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

Olga Sekurova a, Håvard Sletta b, Trond E. Ellingsen b, Svein Valla a, Sergey Zotchev a,*

a UNIGEN Center for Molecular Biology, NTNU, N-7489 Trondheim, Norway
b SINTEF Applied Chemistry, SINTEF, N-7034 Trondheim, Norway

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Abstract

A regulatory gene locus from *Streptomyces noursei* ATCC11455, 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 *PermE* 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-
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 afsR homologue in S. noursei, and that putative overexpression of ssmA positively affects actinorhodin 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\(\cdot\)H\(_2\)O, 90;
- NH\(_4\)NO\(_3\), 2.5;
- corn flour, 3;
- MgSO\(_4\)\(\cdot\)7H\(_2\)O, 0.4;
- KH\(_2\)PO\(_4\), 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\(\cdot\)H\(_2\)O. Trace element solution (mg ml\(^{-1}\)):
- FeSO\(_4\)\(\cdot\)7H\(_2\)O, 5.0;
- CuSO\(_4\)\(\cdot\)5H\(_2\)O, 0.39;
- ZnSO\(_4\)\(\cdot\)7H\(_2\)O, 0.44;
- MnSO\(_4\)\(\cdot\)H\(_2\)O, 0.15;
- Na\(_2\)MoO\(_4\)\(\cdot\)2H\(_2\)O, 0.01;
- CoCl\(_2\)\(\cdot\)6H\(_2\)O, 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

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<td><strong>Bacterial strains and plasmids used in this study</strong></td>
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<td><strong>Strain/plasmid</strong></td>
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<td>S. noursei ATCC11455</td>
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<td>S. lividans 1326</td>
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<td>E. coli DH5(\alpha)</td>
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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 dimethylformamide 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 ClaI 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 KpnI and HindIII, and ligated together with the 3.0 kb KpnI/HindIII 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\(^R\) 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 Sau3A1, size-fractionated on a sucrose gradient, ligated with BamHI-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 SacI/BamHI 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.
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 streptomycte 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

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.
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 ClaI fragment, and A8dO3 by deleting the 354 bp AccI 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 PermE 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

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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.
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-A8d8..) 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 *ssmB*. 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. 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.
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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