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Characterization and analysis of the regulatory network involved in control of lipomycin biosynthesis in *Streptomyces aureofaciens* Tü117

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Abstract Analysis of the α-lipomycin biosynthesis gene cluster of *Streptomyces aureofaciens* Tü117 led to the identification of five putative regulatory genes, which are congregated into a subcluster. Analysis of the *lipReg1–4* and *lipX1* showed that they encode components of two-component signal transduction systems (LipReg1 and LipReg2), multiple antibiotics resistance-type regulator (LipReg3), large ATP-binding regulators of the LuxR family-type regulator (LipReg4), and small ribonuclease (LipRegX1), respectively. A combination of targeted gene disruptions, complementation experiments, lipomycin production studies, and gene expression analysis via RT-PCR suggests that all regulatory *lip* genes are involved in α-lipomycin production. On the basis of the obtained data, we propose that LipReg2 controls the activity of LipReg1, which in its turn govern the expression of the α-lipomycin pathway-specific regulatory gene *lipReg4*. The ribonuclease gene *lipX1* and the transporter regulator *lipReg3* appear to work independently of genes *lipReg1*, *lipReg2*, and *lipReg4*.

Keywords *Streptomyces* · Secondary metabolite · α-Lipomycin · Regulation

Liliya Horbal and Yuriy Rebets equally contributed to this work.

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Introduction

The complex morphogenesis of *Streptomycetes* has made this genus a model prokaryote for studying multicellular differentiation, and the ability to produce a wide variety of secondary metabolites has made them excellent suppliers of biologically active substances (Horinouchi 2007). Secondary metabolites play different roles in a producer strain, and they are usually formed in nature at very low levels, indicating the existence of tight control mechanisms for their biosynthesis (Chen et al. 2008). Control of these processes of morphological and physiological differentiation seems to be operated at several levels. Earlier work has suggested a model in which "higher level" pleiotropic regulators activate "pathway-specific" regulators located within chromosomal gene clusters encoding biosynthesis of individual antibiotics and directly influencing the expression of biosynthetic genes (Dylan and Jensen 1998; Lee et al. 2002). Many pathway-specific regulators belong to a group called *Streptomyces* antibiotics regulatory proteins (SARPs) and are final checkpoint regulating production of certain metabolite (Fernandez-Moreno et al. 1991; Hutchings et al. 2004). Most of antibiotic biosynthetic gene clusters harbor one gene encoding transcriptional regulator. Some clusters, however, include more than one regulatory gene,

as it is the case for gene clusters for biosynthesis of pristinamycin (Folcher et al. 2001), tylosin (Bate et al. 2006), alpomycin (Aigle et al. 2005; Pang et al. 2004), fredericamycin (Chen et al. 2008), daunorubicin (Otten et al. 1995; Sheldon et al. 2002), and pimaricin (Nuria et al. 2004). For instance, TylS, DnrI, AlpV, FdmRI, and PimR are all involved in pathway-specific regulatory cascades that are tuned by pathway-specific regulators and activated or repressed by other regulators in the same cascade (Bate et al. 2006; Pang et al. 2004; Aigle et al. 2005; Fernandez-Moreno et al. 1991; Nuria et al. 2007). Despite the growing body of data on the regulatory pathways in *Streptomyces*, our understanding of secondary metabolism regulation is far from complete. In addition, investigation of regulatory mechanisms of antibiotic production is of great interest, as these studies provide a potential platform for manipulating industrially important strains to increase production of their secondary metabolites.

A gene cluster encoding type one polyketide synthase (PKSI) and nonribosomal peptide synthase (NRPS; Fig. 1) was identified in the chromosome of *Streptomyces aureofaciens* Tü117. This cluster was shown to be responsible for the production of the acyclic polyene antibiotic α-lipomycin (Fig. 2) and consequently was named *lip* (Bihlmaier et al. 2006). Lipomycin is active against gram-positive bacteria but has no effect upon the growth of fungi, yeast, and gram-negative bacteria that is not typical for polyene antibiotics (Bolard 1986). Sequence analysis of the *lip* cluster has revealed the presence of five putative regulatory genes, *lipReg1*, *lipReg2*, *lipReg3*, *lipReg4*, and *lipX1*. They are congregated into subcluster at the left end of the cluster. Such diversity of putative regulatory genes within single biosynthesis gene cluster can reflect the existence of complex regulatory machinery responsible for fine-tuning of lipomycin production. In this report, we describe the generation and analysis of the mutant strains deficient in the *lipReg* genes. On the basis of the obtained data, we propose a putative model for regulation of lipomycin biosynthesis.

Materials and methods

Bacterial strains, plasmids, and culture conditions

All strains and plasmids are listed in Table 1. *Escherichia coli* DH5α (Life Technologies) 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 *Streptomyces* species (Flett et al. 1997; Luzhetskyy et al. 2006). *E. coli* DH5α carrying pSC101 plasmid was used for constructing plasmids for gene inactivation with the use of λRedirect technology (Gust et al. 2002). For plasmid and total DNA isolation, *E. coli* and *S. aureofaciens* strains

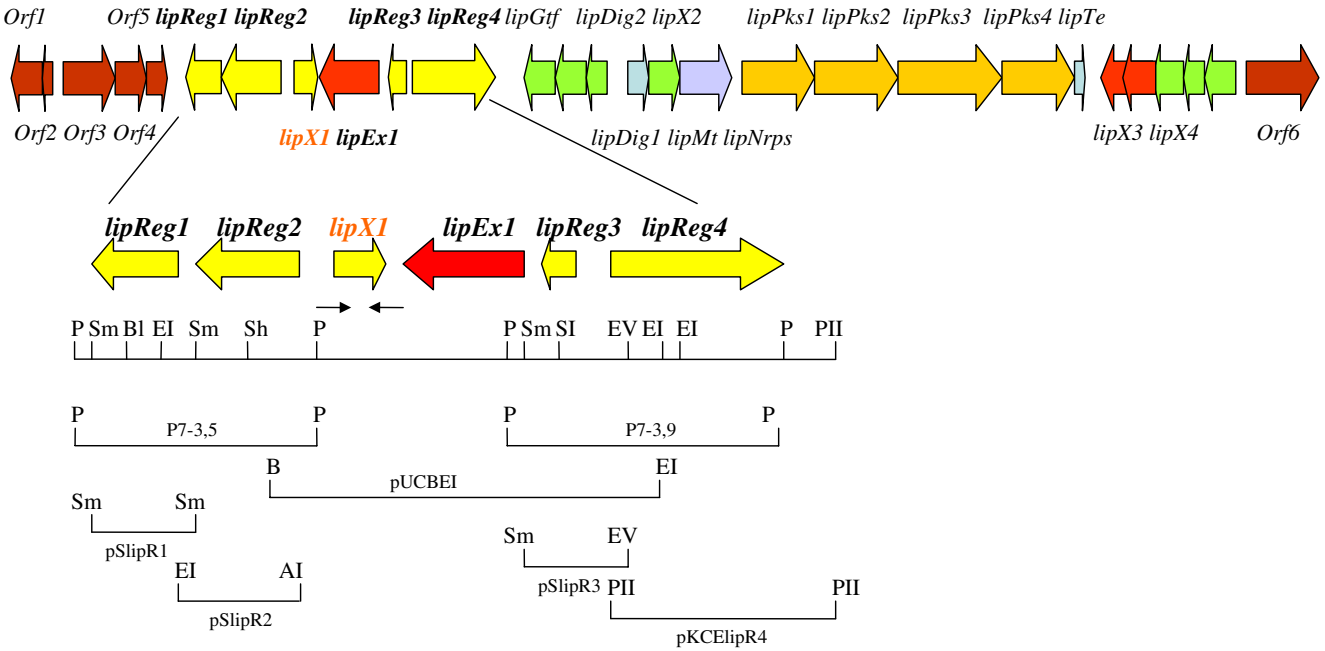

Fig. 1 Schematic representation of the lipomycin biosynthesis gene cluster of *S. aureofaciens* Tü117. Fragments used for gene disruption and expression experiments are shown below the genes. Primers used for *lipX1* gene cloning and PCR are indicated as *arrows*. Restriction endonucleases used in this work are shown: *P Pst*I, *Sm Sma*I, *Sh Sph*I, *SI Sac*I, *Bl Bsl*E, *AI Apa*I, *EI Eco*RI, *EV Eco*RV, *BI Bam*HI, *PII Pvu*II

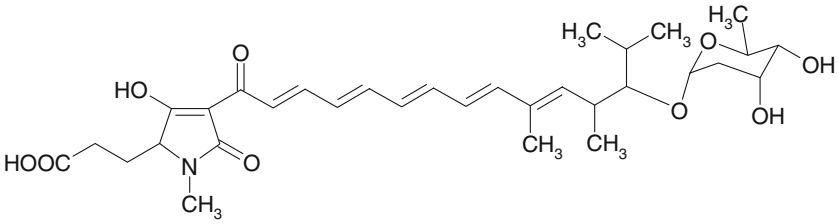

Fig. 2 Chemical structure of the α-lipomycin

were grown as described by Kieser et al. (2000) and Sambrook and Russell (2001). For α-lipomycin production, *S. aureofaciens* strains were grown in liquid HA medium (yeast extract 4 g l^{-1}, maltose 10 g l^{-1}, glucose 5 g l^{-1}, pH prior to sterilization=8.0) at 30°C. *E. coli* ET12567 (pUB307) carrying plasmids for conjugal transfer was grown on LB medium containing kanamycin (Km, 25 μg ml^{-1}), chloramphenicol (Cm, 25 μg ml^{-1}), and apramycin (Am, 50 μg ml^{-1}) for 16 h. For conjugation, spores of *S. aureofaciens* strains were harvested from a sporulated lawn grown on soy-mannitol medium (Kieser et al. 2000). When it was necessary, bacterial strains were grown in the presence of antibiotics. X-gal and IPTG were used as described elsewhere for blue–white colony selection in case of vectors pBluescript, pT7Blue, pSET152, and pKC1218E.

DNA manipulations

Isolation of genomic DNA from streptomycetes and plasmid DNA from *E. coli* were carried out using standard protocols (Kieser et al. 2000). Restriction enzymes and molecular biology reagents were used according to recommendation of suppliers (NEB, MBI Fermentas, Promega). DIG DNA labeling and Southern blot analyses were performed according to the DIG DNA labeling and detection kit (Roche Applied Science).

Construction of plasmids

For lipReg1 inactivation Plasmid p7-3,5 (Fig. 1) was digested with *Bsp*EI, treated with the Klenow enzyme and ligated to a *Sma*I fragment from pHP45Ω containing the spectinomycin resistance cassette *aadA* (Blondelet-Rouault et al. 1997). The resulting plasmid pUClipR1-aadA was digested with *Pst*I, and the 5.5-kb fragment containing *lipReg1::aadA* mutant allele was cloned into pKC1132 to yield pKClipR1::aadA.

For lipReg2 inactivation Plasmid p7-3,5 (Fig. 1) was digested with *Sph*I, treated with the Klenow enzyme and ligated to *Sma*I cassette *aadA*. The resulting plasmid pUClipR2-aadA was digested with *Pst*I, and the 5.5-kb fragment containing *lipReg2::aadA* was cloned into pKC1132 to yield pKClipR2::aadA.

For lipReg3 inactivation Plasmid p7-3,9 (Fig. 1) was digested with *Bsa*AI and ligated to the *aadA* cassette to yield pUClipR3-aadA. *lipReg3::aadA* was digested with *Pst*I fragment, and the 5.9-kb fragment was cloned into *Pst*I-digested pKC1132 to yield pKClipR3-aadA.

For lipReg4 inactivation A *Pst*I–*Sma*I 3.3-kb fragment was cloned from p7-3,9 into the *Pst*I and *Eco*RV sites of pKC1132 to yield pKClipR4. The resulting plasmid was digested with *Eco*RV and ligated to the *aadA* cassette. The obtained plasmid was named pKClipR4-aadA.

For lipX1 inactivation The *lipX1* gene was replaced with the *aadA* cassette (pIJ779) within cosmid 52-2 L13 by the use of the λRed recombination process (Gust et al. 2002). Primers lipR5aadAF and lipR5aadAR used for replacement are listed in Table 2. The resulting cosmid was digested with *Stu*I, and the *lipX1::aadA* mutant allele was cloned into the *Eco*RV-digested pKC1132 to yield pKClipR5-aadA.

Generation of the chromosomal mutants of *S. aureofaciens* Tü117

Five gene disruption plasmids were conjugally transferred from *E. coli* into *S. aureofaciens* Tü117. Exconjugants were selected for resistance to apramycin (10 μg ml^{-1}).

For the generation of *S. aureofaciens* ΔlipReg1, *S. aureofaciens* ΔlipReg2, *S. aureofaciens* ΔlipReg3, *S. aureofaciens* ΔlipReg4, and *S. aureofaciens* ΔlipX1 strains, single-crossover apramycin and spectinomycin resistant mutants were screened for loss of apramycin resistance as a consequence of a double-crossover event. Replacement of the *lipReg1*, *lipReg2*, and *lipX1* genes was confirmed by PCR using primer pairs lipR1-F + lipR1-R, lipR2-F + lipR2-R, and lipReg5-F + lipReg5-R, respectively (see Table 2 for primer sequences). The sizes of PCR fragments were 2.3, 2.8, and 1.8 kb when chromosomal DNAs of *S. aureofaciens* ΔlipReg1, ΔlipReg2, and ΔlipX1 were used, respectively, while the same primers for *lipReg1* and *lipReg2* genes produced amplicons shorter by 2 kb and primers for *lipX1* gene gave 1 kb shorter product when genomic DNA of the wild-type strain was used as a template.

Inactivation of *lipReg3* and *lipReg4* genes was proven by Southern hybridization. *Pst*I digested chromosomal DNA

Table 1 Strains and plasmids used during this work

Bacterial strains and plasmids	Description	Source or reference
E. coli DH5α	*supE44 ΔlacU169(ϕ80lacZΔM15)*	MBI Fermentas
E. coli DH5α/pSC101-BAD-gbaA	*supE44 ΔlacU169(ϕ80lacZΔM15)*, harbors pRedαβγ on the basis pSC101 with thermosensitive replicon; Tet^r	Guzmann et al. 1995; Zhang et al. 2003
E. coli ET12567/pUB307	*hsdR17 recA1endA1gyrA96 thi-1 relA1 dam*-13::Tn9(Cmr) *dcm*-6 *hsd*M; harbors conjugative plasmid pUB307; Cm^r, Km^r	C. P. Smith, UMIST, UK
S. aureofaciens Tu117	Lipomycin α and β producing strain	A. Bechthold, Germany
S. aureofaciens ΔlipReg1	Derivative of *S. aureofaciens* Tu117 with disrupted *lipReg1* gene (*lipReg1::aadA*)	This work
S. aureofaciens ΔlipReg2	Derivative of *S. aureofaciens* Tu117 with disrupted *lipReg2* gene (*lipReg2::aadA*)	This work
S. aureofaciens ΔlipReg3	Derivative of *S. aureofaciens* Tu117 with disrupted *lipReg3* gene (*lipReg3::aadA*)	This work
S. aureofaciens ΔlipReg4	Derivative of *S. aureofaciens* Tu117 with disrupted *lipReg4* gene (*lipReg4::aadA*)	This work
S. aureofaciens ΔlipX1	Derivative of *S. aureofaciens* Tu117 with disrupted *lipX1* gene (*lipX1::aadA*)	This work
pBluescriptKS$^+$	General purpose cloning vector; Ap^r	MBI Fermentas
pHP45Ω	Plasmid carrying Ω interposon with spectinomycin resistance gene cassette *aadA*; Ap^r, Sm^r/Sp^r	Blondelet-Rouault et al. 1997
pT7Blue	*E. coli* T-vector for PCR products cloning Ap^r	Novagen
pSET152	*E. coli*/*Streptomyces* shuttle vector with oC31 integration system for streptomycetes Am^r	Bierman et al. 1992
pKC1218E	pKC1218 derivative expression vector with P*ermE* promoter; Am^r	C. Olano, Univ. de Oviedo, Spain
pKC1132	*E. coli*/*Streptomyces* shuttle vector, Am^r	A. Bechthold, Germany
P7-3,5	pUC19 derivative with cloned 3.5-kb *PstI* fragment of *lip*-cluster *lipReg1 and lipReg2* genes	Bihlmaier et al. 2006
P7-3,9	pUC19 derivative with cloned 3.9-kb *PstI* fragment of *lip*-cluster *lipReg3 and lipReg4* genes	Bihlmaier et al. 2006
pBlueR4	pUC19 derivative with cloned 4.3-kb *Bam*H1–*Xho*I fragment of *lip*-cluster *lipReg4* gene	This work
pKCElipR4	pKC1218E containing lipReg4 gene under the control of erythromycin promoter	This work
pSlipReg1	pSET152 containing *lipReg1* gene	This work
pSlipReg2	pSET152 containing *lipReg2* gene	This work
pSlipR3	pSET152 containing *lipReg3* gene	This work
pKCElipR5	pKC1218E containing *lipX1* gene under the control of erythromycin promoter	This work
pUClipR1-aadA	p7-3,5 derivative with spectinomycin resistance cassette *aadA* inserted into *lipReg1* coding region	This work
pKClipR1-aadA	pKC1132 derivative with cloned *lipReg1::aadA* construction used for *lipReg1* gene inactivation	This work
pUClipR2-aadA	p7-3,5 derivative with spectinomycin resistance cassette *aadA* inserted into *lipReg2* coding region	This work
pKClipR2-aadA	pKC1132 derivative with cloned *lipReg2::aadA* construction used for *lipReg2* gene inactivation	This work
pUClipR3-aadA	p7-3,9 derivative with spectinomycin resistance cassette *aadA* inserted into *lipReg3* coding region	This work
pKClipR3-aadA	pKC1132 derivative with cloned *lipReg3::aadA* construction used for *lipReg3* gene inactivation	This work
pKClipR4	pKC1132 derivative with cloned *Pst*I fragment containing lipReg4 gene	This work
pKClipR4-aadA	pKClipR4 derivative with spectinomycin resistance cassette *aadA* inserted into *lipReg4* coding region	This work
pKClipR5-aadA	pKC1132 derivative with cloned *lipX1::aadA* construction used for *lipX1* gene inactivation	This work

of wild-type *S. aureofaciens* ΔlipReg3 and *S. aureofaciens* ΔlipReg4 strains were probed with the DIG-labeled fragment p7-3,9. A single hybridization signal of the expected size (3.9 kb) was detected in the case of the wild-type strain, and a 5.9-kb fragment was detected in both mutants.

Complementation of the *lipReg1*, *lipReg2*, *lipReg3*, *lipReg4*, and *lipX1* mutants

*Sma*I fragment (1.7 kb) harboring *lipReg1* was retrieved from p7-3,5 and cloned into pSET152 to yield pSlipR1. Gene *lipReg2* was retrieved as a 2.7-kb *Apa*I–*Eco*RI

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

Primer	Nucleotide sequence (5′–3′)	Purpose	Gene names
lipR5aadAF	TTG ACA TGC CAC TGT CTA CGC GCG TCA TCA TGA AGG CAT CCG TAT TTG CAG TAC CAG CG	Construction of *lipX1* mutant strains	*lipX1*
lipR5aadAR	TGC AAC TGT GGG ACC GAT CCG CGG GGG AGG GGG GAC TCA TGT AGG CTG GAG CTG CTT C		
lipReg5-F	GTACGACACCCTCGAACTGATCG	Complementation	*lipX1*
lipReg5-R	TCAGCAGCCGTAGTCGACCAG		
lipR1-F	GTGTCCTTGAGGACGAAGC	RT-PCR	*lipReg1*
lipR1-R	ATCAGACTGCTCCTCGTCG		
lipR2-F	GATGATCTGCCGCAGATCC	RT-PCR	*lipReg2*
lipR2-R	GAACAGTGACAAGCAGGCC		
lipR3-F	GACCCCTTCTCGCTCTTG	RT-PCR	*lipReg3*
lipR3-R	CGTGTTCGCCAACAATCAG		
lipR4-F	TGGATTTGCTCATCGAGAGG	RT-PCR	*lipReg4*
lipR4-R	TCAGCAGCAATGGACTTCCA		
lipR5-F	GTACGACACCCTCGAACTGATCG	RT-PCR	*lipX1*
lipR5-R	TCAGCAGCCGTAGTCGACCAG		
lipNRPS-F	GAGGAGAGCCTCGCCTACTGG	RT-PCR	*lipNrps*
lipNRPS-R	TCCACAGGTGTGTCGCTGTG		
lipDiG3-F	CAGGTTGTACGCGCACTGCTC	RT-PCR	*lipDig3*
lipDiG3-R	AGATCAGCAGATTGTGCGTG		
lipPks1-F	CATCTGGACACCGAGCTGTTC	RT-PCR	*lipPks1*
lipPks1-R	GTGATGAGGCCGAGGAACAC		
lipEx1-F	TGTCTTTATGTGAACGTGC	RT-PCR	*lipEx1*
lipEx1-R	CATGCCGAAGTTGAGCAA		

fragment p7-3,5, treated with Klenow enzyme and ligated into the *Eco*RV-digested pSET152 to generate pSlipR2. Plasmid for *lipReg3* expression (pSlipR3) was obtained by cloning of 1.6-kb *Sma*I–*Eco*RV fragment from p7-3,9 into the *Eco*RV site of the pSET152. Gene *lipReg4* was retrieved as *Bam*HI–*Xho*I fragment from cosmid 52-2 L13 (Bihlmaier et al. 2006) and cloned into the *Bam*HI–*Xho*I sites of pBluescriptIIKS$^+$ resulting in pBlueR4. A *Pvu*II fragment containing *lipReg4* gene was retrieved from pBlueR4 and cloned into the *Eco*RV-digested pKC1218E behind the *ermE** promoter, yielding pKCElipR4. The coding region of *lipX1* was amplified using primers lipReg5-F and lipReg5-R (Table 2) and cloned into pT7Blue using T-cloning (Köseoglu and Kocagöz 2004). Then, *lipX1*-containing *Eco*RV fragment was moved from pT7Blue into the respective site of pKC1218E to yield pKCElipR5.

Analysis of secondary metabolites production

Streptomyces strains were grown in liquid HA medium for 5 days at 30°C in a rotary shaker (180 rpm). The culture broths were extracted with an equal volume of ethyl acetate three times. Extracts were dried in vacuum and dissolved in acetonitrile. The metabolites were analyzed by high-pressure liquid chromatography (Bihlmaier et al. 2006). One milliliter of each culture was taken and lyophilized. The dry weight of each sample was measured, and the α-lipomycin concentration was determined.

Testing of antibiotic sensitivity

The sensitivity of *S. aureofaciens* ΔlipReg3 to different antibiotics, including α-lipomycin, was tested by disk diffusion test (Esser and Elefson 1970). For this purpose, disks with kanamycin (30 μg), gentamicin (10 μg), lincomycin (30 μg), tetracycline (30 μg), ampicillin (10 μg), cefamycin (30 μg), erythromycin (15 μg), oleandomycin (15 μg), polymixin (15 μg), rifampicin (15 μg), streptomycin (30 μg), and α-lipomycin (100 μg) were used. All experiments were performed on the LB–agar medium. The sizes of zones of *S. aureofaciens* growth inhibition were measured after 20 h.

RT-PCR analysis of lip genes expression

Total RNA samples were isolated from culture of *S. aureofaciens* Tü117 and all regulatory mutants grown in liquid HA medium after 5 days of incubation according to instructions of manufacturer of the SV Total RNA Isolation System (Promega). To avoid DNA contamination, samples were treated with RQ1 DNase (Promega). RNA concentra-

tion and purity were determined by measuring the ratio of OD260/OD280, and an equal amount of RNA from each studied strain was used for RT reaction. cDNA was obtained using AMV Reverse Transcriptase (Promega) and random hexanucleotide primers. PCR was performed using Go-Taq DNA polymerase (Promega) and primer pairs specific to each individual *lip* gene (Table 2). As a positive control, the *rrnA* primer pair specific to 16S rRNA of *Streptomyces coelicolor* was used. Negative controls were carried out with *rrnA* primers to confirm the absence of contaminating DNA in the RNA preparations. PCR products were analyzed by electrophoresis in 1.5% agarose gel, and band intensity was established by the ImageJ1.36b software (National Institutes of Health, Bethesda, MD, USA).

Results

Identification of putative regulatory genes within *lip* cluster

Sequence analysis of the *lip* cluster (Fig. 1) has revealed the presence of five putative regulatory genes, named *lipReg1*, *lipReg2*, *lipReg3*, *lipReg4*, and *lipX1* (Bihlmaier et al. 2006). All these genes are located at the left end of the gene cluster and are congregated in a separate subcluster.

Gene *lipReg1* encodes protein with a predicted molecular weight 23.86 kDa. BlastP-assisted homology search showed high similarity of *lipReg1* translation product to response regulatory proteins (RR) of LuxR family. The closest homologues of LipReg1 are proteins SAV_1988 from *Streptomyces avermitilis* (84% of identity) and AbsA2 from *S. coelicolor* (44% of identity). This type of transcriptional factors is known to be components of signal transduction systems, and their genes are located in close proximity to the respective protein kinases (McKenzie and Nodwell 2007). Analysis of LipReg1 deduced a.a. sequence with SwissProt protein motif identification software revealed phosphorylation site on the N terminus of the protein and DNA-binding domain on the C terminus. Based on these results, we suppose that LipReg1 is a target for protein kinase. The partner of LipReg1 is assumed to be a protein with calculated molecular weight 44.47 kDa encoded by *lipReg2* gene that is located 85 bp upstream of *lipReg1*. The closest homologues of LipReg2 are putative histidine kinase from *S. avermitilis* (NP_823166, 73% of identity) and kinase AbsA1 from *S. coelicolor* (31% of identity). In silico analysis showed that LipReg2 contains 5 transmembrane domains, indicating its putative membrane localization. These data let us suggest that LipReg1 and LipReg2 are members of two-component signal transduction system.

Gene *lipReg3* codes for a small protein with calculated molecular weight of 17.03 kDa. LipReg3 shares high level of homology with the group of transcriptional regulators belonging to the multiple antibiotics resistance (MarR) family. The closest similarity was found to putative proteins with unknown function identified during genome sequencing of *S. coelicolor* (NP_625448, 61% of identity) and *S. avermitilis* (NP_822741, 62% of identity). The closest homolog whose function was established is MarR regulator from *E. coli* with the level of identity less than 10%. It is known that transcriptional factors of MarR-type participate in a negative regulation of genes expression. For example, the MarR regulator from *E. coli* represses the expression of the *marA* gene. The *marA* gene product auto-activates expression of the marRAB operon (Alekshun et al. 2001; Grkovich et al. 2002). MarR-type proteins contain a DNA-binding motif that interacts with short conserved regions of target DNA (Alekshun et al. 2001; Grkovich et al. 2002; Saridakis et al. 2008). Analysis of the predicted secondary structure of the LipReg3 revealed the presence of such winged helix motif within the C-terminal domain. Like other MarR proteins, it might control an assortment of biological functions, including resistance to multiple antibiotics, organic solvents, and oxidative stress agents, that are collectively termed the multiple antibiotic resistance (Mar) phenotype (Alekshun et al. 2001; Saridakis et al. 2008). We suppose that LipReg3 regulates *lipEx1* transcription as the deduced a.a. sequence of *lipEx1* shows high similarity to efflux proteins.

Gene *lipReg4* is predicted to encode large protein consisting of 826 a.a. and calculated molecular weight of 90.76 kDa. Analysis of LipReg4 deduced a.a. sequence with SwissProt protein motif identification software revealed two functionally different domains. N terminus contains typical Walker A GXXXGKT (a.a. 44 - 51) and B motifs that are readily found in enzymes that bind and hydrolyze ATP. The C-terminal domain is predicted to fold into helix-turn-helix DNA-binding motif. Such architecture is typical for LuxR-type of transcriptional factors. A BlastP analysis of LipReg4 showed its high homology to known regulatory proteins NysRI from *S. noursei* (26% of identity) and PikD from *Streptomyces venezuelae* (33% of identity). The last one is a large ATP-binding regulator of the LuxR (LAL) family, a transcriptional activator that controls pikromycin biosynthesis (Wilson et al. 2001).

Gene *lipX1* codes for a small protein with calculated molecular mass of 13.84 kDa. BlastP search revealed that the putative protein sequence of *lipX1* exhibits similarity to guanylyl specific endoribonuclease Sa3 from *S. aureofaciens* (73% of identity). Sa3 belongs to the T1 family of microbial ribonucleases (RNase) and does not require a RNA component for its functioning like other RNases. It is known that Sa3 cleaves phosphodiester bonds, which are essential for both non-specific RNA degradation and for numerous

forms of RNA processing (Kennel 2002; Sevcik et al. 2002, 2004). It is worth mentioning that the function of this type of endoribonuclease was established only in vitro. There are no available data about their function in vivo. Hence, it is quite intriguing to find out why such a gene is located within the *lip* cluster and whether it is somehow involved in biosynthesis of lipomycin.

Inactivation, complementation, and overexpression studies

In order to investigate the function of the regulatory *lip* genes found in the cluster, they were replaced with the respective mutant alleles containing insertion of the spectinomycin resistance cassette (*aadA*). Gene inactivations were confirmed by PCR analysis (*lipReg1*, *lipReg2*, and *lipX1*) and Southern hybridization (*lipReg3* and *lipReg4*). In all mutants, growth and morphological characteristics were identical compared to the wild-type strain. Production of α-lipomycin was altered in all mutants as shown in Fig. 3. *S. aureofaciens* ΔlipReg1, *S. aureofaciens* ΔlipReg2, and *S. aureofaciens* ΔlipX1 produced less α-lipomycin (three, four, and two times, respectively) than the wild-type strain, indicating that they might be involved in a positive regulation of antibiotic biosynthesis. *S. aureofaciens* ΔlipReg3 produced four times more of α-lipomycin, indicating that *lipReg3* may be (directly or indirectly) involved in negative regulation of the lipomycin production. No α-lipomycin production was detectable in *S. aureofaciens* ΔlipReg4, indicating that *lipReg4* is main transcriptional activator of α-lipomycin biosynthesis.

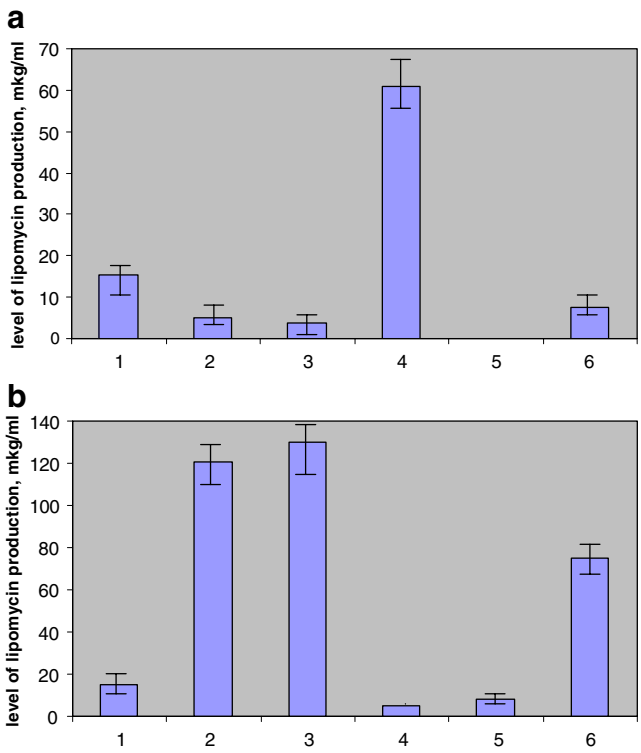

Fig. 3 Level of lipomycin production of *S. aureofaciens* strains. Each value represents the average of three different experiments. In each case, two different clones were analyzed. **a** *1*, *S. aureofaciens* Tü117; *2*, *S. aureofaciens* ΔlipReg1; *3*, *S. aureofaciens* ΔlipReg2; *4*, *S. aureofaciens* ΔlipReg3; *5*, *S. aureofaciens* ΔlipReg4; *6*, *S. aureofaciens* ΔlipX1; **b** *1*, *S. aureofaciens* Tü117; *2*, *S. aureofaciens* Tü117×pSlipReg1; *3*, *S. aureofaciens* Tü117×pSlipReg2; *4*, *S. aureofaciens* Tü117×pSlipR3; *5*, *S. aureofaciens* Tü117×pKCElipR4; *6*, *S. aureofaciens* Tü117×pKCElipR5

Complementation experiments were carried out using plasmids that express individual *lipReg* genes (see Mat and Meth) to exclude any possibility of polar effects and to confirm that the changes in α-lipomycin production were caused by the inactivation of the respective genes. In four cases (ΔlipReg1, ΔlipReg2, ΔlipReg3, and ΔlipX1), the level of α-lipomycin production by the mutants was restored to that by wild-type strain. The overexpression of *lipReg4* in *S. aureofaciens* ΔlipReg4 strain restored lipomycin production, but its level was significantly lower compared to the wild type. It could be because of an inappropriate regulation of the transcription and translation of the regulatory genes from the multicopy vectors with constitutive promoters.

Overexpression of the *lipReg* genes in the wild-type strain was also performed. Recombinant strains harboring additional copies of *lipReg1* or *lipReg2* or *lipX1* produced five to ten times more α-lipomycin compared to the wild-type strain (Fig. 3). Additionally, overexpression of *lipX1* was also leading to the accumulation of desdigitoxy-α-lipomycin, an intermediate to α-lipomycin.

A recombinant strain harboring additional copies of *lipReg3* produced three times less α-lipomycin compared to the wild-type strain, and a recombinant strain carrying additional copies of *lipReg4* produced α-lipomycin at the level of the wild type.

It is known that transcriptional factors of MarR type are usually involved in modulation of gene expression responsible for resistance to different antibacterial agents (Alekshun et al. 2001; Grkovich et al. 2002). Therefore, the level of resistance of *S. aureofaciens* ΔlipReg3 against different antibiotics, including α-lipomycin, was tested by a disk diffusion test (Esser and Elefson 1970). No obvious differences in resistance were revealed between the mutant and wild-type strain. We suppose that LipReg3 regulates *lipEx1* transcription. Gene *lipEx1* that has a high level of homology to efflux proteins is located upstream from the *lipReg3* and might be involved in export of α-lipomycin from the cell. The level of lipomycin in the culture broth of lipReg3 mutant was four times higher than in the case of wild-type strain.

RT-PCR analysis of *lip* genes expression in respective regulatory mutants

RT-PCR was performed to elucidate in more details the effect of *lip* regulatory genes inactivation on expression of structural *lip* genes as well as to reveal the interactions between individual regulatory genes. Total RNA was isolated from the *S. aureofaciens* wild-type strain and from all regulatory mutants (ΔlipReg1, ΔlipReg2, ΔlipReg3, ΔlipReg4, and ΔlipX1) during antibiotic biosynthesis and used as a template for cDNA synthesis in RT reaction. Primers complementary to regulatory genes (*lipReg1*, *lipReg2*, *lipReg3*, *lipReg4*, and *lipX1*), polyketide synthase gene (*lipPks1*), 3-ketoreductase gene (*lipDig3*), nonribosomal peptide synthetase gene (*lipNrps*), and transporter gene (*lipEx1*) were used to study the transcription of the respective genes (Table 2). All genes were found to be actively transcribed in the wild-type strain. In *S. aureofaciens* ΔlipReg1, we detected the presence of *lipReg2*, *lipReg3*, and *lipReg5* transcripts. In *S. aureofaciens* ΔlipReg2, transcripts of *lipReg1*, *lipReg3*, and *lipReg5* were identified. The level of expression of *lipReg4* gene in both mutants was much more lower than in the case of wild type (Fig. 4a), indicating that LipReg1–LipReg2 regulatory pair is likely to modulate transcription of *lipReg4*. In *S. aureofaciens* ΔlipReg4, all regulatory genes (except *lipReg4*) were active (Fig. 4b), implying that LipReg1 together with LipReg2 occupy a hierarchically higher position and can modulate *lipReg4* transcription. The *lipPks1*, *lipDig3*, and *lipNrps* transcripts were not detectable (Fig. 4c) in ΔlipReg4 mutant, again showing that LipReg4 directly controls the expression of structural *lip* genes, presumably via binding to the respective promoter regions. Taking into consideration that there was no lipomycin production in ΔlipReg4 mutant and that *lipReg1* and *lipReg2* genes were actively transcribed in ΔlipReg4, we suggest tentatively that only *lipReg4* is the only direct activator of the expression of structural *lip* genes. In *S. aureofaciens* ΔlipReg3 and *S. aureofaciens* ΔlipX1, transcripts of all other regulatory and structural genes were detectable, and no significant changes in the level of their expression were detected. In *lipReg3* mutant, the level of *lipEx1* transcript increased significantly in comparison to wild-type strain (Fig. 4d), indicating that the effect of *lipReg3* deletion on the level of lipomycin production stems from uncontrolled efflux of the antibiotic by LipEx1.

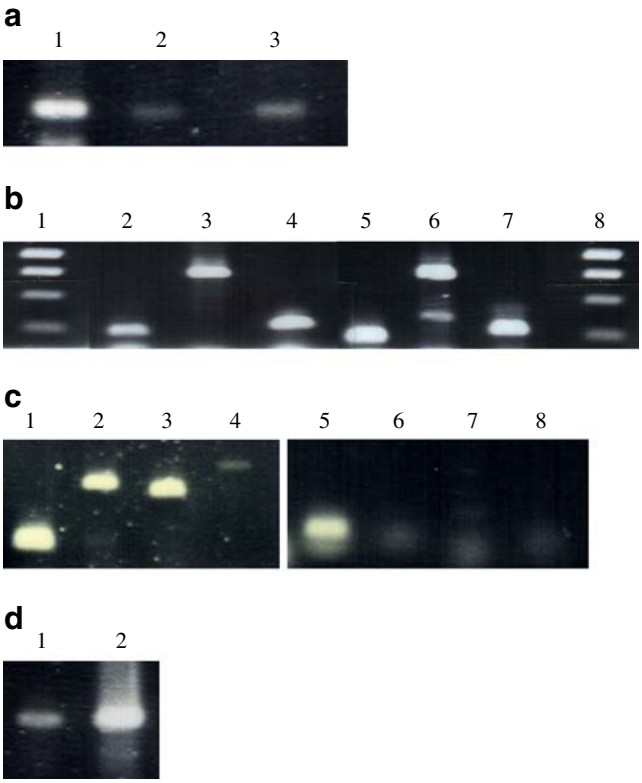

Fig. 4 Agarose gel electrophoresis of fragments obtained in RT-PCR reaction with total RNA samples of **a** *S. aureofaciens* Tü117 (*lipReg4* (*1*)), ΔlipReg2 (*lipReg4* (*2*)), ΔlipReg1 (*lipReg4* (*3*)); **b** *S. aureofaciens* Tü117 (*lipReg1* (*2*), *lipReg2* (*3*), and *lipReg3* (*4*)), ΔlipReg4 (*lipReg1* (*5*), *lipReg2* (*6*), and *lipReg3* (*7*)), *lane 1*, 8–1-kb DNA ladder; **c** *S. aureofaciens* Tü117 (*lipX1* (*1*), *lipPks1* (*2*), *lipNrps* (*3*), *lipDig3* (*4*)), ΔlipReg4 (*lipX1* (*5*), *lipPks1* (*6*), *lipNrps* (*7*), and *lipDig3* (*8*)); **d** *S. aureofaciens* Tü117 (*lipEx1* (*1*)), ΔlipReg3 (*lipEx1* (*2*))

Discussion

Most of antibiotic biosynthetic gene clusters harbor one or more regulatory genes. The lipomycin cluster from *S. aureofaciens* Tü117 contains several regulatory genes (Fig. 1), and our work allowed us for the first time to propose their roles as it is summarized in Fig. 5. Particularly, LipReg4 appears to directly control lipomycin production since inactivation of *lipReg4* completely abolished transcription of structural *lip* genes and antibiotic production. Transcripts of all other regulatory genes were identified in ΔlipReg4 mutant, indicating their hierarchically higher position compared to *lipReg4*. Gene *lipReg4* encodes a large protein possessing typical structural organization characteristic for the LAL family of transcriptional factors. It contains two Walker motifs in the N-terminal part responsible for binding and hydrolysis of ATP, while the C terminus is predicted to fold into a DNA-binding structure. The presence of Walker A and B motifs in the structure of LipReg4 is a strong hint that it activates RNA polymerases and consequently promotes the transcription of biosynthetic genes. Like guanilate cyclases and the LAL regulatory protein of the maltose regulon in *E. coli* MalT (Boos and Shuman 1998), LipReg4 probably requires energy of ATP hydrolysis for transcriptional activation.

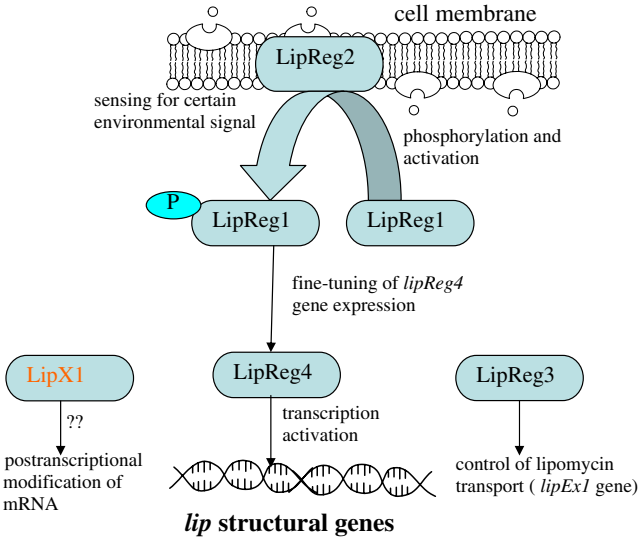

Fig. 5 Model for regulation of lipomycin biosynthesis (for details, see text)

As the LipReg1 and LipReg2 are similar to response regulators and sensor histidine kinases, respectively, they are postulated to form a two-component regulatory system. Disruption of either *lipReg1* or *lipReg2* led to a decrease in α-lipomycin production, while additional copies of *lipReg1* and *lipReg2* in *S. aureofaciens* Tü117 led to an increase in α-lipomycin production. Therefore, we believe that both proteins are responsible for fine tuning the level of α-lipomycin production.

LipReg3, a member of the MarR family, is a repressor of lipomycin export. Several lines of evidence support our conclusion. Particularly, deletion of *lipReg3* increases the transcription of exporter gene *lipEx1* and, consequently, causes greatly increased extrusion of alpha-lipomycins from producing cells. In the same time, resistance of *S. aureofaciens* ΔlipReg3 and wild-type strains to different classes of antibiotics (including alpha-lipomycin) was similar, indicating that LipEx1 does not confer high-level resistance to antibiotics (although small effects of *lipEx1* expression on alpha-lipomycin resistance cannot be excluded). On combining the results of *lipReg3* disruption, overexpression, measurements of lipomycin distribution, and RT-PCR analyses, we believe that the main role of LipEx1 is to export the final product of lipomycin pathway. Enhanced expression of *lipEx1* in ΔlipReg3 strain is likely to create favorable conditions for repeated rounds of production of lipomycin, while its accumulation inside of wild-type cells might have limiting effect on biosynthesis. It is known from literature that MarR-type regulators function is usually modulated by binding of small ligands that are substrates for the transporter system (Grkovich et al. 2002; Hopwood 2007; Tahlan et al. 2007). The high homology between LipReg3 and other MarR proteins lets us predict that LipReg3 activity can be controlled by the binding of lipomycin or some of its intermediates. Thus, we suggest that *lipReg3* could provide a feed-back control of lipomycin production.

LipReg5, belonging to the Sa-type of extracellular guanilyl specific RNases (Sevcik et al. 2002, 2004), is also involved in α-lipomycin production. Disruption of *lipX1* decreased antibiotic production. Interestingly, when *lipX1* was expressed in the wild-type strain under the control of the erythromycin promoter, a strong increase in α-lipomycin production was observed. In addition, desdigitoxy-α-lipomycin was accumulated by the strain.

One of the most striking characteristics of the regulation of secondary metabolite production in actinomycetes is its complexity and diversity. Production of α-lipomycin in *S. aureofaciens* Tü117 is also regulated by a complex and very unique set of proteins (Fig. 5). Based on sequence information and on experiments described in this study, we believe that LipReg2 is sensing for environmental signals and transducing them to LipReg1 upon phosphorylation. The phosphorylated form of LipReg1 is active and can modulate the expression of *lipReg4* gene by enhancing its transcription. LipReg3 controls the amount of α-lipomycin by downregulating the export mechanism (*lipEx1* gene expression). Although not identical, tylosin biosynthesis is also controlled by a regulatory cascade consisting of TylP, TylQ, and TylR. Here, TylP represses its own synthesis, permitting expression of *tylQ*. TylQ represses transcription of *tylR*. Once TylP is expressed, TylQ is downregulated, allowing the synthesis of TylR, which is required for tylosin biosynthesis. TylS, which is also required for *tylR* expression, is also required for the expression of some tylosin biosynthetic genes, and it is acting independently of TylR (Bate et al. 2002, 2006). In lipomycin biosynthesis, LipX1 is also positively influencing antibiotic production. The LipX1 mode of influence is still poorly understood. However, it is not a first-known example of involvement of RNase in the control of secondary metabolism. A mutant deficient in *absB*, encoding a RNase, displays severely impaired antibiotic production (Gravenbeek and Jones 2008; Price et al. 1999) and sporulation (Sello and Buttner 2008).

Summarizing all of the above, we can conclude that the *lip* cluster contains a specific group of genes forming hierarchically organized network that orchestrate lipomycin production. Some features of this system were elucidated during this work, yet additional experiments are required for complete and detailed understanding of interaction between each elements of the network.

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