry of antibiotic biosynthesis in these bacteria (Thompson et al., 1980). In 1983, a complete biosynthetic cluster of antibiotic actinorhodin has been cloned for the first time (Malpartida and Hopwood, 1984) – an extremely important achievement that had a long-term impact on genetics of *Streptomyces* bacteria.

1.2.2. Antibiotic biosynthesis gene clusters from Streptomyces

The powerful tools of molecular biology made it possible not only to clone antibiotic biosynthesis gene clusters, but also to determine the functions of genes involved in the process. Methylenomycin gene cluster, located on SCP1, was shown to contain biosynthetic, regulatory and resistance genes. The first known methylenomycin resistance gene was identified and cloned in *Streptomyces*. It was a part of gene locus that caused the production of methylenomycin when introduced into non-producing strain, and overproduction of antibiotic when inactivated in the original strain, and it was deduced as a regulatory region. This work led to the first demonstration by C. Bruton of what has become a paradigm, the clustering of antibiotic biosynthesis, resistance and pathway-specific regulatory genes (Chater and Bruton, 1985). Further studies revealed the requirement for conjoined actions of enzymes involved in assembly of scaffold (chemical skeleton) of antibiotic molecule, and its modification. Clustering of genes encoded such proteins seems to be logical as it promotes coordinated expression and regulation within a genome of producing organism. As mentioned above, there are several groups of genes, which have been detected, but not all of them necessarily present in every antibiotic biosynthetic cluster.

"Core" of such cluster consists of structural genes encoding enzymes for scaffold biosynthesis, as for example, PKS or NRPS. Genes for modification, as methyltransferases, hydroxylases, acyltransferases, halogenases, and glycosyltransferases are usually localized in the neighborhood of the "core".

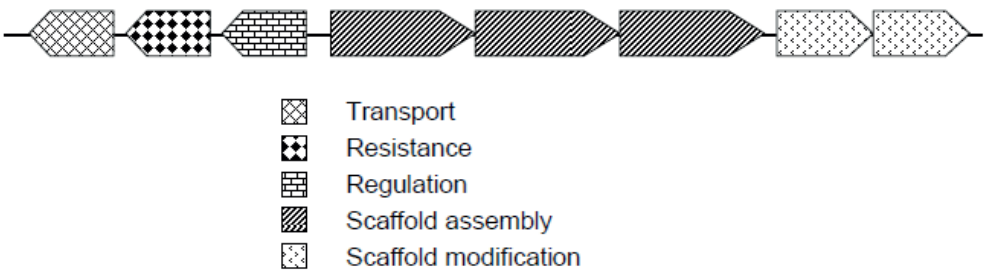

Figure 1.2. Organization of a typical antibiotic biosynthesis gene cluster. (Adapted from Zotchev (2009).

Resistance genes for self-protection against produced antibiotic encode enzymes, which inactivate it or modify the antibiotic target in producing organism. The genes for active transport of the synthesized product are also can be found in the cluster. The enzymes encoded

by these genes are responsible for efflux of antibiotic outside the cell and thus also ensure the protection of organism against it. Pathway-specific regulator/s control the expression of structural, transport and resistance genes and are the part of the antibiotic biosynthesis cluster with some rare exceptions. Typical organization of antibiotic biosynthesis gene cluster is presented on Figure 1.2 (above).

Cloning and analysis of entire biosynthetic clusters is an important step in understanding of antibiotic biosynthesis. The analysis of biosynthetic genes using bioinformatics tools allows determining the whole biosynthetic pathways. Hundreds of antibiotic biosynthesis gene clusters have now been cloned and analyzed. Some important examples are provided in Table 1.2 below.

Table 1.2. Examples of cloned and characterized antibiotic biosynthesis gene clusters.

Gene cluster	Antibiotic class	Application	Producer	Reference
Erythromycin	Macrolide	antibacterial	*Sac. erythraea*	Donadio et al. 1991
Penicillin	Nonribosomal peptide	antibacterial	*Penicillium notatum*	Aharonowitz et al. 1992
Vancomycin	Glycopeptide	antibacterial	*Amycolatopsis orientalis*	Martin et al. 1998
BE-14106	Macrolactam	cytotoxic	*Streptomyces* sp.	Jørgensen et al. 2009
Rapamycin	Macrolide	immunosuppressant	*S. hygroscopicus*	Schweke.et al. 1995
Daunorubicin	Antracycline	cytotoxic	*S. peucitius*	Madduri & Hutchinson, 1995

Avermectin	Macrolide	antihelmintic	*S. avermitilis*	Ikeda et al. 1987
Thiostrepton	Thiopeptide	antibacterial	*S. azureus*	Liao et al. 2009
Validamycin	Aminocyclitol	antifungal	*S. hygroscopicus*	Bai et al. 2006
Novabiocin	Aminocoumarine	antibacterial	*S. spheroides*	Steffensky et al. 2000

In the following section, I will focus on macrolide polyketides synthesized by type I polyketide synthase, since this study was dedicated to nystatin, the representative of this class of antibiotics.

1.2.3 Biosynthesis of type I polyketides

There are three types of polyketide antibiotics: type I, II, and III. They are synthesized by adding small monomer units, such as acetic or malonic acid, to linear oligomer, two atoms at a time, by multifunctional enzymes, polyketide synthases (PKS). PKS are responsible for such characteristics of molecule as chain length, oxidation state, cyclisation, branching, and stereochemistry. Biosynthesis of polyketides occurs by a mechanism reminiscent to fatty acid biosynthesis but with some differences (Hopwood and Sherman, 1990).

Type I polyketide antibiotics are often macrolides differing from each other in the size, modification and composition of lactone ring. The latter is a common characteristic of all macrolide antibiotics. Typical representatives are, for example, erythromycin, rapamycin and nystatin (Figure 1.3). The latter is a representative of polyene macrolides with characteristic large macrolactone ring featuring conjugated double bonds.

The first details on the type I polyketide biosynthesis were revealed for erythromycin (**Ery**), macrolide antibiotic produced by *Saccharopolyspora erythrea,* and this remains the model system for this class of polyketides (Donadio et al., 1991). Ery molecule is composed of the 14-membered macrolactone ring, 6-deoxyerythronolide B (6dEB), with two deoxysugars, L-mycarose and d-desosamine, attached at C-3 and C-5, respectively. The 6dEB core of erythromycin is a result of six successive condensations between a propionyl-CoA starter and

six (2*S*)-methylmalonyl-CoA extenders, which are catalyzed by 6-Deoxyerythronolide B synthase (DEBS) (Marsden et al., 1994).

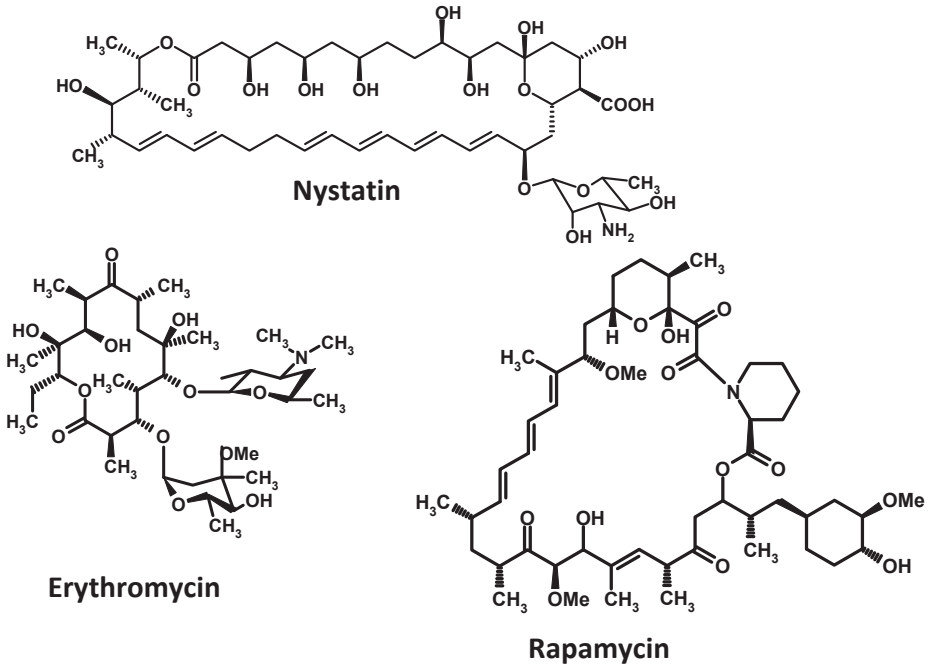

Figure 1.3. Chemical structures of macrolide antibiotics assembled by type I PKS.

Three PKS proteins encoded by *eryA* genes are organized in 6 modules. Each module contains enzymatic functions as separate domains necessary for one of the 6 elongation cycles in the synthesis of the polyketide chain. So, the "modular hypothesis" (Donadio et al., 1991) proposed that: (1) one module is used exclusively for one condensation β-carbonyl processing step in the synthesis of polyketide; (2) the order in which modules appear in DEBS matches the order of use in the biosynthesis of 6dEB. There are three enzymatic activities which are absolutely required for chain elongation. The so-called minimal PKS include:

- an acyl transferase (AT) domain to load the correct extender unit onto the ACP enzyme. Polyketides are composed of a variety of acyl building blocks. At least three AT domains groups according to specificity to extender units can be recognized in macrolide PKS: malonyl- transferring AT, methylmalonyl transferring AT, and AT, recognizing acyl residues derived from monocarboxylic acid. In DEBS, the AT domains of extender modules are

specific for 2-methylmalonyl-CoA. AT domain in the loading module is specific for propionyl-CoA.

- an acyl carrier protein (ACP) is a unit on which the growing carbon chain is tethered;
- kethosynthase (KS) domain is responsible for the Claisen condensation, linking the new extender unit with growing polyketide chain, accompanied by loss of carbon dioxide.

In addition to the "minimal" PKS domains, other domains with different reductive activities may be present.

In DEBS, after each two-carbon unit condensation, the β-carbon can be retained as a ketone (module 3), or modified by ketoreductase (KR) to hydroxyl as in module 2, to double bond by KR + dehydratase (DH) as in module 4, or to saturated bond by the action of KR, DH and enoylreductase (ER), the same module. The growing PKS I carbon chain is assembled and modified according to the order of domains, moving along the protein, interacting with domains in turn, and undergoing reductive changes, corresponding to the set of domains presented in the module. The logic of this model arose from the gene organization of Ery cluster, and the idea that the numerous number of analogs could be produced by alteration of PKS genes, appeared as a logical supposition (Katz, 1997). An overview of 6dEB biosynthesis presented on Figure 1.4 below.

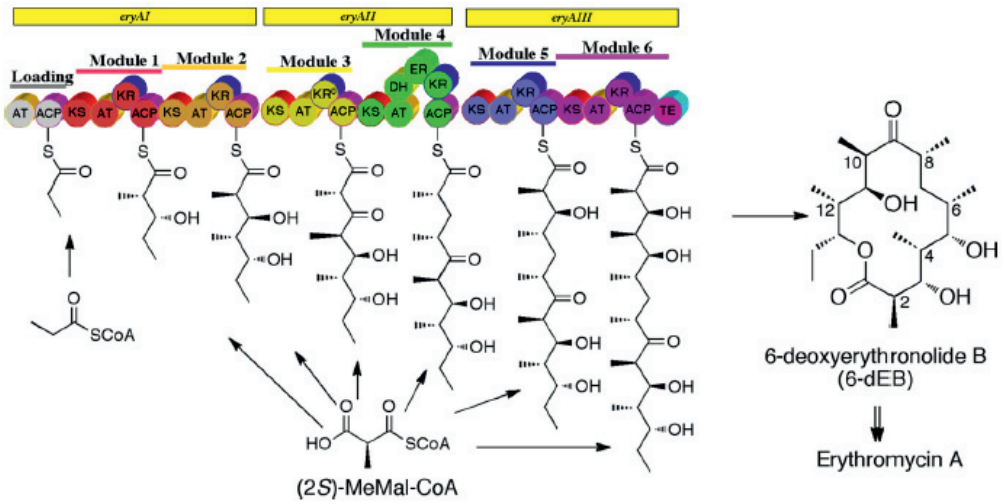

Figure 1.4. Overview of erythromycin biosynthesis – formation of aglycone by type I PKS. Adapted from Cane (2010).

The two final steps in the biosynthesis of polyketide backbone are the chain release and cyclisation into the lactone ring. Thioestherase (TE) domains are found on the C- terminal of PKS. On the end of module 6 in DEBS there is a TE domain, which is defined as type I TE (TE-I). The synthesized full-length polyketide chain covalently links to the TE domain, then released by hydrolysis resulted in Ery lactone macrocycle. It is not determined yet if Ery TE is also responsible for lactone cyclisation or the additional function is needed. Interestingly, some gene clusters for macrolide PKS may contain an additional gene for type II TE, as in nystatin and tylosin gene clusters (Brautaset et al., 2000; Cundliffe et al., 2001). Disruption of TE-II genes from several PKS gene clusters revealed that TE-II plays an important role in keeping the normal levels of antibiotic production. It is also shown that TE-II can perform editing function by removing aberrant intermediates appearing in case of incorrect operation during chain extension (Heathcote et al., 2001).

After the lactone ring is released from the PKS, it is usually enzymatically modified in order to obtain the biologically active molecule. Common in all macrolactone biosynthetic pathways is mono-, di-, or tri-glycosylation. In addition, some other functional groups as methyls, hydroxyls and carbonyls may be added (McDaniel et al., 2005). The first step after 6dEB synthesis is C-6 hydroxylation resulting in production of erythromycin B. This reaction is catalyzed by cytochrome P-450 monooxygenase encoded by *eryF* (Weber et al., 1991).

Many macrolides contain deoxysugar(s) and/or deoxyaminosugar(s), attached to the lactone ring via glycoside linkage, and can play an important role in the bioactivity of a molecule. These deoxysugars are derived from glucose and are from the 6-deoxyhexoses group. The genes encoding enzymes for deoxysugar biosynthesis are located in the PKS clusters. Glycosyltransferases are responsible for the transfer of individual synthesized sugars into the aglycon to form glycoside bond, as for example, TylCV for L-mycarose in tylosin (Merson-Davies and Cundliffe, 1994) and EryCIII for D-desosamine (Summers et al., 1997).

1.2.4 Genetic manipulation of PKS genes

As it was mentioned above, the hypothesis of modular PKS led to the idea that PKS in principle can be manipulated by, for example, introduction of mutations into different domains. Such changes may result in loss of function, alteration of substrate specificity or even in gaining of a new function (Rodriguez and McDaniel, 2001). Theoretically, if we can predict all consequences of such changes, then the number of new PKS of varying length and

composition created by combining of different domains and modules is enormously high. This features of biosynthesis provoked the idea of constructing the libraries of compounds with all possible length and combinations of carbon units. The creation of a combinatorial library of more than 50 different analogs of erythromycin with up to three mutations in one or more domains in antibiotic molecule by Mc Daniel et al. (1999) is a convincing evidence of great potential of combinatorial biosynthesis. Once the library of productive mutants has been prepared, the introduction of additional multiple mutations lead to dramatic increase of the library size, and production of a great diversity of erythromycin analogues.

Later, the work of Menzella et al. (2005) demonstrated the possibility of complete reorganization of PKS and production of functional biosynthetic enzymes. DNA fragments encoding parts of modular PKS from seven different polyketide synthases biosynthetic pathways of streptomycetes and myxobacteria have been synthesized and assembled in 154 bimodular combinations. About half of the bimodular assemblies yielded the triketide lactone product after introduction in *E.coli* strain. The research team from Biotica (Cambridge, UK) constructed a direct biosynthetic pathway for Ivermectin, veterinary antiparasitic drug. Ivermectin is usually produced by costly semi-synthesis (Gaisser et al., 2003). After successful domain exchange in the avermectin PKS it is possible to produce Ivermectin-like compounds by direct fermentation of *S. avermitilis* recombinant strain.

However, polyketide synthase systems are much more complicated then they first appeared. In order to be able to use the advantages of above mentioned technologies in full we have to deal with some potential problems. For example, host cells that would survive after introduction of a new PKS pathway, and have to provide all building blocks and enzymes to synthesize and activate a new compound. Some years ago *Escherichia coli* for production of polyketides was successfully engineered (Pfifer and Khosla, 2001), although high yieds of polyketides have never been achieved in this system.

Substrate preference might be a serious challenge, as altered PKS domains have to interact normally. Once the domain is changed, it must be recognized and correctly processed by following functions. For example, KS domains must interact with any ACP domain and accept any building block in chain extension. Substrates for TE domains are also determined by the action of previous modules/domains. The details about so-called linkers, sequences in size from 20 to over 250 amino acids, which separate domains, as far as details about protein folding in a new PKS, all this information must contribute to the successful creation of the new functional PKS molecules.

1.3 Regulation of antibiotic biosynthesis

Taking in consideration that streptomycetes live in complicated and changing environment, and that the antibiotic biosynthesis is an energy consuming process, it is logical to assume that these organisms might have a comprehensive regulatory network allowing switching on the antibiotic production only in response to certain environmental signals in order not to waste the cells resources unnecessarily. Such factors as nutrient limitation, change of temperature and pH, phage infection, presence of other organisms or organic solvents are known to trigger the antibiotic production (Martin and Liras, 2010). The biosynthesis of secondary metabolites, including antibiotics, by *Streptomyces* is growth phase-dependent, and generally coincides with the development of aerial hyphae in surface-grown cultures. The fact, that one organism has a genetic capacity to produce several secondary metabolites with diverse chemical structure and biological activity means that there might exist a competition for the precursor supply between the biosynthetic pathways, and the decision should be made on which of the pathways should be activated in current circumstances.

Regulatory proteins that control antibiotic production can be divided in several groups according to their role in the above mentioned process. The first includes pleiotropic regulators that influence both secondary metabolism and morphological differentiation (e.g. sporulation). Example is *bldA* gene, which encodes the only tRNA translating rare leucin codon UUA in *Streptomyces* (Lawlor et al., 1987).

The other group consists of pleiotropic regulators, which have little or no effect on differentiation, as, for example, *absA* operon from *S. griseus* and *S. coelicolor* encoding a two-component system that repress antibiotic production in these organisms (Aceti and Champness, 1998; Price et al., 1999; Ishizuka et al., 1992). Usually, such pleiotropic regulators affect several antibiotic biosynthesis pathways simultaneously.

Most of the gene clusters for biosynthesis of secondary metabolites contain regulatory genes, which directly control structural biosynthetic genes. Such “low level” regulators are usually called pathway-specific, and include, for example, *actII-ORF4* of the actinorhodin biosynthetic cluster (Fernandez-Moreno et al., 1991), *cdaR* of the calcium–dependent antibiotic cluster (Ryding et al., 2002), or *redZ* and *redD* genes from undecylprodigiosin cluster, all in *S. coelicolor* (White and Bibb, 1997).

1.3.1 Pleiotropic regulation

Several genes involved in the global regulation of antibiotic production have been identified in *S.coelicolor*. One such group of genes *is afsK-afsR-afsS*, which globally controls antibiotic synthesis under certain conditions. AfsR is a pleiotropic regulator which controls actinorhodin, undecylprodigiosin and CDA production, and it shares homology with the SARP (Streptomyces Antibiotic Regulatory Proteins) proteins. Although a member of SARP family (details are in the text below), it does not directly control antibiotic biosynthesis genes, but activates AfsS, a regulator, which stimulates transcription of the SARP's *redD* and *actII-ORF4* (Tanaka et al., 2007).

AfsR also contains a central ATPase domain and a tetratricopeptide repeat (TPR) associated with some other *Streptomyces* regulatory proteins and possibly mediating protein-protein interactions (Tanaka et al., 2007; Sekurova et al., 2004). AfsR itself is a subject to phosphorylation by the AfsK, a serine/ threonine kinase, which is located on the inner side of the cell membrane. Two more serine/threonine kinases, AfsL and PkaG, take part in phosphorylation of AfsR. AfsK autophosphorylates in response to unknown signal (Matsumoto et al., 1994), and its activity is modulated by KbpA, an AfsK-binding protein, which level in cells is enhanced after the onset of antibiotic biosynthesis and its role is probably to prevent unlimited antibiotic production (Umeyama and Horinouchi, 2001).

As a transcriptional activator, AfsR-P (phosphorylated form of AfsR) has greatly enhanced DNA-binding activity towards the *afsS* promoter. *afsS* gene of *S.coelicolor (afsR2* in *S.lividans)* is located immediately 3' to *afsR,* and encodes a 63aa "sigma-like" protein, containing certain conserved sequences of the domain 3 of sigma-factors (Kim et al., 2006). Two AfsR monomers bind to the direct repeats at the *afsS* promoter and recruit RNAP (RNA polymerase). The DNA-AfsR-RNAP complex is formed, allowing binding of RNAP to the *afsS* promoter. In the absence of AfsR this binding doesn't occur. Activated AfsS in its turn stimulates transcription of the SARPs *redD* and *actII-ORF4* in *S.coelicolor* in as yet unknown manner (Vogtli et al., 1994; Floriano and Bibb, 1996; Lee et al., 2002). AfsS-like proteins are relatively rare in streptomycetes, and as of today have only been identified in *S. coelicolor, S. lividans, S. griseus*, and *S. noursei.*

Recently, it has been shown by Lian et al. (2008) that AfsS regulates both antibiotic biosynthesis and nutrition starvation response genes in *S. coelicolor.* Microarray analysis was used to analyze the expression profile in the *afsS* disruption mutant. More than 117 genes were perturbed in the mutant strain, and the great majority of them were downregulated, proving

that AfsS is a pleiotropic transcriptional regulator for many genes. In the *afsS* disruption mutant the phosphate starvation mechanism was shown to be affected, leading to complete abolishment of actinorhodin production. In most cases, the effect of AfsS regulation was not visible until mid/late stationary phase. In this respect activity of AfsS resembles sigma factors, the great number of which is involved in stress response and growth phase adaptation in bacteria.

The antibiotic production is a complex process involving several levels of regulation. Earlier works have suggested an elegant model of regulatory cascades involving signaling molecules γ–butyrolactones, which bind to the receptor proteins, usually repressors, and thus allowing the expression of positive regulators. Activators, in their turn, can directly or through pathway-specific regulators, induce the antibiotic production, and also can be involved in morphological differentiation.

A-factor in *Stretomyces griseus* is so far the most characterized γ–butyrolactone (Horinouchi, 2002). A-factor acts to induce the transcription of a pleiotropic regulator AdpA, which controls antibiotic production (streptomycin and grixazone), and formation of aerial mycelium in *S. griseus* (Ohnishi et al., 2005). When the concentration of A-factor, synthesized in a growth-dependent manner, reaches a certain level, it binds to the protein ArpA, repressor of transcription of *adpA,* the key transcriptional factor for the *adpA* regulon. ArpA dissociates from the *adpA* promoter, and AdpA activates, among others, *strR,* pathway-specific regulator for all streptomycin biosynthesis genes, causing the onset of streptomycin biosynthesis.

A-factor homologs, with a γ-butyrolactone-like structure, have been identified in many streptomycetes, as, for example, in *S. coelicolor* A(3)2, *S. bikiniensis*, *S. cyaneofuscatus, S. lavendulae*, *S. viridochromogenes* (Takano et al., 2005; Nishida et al., 2007). These strains contain genes homologous to *afsA* and *arpA*, encoding an A-factor biosynthesis enzyme and A-factor receptor, respectively. The latter suggests that these molecules play an important role as "bacterial hormones" that switch on certain genes for morphological differentiation and antibiotic biosynthesis in streptomycetes, and that the mechanism for regulation by these γ-butyrolactones are similar to the A-factor regulation, where *afsA* and *arpA* are involved.

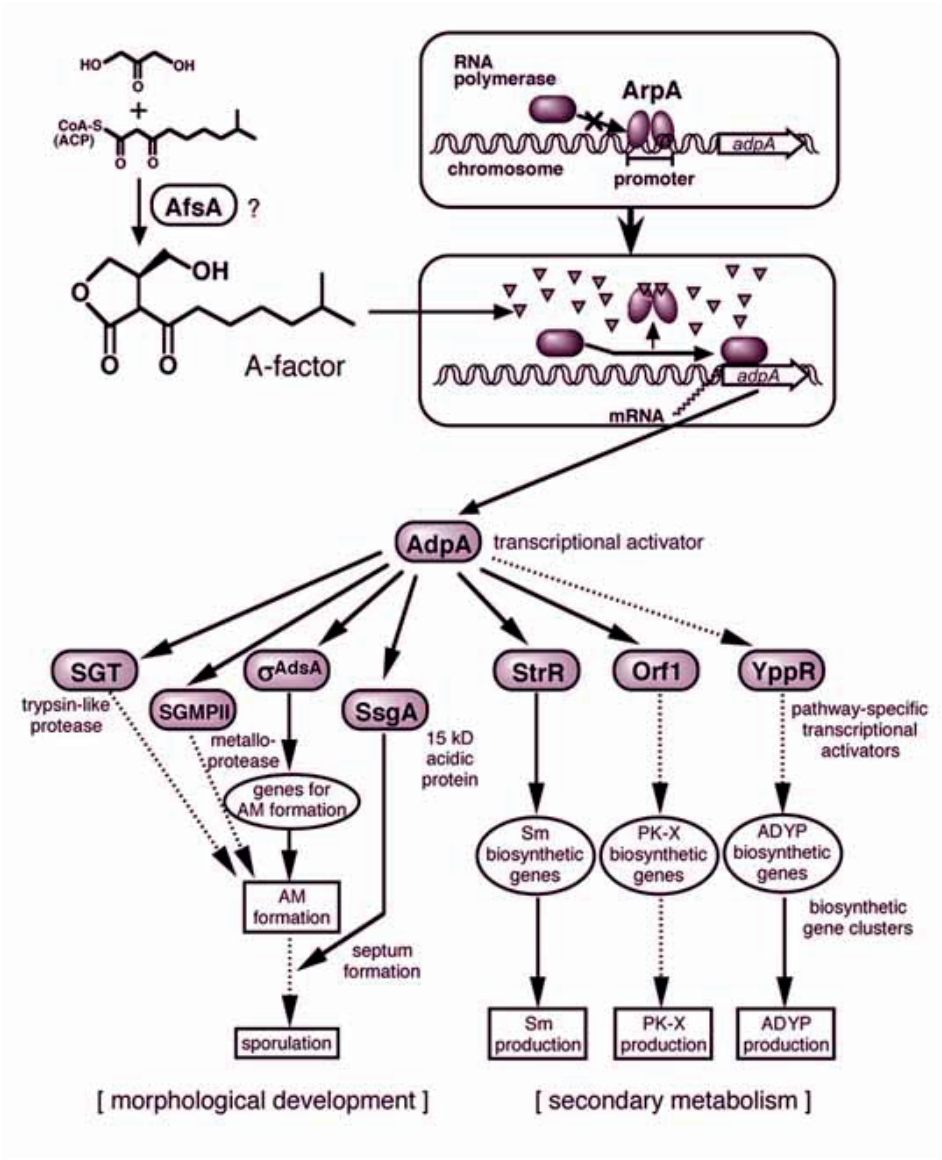

Figure 1.5. A-factor regulatory cascase in *S. griseus.* Adapted from Horinouchi (2002).

1.3.2 Pathway –specific regulation

Regulatory proteins encoded by the genes located within the antibiotic biosynthesis gene clusters in streptomycetes are referred to as pathway-specific regulators or so-called cluster-specific regulators (CSR). With very few exceptions, these regulators cotroll exclusively

expression of genes in their own cluster. This group of regulators is not uniform, but the most studied ones belong to the *Streptomyces* antibiotic regulatory proteins (SARP), which are transcriptional regulators. Representatives of this protein family have a characteristic N-terminal OmpR-like helix-turn-helix DNA-binding motif, and accompanying bacterial transcription activation domain (BTAD). At least some of the SARPs were shown to recognize and bind heptameric direct repeats in the promoter regions of genes they regulate (Wietzorrek and Bibb, 1997; Tanaka.et al., 2007).

SARP regulators often control transcription of some or all genes for antibiotic biosynthesis in the cluster. The operon organization of genes allows SARP to regulate multiple genes through just few promoters. *actII-ORF4* and *redD* from *S.coelicolor* are represnetatives of the SARP regulators family, controlling actinorhodin and undecylprodigiosin production, respectively. Both of them are located within the clusters they regulate **(**Arias et al., 1999; Takano et al., 1992), and are essential for antibiotic biosynthesis. Microarray analysis revealed that many genes for undecylprodigiosin production are activated by RedD (Huang et al., 2005). As in case of *actII-ORF4*, transcription of *redD* is also growth-phase dependent, and maximum level of transcript must be present to activate undecylprodigiosin biosynthesis genes (Gramajo et al., 1993). In both cases the overexpression of SARP regulators leads to increased levels of antibiotic production. Expression of *redD* itself is activated by another pathway-specific regulator, also located in the cluster, the response regulator-like protein RedZ (White and Bibb, 1997).

SARP family regulators have been found in gene clusters for biosynthesis of aromatic polyketides, PKS I gene clusters, ribosomally and non-ribosomally synthesized peptides, and some other classes of antibiotics. SARP proteins are specific for actinomycetes, and most of them have been found in *Streptomyces* (reviewed in Bibb, 2005).

In contrast to the SARP family regulators common among various classes of antibiotic biosynthesis gene clusters, the representatives of LAL (Large ATP- binding regulators of the LuxR type) family of transcriptional regulators have been found almost exclusively in the PKS I gene clusters, and some of these clusters contain multiple LAL regulators (Brautaset T et al., 2000; Campelo and Gil, 2002; Rascher et al., 2003). Proteins of the LAL family are large (872 to 1,159 amino acids) ATP-binding transcriptional regulators characterized by C-terminal helix-turn-helix DNA-binding motif of LuxR type (De Schrijver and De Mot, 1999). They also contain NTP (nucleotide triphosphate) binding domain on the N- terminus represented by Walker A and B motifs. When deletion was introduced into the NTP-binding

domain in PikD, positive regulator of pikromycin biosynthesis from *S.venezuelae*, the pikromycin production was abolished, suggesting that binding ATP is essential for its activity as a regulator (Wilson et al., 2001). However, the exact mode of action of these regulators is not yet understood.

1.3.3 Tylosin biosynthesis in S .fradiae: an example of regulation of macrolide antibiotic biosynthesis gene cluster

Tylosin is an antibacterial macrolide antibiotic produced by *S. fradiae.* The tylosin biosynthetic gene cluster contains multiple regulatory genes, *tylP, tylQ, tylS and tylU,* organized in the *"*regulatory island" downstream the PKS genes, and one more regulator, *tylR*, which is located on the opposite side of PKS genes block (Bate et al., 1999). Tylosin biosynthesis was shown to be controlled in a cascade-like manner by these five regulators. *tylP* and *tylQ* encode homologues of γ-butyrolactone- binding proteins, *tylS* product belongs to SARP family, *tylR* is an ortholog of *acyB2,* the novel transcriptional activator from *S. thermotolerans* (Arisawa et al., 1995). On top of the regulatory cascade is the TylP repressor, which in the absence of yet unidentified γ-butyrolactone represses its own synthesis, thus allowing synthesis of another repressor, TylQ, which gene it is otherwise targeting. TylQ inhibits expression of *tylR,* the positive regulator of tylosin biosynthetic genes.

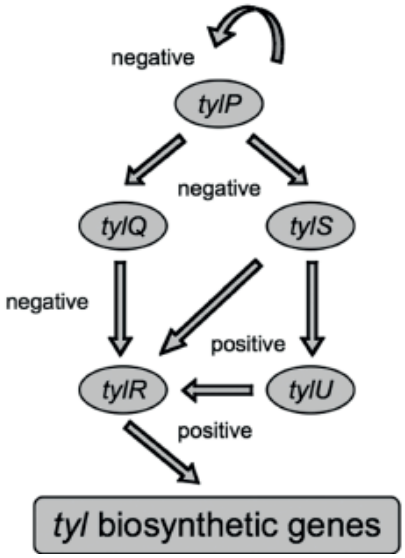

Figure 1.6. Regulatory cascade controlling biosynthesis of tylosin in *S. fradiae*. Adapted from Cundliffe (2008).

TylP also negatively controls expression of *tylS*. Authors suggested that, when γ-butyrolactone concentration reaches certain level, TylP represses expression of TylQ, and expression of *tylR* is activated via interplay of two activators TylS and TylU. So, the derepression of *tylP* due to the *tylP*-specific γ-butyrolactone results in the onset of tylosin production.

Interestingly, there was at first no special interest in the *tylU* regulatory role until RT-PCR experiments, where this gene was shown to be silent together with *tylR* in the *tylS*-deficient mutant (Stratigopoulous et al., 2004). When *tylU* gene was inactivated, the production of tylosin in the mutant strain was reduced by 80%. It was restored to the WT level by overexpression of *tylR*. Based on these data, *tylU* was defined as the first "SARP-helper" (Bate et al., 2006).

1.3.4 Cross-talk between antibiotic biosynthetic pathways

As described above, the earlier works have suggested a model of regulation, in which pleiotropic "higher level" regulators control individual genes from antibiotic biosynthesis pathways through cluster-specific "lower level" regulators. However, the question of how the coordination between different pathways in an organism producing many secondary metabolites is achieved still remains open. New investigations based on microarray data and availability of genome sequences of several streptomycetes, and particularly of *S.coelicolor*, made it possible to reveal some interesting details about cross-talk between regulators of different antibiotic biosynthesis gene clusters.

The *absB* gene from *S.coelicolor* encoding an RNase III homologue is a pleiotropic regulator, which controls expression of *act-ORF4* and *redD* pathway-specific regulatory genes in Act and Red clusters respectively (Aceti and Champness, 1998). Huang et al. (2005) studied the effects of cluster-specific regulators (CSRs) on biosynthetic pathways in the wild-type (WT) strain, and a mutant deficient in the AbsB regulation. For this purpose, *cdaR* (CSR for calcium- dependent antibiotic), *actII-ORF4*, *redD* and *redZ* pathway-specific regulatory genes were individually expressed in the WT and *absB*-deficient mutant strains. The results obtained from these experiments revealed that CSRs can communicate with genes in different antibiotic biosynthesis gene clusters. It has been shown that *redZ* activated expression of *red, act* and *cda* genes in both the WT and mutant strains. Deletion of *redZ* abolished expression of *red* genes, but also expression of genes for two other antibiotics was delayed. Remarkably, this study revealed that CSRs from one pathway can regulate expression of CSRs in the other

cluster(s), and also are able to influence the global regulation. Microarray analysis revealed the increased abundance of mRNA for *afsR2* (*afsS*), the pleiotropic regulator, in the strain overexpressing *redZ.* Interestingly, *redZ* exhibits the growth phase-dependent control over *afsR2*, as the regulatory effects of *afsR2* were not observed until 60 h of growth. Authors suggested that in addition to AfsR2-mediated control of antibiotic production, other cellular factors might be needed for initiation of the CSRs expression. These results demonstrate the example of a cross-talk between clusters for different secondary metabolites and reveal that the role of CSRs is much more complicated than in common model for the cascade-like regulation of secondary metabolites biosynthesis.

2. AIMS OF THE STUDY

Fungal infections represent a serious medical problem for patients with weakened immune system. The number of antifungal compounds used in medical treatment of such infections is limited. Polyene macrolides are efficient antifungal agents that have been used for over 50 years to treat fungal infections. However, their use is limited due to serious side effects, such as nephrotoxicity. Development of a new polyene macrolide with retained antifungal activity and reduced toxicity would provide a substantially improved antifungal antibiotic. Although chemical derivatization of polyene macrolides has been extensively tried out for many years, no improved polyene macrolide appeared on the market. Genetic engineering of antibiotic biosynthesis genes could provide an alternative to medicinal chemistry in changing the molecular structure of polyene macrolides and, hopefully, improving their pharmacological properties. To use this approach, however, biosynthetic genes for polyene antibiotic must be cloned and studied.

The aim of this study was to clone, analyze and manipulate genes involved in the biosynthesis of polyene antifungal antibiotic nystatin produced by a bacterium *Streptomyces noursei*. Prior to this study, nothing was known about the genetics and biochemistry of nystatin biosynthesis. Besides cloning of the biosynthetic genes, the aim was also to study the regulation of the biosynthesis, both by pleiotropic and pathway-specific regulators. Understanding of the regulation could prove useful in improving the yield of antibiotic, thus having direct relevance to its industrial production. Another important aim of this research was to produce nystatin analogs by manipulation of its biosynthetic genes. The idea was to use knowledge on the structure-activity relationship of polyene macrolides and on the biosynthesis of nystatin in order to rationally engineer novel analogs with improved properties.

3. LIST OF PAPERS

Papers included in this thesis:

PAPER I: Sekurova O., Sletta H., Ellingsen T., Valla S., Zotchev S. B. (1999). Molecular cloning and analysis of a pleiotropic regulatory gene locus from the nystatin producer *Streptomyces noursei* ATCC11455. *FEMS Microbiol. Lett.* **177**: 297-304.

PAPER II: Brautaset T., Sekurova O.N., Sletta H., Ellingsen T.E., Strøm A.R., Valla S., Zotchev S.B. (2000). Biosynthesis of the polyene antibiotic nystatin in *Streptomyces noursei* ATCC 11455: analysis of the gene cluster and deduction of the biosynthetic pathway. *Chem Biol* **7:** 395-403.

PAPER III: Sekurova, O.N., Brautaset, T., Sletta, H. Borgos, S.E.F., Jakobsen, Ø.M., Ellingsen, T.E., Strøm, A.R., Valla, S., Zotchev, S.B. (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.

PAPER IV: Brautaset, T., Sletta, H., Nedal, A., Borgos, S.E.F., Degnes, K.F., Bakke, I., Volokhan, O., Sekurova, O.N., Treshalin, I.D., Mirchink, E.P., Dikiy, A., Ellingsen, T.E., Zotchev, S.B. (2008). Improved anti-fungal polyene macrolides via engineering of the nystatin biosynthetic genes in *Streptomyces noursei*. *Chem Biol* **15**:1198-1206.

Papers/published materials not included in this thesis:

Zotchev S.B., Haugan K., Sekurova O.N, Sletta H., Ellingsen T.E. & Valla S. (2000). Identification of a gene cluster for antibacterial polyketide-derived antibiotic biosynthesis in the nystatin producer *Streptomyces noursei* ATCC 11455. *Microbiology* **146**: 611-619.

Zotchev, S.B., Brautaset, T., Sekurova, O.N., Sletta, H., Fjærvik, E., Ellingsen, T.E., Strøm, A.R., Valla, S., Gulliksen, O.-M. (2001). Novel genes encoding a nystatin polyketide synthase

and their manipulation and utility. Patent application WO0159126 (granted in USA and Australia).

Sletta, H., Borgos, S.E.F., Bruheim, P., Sekurova, O.N., Grasdalen, H., Ellingsen, T.E., Zotchev, S.B. (2005). Nystatin biosynthesis and transport: the *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**: 4776-4583.

Volokhan, O., Sletta, H., Sekurova, O.N., Ellingsen, T.E., Zotchev, S.B. (2005). An unexpected role for the putative 4'-phosphopantetheinyl transferase-encoding gene *nysF* in the regulation of nystatin biosynthesis in *Streptomyces noursei* ATCC 11455. *FEMS Microbiol Lett* **249**: 57-64.

Nedal, A. Sletta, H., Brautaset, T., Borgos, S.E.F., Sekurova, O.N., Ellingsen, T.E., Zotchev, S.B. (2007). Analysis of the mycosamine biosynthesis and attachment genes in the nystatin biosynthetic gene cluster of *Streptomyces noursei* ATCC 11455. *Appl Environ Microbiol* **73**:7400-7407.

4. SUMMARY OF RESULTS AND DISCUSSION

4.1. Pleiotropic regulatory gene locus from *S. noursei* involved in a regulatory network affecting nystatin biosynthesis (Paper I)

As mentioned in the Introduction, the detailed understanding of the regulatory networks influencing biosynthesis of antibiotics is still far from being complete. This is despite the fact that a huge amount of information have been obtained nowadays from sequencing and analyzing of complete genomes of *Streptomyces*, as well as metabolomes and transcriptomes (Alam et al., 2010; Castro-Melchor et al., 2010).

By the time this study has been initiated, nothing was known about the regulation of antibiotic production in *S. noursei*, but some interesting works on regulatory genes and proteins sensing environmental changes, reacting on it, and thus regulating antibiotic production in other streptomycetes, have been published (see Introduction). For example, *afsR*, when overexpressed, enhances transcription of pathway-specific regulatory genes from Act and Red biosynthetic gene clusters in *S. coelicolor*. Small gene *afsS* situated downstream of *afsR* in both *S. coelicolor* and *S. lividans,* can also stimulate Act and Red production when cloned on a multicopy vector. With the initial aim of enhancing nystatin production through similar approach, we intended to identify possible positive regulators in *S. noursei*.

4.1.1 Regulatory gene locus from S. noursei capable of stimulating enhanced actinorhodin production in S.lividans

Total DNA from *S. noursei* was isolated, partially digested with *Sau*3AI, size-fractionated on sucrose gradient, ligated into a multi-copy *Escherichia coli-Streptomyces* shuttle vector pWHM4 (Vara et al.,1989), and introduced into *S. lividans.* Several transformants producing blue pigment (Act) were isolated, and one of them, designated A8, was chosen for further analysis. Recombinant plasmid pA8, containing a 4.8kb DNA insert, caused the Act overproduction when reintroduced into *S. lividans*. In addition, pA8 had week, but detectable growth inhibition effect on recombinant *S. lividans* strain.

The deletion analysis of the A8 fragment in order to determine precisely the location of regulatory gene, revealed the 1.8kb fragment, A8d8, which was found to be sufficient for eliciting Act overproduction phenotype in *S. lividans*.

The complete sequence of the fragment A8d8 revealed two complete (ORF2 and ORF3) and two incomplete (ORF1 and ORF4) open reading frames within it. An incomplete ORF1 was shown to encode a C-terminal part of peptide homologous to the AfsR protein from *S. coelicolor* A3 (2). Downstream of ORF1, a small ORF2 (164 nt) was detected, which deduced product of 55 aa showed some homology to the C-terminal part of CREA catabolite repressor from *Aspergillus nidulans* (Dowzer and Kelly, 1991). Interestingly, detailed analysis of the putative ORF2 product revealed two repeats homologous to those from the peptide encoded by *afsR2* (*afsS*) from *S.lividans.* Homology search and analysis of the putative ORF3 product (54 aa), showed 59% identity of its 27 N-terminal aa to the putative signal peptide of the secretable metalloproteinase from *S. coelicolor.* Finding of the putative transmembrane helix and the AxA conserved motif typical for streptomycete signal peptides (Gilbert et al., 1996) also supported the suggestion that ORF3 encodes a secretable peptide. Truncated product of the incomplete ORF4 located downstream of ORF3 shows similarity to peptide synthetases or acetyl-CoA synthetases from different organisms.

4.1.2 Individual roles for ORF2 and ORF3 in regulation of Act production in S. lividans.

It was logical to suggest that ORF2 (designated *ssmA*) or ORF3 (*ssmB*), or both of them, take part in the regulation of Act production in *S. lividans*, since ORF1 and ORF4 were incomplete. Recombinant *S. lividans* strains carrying A8d8, A8DO2 (*ssmA* deletion) and A8DO3 (*ssmB* deletion) fragments on either multi-copy vector pWHM4 or pSET152 (introduces one additional copy of gene per genome via site-specific integration) were constructed to check individual roles of *ssmA* and *ssmB* in the regulation of Act biosynthesis. In addition, a recombinant strain was made by introduction of pSET152 carrying additional copy of *ssmA* under control of the strong constitutive promoter P_{ermE*} from *Saccharopolyspora erythrea* (fragment PA8DO3). Recombinant *S. lividans* strains were tested for Act production in liquid media.

According to the results of the experiments with strains containing abovementioned fragments on the multi-copy vector, *ssmA* was required for Act overproduction, as its deletion leads to abrogation of this phenotype. Deletion of *ssmB* from the *ssmA*-containing multicopy plasmid resulted in enhancement of Act production, while multiple copies of the *ssmB* suppressed the enhancing effect of *ssmA*. It seems therefore that *ssmB* is involved in the negative regulation of antibiotic production.

As for *S. lividans* strains containing the same fragments cloned into pSET152, the results confirmed that *ssmA* is responsible for overproduction of Act as even its single copy conferred this phenotype. No Act overproduction was detected in *ssmA* deletion mutant (construct pSET-A8dO2). Strain containing pSET-PA8dO3 produced double amount of Act compared to pSET-A8d8, as a result of P_{ermE*} -driven overexpression of *ssmA*. The fact that overexpression of *ssmB* in a construct where one additional copy of it was introduced in *S. lividans* (pSET-PA8dO3) had a negative effect on Act production further confirms the negative regulatory role of *ssmB* in Act production in *S. lividans.*

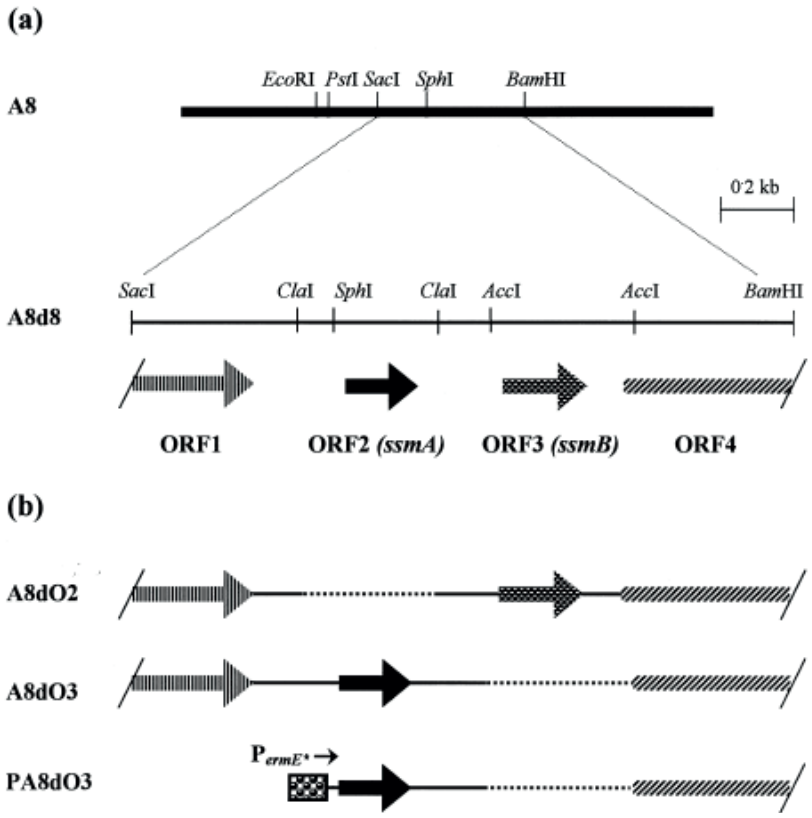

Figure 4.1. (a) A8 DNA fragment from *S.noursei*, and its derivative A8d8, carrying genes *ssmA* and *ssmB* essential for activation of actinorhodin production in *S.lividans.* (b) Schematic representation of the A8d8 DNA fragment deletion derivatives. Deletion regions are indicated with dotted line (adapted from Sekurova et al., (1999).

4.1.3. SsmA might be a part of a system sensing the carbon source in S. noursei.

In order to investigate the involvement of *ssmA* in regulation of nystatin production in *S. noursei,* and possible effect of *ssmA* overexpression on this process, the *ssmA* deletion mutant DO221 and strain where *ssmA* was placed under control of P_{ermE*} , PA8DO32, have been generated. As demonstrated by Shroff et al. (1996), CREA protein is a catabolite repressor for the ethanol regulon in *Aspergillus nidulans*, and it negatively affects the ethanol utilization genes on a glucose-containing media. The fact of homology between SsmA and CREA protein let us speculate that the function of SsmA might be also dependent on the carbon source. Experiments on nystatin production by the *S. noursei* WT, DO221, PA8DO32, and WT (pSET152) strains in semi-defined media with two different carbon sources were performed.

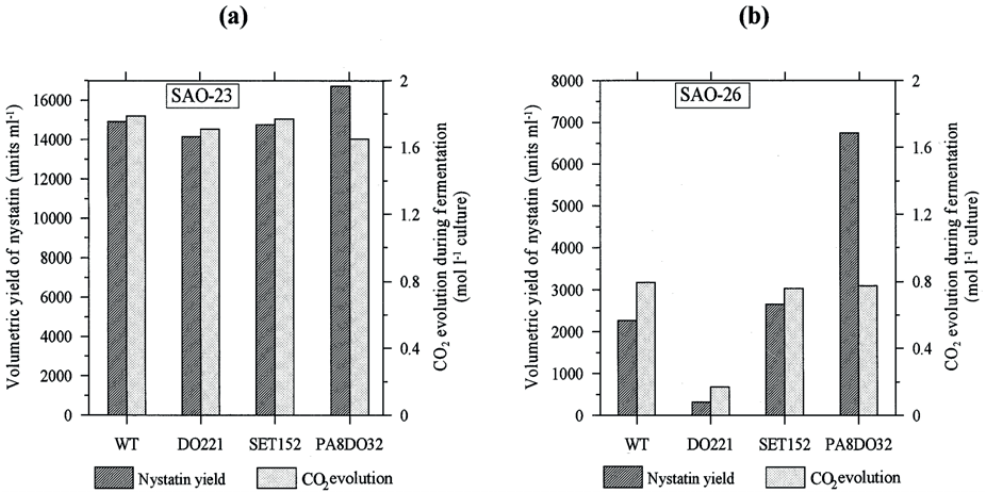

Figure 4.2. Analysis of the nystatin production and respiration rate by the *S. noursei* WT, WT (pSET152), DO221, and PA8DO32 strains in SAO-23 (a) and SAO-26 (b) media in 3 l fermentors. Average values from two independent experiments are presented (adapted from Sekurova et al., (1999)

No significant differences were observed on glucose-containing medium either for *ssmA* deletion or for its overexpression mutants when compared to WT (Fig. 4.2a). However, on starch-containing medium, nystatin volumetric yield by the deletion mutant DO221 was 86 % lower compared to the WT. As for the overexpression mutant PA8DO32, we observed very significant (250 %) increase in both volumetric and specific nystatin production. There were

no differences in growth characteristics between this strain and the one containing empty pSET152 vector, strongly suggesting that the increase of nystatin production occurred due to more efficient expression of *ssmA*.

Interestingly, the fermentations on glucose-containing medium were run for 142 h until all the glucose was consumed, and in starch-containing media – until 288 h, because of slow starch consumption. *ssmA* deletion mutant also showed slow starch consumption, and very low respiration rate (app. 4, 5 times lower) compared to other strains, which indicates an impairment in assimilation of starch as a carbon source by this mutant.

The obtained results suggest that SsmA might be a part of a system sensing the carbon source, and transmitting the signal to the pathway-specific regulators of the nystatin biosynthetic gene cluster in *S. noursei***.** As mentioned in the Introduction, in a microarray study of the disruption mutant of *afsS,* the *ssmA* homologue in *S. coelicolor*, it was shown that AfsS is a pleiotropic regulator, affecting both nutrition starvation response genes and antibiotic biosynthesis. This fact, together with our observations further supports the idea about the important pleiotropic regulatory role of SsmA in nystatin production.

4.2. The genetics and biochemistry of nystatin biosynthesis in *Streptomyces noursei* (Paper II)

4.2.1 Cloning of the nystatin biosynthetic gene cluster

The *S. noursei* gene library constructed previously (Zotchev et al., 2000) was screened with a labeled DNA fragment encoding type I PKS to identify and isolate nystatin biosynthetic genes. Probe was produced via PCR reaction using oligonucleotide primers corresponding to conserved amino acid sequences in ACP and KS domains in known type I PKSs. PCR product was cloned and sequenced in order to confirm that it encodes a part of a PKS type I enzyme. To prove the involvement of the cloned gene fragment in nystatin biosynthesis, it was used in the construction of a suicide conjugative plasmid, and the latter was introduced into *S. noursei.* The plasmid was integrated into the genome via homologous recombination, and the resulting disruption mutant obtained was unable to produce nystatin, thus proving the requirement of the identified gene for nystatin biosynthesis. Later, this fragment was shown to be a part of the *nysC* PKS gene. The “chromosome walking” technique allowed to isolate a complete nystatin

biosynthetic cluster encompassing ca. 125 kb and containing 20 genes (Figure 4.3, and Table 4.1). The sequencing and mutational analysis of the cluster was subsequently performed.

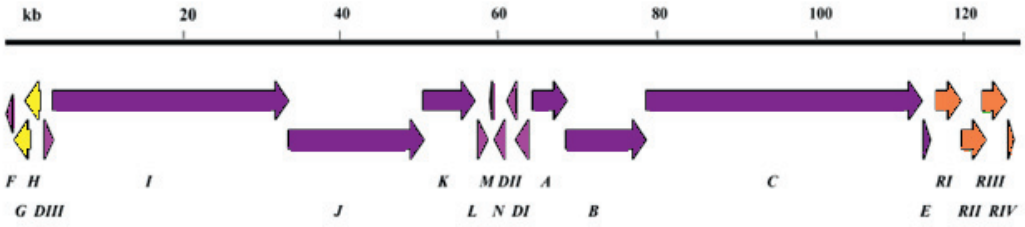

Figure 4.3. Organization of the nystatin biosynthetic gene cluster in *S. noursei* ATCC 11455. PKS genes are in violet, genes for post-PKS modifications are in pink, transporters marked in yellow and genes for regulation are in orange.

4.2.2 Putative genes and their functions in the nystatin biosynthetic gene cluster

Using the BLAST search and amino acid sequence alignments, six genes encoding PKS type I, *nysA*, *nysB*, *nysC*, *nysJ*, *nysI* and *nysK*, were identified. All proteins were shown to have a significant homology to rapamycin and rifamycin PKSs and predictions of their functions were based on comparisons to the known polypeptides.

The NysA protein apparently represented a loading module for the nystatin PKS, involved in the initiation of the nystatin aglycone biosynthesis. AT domain of NysA is acetate-specific, according to the comparisons with ATs from different PKSs with known substrate specificity, and this correlates well with the nystatin molecular structure. In many PKS I loading module utilizes acetyl CoA or malonyl CoA (subsequently decarboxylated) to initiate the antibiotic biosynthesis. It has been shown that KS, if present in the loading module, is essential for decarboxylation, and contains glutamine residue instead of cysteine in the active site (Bisang et al., 1999). Interestingly, KS domain in NysA contains active-site serine residue instead of conserved cysteine or typical loading module-type glutamine. Later, NysA was studied more closely (Brautaset at al., 2003) in our group. Substitution of serine with either cysteine or glutamine had no effect on the nystatin production. Further experiments with hybrid loading modules suggested that NysA can utilize both acetyl CoA and malonyl CoA (after decarboxylation) to start the nystatin synthesis. In addition, NysA contains ACP module and a

seemingly intact DH domain. The latter seems not to have any function in nystatin biosynthesis, and might be an evolutionary remnant.

Table 4.1. Genes in the nystatin biosynthetic gene cluster of *S. noursei* and their putative functions.

nysF	Putative phosphopantetheine transferase
nysG	ABC transporter
nysH	ABC transporter
nysDIII	GDP-mannose dehydratase
nysI	Nystatin PKS, modules 9-14
nysJ	Nystatin PKS, modules 15-17
nysK	Nystatin PKS, module 18+ thioesterase I
nysL	P 450 monooxygenase
nysM	Ferredoxin
nysN	P 450 monooxygenase
nysDII	Aminotransferase
nysDI	Glycosil transferase
nysA	Nystatin PKS loading module
nysB	Nystatin PKS modules 1-2
nysC	Nystatin PKS modules 3-8
nysE	Thioesterase II
nysRI	Transcriptional regulator
nysRII	Transcriptional regulator
nysRIII	Transcriptional regulator
nysRIV	Transcriptional regulator

NysB represents modules 1and 2, and is involved in two first elongation steps in biosynthesis of the nystatin aglycone. DHs in both modules are inactive, as they lack the characteristic active-site sequence.

NysC is a gigantic, one of the largest known bacterial polypeptides, representing 6 extension modules, and responsible for elongation steps 3 to 8 in the aglycone biosynthesis.

Interestingly, both rapamycin and amphotericin gene clusters from *S. hygroscopicus* and *S. nodosus* respectively, encode RAPS2 and AmphC PKSs very similar to the NysC protein. All three of them are hexamodular large type I PKSs, and the overall number of domains, and their positions in the protein sequences are also similar. Nevertheless, both, RAPS2 (Rascher A. et al. 2003) and AmphC are shorter then NysC. This difference can be explained by more compact structure of domains or interdomain linkers in these proteins. For example, the interdomain linker preceding ER5 in AmphC is 45aa shorter than correspondent sequence in NysC (Caffrey et al., 2001). ER domains are responsible for the appearance of a saturated C-C bond on the antibiotic molecule. Caffrey et al. suggested that shortening of linker can lead to some spatial movements restrictions for ER5 domain, and production of both amphotericin A (tetraene) and B (heptaene) by *S. nodosus*, as a result. Taking into consideration all the similarities between these three proteins, and that they represent PKSs type I for polyene macrolides, it is logical to suggest that all of them might have evolved from a common ancestor.

NysI protein is a hexamodular type I PKS which is responsible for the elongation steps 9-14. The DH domains in modules 10, 11, 12, 13 and 14, and KR domain in module 13 are inactive. NysJ is a trimodular type I PKS protein. DH domains in modules 16 and 17 appear to be inactive. This enzyme is responsible for the elongation steps 15-17 in nystatin aglycone biosynthesis.

Unimodular NysK PKS completes the synthesis of the nystatin polyketide chain. It lacks a KR domain, and its DH domain is inactive. A TE domain is localized at the C- terminus of the protein. The latter suggests that in addition to the last condensation step NysK takes part in the release of the mature polyketide chain, and its cyclization.

In order to confirm the involvement of genes *nysA*, *nysB*, *nysI*, *nysJ* in nystatin biosynthesis, disruption mutants inactivating these genes were obtained. All of them appeared to be nystatin non-producers, suggesting the essential roles of these genes in the biosynthesis of this antibiotic.

nysE gene located immediately downstream *nysC* encodes another thioesterase in the same cluster. NysE shows high degree of similarity to the TEs type II, which function as “editing” enzymes during biosynthesis of polyketides. (Buttler et al., 1994; Kim et al., 2002). We have later performed inactivation of *nysE*, which led to considerable (ca. 65%) decrease in nystatin biosynthesis (Master Thesis, Anne S. Mortensen, 2002), which correlated well with the suggested editing function for NysE.

In order to obtain a biologically active molecule, a PKS-assembled macrolactone ring most often has to be modified. According to the nystatin chemical structure, and organization of the nystatin gene cluster, at least three modifications have to be performed after macrolactone ring synthesis is completed. Several genes for post-PKS modifications have been found in the nystatin cluster. *nysL* and *nysN* genes encode P450 monooxygenases, and have been suggested to be responsible for C10 hydroxylation, and oxidation of a C16 methyl group into a carboxyl, respectively (Brautaset et al., 2000). The putative role of NysL as hydroxylase was confirmed in later study by Volokhan et al. (2006). Inactivation of *nysL* gene in *S. noursei* resulted in a recombinant strain producing 10-deoxynystatin as a main polyene macrolide product. *nysM* encodes a ferredoxin, which presumably is a part of one or both P450 monooxygenase systems, and serves as an electron donor.

The *nysH* and *nysG* genes encode putative ABC-type III transporter proteins, whose role is an active efflux of nystatin out of the cell. Later, nystatin transporters were studied in more details in our group. Sletta et al. (2005) have shown that in-frame deletions of either *nysH* or *nysG* had quantitatively the same negative effect on nystatin production in *S. noursei,* while both mutants overproduced nystatin analogue identified as 10-deoxynystatin. Transporter mutants continued to efficiently efflux both nystatin and 10-deoxynystatin out of the cells, and this fact strongly supports the hypothesis on the existence of alternative transport system(s) for the nystatin-related antibiotics. *nysG* and *nysH* are transcriptionally coupled, and probably act in a concert as a heterodimer. As already mentioned before, accumulation of 10-deoxynystatin was observed also as a result of inactivation of NysL. This observation suggests a possible link between the C-10 hydroxylation and active efflux of nystatin out of the cell. Most probably, NysH-NysG transporter system provides favorable conditions for NysL-catalyzed hydroxylation.

nysDI, *nysDII*, and *nysDIII* are three genes in the nystatin biosynthetic cluster presumably involved in the synthesis and attachment of deoxyaminosugar mycosamine moiety to the nystatin aglycon. The glycosyltratransferase NysDI is presumably responsible for the attachment of the mycosamine moiety to the nystatin aglycon at C-19. NysDII represents an aminotransferase and is involved in mycosamine biosynthesis, while *nysDIII* encodes a protein similar to GDP-mannose-4, 6-dehydratases and probably takes part in one of the initial steps in the biosynthesis of mycosamine. The predicted roles for NysDI and NysDII were confirmed in a later study by Nedal et al. (2007). Genes *nysDI* and *nysDII* were inactivated and expressed in-*trans* in *S. noursei.* Analysis of the resulting mutants supported the predicted

roles for the both enzymes. NysDIII protein was expressed in *Escherichia coli*, purified, and its *in vitro* GDP-mannose dehydratase activity was confirmed. The genes for the other enzymes involved in deoxysugar formation are missing in the cluster, and can be, in principle, recruited from primary metabolism.

nysF gene, situated downstream of *nysG,* represents the 5' border of the nystatin biosynthetic cluster. The product of the gene was identified as a putative 4'-phosphopantheteine transferase. Such enzymes are responsible for the post-translational modification of the ACP domains on the PKSs (attachment of the phosphopantheteine moiety), and are required for their functionality (Kealey et al., 1998).

In order to test the involvement of *nysF* in modification of the nystatin PKS, this gene was inactivated, and we observed an unexpected increase (approx. 60 %) of nystatin production by the mutant strains, suggesting a negative regulatory role of *nysF* in the nystatin biosynthesis (Volokhan et al., 2005). *xylE* reporter gene was expressed in the *nysF* deletion mutant under the control of different promoters from the nystatin cluster. The obtained results showed an enhanced expression of *xylE* from several regulatory and structural genes thus proving that NysF negatively regulates the biosynthesis of nystatin. It has been suggested that regulation of nystatin biosynthesis may be mediated by a signaling molecule synthesized via NysF-dependent pathway involving NysF.

4.2.3 Model for the nystatin biosynthesis in S. noursei

Based on the data described above, the following model of nystatin biosynthesis was suggested (Figure 4.4). Synthesis of the nystatin macrolactone ring begins with the loading and chain initiation by NysA. NysB catalyses the elongation of the chain via adding two propionate extender units to the acetate starter. Elongation is then performed sequentially by NysC (6 modules), NysI (6modules), NysJ (3 modules), and NysK, which adds the last acetate extender unit, and is responsible for the cyclisation of the mature polyketide chain.

According to the chemical structure of nystatin and organization of its PKS, it could be suggested that at least 3 post-PKS modification steps have to be performed to finish the synthesis of completed active nystatin molecule. These modifications include, as mentioned above, oxidation of a C16 methyl group to a carboxyl (NysN), C10 hydroxylation (NysL), and formation of a glycosidic bond between the aglycone and mycosamine (NysDI).

Two transporters, NysH and NysG, acting most likely, as a heterodimer, provide for the active transport of nystatin out of the producing organism.

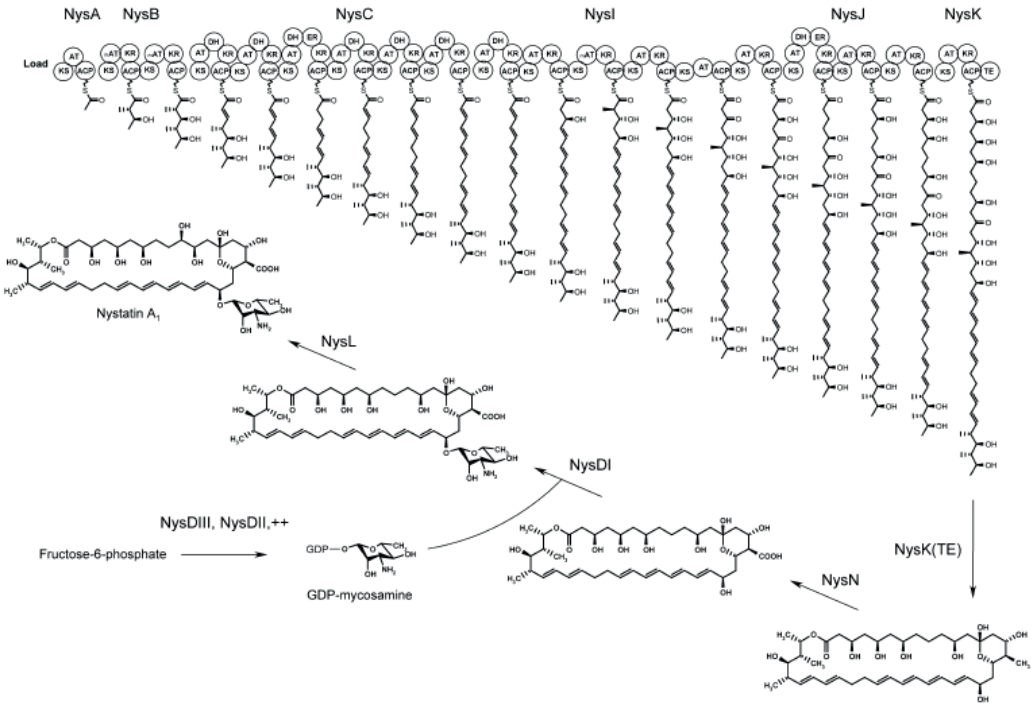

Figure 4.4. Model for nystatin biosynthesis in *S. noursei* ATCC11455 (adapted from Fjærvik and Zotchev (2005).

At least four regulators, NysRI-IV, acting in a cascade manner (see paper III), regulate the process presumably via initiating transcription of the key structural genes for nystatin biosynthesis initiation and transport, and also accomplishing the autoregulation of the regulatory operon.

4.3 Analysis of the regulatory genes in the nystatin biosynthetic gene cluster of *Streptomyces noursei* (Paper III)

4.3.1 Regulatory gene locus in the nystatin biosynthetic cluster of S. noursei

A set of 6 regulatory genes was found on the right flank of nystatin biosynthesis gene cluster. Genes were designated as *nysRI*, *nysRII*, *nysRIII*, *nysRIV*, and *orf2* and *orf3* (Figure 4.5). *nysRI*, *nysRII*, and *nysRIII* are organized in an operon-like structure, and located downstream of the *nysE* gene encoding putative thioesterase II. The *nysRIII* gene's start codon overlaps by 11 nt with 3' end of the *nysRII* gene. The *nysRIV* gene is located 404 nt downstream of *nysRIII,* and appears to be transcribed separately. *orf3* and *orf2* located downstream of *nysRIV,* and *orf2* is transcribed in the direction opposite to that of all the other putative regulatory genes.

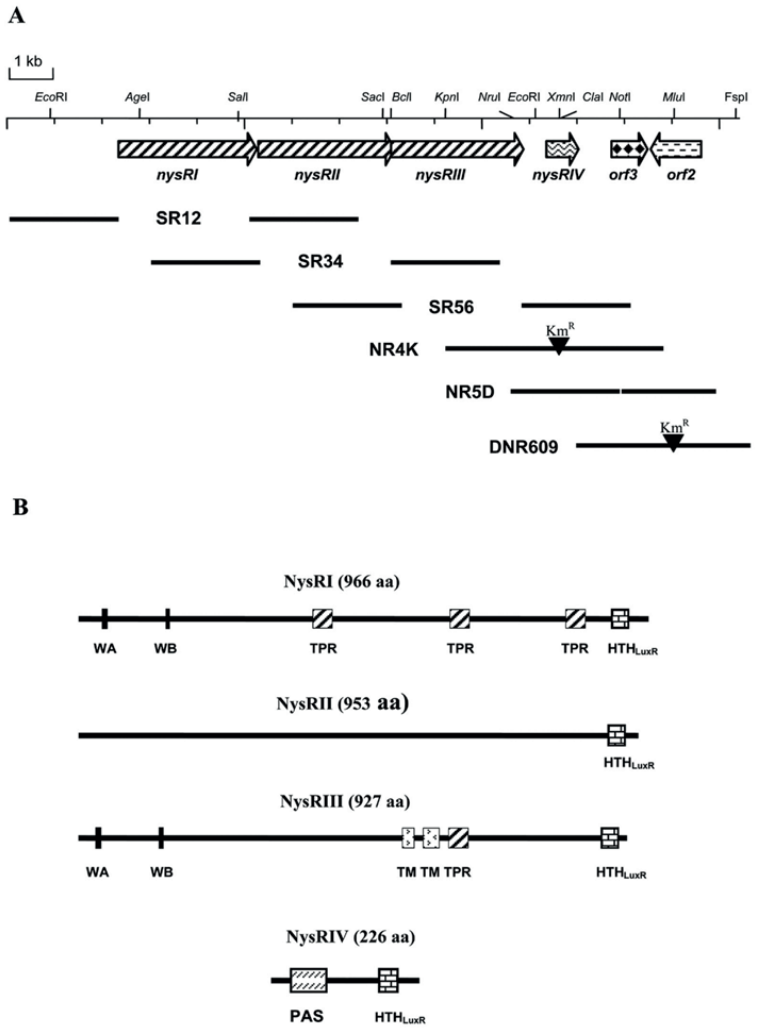

Figure 4.5 (A) Organization of the regulatory gene locus associated with the nystatin biosynthetic gene cluster in *S. noursei* ATCC 11455. (B) Putative functional features

predicted for the NysRI, NysRII, NysRIII, and NysRIV proteins: WA and WB, Walker A and B NTP binding motifs; PAS, PAS-like domain (adapted from Sekurova et al. (2004).

Analysis of the protein sequences encoded by *nysRI*, *nysRII* and *nysRIII* revealed their similarity to each other, and to a number of putative transcriptional activators of the LAL subfamily (De Shriever and De Mot, 1999). All three predicted proteins have C termini – located HTH DNA-binding motifs of the LuxR type and Walker A and B NTP binding motifs at the N termini of NysRI and NysRIII. TPR (tetratricopeptide repeats), which have been implicated into protein-protein interactions (D'Andrea and Regan, 2003) have been also found in both NysRI and NysRIII polypeptides. In addition, two putative transmembrane regions were predicted in the central part of NysRIII. Proteins similar to NysRI-III are found mostly in actinomycetes, and act as transcriptional activators for macrolide antibiotic biosynthesis.

nysRIV gene encodes a 226 aa protein with high degree identity (63%) to the regulatory protein PteR from pentaene macrolide antibiotic gene cluster of *Streptomyces avermitilis* (Omyura et al., 2001). It has an N-terminal PAS domain and C-terminal DNA- binding HTH motif of the LuxR type. NysRI, NysRII, NysRIII, and NysRIV orthologs have been found in amphotericin, candicidin/FR-008 and pimaricin antibiotic biosynthesis gene clusters.

orf3 encodes a 233 aa protein similar to transcriptional repressors of the DeoR family, and the product of *orf2* gene is a 354 aa polypeptide similar to transcriptional regulators of the AsnC type.

4.3.2 Four regulatory genes are essential for efficient nystatin biosynthesis

Mutational analysis of all 6 regulatory genes was performed in order to determine their individual roles in nystatin biosynthesis. Disruption of *nysRI* gene has led to elimination of nystatin production in the mutant strain. Because of operon-like organization of *nysRI, nysRII,* and *nysRIII* genes the polar effect of this mutation on downstream genes has to be taken into consideration.

In-frame deletion mutants were generated, and mutant strains SR12 (Δ*nysRI*), SR34 (Δ*nysRII*), SR56 (Δ*nysRIII*) were analyzed for nystatin production (Table 4.2). Only 0, 5 % of the WT nystatin biosynthesis level was obtained in SR12. This result confirmed our previous conclusion on polar effect of this mutation on downstream genes. Nystatin production in SR34 and SR56 mutants was also very low: 7 and 9% of WT strain, respectively.

Table 4.2. Nystatin production by the recombinant *S. noursei* strains with inactivated or overexpressed regulatory genes.

Strain/Background	Genotype	Complementation	Nystatin production, % of WT
SR12	*ΔnysRI*	-	0.5
SR12	*ΔnysRI*	pNRE2 (*ermE**P::*nysRI*)	59
SR34	*ΔnysRII*	-	7
SR34	*ΔnysRII*	pC3A1 (*ermE**P::*nysRII*)	100
SR56	*ΔnysRIII*	-	9
SR56	*ΔnysRIII*	pNRT3 (*ermE**P::*nysRIII*)	100
NR4K	*nysRIV::*Km^R	-	2
NR4K	*nysRIV::*Km^R	pNR4ES (*ermE**P::*nysRIV-S*)	2.5
NR4K	*nysRIV::*Km^R	pNR4EL (*ermE**P::*nysRIV-L*)	57
ATCC 11455	wild type	pSOK804	100
ATCC 11455	wild type	pNRE2 (*ermE**P::*nysRI*)	104
ATCC 11455	wild type	pC3A1 (*ermE**P::*nysRII*)	121
ATCC 11455	wild type	pNRT3 (*ermE**P::*nysRIII*)	100
ATCC 11455	wild type	pNR4EL (*ermE**P::*nysRIV*)	136

Inactivation mutant for *nysRIV* gene NR4EL was obtained by insertion of the Km^r gene into the coding sequence of the gene. Mutant strain produced only ca. 2% nystatin of the WT level. As for the inactivation *orf2* and *orf3*, no significant effect of these mutations on nystatin biosynthesis was observed. This result suggests that neither *orf2* nor *orf3* genes are directly involved in the regulation of nystatin production under conditions tested.

From the data obtained in these experiments we can conclude that four genes, *nysRI*, *nysRII*, *nysRIII*, and *nysRIV,* are essential for efficient nystatin biosynthesis in *S. noursei,* and probably represent transcriptional activators for the structural genes in the cluster.

4.3.3 Complementation and cross-complementation experiments

pSOK804, an integrative *E. coli-Streptomyces* shuttle vector, which is able to integrate site-specifically into one site in the genome of *S.noursei*, was constructed. Five expression vectors, where regulatory genes were placed into pSOK804 under control of strong *ermE**p promoter, were obtained, used for the complementation of various *nysR* mutants, and introduced into WT *S.noursei.* The results of experiments are shown in Table 4.2.

According to the results obtained, nystatin biosynthesis was partially or fully restored in the *ΔnysRI*, *ΔnysRII*, *ΔnysRIII*, and *ΔnysRIV* mutants, when the corresponding *nysR* genes under control of *ermE**p promoter were introduced into the mutants. In case of introduction of the expression *nysR* vectors into WT *S. noursei*, we observed no effect on nystatin production with *nysRI* and *nysRIII,* 21% increase with *nysRII*, and the strongest effect was observed with *nysRIV*: 36% increase in the nystatin production compared to WT (pSOK804).

It was interesting to understand the hierarchy among the *nysR* genes in terms of their regulation of nystatin biosynthesis. The idea of cross-complementation experiments was to check if regulators, expressed from a constitutive *ermE**p promoter, could substitute for each other in the *nysR* mutants.

Expressed under *ermE**p promoter, *nysRII*, *nysRIII*, and *nysRIV* genes could restore nystatin production in the *ΔnysRI* mutant to approximately same extent (Table 4.3). No cross-complementation was observed between *nysRII* and *nysRIII.* This fact suggests that these two genes could be placed on the same level in hierarchy of regulatory genes. Expression of *nysRIV* restored nystatin production in all regulatory mutants, while none of the other *nysR* genes could complement the *ΔnysRIV* mutant. This indicates that *nysRIV* can be placed on the lowest level in the hierarchy, and probably directly controls the expression of structural genes in the nystatin biosynthetic cluster.

Table 4.3. Restoration of nystatin production in cross-complementation experiments with the regulatory mutants (see text for details).

Mutant	Nystatin production, % WT			
	pNRE2 (*ermE**p::*nysRI*)	pC3A1 (*ermE**p::*nysRII*)	pNRT3 (*ermE**p::*nysRIII*)	pNR4EL (*ermE**p::*nysRIV*)
SR12 (*ΔnysRI*)	60	58	68	62
SR34 (*ΔnysRII*)	5.4	100	5.6	77
SR56 (*ΔnysRIII*)	8.5	11	98	87
NR4K (*nysRIV::Km*^{R})	2.5	2.0	2.5	60

4.3.4 Promoter activity studies

To obtain the deeper insight into regulatory machine of the nystatin biosynthesis, particularly the individual contributions of *nysR* genes into the process, and also their target genes, the promoter activity studies with regulatory mutants were performed.

The seven putative promoter regions for regulatory and structural genes (Figure 4.6) were fused to the promoterless *xylE* reporter gene, and the reporter cassettes were cloned into the pSOK804 vector. The constructs were introduced into wild-type *S.noursei*, as well as into the *nysR* mutants, and XylE catechol dioxygenase activity assay was used to measure the promoters' activities (see Paper III for the results of XylE assays).

In the wild-type background, the XylE expression was registered for all promoter-probe constructs.

The promoter region for the first three PKS genes *nysA, nysB* and *nysC* is relatively strong, as the level of XylE expression from *nysAp* appeared to be approximately 35 times higher than that for the three last PKS genes *nysI*, *nysJ,* and *nysK,* and absolutely dependent on each of the

four regulators. *nysA* encodes the loading module for the nystatin PKS, and has the crucial role in the initiation of nystatin biosynthesis (Paper II). The mutations in all *nysR* genes had the most significant effect on *nysAp* among all other studied promoter regions, suggesting that initiation of nystatin biosynthesis is a primary target for the regulators.

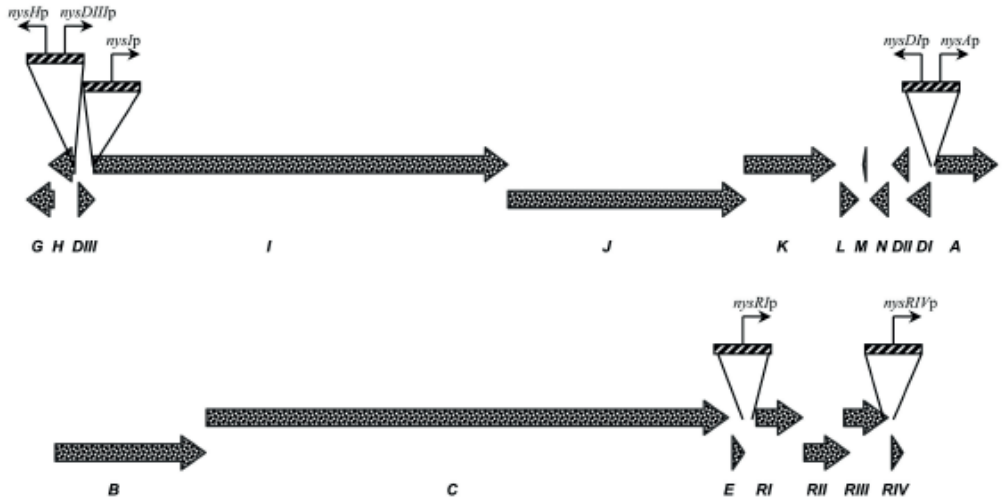

Figure 4.6. Promoter regions in the nystatin gene cluster used in this study (adapted from Sekurova et al.(2004).

As for the *nysIp* promoter region, its slightly reduced activity in all *nysR* mutants indicates that expression of these genes is less dependent on the pathway-specific regulators.

According to our results, promoters driving expression of the *nysDI-nysDII-nysN*, and *nysDIII* genes, taking part in biosynthesis and attachment mycosamine, and C-16 methyl oxidation are only slightly dependent on the *nysR* regulators.

The promoter for the transporter genes *nysH* and presumably co-transcribed *nysG* was dependent on NysRI at a very low degree, while its activity was strongly suppressed in *nysRII*, *nysRIII* and *nysRIV* mutants. This fact suggests that transporter genes are another (after *nysAp*) important target for the three last activators.

The promoter region upstream of the regulatory gene *nysRI*, *nysRIp*, seems to be relatively strong compared to the other studied promoter regions from the cluster, and stronger then *nysAp*. A complete *nysRI-nysRII-nysRIII* operon is presumably transcribed from the *nysRIp*

promoter, and is autoregulated by NysRI and NysRII, as it is strongly dependent on both of these regulators. It also has some low dependence on the NysRIV and NysRIII regulators.

As mentioned above, *nysRIV* has its own separate promoter *nysRIVp,* which provided the highest level of *XylE* expression achieved with *nys* promoters in this study, and is regulated by all three other activators NysRI, NysRII, and NysRIII. Interestingly, NysRIV seems to moderately autoregulate its own promoter.

The efficiency of the *ermE**p in *S.noursei* was also estimated in the control experiment. For this purpose, XylE activity was measured in crude cell extracts from WT strains where *xylE* was expressed from *nysAp,* and *ermE**p promoters. It appeared, that *ermE**p is stronger (ca.12 times) than *nysAp,* and any other promoters in nystatin cluster.

According to the results obtained from the mutational analysis of the regulators, complementation and cross-complementation experiments, and XylE assay, we can conclude that NysRIV plays the central role in the control of nystatin biosynthesis. In *nysRIV* inactivation mutant the level of nystatin production dropped dramatically, while in overexpression mutant we observed the most significant increase (36%) of nystatin production level in the wild-type strain. *nysRIV* can complement all *nysR* mutants, while none of the other 3 regulators can complement *nysRIV*. From promoter probe experiment it is evident that *nysRIVp* is downregulated in all *nysR* mutants, and is the strongest promoter among all studied promoters from the cluster. The fact that even in the *nysR* mutants its activity is not lower than that of *nysIp* and *nysDIIIp*, suggests that high level of *nysRIV* transcription is necessary to achieve its regulatory function in the nystatin biosynthesis.

4.3.5 The model of the regulatory cascade for the nystatin biosynthesis in S. noursei

As a result of our study, the following model of the regulatory cascade for nystatin biosynthesis in *S. noursei* can be suggested (Figure 4.7).

NysRI presumably responds to some external signal and activates the transcription of the LAL regulatory operon. It seems that NysRII is also required for the efficient transcription from *nysRIp,* and it is logical to suggest that NysRI and NysRII function in concert to autoregulate the operon *nysRI-nyRII-nysRIII.*

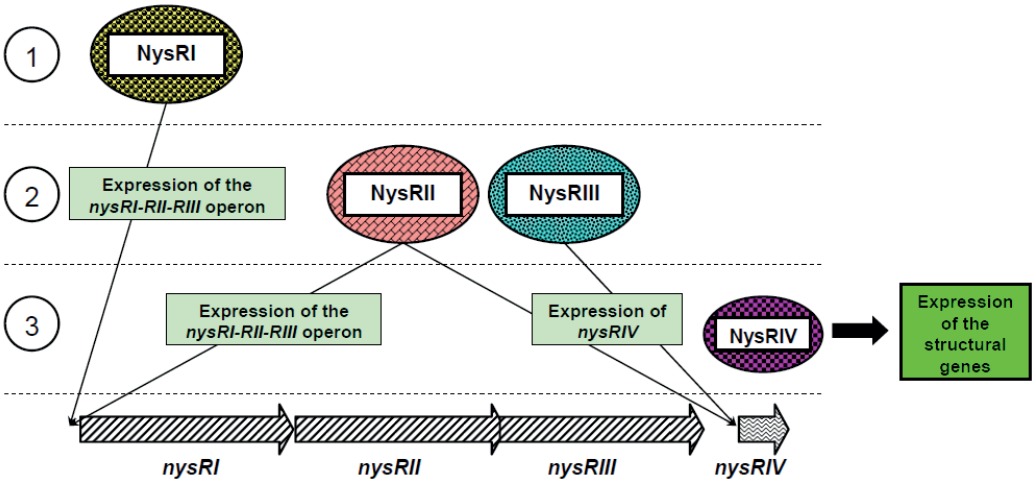

Figure 4.7. A model of the regulatory cascade controlling nystatin biosynthesis in *S. noursei* ATCC11455.

The products of all three genes are presumably required to activate transcription of *nysRIV*. NysRIV, in turn, seems to moderately activate its own promoter, and activates the transcription from promoters for the different biosynthetic and transport operons, thus directly controlling the nystatin biosynthesis. The structure of NysRIV, in particular the presence of PAS domain, suggests that transcriptional activation by NysRIV may also be dependent on energy levels within the cell. PAS-like domains are found in many signaling proteins, and serve as signal sensor domains (Galperin, 2004). PimM, a transcriptional activator of the LuxR type with a PAS sensory domain from pimaricin gene cluster, is an ortholog of NysRIV (Anton et al., 2007). Experiments on gene expression of the pimaricin gene cluster showed that the main targets for regulation for PimM are genes for initiation and first elongation cycles for polyketide biosynthesis. These results suggest that PimM and NysRIV follow similar pattern in their regulatory activity.

As mentioned above, homologs for *nysRI-III* regulatory genes are found in different macrolide antibiotic biosynthetic clusters. The analogs for all 4 *nysR* regulators have been found in amphotericin gene cluster. The organization of *amphRI-RIV* genes is identical to that of *nysRI-RIV* (Carmody et al., 2004**).**

4.4 Engineering of the nystatin biosynthesis genes to produce improved antifungal antibiotics (Paper IV)

Understanding of antibiotic biosynthesis machinery provides an opportunity for the development of new antibiotic analogues with improved properties through biosynthetic engineering. However, background knowledge on the structure-activity relationship of target antibiotic is often required.

4.4.1 Engineering of specific changes in the polyol region of nystatin

The polyol region on the polyene macrolide molecule is a promising target for obtaining a new antifungal compounds via genetic engineering. Recent study of Caffrey and colleagues showed the importance of this region for antifungal and hemolytic activity of polyene macrolides, in particular amphotericin B (AmB), which biosynthesis is almost identical to that of nystatin (Power et al., 2008). This group performed the replacement of the C-7 hydroxyl on amphotericin B molecule with a keto group via inactivation of a ketoreductase domain in module 16, and observed a significant reduction of in vitro hemolytic activity although the antifungal activity was also reduced.

The C-1-C-15 polyol region on nystatin molecule is interrupted by a saturated C-9-C-10 bond, and KR15, DH15 and ER15 domains of NysJ are involved in the formation of this bond. To get a better understanding of structure-activity relationship of nystatin, we performed an inactivation of DH15 domain of NysJ by site-specific mutagenesis. The resulting mutant was shown to produce a molecule 9-hydroxy-10-deoxy nystatin, denoted as BSG002. Purified BSG002 was found to be at least 2-fold less hemolytic compare to nystatin, but at the same time, its antifungal activity was reduced at approximately 4-fold.

We have been working in parallel with a similar strategy for nystatin biosynthesis (Figure 4.8), and decided to replace the C-5 and C-7 hydroxyls with keto groups on the S44HP nystatin analogue, which was generated in our group earlier (Bruheim et al., 2004). The antifungal activity of the S44HP obtained through inactivation of ER5 domain in NysC is considerably higher than that of nystatin and equal to that of AmB. S44HP is ca. 10 times more soluble and has a wider therapeutic window (MTD-LD_{50} dose interval) compared to AmB (Treshalin et al., 2005). Because of these improved properties S44HP was considered to be a good candidate for further genetic manipulations.

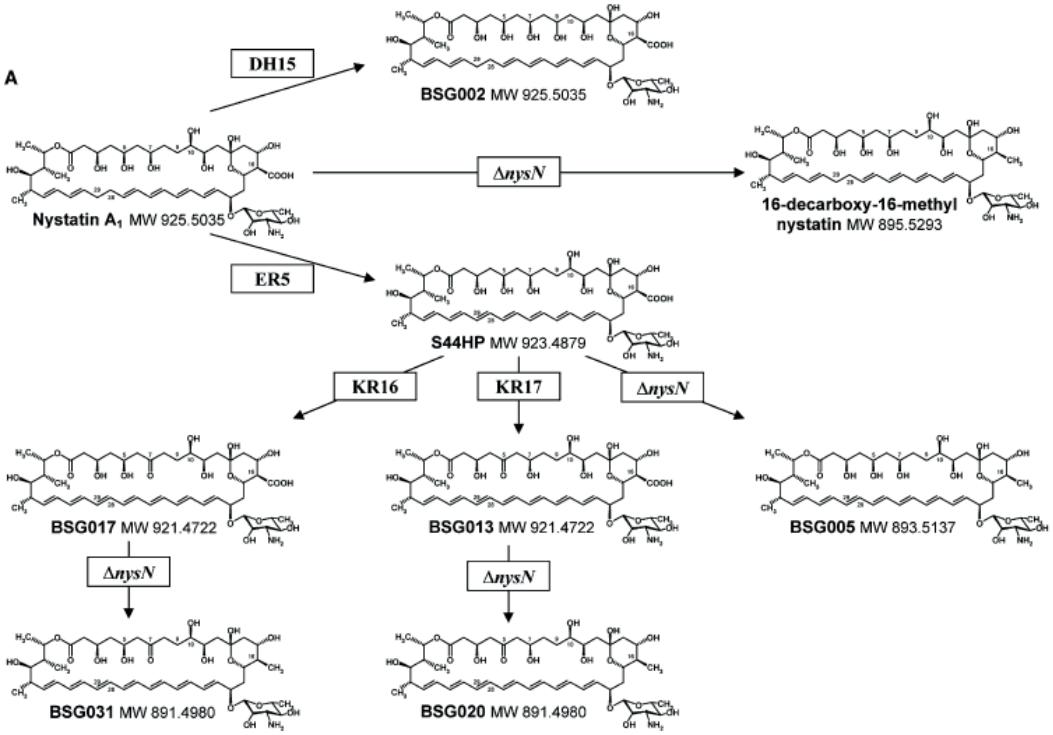

Figure 4.8. Genetically engineered nystatin analogues produced via manipulation of nystatin PKS and post-PKS modification genes (adapted from Brautaset et al. (2008).

The KR16 and KR17 mutations were individually introduced into the S44HP-producing mutant in order to achieve production of 7-oxo and 5-oxo S44HP analogues, respectively. Polyene macrolides produced by these mutants, designated as BSG017 and BSG013, respectively, were purified and tested for toxicity and antifungal activity. In both cases the antifungal and hemolytic activities, compared to S44HP, were decreased. These data corresponded to those obtained for AmB with C-5 and C-7 hydroxyls substituted with keto groups.

4.4.2 C-16 carboxyl is important for toxicity of the nystatin and its analogues

As was described above, S44HP, the nystatin analog with significantly increased antifungal activity, is an attractive candidate for further chemical modifications in order to obtain molecule with better pharmacological properties. Based on the data on polyene macrolides

with modified exocyclic carboxy group, we assumed that replacement of C-16 carboxyl by methyl via inactivation of *nysN* could reduce toxicity of S44HP. The resulting mutant produced expected compound 16-decarboxy-16-metyl-28, 29-didehydro nystatin, designated BSG005. In vitro assays showed its approx. 20-times and 2-times increased antifungal activity, compared to nystatin and S44HP, respectively. Simultaneously, its hemolytic activity was reduced approx. 1, 5-times, compared to S44HP.

Taking into consideration that C-5 and C-7 keto modifications in polyol region, and also C-16 modification on nystatin molecule resulted in less toxic molecules, we attempted to combine these mutations on one molecule in order to obtain even less toxic analog(s). This time we introduced *nysN* mutation into BSG013-producing mutant (C-5-oxo) and BSG017-producing mutant (C-7-oxo). Expected analogues 5-oxo-5-deoxy-16-decarboxy-16-methyl-28, 29-didehydro nystatin and 7-oxo-7-deoxy-16-decarboxy-16-methyl-28, 29-didehydro nystatin, designated BSG020 and BSG031, respectively, were produced by the mutants. Remarkably, production of these compounds was not reduced dramatically, which means, that it is possible to combine multiple mutations in one molecule, and introduce them into pathway simultaneously without significant reduction in the production level.
The results of the in vitro activity test and hemolytic assay revealed reduced toxicity, compared with S44HP, and increased antifungal activities, compared to BSG013 and BSG017, for both compounds.
We could then conclude that mutations in the polyol region combined with C-16 modification on the S44HP background can lead to the production of compound with the better therapeutic properties, compared to S44HP.

4.4.3 In vivo analysis of three engineered nystatin analogs

Three nystatin analogs, BSG002, BSG005 and BSG020, which performed best in the in vitro tests, have been chosen for in vivo studies on acute toxicity and antifungal activity against disseminated candidosis in a mouse model.
BSG002 was shown to have considerably decreased acute toxicity, compared to nystatin. However, in the experiment with animals infected with *Candida albicans,* BSG002 displayed almost complete loss of its antifungal activity. This fact suggests that the stability in vivo and

pharmacokinetics of new molecules determine the utility of new compounds for therapeutic use.

The results for BSG005 and BSG020 clearly showed that both compounds have considerably lower toxicity in vivo. As for the antifungal activity experiments, they correlated well with those obtained in the in vitro tests. BSG005 performed slightly better than AmB and S44HP, while BSG020 displayed a slightly reduced antifungal activity compared to other compounds. Remarkably, the in vivo antifungal activities for BSG005 and BSG020 were observed at doses which are much lower (10% of Maximal Tolerated Dose, MTD), than those for AmB and S44HP (40% and 94% of MTD doses, respectively). This fact suggests that both compounds might have better then AmB therapeutic properties, and thus can become candidates for further development as new antifungal agents.

5. CONCLUDING REMARKS

This study is a part of detailed investigation of the nystatin biosynthetic machinery, which was started first as an attempt for the strain improvement in order to increase the nystatin production in *S.noursei* ATCC 11455, and ended up as an alternative approach (targeted genetic engineering) for generation of improved antifungal polyene macrolides. I have been lucky to take a part in the work with such a unique and interesting cluster from the very beginning, e.g. cloning and unravelling the genes functions in the biosynthesis of nystatin.

Earlier studies on the regulation of antibiotic biosynthesis in bacteria have revealed a number of regulators of different levels, but still the whole picture of regulation of biosynthesis for such a complex compounds as polyketides remains unclear. The pleiotropic regulatory locus from *S.noursei*, which is able to activate production of actinorhodin in *S.lividans,* has been identified and characterized during our initial studies on nystatin biosynthesis. *ssmA* gene from this locus was shown to regulate nystatin biosynthesis in *S.noursei* in carbon source-dependent manner. Remarkably, *ssmA* has some homology to *afsR2 (afsS* in *S.coelicolor),* the pleiotropic regulator from *S.lividans*. Recently, it was shown that *afsS/afsR2* affects both antibiotic biosynthesis and nutrition starvation response genes in *S.coelicolor* (see Introduction). These facts correlate well with our conclusions that SsmA might be a part of a system sensing the carbon source, and transmitting the signal to the pathway-specific regulators of the nystatin biosynthetic gene cluster in *S. noursei.*

This work was shortly followed by the cloning and comprehensive analysis of the nystatin biosynthetic gene cluster. It can be mentioned that this was the first ever publication of a complete gene cluster for a polyene macrolide antibiotic (Brautaset et al., 2000). Later studies on the cluster, where I participated, were focusing on nystatin transport, synthesis and attachment of the mycosamine moiety, and regulation of the biosynthesis. The latter works have revealed a complex hierarchy among the pathway-specific regulators, regulatory targets within the cluster, and allowed to suggest a model for the regulation. It should be noted that it was the first publication on such a unique regulatory system with multiple regulatory genes acting in a cascade manner in the streptomycetes producing polyene macrolide antibiotics. Interestingly, an unexpected role of a cluster-associated gene encoding a PKS modification enzyme (phosphopantetheinyl transferase) was revealed. Data obtained suggested that this

gene is most likely involved in the biosynthesis of a signaling molecule that negatively regulates nystatin biosynthesis. Together, these findings provide a basis for rational engineering of *S. noursei* for the increased production of nystatin and its analogues.

Finally, the work on the nystatin cluster has culminated in biosynthetic engineering of new analogues with improved pharmacological properties. It is worth noting that one of these analogues is now being further developed in a biotech start-up company as an antifungal drug for treatment of systemic fungal infections. Overall, the impact of this work on the field of antibiotic biosynthesis and biosynthetic engineering has been considerable, and it was very exciting to paricipate in this project.

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Paper I

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Molecular cloning and analysis of a pleiotropic regulatory gene locus from the nystatin producer *Streptomyces noursei* ATCC11455

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Abstract

A regulatory gene locus from *Streptomyces noursei* ATCC14455, the producer of the antifungal antibiotic nystatin, was cloned in *Streptomyces lividans* based on its ability to activate actinorhodin (Act) production in this host. Deletion and DNA sequencing analyses showed that a small gene, designated *ssmA*, located downstream of an *afsR* homologue (a known pleiotropic regulator) was responsible for the Act overproduction in *S. lividans*. Database searches for the *ssmA* gene product revealed its limited similarity to the AfsR2 regulatory protein from *S. lividans* and CREA catabolite repressor from *Aspergillus nidulans*. To study the effect of *ssmA* on nystatin production, this gene was either deleted from *S. noursei* genome, or placed under control of P_{ermE*} promoter and introduced in *S. noursei*. The properties of the corresponding strains indicate that *ssmA* is involved in regulation of growth and antibiotic production only in the media with certain carbon sources.

Keywords: Pleiotropic regulator; Antibiotic production; *Streptomyces noursei*; *Streptomyces lividans*

1. Introduction

Filamentous soil bacteria belonging to the genus *Streptomyces* are capable of producing a wide variety of antibiotics commonly used in medicine and agriculture. The antibiotic biosynthesis is an energy-consuming process, which must be tightly regulated in order not to waste the cells resources unnecessarily. It follows then that the streptomycetes might have developed a comprehensive regulatory network allowing to switch on the antibiotic production only in response to certain environmental signals. Recent studies show that the antibiotic biosynthesis in *Streptomyces* is indeed regulated on several levels, and in many cases the start of antibiotic production is mediated by such environmental factors as growth media components, heat shock, media pH, phage infection, etc. [1]. The scheme for the network regulating antibiotic biosynthesis in *Streptomyces* is still far from being complete. One of the most interesting parts of the network includes genes regulating antibiotic pro-

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duction only under certain conditions. These genes are the most likely candidates for being parts of the system sensing the changes in the environment and allowing an organism to promptly react. For example, multiple copies of the *afsQ1* and *afsQ2* genes from *Streptomyces coelicolor* A3(2), apparently representing a two-component regulatory system most probably responding to environmental factors, stimulate antibiotic production [2]. Another gene, originally isolated from *S. coelicolor* A3(2) and designated *afsR*, enhances production of the antibiotics actinorhodin (Act) and undecylprodigiosin (Red) in both *S. coelicolor* and *Streptomyces lividans* when overexpressed [3]. No plausible function for *afsR* has been assigned yet, although its overexpression was shown to markedly enhance transcription of pathway-specific regulatory genes from Act and Red biosynthetic gene clusters. A small gene *afsR2* (*afsS*), encoding a 63 aa peptide and located just downstream of *afsR* in both *S. lividans* and *S. coelicolor*, can also stimulate Act and Red production when cloned on a multicopy vector [4].

In the present paper we describe cloning and analysis of two small genes, designated *ssmA* and *ssmB*, from *Streptomyces noursei*, the producer of the polyene antifungal antibiotic nystatin. DNA sequence analysis revealed that both genes are located downstream of the *afsR* homologue in *S. noursei*, and that putative overexpression of *ssmA* positively affects actonorhodin production in *S. lividans*. We also demonstrate that *ssmA* exerts a positive effect on nystatin biosynthesis in *S. noursei*, and that this effect depends on the carbon source used for cell growth.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth media

Bacterial strains and plasmids used in this study are listed in Table 1. New strains and plasmids developed in the course of this study are described in Section 3. *S. noursei* strains were maintained on ISP2 agar media (Difco), and *S. lividans* strains were maintained on R2 media [5]. *Escherichia coli-Streptomyces* conjugation experiments were carried out essentially as described elsewhere [6]. Tests for actinorhodin production by *S. lividans* strains were performed in the R2 medium prepared according to [5], but without agar.

2.2. Fermentations

Fermentations were performed in Applicon 3-l fermentors containing initially 1.3 l SAO-23 or SAO-26 medium. SAO-23 (g l^{-1}): glucose·H_2O, 90; NH_4NO_3, 2.5; corn flour, 3; $MgSO_4 \cdot 7H_2O$, 0.4; KH_2PO_4, 0.2; $CaCO_3$, 7; trace element solution, 3 ml. SAO-26 had the same composition except that it contained 60 g l^{-1} starch instead of 90 g l^{-1} glucose·H_2O. Trace element solution (mg ml^{-1}): $FeSO_4 \cdot 7H_2O$, 5.0; $CuSO_4 \cdot 5H_2O$, 0.39; $ZnSO_4 \cdot 7H_2O$, 0.44; $MnSO_4 \cdot H_2O$, 0.15; $Na_2MoO_4 \cdot 2H_2O$, 0.01; $CoCl_2 \cdot 6H_2O$, 0.02; HCl, 50. The fermentations were performed at 28°C with pH controlled at 6.5–7.0 by HCl (2 M) and NaOH (2 M). The dissolved oxygen was controlled at $>40\%$ of saturation by the agitation (300–900 rpm) and

Table 1
Bacterial strains and plasmids used in this study

Strain/plasmid	Characteristics	Source/reference
S. noursei ATCC11455	wild-type (WT), nystatin producer	ATCC
S. lividans 1326	wild-type	[20]
E. coli DH5α	general cloning host	[8]
E. coli ET12567	*dam*$^-$*dcm*$^-$ host for *E. coli*/*Streptomyces* conjugation	D. MacNeil
pUZ8002	helper plasmid providing RK2 transfer functions	D.H. Figurski
pGEM3Zf	*E. coli* cloning vector	Promega, USA
pWHM4	*E. coli*/*Streptomyces* cloning vector	[21]
pSET152	*E. coli*/*Streptomyces* integrative vector	[16]
pGEM7ermELi	pGEM7Zf plasmid (Promega) containing P_{ermE*} promoter	C.R. Hutchinson

aeration (0.25 vvm). Inocula for the fermentations (3 vol%) with SAO-23 and SAO-26 medium were grown in respectively TSB medium (TSB, Oxoid CM129, 37 g l^{-1}) and Medium-SAO-27 at 28°C in shake flasks (500 ml baffled Erlenmeyer flasks with 100 ml medium; 200 rpm). SAO-27 (g l^{-1}): starch, 10; corn flour, 2; TSB, 2; yeast extract, 2. Each shake flask was inoculated with 0.2 ml spore suspension and incubated for 18–20 h. Nystatin production was assayed by HPLC of the dimetylformamide extracts of the cultures after fermentations [7].

2.3. DNA manipulation

Standard DNA manipulation techniques were performed as described previously in [5,8]. DNA sequencing was performed at MediGene (Germany) and the data were analyzed with the GCG software [9].

2.4. Gene replacement procedure

To construct the vector for gene replacement, the 413 bp *Cla*I fragment containing complete *ssmA* gene was deleted from the A8 fragment subcloned in the pGEM3Zf vector. The insert from the resulting plasmid was then excised with *Kpn*I and *Hin*dIII, and ligated together with the 3.0 kb *Kpn*I/*Hin*dIII fragment from vector carrying *oriT*, the apramycin resistance gene (Am^R), and the ColEI replication origin [10]. These manipulations yielded the pCDO22 plasmid, which was introduced into *S. noursei* by conjugation from the *E. coli* ET12567 (pUZ8002). One of the clones carrying pCDO22 integrated into the chromosome via homologous recombination was subjected to three rounds of sporulation on Am-free medium, and the progeny was tested for the loss of the Am^R marker. Southern blot analysis of DNA isolated from 10 Am^S strains with the A8d8 probe revealed that five of them contained the desired *ssmA* deletion (data not shown).

2.5. Analysis of the actinorhodin production

S. lividans strains were grown in 5 ml of YEME medium [5] for 48 h at 30°C under appropriate antibiotic selection (thiostrepton 10 μg ml^{-1}, apramycin 20 μg ml^{-1}). Three ml of cells were then transferred into 30 ml of liquid R2 medium in 250 ml shake flasks supplemented with an appropriate antibiotic and incubated with shaking at 30°C. One ml samples were removed every 24 h, and 0.5 ml of 3 M KOH was added to the suspension. After vortexing, samples were centrifuged and the absorptions at 640 nm (A_{640}) of the supernatants were measured.

3. Results

3.1. Cloning of an S. noursei DNA fragment causing enhanced actinorhodin production in S. lividans

Total DNA isolated from *S. noursei* was partially digested with *Sau*3AI, size-fractionated on a sucrose gradient, ligated with *Bam*HI-digested DNA of the multicopy vector pWHM4, and introduced into *S. lividans*. Several transformants exhibiting overproduction of blue pigment (most probably actinorhodin, Act) on the R2 medium were isolated and one of them, designated A8, was chosen for further analysis. Recombinant plasmid isolated from the latter clone (designated pA8) contained a 4.8 kb DNA insert and conveyed the Act overproduction phenotype (blue colonies) upon retransformation into *S. lividans*. In addition to the Act overproduction, the pA8 plasmid imposed a weak, but detectable growth inhibition effect on the recombinant *S. lividans* strain. To determine more precisely the location of a putative positive regulator within the A8 fragment, its deletion analysis was performed. The latter resulted in identification of the 1.8 kb *Sac*I/*Bam*HI fragment, designated A8d8 (Fig. 1a), as sufficient for eliciting the Act overproduction phenotype in *S. lividans*.

3.2. DNA sequence analysis of the A8d8 fragment and construction of its deletion derivatives

The complete DNA sequence of the A8d8 fragment was determined. Analysis of the 1795 bp sequence showed the presence of two complete and two incomplete open reading frames (ORFs) (Fig. 1a), the putative products of which were compared to the proteins in the SWISSPROT and TREMBL databases. An incomplete ORF1 located at nt 1–420 was shown to encode a C-terminal part of a peptide sharing 58% identity with the AfsR protein from *S.*

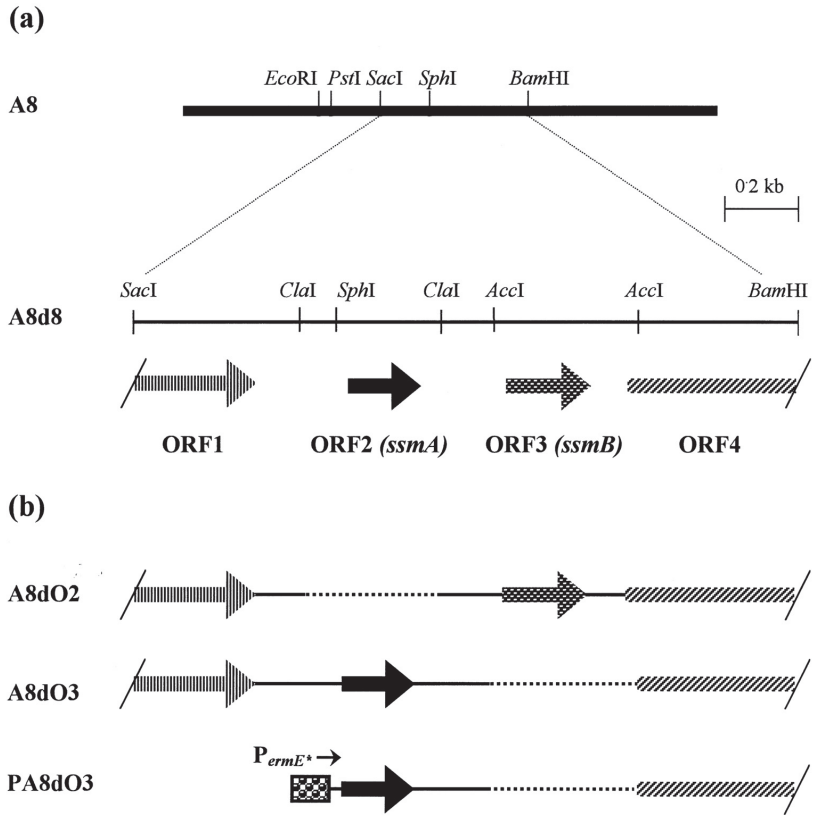

Fig. 1. a: A8 DNA fragment from *S. noursei* which stimulates actinorhodin production in *S. lividans*, and its derivative A8d8 identified by deletion analysis as carrying all functions necessary for activation. Genes identified within A8d8 DNA fragment are indicated with arrows. DNA sequence of the A8d8 DNA fragment was deposited into the GenBank under accession number AF118856. b: Schematic representation of gene organization within the A8d8 DNA fragment deletion derivatives. Deleted regions are indicated with dotted line.

coelicolor A3(2) [11]. Downstream of the ORF1, a small ORF2 (nt 636–800) was identified, which deduced product of 55 aa did not show significant matches with the proteins in the databases. Some homology (34% identity in 47 aa overlap) was found between the ORF2 product and the C-terminal part of the CREA catabolite repressor from *Aspergillus nidulans* [12] (Fig. 2a). Detailed analysis of the putative ORF2 product revealed the presence of two repeats homologous to those present in the *S. lividans* peptide encoded by the *afsR2* gene [4] (Fig. 2b). Beside the repeats, no considerable homology could be found between the ORF2 product and AfsR2. Downstream of ORF2, a small ORF3 (nt 1015–1176) was identified. Homology search and analysis of the putative ORF3 product (54 aa) with the TMPRED program [13] suggested that it is secretable peptide. This was supported by considerable homology (59% identity) of its 27 N-terminal aa to the putative signal peptide of the secretable metalloproteinase from *S. coelicolor* [14], and by finding a putative transmembrane helix between aa 6 and 27. The aa 25–27 of the ORF3 product (AIA) matched the AxA conserved motif usually located in the streptomycete signal peptides just before the cleavage site [15]. ORF4, located downstream of ORF3, starts at nt 1347, and is incomplete. Its predicted truncated product of 149 aa shows homology to the proteins

which bind AMP, such as peptide synthetases and acetyl-CoA synthetases from different organisms (up to 36% identity over 147 aa overlap). However, an AMP-binding domain itself was not found within the sequence.

3.3. Actinorhodin production by the recombinant *S. lividans* strains carrying A8 derivatives

Since both ORF1 and ORF4 are incomplete, it was logical to assume that either ORF2 or ORF3 (or both) are responsible for Act overproduction in *S. lividans*. To establish the roles for ORF2 and ORF3, constructs were made where either of the above genes was deleted from the A8d8 (Fig. 1b). A8dO2 was made by deleting the 413 bp *Cla*I fragment, and A8dO3 by deleting the 354 bp *Acc*I fragment from the A8d8 fragment. The resulting fragments were subcloned in both pWHM4 and pSET152 vectors. The latter is able to integrate site-specifically into the chromosomes of many streptomycetes providing one copy of the cloned gene per genome [16]. In addition, the strong constitutive promoter P_{ermE*} from *Saccharopolyspora erythraea* [17] was inserted upstream of ORF2, giving the PA8dO3 fragment (Fig. 1b). The latter could only be assembled on the pSET152 vector, and attempts to subclone the insert from the resulting construct pSET-PA8dO3 into pWHM4 were unsuccessful, as recombinant plasmids suffered deletions in *E. coli* DH5α.

S. lividans strains carrying fragments A8d8, A8DO2, and A8DO3 cloned into the pWHM4 multicopy vector (and designated pA8...) were tested for Act production in liquid R2 media (Fig. 3a). The results clearly indicated that the ORF2 is required for the Act overproduction since its deletion in pA8dO2 abolished the above phenotype. Comparison of the data obtained for pA8d8 and pA8dO3 revealed that the deletion of ORF3 leads to enhancement of Act production. Because of the effects imposed by both ORF2 and ORF3 on Act production

a

```
                10         20         30         40

SsmA   MATENKAPEANERDIVKPMDNHTPIAAPGLGKKKAPRKPGEMLP-----ADNHTPIINPR
       :.:  ..:  .. : ..:  .:::.:.:. . .  :   :  ::     . .::: . :
CREA   MSTAPSSPTFSH-DSLSPTPDHTPLATPAHSPRLRPYGGGYDLPGIRNLSLHHTPALAPM

        290        300        310        320        330        340
```

b

```
CREA  R1    P T P D H T P L A T P
                  : : :       :
CREA  R2    L S L H H T P A L A P
                  : : :     : :
SsmA  R1    P M D N H T P I A A P
            :   : : : : : :     :
SsmA  R2    P A D N H T P I I N P
            :   : : :   :       :
AfsR2 R1    P Q D N H M P T P - P
                : : : : :       :
AfsR2 R2    T L D N H M P S G - P
            :   : : : : :       :
AfsR2 R3    T M D N H M P A P - P
```

Fig. 2. Similarity of the SsmA peptide to the known proteins. a: Alignment of the SsmA and CREA proteins (see text for details). Conserved aa residues within repeats are underlined. b: Alignment of the putative repeats (R) in CREA, SsmA and AfsR2 peptides.

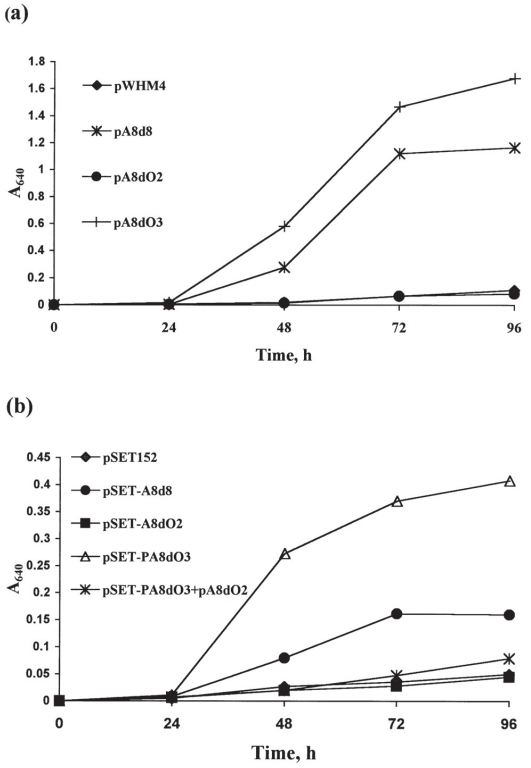

Fig. 3. Shake flask experiments on actinorhodin production by the recombinant *S. lividans* strains carrying A8d8 derivatives on the multicopy vector pWHM4 (a), or integrated into the chromosome in one copy when cloned into pSET152 (b) (see text for constructs description). Average values from three independent experiments are presented.

in *S. lividans*, the latter genes were designated *ssmA* and *ssmB* (for stimulation of secondary metabolism), respectively.

Next, the *S. lividans* transformants carrying the same fragments cloned into pSET152 (and designated pSET-A8...) were tested for Act production in liquid R2 medium. The results obtained (Fig. 3b) revealed that a single copy of *ssmA* is sufficient for Act overproduction in *S. lividans*, although the production level was much lower compared to that achieved with the multicopy vector. No Act overproduction could be detected with the pSET-A8dO2 construct, in which *ssmA* was deleted. Strain carrying the pSET-PA8dO3 produced double amount of Act compared to the pSET-A8d8-containing strain, presumably due to the P_{ermE*}-driven overexpression of *ssmA*. To test the possible effect of multiple copies of *ssmB* on Act production, the pA8dO2 plasmid was introduced into the *S. lividans* (pSET-PA8dO3) strain. The fact that the Act production was substantially reduced in the resulting recombinant strain further supported the idea of *ssmB* overexpression negatively affecting antibiotic production.

3.4. Involvement of ssmA in regulation of nystatin production in S. noursei

The autonomously replicating plasmids based on pIJ101, SCP2* or pSG5 replicons could not be established in *S. noursei* wild-type (WT) strain. However, integration of the suicide mobilizable vector carrying homologous DNA, as well as site-specific integration of the pSET152 vector into the *S. noursei* chromosome was demonstrated upon conjugation from *E. coli* ET12567 (pUZ8002) [10]. To study the possible effect of *ssmA* overexpression on nystatin biosynthesis, the plasmid pSET-PA8dO3 was introduced into *S. noursei*, generating strain PA8DO32. The latter displayed slightly inhibited growth and colony development compared to the WT (pSET152). In order to test the involvement of *ssmA* in regulation of nystatin production, this gene was deleted from the *S. noursei* chromosome via gene replacement (as described in Section 2) yielding the *ssmA*$^-$ strain DO221.

Nystatin production by the *S. noursei* WT, DO221, WT (pSET152), and PA8DO32 strains in semi-defined media with different carbon sources was assessed in two parallel experiments (for each medium) with 3 l fermentors. In the SAO-23 medium containing glucose as a main carbon source, nystatin production by strain DO221 was 5% lower than that by the WT (Fig. 4a). On the same medium, PA8DO32 strain produced 12% more nystatin when compared with the WT (pSET152). No significant differences in glucose consumption and respiration rates on SAO-23 medium could be observed between the strains tested. When the SAO-26 medium was used, where glucose was substituted with starch, nystatin volumetric yield by the deletion mutant DO221 was 86% lower compared to the WT strain (Fig. 4b), while PA8DO32 produced approximately 250% more nystatin compared to the WT

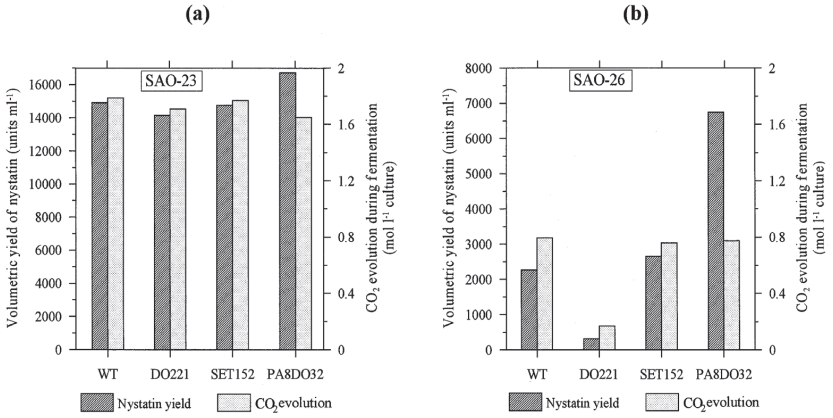

Fig. 4. Analysis of the nystatin production and respiration rate by the *S. noursei* WT, WT (pSET152), DO221, and PA8DO32 strains in SAO-23 (a) and SAO-26 (b) media in 3 l fermentors. Average values from two independent experiments are presented. See text for strain description and Section 2 for media composition.

(pSET152). It should be noted that fermentations on the SAO-23 medium were run for 143 h (until all the glucose was consumed), while the fermentations on SAO-26 were continued until 288 h (slow starch consumption was observed). The DO221 mutant showed extremely slow starch consumption, and approximately 4.5 times lower respiration rate on the SAO-26 medium compared to the other strains, suggesting that the former is impaired in assimilation of starch as a carbon source.

4. Discussion

We have cloned and characterized a gene locus from the nystatin-producing organism *S. noursei*, which stimulates actinorhodin production in *S. lividans*. DNA sequence and deletion analysis revealed that two small genes, *ssmA* and *ssmB*, are involved in implementation of this phenomena. The *ssmA* gene, which product shows limited similarity to both the C-terminal region of the *A. nidulans* catabolite repressor CREA [12], and AfsR2 peptide from *S. lividans* [4], is responsible for Act overproduction. The gene *ssmB* found immediately downstream of *ssmA*, and seemingly coding for a secretable peptide, is likely to be involved in negative regulation of antibiotic production. The *ssmB* deletion from the *ssmA*-containing multicopy plasmid further stimulates Act production in *S. lividans*, while multiple copies of the *ssmB* suppress stimulating effect of *ssmA*. We were unable to confirm involvement of *ssmB* in regulation of nystatin production, however, as attempts on deleting *ssmB* from *S. noursei* chromosome have so far been unsuccessful [10].

The homology between SsmA and CREA proteins prompted us to speculate that the function of the former might be modulated by the carbon source. The CREA protein functions as a catabolite repressor for the ethanol regulon in *A. nidulans*, repressing the ethanol utilization genes on the media containing glucose [18]. Although the exact function of the SsmA homologous CREA domain is not clear, truncation of the CREA protein for this region results in the loss of its repressing activity [19]. The data obtained for the recombinant *S. noursei* strains indicated that on the glucose-containing medium neither *ssmA* deletion nor its presumable overexpression significantly affects growth and nystatin production. However, in the medium containing starch, *ssmA* overexpression led to substantial increase in both volumetric and specific nystatin production, while deletion of this gene resulted in much lower volumetric antibiotic production due to the poor cell growth. The latter is probably caused by the impaired assimilation of starch in the *ssmA*$^-$ mutant, suggesting that the wild-type level of *ssmA* expression is required for normal growth of *S. noursei* on this car-

bon source. At the same time, similar growth characteristics of the wild-type strain carrying empty vector, and the one overexpressing *ssmA*, suggest that the stimulation of nystatin production in the latter is probably due to the more efficient expression of the pathway-specific regulatory gene(s). We suggest that SsmA might be a part of a system sensing the carbon source, and presumably transmitting the signal to the specific regulators. Transcriptional analysis of the pathway-specific regulatory genes in both *S. lividans* and *S. noursei* strains overexpressing *ssmA* will help to reveal the mechanism of *ssmA*-mediated stimulation of antibiotic production in more detail.

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Paper II

Biosynthesis of the polyene antifungal antibiotic nystatin in *Streptomyces noursei* ATCC 11455: analysis of the gene cluster and deduction of the biosynthetic pathway

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Background: The polyene macrolide antibiotic nystatin produced by *Streptomyces noursei* ATCC 11455 is an important antifungal agent. The nystatin molecule contains a polyketide moiety represented by a 38-membered macrolactone ring to which the deoxysugar mycosamine is attached. Molecular cloning and characterization of the genes governing the nystatin biosynthesis is of considerable interest because this information can be used for the generation of new antifungal antibiotics.

Results: A DNA region of 123,580 base pairs from the *S. noursei* ATCC 11455 genome was isolated, sequenced and shown by gene disruption to be involved in nystatin biosynthesis. Analysis of the DNA sequence resulted in identification of six genes encoding a modular polyketide synthase (PKS), genes for thioesterase, deoxysugar biosynthesis, modification, transport and regulatory proteins. One of the PKS-encoding genes, *nysC*, was found to encode the largest (11,096 amino acids long) modular PKS described to date. Analysis of the deduced gene products allowed us to propose a model for the nystatin biosynthetic pathway in *S. noursei*.

Conclusions: A complete set of genes responsible for the biosynthesis of the antifungal polyene antibiotic nystatin in *S. noursei* ATCC 11455 has been cloned and analyzed. This represents the first example of the complete DNA sequence analysis of a polyene antibiotic biosynthetic gene cluster. Manipulation of the genes identified within the cluster may potentially lead to the generation of novel polyketides and yield improvements in the production strains.

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Introduction

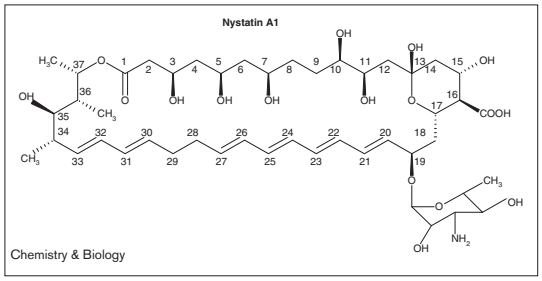
Polyketides are natural products, many of which have applied potential as pharmaceuticals. Examples of such polyketides include erythromycin (antibacterial), nystatin (antifungal), avermectin (antiparasitic), rapamycin (immunosuppressant) and daunorubicin (antitumor). The Gram-positive bacteria of the genus *Streptomyces* are the main producers of polyketides, and the genetics and biochemistry of polyketide biosynthesis in these organisms are relatively well characterized [1]. Macrolide polyketide compounds are formed via repeated condensations of simple carboxylic acids by modular (type I) polyketide synthases (PKSs) in a manner similar to fatty acid biosynthesis. The modular hypothesis proposed by Donadio *et al*. [2] suggested that type I PKSs are organized into repeated units (modules), each of which is responsible for one condensation cycle in the synthesis of a polyketide chain. This was proven to be correct by manipulations of type I PKS genes resulting in predictable changes in the chemical structures of macrolides [3]. Besides condensation of the next carboxylic acid onto the growing polyketide chain, ensured by the catalytic activity of a β-ketoacyl synthase (KS) domain, modules of PKSs type I may contain domains with β-ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) activities, which determine the reduced state of the incorporated extender unit. The acyltransferase (AT) and acyl carrier protein (ACP) domains present in each module are responsible for the choice of extender unit and retention of the growing polyketide chain on the PKS, respectively. Upon completion of synthesis, the polyketide chain is released from the PKS via the action of a thioesterase (TE), which is probably also involved in cyclization of the final product. The type I PKSs therefore represent an assembly line for polyketide biosynthesis that can be manipulated by changing the number of modules, their specificities towards carboxylic acids, or by inactivating or inserting domains with reductive activities [3]. After the polyketide moiety is synthesized and cyclized to form a macrolactone ring, it is usually modified via hydroxylation,

glycosylation, methylation and/or acylation. These modifications are believed to be crucially important for the biological activities of macrolides.

The genes for macrolide antibiotics biosynthesis in *Streptomyces* are organized in clusters, making isolation of complete sets of such genes relatively straightforward. Indeed, exploitation of recombinant DNA technology makes it possible to isolate complete antibiotic biosynthetic gene clusters by screening gene libraries with DNA probes encoding PKS fragments [1]. The molecular cloning and complete DNA sequencing has been described for several macrolide antibiotics produced by streptomycetes, including those for avermectin, pikromycin and rapamycin [4–6]. The cloning and partial DNA sequencing of the gene cluster for the polyene macrolide antibiotic pimaricin has recently been reported [7].

Polyene macrolide antibiotics have 20–44-membered lactone rings containing three to eight conjugated double bonds. The latter structural feature most probably contributes to the mode of action of these compounds, determined to be an interaction with sterols present in the membranes of fungi [8]. Such an interaction leads to formation of polyene–sterol complexes capable of organizing themselves into transmembrane channels making the membrane permeable to water and ions, leading to cell death. Beside being antifungal agents, some of the polyene antibiotics have been shown to have antibacterial, antiviral and immunostimulating activities [9,10]. The antibiotic nystatin A1 (Figure 1) is produced by the Gram-positive bacterium *Streptomyces noursei* ATCC 11455, and is used as an antifungal agent. The chemical structure of nystatin suggests involvement of a type I PKS in the biosynthesis of its macrolactone ring. Furthermore, it can be predicted that the synthesis of the nystatin polyketide chain starts with acetyl-CoA, and proceeds through condensation of three methylmalonyl-CoA and 15 malonyl-CoA extender units. In this paper we describe the cloning and analysis of the complete nystatin biosynthetic gene cluster of *S. noursei*.

Figure 1



Structure of the nystatin A1 molecule according to Lancelin and Beau [36].

Results and discussion

Cloning of the nystatin biosynthetic gene cluster

In order to isolate the nystatin biosynthesis genes, we probed a *S. noursei* gene library with labelled DNA encoding a PKS. To obtain such a DNA probe, two degenerate oligonucleotide primers were designed, corresponding to conserved amino acid regions within KS and ACP domains of known modular PKSs (see the Materials and methods section). The polymerase chain reaction (PCR)-assisted amplification of a DNA fragment from the *S. noursei* genome with these primers was performed. Subsequent cloning and DNA sequence analysis of the resulting 0.7 kb PCR product (see the Materials and methods section) confirmed that it encodes part of a type I PKS. This DNA fragment was used for screening the *S. noursei* gene library constructed previously [11], and one recombinant phage, designated DASHII-N1, which hybridized to the probe, was isolated.

Preliminary DNA sequence analysis of the N1 DNA insert showed that it encodes a type I PKS. To confirm the involvement of the cloned DNA in nystatin biosynthesis, a 4.2 kb *Bam*HI DNA fragment from the N1 insert was used in a gene-disruption experiment as a part of the suicide conjugative plasmid pKO(4.2)–1 (Table 1). One of the resulting *S. noursei* disruption mutants, NCD3, carrying pKO(4.2)–1 integrated into its genome (data not shown) was unable to produce nystatin, thereby confirming the requirement of the identified PKS gene for nystatin biosynthesis. This fragment was later shown to be an internal part of the *nysC* gene (see below).

In order to clone a larger portion of the nystatin biosynthetic gene cluster, the DNA fragments derived from the ends of the DNA insert in phage N1, as well as those fragments from overlapping recombinant phages, were used as probes for screening the gene library. This 'chromosome walking' resulted in isolation of recombinant phages encompassing ~125 kb of the *S. noursei* genome (Figure 2).

DNA sequence and mutational analyses of the nystatin PKS genes

The complete DNA inserts from recombinant phages were subcloned in *Escherichia coli* and sequenced. Computer-assisted analysis of the DNA sequence (123,580 bp) of the cloned region led to identification of the genes shown in Figure 2 and listed in Table 2. Six genes, *nysA*, *nysB*, *nysC*, *nysI*, *nysJ* and *nysK*, encoding a type I PKS were identified. The amino acid (aa) sequences of the deduced products encoded by these genes were analysed by comparing them to the aa sequences of known type I PKSs. Because all six proteins were shown to share considerable homology with rifamycin and rapamycin PKSs [12,13],

Table 1

Bacterial strains and plasmids used in this study.

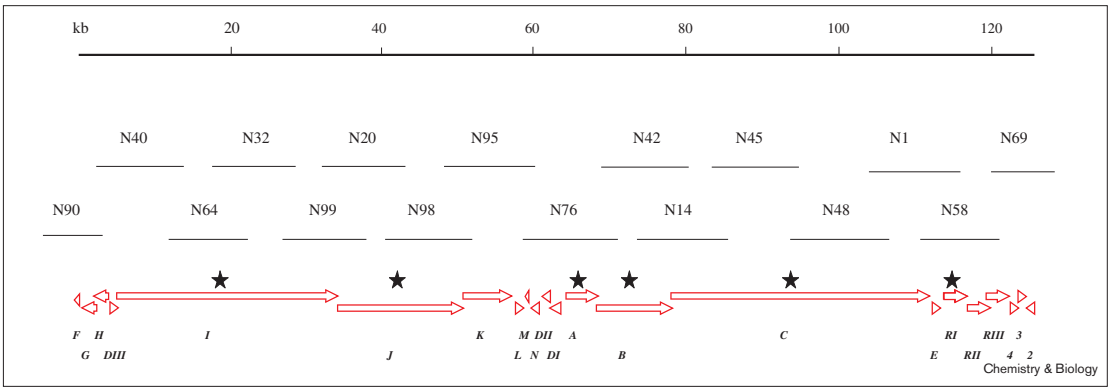
Strain or plasmid	Properties	Source or reference
E. coli XL-1 Blue MRA(P2)	Host for maintaining the gene library	Stratagene
E. coli DH5α	General cloning host	BRL
E. coli ET12567	Strain for intergeneric conjugation	[37]
S. noursei ATCC 11455	Wild type (WT), nystatin producer	ATCC
S. noursei NAD1	WT derivative with *nysA* disrupted by integration of pKNA1, nystatin nonproducer	This work
S. noursei NBD2	WT derivative with *nysB* disrupted by integration of pKNB1, nystatin nonproducer	This work
S. noursei NCD3	WT derivative with *nysC* disrupted by integration of pKO(4.2)–1, nystatin nonproducer	This work
S. noursei NID1	WT derivative with *nysI* disrupted by integration of pKNI1, nystatin nonproducer	This work
S. noursei NJD1	WT derivative with *nysJ* disrupted by integration of pKNJ1, nystatin nonproducer	This work
S. noursei NRD2	WT derivative with *nysRI* disrupted by integration of pNRD2, nystatin nonproducer	This work
pGEM3Zf(–)	ColEI replicon, Ap^R, 3.2 kb	Promega
pGEM11Zf(–)	ColEI replicon, Ap^R, 3.2 kb	Promega
pUZ8002	RK2 derivative, Km^R, Tc^R	D.H. Figurski*
pSOK201	pSG5 and ColEI replicons, *oriT* and Am^R, 7.1 kb	[11]
pPKS72	0.7 kb PCR product amplified from the *S. noursei* genome cloned in pUC18 (see the Materials and methods section)	This work
pL76S1.3	1.4 kb *Sal*I fragment of *nysA* cloned in pGEM3Zf(–)	This work
pKNA1	1.4 kb *Eco*RI–*Hin*dIII fragment from pL76S1.3 ligated with the 3.0 kb *Eco*RI–*Hin*dIII fragment from pSOK201	This work
pL76NB2.8	2.8 kb *Not*I–*Bam*HI fragment of *nysB* cloned in pGEM11Zf(–)	This work
pKNB1	2.8 kb *Bam*HI–*Hin*dIII fragment from pL76NB2.8 ligated with the 3.0 kb *Bam*HI–*Hin*dIII fragment from pSOK201	This work
pL64EB3.8	3.8 kb *Bam*HI–*Eco*RI fragment from N64 (internal for *nysI*) cloned in pGEM3Zf(–)	This work
pKNI1	3.8 kb *Eco*RI–*Hin*dIII fragment from pL64EB3.8 ligated with the 3.0 kb *Bam*HI–*Hin*dIII fragment from pSOK201	This work
pL20EB3.7	3.7 kb *Bam*HI–*Eco*RI fragment from N20 (internal for *nysJ*) cloned in pGEM3Zf(–)	This work
pKNJ1	3.7 kb *Eco*RI–*Hin*dIII fragment from pL20EB3.7 ligated with the 3.0 kb *Bam*HI–*Hin*dIII fragment from pSOK201	This work
pGEM4.2–1	4.2 kb *Bam*HI fragment of *nysC* cloned in pGEM11Zf(–)	This work
pKO(4.2)–1	4.2 kb *Eco*RI–*Hin*dIII fragment from pGEM4.2-1 ligated with the 3.0 kb *Eco*RI–*Hin*dIII fragment from pSOK201	This work
pNRD1	1.4 kb *Apa*I fragment of *nysRI* cloned in pGEM11Zf(–)	This work
pNRD2	1.4 kb *Eco*RI–*Hin*dIII fragment from pNRD1 ligated with the 3.0 kb *Eco*RI–*Hin*dIII fragment from pSOK201	This work

Am, apramycin; Ap, ampicillin; Km, kanamycin; Tc, tetracycline. *Personal communication.

presumptive functional analysis of the nystatin PKSs was based on the comparison to the former polypeptides. The predicted functional features of the NysA, NysB, NysC, NysI, NysJ and NysK proteins are shown in Figure 3.

The NysA protein most probably represents a loading module involved in the initiation of the nystatin aglycone biosynthesis. NysA contains a KS domain (KS^S) similar to that found in the presumed loading module of the pimaricin PKS PIMS0 [7]. Both the NysA and PIMS0 proteins are unusual in a sense that they represent the loading modules only, whereas in all other type I PKSs characterized so far, loading modules are fused to the first condensing module in multimodular polypeptides. The conserved active-site cysteine residue in both NysA and PIMS0 KS domains is replaced with a serine residue. In all the other known inactive KSs (KS^Q) in the loading modules of type I PKSs, the conserved cysteine is replaced by glutamine [14], except for the epothilone PKS loading module KS, which bears a Cys→Tyr replacement [15]. Because the aa sequence homology between the KS^S domains in NysA and PIMS0 (62.8%) is not higher than between the NysA KS^S and KS domains from other modular PKSs (ranging from 49.9% to 64.6%), it seems unlikely that these proteins have a recent common ancestor. It is tempting to speculate that the Cys→Ser replacement in the active sites of KS^S domains might have some significance for NysA and PIMS0 functioning as separate loading polypeptides. The latter might be related to the decarboxylase activity shown for the KS^Q domains to be largely

Figure 2



Gene organization within the *S. noursei* ATCC 11455 nystatin biosynthetic gene cluster. The inserts from the overlapping recombinant phages encompassing the cloned region are shown above the physical/genetic map. The *nys* genes are designated with capital letters in italics, other ORFs are numbered. Stars above certain ORFs indicate that these genes were successfully disrupted (see the text and Table 1 for details).

dependent on the presence of a glutamine residue in the active site [14]. It has been suggested that KS^Q domains in the loading modules of modular PKSs might provide the starter unit through the specific decarboxylation of enzyme-bound extenders [14]. Because the significance of the serine residue in the active sites of KS^S domains for their putative decarboxylase activity is not apparent, we are planning to address this question experimentally. Comparison of the NysA AT domain with its counterparts from different PKSs suggested that it is acetate specific, which correlates well with the structure of the nystatin molecule. Interestingly, NysA contains an apparently intact DH domain, which seems to serve no function, and might just have been retained in this protein in the process of evolution.

NysB apparently represents modules 1 and 2. The DH domains in both of these modules lack the conserved active-site motif $H(X_3)G(X_4)P$, due to large internal deletions, and therefore must be inactive. The AT domains identified within NysB display characteristic features of the propionate-specific AT domains (mAT) [13,16]. These features of NysB support the assumption about its involvement in first two elongation steps of the nystatin polyketide moiety biosynthesis.

The NysC protein, to our knowledge the largest bacterial polypeptide discovered to date, is composed of six modules apparently responsible for elongation steps 3–8 in the nystatin polyketide chain formation. All AT domains in NysC modules have features typical of the acetate-specific ATs (data not shown). This correlates well with the assumption that NysC incorporates six malonyl-CoA extenders (C-32 to C-21) into the nystatin aglycone. The only other protein with an organization similar to NysC, the RAPS2 PKS of *S. hygroscopicus*, comprising six modules and involved in rapamycin biosynthesis, is 873 aa shorter than NysC. This difference can be explained by the more compact structure of the RAPS2 domains and interdomain linkers, because the overall number of modules and domains, as well as their relevant positions in NysC and RAPS2 match perfectly. The end-to-end alignment of the NysC and RAPS2 aa sequences shows 49% identity — an unusually high number considering the lengths of these polypeptides. This, and the fact that rapamycin has an antifungal activity and initially was classified as an atypical polyene macrolide [17], might indicate that similarities between NysC and RAPS2 are due to a common ancestor from which the two proteins have evolved.

The NysI protein is probably responsible for elongation steps 9–14 of the nystatin polyketide backbone biosynthesis. The presence of an mAT domain in module 11 is consistent with incorporation of methylmalonyl-CoA extender at this elongation step. The DH domains in modules 10, 11, 12, 13 and 14 seem to be inactive due the large internal deletions encompassing the active-site motif (see above). The KR domain in module 13 of NysI lacks the conserved motif aSRrG, and thus appears to be inactive. The latter feature, together with inactive DH domain in module 11, most probably account for the presence of a six-membered ketalic ring (between C-13 and C-17) on the nystatin molecule (Figure 1). As judged from the organization of modules in NysJ, the latter is required for elongation steps 15–17 in nystatin macrolactone ring assembly. The DH domain in module 16 contains a large

Table 2

Putative genes identified in the nystatin biosynthetic gene cluster of *S. noursei*.

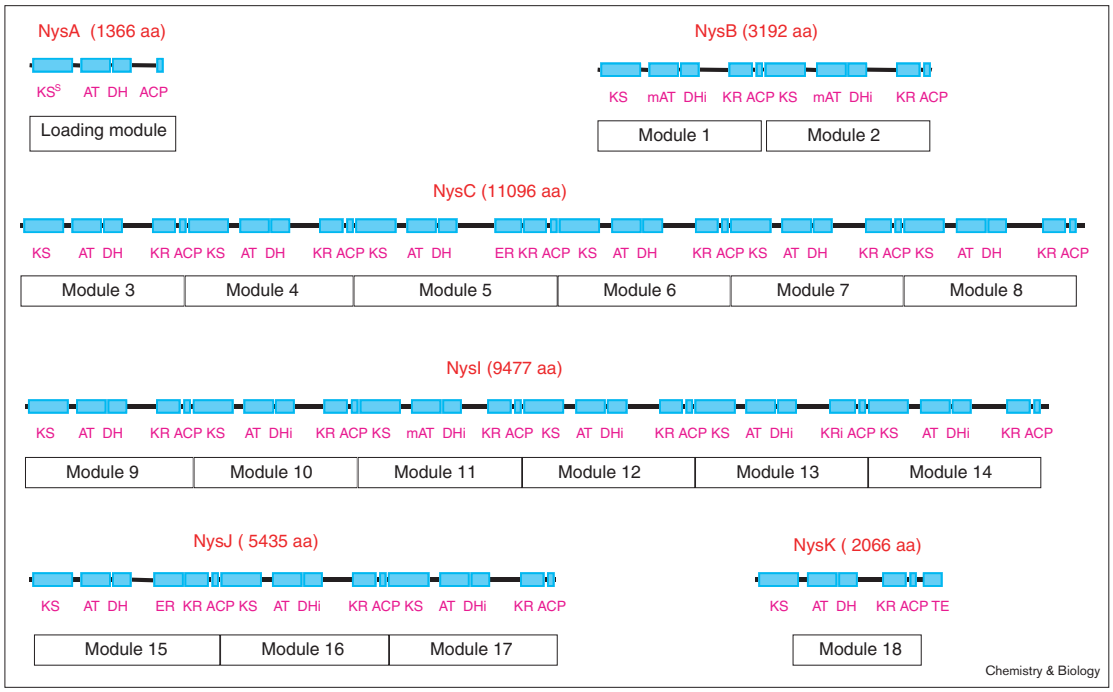
Designation	Product	Putative function
nysA	Type I PKS	Nystatin PKS (loading module)
nysB	Type I PKS	Nystatin PKS (modules 1 and 2)
nysC	Type I PKS	Nystatin PKS (modules 3–8)
nysDI	Glycosyltransferase	Attachment of mycosamine
nysDII	Aminotransferase	Mycosamine biosynthesis
nysDIII	GDP-mannose-4,6-dehydratase	Mycosamine biosynthesis
nysE	Thioesterase	Release of polyketide chain from PKS
nysF	4′-Phosphopantheteine transferase	Post-translational PKS modification
nysG	ABC transporter	Efflux of nystatin
nysH	ABC transporter	Efflux of nystatin
nysI	Type I PKS	Nystatin PKS (modules 9–14)
nysJ	Type I PKS	Nystatin PKS (modules 15–17)
nysK	Type I PKS	Nystatin PKS (module 18 + TE)
nysL	P450 monooxygenase	Hydroxylation at C-10
nysM	Ferredoxin	Electron transfer in P450 system
nysN	P450 monooxygenase	Oxidation of methyl group at C-16
nysRI	Transcriptional activator	Regulation of nystatin production
nysRII	Transcriptional activator	Regulation of nystatin production
nysRIII	Transcriptional activator	Regulation of nystatin production
ORF4	Transcriptional activator	Regulation
ORF3	Transcriptional repressor	Regulation
ORF2	Transcriptional activator	Regulation

internal deletion encompassing the active site, and the conserved histidine residue in the module 17 DH domain active site $H(X_3)G(X_4)P$ is replaced with tyrosine. The DH domains in modules 16 and 17 within NysJ therefore appear to be inactive. The ER domain localized in module 15 is probably responsible for the reduction of a double bond between C-8 and C-9. The final 18th module in the nystatin PKS system is represented by the NysK protein, which contains an apparently intact DH domain whose activity should not be required at the last condensation step. We note that a similar observation was made during analysis of the rifamycin PKS, in which intact DH domains in modules 6 and 7 seem to be nonfunctional in the producing organism [13]. A TE domain was identified at the carboxyl terminus of NysK, suggesting that in addition to the condensation of the last extender unit, this protein also participates in the release of the mature nystatin polyketide chain from the PKS complex.

To confirm the involvement of *nysA*, *nysB*, *nysI* and *nysJ* in nystatin biosynthesis, these genes were disrupted in *S. noursei* via homologous recombination using the conjugative suicide vectors pKNA1, pKNB1, pKNI1, and pKNJ1 (Table 1). All the disruption mutants obtained (Table 1) were unable to produce nystatin, suggesting that the genes mentioned above are essential for nystatin biosynthesis. Even though the polar effects of the disruptions mentioned above cannot be ruled out, such effects would imply that the genes affected are transcribed as part of a polycistronic mRNA together with the disrupted genes (see Figure 2). It seems highly unlikely, therefore, that the disrupted genes themselves have no role in nystatin biosynthesis.

Immediately downstream of the *nysC* gene, a coding sequence (*nysE*) for a TE was identified, providing yet another example of two TE activities being localized in the same antibiotic biosynthetic gene cluster. The 251 aa NysE protein shows a high degree of homology (45%) to the TEs encoded by the independent genes in the pikromycin and tylosin biosynthetic gene clusters of *Streptomyces venezuelae* and *Streptomyces fradiae* [6,17]. The NysE polypeptide, however, does not display substantial homology to the TE domains located at the carboxyl termini of several modular PKSs. The precise role for the putative TE encoded by *nysE* in nystatin biosynthesis remains uncertain. Both the pikromycin and tylosin biosynthetic gene clusters encode two TE activities, one of them embedded in the PKS [6,18]. Although the exact functions of these TEs are not clear, it was proposed that one of them might be a 'proof-reading' enzyme, clearing off certain substrates that would block further extension of the chain by PKS [19,20].

Figure 3



Functional organization of the nystatin PKS including the NysA, NysB, NysC, NysI, NysJ and NysK proteins. KS, ketosynthase; KS^S, ketosynthase with the Cys→Ser substitution in the active site; AT, acetate-specific acyltransferase; mAT, propionate-specific acetyltransferase; DH, dehydratase; DHi, inactive dehydratase; ER, enoyl reductase; KR, ketoreductase; KRi, inactive ketoreductase; ACP, acyl carrier protein.

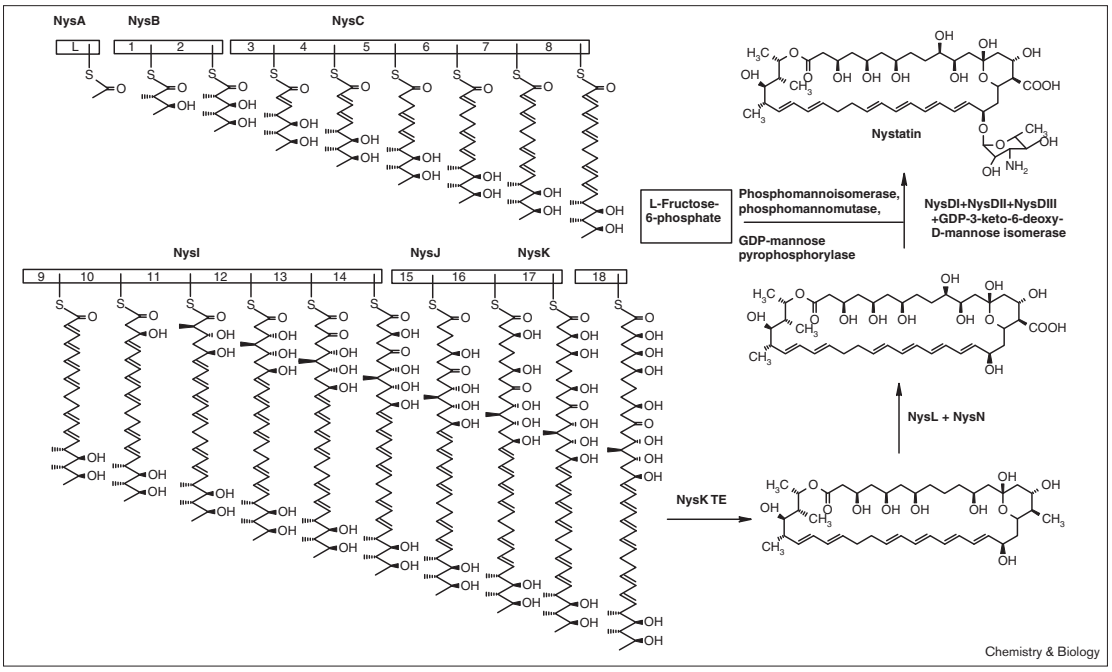
Genes in the regulatory region

The putative regulatory genes *nysRI*, *nysRII* and *nysRIII* encoding polypeptides of 966 aa, 953 aa and 927 aa, respectively, were identified downstream of *nysE*. The deduced products of these genes are homologous to the transcriptional regulators found in the vicinity of the cholesterol oxidase–cytochrome P450 operon of *Streptomyces* sp. SA-COO [21]. To confirm the involvement of *nysRI* in nystatin biosynthesis, this gene was disrupted in *S. noursei* with the suicide vector pNRD2 (Table 1). Analysis of the secondary metabolites produced by the corresponding mutant NRD2 revealed that it cannot synthesize nystatin. Because the *nysRII* and *nysRIII* genes are located just downstream of *nysRI*, and are transcribed in the same direction, it is plausible that the *nysRI* disruption has a polar effect on transcription of these genes.

Downstream of *nysRIII*, three ORFs (ORFs 2–4) were identified that might be involved in the regulation of nystatin biosynthesis. The deduced *ORF4* product of 210 aa is similar to the transcriptional activators of response regulator type, whereas a 253 aa polypeptide encoded by *ORF3* shows considerable homology to the transcriptional repressors of the DeoR family [22]. *ORF2*, located downstream of *ORF3*, and transcribed in the opposite direction, encodes a putative polypeptide of 354 aa that is similar to the transcriptional activators of the AsnC family [23]. Experiments aimed at mutational analysis of all the regulatory genes associated with the gene cluster are currently under way, and will provide the answer as to their involvement in the process of nystatin biosynthesis.

Sequencing of an additional 5 kb DNA from the phage N69 insert extending the cloned region to the right from *ORF2* (data not shown) identified several genes for peptide metabolism (T.B. and S.B.Z., unpublished observations) for which no role in nystatin biosynthesis could be assigned. This observation suggests that the right border of the nystatin biosynthetic gene cluster has been identified.

Figure 4



Proposed model for nystatin biosynthesis in *S. noursei*.

Putative mycosamine biosynthesis genes

Three genes presumably involved in biosynthesis and attachment of the mycosamine moiety were found in the cluster (Figure 2; Table 2). The 506 aa-long *nysDI* product shows considerable homology to the eukaryotic UDP-glucuronosyltransferases. The latter enzyme belongs to the UDP-glycosyltransferase family, and is involved in eliminating potentially toxic xenobiotics by the way of their glycosylation [24]. It seems likely that NysDI represents a glycosyltransferase responsible for the attachment of the deoxysugar moiety (mycosamine) to the nystatin aglycone at C-19. The deduced product of *nysDII* (352 aa) is highly similar to perosamine synthetases from different bacteria. The latter enzyme catalyzes conversion of GDP-4-keto-6-deoxy-D-mannose to 4-NH_2-4,6-dideoxy-D-mannose in the biosynthesis of perosamine, which constitutes the backbone structural unit of the lipopolysaccharide O-antigens in pathogenic bacteria [25]. Because the nystatin deoxysugar moiety mycosamine contains an amino group, it is plausible that NysDII represents an aminotransferase involved in mycosamine biosynthesis. The *nysDIII* gene encodes a 344 aa protein similar to GDP-mannose-4,6-dehydratases, and is therefore probably responsible for one of the initial steps in mycosamine biosynthesis. The fact that NysDIII more closely resembles the GDP-mannose-4,6-dehydratases than the TDP-glucose-4,6-dehydratases (Gdh) suggests that the mycosamine biosynthetic pathway in *S. noursei* differs from those of other antibiotics' deoxysugar moieties. This would also explain why our initial attempt to identify the nystatin gene cluster using a Gdh-specific DNA probe failed [11]. The presence of only two deoxysugar biosynthesis genes in the cluster might be explained by the key roles played by the corresponding enzymes in mycosamine biosynthesis. All other enzymes required for this deoxysugar formation can, in principle, be recruited from different (i.e. primary) metabolic pathways.

Modification and transport genes

Three genes encoding proteins presumably involved in modification of the nystatin molecule were identified between *nysK* and *nysDII* (Figure 2). Both the *nysL* and *nysN* genes encode P450 monooxygenases of 394 aa and 398 aa, respectively, that are probably responsible for hydroxylation of the nystatin polyketide moiety at C-10, and oxidation of the methyl group at C-16. Which protein is responsible for which reaction is not clear at the moment, and additional experiments are required for exact placement of NysL and NysN in the nystatin biosynthetic pathway. The *nysM* gene apparently encodes

a ferredoxin of 64 aa, which presumably constitutes a part of one or both P450 monooxygenase systems, and serves as an electron donor [26].

The *nysH* and *nysG* genes localized upstream of *nysDIII* encode 584 aa and 605 aa polypeptides, respectively. Both NysH and NysG display high degrees of similarity to transporters of the ABC family [27], and thus might be involved in ATP-dependent efflux of nystatin.

Downstream of *nysG*, a putative *nysF* gene was identified whose 245 aa product is homologous to the 4′-phosphopantheteine transferases. The latter enzyme carries out the post-translational modification of the ACP domains on the PKSs, which is required for their full functionality [28]. It seems likely, therefore, that the NysF protein functions in modification of the nystatin PKS and is important for the nystatin biosynthesis. DNA sequencing and analysis of the insert in phage N90, which extends the characterized region by ~10 kb to the left of *nysF*, identified genes for signal peptidase, and other proteins with no possible role in nystatin biosynthesis (T.B. and S.B.Z., unpublished observations). The putative *nysF* gene therefore marks the left border of the nystatin biosynthetic gene cluster in *S. noursei*.

Proposed model for the nystatin biosynthetic pathway of *S. noursei*

Based on the information derived from the analysis of the genes found in the nystatin biosynthetic gene cluster we propose a model for synthesis of this antibiotic in the producing organism *S. noursei* (Figure 4). The synthesis starts with loading of the acetyl-CoA onto the NysA protein, and proceeds through condensation of three methylmalonyl-CoA and 15 malonyl-CoA extender units by NysB, NysC, NysI, NysJ and NysK PKS. After cleavage of the mature polyketide chain from the PKS complex by the TE domain of NysK, the chain is cyclized to form the nystatin aglycone. The next two steps in the nystatin biosynthesis are probably accomplished by the NysL and NysN monooxygenases, which perform hydroxylation and oxidation of the macrolactone ring at C-10 and C-16, respectively.

Biosynthesis of mycosamine presumably starts with the L-fructose-6-phosphate, which is converted to GDP-D-mannose through the action of a phosphomannoisomerase, phosphomannomutase, and a GDP–mannose pyrophosphorylase. These enzymes are involved in primary metabolic pathways for cell-surface lipopolysaccharide biosynthesis in many bacteria [25], and the genes for the two former have been located on the chromosome of *S. coelicolor* A3(2) during the genome sequencing effort. It seems likely, therefore, that there is interplay between the primary and secondary metabolisms in *S. noursei* during mycosamine biosynthesis, although it is not apparent how the regulation of such genes is coordinated. The GDP-D-mannose probably serves as a substrate for the NysDIII protein, which converts it to the GDP-4-keto-6-deoxy-D-mannose. A GDP-3-keto-6-deoxy-D-mannose isomerase must perform the next step in mycosamine biosynthesis, probably followed by the NysDII-mediated amidation leading to formation of GDP-mycosamine. The NysDI protein, a putative glycosyltransferase, presumably completes the pathway by attaching the mycosamine moiety to the modified nystatin aglycone.

The model presented above is based on the assumption that both monooxygenases modify the nystatin macrolactone ring prior to its glycosylation. Whether this is true will be verified by the ongoing experiments on inactivation of the *nysL* and *nysN* genes, and structural analysis of the nystatin derivatives produced by the corresponding mutants.

Significance

Analysis of the *Streptomyces noursei* nystatin biosynthetic gene cluster carried out in this study provides useful information with regard to the genes governing synthesis of this important antifungal agent. Functional assignments for the proteins encoded within the cluster suggest a model for the nystatin biosynthetic pathway, and open possibilities for genetic manipulations with the aim of producing novel nystatin derivatives. Identification of several putative regulatory genes associated with the cluster, along with data on inactivation of one potential regulator, imply that they can be used for enhancing the yield of nystatin (and probably its derivatives) in fermentations.

Materials and methods

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *S. noursei* ATCC 11455 and its mutants were grown on solid ISP2 medium (Difco), and in liquid TSB medium (Oxoid). Intergeneric conjugation from *E. coli* ET12567 (pUZ8002) into *Streptomyces* strains was done as reported elsewhere [29], but with the 'heat shock' time reduced to 5 min. *E. coli* strains were grown and transformed as described by others [30], except for *E. coli* ET12567 (pUZ8002), which was maintained on media with 20 μg/ml chloramphenicol and 50 μg/ml kanamycin. Analysis of the nystatin production by *S. noursei* strains was performed in SAO-23 liquid medium [31].

DNA manipulations

Plasmid, phage and total DNA preparations, endonuclease digestions and ligations were performed as described previously [30,32]. DNA fragments were isolated from agarose gels using the QIAGEN Kit (QIAGEN GmbH, Germany), labelled with the use of the digoxygenin kit from Boehringer Mannheim, and used for Southern blot analysis according to the manufacturer's instructions. Genotypes of all disruption mutants obtained in the course of this study (Table 1) were verified by Southern blot analysis (data not shown). DNA sequencing was performed at QIAGEN GmbH, and the data were analyzed with the FramePlot 2.3 online program [33] and GCG software [34].

Amplification of a PKS-encoding DNA fragment used for screening of the S. noursei *gene library*

Degenerate primers used for amplification of a PKS-encoding DNA fragment corresponded to the conserved aa motifs in ACP and KS domains in known type I PKSs, and were designed according to the

codon usage table for *Streptomyces* [35]. The ACP oligonucleotide primer (sense) had the sequence 5′-GAG/C CTG/C GGC/G T/CTG/C GAC TCC/G CTG/C-3′, and the KS oligonucleotide primer (antisense) had the sequence 5′-G/CGA G/CGA G/ACA G/CGC C/GGT GTC G/CAC-3′. The 50 µl PCR mixture contained: 0.1 µg *S. noursei* ATCC 11455 genomic DNA, 25 pm each ACP and KS oligonucleotide primers, dNTPs (final concentration 350 µm), 1× PCR buffer from Expand High Fidelity PCR System (Boehringer Mannheim), and 1.5 U of the DNA polymerase mixture from the same system. The PCR was performed on the Perkin Elmer GeneAmp PCR System 2400 with the following program: 1 cycle of denaturation at 96°C (4 min), 35 cycles of denaturation/annealing/synthesis at 94°C (45 s) and 70°C (5 min), and 1 cycle of final annealing/extension at 72°C (7 min). The 0.7 kb DNA fragment obtained with this procedure was cloned in pUC18 with the use of SureClone Ligation Kit (Pharmacia). One of the resulting recombinant plasmids, pPKS72, was subjected to DNA sequence analysis, and later used for primary screening of the *S. noursei* gene library.

Accession numbers

The DNA sequence reported here was deposited in GenBank under the accession number AF263912.

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Paper III

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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

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Six putative regulatory genes are located at the flank of the nystatin biosynthetic gene cluster in *Streptomyces noursei* ATCC 11455. Gene inactivation and complementation experiments revealed that *nysRI*, *nysRII*, *nysRIII*, and *nysRIV* are necessary for efficient nystatin production, whereas no significant roles could be demonstrated for the other two regulatory genes. To determine the in vivo targets for the NysR regulators, chromosomal integration vectors with the *xylE* reporter gene under the control of seven putative promoter regions upstream of the nystatin structural and regulatory genes were constructed. Expression analyses of the resulting vectors in the *S. noursei* wild-type strain and regulatory mutants revealed that the four regulators differentially affect certain promoters. According to these analyses, genes responsible for initiation of nystatin biosynthesis and antibiotic transport were the major targets for regulation. Data from cross-complementation experiments showed that *nysR* genes could in some cases substitute for each other, suggesting a functional hierarchy of the regulators and implying a cascade-like mechanism of regulation of nystatin biosynthesis.

Antibiotic production by *Streptomyces* bacteria has received much attention in recent years due to the problems associated with a constantly increasing incidence of multiresistant microbial pathogens. Considerable efforts are directed toward understanding the antibiotic biosynthetic pathways in *Streptomyces* spp. and manipulating the corresponding gene clusters in order to produce novel compounds with improved properties (17). In addition, remarkable progress is being made in dissecting the functions of the genes that regulate antibiotic production in streptomycetes (11). Coupling these two fields of research is of great importance both for a fundamental understanding of the antibiotic biosynthesis processes and for the rational engineering of novel antibiotic producers.

Biosynthesis of secondary metabolites, and in particular antibiotics, by *Streptomyces* bacteria is a complex process involving several levels of regulation. Many pleiotropic regulatory genes have been isolated from streptomycetes; in most cases, these genes affect antibiotic biosynthesis by influencing the expression of the pathway-specific regulatory genes (reviewed in reference 11). The latter genes are usually found physically linked to the structural antibiotic biosynthesis genes on the chromosomes of streptomycetes. Both positive and negative regulators directly affecting the expression of structural genes via binding to their promoter regions have been identified in antibiotic biosynthetic gene clusters. In some cases, it has been shown that expression of the pathway-specific regulatory genes is controlled by signaling molecules, such as A-factor, through the action of other regulators encoded by genes located outside of the biosynthetic gene clusters (22). Since antibiotic biosynthesis in *Streptomyces* spp. is linked to the process of cell differentiation (10), it is likely that expression of most of the pathway-specific regulators depends on some sort of signal transmitted via a complex network. Analysis of the regulatory genes in the antibiotic biosynthetic gene clusters is crucial for understanding the mechanisms of regulation, as well as for designing strategies for the construction of strains with enhanced antibiotic production.

Most of the detailed analyses of pathway-specific regulators described in the literature are concerned with the biosynthesis of nonmacrolide antibiotics such as actinorhodin, undecylprodigiosin, and daunorubicin (1, 32, 39). However, several studies where regulatory genes for macrolide antibiotic biosynthesis were analyzed have also been reported. At least some of these regulators must be rather special, since they control the expression of very large polyketide synthase (PKS)-encoding genes, which implies synthesis of unusually long mRNAs. The transcriptional activator SrmR encoded within the spiramycin biosynthetic gene cluster of *Streptomyces ambofaciens* has been shown to be required for the transcription of at least one of the PKS genes involved in assembly of the spiramycin macrolactone ring (14). The *acyB2*-encoded regulator of *Streptomyces thermotolerans* has been shown to activate the expression of the acyltransferase gene involved in biosynthesis of the macrolide antibiotic carbomycin (2). Five regulatory genes associated with the tylosin biosynthetic gene cluster of *Streptomyces fradiae* have been found (3). Gene inactivation experiments have confirmed differential roles for two regulators of the SARP (*Streptomyces* antibiotic regulatory protein) family, TylS and TylT, in controlling tylosin production (4). TylS was shown to control the expression of a global regulator (TylR) for the tylosin cluster, while TylT appeared not to be essential for

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antibiotic biosynthesis. In a separate report, the transcriptional repressor TylQ was found to play a central role in controlling tylosin biosynthesis in *S. fradiae* (31).

A detailed genetic analysis of PikD, the positive regulator for the pikromycin biosynthetic gene cluster in *Streptomyces venezuelae*, has recently been reported (40). PikD belongs to a LAL family of transcriptional regulators containing nucleotide triphosphate (NTP) binding motifs and a C-terminally located helix-turn-helix (HTH) motif of the LuxR type (12). Presumably, these functional features are responsible for the ability of LAL regulators to bind DNA and activate the transcription of target genes upon NTP hydrolysis. It was shown that PikD is required for pikromycin biosynthesis, and the ability of this protein to act as a transcriptional activator depends on the presence of functional NTP binding motifs.

The polyene macrolide antibiotic nystatin produced by *Streptomyces noursei* ATCC 11455 is widely used in treatments of fungal infections. Brautaset et al. have previously cloned and sequenced the entire nystatin biosynthetic gene cluster and located six putative regulatory genes within its flanking region (7). In the present work, we describe a comprehensive in vivo analysis of these genes by means of their inactivation in *S. noursei*, determination of targets by use of the *xylE* reporter system, and cross-complementation experiments.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Bacterial strains, plasmids, and recombinant phages 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, Detroit, Mich.) and grown in liquid Trypticase soy broth (TSB) medium (Oxoid) for DNA isolation. *Escherichia coli* strains were handled by standard techniques (26). Conjugation from *E. coli* ET12567(pUZ8002) to *S. noursei* and gene replacement were performed as described previously (13, 28). Nystatin production was assessed by high-performance liquid chromatography (25) of the dimethylformamide extracts of cultures from 500-ml shake flask fermentations in 100 ml of semidefined SAO-23 medium (28).

DNA manipulation and sequence analysis. General techniques for DNA manipulation were used as described elsewhere (16, 26). DNA fragments were isolated from agarose gels with the QIAEX kit (QIAGEN, Hilden, Germany). Southern blot analysis was performed with a DIG High Prime labeling kit (Roche Biochemicals, Mannheim, Germany) according to the manufacturer's manual. Oligonucleotide primers were purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, United Kingdom). Analyses of the amino acid sequences were performed by using the PSORT Prediction (http://psort.nibb.ac.jp/form.html), MEME (http://meme.sdsc.edu/meme/website/meme.html), Pfam (http://www.sanger.ac.uk/Software/Pfam/search.shtml), and MOTIF (http://motif.genome.ad.jp/) search engines.

Construction of plasmids for gene inactivation. (i) *nysRI* in-frame deletion vector. A 1.37-kb DNA fragment designated SR1, encompassing the region upstream of *nysRI* and some of its coding region, was amplified from the phage N58 template by using primers SOS1 (5′-GCAATGAATTCCGTGGCTCG-3′) and SOS2 (5′-GGCTCTAGAGTCAG TAAGCCGGAAGAAC-3′) (restriction enzyme sites are underlined). A 1.50-kb DNA fragment designated SR2, encompassing the 3′ end of *nysRI* and the downstream region, was amplified from the N58 template by using primers SOS3 (5′-GCCTCTAGAGACCAGGACCGCC ACCTCC-3′) and SOS4 (5′-GACAAGCTTCGGTGCTG CGGACGAGTTC-3′). The SR1 and SR2 PCR products were digested with the *Eco*RI/*Xba*I and *Xba*I/*Hin*dIII endonucleases, respectively, and ligated together with the 3.0-kb *Eco*RI-*Hin*dIII fragment from pSOK201, yielding the nysRI replacement vector pSR12. The in-frame deletion affecting the *nysRI* gene within the pSR12 plasmid eliminated the coding sequence for amino acids (aa) 13 to 943 in the NysRI protein, thus affecting all functional features predicted for this polypeptide.

(ii) *nysRII* in-frame deletion vector. A 1.43-kb DNA fragment designated SR3, encompassing the region upstream of *nysRII* and some of its coding region, was amplified from the phage N58 template by using primers SOS5 (5′-GCAGAA TTCGAGTCCGTGCTGCTCATCG-3′) and SOS6 (5′-GCACTGCAGGTGGT CGGTTGGTTCC-3′). A 1.52-kb DNA fragment designated SR4, encompassing the 3′ end of *nysRII* and the downstream region, was amplified from the N58 template by using primers SOS7 (5′-GGCCTGCAGAGCTGTACCTGCTCCT GG-3′) and SOS8 (5′-GACAAGCTTCCTGCCGCACCAACTCGAC-3′). The SR3 and SR4 PCR products were digested with the *Eco*RI/*Pst*I and *Pst*I/*Hin*dIII endonucleases, respectively, and ligated together with the 3.0-kb *Eco*RI-*Hin*dIII fragment from pSOK201, yielding the *nysRII* replacement vector pSR34. The in-frame deletion affecting the *nysRII* gene within the pSR34 construct eliminated the coding sequence for aa 14 to 936 in the NysRII protein, thus affecting most of this polypeptide, including the C-terminal HTH domain.

(iii) *nysRIII* in-frame deletion vector. A 1.42-kb DNA fragment designated SR5, encompassing the region upstream of *nysRIII* and some of its coding region, was amplified from the phage N58 template by using primers SOS9 (5′-GACG AATTCAACTGGTCGCGCTGTTCTG-3′) and SOS10 (5′-GACCTGCAGTC AGGAGGAGCGAGGAGTC-3′). A 1.50-kb DNA fragment designated SR6, encompassing the 3′ end of *nysRIII* and the downstream region, was amplified from the N58 template by using primers SOS11 (5′-GCACTGCAGTGGAGA AGCACCTCACCAG-3′) and SOS12 (5′-GAGAAGCTTGAGTATTCGGAG GCCGCTC-3′). The SR5 and SR6 PCR products were digested with the *Eco*RI/*Pst*I and *Pst*I/*Hin*dIII endonucleases, respectively, and ligated together with the 3.0-kb *Eco*RI-*Hin*dIII fragment from pSOK201, yielding the *nysRIII* replacement vector pSR56. The in-frame deletion affecting the *nysRIII* gene within the pSR56 construct eliminated the coding sequence for aa 29 to 899 in the NysRIII protein, thus affecting all functional features predicted for this polypeptide.

(iv) *nysRIV* and *orf2* insertional inactivation. The plasmids constructed for insertional inactivation of *nysRIV* and *orf2* were designated pNR4K and pLRD6K, respectively (see Table 1 for details).

(v) *orf3* "frameshift" deletion. A 1.3-kb DNA fragment from the *S. noursei* genome encompassing the 3′ ends of *orf3* and *orf2* was amplified by PCR with primers NR5D1 (5′-GCGAGCGGCCGCTTCACCCCGCAACTCA-3′) and NR5D2 (5′-CGCGAAGCTTGGCCGACTGCTCGACGTC-3′). The PCR product was digested with *Not*I and *Hin*dIII and then ligated with a 1.7-kb *Eco*RI-*Not*I DNA fragment from phage N58 (encompassing *nysRIV* and the N-terminal part of *orf3*) and a 3.0-kb *Eco*RI-*Hin*dIII fragment from pSOK201. The resulting plasmid, pNR5D, contained the *S. noursei* DNA fragment with a 43-bp deletion in the coding region of *orf3*. This deletion creates a frameshift mutation within the ORF3 coding region, subsequently leading to truncation of its product. As a result of this truncation, 165 C-terminal amino acid residues of *orf3* were eliminated and replaced with 14 aa encoded by another reading frame (and thus unrelated to *orf3*).

Construction of plasmids for expression of regulatory genes from the *ermEp promoter. (i) *nysRI* expression vector.** A 0.6-kb DNA fragment representing a promoterless 5′ end of *nysRI* was PCR amplified from the phage N1 template by using primers NR1.1 (5′-CGCCGCATGCTGTTCTCACCCCACGT-3′) and NR1.2 (5′-GGCGCGACCGGTTCGGCCT-3′). The PCR product was digested with *Sph*I/*Age*I and then ligated together with a 2.8-kb *Age*I-*Eco*RI DNA fragment from phage N1 into the pGEM7Zf(−) vector digested with *Sph*I/*Eco*RI. From the resulting construct, a 3.4-kb *Sph*I-*Hin*dIII fragment was isolated and ligated together with a 0.3-kb *Eco*RI-*Sph*I fragment from pGEM7ZfErmE*li, containing the *ermE**p promoter, into the *Eco*RI/*Hin*dIII-digested pSOK804 vector (for details, see Results, Table 1, and Fig. 2), resulting in the pNRE2 construct.

(ii) *nysRII* expression vector. A 2.2-kb *Sal*I-*Bcl*I fragment from phage N58 (representing the 3′ end of *nysRII*) was cloned into *Sal*I/*Bam*HI-digested pGEM11Zf(−). A 0.8-kb fragment representing the 5′ end of the *nysRII* gene was PCR amplified from the phage N58 template with primers NSR2.1 (5′-GC CGGCATGCGACGAACAGGACGAGAGGT-3′) and NSR2.3 (5′-GCCGTG GTCGACGAAGG-3′). The PCR fragment was digested with *Sph*I/*Sal*I and then ligated, together with a 2.2-kb *Sal*I-*Hin*dIII fragment from the pGEM11Zf(−)-based construct, into the *Sph*I-*Hin*dIII-digested pGEM3Zf(−) vector. From the latter, a 3.0-kb *Sph*I-*Hin*dIII fragment was isolated and ligated, together with the 0.3-kb *Eco*RI-*Sph*I *ermE**p promoter fragment, into the *Eco*RI/*Hin*dIII-digested pSOK804 vector, resulting in the pC3A1 construct.

(iii) *nysRIII* expression vector. A 2.8-kb *Sac*I-*Nru*I fragment from phage N58, encompassing 89 nucleotides (nt) upstream of the *nysRIII* start codon and a large portion of the coding region, was ligated together with a 0.5-kb *Nru*I-*Eco*RI fragment from the same phage, representing the 3′ end of this gene, into pGEM3Zf(−). The *nysRIII* gene was excised from this construct as a 3.2-kb *Sph*I-*Eco*RI fragment and ligated into pGEM7Zf(−). From the pGEM7Zf(−)-based construct the *nysRIII* gene was excised as a 3.2-kb *Sph*I-*Hin*dIII fragment and ligated together with the 0.3-kb *Eco*RI-*Sph*I *ermE**p promoter fragment into the *Eco*RI/*Hin*dIII-digested pSOK804 vector, resulting in the pNTE3 construct.

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

Strain, plasmid, or phage	Characteristics	Reference or source[a]
Strains		
S. noursei		
ATCC 11455	WT strain, nystatin producer	ATCC
SR12	WT with in-frame deletion in *nysRI*	This work
SR34	WT with in-frame deletion in *nysRII*	This work
SR56	WT with in-frame deletion in *nysRIII*	This work
NR4K	WT with Km^r insertion in *nysRIV*	This work
NR5D	WT with deletion in *orf3*	This work
DNR609	WT with Km^r insertion in *orf2*	This work
E. coli		
DH5α	General cloning host	BRL
JM110	*dam dcm* strain	Promega
ET12567 (pUZ8002)	Strain for intergeneric conjugation	13
DASHII phage vector derivatives		
N1	Recombinant phage (nystatin gene cluster)	7
N40	Recombinant phage (nystatin gene cluster)	7
N58	Recombinant phage (nystatin gene cluster)	7
N69	Recombinant phage (nystatin gene cluster)	7
N76	Recombinant phage (nystatin gene cluster)	7
Recombinant plasmids		
pGEM3Zf(−)	Cloning vector	Promega
pGEM7Zf(−)	Cloning vector	Promega
pGEM11Zf(−)	Cloning vector	Promega
pGEM7ZfErmE*li	pGEM7Zf(−) vector with *ermE**p promoter	C. R. Hutchinson
pKTO2	VWB-based *Streptomyces* integrative vector	37
pSET152	*E. coli-Streptomyces* conjugative vector	6
pSOK101	*E. coli-Streptomyces* conjugative vector	41
pSOK201	*E. coli-Streptomyces* conjugative vector	41
pSOK804	2.3-kb *Sph*I-*Hin*dIII fragment from pKTO2 ligated with a 3.0-kb *Sph*I-*Hin*dIII fragment from pSET152	This work
pSR12	Vector for *nysRI* in-frame deletion	This work
pSR34	Vector for *nysRII* in-frame deletion	This work
pSR56	Vector for *nysRIII* in-frame deletion	This work
pL58KX5	5.0-kb *Kpn*I-*Xba*I fragment from phage N58 cloned into pGEM3Zf(−)	This work
pNR4Km	1.3-kb Km^r marker inserted into the *Cla*I site within the *nysRIV* gene in pL58KX5 after Klenow fill-in	This work
pNR4D	6.3-kb *Kpn*I-*Sph*I fragment from pNR4Km ligated with a 3.0-kb *Kpn*I-*Sph*I fragment from pSOK101	This work
pNR5D	Vector for *orf3* inactivation	This work
pNR6	3.7-kb *Xmn*I-*Fsp*I fragment from phage N69 with *orf2* cloned into the *Sma*I site of pGEM3Zf(−)	This work
pNR6K	1.3-kb Km^r marker inserted into the *Mlu*I site within *orf2* in pNR6 after Klenow fill-in	This work
pLDR6K	5.0-kb *Eco*RI-*Xba*I fragment from pNR6K ligated with a 3.0-kb *Eco*RI-*Xba*I fragment from pSOK804	This work
pNRE2	Vector for *nysRI* expression	This work
pC3A1	Vector for *nysRII* expression	This work
pNR3T	Vector for *nysRIII* expression	This work
pNR4EL	Vector for expression of a 226-aa version of NysRIV	This work
pNR4ES	Vector for expression of a 210-aa version of NysRIV	This work
pIJ4081	Vector with promoterless *xylE* gene	J. White
pGEM-XylE1	pGEM3Zf(−)-based vector with promoterless *xylE* gene	This work
pAML8	pSOK804-based vector with *xylE* under the control of *nysHp*	This work
pAML9	pSOK804-based vector with *xylE* under the control of *nysAp*	This work
pAML10	pSOK804-based vector with *xylE* under the control of *nysDIIIp*	This work
pAML11	pSOK804-based vector with *xylE* under the control of *nysRIp*	This work
pAML12	pSOK804-based vector with *xylE* under the control of *nysIp*	This work
pAML13	pSOK804-based vector with *xylE* under the control of *nysDIp*	This work
pAML14	pSOK804-based vector with *xylE* under the control of *nysRIVp*	This work

[a] ATCC, American Type Culture Collection; BRL, Bethesda Research Laboratories.

(iv) *nysRIV* expression vectors. The long (L) and short (S) versions of the *nysRIV* gene were PCR amplified from N58 recombinant phage DNA with primers NR4P3 (5′-CTCAGCATGCCGAAAGGATGGCG-3′) and NR4P5 (5′-AGGCAAGCTTCGGCGACACGGGCGT-3′) or primers NR4P4 (5′-CTCAGCATGCGTACGACCGGCGGG-3′) and NR4P5, respectively. The corresponding PCR products of 0.78 (NR4L) and 0.73 (NR4S) kb were digested with *Sph*I and *Hin*dIII and then ligated, together with the 0.3-kb *Eco*RI-*Sph*I fragment containing the *ermE**p promoter, with the *Eco*RI-

*Hin*dIII-digested pSOK804 vector, yielding vectors pNR4EL and pNR4ES, respectively.

PCR amplification of putative promoter regions. Seven intergenic regions from the nystatin biosynthetic cluster that might contain promoters have been amplified by PCR (see Fig. 4). A 315-bp DNA fragment designated *nysHp* and containing the region between the *nysH* and *nysDIII* genes was amplified from the N40 template by using primers NHP1 (5′-GCAGTCTAGAGAGGAACAC CCCGGTTGAC-3′) and NHP2 (5′-GCAGAAGCTTGGCAAACCCTTCTCG AACAC-3′). In PCR a 315-bp intergenic fragment designated *nysDIIIp* was amplified from the N40 template by using primers ND31 (5′-GCAGTCTAGA GGCAA ACCCTTC TCGAACAC-3′) and ND32 (5′-GCAGAAGCTTGAGG AACACCCCGGTTGAC-3′). A 202-bp fragment encompassing the region between the *nysDIII* and *nysI* genes and designated *nysIp* was amplified from the same template with the help of primers NIP1 (5′-GCCAACTGGTAG CAGTT CTCAAGCTTTCG-3′) and NIP2 (5′-GCGGTCTAGACTCAACTCAACCCA TCTCG-3′). The primers for *nysAp*, the intergenic region upstream of the *nysA* gene, were NSAP1 (5′-GCAGAAGCTTCGGTTACTTGGTCTCATGC-3′) and NSAP2 (5′-GCAGTCTAGAGCCTTGCTCACCCCTGCGG-3′); the 212-bp PCR product was amplified from the N76 template. A 212-bp fragment encompassing the region upstream of the *nysDI* gene and designated *nysDIp* was amplified from the N76 template by using primers ND11 (5′-GCAGTCTAGA CGGTTACTTGGTCTCA TGC-3′) and ND12 (5′-GCAGAAGCTTGCCTTG CTCACCCCTGCGG-3′). The 351-bp *nysRIp* and *nysRIVp* DNA fragments upstream of the *nysRI* and *nysRIV* genes, respectively, were amplified from the N58 template by using primers NR11 (5′-GCAGAAGCTTGAGACGGCACCATG CCAC-3′) and NR12 (5′-GCAGTCTAGACACGCGTTCCTCCACGTG-3′) for the *nysRIp* fragment and primers NR41 (5′-GCAGAAGCTTGTCGTACG CCCGTCCGG-3′) and NR42 (5′-GCAGTCCAGAGAGACGCGCATCCTTT CGG-3′) for the *nysRIVp* fragment.

Construction of *xylE*-based promoter probe vectors. PCR-amplified fragments of intergenic regions were digested with the *Hin*dIII and *Xba*I endonucleases and ligated into the pGEM3Zf(−) vector. The 1.5-kb *Xba*I-*Bgl*II fragment with the promoterless *xylE* gene was excised from the pIJ4081 vector and subcloned into *Xba*I/*Bam*HI-digested pGEM3Zf(−), resulting in pGEM-XylE1. The *Hin*dIII-*Xba*I promoter-containing fragments from constructs based on pGEM3Zf(−) were ligated, together with the 1.5-kb *Xba*I-*Eco*RI fragment containing the reporter gene *xylE* from pGEM-XylE1, into the *Hin*dIII/*Eco*RI-digested integrative vector pSOK804, resulting in seven constructs (Table 1). Each of the pSOK804-based constructs contains one of the seven intergenic regions upstream of the reporter gene *xylE*.

The resulting promoter-probe constructs were introduced by conjugation into the *nysRI*, *nysRII*, *nysRIII*, and *nysRIV* mutants for the XylE assay experiments.

Assay for XylE activity. For quantitative enzymatic assays, protein extracts from *S. noursei* cultures were prepared. For the precultures, 20 ml of liquid TSB medium in 250-ml shake-flasks containing 3 g of 3-mm-diameter glass beads was inoculated with spore suspensions and incubated overnight at 30°C with shaking at 250 rpm. On the next day, 50 ml of MP5 medium (containing, per liter, 25 g of glycerol, 3 g of yeast extract, 2 g of NaCl, 0.2 g of K_2HPO_4, 0.2 g of $MgSO_4$, and 0.02 g of $FeSO_4$ [pH 7.2]) was inoculated with 1.5 ml of precultures and incubated at 30°C for 24 or 48 h with shaking at 250 rpm. Cells were harvested by centrifugation for 10 min at 5,000 rpm (Sorvall), washed with 10 ml of 20 mM phosphate buffer (pH 7.2), and resuspended in 10 ml of sample buffer (100 mM phosphate buffer [pH 7.5]–10% acetone [vol/vol]–20 mM EDTA [pH 8.0]). A 3-ml volume of cell suspension was sonicated for 2 min, and 10 μl of 10% Triton-100 was added per ml of extract. Extracts were placed on ice for 15 min and then centrifuged for 10 min at 15,000 rpm, and cell supernatants were used for XylE assays.

The reaction mixture for measurement of catechol dioxygenase activity consisted of 1.9 ml of assay buffer (100 mM phosphate buffer [pH 7.5], 0.2 mM catechol) preincubated at 37°C for 1 min and 100 ml of cell extract. The optical density at 375 nm was measured over 6 min.

Protein concentrations in extracts were measured according to the Bio-Rad Protein Assay method, by using bovine serum albumin as the standard. The catechol dioxygenase activity was calculated as the rate of change in optical density at 375 nm per minute per milligram of protein.

RESULTS

In silico analysis of putative regulatory genes and their deduced gene products. Three genes, designated *nysRI*, *nysRII*, and *nysRIII*, are located downstream of the *nysE* gene, encoding putative thioesterase, in the nystatin biosynthetic gene cluster of *S. noursei* ATCC 11455 (7) (Fig. 1A). The *nysRIII* gene's putative start codon overlaps by 11 nt with the 3′ end of the *nysRII* gene, suggesting that these two genes might be translationally coupled. Analysis of the deduced primary sequences of the proteins encoded by the *nysRI*, *nysRII*, and *nysRIII* genes revealed their significant similarity to each other and to a number of putative transcriptional activators of the LAL family (12) (data not shown). Several functional features were predicted for the NysRI, NysRII, and NysRIII polypeptides (Fig. 1B). Those include HTH DNA binding motifs of the LuxR type (15) located at the C termini of all three proteins (Fig. 2B) and Walker A and B NTP binding motifs (38) at the N termini of NysRI and NysRIII (Fig. 2A). In addition, two putative transmembrane regions were predicted in the central part of NysRIII, while tetratricopeptide repeats (TPR) (20) were detected in both the NysRI and NysRIII polypeptides (see Discussion).

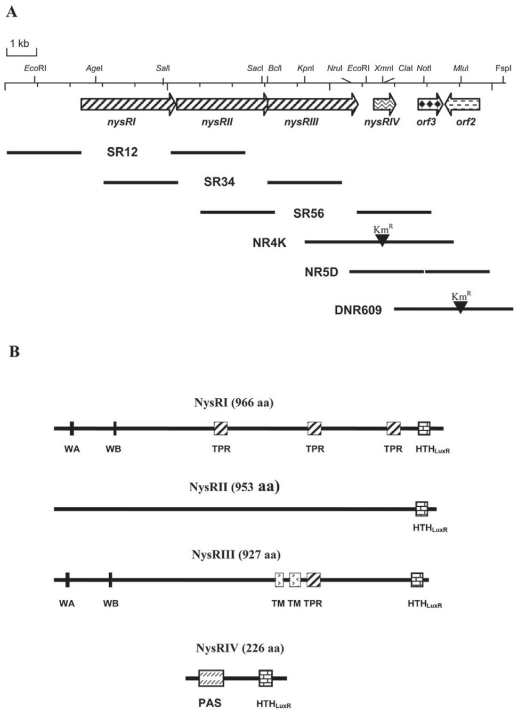

FIG. 1. (A) Organization of the regulatory gene locus associated with the nystatin biosynthetic gene cluster in *S. noursei* ATCC 11455 (GenBank accession number AF263912). The only restriction enzyme sites indicated are those used for vector construction as described in Materials and Methods and Table 1. Heavy solid lines represent DNA fragments used in gene replacement experiments. (B) Putative functional features predicted for the NysRI, NysRII, NysRIII, and NysRIV proteins. WA and WB, Walker A and B NTP binding motifs; PAS, PAS-like domain.

The *nysRIV* gene, previously designated *orf4* (7), is located 404 nt downstream of *nysRIII*. The start codon for *nysRIV* has been reassigned, according to a better match of an upstream

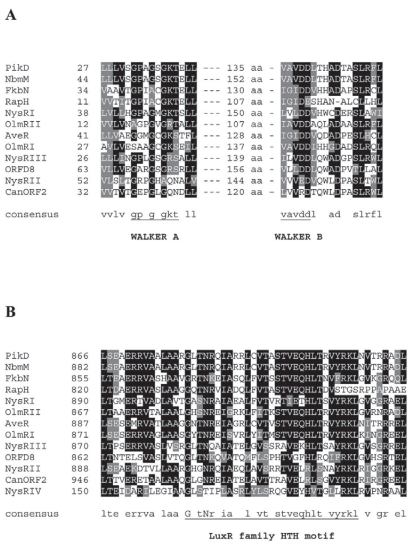

FIG. 2. Amino acid sequence alignment. (A) Walker A and B NTP binding motifs in the N termini of LAL-family regulators. (B) LuxR-type HTH DNA binding motifs at the C termini of LAL-family regulators.

sequence (AGGA) to the consensus Shine-Dalgarno sequence (30), and is likely to be located 48 nt upstream of the start codon originally proposed (7). Thus, *nysRIV* presumably encodes a 226-aa rather than a 210-aa protein (see below). A database search showed a high degree of NysRIV sequence identity (63%) to the regulatory protein PteR encoded within the pentaene macrolide antibiotic gene cluster of *Streptomyces avermitilis* (23). Detailed sequence analysis revealed the presence of a PAS-like domain at the N terminus of NysRIV (33) and a putative C-terminal HTH motif of the LuxR type (Fig. 1B and 2B).

orf3, located downstream of *nysRIV*, encodes a protein of 253 aa similar to transcriptional repressors of the DeoR family (36). *orf2*, which is transcribed in the direction opposite that of all the other putative regulatory genes, encodes a 354-aa polypeptide similar to transcriptional regulators of the AsnC type (19).

Inactivations of the regulatory genes and their effects on nystatin biosynthesis. Disruption of the *nysRI* gene, described previously, has led to complete elimination of nystatin biosynthesis in the *S. noursei* mutant NRD2 (7). However, judging from the operon-like organization of the *nysRI*, *nysRII*, and *nysRIII* genes (Fig. 1A), this mutation very likely had a polar effect. In order to determine the individual roles of these three genes in the regulation of nystatin biosynthesis, we constructed in-frame deletion mutants. The deletions were generated via selection of a second crossover event after integration of the pSR12, pSR34, and pSR56 gene replacement vectors (see Materials and Methods) into the genome of the *S. noursei* wild-type (WT) strain. The resulting mutant strains, SR12, SR34, and SR56 (see Fig. 1A for genotypes), were analyzed for nystatin production.

Mutant SR12 (Δ*nysRI*) produced nystatin at a severely reduced level, i.e., 0.5% of that in the WT (Table 2). This result confirmed the assumed polar effect of the *nysRI* disruption in the NDR2 mutant, since not even traces of nystatin could be detected upon fermentation of the latter (7). Nystatin production in mutants SR34 (Δ*nysRII*) and SR56 (Δ*nysRIII*) was reduced by 93 and 91%, respectively, compared to that in the WT strain (Table 2).

The *nysRIV* gene was inactivated by insertion of the Km^r gene into its coding sequence via a gene replacement procedure using plasmid pNR4D (Table 1; Fig. 1A). The resulting NR4K mutant produced nystatin at a level of ca. 2% of WT production (Table 2).

orf3 and *orf2* were inactivated by deletion and disruption

TABLE 2. Nystatin production by recombinant *S. noursei* strains with inactivated or overexpressed regulatory genes

Strain or background (genotype)	Complementation	Nystatin production (% of WT)[a]
SR12 (Δ*nysRI*)		0.5
	pNRE2 (*ermE**p::*nysRI*)	59
SR34 (Δ*nysRII*)		7
	pC3A1 (*ermE**p::*nysRII*)	100
SR56 (Δ*nysRIII*)		9
	pNRT3 (*ermE**p::*nysRIII*)	100
NR4K (*nysRIV*::Km^r)		2
	pNR4ES (*ermE**p::*nysRIV-S*)	2.5
	pNR4EL (*ermE**p::*nysRIV-L*)	57
ATCC 11455 (WT)	pSOK804	100
	pNRE2 (*ermE**p::*nysRI*)	104
	pC3A1 (*ermE**p::*nysRII*)	121
	pNRT3 (*ermE**p::*nysRIII*)	100
	pNR4EL (*ermE**p::*nysRIV*)	136

[a] Values are means from three independent experiments. In general, variations were within 7% of the mean.

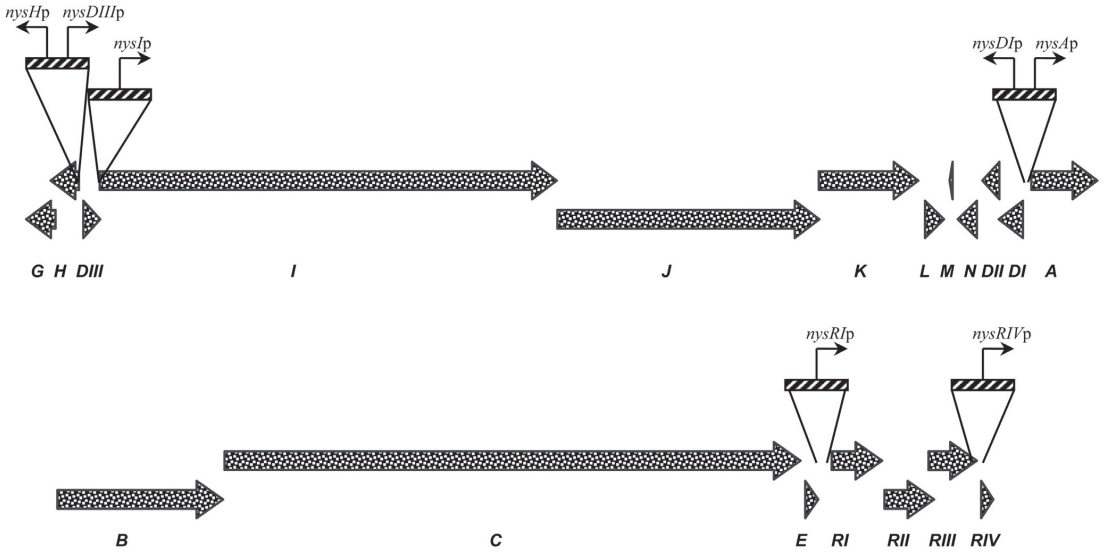

FIG. 3. Putative promoter regions in the nystatin biosynthesis gene cluster used in these studies.

with the Km^r cassette by using plasmids pNR5D and pLDR6K, respectively, creating frameshift mutations (see Materials and Methods) (Table 1). Neither the *orf3* nor the *orf2* mutation had a significant effect on nystatin biosynthesis, suggesting that these genes are not directly involved in the regulation of nystatin biosynthesis, at least under the conditions tested (data not shown).

Complementation of the *nysRI*, *nysRII*, *nysRIII*, and *nysRIV* mutants by expression of the regulatory genes from the *ermEp promoter.** The pSOK804 plasmid vector, containing an integration function (an integrase gene and AttP) from the streptomycete temperate phage VWB (37) and part of the pSET152 vector (6), was constructed (Table 1). Plasmid pSOK804 was able to integrate site-specifically into one site in the genome of *S. noursei* (data not shown), at a frequency about 2 orders of magnitude higher than that for the pSET152 vector previously used for gene expression in *S. noursei* (41). pSOK804-based integration vectors were assembled for the expression of the *nysRI*, *nysRII*, *nysRIII*, and *nysRIV* genes in *S. noursei* (see Materials and Methods and Table 1). To circumvent potential problems related to self-regulation of these genes' endogenous promoters, we chose to use the constitutive *ermE**p promoter (5) for their expression. Five integrative expression vectors were constructed (see Materials and Methods and Table 1) and used for complementation of the corresponding *S. noursei* mutants. The results of these experiments are summarized in Table 2. Nystatin synthesis was either partly or fully restored in the SR12, SR34, SR56, and NR4K mutants upon introduction of the vectors expressing the respective regulatory genes, suggesting that the mutations did not have polar effects. Only vector pNR4EL, expressing the longer, 226-aa version of NysRIV, was able to complement NR4K, thus corroborating the new assignment of the *nysRIV* start codon (see above).

The vectors used in complementation experiments were also introduced into WT *S. noursei* in order to test whether potential overexpression of the regulators might increase nystatin production. Interestingly, while no effect was observed with *nysRI* and *nysRIII*, additional expression of *nysRII* from *ermE**p provided for a 21% increase in nystatin production (Table 2). Expression of *nysRIV* from the pNR4EL vector in the WT *S. noursei* strain had the strongest positive effect on nystatin synthesis: the resulting recombinant strain produced nystatin at a level 36% above that of the WT (pSOK804) (Table 2).

Promoter activity studies with the regulatory mutants. Although definitive roles in controlling nystatin biosynthesis were established for LAL-family regulators and NysRIV by the experiments described above, their individual contributions to the process, as well as the target genes, remained unknown. To address these questions, seven putative promoter regions for the structural and regulatory genes from the nystatin cluster (Fig. 3) were cloned upstream of a promoterless *xylE* reporter gene (see Materials and Methods). Since we deduced that the *nysH-nysDIII* and *nysDI-nysA* intergenic regions contain divergent promoters, these regions were cloned in two alternative orientations to allow the assessment of both promoters. The reporter cassettes were cloned into the pSOK804 integrative vector, and the resulting plasmids were introduced into the *S. noursei* WT strain and the regulatory mutants. XylE activity assays of crude extracts prepared from the recombinant strains were used to monitor relative expression levels from the various promoters (Fig. 4).

In the WT background, XylE activity could be detected for all promoter-probe vectors. Data from the XylE assay (Fig. 4) showed that expression from the promoter for the putative mycosamine transferase gene *nysDI* (and probably for the cotranscribed *nysDII* and *nysN* genes) was 2 times higher in the

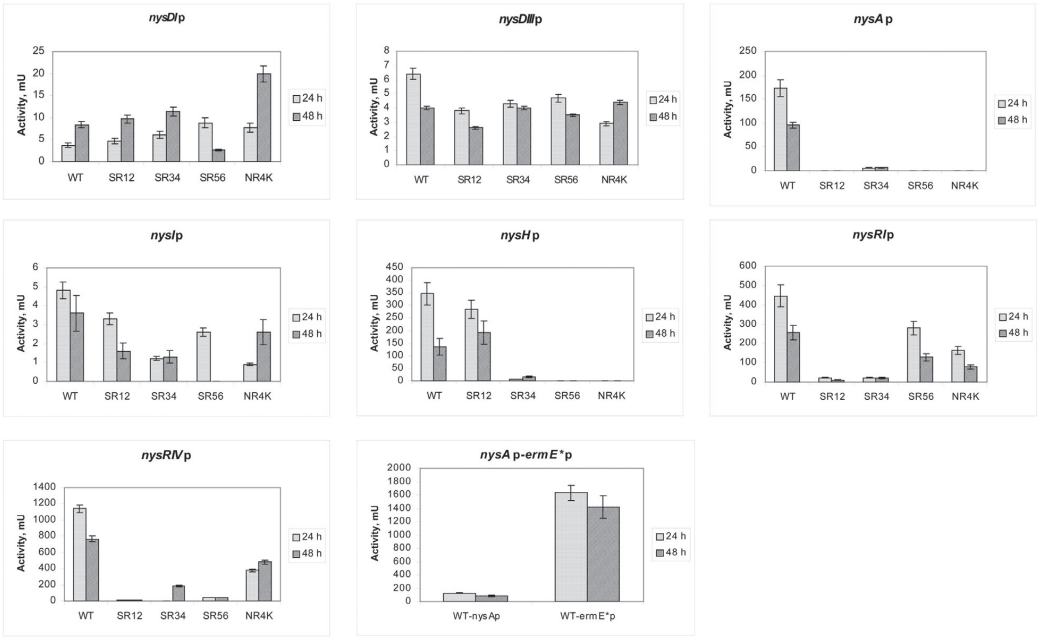

FIG. 4. XylE activities in the protein extracts of WT *S. noursei* and regulatory mutants expressing *xylE* from different promoters. The last diagram shows a comparative analysis of *xylE* expression from the *nysAp* and *ermE*∗p promoters in *S. noursei* ATCC 11455. Variations from the mean in each data series are represented by error bars.

SR56 and NR4K mutants than in the WT background at the 24-h time point. Interestingly, this pattern was changed after 48 h: XylE activity in the SR56 mutant was reduced to ca. 30% of that in the WT, while that in the NR4K mutant continued to increase. *nysDIIIp*, the promoter for a putative GDP-mannose dehydratase gene, depended only weakly on the regulators: the XylE activity measured for the *nysDIIIp*::*xylE* construct in the regulatory mutants was ca. 20 to 50% lower than that in the WT. This difference was most visible at the 24-h time point and was less profound after 48 h.

Expression from the *nysAp* promoter for the PKS loading module gene *nysA* (and presumably for the cotranscribed *nysB* and *nysC* genes) was very strongly dependent on all four regulators. Essentially no XylE activity was observed in the corresponding protein extracts except for the SR34 mutant, where only very low XylE activity (ca. 3% of the WT level) was detected. Compared to the promoters for mycosamine biosynthesis and attachment genes, *nysAp* provided for ca. 30-times-higher XylE expression in the WT.

nysIp, the promoter presumably driving the expression of the NysI, NysJ, and NysK PKS proteins, responsible for further elongation and termination of synthesis of the nystatin polyketide chain, showed limited dependence on the presence of the regulators. The strongest effects observed were those in the SR34 and NR4K mutants at 24 h, where XylE activity due to expression from *nysIp* was ca. 60 to 70% lower than that in the WT background. Interestingly, *nysIp* seemed to be dependent on the NysRIII regulator at 48 h, while no such trend could be observed when XylE activity in the protein extract from the 24-h culture was measured (Fig. 4). Also, the XylE activity in the NR4K mutant almost reached the level of that in the WT at 48 h.

According to the XylE assay, the promoter for the transporter gene *nysH* (and presumably for the cotranscribed *nysG* gene) was essentially independent of NysRI, while its activity was greatly diminished in the *nysRII*, *nysRIII*, and *nysRIV* mutants (see Discussion and Fig. 4).

nysRIp (the promoter region upstream of the regulatory gene *nysRI*) showed only moderate dependence on the NysRIII and NysRIV regulators, since the XylE activities in the corresponding mutants were diminished by ca. 50 to 60% from that in the WT. At the same time, *nysRIp* was strongly dependent on NysRI and NysRII (Fig. 4). This result suggested that NysRI regulates its own expression and that NysRII is involved in this process as well. The *nysRIp* promoter seemed to be very strong and was superseded only by *nysRIVp*, which provided the highest level of XylE expression demonstrated in these experiments. The activity of the *nysRIVp* promoter was greatly affected in all three LAL regulatory mutants, while NysRIV seemed to be moderately autoregulating its own expression, as XylE activity in the NR4K mutant was diminished by ca. 60%.

In order to gain more insight into the results of the complementation experiments described in the preceding section, a control experiment designed to assess the efficiency of the *ermE*∗p promoter in *S. noursei* was performed. In this experiment, XylE activity was measured in protein extracts from the WT strain expressing *xylE* from the *nysAp* and *ermE*∗p promoters. Apparently, *ermE*∗p provided for a much more effi-

TABLE 3. Restoration of nystatin production in cross-complementation experiments with the regulatory mutants[a]

Mutant	Nystatin production (% of WT)[b] with complementation by:			
	pNRE2 (*ermE**p::*nysRI*)	pC3A1 (*ermE**p::*nysRII*)	pNRT3 (*ermE**p::*nysRIII*)	pNR4EL (*ermE**p::*nysRIV*)
SR12 (Δ*nysRI*)	60	58	68	62
SR34 (Δ*nysRII*)	5.4	100	5.6	77
SR56 (Δ*nysRIII*)	8.5	11	98	87
NR4K (*nysRIV*::*Km*^{r})	2.5	2.0	2.5	60

[a] See the text for details.
[b] Values are means from two independent experiments. Variations were within 11% of the mean.

cient (ca. 12-times-higher) expression of *xylE* than *nysAp* (Fig. 4). *ermE**p appears to be the strongest of the promoters investigated in this study.

Cross-complementation experiments. The *xylE* promoter fusion experiments provided important clues on the target genes controlled by the four NysR regulators in the nystatin gene cluster. However, the possible hierarchy of the regulators remained obscure. In order to gain deeper insight into the mechanism of regulation of nystatin biosynthesis, cross-complementation experiments were carried out. The idea behind these studies was to test which of the regulatory genes, when expressed from the heterologous *ermE**p promoter, could substitute for each other in the regulatory mutants.

Accordingly, pSOK804-based expression vectors containing four pathway-specific regulatory genes were introduced into the SR12, SR34, SR56, and NR4K mutants, and nystatin production by recombinant strains was assessed (Table 3). Nystatin production in the SR12 mutant could be restored to the same extent (ca. 60% of the WT level) by introduction of any of the four regulatory genes. Interestingly, both *nysRII* and *nysRIII* were able to complement the SR12 mutant, while no cross-complementation was observed between the *nysRII* and *nysRIII* genes, suggesting that these regulatory genes can be placed on the same hierarchy level (see Discussion). *nysRIV* was able to restore nystatin biosynthesis to a significant level (60 to 87% of the WT) in all regulatory mutants.

DISCUSSION

The results obtained in the gene inactivation experiments clearly show that at least four regulatory genes control nystatin production in *S. noursei*. The *nysRI*, *nysRII*, *nysRIII*, and *nysRIV* genes are required for efficient nystatin biosynthesis and probably represent transcriptional activators for the gene cluster. Clearly, *nysRII* and *nysRIII* are not as important in this respect as *nysRI*, since their inactivation has less profound effects on antibiotic production. This notion is exemplified by the fact that, according to the *xylE* fusion experiments, NysRI regulates an endogenous promoter for its own gene, which is also likely to drive the expression of *nysRII* and *nysRIII*. The operon-like structure of *nysRI-nysRII-nysRIII* and the apparent polar effect of *nysRI* disruption imply that these three genes might be transcribed from the same promoter located upstream of *nysRI*. *nysRIp* is the second strongest (after *nysRIVp*) of the *nys* promoters and remains at least partially active in all regulatory mutants. Both *nysRIp* and *nysRIVp* seem to be weaker than the *ermeE**p promoter when used for the expression of *xylE* in WT *S. noursei* (Fig. 4). This fact, along with the results from the complementation experiments (Table 2), suggests that a high level of expression of the regulatory genes alone does not ensure a high level of nystatin production, since only partial complementation was observed when *nysRI* and *nysRIV* were expressed from *ermE**p. It seems plausible, therefore, that the mechanism governing gene expression in the nystatin biosynthetic cluster requires coordinated expression of the regulatory genes, which is provided through the intrinsic regulatory genes' promoters.

Remarkably, XylE expression from the *nysRIp* promoter is strongly reduced only in Δ*nysRI* and Δ*nysRII* mutants, suggesting that transcription of the *nysRI-nysRII-nysRIII* genes is autoregulated. Taking the above into consideration, it is conceivable that in both of these mutants, neither of the regulators is efficiently expressed. It seems, however, that the basal level of expression of NysRII and NysRIII in the Δ*nysRI* mutant is sufficient to activate the *nysHp* promoter (Fig. 4). The latter appears to be strongly dependent on the availability of the NysRII, NysRIII, and NysRIV regulators.

The NysRI, NysRII, and NysRIII proteins seem to derive from a common ancestor, since they contain homologous regions and their domain organization, especially for NysRI and NysRIII, is similar (Fig. 1B). Proteins with significant similarity to NysRI, NysRII, and NysRIII are found mostly in actinomycetes and are proposed to be transcriptional activators for antibiotic biosynthesis, lipase, and cholesterol oxidase genes (18, 21, 27, 29). Genes encoding proteins similar to NysRI to -III are also located in the biosynthetic gene cluster for the polyene antibiotic candicidin (9). Most of these proteins might be considered members of the LAL subfamily of transcriptional regulators proposed by De Schrijver and De Mot (12), on the basis of their size and the presence of the N-terminal NTP binding and C-terminal LuxR HTH motifs. The NTP binding motifs in the transcriptional regulator PikD have been shown to be required for its activity (40). Therefore, it seems likely that NysRI and NysRIII also require NTP binding and hydrolysis for their function. The presence of the TPRs, which are implicated in protein-protein interactions (35), in NysRI and NysRIII suggests that these proteins might interact with other proteins. It is well documented that protein-protein interactions play an important role in transcriptional control in bacteria (34).

All experimental data obtained for the *nysRIV* gene (see below) point to its central role in controlling nystatin biosynthesis. Indeed, very little nystatin is produced by the *nysRIV* disruption mutant, while expression of *nysRIV* from the *ermE**p promoter results in significant stimulation of nystatin

production in the WT strain. Also, *nysRIV* can complement all *nysR* regulatory mutants. Detection of a PAS-like domain within NysRIV suggests that this protein might respond to the energy levels in the cell. PAS domains are found in many signaling proteins, where they serve as signal sensor domains (24). Promoter probe studies clearly demonstrate that *nysRIVp* is strongly downregulated in all three LAL regulator mutants. However, the *nysRIVp* promoter appears to be the strongest of the seven *nys* promoters studied, and even in the absence of LAL regulators, its activity is at the level of the *nysDIIIp* and *nysIp* promoters. The latter fact suggests that a relatively high level of *nysRIV* transcription is required for this gene's product to exert a positive effect on nystatin biosynthesis.

The *xylE* fusion experiments with the promoters for the nystatin structural genes provided the first clues on the regulatory mechanism controlling nystatin biosynthesis in *S. noursei*. Apparently, promoters driving the expression of *nysDIII* and *nysDI-nysDII-nysN* (presumably cotranscribed) are only weakly dependent on the NysR regulators.

Regulation of the promoters driving the expression of PKS genes, *nysIp* and *nysAp*, is strikingly different. First, the level of XylE expression from the *nysAp* promoter in the WT strain seems to be at least 35 times higher than that from *nysIp*. This is not surprising, since *nysA* encodes the loading module of the nystatin PKS, expression of which is pivotal for initiation of biosynthesis (8). The mutations in the *nysR* regulatory genes have much stronger effects on *nysAp* than on *nysIp* (see Fig. 4), suggesting that initiation of nystatin biosynthesis promoted by the *nysA* gene product is the primary target for the regulators.

Cross-complementation experiments helped to establish a hierarchy among the four NysR regulators of the nystatin gene cluster. Differences in the degree of complementation observed in these experiments could most probably be attributed to the expression of the genes in *trans* from the heterologous promoter. The ability of *nysRII* and *nysRIII* to complement the Δ*nysRI* mutant implies that NysRII and NysRIII can each substitute for NysRI and that expression of these proteins in the SR12 mutant is severely affected. It is not clear, however, why both *nysRII* and *nysRIII* can complement the Δ*nysRI* mutant, since deletion of either of these genes has a detrimental effect on nystatin biosynthesis, and these genes cannot substitute for each other in cross-complementation experiments (Table 3). The answer to this question probably lies in the plausibility of concerted action of the regulators or might be that such complementation is due to the use of a nonnatural constitutive promoter, *ermE*∗p, in the complementation experiments.

Based on the data from promoter analysis and cross-complementation experiments, the following tentative model can be suggested. Expression of the LAL regulatory operon would start with NysRI, which positively regulates its own promoter. However, it seems that NysRII is required for efficient transcription from *nysRIp*, while NysRIII is not essential (Fig. 4). It is thus logical to assume that NysRI and NysRII function in concert as autoregulators of the LAL operon, ensuring its efficient transcription. NysRII seems to play a pivotal role here, as expression of this protein can alleviate the effect of Δ*nysRI* mutation. Since no cross-complementation is observed in the case of *nysRII* and *nysRIII*, it seems likely that their products are both required for efficient *nysRIV* expression. Since *nysRIV* can complement all regulatory mutants, but none of the other regulators can complement NR4K, *nysRIV* most probably directly controls the expression of nystatin biosynthetic genes.

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Paper IV

Chemistry & Biology
Article

Improved Antifungal Polyene Macrolides via Engineering of the Nystatin Biosynthetic Genes in *Streptomyces noursei*

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SUMMARY

Seven polyene macrolides with alterations in the polyol region and exocyclic carboxy group were obtained via genetic engineering of the nystatin biosynthesis genes in *Streptomyces noursei*. In vitro analyses of the compounds for antifungal and hemolytic activities indicated that combinations of several mutations caused additive improvements in their activity-toxicity properties. The two best analogs selected on the basis of in vitro data were tested for acute toxicity and antifungal activity in a mouse model. Both analogs were shown to be effective against disseminated candidosis, while being considerably less toxic than amphotericin B. To our knowledge, this is the first report on polyene macrolides with improved in vivo pharmacological properties obtained by genetic engineering. These results indicate that the engineered nystatin analogs can be further developed into antifungal drugs for human use.

INTRODUCTION

Systemic fungal infections represent a serious problem in medical care, as they usually affect patients whose immune systems have been compromised as a result of HIV infection, anticancer therapy, or immunosuppressive therapy after organ transplantation (Sims et al., 2005; Maschmeyer et al., 2007; Silveira and Husain, 2007). The number of antifungal agents that can be used to treat such infections is currently limited to azoles (e.g., voriconazole), echinocandins (e.g., caspofungin), and polyene macrolides (e.g., amphotericin B). All the above-mentioned antifungals have their own therapeutic limitations because of drug-drug interactions, development of resistance, narrow spectrum of activity, and toxicity (Scott and Simpson, 2007; Perlin, 2007; Spanakis et al., 2006; Zotchev, 2003). The polyene macrolide amphotericin B (AmB) has been used for treatment of systemic fungal infections for several decades, showing excellent efficacy against a number of fungal pathogens. At the same time, severe side effects, such as nephrotoxicity, and suboptimal pharmacokinetics, undermine therapeutic value of this antibiotic. A number of AmB analogs, both semisynthetic and genetically engineered, have been generated over the last 20 years in an attempt to reduce its toxicity and improve solubility (Falk et al., 1999; Paquet and Carreira, 2006; Seco et al., 2005; Carmody et al., 2005). Despite that, no new AmB-based antifungal has appeared on the market, except for the lipid and liposomal formulations. However, studies of the AmB analogs have generated important data on the structure-activity relationship of polyene macrolides that may also be at least partially applicable to other scaffolds belonging to the same type of compounds. According to these studies, the exocyclic carboxyl and the amino group of mycosamine seem to be particularly important for selective toxicity and activity (Mazerski et al., 1995; Borowski, 2000). Moreover, Power et al. (2008) have recently generated a mutant of *Streptomyces nodosus* with inactivated ketoreductase (KR) domain in module 16 of the AmB polyketide synthase (PKS) that was shown to produce 7-deoxy-7-oxy AmB. The latter compound has been shown to retain antifungal activity, while having reduced hemolytic activity, compared with that of AmB. To our knowledge, none of these AmB analogs has been tested in vivo.

Polyene macrolides are mostly produced by *Streptomyces* bacteria and exert their fungicidal action via interaction with membrane sterols, resulting in the formation of highly organized hydrophilic channels, through which small molecules and ions can leak out (Ōmura and Tanaka, 1984; Teerlink et al., 1980; Baginski et al., 2006). These antibiotics appear to have a higher affinity toward membranes containing ergosterol (e.g., in fungi and some parasites), compared with cholesterol-containing membranes (mammalian cells) (Gagos et al., 2005). Still, polyene macrolides bind to the cholesterol-containing membranes to some extent, thus causing lysis of particularly vulnerable cells (e.g., in kidneys) and subsequent organ damage.

The polyene macrolide antibiotic nystatin produced by *Streptomyces noursei* ATCC 11455 is an important antifungal agent used in human therapy for treatment of superficial mycoses. Its use for treatment of systemic mycoses is precluded by toxicity,

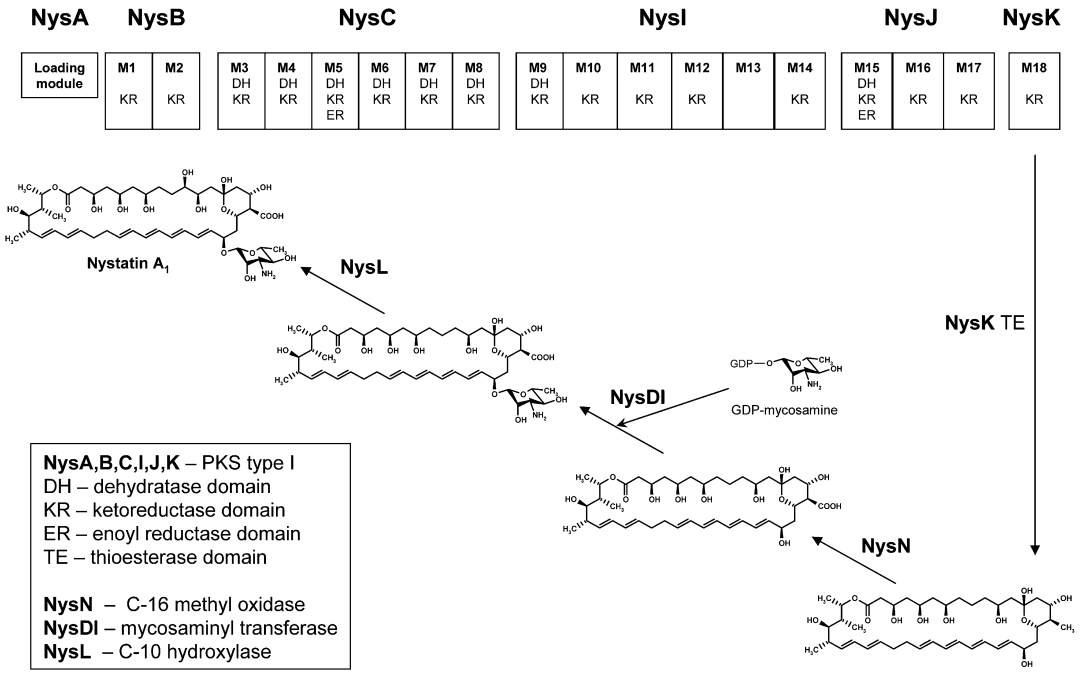

Figure 1. Proposed Nystatin Biosynthesis in *Streptomyces noursei*
Only reductive domains in the nystatin PKS modules are shown.

solubility problems, and lower antifungal activity, compared with that of AmB (Hamilton-Miller, 1973). The nystatin biosynthetic pathway has been established after cloning of the entire biosynthetic gene cluster (Brautaset et al., 2000). It comprises assembly of the 38-member macrolactone ring, followed by oxidation of a C-16 methyl group, attachment of deoxysugar mycosamine at C-19, and hydroxylation at C-10 (Fjærvik and Zotchev, 2005) (Figure 1).

Using genetic engineering, we have recently obtained a heptaene nystatin analog S44HP with antifungal activity considerably higher than that of nystatin and equal to that of AmB (Bruheim et al., 2004). S44HP is structurally similar to AmB but has significantly different properties. For example, S44HP was found to be ~10 times more soluble than AmB and, despite having a lower maximal tolerated dose (MTD) value compared with the latter, seems to have a wider therapeutic window (MTD-LD_{50} dose interval) (Treshchalin et al., 2005). We hypothesized that further derivatization of S44HP might yield new polyene macrolides with improved pharmacological properties, which can be useful for development of safer antifungals. In the present work, we report the generation of new nystatin and S44HP analogs by means of manipulation of the nystatin biosynthetic genes and their biological characterization. Two of these analogs tested in vivo displayed high efficacy and considerably lower acute toxicity, compared with AmB, suggesting that they can become lead compounds for development of new antifungal drugs for human use.

RESULTS AND DISCUSSION

Specific Change in the C-9–C-10 Polyol Region of Nystatin Leads to a Significant Reduction of Hemolytic Activity

The polyol region on the polyene macrolide molecule has recently become a potentially interesting target for modification through biosynthetic engineering. Caffrey and co-workers have demonstrated that replacement of the C-7 hydroxyl group on the AmB molecule with a keto group leads to over 10-fold reduction of in vitro hemolytic activity and ~4-fold reduction of antifungal activity (Power et al., 2008). On the nystatin A_1 molecule, the C-1–C-15 polyol region is interrupted by a saturated C-9–C-10 bond formed as a result of the activity of KR, dehydratase (DH), and enoyl reductase (ER) domains in the module 15 of the nystatin PKS NysJ (Figure 1). To gain a better insight into structure-activity relationship of nystatin, we inactivated DH15 domain of NysJ by site-specific mutagenesis of the active site His followed by gene replacement in *S. noursei* NDA59, yielding mutant NJDH15 (see Table S1 available online). The host strain NDA59 has a *nysA* deletion, and nystatin production can be restored to almost 100% by complementation with *nysA* gene (Brautaset et al., 2003).

Mutant NJDH15 was complemented with the *nysA* gene, cultivated in the fermentor, and DMSO extracts were assayed for polyene production by liquid chromatography (LC)-diode array detector (DAD)-time of flight (TOF). The analysis unraveled the

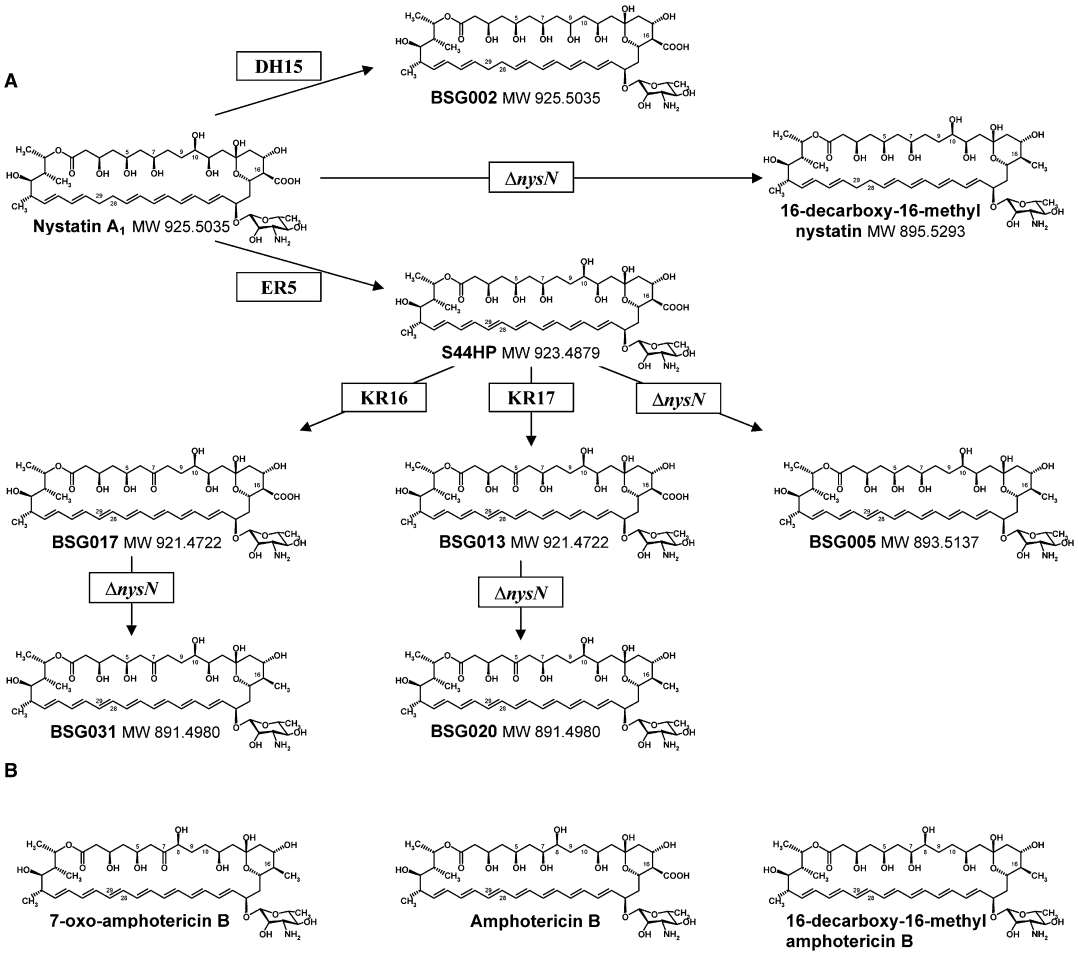

Figure 2. Genetically Engineered Polyene Macrolides

(A) Molecular structures of nystatin and its analogs generated by biosynthetic engineering.

(B) Molecular structures of amphotericin B analogs produced via manipulation of biosynthetic genes (Carmody et al., 2005; Power et al., 2008).

production of an analog with a mass (m/z) corresponding to the stoichiometric formula of nystatin (less than 2 ppm difference from the theoretical mass) in both ESI+ (positive ionization) and ESI− (negative ionization) modes (Figure S1). The volumetric production yield of this analog, denoted BSG002 (0.88 ± 0.04 g/l) was about 30% of the nystatin production level obtained in the wild-type strain under the same conditions (Table S2).

Since the expected change shall affect the C-9–C-10 region, we hypothesized that the NysL hydroxylase (Volokhan et al., 2006) might not recognize the altered C-9–C-11 region on the new molecule, thus failing to perform C-10 hydroxylation. Thus, a new analog with a molecular weight identical to that of nystatin can be explained if this compound has acquired a C-9 hydroxyl, but lacks C-10 hydroxyl group. To verify this hypothesis, the major compound produced by the NJDH15 mutant was purified using preparative high-pressure liquid chromatography (HPLC), and the concomitant nuclear magnetic resonance (NMR) analysis (see Supplemental Text and Figure S2) clearly supported our hypothesis that BSG002 indeed represents 9-hydroxy-10-deoxy nystatin (Figure 2).

Purified BSG002 (purity >95% and concentration of active principle adjusted according to the purity) was then subjected to the in vitro tests for antifungal and hemolytic activities (see Experimental Procedures). BSG002 was found to be at least 2-fold less hemolytic, compared with nystatin. However, its antifungal activity was simultaneously reduced ~4-fold (Table 1). According to the model of the channel formed by polyene macrolides, the hydroxyl groups in the polyol region create a hydrophilic

Table 1. In Vitro Determined Antifungal and Hemolytic Activities of the Genetically Engineered Nystatin Analogs

Compound	MIC_{50} (μg/ml) [a]	MIC_{90} (μg/ml) [a]	HC_{50} (μg/ml) [b]
Nystatin	1.2 ± 0.2	2.0 ± 0.3	85
BSG002	4.8 ± 0.4	10.5 ± 3.5	180
16-DecNys	1.3 ± 0.4	1.8 ± 0.5	175
AmB	0.12 ± 0.03	0.19 ± 0.03	2.6
S44HP	0.12 ± 0.03	0.20 ± 0.03	2.5
BSG005	0.07 ± 0.02	0.12 ± 0.03	4.0
BSG013	0.25 ± 0.07	0.43 ± 0.07	3.0
BSG017	0.47 ± 0.15	0.92 ± 0.03	3.3
BSG020	0.15 ± 0.03	0.19 ± 0.03	9.0
BSG031	0.18 ± 0.06	0.37 ± 0.07	3.8

The ± values represent maximum deviation from the mean.
[a] Tested as described in Nedal et al., (2007) using *Candida albicans* ATCC 10231 as a test organism.
[b] Tested by using horse blood erythrocytes and antibiotic concentrations ranging between 0 and 200 μg/ml.

environment allowing for leakage of ions from the affected cells. One could expect that the addition of a hydroxyl group at C-9 might increase the conductance of the channel, and hence increase antifungal activity, while removal of C-10 hydroxyl should have no effect, as suggested by the data on 10-deoxy nystatin (Volokhan et al., 2006).

Replacement of the C-16 Carboxyl on the Nystatin Molecule with a Methyl Group Reduces Hemolytic Activity Without Affecting the Antifungal Activity

Replacement of a C-16 carboxyl with a methyl group on the AmB molecule by means of inactivation of the methyl oxidase AmphN yielded a less hemolytic analog with retained antifungal activity (Carmody et al., 2005). The *S. noursei* nystatin gene cluster contains a *nysN* gene encoding a putative P450 monooxygenase earlier implicated in oxidation of the C-16 methyl to afford a carboxyl group on the mature antibiotic molecule (Brautaset et al., 2000). Accordingly, *S. noursei* mutant CL346AS, carrying a mutation in the *nysN* gene replacing the conserved Cys residue (Cys346) of NysN presumed to be responsible for heme binding with Ala, was constructed (Table S1). This mutation was predicted to abolish activity of NysN on the basis of the data reported for another P450 monooxygenase (Vatsis et al., 2002). Mutant strain CL346AS was cultivated in the fermentor and analyzed for production of polyene macrolides, as described above. The expected mass corresponding to the stoichiometric formula of 16-decarboxy-16-methyl nystatin ($C_{47}H_{77}NO_{15}$) was identified (<3 ppm difference from the theoretical mass) in both ESI+ and ESI− modes in the culture extract (Figure S3). The production level of 16-decarboxy-16-methyl nystatin (16-DecNys) was very low (0.06 ± 0.01 g/l) and represented less than 2%, compared with the nystatin production in the wild-type strain (Table S2). Similarly low production yield of 16-decarboxy-16-methyl AmB analog has been observed in *S. nodosus* upon inactivation of the *amphN* gene (Carmody et al., 2005). Since we did not observe any abnormal growth for the CL346AS mutant, it seems unlikely that 16-DecNys is toxic to the producing organism. Potentially, accumulation of the latter analog may cause feedback inhibition of the nystatin biosynthetic pathway by an unknown mechanism (see below).

In vitro hemolytic assay of the purified 16-DecNys (purity 75% and concentration of active principle adjusted according to the purity) revealed about 2-fold increased HC_{50} value for this compound, compared with the parental antibiotic nystatin (Table 1). At the same time, its antifungal activity remained unchanged, as judged from the MIC_{50}/MIC_{90} values for *Candida albicans*, which were 1.2 ± 0.2/2.0 ± 0.3 μg/ml and 1.3 ± 0.4/1.8 ± 0.5 μg/ml for nystatin and 16-DecNys, respectively (Table 1). Combined, these data imply that replacement of the C-16 carboxyl with a methyl group reduces the toxicity of nystatin, while having no effect on its antifungal activity. These data are in good agreement with the analogous result previously documented for 16-decarboxy-16-methyl AmB (Carmody et al., 2005). Because of the low yield, we were unable to produce 16-DecNys in quantity and purity sufficient for NMR analysis of the structure and in vivo animal experiments.

C-16 Modification of the Heptaene Nystatin Analog S44HP Yields Less Hemolytic and More Active Polyene Macrolide

Encouraged by the in vitro data obtained for 16-DecNys (see above), we introduced identical C-16 modification on the molecule of the heptaene nystatin analog S44HP, which was shown to have 6-fold higher in vitro antifungal activity than nystatin (Bruheim et al., 2004). The CL346AS mutation in the *nysN* gene was introduced into the S44HP-producing mutant *S. noursei* GG5073SP (Borgos et al., 2006a), generating mutant BSM1 (Table S1). BSM1 was cultivated in the fermentor and assayed for polyene macrolides by LC-DAD-TOF. The expected mass corresponding to the stoichiometric formula of 16-decarboxy-16-methyl-28,29-didehydro nystatin ($C_{47}H_{75}NO_{15}$), was identified in the culture extract (<1.5 ppm difference from the theoretical mass) in the ESI− mode (Figure S4), and this compound was designated BSG005 (Figure 2). Interestingly, the BSG005 production level by BSM1 in the fermentor (0.53 ± 0.01 g/l) was about 40%, compared with the S44HP production level by parental strain GG5073SP (Table S2). This was ~20 times higher than the relative production level of 16-DecNys by the analogous *nysN* mutant CL346AS constructed on the wild-type background (see above), and the biological reason for this discrepancy is unknown. It is possible that the C28-29 double bond in BSG005 somehow negatively affects its ability to inhibit the nystatin biosynthetic pathway, whereas 16-DecNys, which has a saturated C28-C29 bond, has much higher potential for inhibiting the pathway. Chemical structure of the expected BSG005 analog, 16-decarboxy-16-methyl-28,29-didehydro nystatin, was confirmed by NMR experiments on purified compound (Figures S5 and S6).

In vitro assay for the purified BSG005 (purity >95%) revealed its ~2-fold and 20-fold increased antifungal activity, compared with S44HP and nystatin, respectively (Table 1; Figure S7). At the same time, its hemolytic activity was reduced ~1.5-fold, compared with S44HP (Table 1). Together, these data imply that replacement of the C16-carboxyl group on S44HP with a methyl has a positive effect on the pharmacological properties of this compound under the conditions tested. These results are in agreement with the similar data reported for noncarboxylated analogs of both AmB and rimocidin (Seco et al., 2005; Carmody

et al., 2005; Palacios et al., 2007), although the MIC values for the latter two compounds have not been reported.

C-5 and C-7 Keto Modifications in the S44HP Polyol Region Yield Analogs with Reduced Antifungal and Hemolytic Activities

According to the current model, the polyol regions of antibiotic molecules with multiple hydroxyl groups outline the inner part of the channel piercing the target cell membrane (Baginski et al., 2002). The hydrophilic properties of the channel are most likely affected by the number and position of the hydroxyls, and thus variation of these properties might theoretically affect the channel's ion selectivity and conductivity. Power et al. (2008) have reported biosynthetic engineering of the 7-oxo-AmB analog with reduced hemolytic activity. We decided to generate S44HP analogs, where either C-5 or C-7 hydroxyls in the polyol region were replaced with keto groups, and to compare their biological properties. The KR17 domain of the nystatin PKS NysJ protein is responsible for catalyzing the reduction the C-5 keto group to afford the C-5 hydroxyl during the biosynthesis of the nystatin macrolactone ring (Brautaset et al., 2000) (Figure 1). To generate an S44HP analog with retained C5-keto group, we introduced the double mutation TA5145FE affecting the proposed KR17 active site Tyr residue in the nystatin PKS NysJ. The KR16 domain of the NysJ PKS is responsible for catalyzing the reduction the C-7 keto group to afford the C-7 hydroxyl on the nystatin molecule. To generate an S44HP analog with retained C7-keto group, we introduced the double mutation YA3404FE affecting the KR16 active site Tyr3404 residue in NysJ. The KR17 and KR16 mutations were individually introduced to the *S. noursei* mutant GG5073SP, generating mutants BSM2 and BSM4, respectively (Table S1).

The two latter strains were cultivated in the fermentor and assayed for polyene macrolides by LC-DAD-TOF. Accurate masses, which correlated well with the expected 5-oxo-5-deoxy-28,29-didehydro nystatin and 7-oxo-7-deoxy-28,29-didehydro nystatin (both with stoichiometric formula $C_{47}H_{71}NO_{17}$) were found in ESI− and ESI−/ESI+ modes, respectively, (<5 ppm difference from the theoretical mass, Figures S8 and S9). The compounds produced by the BSM2 and BSM4 mutants were designated BSG013 and BSG017, respectively (Figure 2). The volumetric production yields of BSG013 (0.73 ± 0.01 g/l) and BSG017 (0.63 ± 0.07 g/l) correspond to about 56% and 49%, respectively, of the S44HP production yield obtained for the parental strain GG5073SP (Table S2). Chemical structure of BSG013 was also confirmed by NMR experiments (Figure S5). Structurally, BSG013 and BSG017 seem to be identical to the previously reported polyene macrolides mycoheptin (Borowski et al., 1978) and candidin (Volpon and Lancelin, 2002), respectively.

Next, purified BSG013 and BSG017 (purity >95%) were tested in vitro for hemolytic and antifungal activities, as described above. Compared to S44HP, BSG013 showed ~2-fold reduced antifungal activity with concomitantly 1.2-fold increased HC_{50} value (Table 1; Figure S7). BSG017 showed a 4-fold reduced antifungal activity and 1.3-fold increased HC_{50} value compared to S44HP (Table 1; Figure S7). These data indicated that substitutions of C-5 or C-7 hydroxyls with keto groups reduce both antifungal and hemolytic activities of the S44HP analogs. These data were consistent with those reported for 7-oxo AmB analog, where reduction of both antifungal and hemolytic activities have been observed (Power et al., 2008). However, reduction of hemolytic activity upon replacement of C-5 and C-7 hydroxyls with keto groups on S44HP was not as dramatic as in the case of 7-oxo AmB.

Combination of C-16 with C-5 or C-7 Keto Modifications on S44HP Results in Analogs with Further Reduced Hemolytic Activity and High Antifungal Activity

Considering that BSG005, BSG013, and BSG017 were all less hemolytic than S44HP in vitro, we decided to combine the corresponding modifications on these molecules in an attempt to obtain an analog with further reduced toxicity. The CL346AS *nysN* mutation that was used to construct mutant BSM1 was introduced into the BSG013- and BSG017-producing mutants BSM2 and BSM4, yielding recombinant strains BSM3 and BSM5, respectively (Table S1). The two latter strains were cultivated in the fermentor, and production of polyene macrolides was assessed by LC-DAD-TOF. Accurate masses which correlated well with the 5-oxo-5-deoxy-16-decarboxy-16-methyl-28,29-didehydro nystatin and 7-oxo-7-deoxy-16-decarboxy-16-methyl-28,29-didehydro nystatin (both with stoichiometric formula $C_{47}H_{73}NO_{15}$), were found in ESI− and ESI+ modes (all with <3 ppm difference from the theoretical masses, Figures S10 and S11). The analogs identified were designated BSG020 and BSG031, respectively (Figure 2). In addition, tandem mass spectrometer (MS)-MS data were used to confirm the chemical structure of BSG020 (Figure S12). The volumetric production yields of BSG020 (0.26 ± 0.02 g/l) and BSG031 (0.37 ± 0.04 g/l) corresponded to ~20% and 29%, respectively, of that of S44HP produced by the parental strain GG5073SP (Table S2). These data demonstrate that several alterations can simultaneously be introduced into the nystatin biosynthetic pathway without dramatically affecting production yields of new analogs.

The purified compounds BSG020 and BSG031 (93% and 80% pure, respectively) were subjected to in vitro activity and toxicity assays as described above. The data from these experiments showed that BSG020 and BSG031 had ~3.5- and 1.5-fold increased HC_{50} values, compared with S44HP, respectively, and that their antifungal activities were improved compared to those of their parental oxo-analogs. (Table 1 and Figure S7). Together, these data indicate that the combination of the oxo substitutions in the polyol region and C-16 methyl group can potentially be beneficial for improvement of therapeutic index of S44HP. It should be noted that AmB analogs with combined C-15-oxo and C-16-methyl modifications have been obtained by Power et al. (2008). Unfortunately, yields of these analogs had apparently been too low for purification of substantial amounts of materials, although crude samples clearly exhibited antifungal activity. We have failed to generate a mutant producing C-15-oxo analog of S44HP in detectable amounts, although two alternative mutations for KR12 inactivation in the nystatin PKS NysI were attempted (data not shown).

BSG005 and BSG020 Have Considerably Reduced Toxicity, and BSG005 also Displays Improved Antifungal Activity, Compared with AmB In Vivo

On the basis of all the accumulated in vitro data (see above), one nystatin analog (BSG002) and two S44HP analogs (BSG005 and

Table 2. In Vivo Acute Toxicity and Antifungal Activity of Engineered Nystatin Analogs Tested in a Mouse Model of Disseminated Candidosis

Compound	MTD mg/kg	LD_{50} mg/kg	*Candida* kidney load, CFU/g (% MTD)[a,b]
Control	NA	NA	6.5×10^4
Nystatin	7.1	8.5	3.4×10^3 (56%)
BSG002[c]	53.7	61.7	6.3×10^4
S44HP	0.6	2.1	4.2×10^3 (94%)
BSG005	7.8	15.4	2.9×10^3 (10%)
BSG020	7.9	10.5	6.5×10^3 (10%)
AmB	2.0	2.8	4.0×10^3 (40%)

[a] After 4 days of single dose administration of 0.8 mg/kg, except for nystatin (4 mg/kg), BSG002 (32 mg/kg) and S44HP (0.6 mg/kg).
[b] Average data for 3 animals.
[c] No activity demonstrated for doses up to 32 mg/kg.

BSG020) were chosen for in vivo studies on acute toxicity and antifungal activity against disseminated candidosis in a mouse model. First, BSG002 was investigated and compared to its parental compound nystatin. Preparations containing different antibiotic concentrations were administered intravenously, and median lethal and maximum tolerated doses (LD_{50} and MTD values, respectively) were calculated from the experimental data. BSG002 was shown to have considerably lower acute toxicity than nystatin in these experiments; the MTD/LD_{50} values for BSG002 and nystatin were 53.7/61.7 mg/kg and 7.1/8.5 mg/kg, respectively (Table 2). These data were in good agreement with the corresponding in vitro data (Table 1). BSG002 and nystatin were then used in a mouse candidosis model in order to assess their antifungal activity in vivo (see Experimental Procedures). The fungal load in the kidneys of *C. albicans* infected animals treated with antibiotics was determined and compared with that of the untreated animals. In this experiment, nystatin demonstrated in vivo antifungal activity (reduction of *Candida* colony-forming units [CFU] in kidneys by a factor of >10) at doses equal or higher than 4 mg/kg, whereas no activity was shown for BSG002 at doses up to 32 mg/kg (Table 2). The fact that BSG002 lacks activity in a mouse model of candidosis, while having reduced but still significant activity in vitro, suggests that such factors as stability in vivo and pharmacokinetics might play a decisive role for therapeutic usefulness of polyene macrolides.

Next, BSG005 and BSG020 were tested in similar experiments, and the data obtained for both analogs clearly demonstrated higher MTD and LD_{50} values, compared with those for both S44HP and AmB, suggesting that they have considerably lower toxicity in vivo (Table 2). Compared with S44HP, the MTD value was increased ~13-fold for both BSG005 and BSG020, indicating significant reduction of toxicity. These data were in some agreement with the in vitro data on hemolytic activities of these compounds, whereas the effects in vivo were much more profound (Table 1). When assessed for efficacy in a mouse candidosis model, the effective doses for AmB, S44HP, BSG005, and BSG020 (Table 2) were considerably lower than that for nystatin and correlated well with the in vitro data for antifungal activity as presented in Table 1. BSG005 displayed in vivo antifungal activity slightly higher than those of both AmB and S44HP, whereas the antifungal activity of BSG020 was somewhat reduced compared to these control compounds. Again, these data were in agreement with the in vitro antifungal activity tests showing that BSG020 displays a lower antifungal activity than does BSG005 (Table 1). Most importantly, these in vivo antifungal activities were observed at doses corresponding to only ~10% of MTD for both BSG005 and BSG020. At the same time, 40% and 94% MTD doses for AmB and S44HP, respectively, had to be used to achieve the same efficacy in reducing fungal kidney load. The latter data confirm that both BSG005 and BSG020 have favorable activity/toxicity properties, compared with AmB, under these conditions and suggest that they may become promising lead compounds for further development as antifungal agents.

Structure-Activity-Toxicity Relationship of Engineered Nystatin Analogs

The impact of modifications introduced into three different regions of the nystatin molecule on its antifungal activity and toxicity has been investigated both in vitro and in vivo in this study. In particular, the polyene region (C-28–C-29), the exocyclic carboxyl group (C-16), and the polyol region (C-5, C-7, C-9, and C-10) were targeted for modifications. The data obtained for S44HP supports our previous observation (Bruheim et al., 2004) that modification of the polyene region resulting in the appearance of seven instead of four conjugated double bonds increases both antifungal activity and toxicity. This result is consistent with significantly higher antifungal activity and toxicity reported for the heptaene polyene macrolide AmB, compared with that of nystatin. The data obtained for C-16 methyl substituted S44HP analog demonstrated that this modification leads to increased antifungal activity and reduced toxicity, whereas the analogous modification reported for AmB caused reduced toxicity while leaving antifungal activity presumably unchanged (Carmody et al., 2005). Modifications in the polyol region of nystatin (C-5, C-7, and C9+C10) yielded compounds with both reduced antifungal activity and toxicity, whereas the effect varied among these modifications. Interestingly, the C-7 modification in AmB (Power et al., 2008) had no effect on antifungal activity, while causing reduced toxicity, thus further confirming differences between AmB and S44HP in terms of structure-activity relationship. Taken together, our data suggest that the polyol and polyene region, as well as C-16 exocyclic, are all important for the antifungal activity and toxicity of nystatin and S44HP. Moreover, our data obtained for the analogs combining different modifications indicate that simultaneous introduction of C-16 methyl and C-5 keto group on S44HP may be beneficial for the activity/toxicity profile. Although some of these results could potentially have been expected on the basis of the data available for the genetically engineered AmB analogs (Carmody et al., 2005; Power et al., 2008), certain modifications in the polyol region of nystatin, such as at C-7 and C-9/C-10, had a significant negative effect on antifungal activity, while contributing little to the reduction of toxicity.

SIGNIFICANCE

There is an urgent need for efficient and safe antifungal agents because of the growing number of life-threatening systemic fungal infections. Polyene macrolide antibiotics

are potent fungicidal agents, but their medical usefulness is hampered by considerable toxicity. A series of new analogs of the polyene macrolide antibiotic nystatin was generated by means of biosynthetic engineering. Testing of these analogs in vitro for antifungal and hemolytic activities provided important new data on structure-activity-toxicity relationship and allowed selection of the most active and least toxic analogs. In vivo experiments in a mouse model revealed that two new nystatin analogs, BSG005 and BSG020, are at least as efficient as AmB, the only polyene macrolide currently used for treatment of systemic fungal infections, against disseminated candidosis, while being considerably less toxic. The two analogs might therefore represent promising lead compounds for further development of antifungal drugs for human therapy.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Media, and Growth Conditions

Plasmids, phages, and bacterial strains used in the present study are described in Table S1. *S. noursei* and *Escherichia coli* strains were maintained and genetically manipulated as described elsewhere (Sekurova et al., 1999; Sambrook et al., 1989). Cultivations of *S. noursei* strains for production of analogs were performed in fed-batch fermentation essentially as described elsewhere (Borgos et al., 2006a), except for the use of SAO-50 medium, which contained double amounts of all nutrients, compared to SAO-40 (Borgos et al., 2006a). Analysis of polyene macrolides produced was done as reported elsewhere (Bruheim et al., 2004). The gene replacements were performed through conjugation of the corresponding vectors (see below) into the *S. noursei,* verification of chromosomal integration, and selection for double homologous recombination, as described elsewhere (Sekurova et al., 1999). Gene replacements were performed in *nysA*-deficient mutants NDA59 (Brautaset et al., 2003) and GG5073SP (Borgos et al., 2006a), and the resulting recombinant strains were complemented with the *nysA* gene to restore the polyene macrolide biosynthesis.

DNA Manipulation, Sequencing, and PCR

General DNA manipulations were performed as described elsewhere (Sambrook et al., 1989). DNA fragments from agarose gels were purified using QIAEX II kit (QIAGEN, Germany). Southern blot analyses were performed with the digoxigenin-11-dUTP High Prime labeling kit (Roche Molecular Biochemicals) according to the manufacturer's instruction. Oligonucleotide primers were purchased from MWG Biotech (Germany). The PCRs were performed with the GC Rich PCR System (Roche Molecular Biochemicals) on Eppendorf Mastercycler (Eppendorf, Germany), using conditions described elsewhere (Brautaset et al., 2003). DNA sequencing was performed at MWG Biotech (Germany).

Construction of Gene Replacement Vectors

Inactivation of NysJ DH15

The 3.33 kb *Bcl*I/*Sph*I fragment of recombinant phage N20 (Table S1) was ligated into the *Bam*HI/*Sph*I sites of pGEM-11zf+. From the resulting plasmid, the 3.34 kb *Eco*RI/*Hin*dIII fragment was isolated and ligated into the corresponding sites of plasmid pGEM-3zf+, yielding pDH15-B. The latter plasmid was used as a template for site-directed mutagenesis to introduce the DH15 mutation H966F using the mutagenic oligonucleotides Mut-DH15-1: 5′-CACCCCTGGCTCGCCGACT**TC**GTCGTCGGCGGCATGGTC−3′ (sense) and Mut-DH15-2: 5′-GACCATGCCGCCGACGAC**GA**AGTCGGCGAGCCAGGGGTG−3′ (antisense). Altered nucleotides are indicated in bold, and the introduced mutation eliminated the originally present *Brt*I recognition site (not shown). Mutation was first confirmed by *Brt*I restriction digestion and then verified by DNA sequencing. The plasmid-containing mutation was designated pDH15-mut. The 1.6 kb *Eco*RI/*Sac*I fragment of pDH15-B, the 1.1 kb *Hin*dIII/*Bgl*II fragment of pDH15-B, and the mutated 0.64 kb *Sac*I/*Bgl*II fragment from pDH15-mut were isolated and ligated together with the 3.1 kb *Eco*RI/*Hin*dIII fragment of pSOK201 (Zotchev et al., 2000) (Table S1), yielding DH15 inactivation vector pSH15-123.

Inactivation of nysN

The 4.1 kb *Nco*I/*Xba*I fragment from the recombinant phage N95 (Table S1) was cloned into the corresponding sites of the plasmid pLITMUS28. From the resulting construct, the entire 4.1 kb insert was excised with *Eco*RI/*Hin*dIII and cloned into the corresponding sites of pGEM11-zf+. From the resulting plasmid, pGEM11nysN4.1, the 1.5 kb region including the *nysN* active site was PCR-amplified with primers conA-1F: 5′-TTTTGAATTCTTCAAGCCGATGAGCC-3′ and conA-1R: 5′-TTTTAAGCTTGGTCGAAC AGGTCCGG-3′, introducing *Eco*RI and *Hin*dIII sites (underlined) into the PCR product. These sites were used to clone the PCR fragment into pGEM11-zf+, yielding plasmid pGEM11nysN1.5. The latter plasmid was used as a template for site-directed mutagenesis (QuickChange kit, Stratagene) to introduce *nysN* mutations CL346AS using the oligonucleotides CL346AS-F: 5′-TCGGCTACGGTGTCCAC**GCTA**GCCT GGGCCAGAAC CTGG-3′ and CL346AS-R: 5′-CCAGGTTCTGGCCCAG**GC TAGC**GTGGACACCGTAG CCGA-3′. Altered nucleotides are indicated in bold while the new *Nhe*I restriction site introduced is underlined. Mutation was first confirmed by restriction analysis and then verified by DNA sequencing. The mutated 1.3 kb *Fsp*AI/*Bpu*1102I fragment from the resulting plasmid was cloned back into the corresponding sites of pGM11nysN4.1, yielding pGM11nysN4.1-CL346AS. From the latter construct, the 4.1 kb insert containing mutated *nysN* genes was excised with *Eco*RI/*Hin*dIII and ligated with the 3.1 kb *Eco*RI/*Hin*dIII fragment of pSOK201, yielding plasmid pKOnysN-CL346AS, which was used for *nysN* replacement in *S. noursei*.

Inactivation of NysJ KR17

The 4.0 kb *Pml*I/*Bam*HI fragment from the recombinant phage N98 (Table S1) was excised and ligated into the *Hin*cII/*Bam*HI sites of vector pGEM3-zf+, yielding plasmid pBB4.0. A 1.5 kb DNA fragment, including the KR17 active site region, was PCR amplified from pBB4.0 using primers KR17-F: 5′-TTTTCTGCAGGCCGCGGTGCGCGC-3′ and KR17-R: 5′-TCCGGC ATGGTCCGTGAAACC-3′. The PCR product was digested with *Pst*I (site underlined) and *Sac*I (recognition site in the amplified DNA fragment), and the 1.4 kb fragment was ligated into the corresponding sites of pLITMUS28. The resulting plasmid, pLIT1.4, was used as a template for site-directed mutagenesis with oligonucleotides KR17-mut1: 5′-GCCCCGGCCAGGGCA AC**TTCGAA**GCCGGCAACAC GTTCC-3′ and KR17-mut2: 5′-GGAACGTGTTGCCGG C**TTCGAA**GTTGCCCT GGCCGGGGC-3′. Altered nucleotides are indicated in bold while new *Bst*BI restriction site introduced is underlined. Correct mutation was verified with *Bst*BI digestion, and the entire insert of the mutated plasmid was verified by DNA sequencing. From the resulting plasmid, pLIT1.4 m, the 1072 bp *Bcl*I/*Acc*III fragment, was excised and used to replace the corresponding fragment in pBB4.0, yielding plasmid pBB4.0 m. The entire 4.0 insert of pBB4.0 m was excised with *Eco*RI and *Hin*dIII and ligated together with the 3.1 kb *Eco*RI/*Hin*dIII fragment of pSOK201, yielding the KR17 inactivation vector pKR17m.

Inactivation of NysJ KR16

The 14 kb *Xba*I insert of the recombinant phage N20 (Table S1) was ligated into plasmid pGEM-3zf, and from the resulting plasmid, pL20X, the 3.7 kb *Bam*HI fragment was excised and ligated into pGEM-3zf+, yielding plasmid pGEMB3.7. A 0.8 kb DNA fragment (encompassing the codon for the KR16 active site residue Y3404) was PCR-amplified from pGEMB3.7 with primers KR16-F1: 5′-ttttctgCAGCCGACCGGCACCGTCC-3′, and KR16-1R: 5′-ttttaaGCTTCCTGGAC CGCGCGGG-3′. The PCR product was digested with *Pst*I and *Hin*dIII (recognition sites underlined in the two PCR primers) and ligated into the corresponding sites of pLITMUS28, yielding pLITPH0.8. The latter plasmid served as a template for site-directed mutagenesis to introduce the KR16 double mutation YA3404FE using the mutagenic oligonucleotides mutKR16-1F: 5′-CCCCGGCCAGGCCGGCT**TCGAA**GCCG CCAACGCGGTC C-3′ (sense) and mutKR16-1R: 5′-GGACCGCGTTGGCGGC**TTCGAA** GCCGG CCTGGCCGGGG-3′ (antisense). Mutated nucleotides are shown in bold, and the new *Bst*BI recognition site introduced is underlined. The correct mutation was identified by *Bst*BI digestion and verified by DNA sequencing. From the plasmid containing desired mutation, the 627 bp *Bpu*102I/*Bpu*10I fragment was excised and used to replace the corresponding fragment in pGEMB3.7, yielding plasmid pGEMB3.7 m. From the latter plasmid, the entire 3.7 kb insert was excised with *Eco*RI/*Hin*dIII and ligated together with the 3.1 kb *Eco*RI/*Hin*dIII fragment of pSOK201, yielding KR16 inactivation vector pKR16m. The latter vector was introduced into the *nysA*-deficient *S. noursei* mutant GG5073SP (Borgos et al., 2006a) by double homologous recombination.

Gene Replacements in *S. noursei* Strains

The constructed gene replacement vectors (see above) were introduced to the *S. noursei* strains by conjugation, and gene replacements were selected after double homologous recombination, as described elsewhere (Sekurova et al., 1999). The correct chromosomal mutations were verified by PCR, DNA sequencing of PCR products, and Southern blot analyses. Polyene macrolide production was restored in the recombinant strains by introducing the *nysA* gene as described elsewhere (Brautaset et al., 2003).

Preparative LC-MS Purification of Genetically Modified Polyenes, MS/MS, and NMR Experiments

The LC-MS-guided purification was performed essentially as described elsewhere (Bruheim et al., 2004; Borgos et al., 2006b), but with methanol instead of acetonitrile as the mobile phase organic constituent (from 70% up to 80% methanol, depending on the polyene to be purified). Purity and concentration of the engineered polyenes was determined by reference to USP standards of nystatin and AmB for tetraenes and heptaenes, respectively, assuming that molar extinction coefficients in the spectral regions of interest were unaltered. Peak UV absorption at 309 nm and 386 nm, arising from the polyene region, was used for tetraenes and heptaenes, respectively. Samples for MS/MS analyses were prepared by dissolving purified compounds in DMSO to a final concentration of 5 mg/ml. The MS/MS analysis was performed using the Agilent 1200 series LC/Qtof system. The eluent was 30% acetonitrile in water at a flow rate of 0.4 ml/min. An Agilent Zorbax Bonus-RP 2.1 × 50 mm column was used. The Qtof mass spectrometer was operated with the electrospray ionization source in positive ionization mode. Drying gas flow was 11 l/min, and nebulizer pressure was 45 psi. Drying gas temperature was 350°C and the fragmentor voltage was 175 V. During MS/MS spectra acquisition, a fixed collision energy of 25 V was used. Samples for NMR spectroscopy were prepared by dissolving freeze-dried compounds purified by preparative HPLC in d_6-DMSO at 1 mM final concentration. To be able to perform direct comparison of NMR assignments reported previously (Bruheim et al., 2004) and obtained in the present study, both sample and further NMR experimental conditions were maintained analogous to those already described. All NMR experiments were recorded at 298 K on a Bruker DRX600 spectrometer equipped with a 5-mm z-gradient TXI (H/C/N) cryogenic probe. Proton and carbon chemical shifts were referenced to TMS signal. To monitor the chemical structure of the investigated compounds, both one-dimensional ^{1}H and two-dimensional COSY and ^{1}H-^{13}C HSQC spectra were recorded. Detailed description and analysis of the results are given in the Supplemental Material.

Determination of In Vitro Antifungal and Hemolytic Activity

Determination of in vitro antifungal activity was done by cultivating *C. albicans* in 96-well plates and monitoring cell growth in the presence of 24 different concentrations of antibiotics, as described elsewhere (Borgos et al., 2006b; Nedal et al., 2007). MIC_{50} and MIC_{90} values, representing minimum inhibitory concentrations causing 50% and 90% inhibition of cell growth, respectively, were calculated from at least 3 parallel experiments. Hemolytic assays for the genetically modified polyene macrolides were performed by monitoring their ability to cause lysis of defibrinated horse blood erythrocytes washed from plasma proteins with PBS buffer, as described elsewhere (Borgos et al., 2006b; Nedal et al., 2007). Antibiotic solutions were prepared in DMSO to achieve final concentrations between 0 and 200 μg/ml, and the mixtures were incubated at 37°C for 1 hr before the optical densities of supernatants at 545 nm were measured. The results presented are mean values from 3 independent measurements. Hemolytic concentrations causing 50% hemolysis, HC_{50} values, were determined from the generated plots (Figure S12).

In Vivo Testing of Acute Toxicity and Antifungal Activity

All animal experiments were performed in accordance with the Guidelines for Drugs Toxicity Testing on Animals approved by the Russian Academy of Medical Sciences (RAMS). Prior to initiation of experiments, the protocols were reviewed and approved by the Ethical Committee of the Gause Institute of New Antibiotics (RAMS). Male mice (weight, 19–22 g) of first generation hybrids BDF1 (C57Bl × DBA2) received from the Central Nursery of RAMS were used in the experiments. The animals were kept on a standard diet consisting of briquette forages with easy access to potable water in the animal nursery of the Gause Institute of New Antibiotics. After the fortnight quarantine, healthy animals were divided into groups with six individuals in each and were entered into the experiments.

Nystatin, AmB, S44HP, BSG002, BSG005, and BSG020 (5 mg) were each mixed with dry sodium deoxycholate (4.1 mg) in a sterile glass vial. Ten milliliters of phosphate buffer (NaH_2PO_4, 1.59 g; Na_2HPO_4, 0.96 g; and H_2O, to 100 ml) was added and immediately subjected to vigorous shaking for 10 min until homogeneous suspensions were formed. The obtained suspensions were placed into the new sterile glass vials, 5% neutral sterile glucose solution was added, and the resulting solutions (up to 0.8 mg/ml) were used for intravenous administration.

Acute Toxicity

Freshly prepared antibiotic solutions were individually injected into the mouse's tail vein at <0.5 ml per minute. Each antibiotic was used in a range of doses resulting in 0% to 100% lethality and a minimum of three intermediate doses. Toxicity-characterizing doses MTD and LD_{50} were calculated with the method of "probit" analysis, according to Litchfield and Wilcoxon (1949), using the statistical analysis program StatPlus (version 3.5.0., 2005).

Antifungal Activity

Mice were infected intravenously with 10^6 CFU of *C. albicans* ATCC 14053 per mouse (0.1 ml). Thirty minutes after infection, antibiotics (six doses, ranging from 1% to 95% MTD, three mice per dose per antibiotic) were administered through the lateral tail vein. Each dose was administered once a day for four days, including the day of infection (0, 1, 2, and 3 days). As a control, a group of untreated infected mice and a placebo group of noninfected animals, which were intravenously administered 0.2 ml of the solvent (phosphate buffer + 5% glucose [1:1]), were used. On the fifth day of the experiment, mice were weighed and sacrificed. The *C. albicans* kidney burden for each mouse was determined by counting CFUs in homogenates from the kidneys. The kidneys were removed aseptically, weighed, and pounded in porcelain mortars with sterile corundum; dilutions of the resulting suspensions were prepared and plated on Sabouraud dextrose agar. The plates were incubated for up to 72 hr at 35°C, colonies of *C. albicans* were counted, and the load was estimated per 1 g of kidney tissue. Statistical analysis was performed with Microsoft Office Excel 2003. Significant distinctions had a $p \leq 0.05$ at comparison by Student T-criterion.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, two tables, and twelve figures and can be found with this article online at http://www.chembiol.com/cgi/content/full/15/11/1198/DC1/.

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