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A. KOBYLYANSKYY, B. OSTASH, V. FEDORENKO
Ivan Franko National University of Lviv,
Department of Genetics and Biotechnology
Hrushevskyy str. 4, Lviv 79005, Ukraine,
E-mail: natureson@rambler.ru

HETEROLOGOUS CROSS-EXPRESSION OF OXYGENASE AND GLYCOSYLTRANSFERASE GENES IN STREPTOMYCETES, PRODUCING ANGUCYCLIC ANTIBIOTICS

The heterologous expression of oxygenase genes urdM, ovmOIII, lanM2, lndZ5 and glycosyltransferase gene urdGT2 in different angucycline producers of genus Streptomyces has been carried out. The introduction of genes urdM, lanM2 and urdGT2 results in the accumulation of new glycosylated compounds in several strains under investigation. A number of processed recombinant strains display streptomycin sensitivity and decrease in total antibacterial activity. The obtained data is an evidence of changes in antibiotics production, resulting from post-polyketide synthase (post-PKS) tailoring gene expression across streptomycete strains. Our study demonstrates the potential of post-PKS tailoring genes for generation of novel bioactive metabolites.

Introduction. The angucyclic polyketide antibiotics are microbial quinone natural products bearing a characteristic four-ring frame of the aglycon moiety, which is assembled in an angular manner [1]. The classification of the angucyclines, which numbers more than a hundred different compounds, is related to the tetracyclic benz[*a*]anthracene system and its derived compounds. Angucyclines show a multitude of valuable biological activities such as anticancer, antibacterial, antiviral, enzyme inhibitory etc. Angucycline producing organisms exclusively belong to order *Actinomycetales*, basically to genus *Streptomyces* – Gram-positive, mycelial, sporulating bacteria.

Though all the angucyclines possess antibiotic, particularly anticancer, activities, the range of their activity greatly depends on the presence of functional groups and glycosyl residues in their molecules [2]. Namely, bioactivity is usually defined by the arrangement and quantity of keto-, hydroxygroups and sugars. These functionalities are introduced by oxygenases and glycosyltransferases (GT's). The need for new polyketides is caused by the emergence of pathogenic strains and tumor cells resistant to traditional chemicals. Modern methods of genetic engineering allow the construction of artificial secondary metabolic pathways by combining the directed mutagenesis and heterologous gene expression approaches in foreign cells (of other species).

Combinatorial biosynthesis anticipates systematic modification and interchange of genes involved in biosynthesis of natural products with the consequential production of 'unnatural' or 'hybrid' natural products [2, 3].

Here, we report the use of combinatorial biosynthesis approach for modification of the biosynthesis pathways of five angucyclic antibiotics, i.e. urdamycin A (*1*), landomycins A and E (*2*), simocyclinone D8 (*3*) and oviedomycin (*4*) (Fig. 1). The aim of current work was to perform heterologous cross-expression of four oxygenase genes *urdM, ovmOIII, lanM2, lndZ5,* descending, respectively, from *S. fradiae* Tu2717, *S. antibioticus* ATCC 11891, *S. cyanogenus* S136, *S. globisporus* 1912 and one glycosyltransferase gene *urdGT2* from *S. fradiae* Tu2717 in these strains and in two *S. globisporus* mutants deficient oxygenase genes. This approach has been used to obtain the collection of recombinants producing novel compounds with altered bioactivity and to investigate the changes in their secondary metabolism.

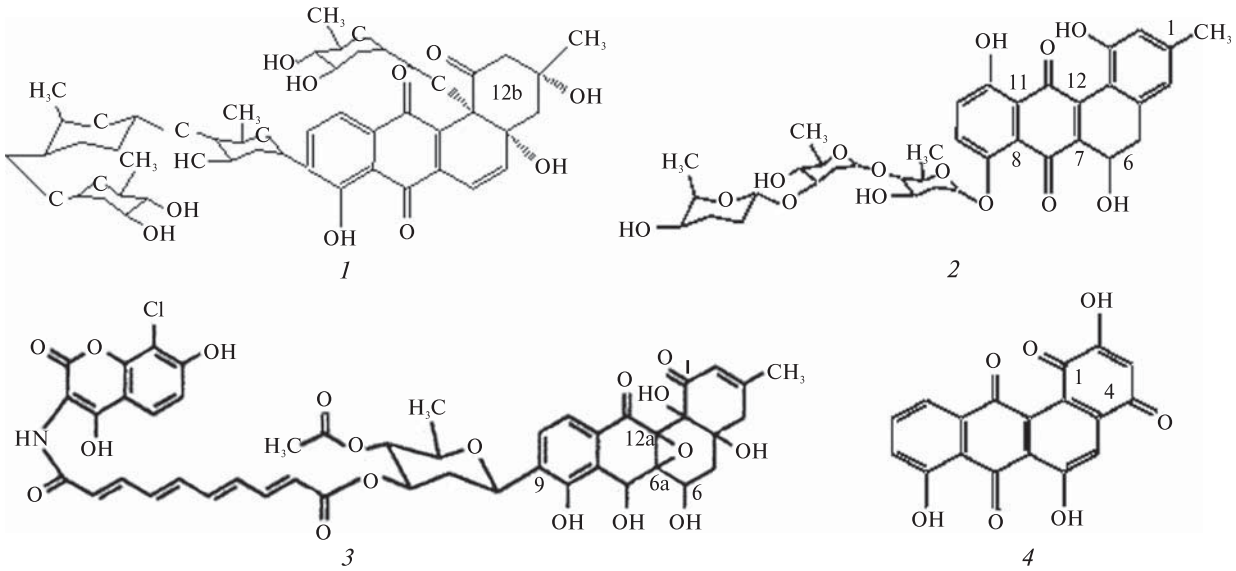

Fig. 1. Structural formulae of urdamycin A (*1*), landomycin E (*2*), simocyclinone D8 (*3*), oviedomycin (*4*)

Materials and methods. Bacterial strains and plasmids used in this work are listed in accompanying table. *Escherichia coli* strains were grown at 37 °C in LB for routine applications [4]. *Streptomyces* strains were grown at 30 °C. Solid oatmeal medium was used for streptomycetes sporulation and plating of *E. coli* – *Streptomyces* matings [5]. Growth dynamics and antibiotic production of *Streptomyces* strains were examined in TSB (120 h of incubation). Where required, strains were grown in the presence of antibiotics and chromogenic substrates as described elsewhere [6]. *Bacillus subtilis, B. cereus, S. albus* and *Sarcina flava* were used as test-cultures for determining antibiotic activity of obtained mutants. Agar plugs were cut from solid media and put on the surface of test culture. The diameter of growth inhibition zones was measured on 16th, 24th and 48th hour of the cultivation. The resistance spectra were analysed by means of antibiotic disc diffusion method.

Plasmid DNA from *E. coli* was isolated using standard protocols [4]. *E. coli* transformation and intergeneric matings (using *E. coli* ET12567 (pUB307) as a donor) were performed as described previously [5, 6]. Enzymes and kits for molecular biological manipulations were purchased from standard commercial sources and used as described by the manufacturer.

Secondary metabolites from the culture medium were extracted with equal volume of ethyl acetate on the 48–72th hour of *Streptomyces* strains growth in 25 ml of SG at 28 °C in a rotary shaker (120 rpm) as described [4]. The combined organic extracts were dried and dissolved in 30 μl of methanol for thin layer chromatography (TLC). TLC analysis was carried out on SilG-25 silica gel plates («Merck», Germany) with chloroform/methanol (9 : 1) as solvent. R_f values are not presented as appearance of blots different from control was crucial and sufficient in these experiments.

In each separate experiment of heterologous gene expression we analysed few exconjugants to ensure unambiguous result.

Results and discussion. ***Heterologous expression of urdM.*** Introduction of angular hydroxyl in 12b position of urdamycin A (*1*) molecule of *S. fradiae* Tu2717 is the unique reaction in angucycline biosynthesis (Fig. 1). Moreover, additional olivose is attached to this group, obviously, intensifying anticancer properties of the antibiotic [10]. The protein UrdM consists of two parts: oxygenase and reductase. It is involved in oxygenation at 12b position of urdamycin precursor. In order to provide effective heterologous expression of *urdM* a 1,5-kb *Xba*I-*Eco*RI fragment of *S. fradiae* Tu2717 DNA containing this gene was cloned into the conjugative expression vector of pKC1218E [6] next to erythromycin resistance gene promoter giving pKC1218E*urdM* (Fig. 2, *a*). By means of intergeneric *E. coli* – *Streptomyces* conjugation pKC1218E*urdM* was

Bacterial strains and plasmids used in this study

Strains/plasmids	Relevant characteristics	Source or reference
E. coli DH5α	F-(φ80d△(*lacZ*)M15 *recA1 endA1 gyrA96 thi1 deoR* (*lacZYA-argF*) U169)	MBI Fermentas
E. coli ET12567 (pUB307)	*dam*-13::*Tn9* (Cmr) *dcm-6 hsdM*; contains RK2-based conjugative plasmid pUB307	C. Smith, UMIST Manchester, UK
S. antibioticus ATCC 11891	Oviedomycin producer	J. Salas, University of Oviedo, Spain
S. antibioticus Tu6040	Symocyclinone producer	A. Bechtold, Albert-Ludwigs-University of Freiburg, Germany
S. cyanogenus S136	Landomycin A producer	A. Bechtold, Albert-Ludwigs-University of Freiburg, Germany
S. globisporus SMY622	Landomycin E overproducer	[7]
S. globisporus M12	*lndM2* disruption mutant of *S. globisporus* 1912	[8]
S. fradiae Tu2717	Urdamycins producer	A. Bechtold, Albert-Ludwigs-University of Freiburg, Germany
S. globisporus E7	*S. globisporus* SMY622 with mutated *lndE*	[9]
pSET152	*E. coli* – *Streptomyces* conjugative vector (ori ColE1, Am^r, *lacZ*, oriT RK2, intPφC31, attPφC31)	P. Leadlay, Cambridge university, England
pKC1139	pUWL201 *E. coli* – *Streptomyces* shuttle expression vector with *ermE* promotor and pIJ101 replicon, Th^r	[6]
pKC1218E	*E. coli* – *Streptomyces* shuttle expression vector with *ermE* promotor and SCP2 replicon, Am^r	C. Olano, University of Oviedo, Spain
pUWL201	*E. coli* – *Streptomyces* shuttle expression vector with *ermE* promotor and pIJ101 replicon, Th^r	[6]
pKC1218E△BX3	pKC1218E, where *lndZ4Z5* cloned downstream to *ermE* promotor	This work
pKC1218E*urdM*	pKC1218E, where *urdM* cloned downstream to *ermE* promotor	»
pSET*ovmOIII*	pSET152, carries *ovmOIII*	»
pSET*urdGT2*	pSET152, carries *urdGT2*	»
pUWL*lanM2*	pUWL201, where *lanM2* cloned downstream to *ermE* promotor	»
pUWL*urdGT2*	pUWL201, where *urdGT2* cloned downstream to *ermE* promotor	»

transferred to the cultures *S. globisporus* Smy622, *S. globisporus* E7, *S. globisporus* M12 and *S. cyanogenus* S136. These cultures were selected on purpose, because none of their secondary metabolites comprises angular hydroxyl in 12b position and such experiments would help us understand better the substrate specificity of UrdM. The analysis of secondary metabolites extracts from obtained four recombinant strains by means of TLC discovered distinct changes in spectra of synthesized colored compounds for *S. globisporus* E7*urdM* and *S. globisporus* M12 *urdM*. Apparently, E7+*urdM* strain no longer produced 2,3-dehydro-UWM6 typical for initial E7 strain. Instead, there are three new compounds present on TLC (Fig. 2, *b*). M12 + *urdM*, generates no aromatic secondary metabolites at all, at least in detectable quantities. E7 + *urdM* strain most likely produces compounds with one, two and three sugars, as judged from changes in mobility and color of compounds. The experiment shows that 11-hydroxylation is advantageous for the glycosyltransfer and this can serve

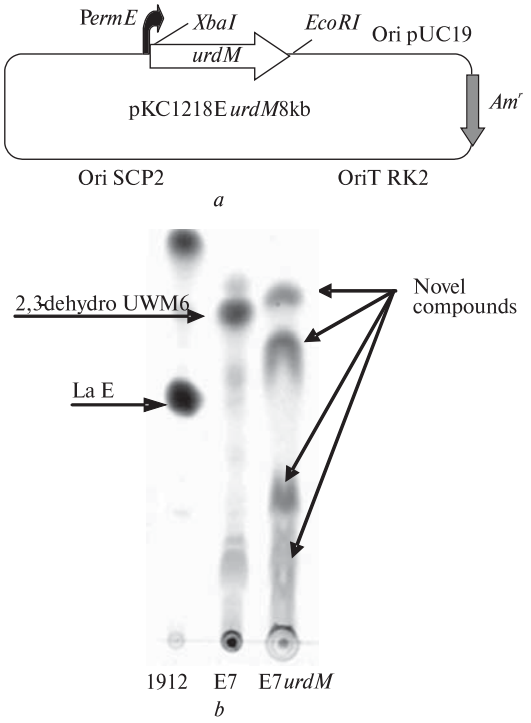

Fig. 2. Recombinant plasmid pKC1218E*urdM* map (*a*) and change in spectrum of secondary metabolites for *S. globisporus* E7*urdM* (*b*); *Am*^{r} – apramycin-resistance gene; LaE – landomycin E

an evidence that the 11-hydroxylation normally precedes the glycosyltransfer in landomycins biosynthesis. It also shows that LndGT2 possesses broadened substrate specificity regarding its aglycon acceptor substrate.

Heterologous expression of lanM2. Over the past decade, two gene clusters encoding landomycin biosyntheses, namely, the trisaccharidal landomycin E (*2*) produced by *S. globisporus* 1912 (Fig. 1) and the hexasaccharidal landomycin A produced by *S. cyanogenus* S136 have been cloned and sequenced [11]. The studies revealed that both clusters display an extraordinarily high degree of identity. Previous investigation of *lndM2* gene descending from *S. globisporus* 1912 [8] asserted the role of its corresponding flavoprotein as oxidoreductase, responsible for the attachment 6-hydroxyl during the biosynthesis of landomycins precursor. With the purpose of *lanM2* heterologous expression the amplifying replicative shuttle plasmid pUWL*lanM2*, where *lanM2* is cloned downstream to erythromycin resistance gene promoter (Fig. 3, *a*) was transferred to the cells of three strains. We selected thiostrepton resistant exconjugants in the matings of *E. coli* (pUWL*lanM2*) with *S. fradiae* Tu2717, *S. antibioticus* Tu6040 and *S. globisporus* E7. 6-hydroxyl group is absent in the molecule of urdamycin A (*1*), produced by *S. fradiae* Tu2717, so we intended to introduce this hydroxyl function, aiming to redistribute electron density in ring B. Unfortunately, we did not reveal changes in the secondary metabolites spectrums for this recombinant, as well as for E7 + *lanM2*. Concerning *S. antibioticus* Tu6040 carrying pUWL*lanM2*, the spectrum differs in principle (Fig. 3, *b*). *S. antibioticus* Tu6040 is the producer of a complex angucyclic antibiotic simocycli-

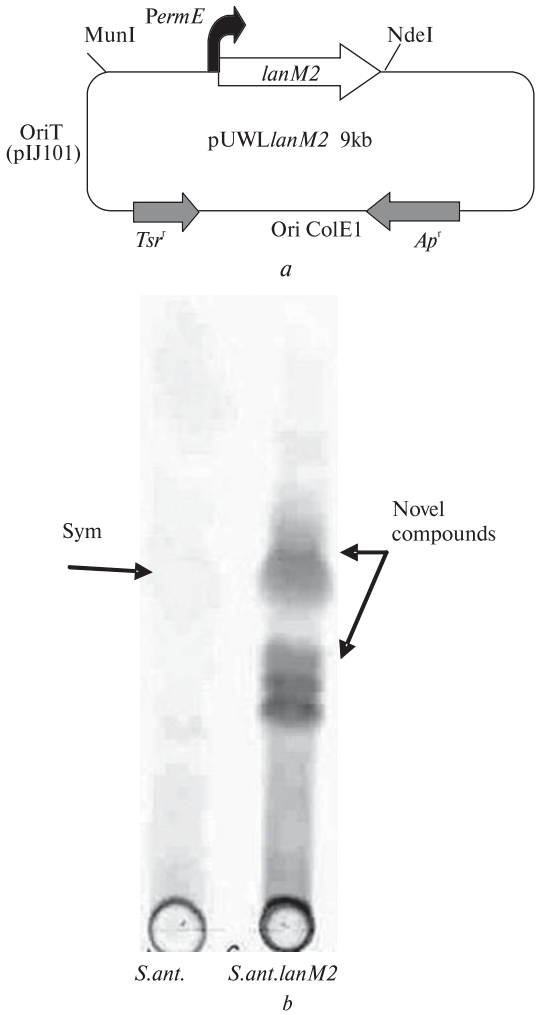

Fig. 3. Recombinant plasmid pUWL*lanM2* map (*a*) and change in spectrum of secondary metabolites for *S. antibioticus lanM2* (*b*); *Tsr*^{r} – thiostrepton-resistance gene; *Ap*^{r} – ampicillin-resistance gene; Sym – symocyclinone D8

none D8 (*3*), which biosynthetic cluster has been cloned and sequenced [16], still little is known about the mechanisms and timing of oxygenation-reduction reactions. One of simocyclinone's drastic peculiarities is a unique epoxyfunction in aromatic ring B (Fig. 1). We surmise that oxygenase domain of *lanM2*, due to its low substrate specificity could facilitate aromatization of this ring and the recombinant strain produces as-yet-unidentified aglyca along with the final compound.

lndZ4/Z5 heterologous expression. The pair of genes *lndZ4* and *lndZ5,* was declared to be responsible for the 11-hydroxylation that occurs during landomycin E (*2*) biosynthesis [13]. The authors also showed that hydroxylation at position 11 is not dependent on the length of the side chain and may occur at different stages during landomycin A biosynthesis. This opportunity encouraged us to introduce this pair of genes, encoding hydroxylase and reductase to *S. fradiae* Tu2717, as urdamycins just lack 11-hydroxyl. Interestingly, landomycin F, was approximately threefold less active against the MCF-7 breast cancer cell line than its corresponding 11-hydroxy analogue, landomycin D [13]. We brought *lndZ4/Z5* consisting of the plasmid pKC1218E△BX3 (Fig. 4, *a*) to the culture *S. fradiae* Tu2717. TLC analysis detected new major metabolite in comparison to wild type strain (Fig. 4, *b*). Previously we concluded that all oxygenations steps except the 11-hydroxylation occur before Lan/LndGT2 adds the first sugar moiety to landomycinone precursor [9], in contrast to urdamycins biosynthesis where first glycosylation step precedes all oxygenations. Therefore, it is interesting to know how the presence of additional hydroxyl would affect UrdGT2's ability to attach first olivose to urdamycinone precursor molecule.

ovmOIII heterologous expression. *ovmOIII* gene originates from *Streptomyces antibioticus* ATCC 11891 oviedomycin (*4*) biosynthesis genes cluster [14]. At least three oxygenations take place in oviedomycin formation. They are the result of *ovmOI*, *OII* and *OIII* expression. OvmOIII reveals similarity to flavin-type hydroxylases of *Rhodococcus* sp., that modify 7-ethoxycumarin and rifampin [14]. Consequently, it is considered that *ovmOIII* is involved in hydroxylation, or ketogroup attachment in the first or fourth positions of the oviedomycin precursor. The presence of oxygen in 4th position of oviedomycin is an unprecedented case among angucyclines. For heterologous expression of *ovmOIII* we engineered an integrating bifunctional plasmid pSET*ovmOIII*. The plasmid was heterologously expressed in four cultures *S. globisporus* Smy622*, S. globisporus* E7, *S. globisporus* M12 and *S. cyanogenus* S136. No legible change in the spectra of secondary metabolites was discovered by TLC analysis. It is, obviously, predefined by inability of *ovmOIII* oxygenase to recognize heterologous substrates, or the fact that gene descends from a «taciturn» cluster and is naturally expressed only at very specific conditions.

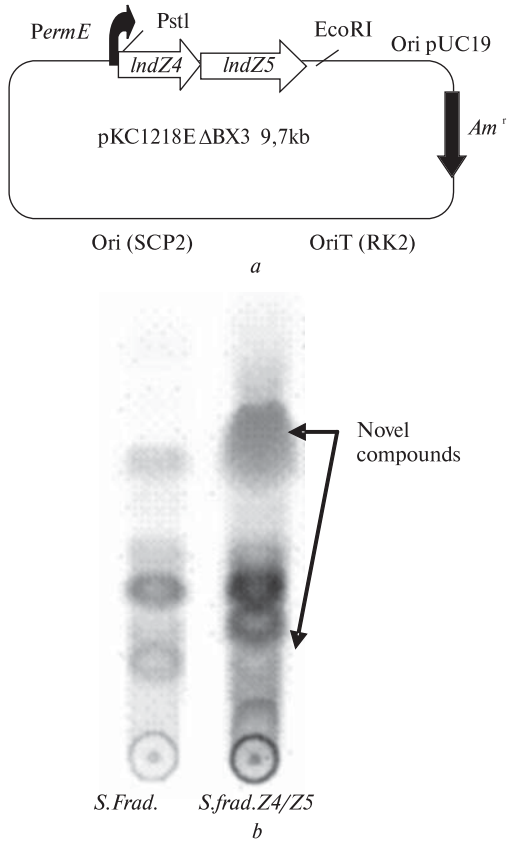

Fig. 4. Recombinant plasmid pKC1218E△BX3 map (*a*) and change in spectrum of secondary metabolites for *S. fradiae lndZ4/Z5* (*b*)

As it is well known, that most oxygenases taking part in aromatic polyketyde antibiotics biosynthesis are not cytochrome P450-dependent (for example LndE/LanE and UrdM are flavin-dependent), this, obviously stipulates for their low substrate specificity, while cytochrome P450-dependent oxygenases are highly tailored [2]. These qualities are very important for the combinatorial biosynthesis of polyketide antibiotics.

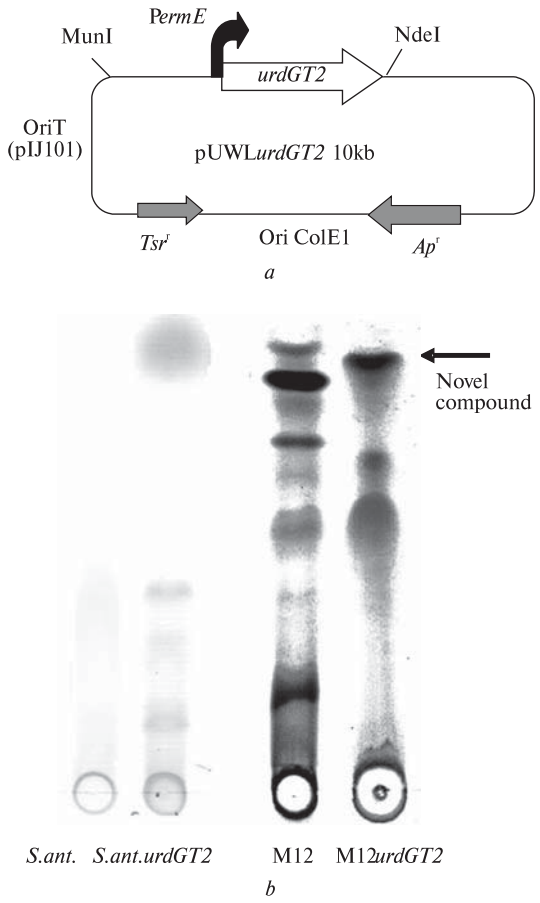

Fig. 5. Recombinant plasmid pUWL*urdGT2* map (*a*); change in spectrums of secondary metabolites for *S. antibioticus urdGT2* and M12 *urdGT2* (*b*)

Heterologous expression of urdGT2. Bioactive natural products frequently include saccharide chains, which contribute to specific interactions with the biological target. Combinatorial biosynthesis approaches are being used in antibiotic-producing actinomycetes to generate derivatives with novel sugars in their architecture. Recent advances in this area indicate that glycosyltransferases involved in the biosynthesis of natural products have substrate flexibility regarding the sugar donor and also, less frequently, with respect to the aglycon acceptor [2, 15].

The first glycosyltransfer step in urdamycins biosynthesis is carried by UrdGT2, which is closely related to Lan/LndGT2. However, the acceptor substrates of these glycosyltransferases appear to be quite different (one is a carbon-GT, and the other is an oxygen-GT). Previously, we have shown that protein UrdGT2 reveals remarkably broadened substrate specificity, as its heterologous expression in the *lndE*-minus mutant of *S. globisporus* 1912 (E7) yielded three novel prejadomycin analogues that differ in their *C*-glycosidically bound moieties [9]. Hence, *urdGT2* turned out to be a promising candidate for combinatorial biosynthesis. Generated in advance pUWL*urdGT2* plasmid (Fig. 5, *a*) was employed to introduce *urdGT2* into three strains – *S. globisporus* Smy622, M12 and *S. antibioticus* Tu6040.

The changes in aromatic secondary metabolites spectra took place in two cases (Fig. 5, *b*). Concerning *S. antibioticus* + *urdGT2*, its spectrum also differs radically from the original strain, but more profound investigation is needed to elucidate the structures of its secondary metabolites.

Traditional studies on antibiotic resistance, antibacterial activity, and mutation maintenance examination for the obtained strains with obvious changes in antibiotic biosynthetic pathways were carried, using wild type strains as controls. To confirm effective replication and passing of heterologously expressed recombinant plasmids that carry *urdM, lanM2* and *urdGT2* genes we cultivated the strains in the course of five generations in nonselective conditions with consequent verification of antibiotic resistance maintenance on media containing selective agents. Approximately 85 % of colonies inherited replicative plasmids after five passages under nonselective conditions ascertained that new features of these recombinants are quite stable. We also analyzed the antibiotic resistance spectra of the mentioned strains against twelve antibiotics of different classes and observed unanticipated streptomycin sensitivity in case of E7 + *urdGT2*. In order to test the changes in antibacterial activity of recombinant strains in comparison with landomycin E (*2*) we used four bacterial species as test-cultures – *Bacillus subtilis, B. cereus, Streptomyces albus* and *Sarcina flava*. The diminution of antibacterial activity against *S. albus* in case of *S. globisporus* E7 carrying *urd*-genes was found. These results apparently point at the deeper changes in physiology of the strains and resistance mechanisms caused by overexpression of *urdGT2* and *urdM* genes under *ermE* gene promoter and undoubtedly can be a subject of future work.

The heterologous expression of *Streptomyces* oxygenase and glycosyltransferase genes is known

to be an effective and beneficial approach to obtain recombinant strains with altered secondary metabolism. Here we report the generation of collection of angucycline-producing strains that carry various heterologous oxygenase and GT genes. We demonstrated here that introduction of certain post-PKS tailoring genes into actinomycete species provokes them to produce modified compounds that could possess valuable activities. The production of these antibiotics testifies low degree of substrate-specificity of oxygenases UrdM, LanM2 and glycosyltransferase UrdGT2. Their genetic determinants are promising candidates for combinatorial biosynthesis of 'hybrid' angucyclines. More detailed chemical analysis and biological assays of mentioned compounds will also clarify the linkage between their structure and bioactivity.

А.Н. Кобылянский, Б.О. Осташ, В.О. Федоренко

ПЕРЕКРЕСТНАЯ ГЕТЕРОЛОГИЧЕСКАЯ ЭКСПРЕССИЯ ГЕНОВ ОКСИГЕНАЗ И ГЛИКОЗИЛТРАНСФЕРАЗЫ У СТРЕПТОМИЦЕТОВ, ПРОДУЦИРУЮЩИХ АНТИБИОТИКИ АНГУЦИКЛИНОВОГО РЯДА

Проведена гетерологическая экспрессия генов оксигеназ *urdM, ovmOIII, lanM2, lndZ5* и гликозилтрансферазы *urdGT2* в разных видах продуцентов ангуциклинов из рода *Streptomyces*. Введение генов *urdM, lanM2* и *urdGT2* в некоторые из исследуемых штаммов обусловливает накопление ими новых гликозилированных соединений. Обнаружено появление чувствительности к стрептомицину и резкое снижение общей антибактериальной активности у некоторых из полученных рекомбинантных штаммов. Представленные данные свидетельствуют о модификации продукции антибиотиков, которая является следствием экспрессии генов постполикетидного синтеза (пост-ПКС) в штаммах стрептомицетов. Наши исследования демонстрируют потенциал генов пост-ПКС в создании новых биологически активных метаболитов.

А.М. Кобилянський, Б.О. Осташ, В.О. Федоренко

ПЕРЕХРЕСНА ГЕТЕРОЛОГІЧНА ЕКСПРЕСІЯ ГЕНІВ ОКСИГЕНАЗ ТА ГЛІКОЗИЛТРАНСФЕРАЗИ У СТРЕПТОМІЦЕТІВ, ЩО ПРОДУКУЮТЬ АНТИБІОТИКИ АНГУЦИКЛІНОВОГО РЯДУ

Здійснено гетерологічну експресію генів оксигеназ *urdM, ovmOIII, lanM2, lndZ5* та глікозилтрансферази *urdGT2* у різних видів продуцентів ангуциклінів з роду *Streptomyces*. Введення генів *urdM, lanM2* та *urdGT2* у деякі з досліджуваних штамів зумовлює накопичення ними нових глікозильованих сполук. Виявлено виникнення чутливості до стрептоміцину та різке зниження загальної антибактерійної активності у деяких із одержаних рекомбінантних штамів. Отримані дані є свідченням модифікації продукції антибіотиків, яка є наслідком експресії генів постполікетидного синтезу (пост-ПКС) у штамах стрептоміцетів. Наші дослідження демонструють потенціал генів пост-ПКС у створенні нових біологічно активних метаболітів.

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