DNA-binding activity of LndI protein and temporal expression of the gene that upregulates landomycin E production in *Streptomyces globisporus* 1912

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The gene *lndI* is involved in the pathway-specific positive regulation of biosynthesis of the antitumour polyketide landomycin E in *Streptomyces globisporus* 1912. LndI was overexpressed in *Escherichia coli* as a protein C-terminally fused to the intein-chitin-binding-domain tag and purified in a one-step column procedure. Results of *in vivo* LndI titration, DNA gel mobility-shift assays and promoter-probing experiments indicate that LndI is an autoregulatory DNA-binding protein that binds to its own gene promoter and to the promoter of the structural gene *lndE*. Enhanced green fluorescent protein was used as a reporter to study the temporal and spatial pattern of *lndI* transcription. Expression of *lndI* started before cells entered mid-exponential phase and peak expression coincided with maximal accumulation of landomycin E and biomass. In solid-phase analysis, *lndI* expression was evident in substrate mycelia but was absent from aerial hyphae and spores.

INTRODUCTION

Secondary metabolism in streptomycetes is tightly controlled by regulatory networks that sense different cellular and environmental signals (Chater, 1993). Genes encoding pathway-specific transcriptional activators constitute the bottom line in the regulatory hierarchy. Their expression is crucial for the initiation of secondary metabolite production. There are a few reports showing that certain mutations in such genes or alterations in their expression lead to increased antibiotic production (Stephanopoulos, 2002; Stutzman-Engwall et al., 1992). Studies aimed at furthering our understanding of how regulators exert their function are therefore of great interest for industrial applications.

The focus of this research is *Streptomyces globisporus* 1912, the producer of the potent antitumour drug landomycin E (LaE) (Fig. 1a). It shows an interesting spectrum of antitumour activities (Krohn & Rohr, 1997; Polishchuk et al., 1996). The LaE biosynthetic gene cluster (*lnd*) has been cloned (Fedorenko et al., 2000) and the gene *lndI*, encoding a putative transcriptional activator responsible for the activation of all other *lnd* genes, has been identified via gene disruption and complementation studies (Pankevych et al., 2001; Rebets et al., 2003).

The putative LndI protein features characteristics that make it attractive for further studies. It falls into a specific group of ‘angucyclic’ regulators, a separate branch of the *Streptomyces* antibiotic regulatory protein (SARP) family (Wietzorreck & Bibb, 1997). LndI and its homologous proteins contain a unique winged-helix DNA-binding motif (Martínez-Hackert & Stock, 1997), commonly found in eukaryotic transcription factors instead of the more usual helix–turn–helix motif. The DNA-binding motif is located in the C-terminal region of LndI while it is found in the

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**Abbreviations:** CBD, chitin-binding domain; EGFP, enhanced green fluorescent protein; EMSA, electrophoretic mobility-shift assay; LaE, landomycin E; PI, propidium iodide; SARP, *Streptomyces* antibiotic regulatory protein.

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is AY662671.
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A gene very similar to lndI has been identified in the landomycin A biosynthetic gene cluster of Streptomyces cyanogenus S136. Both genes have been proven to be functionally interchangeable (Rebets et al., 2003). Interestingly these proteins contain a set of different amino acids in the a-loops of their winged-helix domains, which are thought to be crucial for DNA recognition and RNA polymerase recruitment (Wietzorreck & Bibb, 1997). These differences might cause a higher level of landomycin production by S. cyanogenus S136 (Rebets et al., 2003). Detailed studies of the mechanisms of transcriptional activation of secondary metabolism pathways will help (i) to elucidate the complex interaction of regulatory proteins during physiological differentiation of Streptomyces, (ii) the understanding of the regulation of production of angucycline antibiotics and (iii) the development of rational approaches towards the generation of a LaE overproducer.

Here we report results of experiments supporting our theory of Lndl as an autoregulatory protein that binds to a promoter region of its own gene lndl and to promoter regions of structural lnd genes thus triggering LaE biosynthesis. Using the enhanced green fluorescent protein (EGFP) reporter system we have studied the temporal and spatial expression profile of the regulatory gene lndl.

METHODS

Bacterial strains and culture conditions. Bacterial strains and plasmids used in this work are listed in Table 1. Escherichia coli strains were grown at 37 °C in LB, 2 × TY or TB (Sambrook & Russell, 2001) for routine applications. S. globisporus strains were grown at 30 °C. Solid oatmeal medium (OM) (Luzhetskii et al., 2001) was used for S. globisporus sporulation and plating of E. coli-Streptomycetes matings. Growth dynamics, EGFP expression analysis and LaE production of S. globisporus 1912 strains were examined in TSB (120 h of incubation) and Hopwood’s minimal medium (MM; 120 h of incubation) (Kieser et al., 2000). For total- and plasmid-DNA isolation, S. globisporus strains were grown in YEME for 48 h (Kieser et al., 2000). Where required, S. globisporus and E. coli strains were grown in the presence of antibiotics and chromogenic substrates as described elsewhere (Kieser et al., 2000; Sambrook & Russell, 2001).

DNA manipulation. Genomic and plasmid DNA from Streptomycetes and plasmid DNA from E. coli were isolated using standard protocols (Kieser et al., 2000; Sambrook & Russell, 2001). E. coli transformation and intergeneric E. coli-Streptomycetes matings were performed as described previously (Kieser et al., 2000; Luzhetskii et al., 2001). Enzymes and kits for molecular biological manipulation were purchased from standard commercial sources and used as described by the manufacturer. PCRs were performed using a thermal cycler iCycler (Bio-Rad). PCR mixtures (50 μl) contained 10 ng of template DNA (pS12-9), Taq polymerase (1 U), 0.5 mM (each) primer and deoxynucleoside triphosphate (25 μM of each). PCR products were verified by sequencing. DNA sequencing was performed by the Sanger method using a CEQ2000 sequencer (Beckman Coulter). Sequences were analysed using DNASIS software (version 2.1; Hitachi Software Engineering).

Plasmid construction

(i) For lndl expression in Streptomycetes. For simultaneous expression of the lndl gene and the EGFP gene fused to the lnd lpromoter regions, pKC1218Ea was generated. The spectinomycin-resistance (Sp') gene cassette aadA was excised from pHP45d (Frenk & Krisch, 1984) as a HindIII fragment. It was treated with the klenow fragment and cloned into the blunt-ended XhoI site of pKC1218E

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Table 1. Strains and plasmids used during this work

<table>
<thead>
<tr>
<th>Bacterial strain or plasmid</th>
<th>Description</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>E. coli DH5x</td>
<td><em>supE44 Δ lacU169 (φ80lacZAM15) hsdRI7 recA1 endA1 gyrA96 thi-1 relA1</em></td>
<td>MBI Fermentas</td>
</tr>
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<td>E. coli BL21(DE3)</td>
<td>F− ompT1 hsdS gal [dcm] [F− casDS6 (r23 m21 ; an E. coli B strain) with DE3, a λ pro phage carrying T7 RNA polymerase gene]</td>
<td>Novagen</td>
</tr>
<tr>
<td><em>S. globisporus</em> 1912</td>
<td>Producer of landomycin E, a variant with increased LaE production</td>
<td>L. Basiliya, L’viv University, Ukraine</td>
</tr>
<tr>
<td><em>S. globisporus</em> 12-1</td>
<td><em>lndI</em>-disrupted mutant (<em>lndI::aphII</em>)</td>
<td>Rebets et al. (2003)</td>
</tr>
<tr>
<td>pBlueScriptISK−</td>
<td>General purpose cloning vector; Ap′</td>
<td>MBI Fermentas</td>
</tr>
<tr>
<td>p77Blue</td>
<td>E. coli T-vector for PCR products cloning; Ap′</td>
<td>Novagen</td>
</tr>
<tr>
<td>pTYB2</td>
<td>Expression vector to purify proteins fused to intein-chitin-binding domain ([IMPACT-CN system]; Ap′)</td>
<td>NEB</td>
</tr>
<tr>
<td>pSOK101</td>
<td><em>E. coli</em> Streptomyces shuttle vector with pJ101 replicon; Am′</td>
<td>Zotchev et al. (2000)</td>
</tr>
<tr>
<td>pKC1218E</td>
<td><em>E. coli</em> Streptomyces shuttle expression vector with <em>p ermE</em> and SCP2 replicon, derivative of pKC1218 (Kieser et al., 2000); Am′</td>
<td>C. Olano, University de Oviedo, Spain</td>
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<td>pH450</td>
<td>Plasmid carrying Ω interposon with spectinomycin-resistance gene cassette <em>aadA</em>; Ap′ Sm′/Sp′</td>
<td>Prentki &amp; Krisch (1984)</td>
</tr>
<tr>
<td>pKC1218Ea</td>
<td>pKC1218E derivative with <em>aadA</em> marker gene instead of <em>Am′</em> gene <em>aac(3)IV</em>; Sm′/Sp′</td>
<td>This work</td>
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<td>pIJ8660</td>
<td>pC31-based promoter probe vector using enhanced green fluorescent protein (EGFP) gene as a reporter; Am′</td>
<td>M. J. Bibb, JIC, UK</td>
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<td>pT7BluelndI−2</td>
<td>pT7Blue T-vector carrying <em>lndI</em> gene used for LndI overproduction as LndI−intein−CBD fused protein</td>
<td>This work</td>
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<td>pSI2-9</td>
<td>pSET152 containing <em>lndI</em> gene</td>
<td>Rebets et al. (2003)</td>
</tr>
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<td>pKC1Eal</td>
<td>pKC1218Ea containing <em>lndI</em> gene under control of <em>P ermE</em></td>
<td>This work</td>
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<td>pSIZT4</td>
<td>pTYB2 containing <em>lndI</em> gene</td>
<td>This work</td>
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<td>pBLH</td>
<td>pBluecriptISK− containing <em>lndI</em> promoter region (<em>P indI)</em></td>
<td>This work</td>
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<tr>
<td>pBLE</td>
<td>pBluecriptISK− containing <em>lndI</em> promoter region (<em>P indI)</em></td>
<td>This work</td>
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<td>pBlue1.8K</td>
<td>pBluecriptISK− with cloned 1.8 kbp <em>KpnI</em> fragment containing <em>lndE−lndF</em> intergenic region</td>
<td>Ostash et al. (2003)</td>
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<td>pSOH</td>
<td>pSOK101 containing <em>P indI</em></td>
<td>This work</td>
</tr>
<tr>
<td>pSOE</td>
<td>pSOK101 containing <em>P indI</em></td>
<td>This work</td>
</tr>
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<td>pIJ8660H</td>
<td>pIJ8660 with 0-47 kbp <em>EcoRI-KpnI</em> fragment, containing <em>lndI</em> promoter, inserted upstream of EGFP gene</td>
<td>This work</td>
</tr>
<tr>
<td>pIJ8660N</td>
<td>pIJ8660 with 0-27 kbp <em>Ndel</em> fragment containing <em>lndI</em> promoter, inserted upstream of EGFP gene</td>
<td>This work</td>
</tr>
<tr>
<td>pIJ8660E</td>
<td>pIJ8660 with 0-57 kbp <em>EcoRI-BamHI</em> fragment, containing <em>lndI</em> promoter, inserted upstream of EGFP gene</td>
<td>This work</td>
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(Rodriguez et al., 2002), which is located within the apramycin-resistance gene *aac(3)IV*. For the generation of pKCE1 *lndI* was cloned on a 3 kbp *EcoRI* fragment (Fig. 1b) into pKC1218Ea.

(ii) For in vivo titration of LndI. Putative promoter sequences were searched with GENETYX-MAC8.1 and DNASTAR software. Promoter regions of the *lndI* (*P indI*) and *lndE* (*P indE*) genes were subcloned from pSI2-9 as HindIII–HindIII and *EcoRV*–*EcoRI* fragments (Fig. 1b; second HindIII site, adjacent to *EcoRI* at the end of *lndI* comes from polylinker of pSI2-9), respectively, into pBluecriptISK− to give pBLH (containing *P indI*) and pBLE (containing *P indE*). The GenBank accession number for *lndI* and its promoter region sequence is AF599998. The sequence of the *lndE* upstream region has been submitted to GenBank under accession number AF562671. To generate pSOH *P indI* was retrieved from pBLH as an *EcoRI*–*BamHI* fragment and cloned into pSOK101. To generate pSOE an *EcoRV*–*EcoRI* fragment from pBLE containing *P indE* was subcloned into *EcoRV*–*EcoRI*-digested pSOK101 (Fig. 1b).

(iii) For expression of LndI as a CBD-fusion protein. For LndI expression using the pTYB2 IMPACT-CN system, primers Lndl and Lec1Rev (5′-ATCATATAGGGAGCGCTGCG and 5′-AATGATATCGAGATCGGGCGGAA, respectively; *Ndel* and *EcoRV* sites underlined) were used to amplify *lndI*. The PCR product was cloned into pT7Blue T-vector to generate pT7BluelndI-2. The *lndI* gene was excised from this plasmid as a *Ndel*–*EcoRV* fragment and cloned into the *Ndel*–*Smal*-digested vector pTYB2 to fuse *lndI* with the intein-chitin-binding domain (CBD) coding region. The generated plasmid was named pSIZT4.

(iv) For studying transcriptional activity of *ind promoters*. The *EcoRV*–*KpnI* fragment carrying *P indI* was excised from pBLH and ligated into the respective sites of pIJ8660 to generate a transcriptional unit with *P indI* and the EGFP gene. The plasmid was named pIJ8660H. To shorten the *lndI* gene promoter region to a final length of 270 bp an *Ndel* fragment was cloned from pIJ8660H into the respective site of pIJ8660 to create pIJ8660N. *P indE* was
subcloned as an EcoRV–BamHI fragment from pBLE into pJ8660 to generate pJ8660E.

**Overexpression and purification of Lndl.** *E. coli* BL21(DE3) strains harbouring plasmids for Lndl expression were grown in TB at 36 °C to an optical density at 600 nm of 0.5, induced with IPTG (0.4 mM) and incubated for an additional 17 h at 16 °C. His-tagged Lndl purification was performed essentially as recommended by commercial suppliers for native conditions (Novagen).

Cells of *E. coli* BL21(DE3) harbouring pSIZT4 were resuspended in binding buffer [10 mM Tris/HCl (pH 7-5), 500 mM NaCl, 0-5 mM EDTA]. After disruption by sonication, proteins were loaded onto a chitin-agarose column (NEB) and washed with 10 vols of binding buffer. CBD-fusion-protein purification was performed as directed by the manufacturer (NEB).

**Protein analysis.** Protein concentrations were determined by the BCA (bicinchoninic acid) protein assay (PIERCE) with bovine serum albumin (BSA) as standard. SDS-PAGE was performed according to the method of Laemmli (Sambrook & Russell, 2001), and the gels were Coomassie-blue-R-stained. For total protein analysis, according to the method of Laemmli (Sambrook & Russell, 2001), samples were centrifuged and incubated in 0-4 M NaOH for 12 h.

**DNA-protein-binding assays.** A 467 bp EcoRI–HindIII fragment from pBLH (P_{lndl}), a 557 bp EcoRI–EcoRV fragment from pBLE (P_{ble}) and a 313 bp KpnI–PstI fragment from pBluel8K (Fig. 1b) were used in the assays. The GenBank accession number of the sequence for *lndE* and the surrounding regions is AY659997. All fragments were labelled from both ends with fluorescein using a 5-IAF-labelling kit as described by the supplier (Amersham Biosciences). DNA-binding tests were performed by the electrophoretic mobility-shift assay (EMSA) (Taylor et al., 1994) with 5-0 to 0-7 µg of Lndl preparation. Fluorescein-labelled DNA (2-5 ng) was then added, and the reaction mixture was incubated for 20 min at 27 °C. The gels were analysed by scanning with a Typhoon 9400 Variable Mode Imager (Amersham Biosciences). In competition experiments the assay was performed with various amounts of unlabelled competitor DNA.

**LaE production analysis.** A pure sample of LaE, kindly provided by Professor J. Rohr (College of Pharmacy, University of Kentucky, KY, USA), was used as an HPLC standard. LaE production was analysed via HPLC as described (Rebets et al., 2003). The levels of LaE production in our experiments are related to equal proportions of total protein in different strains. Measurements were taken in triplicate and the means of the data were found.

**Sample preparation and confocal microscopy.** For EGFP-protein production analysis, mycelia grown in TSB media were washed with deionized water and applied to glass slides. To study cell viability, propidium iodide (PI) was used as an indicator in a procedure described elsewhere (Khetan et al., 2000; Kyung et al., 2001).

For solid-phase observations, single colonies were grown on thin MM plates (Kieser et al., 2000) containing mannitol instead of glucose, and thin longitudinal slices were removed with a sharp blade and covered with water.

A Fluoroview confocal system (Olympus) with an Olympus OL BX50 microscope and an argon laser (providing excitation at 488 nm) was used to observe *S. globisporus* strains with GFP expression and PI staining. FITC- (emission at 506–535 nm) and PI- (emission at 600–615 nm) filter sets were used to observe green and red fluorescence, respectively. Green fluorescence and transition or PI images were obtained simultaneously by using separate detectors. To gain high reliability in quantitative analysis of captured images the same operation parameters were used for samples at the same time point. The confocal images were saved as TIFF files and image analysis was performed by FLUOROVIEW 2.1 software (Olympus). EGFP expression was quantified by measuring the fluorescence intensity within a rectangle on each image (Kataoka et al., 1999).

**RESULTS**

**Titration of Lndl by the *lndl* and *lndE* promoter regions results in lower production of LaE**

Due to amino acid sequence homology to other regulatory proteins the *lndl* gene product was thought to be a transcriptional activator (Pankevych et al., 2001). The Lndl protein could perform its regulatory function via binding to promoter regions of *lnd* genes. Putative promoter regions have been detected in the biosynthetic gene cluster. A DNA fragment upstream of the *lndl* coding sequence was found to contain putative promoter sequences characteristic of streptomycete −10 and −35 promoter regions (5'-TAGTTT and 5'-TTGACA, respectively; 46 bp upstream of the *lndl* translation start codon) and specific tandemly arrayed heptamer sequences (with consensus 5'-TGCGCG(T/A)(G/C)G(C/A)5(C/A)5; 151 bp upstream of the *lndl* translation start codon), which are possible recognition elements for a regulatory protein (Bourn & Babb, 1995; Ostash et al., 2003b). We failed to identify sequences resembling consensus −10 and −35 promoter regions upstream of *lndE*. However, one can suppose tentatively that *lndE* as the first structural gene of the cluster should contain its own promoter. Thus we used the *lndE* 557 bp upstream region (which includes 325 bp preceding the *lndE* start codon) in our experiments and referred to it as the promoter solely for the purpose of convenience. The intergenic regions *lndE–*lndF and *lndF–*lndA are too short (37 bp and 29 bp, respectively) to contain promoters (Luzhetskii et al., 2001; Ostash et al., 2003b). Therefore it seems likely that transcription of the *lndEFABCD* genes can be driven from the *lndE* gene promoter.

To prove that Lndl is a central regulatory protein in vivo Lndl titration experiments were undertaken. P_{ble} and P_{lndl} were chosen for these studies. pJ101-based plasmids pSOH and pSOE, containing P_{lndl} and P_{lndE} respectively, were introduced into *S. globisporus* 1912. LaE production was significantly reduced in both pSOH- (to 71% of the wild-type level) and pSOE- (to 43% of the wild-type level) harbouring strains. This effect can be explained by titration of Lndl by the plasmid-borne P_{lndl} and P_{lndE}. The negative effect on LaE production was more pronounced in strain 1912(pSOE). More efficient competition of P_{lndE} for the Lndl protein possibly reflects higher affinity of Lndl for...
Characterization of *S. globisporus* LndI protein

**Fig. 2.** SDS-PAGE analysis of purified CBD-fused LndI protein. Lanes: 1, protein molecular size marker; 2, total soluble extract from cell lysate of *E. coli* BL21(DE3)(pSiZT4); 3, insoluble proteins from cell lysate of *E. coli* BL21(DE3)(pSiZT4); 4, purified LndI protein; 5, chitin resins with remaining proteins after LndI elution. LndI, CBD and the CBD–LndI fusion protein and their molecular masses are indicated.

LndI was overexpressed in *E. coli* and purified as an intein-CBD-tagged protein

All attempts to purify sufficient quantities of soluble 6-His-tagged LndI were unsuccessful. The level of recombinant LndI production was low and, under conditions stated, less than half of the protein was present in soluble fraction (confirmed by SDS-PAGE; data not shown). To avoid the problems of low protein solubility we pursued a strategy to clone and express LndI as a C-terminal CBD-fusion protein. Using LndI–intein–CBD fusion, we obtained highly soluble LndI, which was easily purified by a one-step column procedure. A high concentration of recombinant LndI protein was achieved and purified LndI protein; 5, chitin resins with remaining proteins after LndI elution. LndI, CBD and the CBD–LndI fusion protein and their molecular masses are indicated.

LndI binds to the promoter of its own gene *lndI* and to the promoter located upstream of the oxygenase gene *lndE* but does not interact with the *lndE–lndF* intergenic region

Purified LndI was used to assess *in vitro* its DNA-binding activity. Fluorescein-labelled P<sub>lndI</sub> and P<sub>lndE</sub> were used in EMSA, and in both cases LndI caused clear retardation of DNA mobility (Fig. 3). To investigate whether complex formation between the promoter regions and LndI was highly specific, various amounts of unlabelled P<sub>lndI</sub> and P<sub>lndE</sub> DNA were added to the reaction mixture. Indeed, the unlabelled P<sub>lndI</sub> and P<sub>lndE</sub> competed efficiently for LndI depending on the amount of the competitor DNA added (Fig. 3).

To confirm the absence of a LndI-binding region located between *lndE* and *lndF* we performed EMSA using the labelled *lndE–lndF* intergenic fragment. No shift was observed upon addition of various amounts of LndI in different experiments (data not shown). This supports our speculation about the absence of *lndI*-regulated promoters upstream of the *lndF* gene.

Results of *in vitro* analysis of LndI-binding activity are in good agreement with the LndI *in vivo* titration experiment. We assume LndI to be a DNA-binding regulatory protein that controls transcription of the structural *lnd* genes and its own *lndI* gene.

**Transcriptional fusion of the *lndI* promoter region to the EGFP reporter gene allows analysis of temporal and spatial *lndI* expression**

EGFP has been shown to be a convenient reporter system in studies on the temporal and spatial expression of various streptomycete genes (Sun *et al.*, 1999). EGFP expression levels can be quantified reliably (Kataoka *et al.*, 1999), and its use does not cause any artifacts that would result from a highly stable reporter protein (Khetan *et al.*, 2000; Kyung *et al.*, 2001). To analyse the spatial and temporal expression pattern of the *lndI* gene the *C31*-based plasmid pI8660H, carrying P<sub>lndI</sub> transcriptionally fused to the EGFP gene, was constructed. pI8660H was introduced into *S. globisporus* 1912. Integration was confirmed by Southern blot analysis (data not shown). Spore suspensions of strains 1912(pI8660H) and 1912(pI8660) were inoculated into 250 ml baffled flasks with 50 ml TSB, and samples of the strains were taken at different culture times for confocal scanning microscopy, and total protein and LaE production determination. Results of these experiments are presented in Fig. 4 and summarized in Fig. 5.

To assess the potential use of EGFP as a reporter system for studying *S. globisporus* gene expression, wild-type strain harbouring vector pI8660 was examined for autofluorescence with both FITC and PI filter sets. The level of fluorescence of mycelia was insignificant (data not shown) and did not cause any artifacts with EGFP expression or PI-staining detection.

**Fig. 3.** Gel mobility-shift assay of fluorescein end-labelled DNA fragments containing *lndI* (a) and *lndE* (b) promoter regions with purified LndI protein. All samples contained 5 ng of labelled target DNA. Lanes: 1, without LndI; 2, with LndI only; 3–5, with LndI and an appropriate amount of unlabelled competitor DNA added.
In the batch culture of *S. globisporus* 1912(pIJ8660) no fluorescence was detectable during the first 12 h of growth. After 12 h slight EGFP production was detectable in culture samples. It was much higher after 24 h, with a maximum after 48 to 60 h. After 60 h the intensity of the fluorescence decreased strongly. LaE synthesis was not detectable during

**Fig. 4.** (a) Autofluorescence of a 48-h-old culture of *S. globisporus* 1912(pIJ8660). (b) Distribution of GFP expression from *IndI* gene promoter in *S. globisporus* 1912(pIJ8660H). Fluorescence (left) and transmission (right) images at different time points of culture growth are shown. Rectangles indicate regions of quantitative analysis.
very weak level of fluorescence was observed 48–60 h after

Spatial EGFP expression driven from the lndI promoter region was studied using S. globisporus colonies harbouring pIJ8660H grown for 120 h on solid MM. No fluorescence was observed in spores and aerial mycelia, while a high level of GFP expression was detected in substrate mycelia (Fig. 7). No fluorescence was observed in substrate mycelia in the case of S. globisporus 1912(pIJ8660). This indicates that neither lndI nor lndE gene promoters are active in aerial mycelia and spores, and that they function only in substrate hyphae.

**lndI activates EGFP expression from the lndE and lndI promoter regions and LaE production in S. globisporus mutant I2-1**

The interactions of LndI with the lndE and lndI promoters were investigated as follows. Plasmid pIJ8660E, containing the P<sub>lndE</sub>-EGFP fusion, was introduced into mutant S. globisporus I2-1, which lacks lndI (Rebets et al., 2003). Only a very weak level of fluorescence was observed 48–60 h after inoculation, although it was clearly higher than the level of autofluorescence of the wild-type strain (data not shown). Plasmid pKCEaI, containing lndI, was then used to transform S. globisporus I2-1 containing pIJ8660E. A strong increase in fluorescence intensity (about 4.5-fold; Table 2) was detectable after 48–60 h of incubation, indicating elevated expression of EGFP from the lndI promoter. LaE production was restored too (data not shown). These data clearly show that lndI is a positively acting regulatory gene governing the expression of structural lnd genes. LndI also increased expression of EGFP from the lndI promoter (pIJ8660H) albeit to a much lower extent (1.35-fold increase; Table 2), showing that LndI appears to act as an activator of its own gene.

**Direct repeats upstream of the lndI gene promoter are not essential for its activity**

Upstream from the lndI gene promoter, heptameric repeats have been described (Ostash et al., 2003b). To study the role of this region in lndI gene expression plasmid pIJ8660N was constructed by subcloning from pIJ8660H a fragment containing P<sub>lndI</sub> but lacking repeats into pIJ8660 (Fig. 1b). In S. globisporus 1912 strains carrying pIJ8660H and pIJ8660N the level of P<sub>lndI</sub> activity established by the level of fluorescence was the same. Furthermore, no differences in the temporal pattern of EGFP gene expression from pIJ8660H and pIJ8660N in S. globisporus 1912 were found. This indicates that the tandemly arrayed repeats upstream of the lndI gene are dispensable for its expression.

**DISCUSSION**

Pathway-specific regulatory genes have been found in many gene clusters for antibiotic biosynthesis (Fernandez-Moreno et al., 1991; Narva & Feitelson, 1990; Paradkar et al., 1998; Stutzman-Engwall et al., 1992). Most of them fall into the specific family of Streptomyces antibiotic regulatory proteins (SARPs; Wietzorreck & Bibb, 1997). All SARPs show high homology to the DNA-binding domain of the E. coli osmotic regulator OmpR (Martinez-Hackert & Stock, 1997). The best-studied representatives of the SARP family are ActII-ORF4, involved in actinorhodin biosynthesis (Arias et al., 1999), DnrI, involved in daunorubicin biosynthesis (Tang et al., 1995), and CcaR, involved in cephaparin biosynthesis (Santamarta et al., 2002).

Recently several gene clusters for angucycline antibiotics have been cloned and sequenced. Genes encoding putative transcriptional activators were identified within these clusters (Fedorenko et al., 2000; Trefzer et al., 2002; Westrich et al., 1999; Yang et al., 2001). They are distinct from other members of the SARP family in that they contain a winged helix–turn–helix DNA-binding domain in their C-terminal region (Wietzorreck & Bibb, 1997). Promoter regions of angucycline biosynthetic genes appear to not contain typical SARP-like target DNA sequences, suggesting a different mechanism for controlling the production of these compounds.

**Fig. 5.** Dynamics of growth established as total protein accumulation (µg ml⁻¹; b, ◆), landomycin E production (µg ml⁻¹; b, ■) and mean fluorescence intensity (a, ▲) by S. globisporus 1912(pIJ8660H).
LaE is a highly cytotoxic angucycline showing also moderate antibacterial activity (Krohn & Rohr, 1997; Polishchuk et al., 1996). It is produced at a low level in the wild-type strain. As a potentially toxic compound, LaE production should be under strict control to avoid its undesired overproduction. LndI has been identified as a putative transcriptional activator of the LaE cluster. Interestingly, LndI contains the rare TTA codon that might limit LndI expression (Pankevych et al., 2001) and is a characteristic for other antibiotic biosynthesis regulators. Our data show that, besides temporal regulation, LndI expression is also the subject of spatial control as is evident from the analysis of PlndI-driven EGFP expression in S. globisporus colonies grown on solid medium.

Based on in vivo LndI titration experiments, gel mobility-shift assays and promoter-probing studies LndI can be classified as an autoregulatory DNA-binding protein. To our knowledge, this is the first research work on an autoregulatory pathway-specific transcriptional factor involved in polyketide biosynthesis in actinomycetes. A similar function has been described for CcaR, a regulatory protein involved in the activation of the β-lactam supercluster (Santamarta et al., 2002). However, the extent of lndI gene activation by LndI is much lower than that of lndE. Thus the biological significance of LndI autoregulation should be addressed in additional experiments. We showed here that LndI binds to the lndE promoter region and to the lndI promoter, and that direct repeats tandemly localized upstream of lndI are not involved in lndI regulation. However, additional biochemical experiments are still required to understand the DNA-binding features of LndI.

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**REFERENCES**


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**Table 2.** Fluorescence intensity in 48 h culture of lndI-deficient strain *S. globisporus* I2-1 containing EGFP-fused lndI (pIJ8660H) or lndE (pIJ8660E) promoter regions in presence and absence of lndI gene (pKCEaI)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Size of intensity measuring rectangle (μm²)*</th>
<th>Mean fluorescence intensity (units)†</th>
<th>Maximal fluorescence intensity (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. globisporus</em> I2-1(pIJ8660H)</td>
<td>2013</td>
<td>2287</td>
<td>2642</td>
</tr>
<tr>
<td><em>S. globisporus</em> I2-1(pIJ8660H, pKCEaI)</td>
<td>2033</td>
<td>3102</td>
<td>3809</td>
</tr>
<tr>
<td><em>S. globisporus</em> I2-1(pIJ8660E)</td>
<td>2038</td>
<td>750</td>
<td>1036</td>
</tr>
<tr>
<td><em>S. globisporus</em> I2-1(pIJ8660E, pKCEaI)</td>
<td>2040</td>
<td>3677</td>
<td>4025</td>
</tr>
</tbody>
</table>

*Ratio of total fluorescence level to size of measuring rectangle.

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**Fig. 6.** Distribution of green fluorescence (right) caused by EGFP and red fluorescence (left) caused by PI staining at different time points of growth of *S. globisporus* 1912(pIJ8660H) culture.

**Fig. 7.** Distribution of GFP expressed from the lndI promoter region in *S. globisporus* 1912(pIJ8660H) growing on solid medium MM (vertical slice through *S. globisporus* 1912(pIJ8660H) colony). A, aerial mycelia and spores; B, substrate mycelia.


