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**STREPTOMYCES GLOBISPORUS 1912 MUTANTS WITH ALTERED  
LANDOMYCIN E BIOSYNTHESIS**

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Ten *Streptomyces globisporus* stable mutants with interrupted or ceased synthesis of novel angucycline antibiotic landomycin E (LE) were examined by thin layer chromatography, spectrophotometry, and Southern hybridization. Four strains (LND23, LND29, LND81, LND901) with completely abolished LE production and three strains (LND317, LND801, LND900) with altered were identified. Strains LND23, LND900, LND901, W1 carry deletions within *lnd*-cluster. In case of strain W1, we did not observe hybridization of W1 total DNA restricts with all DNA-probes used; LND900 and LND901 strains carry deletion of M-fragment (*lndI* gene, possible transcriptional activator of *lnd*-genes) of *lnd* -cluster, LND23 – deletions of M- and N-fragments (genes: *lndI*; *lndE*- putative oxygenase, *lndA*, *B* – minimal polyketide synthase).

*Keywords:* landomycin E, *Streptomyces*, angucyclines, mutants.

Polyketide antibiotics form a huge class of natural products synthesized mainly by streptomycetes with wide range of biological activities [5,11]. Angucycline antibiotics group is one of the largest group among aromatic polyketides. All members of the group possess antitumor activity against different types of tumor cells [6,10,11]. Angucyclines are recently discovered group of compounds. Genetic control of angucycline biosynthesis as well as the mechanisms of their antitumor activity are not well studied [10]. Detailed examination of angucycline antibiotics producers is very important today because of worldwide occurrence of multidrug resistant tumors [3,7]; screening for novel antibiotics also pursues the aim to reveal agents capable of inhibiting such tumor cells growth for curing of which effective drugs are still unknown.

The object of our research is strain *Streptomyces globisporus* 1912, the producer of novel angucycline antitumor antibiotic landomycin E (LE). LE biosynthetic gene cluster (*lnd*-cluster) has been cloned and collection of *Streptomyces globisporus* mutants with altered LE production (*Lnd*<sup>-</sup>-mutants) was obtained [9]. The aim of the work lies in analysis of changes of LE synthesis in selected *Lnd*<sup>-</sup>-mutants using methods of thin layer chromatography (TLC), spectrophotometry (SP) and detection of possible changes at genetic level in given *Lnd*<sup>-</sup>-mutants via DNA-DNA hybridization.

Strains *S. globisporus* 1912 and W1 (control LE – nonproducing mutant) were kindly provided by Prof. Matselukh B. P. (D.K. Zabolotniy Institute of Microbiology and Virology, NAS of Ukraine, Kyiv). Other *Lnd*<sup>-</sup> mutants used in the work were obtained via induced with ultraviolet rays (UV) or N-methyl-N'-nitro-N-nitrosoguanidine (NG) mutagenesis according to Hopwood D.A. et al. [4]. LE extraction, TLC, and SP analysis of LE crude extracts in visible light, determination of *Lnd*<sup>-</sup> mutants resistance to antibiotics were carried out as described [2]. Three DNA fragments of *lnd*-cluster (fig.1) isolated from previously obtained plasmids pKM2, pKN1, pKJ1[9] were used as probes for hybridization - 6,5kb M-fragment (contains gene *lndI*, possible transcriptional activator of *lnd*-genes; M-probe), 7,2kb N-fragment (genes *lndE*, *lndA*, *lndB* – putative oxygenase,  $\alpha$  and  $\beta$  subunits of minimal polyketide synthase respectively; N-probe) 4,5kb J-fragment (genes *lndQ*, *S*, *T* probably involved in LE's deoxysugar synthesis; J-probe). Southern hybridization, non-radioactive DIG-DNA labelling, and all manipulations with total and plasmid DNA *in vitro* were performed according to described techniques [4,13].

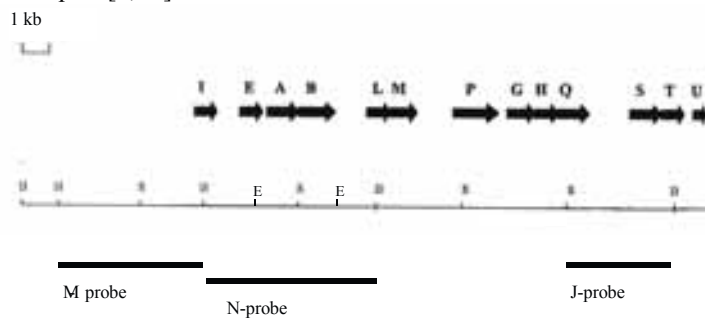


Fig. 1. Fragment of *S. globisporus* 1912 landomycin E (LE) biosynthetic gene cluster (*lnd*-cluster). Putative functions of cloned genes: *lnd I* – transcriptional activator of *lnd*-cluster genes; *lndE* – oxygenase; *lndA* – ketosynthase; *lndB* – chain initiation factor; *lndL* – cyclase-dehydrase; *lndM* – hydroxylase; *lndP* – acyl-CoA-decarboxylase; *lndG*, *lndH*, *lndQ*, *lndS*, *lndT*, *lndU* – genes involved in LE deoxysugar part synthesis. Restriction sites: B-BamHI, S- SacI, E- Eco321. Black arrows represent length and direction of identified ORFs in the *lnd*-cluster. Bold lines under the map indicate *lnd*-cluster fragments used as hybridization probes.

After three passages among 50 *Lnd*<sup>-</sup> mutants 9 stable mutants (LND23, LND29, LND81, LND317, LND602, LND801, LND804, LND900, LND901) were taken for further study. All mutants were obtained after one-step mutagenesis: LND23 has been obtained via NG treatment of spore suspension, other strains were selected after UV-mutagenesis. Levels of resistance to penicillin, ampicillin, cefalotin, erythromycin,

oleandomycin, kanamycin, streptomycin, neomycin, gentamycin, tetracycline and rifampycin in LND-mutants and *S. globisporus*1912 were identical. The only exception is strain LND901. It exhibits increased resistance to erythromycin and streptomycin. Titration of LND901 spores onto media with increased concentrations of given antibiotics reveals 100% survival at 10µg/ml of erythromycin and 50µg/ml of streptomycin (such concentrations of the antibiotics result in less than 1% survival of wild type spores) [1].

We performed TLC of compounds extracted from studied Lnd<sup>-</sup>-mutants and SP of the crude extracts. Results of these experiments are given in a table. TLC of polyketide metabolites shows that mutants LND602, LND804 produce trace quantities of LE. We failed to detect LE production by these strains using SP. Low level of LE production in examined strains can be explained by specific character of the mutations (leaky-mutations, impaired regulation of LE synthesis) or accumulation of revertants to Lnd<sup>+</sup>-phenotype. TLC and SP show the absence of any angucyclic compounds in extracts from strains LND23, LND29, LND81, LND901, W1. This tells about damages in genes controlling regulation and first steps of LE synthesis. Strains LND317, LND801, LND900 produce new compounds (possible intermediates in LE synthesis) that can be detected using TLC and detection in visible light. SP revealed additional absorption peaks in extracts from these strains (LND317 – 370, 400nm, LND801 – 400nm, LND900 – 325nm). Coupled double bonds of quinone-like part of angucycline antibiotics is shown to provide light absorption in visible region of spectrum [10]. So, we concluded that in strains LND317, LND801, LND900 genes supervising polyketide carcass formation and first cyclization remained intact. Possibly, mutations are localized in genes for deoxysugar synthesis or modification of LE's nascent polyketide skeleton. Further studying of these strains and compounds they produce shall allow us to answer the question about character and precise localization of respective mutations.

To uncover possible changes in studied LND-mutants at genetic level, we performed Southern hybridization of total DNA restricts with fragments of *lnd*-cluster. Results of hybridization are summarized in the table and illustrated in fig.2. In six mutants (LND29, LND81, LND317, LND602, LND801, LND804) and initial strain 1912 sizes of total DNA fragments hybridizing with M-, N-, J-probes are identical. So, Lnd<sup>-</sup>-phenotype of given strains is not caused by big deletions in analyzed regions of *S. globisporus* chromosome. We suppose that these mutants carry point mutations in *lnd*-genes or small deletions at the edges of analyzed regions of *lnd*-cluster.

Complete deletion of M- and N-fragments of *lnd*-cluster is detected in strain LND23 that correlates with Lnd<sup>-</sup>-phenotype of the mutant revealed by TLC and SP. In strain W1 used as control LE-nonproducing mutant, deletions of M-, N- and J-fragments are observed. Strains LND900, LND901 carry deletion of M-fragment (Fig.2C). These two strains differ in their Lnd<sup>-</sup>-phenotype. Mutant LND900 produces 2 new lemon-yellow compounds and one of them has TLC mobility similar to that of the LE, whereas LND901 is characterized by abolished LE and other intermediates production. Data presented above allow us to point out a vital role of *lndI* gene in normal LE synthesis.

New compounds produced by LND900 are putative intermediates in the LE biosynthetic pathway, and they can't be converted to LE because some essential genes for LE synthesis are not transcribed in absence of activating function of LndI protein. Similar situation was observed for biosynthesis of other polyketide antibiotics [12]. Absence of LE and intermediates to LE in strain LND901 can be explained by combination of *lndI* deletion and putative point mutations in structural *lnd*-genes.

Results of Southern hybridization of *lnd*-cluster DIG-labelled fragments with total DNA of *S. globisporus* LND-mutants, their TLC and SP analysis.

Lnd mutants	M-probe		N-probe		J-probe		Results of TLC	Results of SP <sup>(1)</sup> (nm)
	DNA fragments	Fragments size, which hybridize with probe, kb.	DNA fragments	Fragments size, which hybridize with probe, kb.	DNA fragments	Fragments size, which hybridize with probe, kb.		
23	B	- <sup>(2)</sup>	B	-	B	13	-	-
					S	10		
29	B	6,5	E	3,8	B	13	-	-
					S	10		
81	B	6,5	B	7,2	B	13	-	-
317	B	6,5	B	7,2	B	13	-	-
					S	10		add. peak 370, 400
602	B	6,5	B	7,2	B	13	trace	-
801	S	7,5; 6,0	B	7,2	S	10	Spot in UV in place of LE	-; add. peak- 400
804	B	6,5	B	7,2	B	13	trace	-
900	S	-	B	7,2	B	13	-	-; add. peak -325
					S	10		
901	S	-	E	3,8	B	13	-	-
					S	10		
w1	B	-	B	-	B	-	-	-
1912	B	6,5	B	7,2	B	13	LE	Peak - 442
	S	7,5; 6,0	E	3,8	S	10		

(1) – Absorption spectra were measured from 300 to 500nm. (2) “-“ – absence of hybridization signal or absence of LE in extracts. Abbreviations: B- BamHI, S- SacI, E- Eco321; add. – additional.

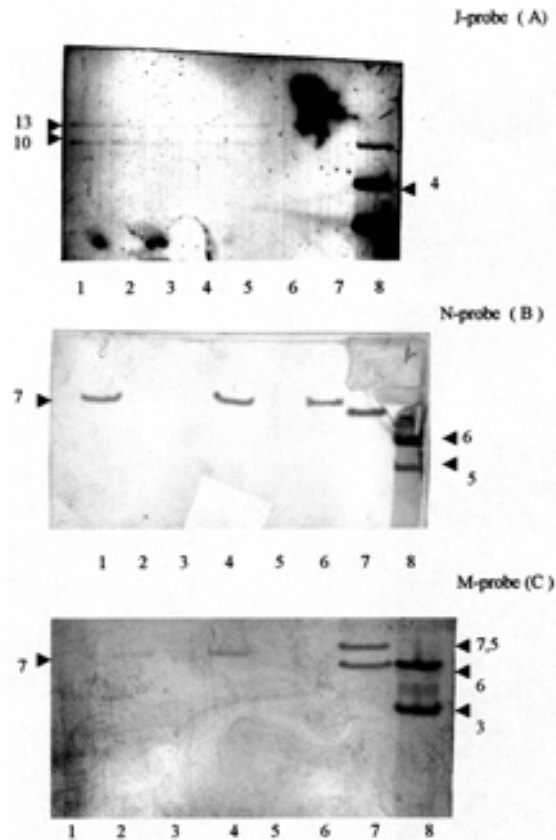


Fig.2. Southern hybridization of *S. globisporus* LND-mutants total DNA restricts with fragments of Ind-cluster (digitals near triangles mean fragments size, in kb). A. (J-probe) Lanes:1.*S. globisporus* LND23 total DNA, cleaved with restriction endonucleases *Bam*HI and *Sac*I (LND23/*Bam*HI *Sac*I) 2. LND29/*Bam*HI *Sac*I 3. LND901/*Bam*HI *Sac*I 4.LND317/*Bam*HI *Sac*I 5. 1912/*Bam*HI *Sac*I 6. *S. coelicolor* A3(2) total DNA, cleaved with *Bam*HI and *Sac*I (negative control) 7. *S. globisporus* W1 total DNA, cleaved with *Bam*HI and *Sac*I 8. pKJ1 plasmid, cleaved with *Bam*HI. B. (N-probe) 1. 1912/*Bam*HI 2. Negative control 3. LND23/*Bam*HI 4. LND29/*Bam*HI 5. W1/*Bam*HI 6. LND900/*Bam*HI 7. LND901/*Sac*I 8. Plasmids pSET152 and pJWH19, cleaved with *Bam*HI. C. (M-probe) 1. LND23/*Bam*HI 2. LND29/*Bam*HI 3. W1/*Bam*HI 4. 1912/*Bam*HI 5. LND900/*Sac*I 6. LND901/*Sac*I 7. 1912/*Sac*I 8. Plasmids pKM2 and pKJ1, cleaved with *Bam*HI.

We have analyzed 10 *S. globisporus* strains deficient in LE production and carried out DNA-DNA hybridization to uncover changes in *lnd*-cluster organization of studied LND-mutants. Obtained results confirm that cloned genes are involved in LE biosynthesis. Our data also provide a proof for *lndI* gene role in LE synthesis. Combining the results of multiple sequence comparisons [9] and ones obtained in this study, we can say that *lndI* gene is necessary for LE production and regulates it, possibly, through transcription activation of other *lnd*-genes. Mutant strains can be used as hosts for heterologous expression of genes for polyketide synthesis from other producers of anticancer antibiotics and obtaining of novel, biologically active compounds. The determination of chemical structure of LE intermediates in strains LND317, LND804, LND900 will give us a clue about the sequence of biosynthetic reactions leading to angucycline antibiotics formation and will allow us to develop rational approach to biotechnological improvement and construction of hybrid antibiotics producers.

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#### МУТАНТИ *STREPTOMYCES GLOBISPORUS* 1912 ІЗ ЗМІНЕНИМ БІОСИНТЕЗОМ ЛАНДОМІЦИНУ E

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Десять стабільних мутантів *S. globisporus* 1912 із зміненим біосинтезом нового ангуциклічного антибіотика ландоміцину E (LE) проаналізовані методами тонкошарової хроматографії, спектрофотометрії та ДНК-ДНК гібридизації. Чотири штами (LND23, LND29, LND81, LND901) характеризуються повним припиненням продукції LE та його інтермедіатів, а три штами (LND317, LND801, LND900) – зміненим синтезом полікетидних сполук. Штами LND23, LND900, LND901, W1 несуть делеції в межах *lnd* –кластеру. У випадку штаму W1 ми не спостерігали гібридизації з жодним із зондів, що використовувались. У штаммах LND900, LND901 делетований район *lnd*–кластеру, що містить ген *lndI*, ймовірний транскрипційний активатор *lnd*-генів, у штаммах LND23 – M- і N-фрагменти *lnd*–кластеру (ген *lndI*; гени *lndE*, *lndA*, *lndB* – оксигеназа,  $\alpha$ - і  $\beta$ -субодиниці кетосинтази, відповідно).

*Ключові слова:* ландоміцин E, *Streptomyces*, ангуцикліни, мутанти.

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