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ORIGINAL PAPER

Function of *lanI* in regulation of landomycin A biosynthesis in *Streptomyces cyanogenus* S136 and cross-complementation studies with *Streptomyces* antibiotic regulatory proteins encoding genes

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Abstract The transcriptional regulator of landomycin A biosynthesis encoded by *lanI* gene has been inactivated within the chromosome of *Streptomyces cyanogenus* S136. The obtained mutant strain did not produce landomycin A and its known intermediates. Loss of landomycin A production caused significant changes in morphology of the *lanI* deficient strain. RT-PCR analysis confirmed complete cessation of transcription of certain *lan* genes, including *lanJ* (encoding putative proton dependent transporter) and *lanK* (presumably involved in *lanJ* expression regulation). Introduction of either *lanI* or *lndI* [*lanI* homologue controlling landomycin E biosynthesis in *Streptomyces globisporus* 1912, both encoding Streptomyces antibiotic regulatory proteins (SARPs)] restored landomycin A production in the mutant strain. Chimeric constructs *ladI* and *ladR* were generated by exchanging the DNA sequences corresponding to N- and C-terminal parts of LndI and LanI. None of these genes were able to activate the production of landomycins in regulatory mutants of *S. cyanogenus* and *S. globisporus*. Nevertheless, the production of novel unidentified compound was observed in the case of *S. cyanogenus* harboring *ladI* gene. Various genes encoding SARPs have been expressed in *S. globisporus* and *S. cyanogenus* regulatory mutants and the results of these complementation experiments are discussed.

Keywords *Streptomyces* · Antibiotics · Polyketide · SARP · Transcriptional regulators

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Introduction

Efforts in streptomycete gene cloning lead to identification and studying of huge number of biosynthetic gene clusters governing the production of important secondary metabolites. Among them there are a large group of aromatic polyketides including angucyclines (for reviews see Rohr and Thiericke 1992; Hertweck et al. 2007). Despite the fact that angucyclines are known to possess broad spectrum of biological activities ranging from bactericidal to specific enzyme inhibition, the biological role of these secondary metabolites in the producing strains is still poorly understood.

In general, the production of antibiotics is strictly coordinated with the growth and environmental conditions. The regulation of secondary metabolism of streptomycetes

depends on function of complicated, sensitive and flexible network of diverse regulatory elements (Bibb 2005). The final decision about onset of antibiotic production is made by transcriptional factors which genes are clustered with the respective antibiotic biosynthesis genes. Members of this big group of proteins resembles the OmpR transcriptional regulator of *Escherichia coli* phosphate regulon particularly in the region of DNA-binding domain formed by "winged" helix-turn-helix structure (Martinez-Hackert and Stock 1997; Harrison-McMonagle et al. 1999). Therefore, these transcriptional factors were grouped into SARP family (for *Streptomyces* antibiotics regulatory proteins, Wietzorrek and Bibb 1997). SARP genes were found within almost all biosynthesis gene clusters governing aromatic polyketides production. Function of some of these genes and their products is well studied. The DNA-binding features as well as the target sequences were determined for DnrI and ActII-ORF4 proteins, controlling production of daunorubicin and actinorhodin, respectively (Tang et al. 1995; Sheldon et al. 2002; Arias et al. 1999). In contrast, the SARPs involved in angucycline biosynthesis are less studied; in fact, there is some information available only about *lndI*, *jadR1*, *aur1P* and *alpV* (Rebets et al. 2003; Yang et al. 2001; Aigle et al. 2005; Novakova et al. 2005).

Previously we reported the cloning and characterization of two SARPs coding genes *lndI* and *lanI* (corresponding proteins share 61% identical amino acids) from *S. globisporus* 1912 and *S. cyanogenus* S136 landomycins E and A biosynthesis gene clusters, respectively (Rebets et al. 2003). The function of *lndI* gene was studied by gene replacement, reporter gene transcriptional fusion and DNA-binding assay (Rebets et al. 2003, 2005). Additionally, importance of TTA codon responsible for the temporal regulation of *lndI* expression was demonstrated (Rebets et al. 2006). Due to technical difficulties in genetic manipulations with the *S. cyanogenus* the function of *lanI* gene was shown only by the ability to restore landomycin E production in *lndI* mutant and to activate expression of *lan* genes in heterologous host (Rebets et al. 2003; von Mulert et al. 2004). However, no experimental evidences were shown for the *lanI* function in the native strain. Here we report the results of *lanI* replacement and analysis of obtained mutant strain with respect to landomycins production and morphology of the colonies, as well as the results of complementation studies in regulatory mutants with *lanI*/*lndI* and some other regulatory genes.

Materials and methods

Bacterial strains, plasmids and growth conditions

The *Streptomyces* strains and plasmids used in this work are listed in the Table 1. *Escherichia coli* DH5α (Hanahan 1983) was used for routine subcloning. *E. coli* ET12567 (dam-13::Tn9 (Cmr), dcm-6, hsdM) harboring the conjugative plasmid pUB307 (gift from C. P. Smith, UMIST, Manchester, UK) was used to perform intergeneric conjugation from *E. coli* to *Steptomyces* species (Flett et al. 1997).

For plasmid DNA isolation *E. coli* strains were grown in LB medium at 37°C for 18 h as described previously (Sambrook and Russel 2001). For *Streptomyces* total DNA isolation, strains were grown in TSB for 3–4 days at 28°C (Kieser et al. 2000). For antibiotics production, streptomycetes were grown in liquid SG medium or on R5 plates at 30°C. The *E. coli* strain ET 12567 (pUB307) carrying plasmids for conjugal transfer was grown on LB agar as described previously (Luzhetskyy et al. 2002). Spores of *Streptomyces* strains for conjugation were harvested from a sporulated lawn grown on oatmeal agar plates. When antibiotic selection of bacteria was needed, strains were incubated with apramycin (50 μg ml^{-1}), ampicillin (100 μg ml^{-1}), spectinomycin (50μg ml^{-1}), kanamycin (35 μg ml^{-1}) and chloramphenicol (35 μg ml^{-1}). Chromogenic substrates X-gal and IPTG were used as described elsewhere (Sambrook and Rusell 2001).

DNA manipulations

Genomic DNA from *Streptomyces* strains and plasmid DNA from *E. coli* were isolated using standard protocols (Kieser et al. 2000). Klenow fragment DNA polymerase, T4 DNA ligase and restriction enzymes were used according to recommendation of suppliers (NEB, MBI Fermentas). Other DNA manipulations were performed following standard procedures as specified by manufacturers (NEB, MBI Fermentas, Roche Applied Science). Intergeneric matings were performed as previously described (Luzhetskyy et al. 2002, 2006). Southern blot analysis and DIG DNA labeling were carried out according to instructions of manufacturer of DIG DNA labeling and detection kit (Roche Applied Science).

Comparison of amino acid sequences of SARPs

The amino acid sequences of SARPs were obtained from the Swiss-Prot protein database (Swiss Institute of Bioinformatics) for ActII–Orf4, DnrI, SimR1, OvmR1, JadR1, AlpV, MtmR, OmpR and deduced from the nucleotide sequences of corresponding genes for LndI and LanI, respectively. The sequences were stored in FASTA format and compared with the Clustal W method using the DNASTAR software package. For the alignment, the following parameters were used in all cases: gaps penalty −5.00, gaps length penalty—0.10, protein weight matrix—BLOSUM Series. For the alignment of putative DNA-binding domains, the first 100 amino acids of ActII–ORF4, DnrI, MtmR, AlpV and last 100 amino acid residues of OmpR, JadR1, SimR1, LndI and LanI were used for comparison.

Table 1 Strains and plasmids used during this work

Bacterial strains and plasmid	Description	Source or reference
E. coli DH5α	supE44 ΔlacU169(φ80lacZΔM15)hsdR17 recA1endA1gyrA96 thi-1 relA1	Hanahan (1983)
E. coli ET12567 (pUB307)	*dam*-13::Tn9(Cm^r) *dcm*-6 *hsd*M; harbors conjugative plasmid pUB307; Cm^r, Km^r	C. P. Smith, UMIST, UK
S. cyanogenus S136	Landomycin A producing strain	Westrich et al. (1999)
S. cyanogenus ΔlanI7	Derivative of *S. cyanogenus* S136 with disrupted *lanI* gene (*lanI::aadA*)	This work
S. globisporus I2-1	Derivative of *S. globisporus* 1912 with mutated *lndI* gene (*lndI::aphII*)	Rebets et al. (2003)
pUC19	General purpose cloning vector; Ap^r	Yanisch-Perron et al. (1985)
pT7Blue T- vector	PCR products T-cloning vector; Ap^r	Novagen
pHP45Ω	Plasmid carrying Ω interposon with spectinomycin resistance gene cassette *aadA*; Ap^r, Sm^r/ Sp^r	Blondelet-Rouault et al. (1997)
pSET152	*E. coli/Streptomyces* shuttle vector with ØC31 integration system for streptomycetes; Am^r	Bierman et al. (1992)
pKC1218	*E. coli/Streptomyces* shuttle vector with SCP2* replicon; Am^r	Kieser et al. (2000)
pKC1218E	pKC1218 derivitive expression vector with P_{ermE} promoter; Am^r	C. Olano, Univ. de Oviedo, Spain
pKC1139	*E. coli/Streptomyces* shuttle vector with temperature sensitive pSG5 replicon, Am^r	Muth et al. (1989)
pKlanI	pBluescriptIIKS⁻ derivative with cloned 4.8 kb *Kpn*I fragment of *lan*-cluster *lanI* gene	Rebets et al. (2003)
pUlanI	pUC19 derivative with cloned 4.8 kb *Kpn*I fragment of *lan*-cluster containing *lanI* gene	This work
pUlanIaadA	pUlanI derivative with spectinomycin resistance cassette *aadA* inserted into *lanI* coding region	This work
pKClanI	pKC1139 derivative with cloned *lanI::aadA* construction used for *lanI* gene inactivation	This work
pSlanI	pSET152 containing *lanI* gene	Rebets et al. (2003)
pSI2-9	pSET152 containing *lndI* gene	Rebets et al. (2003)
pKCEsimR1	pKC1218E with cloned *simR1* gene	This work
pKCEjadR1	pKC1218E with cloned *jadR1* gene	This work
pKCEmtmR	pKC1218E with cloned *mtmR* gene	This work
pKCEovmR	pKC1218 with cloned *ovmR* gene under control of P_{ErmE} promoter	This work
pT7Ln1	pT7Blue derivative with cloned 5′ terminal fragment of *lndI* gene	This work
pT7Ln2	pT7Blue derivative with cloned 3′ terminal fragment of *lndI* gene	This work
pT7La1	pT7Blue derivative with cloned 5′ terminal fragment of *lanI* gene	This work
pT7La2	pT7Blue derivative with cloned 3′ terminal fragment of *lanI* gene	This work
pSladI7	pSET152 derivative containing *ladI* gene encoding N-terminal domain of LndI and C-terminal half of LanI	This work
pSladR4	pSET152 derivative containing *ladR* gene encoding N-terminal domain of LanI and C-terminal half of LndI	This work

Disruption of *lanI* gene

Approximately, 4.8 kb *Kpn*I fragment carrying the entire *lanI* gene and its flanking regions were cloned from pKlanI (Rebets et al. 2003) into *Kpn*I site of pUC19 to yield pUlanI with a unique *Xho*I site located 298 bp downstream of putative *lanI* gene start codon (Fig. 1a). pUlanI was digested with *Xho*I, treated with Klenow fragment and ligated with spectinomycin resistance cassette *aadA* that was retrieved as *Sma*I fragment from pHP45Ω (a gift from J-L Pernodet University Paris-Sud, Orsay, France) (Blondelet-Rouault et al. 1997). The obtained construct, named pUlanIaadA, was digested with *Kpn*I and the 6.8 kb *lanI::aadA* fragment was blunt-ended with Klenow fragment and cloned into *Eco*RV site of pKC1139 to yield pKClanI. pKClanI was transferred into *S. cyanogenus* S136 from *E. coli* by conjugation. Single crossover between *S. cyanogenus S136* chromosome and pKClanI was promoted as described for pSG5-based vectors (Muth et al. 1989). After few passages under nonselective conditions Am^S and Sp^R colonies were selected. One of the obtained clones named *S. cyanogenus* ΔlanI7 that presumably carry *lanI::aadA* allele was subjected

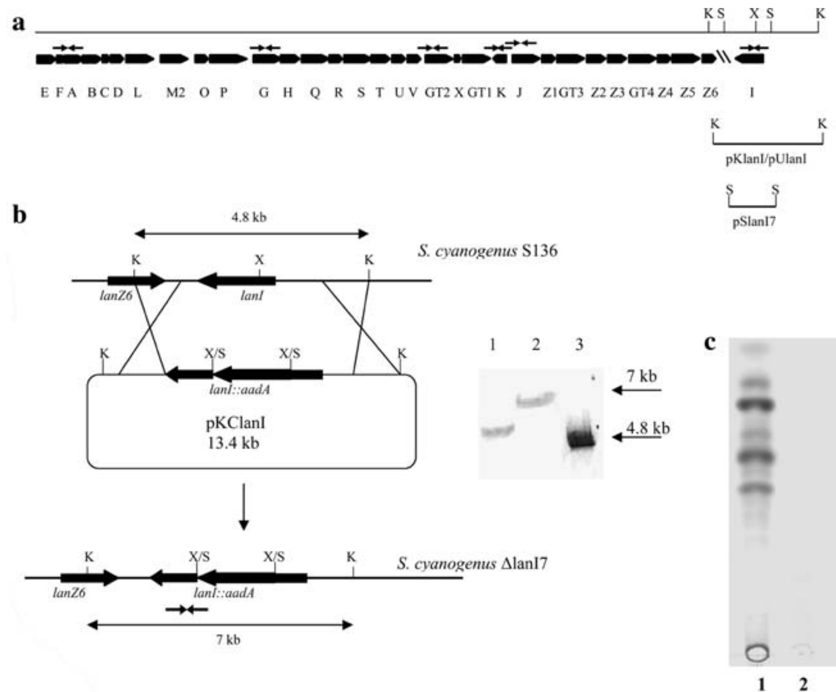

Fig. 1 **a** Schematic representation of landomycin A biosynthesis gene cluster of *S. cyanogenus* DNA fragments used for *lanI* gene disruption and expression experiments. Primers used for genes cloning and RT-PCR are indicated as *arrows*. Only sites for restriction endonucleases used in this work are shown: *Kpn*I (*K*), *Sma*I (*S*), *Xho*I (*X*). **b** Schematic representation of *lanI* gene disruption and the results of Southern hybridization of *Kpn*I digested total DNA samples from *S. cyanogenus* S136 (*1*), ΔlanI7 (*2*) and pKlanI (*3*) with 4.8 kb DNA fragment harbouring *lanI* gene. (*c*) TLC analysis of secondary metabolites produced by *S. cyanogenus* S136 (*1*), ΔlanI7 (*2*) strains

to Southern-blot analysis. *Kpn*I-digested genomic DNA of wild type *S. cyanogenus S136* and ΔlanI7 mutant strains were probed with DIG-labeled 4.8 kb *KpnI* fragment from pKlanI containing *lanI* gene (Fig. 1).

Plasmid construction and complementation studies in Streptomyces *regulatory mutants*

For the complementation of *S. cyanogenus* ΔlanI7 strain, the pSET152-based plasmids pSlanI (carrying *lanI*) and pSI2-9 (*lndI* gene) were used (Rebets et al. 2003). For studying other SARPs encoding genes plasmids were created as follows. The *simR1* gene was retrieved from plasmid 5J10B4 (Trefzer et al. 2002) as 2.3 kb *Pst*I–*Eco*RV fragment and cloned into corresponding sites of pKC1218E under control of P_{ermE} promoter to yield pKCEsimR1. The *jadR1* gene was transfered as 1.2 kb *Bam*HI fragment from pJV73A (Yang K et al. 2001) to pKC1218E to yield pKCE-jadR1. The *mtmR* gene (Lombo et al. 1999) was retrieved from pFL3R as 0.86 kb *Xba*I fragment and cloned into pKC1218E in appropriate orientation to yield pKCEmtmR. OvmR gene was retrieved together with the P_{ErmE} promoter as *Hind*III–*Eco*RI fragment from pFL768 and cloned into corresponding sites of pKC1218 to yield pKCEovmR (Lombo et al. 2004).

Construction of LndI/LanI hybrids and their expression

A 898 bp fragment of *lndI* gene including its promoter sequence and region encoding first 196 amino acids was amplified by PCR using the LnIF1/R1 primers pair (primers used in this work are listed in Table 2). The 3′ terminal 260 bp fragment of *lanI* gene including region coding for last 65 amino acid residues of LanI protein putatively folding into α2 and α3 structures of DNA-binding domain were amplified with the LaIF2/R2 primer sets. Obtained fragments were cloned into pT7Blue T-vector to generate pT7Ln1 and pT7La2, respectively. The *ladI* chimeric gene was constructed by cloning of *Eco*RI–*Nhe*I fragment from pTLn1 into *Eco*R1–*Nhe*I digested pT7La2. Obtained hybrid gene encoding N-terminal domain of LndI and C-terminal half of LanI was cloned into pSET152 as *Eco*R1–*Eco*RV fragment, designated as pSladI7.

The 5′ terminal 916 bp fragment of *lanI* gene including its promoter and region encoding first 196 amino acids and 3′ terminal region of *lndI* (317 bp fragment, including region coding for last 65 amino acid residues of LndI protein) were amplified with the LaF1/R1 and LnIF2/R2 primer sets, respectively. Fragments were also cloned to pT7Blue T-vector resulting in pT7La1 and pT7Ln2. The *ladR* gene encoding N-terminal part of LanI and C-terminal domain of LndI was created by cloning of *Xba*I–*Nhe*I fragment containing 3′ region of *lndI* into *Xba*I–*Nhe*I sites of pTLaI1. The resulted chimeric gene was subcloned into *Eco*RV–*Xba*I sites of pSET152 to generate pSladR4. All final plasmids were verified by DNA sequencing.

Analysis of secondary metabolites production

Streptomycetes strains were grown in SG medium for 4 days in a rotary shaker (250 rpm) or on R5 plates (25 ml/plate) for 5 days. The culture broth or solid media were

Table 2 Primers, used during this work for gene cloning and RT-PCR analysis

Primer name	Nucleotide Sequence	Restriction site/*lan*-gene amplified with
LnIF1	GAATTCTTGTTGTTCTTGCC	*Eco*RI
LnIR1	GGACGCTAGCAGGTAAGAAG	*Nhe*I
LnIF2	CTTCTTACCTGCTAGCGTCC	*Nhe*I
LnIR2	CATGACCGAACATCTCTAGACT	*Xba*I
LaIF1	AACCGATATCGCGAGAATT	*Eco*RV
LaIR1	GGAAGCTAGCAGTGTGAGGAG	*Nhe*I
LaIF2	CTCCTCACACTGCTAGCTTCC	*Nhe*I
LaIR2	GATATCCTGGCACGGTGAGC	*Eco*RV
LIF	GAATGAGTGAGCTCGCGCA	*lanI*
LIR	GTACGGTGACGATCCAGCT	
LAF	CAGCACCTGTACAACCACTT	*lanA*
LAR	TGCGCGTTGATGTAGTCGAT	
LGF	TTCAGCCACTCGATGCCGAA	*lanG*
LGR	GTGAAGAAGTAGACGCCGAT	
LG2F	ACATGGTTCCTTACATCGCG	*lanGT2*
LG2R	TCGCGAACACCAAACGGCT	
LJF	CTACTGCTTCTACGTCAACG	*lanJ*
LJR	GATCGCGTACAGCTTGAAGT	
LKF	GACAAGATCCAGTCCGTCG	*lanK*
LKR	GTACATCAGCTCACCGATG	
rrnAF	CACATGCAAGTCGAACGATG	16S rRNA of *S. Coelicolor*
rrnAR	GCTGCTGGCACGTAGTTAG	

extracted with an equal volume of ethyl acetate with 1% acetic acid three times. Extracts were dried in vacuum and dissolved in methanol. The metabolites were analyzed by thin layer chromatography (TLC) on silica gel plates using solvent system methanol:chloroform = 9:1 or by the HPLC as described previously (Rebets et al. 2003). Extracts of *S. cyanogenus* S136, *S. cyanogenus* ΔlanI7 pSET152$^+$ and *S. cyanogenus* ΔlanI7 pSladI$^+$ were also analyzed by HPLC–MS as described (Luzhetskyy et al. 2005).

To determine the bactericidal activity of secondary metabolites produced by the studied streptomycetes strains *E. coli* W3110 and *Sarcina flava* were used as a test cultures. Equal amounts of dry extracts from *S. cyanogenus* S136, *S. cyanogenus* ΔlanI7 pSET152$^+$ and *S. cyanogenus* ΔlanI7 pSladI$^+$ were dissolved in methanol and applied to Ø5 mm Whatman disks which were left to dry for 6 h at 4°C; 200 µl of cells suspension of either test-culture (ca. 10^7 cfu/ml) were mixed with 3 ml of soft agar and plated on solid LB medium. Then disks were stacked on soft agar. As an alternative the streptomycetes strains were grown at solid SMA medium for 6 days. Ø7 mm columns with respective culture were cut out from medium and stacked on LB agar plates with previously inoculated *E. coli* or *S. flava*. Diameters of growth inhibition zones were measured after 24 and 36 h of incubation. The experiment was triplicated and data have been averaged.

Semiquantitative RT-PCR analysis

For total RNA isolation, *S. cyanogenus* strains were harvested from SM agar media after 5 days of incubation. Mycelia were scraped with loop and resuspended in TE buffer. Total RNA samples were isolated using the Ultraspec™ total RNA isolation system (Biotecx) followed by RQ1 DNase treatment (Promega). Samples concentration and purity were determined by measuring OD at 260/280 nm. Equal amounts of RNA from each sample were used for RT-PCR. First strand DNA synthesis was performed using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas) and random hexanucleotide primers. Second strand synthesis and amplification were performed using Taq DNA-polymerase (NEB) with specific primer pairs for each individual *lan* genes (Fig. 1a, Table 2). Primers for 16S rRNA of *S. coelicolor* were used as a positive control. To ensure the absence of DNA traces, PCR was also performed with 16S RNA specific primers pair and RNA samples as a template. Samples were analysed by electrophoresis in 2% agarose gel.

Electron microscopy

For electron microscopy thin slices of lawn grown on Soy-Mannitol agar plates were prepared and fixed in 1% OsO_4

in cacodylate buffer for 90 min at 0°C. The samples were dehydrated with successive solutions containing increasing concentration of ethanol. The samples were examined with electron microscope Jeol JSM-T220A.

Results and discussion

Disruption of *lanI* and analysis of the mutant strain

Previously we have reported the cloning of pathway-specific regulatory genes *lanI* and *lndI* from landomycins A and E gene clusters of *S. cyanogenus* S136 and *S. globisporus* 1912, respectively. While the function of *lndI* in control of landomycin E production had been studied in details, our knowledge regarding the *lanI* function is based on indirect evidences (Rebets et al. 2003; von Mulert et al. 2004). To elucidate the role of *lanI* gene in landomycin A biosynthesis, its disruption was performed within the chromosome of *S. cyanogenus* S136 strain. The gene was replaced with the mutated allele that was generated by insertion of spectinomycin resistance gene cassette *aadA* at a position adjacent to *lanI* start codon. We succeeded in generation of *lanI* disruption mutant (referred as *S. cyanogenus* ΔlanI7) and verified it by Southern-blot hybridization (Fig. 1b). Single signal of expected size (4.8 kb) was detected in case of *Kpn*I digested total DNA of the wild type strain, whereas 2 kb longer fragment hybridized with the *lanI* probe in case of the mutant strain chromosomal DNA. This corresponds to insertion of *aadA* cassette into respective region of *lanI* gene therefore confirming the *lanI* gene replacement.

The growth rate of the mutant strain in liquid culture was not affected when comparing to the wild type strain. However, production of landomycins was severely affected. *S. cyanogenus* ΔlanI7 failed to accumulate landomycin A and its intermediates (Fig. 1c). These and previous data (Rebets et al. 2003; von Mulert et al. 2004) indicate that the *lanI* might encode the positive regulator of the landomycin E biosynthesis gene cluster. Complementation experiments were performed in order to test whether the deficiency in landomycin A biosynthesis in *S. cyanogenus* ΔlanI7 is caused solely by the *lanI* gene disruption but not by unanticipated polar effect. Introduction of either *lanI* or *lndI* gene into *S. cyanogenus* ΔlanI7 restored antibiotic production, excluding any possibility of polar effect. This data also indicates functional interchangeability of both regulators in their ability to activate landomycin A biosynthesis.

Streptomyces cyanogenus S136 wild type strain has dark blue mycelia colored with the antibiotic and is not able to produce spores (S1). Interestingly, the *lanI* deficient mutant is able to form aerial mycelium and rare spores. Futhermore, the introduction of *lanI* gene into the *S. cyanogenus* ΔlanI7 restores not only landomycin A biosynthesis but also non-sporulating phenotype of the colonies (data not shown). Active sporulation is observed also in other *lan* mutants of *S. cyanogenus* that are deficient in landomycin A production (A. Luzhetskyy, unpublished data). Additionally, overexpression of *lndI* or *lanI* genes in the *S. globisporus* 1912 wild type led to an increase in antibiotics production accompanied with the formation of bald colonies similar to *S. cyanogenus* ones (data not shown). External supplementation of landomycin A to *S. cyanogenus* ΔlanI7 causes restoration of bald phenotype of the strain around the discs with antibiotic. This data allows us to suggest that observed morphological changes are caused by the landomycins accumulation in mycelium rather than by direct effect of regulatory gene themselves. Thus, we assume that the landomycins are influencing the morphological differentiation of the producing strains. We might speculate, that strains responds to the increased accumulation of the landomycins by decreasing the sporulation and by atypical aerial mycelium formation.

RT-PCR analysis of *lanI* mutant and complemented strains

Semiquantitative RT-PCR was performed using total RNA samples from wild type strain and ΔlanI7 mutant in order to prove the function of *lanI* as transcriptional activator of structural *lan* genes in *S. cyanogenus*. The expression of following genes was examined: the ketosynthase gene *lanA*, the NDP-hexose synthetase *lanG*, the glycosyl transferase *lanGT2*, the *tetR* family transcriptional regulator *lanK*, the proton-dependent antiporter *lanJ* and *lanI* (Fig. 1a) (Westrich et al. 1999). In the case of wild type strain, all corresponding transcripts were detected as expected, whereas none of these genes were expressed in *lanI* mutant strain (Fig. 2a, b). These results strongly indicate that LanI is necessary for transcriptional activation of structural *lan* genes. Introduction of *lanI* gene into *S. cyanogenus* ΔlanI7 restored transcription of all studied genes approximately to the level of the wild type strain, leading to restoration of landomycin A biosynthesis (Fig. 2). Interestingly, introduction of *lndI* gene restore antibiotic production at lower level as compared to wild type strain and this coincided with the low level of *lan* genes transcription detected by RT-PCR (approximately two times less from *S. cyanogenus* S136 or *S. cyanogenus* SlanI7 pSlanI + strains). On the other hand, the introduction of *lanI* gene into *S. globisporus* I2-1 regulatory mutant confer higher level of landomycin E production as compared to the wild type strain (Rebets et al. 2003). We suggest that the difference in the level of landomycins production between *S. globisporus* and *S. cyanogenus* strains is caused by the differences in the structure of respective pathway specific transcriptional factors, LndI and LanI.

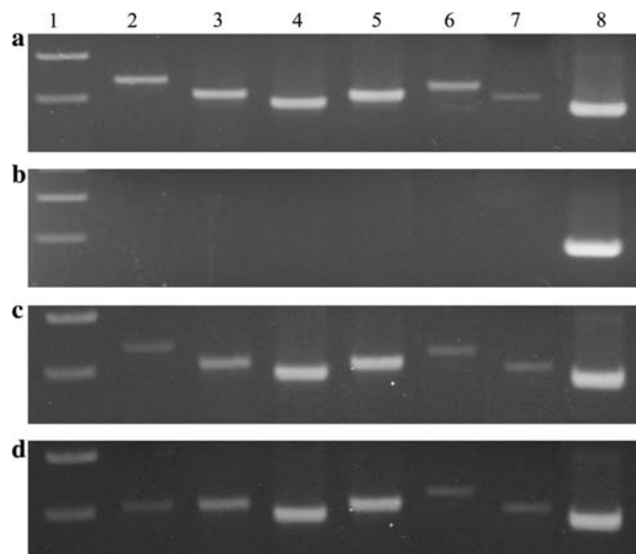

Fig. 2 Agarose gel electrophoresis of fragments obtained in RT-PCR reaction with total RNA samples of *S. cyanogenus* S136 **a**, ΔlanI7 **b**, ΔlanI7pSlanI^{+} **c**, ΔlanI7pSI2-9^{+} **d** and primers specific for *lanI* (*lndI* in the case of ΔlanI7 pSI2-9^{+} strain) (*2*), *lanA* (*3*), *lanG* (*4*), *lanGT2* (*5*), *lanJ* (*6*), *lanK* (*7*) genes and 16S rRNA (*8*). Line 1–100 bp ladder; 7 μl of reaction were loaded on gel for samples from S136, ΔlanI7, ΔlanI7pSlanI strains and 12 μl for ΔlanI7pSI2-9^{+} strain (for sample corresponding to 16S rRNA 7 μl of reaction were used in this case)

LanJ has been presumed to participate in landomycins export from the cell and its gene expression is supposed to be regulated by *lanK* (Ostash et al. 2006, et al. 2007) in the way similar to the *tcmA*/tcm*R* system controlling tetracenomycin resistance in *S. glaucescens* (Guilfoile and Hutchinson 1992). We did not detect *lanK* and *lanJ* transcripts in the case of *S. cyanogenus* ΔlanI7. Additionally, *S. cyanogenus* ΔlanI7 strain is slightly more sensitive to landomycin A in comparison to the wild type strain (data not shown). Due to high similarity of the *lanJ/K* system to the *tcmA*/*R* system we may suggest that the absence of both *lanK* and *lanJ* transcripts can be caused by the function of LanK (Guilfoile and Hutchinson 1992; Ostash et al. 2006). LanK possibly represses *lanJ* and its own gene expression until some intermediate compounds of landomycin A biosynthesis appear in the cell. Once *lanJ* is relieved from repression, *lanI* can boost its expression. Alternatively, LanI can acts through direct activation of *lanJ* expression whereas *lanK* can play role in fine-tuning of *lanJ* transcription level by its repression accordingly to the level of accumulation of landomycin A or its intermediates. However to clarify weather *lanK* is directly controlled by LanI and how these two regulators cooperates additional experiments are required.

lndI/lanI *hybrid gene expression*

Amino acid sequence alignment of LndI and LanI regulators showed that they share 61% identical amino acids. Significant differences can be found in N-terminal portion of both proteins that is consider to act as a putative signal receiving domains (Harrison-McMonagle et al. 1999; Sheldon et al. 2002). Taking the high similarity of both biosynthesis gene clusters into consideration, such differences in N-terminal domains of LanI and LndI might indicate the high evolutionary lability of these regions possibly due to the lack of essential function.

The C-terminal regions of LndI and LanI are more similar to each other than the N-terminus and presumably fold into DNA-binding winged helix-turn-helix structure (Fig. 3). We have generated chimeric genes by combining the regions encoding N-terminal and C-terminal domains of LanI and LndI regulators. Chimeric genes, designated as *ladR* (*lanI* putative promoter region, N-terminus of LanI plus C-terminus of LndI) and *ladI* (*lndI* putative promoter region, N-terminus of LndI and C-terminus of LanI) were expressed in regulatory mutants of *S. globisporus* and *S. cyanogenus*. Although, we did not observe the restoration of landomycins production, *S. cyanogenus* ΔlanI7 strain harbouring *ladI* gene produced unknown brown compound with molecular mass of 255 Da that has TLC mobility similar to that of landomycin A (mol. weight 1,297 Da, mol weight of aglycon 412 Da) (Fig. 4, S2). The antibacterial activity test showed that this compound has no effect on growth of *E. co*li W3110; however slightly inhibit growth of *S. flava* (data not shown). In the same time extract from

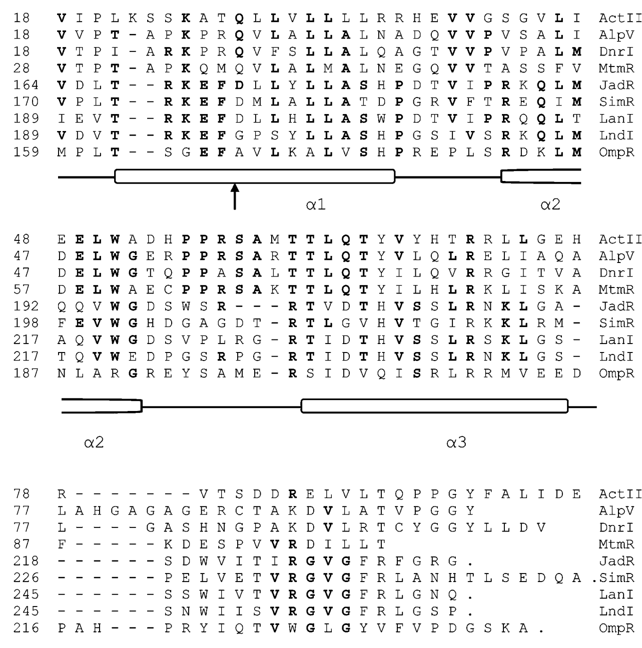

Fig.3 Sequence comparison and predicted structural organization of putative DNA-binding domains of different SARPs and OmpR protein. *Arrow* indicates the position of domains exchange between LndI and LanI proteins

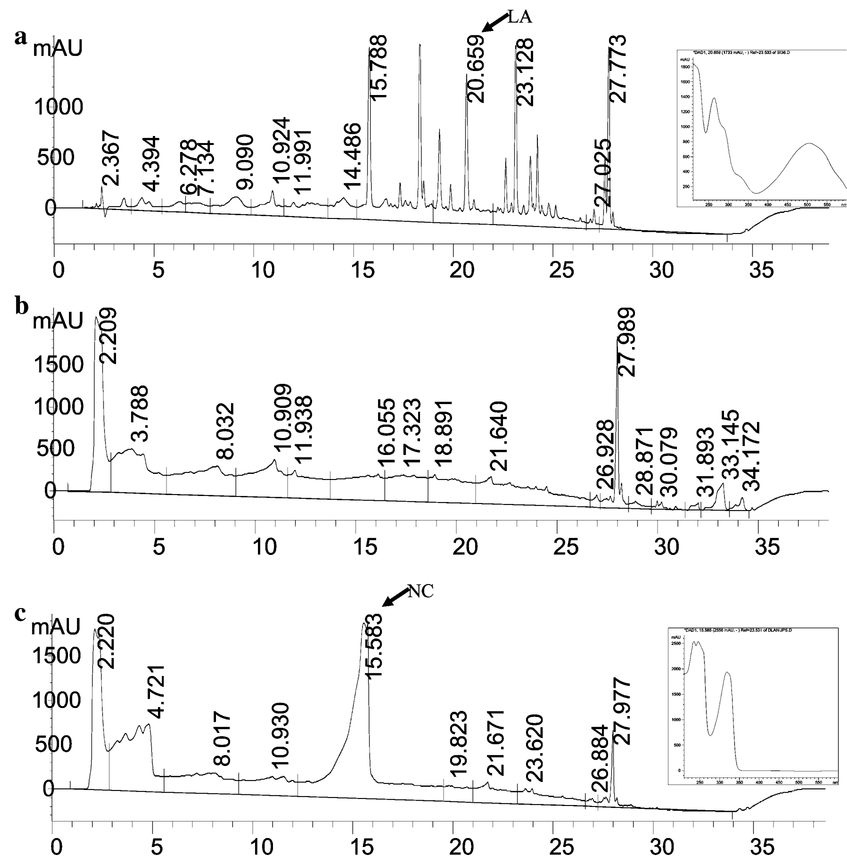

Fig. 4 HPLC–MS analysis of secondary metabolites produced by strains *S. cyanogenus* S136 **a**, *S. cyanogenus* ΔlanI7pSET152^{+} **b** and *S. cyanogenus* ΔlanI7pSladI^{+} **c**. Peaks, corresponding to a novel compound (*NC*) and landomycin A (*LA*), are indicated by *arrow*

S. cyanogenus ΔlanI7 has no activity on both test-cultures. It should be noted that this compound was never found to be produced by the wild type strain as well as by the *S. cyanogenus* ΔlanI7 harbouring *lanI*, *lndI* genes or cloning vector pSET152. RT-PCR analysis showed that *lan* genes are not transcribed in this strain (data not shown), suggesting that this compound may have originated from activation of a silent gene cluster in *S. cyanogenus* S136 genome by some unknown mechanism involving LadI. We may speculate that the LadI protein has changed specificity to binding sites on DNA compare to LanI and LndI that cause its ability to activate this silent biosynthetic pathway.

Cross complementation of regulatory mutants of streptomycetes

Amino acid sequence comparison of different SAPRs showed significant differences between regulators implicated in regulation of angucyclines and other aromatic polyketides production. In LanI, LndI, JadR1 (Yang et al. 2001) and SimR1 (Trefzer et al. 2002) the DNA-binding domain is located in the C-terminal part. In contrast, ActII-ORF4 (Fernandez-Moreno et al. 1991), DnrI (Tang et al. 1995; Sheldon et al. 2002), AlpV (angucycline, Aigle et al. 2005) and MtmR (Lombo et al. 1999) possess N-terminally located DNA-binding domains (Fig. 3). However, most of differences were found in the putative signal receiving domains of these proteins.

It has been known that some of SARPs can restore production of different antibiotics in heterologous hosts (Lombo et al. 1999; Rebets et al. 2003). We tested the ability of *lanI*, *lndI*, *simR1* (angucycline simocyclinone gene cluster), *jadR1* (angucycline jadomycin cluster), *ovmR* (angucycline oviedomycin cluster) and *mtmR* (mithramycin gene cluster) to restore landomycins E and A production in *S. globisporus* I2-1 and *S. cyanogenus* ΔlanI7 regulatory mutants, respectively. Landomycins production was restored upon *lanI*, *lndI* or *jadR1* introduction into *S. cyanogenus* and *S. globisporus* mutants. Regulatory gene *simR1*, which controls hybrid angucycline/aminocoumarine antibiotic simocyclinone D biosynthesis in *S. antibioticus* Tu6040, was not able to activate landomycins production; the same was observed in case of *ovmR* and *mtmR* genes. In the same time, neither *lanI* nor *lndI* were able to restore actinorhodin and mithramycin production in *S. coelicolor* J1681 and *S. argillaceus* R13M1 regulatory mutants, respectively (data not shown). Our data witness the differences between SARPs we tested. Despite the high similarities of the DNA-binding domains of all SARPs, the regulatory sequence recognized by these proteins seems to be different in gene clusters for angucyclines and other aromatic polyketides biosynthesis. Additionally, putative

signal receiving domains of SARPs, quite different among both groups of regulators described above, can contribute to the specific mode of action of proteins.

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