

## Coordination of export and glycosylation of landomycins in *Streptomyces cyanogenus* S136

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### Abstract

In *Streptomyces cyanogenus* S136 gene cluster for biosynthesis of polyglycosylated angucycline landomycin A (LaA), a divergently oriented gene pair for a TetR-family regulator (*lanK*) and an efflux protein (*lanJ*) is located, whose functions remained obscure. Overexpression and disruption studies showed that *lanK* and *lanJ* genes control LaA resistance. Also, a constitutive *lanK* overexpression led to predominant accumulation of LaA precursors bearing shorter glycoside chains. These data as well as the results of *in vitro* and *in vivo* assays of LanK activity are consistent with the idea that LanK represses *lanJ* and some downstream genes involved in conversion of landomycin D (a disaccharide LaA precursor) into LaA. LaA and some of its precursors accumulate in the producing cell and relieve repression by LanK, thus amplifying the biosynthesis and export of landomycins with long glycoside chains. Therefore, the main biological role of LanK appears to be the inhibition of premature extrusion of early LaA precursors from the cells, which in turn creates the optimal conditions for accumulation of LaA as the major landomycin in *S. cyanogenus* S136.

### Introduction

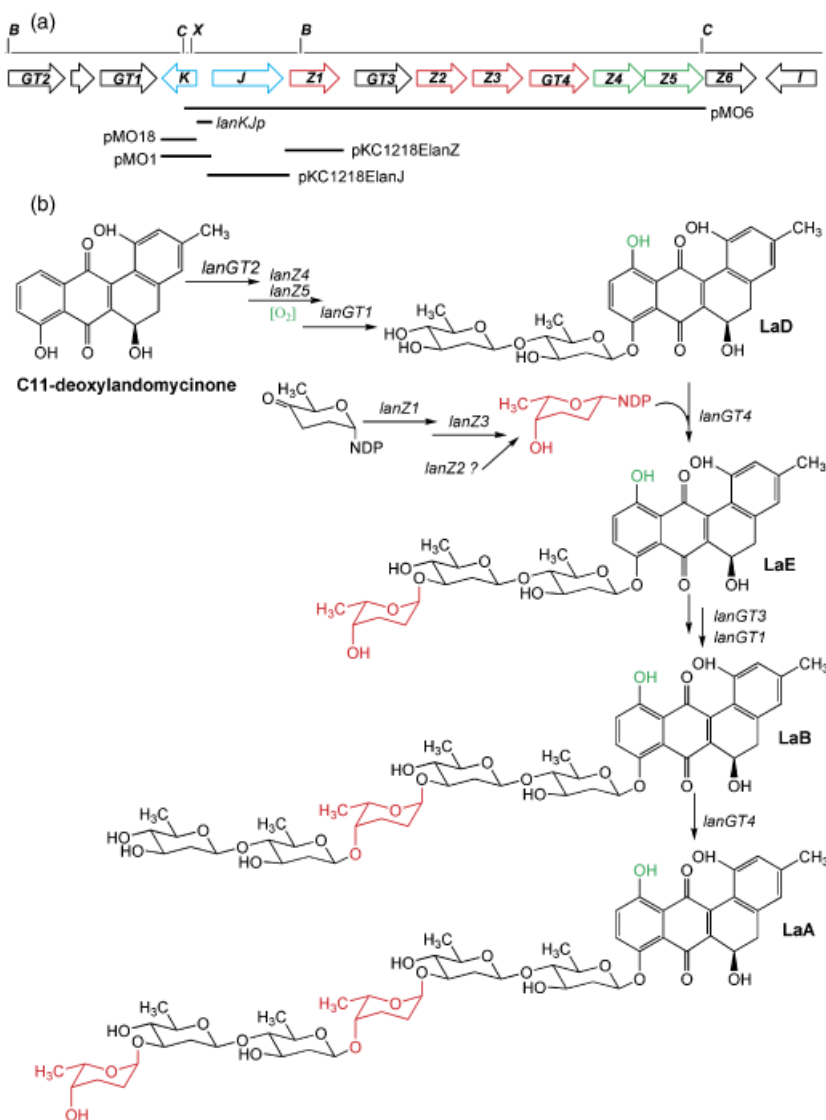
The angucycline antibiotic landomycin A (LaA) (Fig. 1) is the final product of the landomycin (La) biosynthetic pathway in *Streptomyces cyanogenus* S136. This molecule attracts interest because of its unusual hexasaccharide moiety and strong cytotoxicity (Zhu *et al.*, 2007). Two major LaA intermediates that accumulate in *S. cyanogenus* S136 are landomycins D (LaD) and B (LaB), which possess di- and pentasaccharide chains, respectively (Fig. 1). LaA and other landomycins also possess weak antibacterial activity (Matse-liukh *et al.*, 1998; Ostash *et al.*, 2007). Therefore, some La resistance mechanism(s) should operate in *S. cyanogenus* S136. The LaA biosynthetic gene cluster (*lan*) contains a proton-dependent transporter gene *lanJ*, which could be involved in landomycins' resistance/export. Another gene, *lanK*, encoding a putative TetR-family regulator, is divergently located upstream of *lanJ* (Westrich *et al.*, 1999) (Fig. 1). Genes for late steps of LaA biosynthesis flank the *lanJ*–*lanK* pair (Fig. 1).

Although LaA biosynthesis has been studied in detail (Hertweck *et al.*, 2007; Rebets *et al.*, 2008), the function of the *lanK* and *lanJ* is completely unexplored. Here we report that *lanJ* influences La resistance and production and LanK downregulates *lanJ* and some downstream *lan* genes. LanK repression can be relieved by landomycins A, B and E (Fig. 1). The proper functioning of LanK seems to be critical for the production of LaA as a major metabolite of the La pathway in *S. cyanogenus* S136.

### Materials and methods

#### Bacterial strains, plasmids, growth conditions and general methods

Plasmids and *S. cyanogenus* strains are listed in Table 1. *Streptomyces lividans* TK24 was used to test reporter plasmid pMO11 carrying *lanK*–*lanKJp*–*neo* fusion. *Escherichia coli* strains ET12567 (pUB307) and BL21(DE3) were



**Fig. 1.** An overview of the late stage of LaA biosynthesis. (a) Gene pair *lanK*–*lanJ* and the flanking segments of the *lan* cluster. Genes for NDP-L-rhodinose biosynthesis and C11-hydroxylation (Westrich et al., 1999; Hertweck et al., 2007) are shown in red and green, respectively. Fragments of the *lan* cluster used in the experiments are shown below. B, BamHI; C, ClaI; X, XhoI. (b) Pathway leading from C11-deoxylandomycinone to LaA showing the structures of landomycins that are relevant to this study. The L-Rhodinose (in its activated form and as a part of the glycoside chain) and the C11-hydroxyl groups are shown in red and green, respectively.

used for matings and LanK purification, respectively. *Escherichia coli* strains were grown in Luria–Bertani (LB) supplemented with appropriate antibiotics. Solid media oatmeal agar (OM) and soy-mannitol agar (MS) (Kieser et al., 2000; Gromyko et al., 2004) were used for plating of *E. coli*–*Streptomyces* matings and for maintenance of *Streptomyces* strains. *Streptomyces cyanogenus* strains were grown in soy glucose (SG) broth for 72 h at 30 °C (Luzhetskyy et al., 2005). Standard genetic techniques for *E. coli* and *Streptomyces*, and for DNA manipulations were as described (Kieser et al., 2000; Sambrook et al., 2001). All plasmids were introduced into *Streptomyces* strains via intergeneric matings as described (Kieser et al., 2000). Landomycin resistance was analyzed via antibiotic disc diffusion and survival curve methods as described by Ostash et al. (2007) and in the Supplementary material.

### Purification of landomycins

Landomycin E was purified from *Streptomyces globisporus* Smy622 (Gromyko et al., 2004); all other landomycins were extracted from *S. cyanogenus* as described by Luzhetskyy et al. (2005). We used the Agilent 1100 HPLC–MS machine equipped with column Agilent RP-C<sub>18</sub> (4.6 × 250 mm). The mobility of pure [95%, thin layer chromatography (TLC)] landomycins A, B, E, D and landomycinone on TLC, their spectral characteristics and *m/z* values coincided with the published ones (Henkel et al., 1990). For the quantitative analysis of La production, the total extracts were separated using HPLC–MS and mass peaks corresponding to individual landomycins were retrieved and integrated. All values were referred back to equal amounts of dry biomass. The mean values of peak areas (from three independent

**Table 1.** Plasmids and *Streptomyces cyanogenus* strains used in this study

| Plasmid/ <i>S. cyanogenus</i> strain | Genotype/description                                                                                                                                        | Source/reference <sup>†</sup> |
|--------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------|
| pJ486                                | Contains promoterless <i>aphII</i> gene of Tn5; Thio <sup>f</sup>                                                                                           | Kieser <i>et al.</i> (2000)   |
| pHP45Ω                               | Spectinomycin-resistance cassette <i>aadA</i> , Sp <sup>f</sup> Ap <sup>r</sup>                                                                             | Kieser <i>et al.</i> (2000)   |
| pKC1139                              | <i>lacZα</i> ori <sup>pUC19</sup> ori <sup>T<sup>RK2</sup></sup> ori <sup>psG5</sup> <i>aac(3)IV</i> , Am <sup>r</sup>                                      | Kieser <i>et al.</i> (2000)   |
| pSET152                              | ΦC31-based vector; Am <sup>r</sup>                                                                                                                          | Kieser <i>et al.</i> (2000)   |
| pMKI9                                | pKC1139-based vector carrying <i>ermEp*</i> , Am <sup>r</sup>                                                                                               | This study                    |
| pKC1218E                             | SCP2 replicon; pKC1218 plus <i>ermEp*</i> , Am <sup>r</sup>                                                                                                 | Ostash <i>et al.</i> (2007)   |
| pET24b                               | Vector for His-tagged protein expression                                                                                                                    | Novagen                       |
| pMO9                                 | Promoter-probe vector; pKC1139 plus 1.5-kb fragment of pJ486 containing <i>fd</i> terminator, MCS and gene <i>neo</i>                                       | This study                    |
| pK12                                 | <i>lanK</i> disruption plasmid ( <i>lanK::aadA</i> ). Am <sup>r</sup> Sp <sup>f</sup>                                                                       | This study                    |
| pMO1                                 | PMKI9 plus <i>lanK-lanKJp</i> fragment. In pMO1 <i>lanK</i> is under control of tandem promoter <i>ermEp*-lanKJp</i> . Verified via expression in wild type | This study                    |
| pMO6                                 | pSET152 plus 8.5-kb <i>lanJ-lanZ5</i> segment                                                                                                               | This study                    |
| pMO18                                | pMKI9 plus <i>lanK</i>                                                                                                                                      | This study                    |
| pMO7                                 | pET24b plus <i>lanK</i> gene                                                                                                                                | This study                    |
| pKC1218ElanJ                         | pKC1218E plus <i>lanJ</i> gene                                                                                                                              | This study                    |
| pKC1218ElanZ                         | pKC1218E plus <i>lanZ1</i> gene                                                                                                                             | This study                    |
| pMO11                                | pMO9 plus 0.76-kb <i>lanK-lanKJp</i> fragment                                                                                                               | This study                    |
| S136                                 | Wild-type producer of LaA                                                                                                                                   | Westrich <i>et al.</i> (1999) |
| K60                                  | <i>lanK</i> deficient mutant strain                                                                                                                         | This study                    |
| S136(pK12)                           | S136 carrying for <i>lanK</i> disruption                                                                                                                    | This study                    |
| K60(pKC1218ElanJ)                    | K60 carrying <i>lanJ</i> expression plasmid, used for complementation analysis                                                                              | This study                    |
| K60(pKC1218ElanZ)                    | K60 carrying <i>lanZ1</i> expression plasmid, used for complementation analysis                                                                             | This study                    |
| K60(pMO1)                            | K60 carrying <i>lanK</i> expression plasmid, used for complementation analysis                                                                              | This study                    |
| K60(pMO6)                            | K60 carrying <i>lanJ-lanZ5</i> expression plasmid, used for complementation analysis                                                                        | This study                    |
| K60(pMO18)                           | K60 carrying <i>lanK</i> overexpression plasmid                                                                                                             | This study                    |

<sup>†</sup>Details on construction of plasmids and strains can be found in Materials and methods.

experiments) were converted into the amount of given landomycin by means of a calibration curve.

### Disruption of *lanK*

A 3.3-kb BamHI–BglII fragment containing genes *lanG-T1-lanJ* was cloned from cosmid H2-26 (Westrich *et al.*, 1999) into the respective sites of pKC1132 and then subcloned as an XbaI–EcoRI segment into pUC19, giving pIK9. An Sp<sup>r</sup> gene *aadA* was excised from pHP45Ω (Kieser *et al.*, 2000) as an SmaI fragment and ligated into a unique blunt-ended XhoI site within the *lanK* gene of pIK9 to give pIK11. The XbaI–EcoRI fragment from pIK11 was subcloned into the appropriate sites of pKC1139, producing pIK12. In pIK12, the DNA-binding domain of *lanK* is separated from the *lanK* promoter by the *aadA* cassette flanked by transcriptional and translational terminators. This should disable the expression of truncated LanK. The pIK12<sup>+</sup> *S. cyanogenus* transconjugant was obtained and homologous recombination between *S. cyanogenus* chromosome and pIK12 was promoted as described (Kieser *et al.*, 2000). Three Am<sup>s</sup>Sp<sup>r</sup> colonies (an indicative of a double crossover) were selected out of 600 tested. All three candidates exhibited the same phenotype. One of them (referred to as K60) was used throughout this work.

### Complementation plasmids

Because *lanK* and *lanJ* are divergently transcribed, we supposed that the 190-bp intergenic region between them is a bidirectional promoter and marked it as *lanKJp*. Plasmids pKC1218ElanJ, pKC1218ElanZ1, pMO1, pMO6 and pMO18 were constructed for expression studies. pKC1218ElanJ resulted from the cloning of *lanJ* behind the *ermEp\** in pKC1218E. DNA of the *lanJ* gene was amplified from H2-26 cosmid (Westrich *et al.*, 1999) using primers *ajup1* (5'-AAATCTAGAGTCGGCTCGCCAGACCTGA-3') and *ajrp1* (5'-AAAGAATTCTCAGCCGGCGGC-3'). The PCR product was cloned into XbaI–EcoRI sites of pKC1218E. pKC1218ElanZ was constructed by cloning the *lanZ1* PCR product into the XbaI–EcoRI restriction sites of pKC1218E. The *lanZ1* was amplified from H2-26 using primers *az1Xbalup* (5'-AAATCTAGACACGAGGTCGGACCAACCCA-3') and *az1EcoRIrp* (5'-AAAGAATTCTCAGTTGCCGCCGGGC-3'). pMO1 resulted from cloning of the *lanKJp-lanK* fragment into XbaI–EcoRI sites of pMKI9. The vector pMKI9 was constructed by cloning of a 0.2-kb HindIII–XbaI fragment carrying the *ermEp\** from pKC1218E into the respective sites of pKC1139. The *lanKJp-lanK* DNA fragment was obtained by PCR from H2-26 using primers *kupXbaI* (5'-AAATCTAGATCACAGGTCCTCCTCGGC-3') and *krpEcoRI* (5'-AAAGAATTCTCGGT

CTGCTCCCTTGATCAG-3'). pMO6 resulted from subcloning of an 8.5-kb *Cla*I fragment of H2-26 (contained 120 bp of *lanK*, *lanKJp* and *lanJZ1GT3Z2Z3GT4Z4Z5*) into the *EcoRV* site of pSET152. pMO18 was constructed by cloning of the *lanK* gene along with RBS behind *ermEp*\* of pMKI9. The *lanK*-RBS segment was obtained by PCR from cosmid H2-26 using primers *lanKermPXbaup* (5'-AAATCTAGAACACAGGAGAAACCCATGGGC-3') and *krpEcoRI* (described above). All plasmids described in this and the following sections were verified via sequencing.

### Overexpression of LanK

To obtain C-terminally His-tagged LanK, pMO7 was constructed. The entire *lanK* was amplified from cosmid H2-26 using primers *lanKNdeI* (5'-AAACATATGGGCGGCACACCGCACG-3') and *lanKHindIIIrev* (5'-AAAAAGCTTTCACAGTCTCCTCGGCCGA-3). The PCR product was cloned into the pET24b *NdeI*-*HindIII* site, giving pMO7. *Escherichia coli* BL21(DE3)(pMO7) was grown overnight at 37 °C. LB (400 mL) containing 50 µg mL<sup>-1</sup> of kanamycin were inoculated with 2 mL of the overnight culture and incubated at 37 °C until the OD<sub>600nm</sub> reached 0.6. LanK expression was induced with 1 mM isopropyl thio galactoside. After incubation for an additional 4 h, the culture was harvested by centrifugation at 5000 g for 10 min at 4 °C and washed with ice-cold column buffer (20 mM Tris-HCl, pH 8.0, 50 mM NaCl). The cell lysis and native purification of LanK protein on His-tag-binding resin were performed according to Novagen instructions. LanK was eluted with 200 mM imidazole and dialyzed overnight at 4 °C against the storage buffer (50 mM potassium phosphate, pH 8.0, 300 mM NaCl, 10% glycerol). Protein was stored at -80 °C.

### Gel mobility shift assay

A 190-bp DNA fragment containing *lanKJp* and a 600-bp DNA fragment carrying the promoter region of *lanE* (*lanEp*) were used in DNA-binding assays. The DNA fragment for *lanKJp* was amplified from cosmid H2-26 using primers *PlanKup* (5'-GGGTTTCTCCTGTGTGCCG-3') and *PlanKrp* (5'-GGTCTGCTCCCTTGATCAGGT-3'). The 600-bp *lanEp* was amplified from cosmid H2-26 using primers *LanEpup* (5'-GTCGACGACCACCCCGCC-3') and *PlanErp* (5'-TTCGCTCCTTGAATAGGCTGG-3'). One DNA-binding assay contained 50 ng *lanKJp* or *lanEp* DNA and 700 ng LanK protein in a total volume of 30 µL in a buffer (20 mM Tris HCl, pH 8.0; 1 mM EDTA, 1 mM DTT, 100 mM KCl, 10% glycerol). After incubation for 20 min at room temperature, protein-bound and free DNA were resolved by electrophoresis at 4 °C on a 4.5% nondenaturing polyacrylamide gel in 1 × TBE buffer. The gel was stained with ethidium bromide and analyzed using a UV-imaging system

(Fluorochem 5330). LaA (0.05 mM – final concentration in a reaction mixture), LaB (0.05 mM), LaE (0.05 mM), LaD (0.05–7.5 mM), apramycin, tetracycline, spectinomycin, ampicillin and erythromycin (all at 0.5 mM) were tested as LanK ligands.

### Construction of a *lanK*-*lanKJp*::*neo* reporter fusion system

A 1.5-kb DNA fragment carrying the *fd* terminator, MCS and *neo* gene was amplified from pIJ486 (Kieser et al., 2000) using primers *terforNheI* (5'-AAAGCTAGCGTCCGGA TCGCGGCAAC-3') and *neorevMfeI* (5'-AAACAATTGGCGTCTGCTTGGTTCGGTTCATT-3'). The PCR product was inserted into *XbaI*-*EcoRI* sites of pKC1139 to give reporter plasmid pMO9. A 0.76-kb *lanK*-*lanKJp* fragment was amplified from cosmid H2-26 by PCR using primers *KupEcoRI* (5'-AAAGAATTTCGGTCTGCTCCCTTGATCAG-3') and *KrpXbaI* (5'-AAATCTAGATCACAGTCTCCTCGGC-3'). The PCR product was cloned into *XbaI*-*EcoRI* sites of pMO9, giving pMO11. In pMO11, the *neo* expression depends on *lanKJp*.

## Results and discussion

### Overexpression of *lanJ* and *lanK* genes in *S. cyanogenus* S136

*Streptomyces cyanogenus* pKC1218ElanJ<sup>+</sup> strain [referred to as S136(lanJ3)] was more resistant to exogenously added LaA and LaD than the initial strain (Table 2). The resistance of S136(lanJ3) and control strain to erythromycin, tetracycline, nogalamycin and kanamycin was identical. In S136(lanJ3) the average LaD production increased 1.5 times, whereas the amounts of LaA and LaB decreased by three- and twofold, respectively, when compared with the control strain (LC-MS results; see Supplementary material). We also overexpressed the *lanK* gene in S136 [strain S136(pMO18)]. In this strain, LaD was detected as a major product, and the

**Table 2.** Antibiotic disc diffusion assay of resistance of *Streptomyces cyanogenus* strains to landomycins

| Strain      | Diameter of growth inhibition zone (mm)* |                  |
|-------------|------------------------------------------|------------------|
|             | LaA <sup>†</sup>                         | LaD <sup>†</sup> |
| S136        | 12 ± 1                                   | 14 ± 1           |
| S136(lanJ3) | 9 ± 1                                    | 11 ± 1           |
| S136(pMO18) | 14 ± 1                                   | 13 ± 1           |
| K60         | 16 ± 1                                   | 12 ± 2           |
| K60(pMO6)   | 11 ± 2                                   | 14 ± 1           |

\*Diameter of paper discs was 6 mm. Mean values and the SD are obtained from three independent experiments.

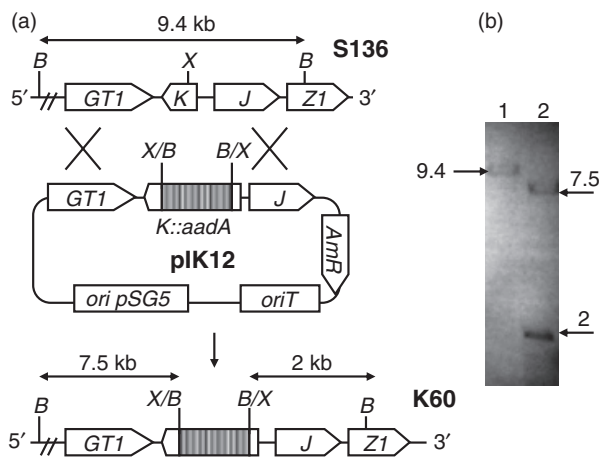
<sup>†</sup>The concentrations of LaA and LaD were 100 and 55 µg per disc, respectively, which are equimolar concentrations (92 mM).

amounts of LaA and LaB were decreased eight- and threefold, respectively (Supplementary material). Disc assay did not reveal a difference in La resistances of wild-type and pMO18<sup>+</sup> strains (Table 2), probably reflecting the low resolution power of this approach. However, the survival curve method revealed that S136(pMO18) is more sensitive to LaA than S136; at the highest concentration tested, the colony survival was  $13 \pm 1\%$  and  $3.0 \pm 0.3\%$  for S136 and S136(pMO18), respectively (Supplementary Fig. S2).

The LaA-resistance profiles of the recombinant strains are consistent with the idea that LanJ exports landomycins thereby increasing the resistance to them, and LanK represses *lanJ* and, consequently, renders S136 more susceptible to LaA. Overexpression of *lanK* had little effect on LaA resistance of S136. Probably, there are as-yet-undiscovered alternative La resistance mechanisms. Changes in the production of intensely colored landomycins provide a reliable phenotypic readout of expression of different regions of the *lan* cluster (Luzhetskyy *et al.*, 2005; Hertweck *et al.*, 2007), and our results suggest that *lanK* overexpression down-regulates some genes for LaD to LaA conversion. Sequence analysis reveals that NDP-L-rhodinose biosynthetic gene *lanZ1* and *lanJ* can form a transcriptional unit. Therefore, the simplest explanation would be that LanK limits the transcription of both *lanJ* and *lanZ1*, and, consequently, halts the biosynthesis of NDP-L-rhodinose necessary for LaD glycosylation (Fig. 1), which ultimately leads to LaD accumulation. However, other scenarios of LanK action cannot be ruled out for the moment. The accumulation of LaD in the *lanJ* overexpressing strain can be explained by the premature export of LaD (and, probably, its precursors) from the cells, which hinders the synthesis of LaA as a major product. A similar situation was observed when the *lanJ* homologue was overexpressed in *S. globisporus* (Ostash *et al.*, 2007).

### Disruption of *lanK*

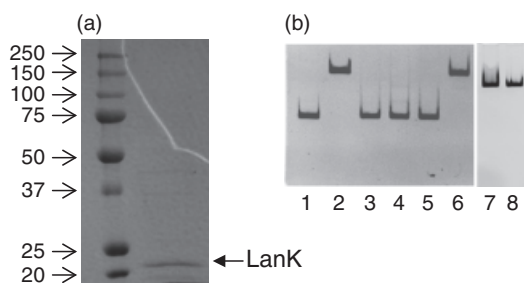
We generated a *lanK*-deficient mutant *S. cyanogenus* K60. The *lanK* was replaced in the *S. cyanogenus* chromosome with the *lanK::aadA* allele (Fig. 2a). The gene replacement event in K60 was verified by Southern hybridization (Fig. 2b). When the 570-bp DNA fragment of *lanK* was used as a DIG-labelled probe, one 9.4-kb hybridizing BamHI band was observed in wild-type digests, while two BamHI bands (7.5 and 2 kb) were observed in the case of the K60 (Fig. 2b). LaD was detected as a major product in the K60 extracts, whereas LaA and LaB production decreased 10-fold when compared with the wild type (see Supplementary material). K60 was also more sensitive to landomycins in comparison with the S136 strain (Table 2, Fig. S2). Because *lanK* down-regulates La resistance and production and its activator



**Fig. 2.** Insertional inactivation of *lanK*. (a) Scheme representing the replacement event in the chromosome of wild-type *Streptomyces cyanogenus* S136 strain produced by a double crossover to construct mutant strain K60. The expected hybridizing restriction fragments and their sizes (in kb) are indicated. B, BamHI; X, XhoI. (b) Southern hybridization using the *lanK* gene DNA fragment as a probe. Lane 1, BamHI-digested chromosomal DNA of the wild-type *S. cyanogenus* strain; lane 2, BamHI-digested chromosomal DNA of the K60 strain. The sizes of bands (in kb) are indicated on the left and the right of the membrane.

function can thus be ruled out, the phenotype of K60 must arise from impaired expression of *lanJ* and downstream genes. Indeed, we failed to complement K60 *in trans* with the *lanK* gene (plasmids pMO1 and pMO18). In contrast, replacement of *lanK::aadA* with the intact *lanK* from pMO1 restored the wild-type phenotype. The expression of either *lanJ* or *lanZ1* did not restore LaA production in K60. Expression of the *lanJ-lanZ5* segment (pMO6) increased the biosynthesis of LaA; nevertheless, the LaA production level was roughly 90% of that in the control strain. Interestingly, the amount of LaB in this strain was twice as much as LaA (Supplementary material). These changes could be due to either the deregulated expression of the *lanJ* gene within pMO6 or the ectopic position of pMO6 (integration into *attB*<sup>φC31</sup>) within the K60(pMO6) genome. K60 (pMO6) was as resistant to LaA and LaD as the wild type (Table 2).

The ability of pMO6 to restore LaA biosynthesis and resistance shows that *lanK* disruption damages the expression of genes within the *lanJ-lanGT4* segment. Additional experiments are needed to determine which genes within the aforementioned region are affected by the polar effect; nevertheless, its mere existence can be deduced from our genetic analysis. The *XhoI* site chosen to insert the *aadA* cassette into *lanK* is located 60 bp downstream of its start codon. Probably, some regulatory element(s) necessary for *lanJ-GT4* genes expression are present within the 5'-end of the *lanK* coding region and they are interrupted in the K60 strain.



**Fig. 3.** Purification of the His-tagged LanK protein from *Escherichia coli* and gel mobility shift analysis of DNA fragment containing *lanKJp*. (a) Lane 1, molecular mass markers; lane 2, purified LanK. (b) Lane 1, *lanKJp* DNA fragment (P); lane 2, P+LanK; lane 3, P+LanK+LaA; lane 4, P+LanK+LaB; lane 5, P+LanK+LaE; lane 6, P+LanK+LaD; lane 7, *lanEp*+LanK (negative control); lane 8, free *lanEp*.

### Export of LaD from the *S. cyanogenus* K60 cells

Strain K60, which predominantly accumulates LaD, was used as a simple model to obtain the evidence that *lanJ* indeed controls La export. K60 overexpressing *lanJ* [K60(*lanJ*3)] was grown in parallel with the control strain (K60 plus empty vector) and the amount of LaD in the spent medium was determined. K60(*lanJ*3) accumulated in the supernatant 2.7 times more LaD than the control strain (see Supplementary material), implying that, at least under over-expression conditions, *lanJ* participates in extrusion of LaD (as speculated above) and, probably, other landomycins.

### DNA-binding activity of LanK

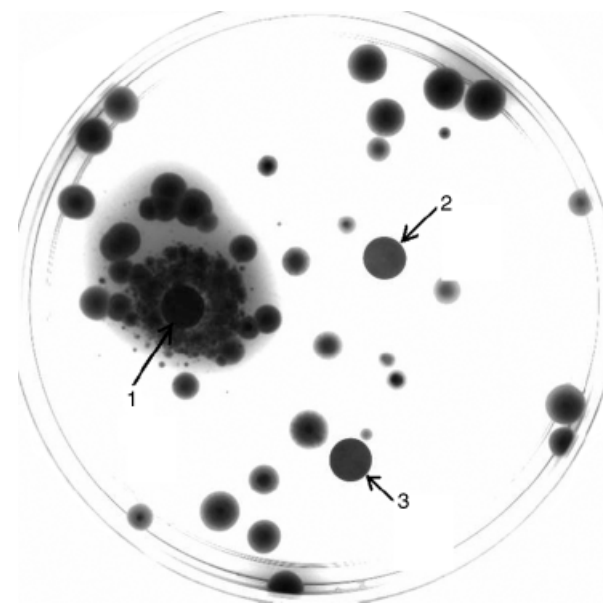
To prove the regulatory role of LanK, we purified it (Fig. 3a) and performed an *in vitro* binding assay using LanK and either 190-bp DNA fragment corresponding to the promoter *lanKJp* (Fig. 1) or 600-bp DNA fragment containing the promoter of oxygenase gene *lanE* (*lanEp*), which is the leftmost one in the *lan* cluster (Westrich et al., 1999). DNA retardation was significant when the *lanKJp* was used in the binding assay, whereas no DNA shift was detected when using the *lanEp* DNA (Fig. 3b). We carried out a set of control assays to demonstrate the specificity of LanK binding to *lanKJp*. For instance, a larger 420-bp fragment containing *lanKJp* and 5'-end of *lanJ* was retarded by LanK, whereas a 41-bp fragment corresponding to the central part of *lanKJp* was not (Supplementary material). Also, LanK did not bind *lanK*, the entire *lanZ2* plus 150-bp upstream region or its internal fragment (data not shown). We carried out the gel DNA retardation assay in the presence of landomycins that possess glycosidic chains of different lengths. 0.05 mM LaA, LaB or LaE abolished LanK–DNA binding, whereas LaD, tetracycline, kanamycin, apramycin, spectinomycin, ampicillin and erythromycin did not (Fig. 3b). LaD did not inhibit LanK–DNA binding even at a 150-fold higher concentration.

Thus, LanK is a DNA-binding protein whose activity is modulated by certain landomycins. LaE, which contains a trisaccharide chain and that has been never detected in extracts of *S. cyanogenus* S136, is the simplest ligand recognized by LanK. It is clear that the glycoside moiety of landomycins is crucial for their ability to interact with LanK. We note that all LanK ligands contain one or two L-rhodinose residues. The importance of this trideoxysugar for LanK–La interaction is under investigation.

### *In vivo* assay of LanK–*lanKJp* interaction

To test LanK behavior *in vivo*, we constructed the plasmid pMO11, in which *lanK*, *lanKJp* and a neomycin reporter gene (*neo*) are fused together. The *neo* expression would be under the control of *lanKJp* and, conceivably, LanK. The spores of the *S. lividans* TK24 (pMO11) strain were plated in soft agar onto plates with neomycin (50 µg mL<sup>-1</sup>). The paper discs soaked in LaA, LaD or landomycinone were then applied onto the plates and incubated for 6 days. LaA elicited growth of TK24(pMO11), whereas LaD and landomycinone did not (Fig. 4), thus confirming that the former is an efficient LanK ligand *in vivo*.

Summarizing all our findings, we propose a model of coordination of glycosylation and export of landomycins. LanK represses the transcription of *lanJ* and one or several genes for sugar chain progression from di- to hexasaccharide



**Fig. 4.** Bioassay of LanK effector ligands. Halo of growth of kanamycin-resistant *Streptomyces lividans* TK24 (pMO11) colonies is seen around the 6-mm paper disc with 100 µg of LaA (1) and no growth is observed in the presence of 100 µg of LaD (2) and 100 µg of landomycinone (3). A background growth of kanamycin-resistant colonies (occurring at frequency  $4 \times 10^{-5}$ ) is observed, apparently due to accidental read through of the *neo* gene.

landomycins. As a result, LaD accumulates in the cell. Basal expression of *lanZ1–lanGT4* genes ensures LaB and LaA production. When the cellular concentration of LaB and/or LaA exceeds a certain threshold level, they relieve *lanKJp* repression by LanK. This induces the expression of *lanJ* and certain downstream *lan* genes, leading to the mass production of LaA and its export.

We described the TetR-like regulatory switch that coordinates landomycin glycosylation and export. This switch includes promoter *lanKJp*, repressor LanK and its effector ligands – landomycins containing at least three deoxysugar residues in their glycoside chain. The data obtained agree with the recently proposed ‘feed-forward’ hypothesis of activation of antibiotic export (Tahlan *et al.*, 2007). New questions emerge and, in our opinion, the most intriguing one is about the advantage(s) that *lanK* confers to *S. cyanogenus* during La biosynthesis. Unlike the wild-type strain, the *lanK*<sup>−</sup> one [K60(pMO6)] produces more LaB than LaA. Hence, the ultimate biological role of *lanK* might be to ensure a high-level LaA production through negative control of extrusion of early LaA precursors. Also, the importance of activator gene *lanI* (Rebets *et al.*, 2008) for expression of the *lanK–lanGT4* region is unclear. Here, *lanK* was shown to function in *S. lividans* in the absence of *lanI*. Probably, *lanI* is not absolutely required to trigger the expression of the aforementioned genes, but it enhances their transcription once the inhibitory function of LanK is cancelled. This and other issues are within the scope of our current research, which will eventually lead to a better understanding of secondary metabolite transport processes.

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## Supplementary material

The following supplementary material for this article is available online:

**Fig. S1.** Qualitative TLC analysis of total extracts of landomycins from equal amounts of biomass of different *S. cyanogenus* strains.

**Fig. S2.** The dependence of *S. cyanogenus* strains survival on landomycin A concentration.

**Fig. S3.** Gel mobility shift analysis of *lanKJp2* DNA fragment.

**Fig. S4.** Gel mobility shift analysis of 41 bp DNA fragment.

**Table S1.** Quantitative analysis of production of different landomycins by *S. cyanogenus* strains.

**Table S2.** Amount of LaD in spent medium of strains *S. cyanogenus* K60(pKC1218E) and K60(lan)3).

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