

An ABC transporter encoding gene *lndW* confers resistance to landomycin E

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Abstract *Streptomyces globisporus* 1912 produces a polyketide antibiotic landomycin E (LaE), which possesses anticancer activity. A 1.8 kb DNA fragment at the end of landomycin E biosynthetic gene cluster was sequenced. DNA sequence analysis of this fragment identified one complete open reading frame, designated *lndW*. The deduced sequence of *lndW* gene product revealed significant similarity to the ATP-binding domains of the ABC (ATP-binding protein cassette) superfamily of transporter-related proteins. Knockout of *lndW* had no significant effect on resistance to LaE and its production. The expression of *lndW* in *S. globisporus* 1912 was proven via transcriptional fusion of *lndW* promoter to EGFP (enhanced green fluorescent protein). Overexpression of *lndW* in *S. lividans* TK24 conferred resistance to LaE. The mechanism of *lndW* function in LaE biosynthesis is discussed.

Keywords *Streptomyces globisporus* 1912 · Landomycin resistance · ABC transporters

Introduction

Angucycline group of antibiotics is one of the largest families of aromatic polyketides. This group comprises more than one hundred secondary metabolites of microbial origin. Angucyclines possess wide spectrum of antitumor activity, in particular against doxorubicin resistant tumor cells (Rohr et al. 1992). The structural genes for angucycline biosynthesis are found clustered with the transporter-encoding genes. The transporter genes are only possible candidates for self-resistance revealed in angucycline-producing streptomycetes (Ostash et al. 2007; Lombo et al. 2004; Novakova et al. 2002; Decker et al. 1995). Considerable progress has been made recently regarding the study of structural genes involved in angucyclines biosynthesis, but function of none of the transporter genes was elucidated (Luzhetskyy et al. 2005; Novakova et al. 2005; Chen et al. 2005; Ostash et al. 2005; Rebets et al. 2005a, b).

Landomycin E (LaE) is a member of angucyclic group of antibiotics with a unique phenylglycoside moiety in the structure. It contains three deoxysaccharide residues (α -L-rhodinose-(1 \rightarrow 3)- β -D-olivose-(1 \rightarrow 4)- β -D-olivose) conjugated to angular tetracyclic quinone (Fig. 1). The mode of LaE action is not understood, but it is known to arrest tumor cell cycle progression (Panchuk et al. 2005; Korynevska et al. 2007). The producer of Landomycin E is *Streptomyces globisporus* 1912. Sequence analysis demonstrated the presence of gene *lndJ*, which encodes a proton-dependent transporter and clustered with LaE biosynthesis genes. The function of *lndJ* gene was described recently. Its overexpression confers resistance to LaE (Ostash et al.

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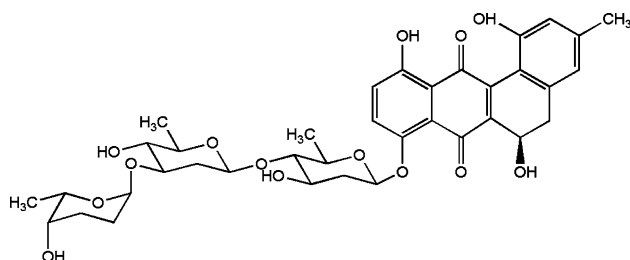


Fig. 1 Landomycin E (LaE) molecule structure

2007). Here we present further characterization of the second transporter gene (*IndW*) found in the LaE biosynthetic gene cluster. The gene *IndW* encodes a putative ATP-binding protein of the ABC transporter superfamily. Its heterologous expression showed that *IndW* might be involved in resistance to LaE.

Materials and methods

Microorganisms, culture conditions and vectors

Streptomyces globisporus 1912, a landomycin E producer was used as a source of DNA and for generation of *IndW* disruption mutant. *Streptomyces globisporus* BI4 (Ostash et al. 2007), *IndJ* mutant strain was used to generate double *IndJ/IndW* knockout. *S. lividans* TK24 was used as a host for heterologous expression. For landomycin E extraction LaE overproducer *S. globisporus* SMY622 (Gromyko et al. 2004) was used. *E. coli* DH5 α was used for DNA manipulations. *E. coli* ET12567 (pUB307) served as a conjugative host strain for introduction of foreign DNA into *S. globisporus* 1912/BI4 and *S. lividans* TK24. The gene disruption and expression were performed with the *E. coli*–*Streptomyces* shuttle vectors pKC1139 (Keiser et al. 2000) and pKC1218E (Ostash et al. 2004; Lombo et al. 2004), respectively. Plasmid pHP45 Ω was used as a source of spectinomycin resistance gene *aadA*. Vector pIJ8660 that contains promoterless EGFP gene (Kieser et al. 2000) was used to probe *IndW* promoter activity. For sporulation, *S. globisporus* strains were grown on OM medium at 30°C (Luzhetskyy et al. 2001). For LaE production, *S. globisporus* strains were grown in SG medium (Ostash et al. 2003). *E. coli* strains were grown under standard conditions (Sambrook et al. 2001).

Plasmids construction

Plasmid for *IndW* gene disruption was generated as follows. A 3 kb of right end of LaE gene cluster was subcloned into pUC18 resulting pUCABC (Fig. 2). This plasmid was used as a DNA source for construction of *IndW* mutant allele.

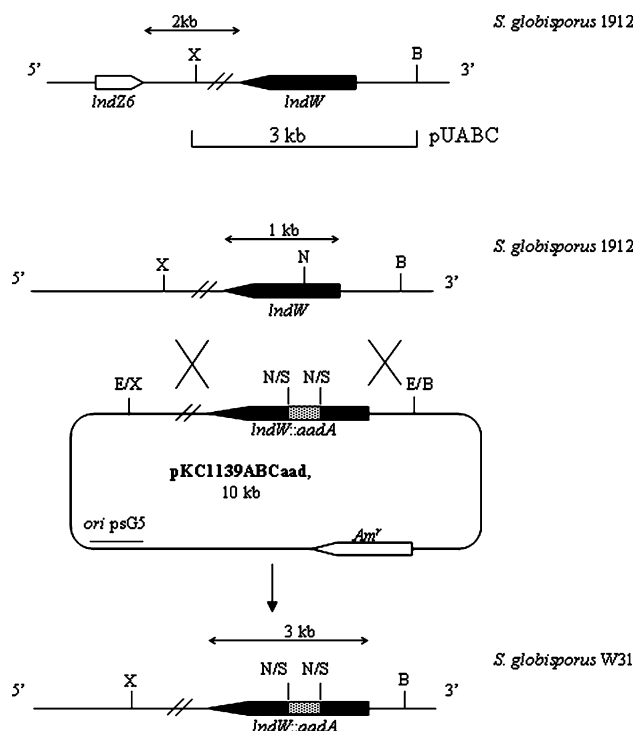


Fig. 2 An insertional inactivation of *IndW*. Double crossover scheme explaining the generation of mutant W31. *Bam*HI (*B*), *Sma*I (*S*), *Xho*I (*X*), *Not*I (*N*), *Eco*RI (*E*) restriction sites, an expected sizes of DNA fragments (in kb) are indicated

The spectinomycin resistance cassette *aadA* was introduced into the unique *Not*I site within *IndW*. Further subcloning into pKC1139 yielded plasmid pKC1139ABCaad carrying *IndW::aadA* allele. Plasmid for *IndW* overexpression was constructed using vector pKC1218E. The 1361 bp DNA fragment consisting of *IndW* gene and its possible promoter region was amplified from genome of *S. globisporus* 1912 using primers wup1 (5'-AAATCTAGACGACCTTCGGC GCGAGCG-3') and wrp1 (5'-AAAGAATTCTCATGGC GCCTGGTCCCTT-3'). Amplified fragment was digested with *Xba*I/*Eco*RI and cloned into corresponding sites of pKC1218E resulting into pKC1218EIndW. *P_{IndW}*-EGFP fusion has been generated by subcloning of 700 bp *Eco*RI/*Bam*HI promoter region from pUABC into pIJ8660, giving pIJ8660W.

Landomycin E resistance determination

To determine the resistance of streptomycetes to LaE, purified sample (as described below) of LaE was used. The resistance of strains to LaE was determined by disc assay and by calculating the colony survival in presence of different LaE concentrations (survival curves method).

Landomycin E was dissolved in methanol and applied onto Whatman disks (\varnothing 5 mm; 100 μ g of antibiotic per disk), which were left to dry for 6 h at 4°C. Two hundred

microliters of spore suspension of streptomycetes strains (ca. 10^8 cfu/ml) were mixed with 3 ml of soft agar and plated on solid LB-medium. Then disks with LaE were stacked on soft agar. Diameters of growth inhibition zones with LaE were measured on third day of incubation. The experiment was triplicated and data have been averaged.

For determination of survival curves, the LaE was added to Bennett agar medium at different concentrations (1, 5, 10, 25, 50 and 100 $\mu\text{g/ml}$). The spore suspensions of streptomycetes strains were seeded at different dilutions onto LaE-containing plates and incubated for 2 days. The percentage of survival was calculated with regard to colony numbers on control plates (no LaE).

Landomycin E extraction and purification

The LaE overproducer strain *S.globisporus* SMY622 (Gromyko et al. 2005) was cultivated in liquid SG medium at 30°C for 24 h with shaking (220 rpm). This preculture was subsequently used to inoculate the main culture of the same composition, which was harvested after 72 h of shaking as above. The culture broth was extracted with equal amount of ethyl acetate. The extract was applied to the gradient silicagel column and eluted with chloroform: methanol (96:4). The identity of extracted LaE was confirmed by TLC using standard LaE (kindly provided by Prof. Jurgen Rohr) and LC-MS analysis. The same extraction procedure was employed to isolate the LaE from *lndW*-deficient mutant and wild type strains. The amount of extracted LaE was referred back to the equal amount of dry biomass.

Visualization of enhanced green fluorescent protein

For the EGFP production analysis the mycelia of *S.globisporus* strains grown in the TSB media were washed with water and applied to glass slides. Fluorescent microscopy was carried out on a Fluoroview confocal system (Olympus) equipped with an Olympus OL BX50 microscope and an argon laser (providing excitation at 488 nm) with EGFP expression. Fluorescein-isothiocyanate (FITC) (emission at 506–535 nm) filters were used to observe a green fluorescence. The green fluorescence and transition images were obtained simultaneously using separate detectors. To ensure a high reliability in the quantitative analysis of the captured images, the same operational parameters were used for each sample at the same time point. The confocal images were saved as TIFF files and the image analysis was done using Fluoroview 2.1 software. The *S.globisporus* strains 1912 (pIJ8660H) and 1912 (pIJ8660E) carrying transcriptional fusions $P_{lndI}::\text{EGFP}$ and $P_{lndE}::\text{EGFP}$, respectively, were used as a positive control of green fluorescence. The *S.globisporus* 1912(pIJ8660)

harboring vector with promoterless EGFP gene was used as a negative control.

Results and discussion

In silico analysis of *lndW*

During additional sequencing of 1.8 kb fragment of right end of LaE gene cluster (GenBank accession number EU128492), two ORFs were detected. The central part of the sequenced region occupies a streptomycete-like ORF marked as *lndW* (Fig. 2). FramePlot and BLAST analyses define 984 bp *lndW* gene, which is the longest possible one; however, alternative start-codons, located closer to stop-codon, cannot be ruled out at the moment. A 5'-end of divergently oriented ORF was detected upstream of *lndW*. It encodes putative trehalose-6-phosphatase, which most likely is involved in primary carbohydrate metabolism. The 160 bp region was sequenced downstream of *lndW*. It carries no signs of either a complete ORF or its part as judged from computer analysis. The deduced product of *lndW* is a protein of 327 amino acid residues. Using Conserved Domain Architecture Retrieval Tool (CDART), BLAST and ExpASY Proteomics server SwissProt (Prosite) one ATP-binding domain was detected within *lndW* sequence. Analysis with Conserved Domains Database, (CDD) BLAST revealed three closest to *lndW* conservative domains, which belong to ATPase of ABC transporters involved in drug resistance (cd03269, cd03230) and carbohydrate transport (cd03259). Among homologues of *lndW* product are ABC transporters with unknown function, wide spread in *S.coelicolor* and *S.avermitilis* genomes. Two *lndW* homologues are associated with antibiotic biosynthetic gene clusters and belong to ATPase protein of ABC transporters. One of them, orf32, (42% identity, 58% similarity) is associated with enduracidin biosynthetic gene cluster and probably is involved in enduracidin transport (Yin et al. 2006). Another *lndW* homologue, *bls orf9*, (43% identity, 59% similarity) revealed in blasticidin gene cluster was shown to be involved in resistance to blasticidin under heterologous conditions (Cone et al. 2003).

Functional elucidation of *lndW*

According to accepted classification, there are three types of ABC transporters. Type I and III contain transmembrane protein besides an ATP-binding subunit. Type II ABC transporters are formed by a hydrophilic protein containing two nucleotide-binding domains (Mendez et al. 2001). However, the presence of two ATP-binding domains for proper functioning is not necessary. For instance, a gene *oleB* encodes type II ABC transporter with two ATP-binding

domains. But it was shown that either the first or the second half of this gene coding for only one ATP-binding domain is sufficient to confer the resistance to oleandomycin (Olano et al. 1995). From bioinformatics analysis it is deduced that *LndW* possesses only one ATP-binding domain and no gene encoding transmembrane subunit for ABC transporter was found within LaE biosynthetic gene cluster. We speculated that *LndW* can belong to type II ABC transporters and possibly is involved in resistance to LaE.

To study the role of *LndW* in LaE biosynthesis, we set out to generate *LndW* deficient mutant of *S. globisporus* 1912. The *LndW* gene disruption was done via double crossover according to procedure (Kieser et al. 2000), using temperature sensitive plasmid pKC1139ABCaadA (Fig. 1). Sp^r Am^s strain of *S. globisporus* W31, which carries replacement of *LndW* with *LndW::aadA* allele, was selected. The incorporation of the mutant allele into chromosome was proven by amplification of *LndW* gene from *S. globisporus* 1912 (wild type) and *S. globisporus* W31 (*LndW*-mutant) strains. Approximately 1 kb DNA fragment corresponding to *LndW* was amplified from 1912, whereas 3 kb DNA fragment was amplified from W31, indicating that wild type copy of *LndW* was replaced by the mutated one (data not shown). No significant differences in the resistance level to LaE and its production were observed between wild type and W31 strains. We supposed that *LndJ* gene could complement the loss of *LndW* in W31 strain. Therefore, we generated double *LndJ/LndW* knockout strain W31BI4, on basis of *LndJ* mutant BI4 (Ostash et al. 2007), but this mutant also did not differ in resistance to LaE in comparison to parental strains. The W31BI4 produced landomycin G as a major product as parental strain BI4 did (Ostash et al. 2007), and no differences in amount of produced landomycin were observed in comparison with control strains (BI4 and GT4.1). Probably, *LndJ* and *LndW* genes are not the only determinants that control the LaE export or resistance to this antibiotic and the presence of other LaE resistance genetic determinants outside of *Lnd*-cluster cannot be excluded. The similar case was observed when disruption of rebeccamycin resistance gene *rebT* in producer did not affect its resistance to rebeccamycin. However, heterologous expression of *rebT* provided resistance to rebeccamycin (Sanchez et al. 2006). Therefore, to prove the function of *LndW* as a hypothetical LaE determinant, heterologous expression of *LndW* in *S. lividans* TK24 was performed. *Streptomyces lividans* TK24 carrying *LndW* under strong promoter (*ermEp**) showed increased resistance to LaE (10 ± 2 mm) when comparing to control TK24 (pKC1218E) strain (19 ± 2 mm). These data correlate positively with the survival curve of the same strains in presence of LaE. Indeed, at the highest tested LaE concentration, the survival of *LndW*⁺ *S. lividans* increased tenfold when comparing to *S. lividans* carrying empty vector (Fig. 3).

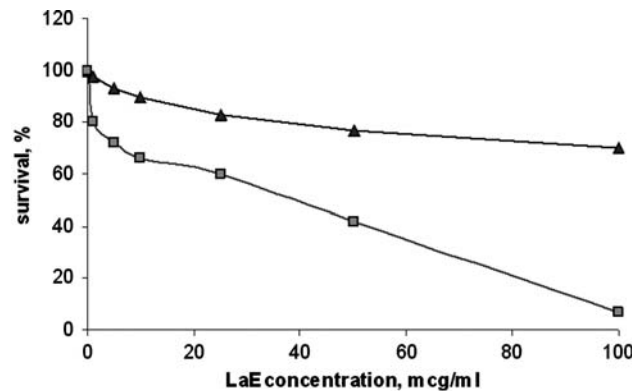


Fig. 3 A survival of pKC1218E⁺ *S. lividans* TK24 (gray squares) and pKC1218ELndW⁺ *S. lividans* TK24 (black triangles) strains in presence of different concentrations of LaE

In order to find out whether *LndW* is indeed expressed in *S. globisporus*, we introduced *P_{LndW}::EGFP* fusion plasmid (pIJ8660W) into 1912 strain. The *P_{LndW}* expression was monitored by observing green fluorescence in mycelia at a different time of growth. No fluorescence was observed in mycelia of *S. globisporus* 1912(pIJ8660). We detected slight fluorescence at 18 h and maximal at 48 h of culture growth (Fig. S1 in Electronic supplementary material). The results showed that *LndW* promoter is active in *S. globisporus* 1912. A temporal character of *P_{LndW}* expression correlates with promoter activity of *LndI* (activator of LaE gene cluster; Rebets et al. 2005a, b) and structural gene *LndE* (Fig. 4). Our data indicate that *LndW* is somehow involved in LaE biosynthesis.

According to results of *LndW* and double *LndW/LndJ* gene knockouts we speculate that alternative genes for LaE transport or other genetic resistance determinants are located outside of LaE biosynthetic gene cluster. Nevertheless, data on heterologous expression of *LndW* showed that it could confer the resistance to LaE. Since expression of *LndW* confers increased resistance to LaE only in heterologous host we suggest that *LndW* is responsible for recognition of

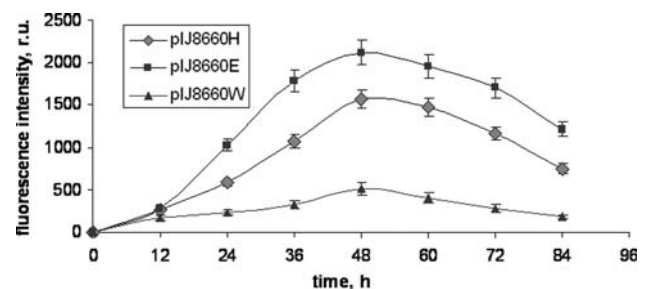


Fig. 4 A temporal pattern of transcription of *LndW* (triangles) in comparison with that of *LndI* (squares) and *LndE* (diamonds). The average fluorescent intensity of respective strains mycelia after every 12 h of growth were measured and calculated and the values were plotted against the X-axis (time of growth)

LaE as a substrate and that another transmembrane protein capable of interacting with LndW could form part of the export system in the *S. lividans* TK24 (pKC1218EIndW). Since *lndW*-EGFP transcriptional fusion proved that *lndW* gene is expressed in *S. globisporus* strain, we propose that *lndW* is active during LaE production and might be involved in LaE export.

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