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**Генетика**

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**OBTAINING AND STUDYING OF THE *STREPTOMYCES GALILAEUS* HKI022  
EXCONJUGANTS WHICH CARRY CONJUGATIVE PLASMIDS pWA1,  
pSET152 AND pWPK**

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Plasmids pSET152 and pWA1 were transferred into strain *Streptomyces galilaeus* HKI022 (producer of aclacinomycins) with frequency  $4,7 \times 10^{-3}$  and  $4,5 \times 10^{-4}$  respectively. *Eshcherichia coli* DH1 (pUB307) and *E.coli* ET12567 (pUB307) were used as donors for mating. Integration of pSET152 into chromosome of *S.galilaeus* HKI022 has negative effects on antibiotic production. Hybridization analysis shows that two copies of pSET152 are integrated into chromosome of *S.galilaeus* HKI022. Plasmid pSET152 was inherited every time under non-selective conditions. *S.galilaeus* HKI022 (pWA1) exhibited a 100% stability of inheritance of the  $Th^r$  marker after two passages, and 83% of clones inherited pWA1 after seven passages under non-selective conditions. Plasmid pWPK, which carries genes of cluster of landomycin E biosynthesis (*Ind*-cluster): *IndA* ( $\beta$ -ketoacylsynthase), *IndB* (chain length determinant), *IndE* (oxygenase) and *IndD* (ketoreductase), was constructed. Exconjugants *S.galilaeus* HKI022 (pWPK) were obtained with frequency  $3,4 \times 10^{-6}$ . Spectrophotometric analysis of extracts from *S.galilaeus* HKI022 (pWPK) shows that this strain produces new compound with maximum of absorption 528 nm.

*Key words:* *Streptomyces*, conjugation, antitumor antibiotics.

Anthracyclines are widely used in medicine as antitumor drugs [3, 10]. Because of their clinical importance, many aspects of the anthracycline, such as their way of activity, chemical synthesis, modifications, screening for new derivatives, and biosynthesis, have been intensively studied.

The strain *S.galilaeus* produces polyketide antibiotics of anthracycline group aclacinomycins A, B, and Y. Aclacinomycin A contains aklavinone, rhodosamine, 2-deoxyfucose, and cinerulose A. These antibiotics showed inhibitory effect on the growth of various tumor cells in culture resulting from specific inhibition of RNA synthesis, and they also have strong antimicrobial activity against bacteria and yeast. The genes of aclacinomycins biosynthesis were cloned and sequenced [10]. *S.galilaeus* was found to

have high capacity to accept foreign compounds such as substrates for further modification as exemplified by the glycosylation of various aglycons. Also, the fact that heterologous streptomycete genes like act genes can function in this host suggests the potential utility of this bacterium as host for expression of genetically modified genes for the production of novel hybrid antibiotics [10].

The aim of our work was to evaluate the ways of introducing of recombinant plasmids into *S.galilaeus* HKI022, to examine the effects of integrating and self-replicating plasmids on aclacinomycins production and strain resistance to antibiotics, to study their inheritance stability under selective and non-selective conditions, to develop the heterologous expression of landomycin E biosynthesis genes.

We used strain *S.galilaeus* HKI022, which we received from the Institute of Investigation of Natural Products (Jena, Germany). *Escherichia coli* ET12567 (pUB307) was received from Manchester University (Great Britan). *E.coli* ET12567 (pUB307) is a methylation defective strain (dam-13::Tn9, dcm-6, hsdM) [11]. Plasmid pUB307, is the derivative of RP1, which is required for mobilisation of conjugative plasmids. We used plasmids pSET152 and pWA1 for conjugative transfer into streptomycetes. Plasmid pSET152 contained an *oriT* fragment from RK2, *E.coli* replication functions from pUC118, a fragment of actinophage  $\phi$ C31 DNA with the attachment site (*attP*), and gene *int* of integrase [5]. The gene *aac(3)IV* encoding resistance to apramycin (Am<sup>r</sup>) in pSET152 was used for selection in both *Streptomyces* and *E.coli*. The plasmid does not contain replicative functions of streptomycete plasmids and can be maintained in strains of this genus in the chromosomally integrated state [8]. Plasmid pWA1 also contains an *oriT* fragment from RK2, streptomycete temperature-sensitive replication function from pSG5. This vector posseses marker genes for selection in streptomycetes (thiostrepton resistance (Th<sup>r</sup>) gene, *tsr*) and in *E.coli* (chloramphenicol resistance gene, *cat*) [9]. Plasmid pWA1 additionally contains the kanamycin resistance gene *aphII* of *Tn5*, which is expressed in both *E.coli* and *Streptomyces* [9].

Isolation of plasmid and chromosomal DNAs from *E.coli* and *Streptomyces* followed the technique described in [2]. Other DNA manipulations, such as restriction, electrophoresis, ligation of DNA fragments, and DNA-DNA hybridization were carried out as in [7]. Stability of plasmid inheritance in exconjugants was determined as the ratio of number of colonies that retained resistance to antibiotics after several passages of the strain under selective and non-selective conditions to the total number of colonies. Antibiotic production was determined using *Sarcina aurantica* as the test-culture. Index of antibiotic production (IP) was determined as ratio of diameter of test-culture inhibition zone to diameter of block with lawn of *S.galilaeus* HKI022. Conjugation between *E.coli* ET12567 (pUB307) and *S.galilaeus* HKI022 was carried out as described in [1]. We used oatmeal medium for mating *E.coli-Streptomyces*. For growing of streptomycetes and *E.coli*, we used corn medium, YEME and LB, LA respectively [2, 4].

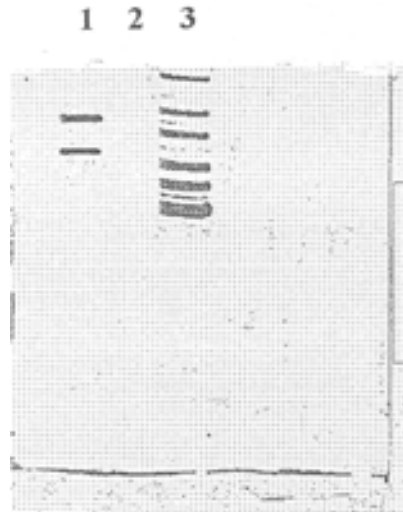


Fig.1. Southern blot hybridization of total DNA from the *S.galilaeus* HKI022 (lane 2) (negative control), *S.galilaeus* HKI022 (pSET152) (lane 1) strains probed with *oriT* from pSET152 and 1-kb ladder (lane 3). The DNA was digested with *Bam*HI.

Plasmid pSET152 was transferred from *E.coli* ET12567 (pUB307) into strain *S.galilaeus* HKI022 with average frequency  $4,7 \pm 0,5 \times 10^{-3}$  per recipient. The frequency of protoplasts transformation of *S.galilaeus* was 10 transformants per  $1\mu\text{g}$  of plasmid DNA [10]. Thus, intergeneric conjugation applied to *S.galilaeus* is more effective than protoplasts transformation of this strain. Plasmid pWA1 could not be selected in *E.coli* ET12567 (pUB307) because it carries the same markers of resistance (*cat* and *aphII*). To omit this inconvenience, we have constructed new donor strain after mating *E.coli* ET12567(pUB307) with streptomycin resistant strain *E.coli* DH1. Exconjugants were selected on the plates with LA supplemented with kanamycin ( $50\mu\text{g}/\text{ml}$ ) and streptomycin ( $100\mu\text{g}/\text{ml}$ ). Plasmid pWA1 was transferred from *E.coli* DH1(pUB307) into *S.lividans* TK24 for testing of conjugative characteristic feature. Exconjugants appeared with average frequency  $5,2 \pm 0,5 \times 10^{-3}$  per recipient. After mating *E.coli* DH1 (pUB307, pWA1) with *S.galilaeus* HKI 022, exconjugants which carry pWA1 were obtained with frequency  $4,5 \pm 0,5 \times 10^{-4}$ . So, frequency of appearing of *S.galilaeus* HKI022 (pSET152) is higher than in the case of *S.galilaeus* HKI022 (pWA1). It can be due to different sizes of plasmids, because the size of pWA1 is twice as large as the size of pSET152. It was shown for *S.fradiae* (producer of tylosin) that the frequency was much higher with small plasmids (approx. 5,5kb) than with large (approx. 15kb) [5]. The fact of increase in the frequency of appearing of *S.galilaeus* HKI022 (pSET152)

exconjugants can be also explained by using different donor strains for mating. It was demonstrated that the methylation deficient donor could yield  $> 10^4$  –fold more *S.coelicolor* exconjugants than the standard methylation proficient donor [6].

Exconjugants of strain *S.galilaeus* HKI022 (pSET152) were analysed for pSET152 plasmid status. To verify the possibility of plasmid incorporation into chromosome of recipient strain, we hybridized *Bam*HI restriction fragments of total DNA from exconjugants with *DIG*-labeled *Pst*I fragment of pSET152, which contains *oriT*. Fig1. shows that *oriT* probe hybridizes with two *Bam*HI fragments of about 6 and 4 kb. Plasmid pSET152 has a unique site for *Bam*HI endonuclease outside of *oriT*. So, we can suggest that chromosome of *S.galilaeus* HKI022 contains two sites for insertion vectors that integrate site-specifically using the bacteriophage  $\phi$ C31 *att/int* system.

The stability of plasmids pSET152 and pWA1 inheritance with respect to the Am<sup>r</sup> and Th<sup>r</sup> markers was analysed in the obtained exconjugants. Plasmid pSET152 was inherited every time under non-selective conditions. *S.galilaeus* HKI022 (pWA1) exhibited 100% stability of the Th<sup>r</sup> marker inheritance after two passages. 96% of clones of *S.galilaeus* HKI022 were resistant to thiostrepton after third passage, and 83% of clones inherited pWA1 after seven passages under non-selective conditions.

A comparative analysis of morphological traits was conducted in the original strain *S.galilaeus* HKI022 and its exconjugants carrying plasmids pSET152 and pWA1. We discovered that the presence of pSET152 had no effects on growth of strains, sporulations, size of colonies, and pigmentation. Spores and mycelium of *S.galilaeus* HKI022 (pWA1) were dyed in red colours.

Influence of the presence of plasmids pSET152 and pWA1 on antibiotics production by *S.galilaeus* HKI022 was examined. We demonstrated that exconjugants *S.galilaeus* HKI022 (pSET152) have smaller IP =  $1,2 \pm 0,1$  in comparison with wild type and exconjugants carrying pWA1 for which IP =  $1,6 \pm 0,1$ . Such decrease in antibiotics production caused by strains which carry plasmids integrated site-specifically was shown for many strains: *S.fradiae* (producer of tylosin), *S.hygroscopicus* (producer of bialaphos), *S.kanamyceticus* (producer of kanamycin) and others [1, 8]. Maybe such effects can be caused by integration of plasmids in important for development of strains structure of genomes (for example, tRNA genes, which can contain *attB* site for phase integration), which have indirect influence on the biosynthesis of antibiotics [8].

Thus, possibility of using conjugative self-replicating plasmids is unlikely because of their relatively high frequency of loss during growth of strain under non-selective conditions. Integrative plasmids can be stably maintained in strains even in the case of the absence of selective pressure. It can be used for the development of stable recombinant producers of antibiotics. Therefore, the possible negative effect of the presence of integrative plasmids on the level of antibiotic production should be considered when integrative vectors are used.

The purpose of this stage of work was to construct a recombinant plasmid, which carries genes of landomycin E biosynthesis, for heterologous expression in *S.galilaeus* HKI 022 to obtain new hybrid compounds. Landomycin E is a polyketide antibiotic of

angucyclic group produced by *S.globisporus* 1912 [3]. Taking into consideration that integration of plasmid pSET152 has negative effect on aclacinomycins biosynthesis, it was decided to construct recombinant plasmid using conjugative vector pWA1.

Plasmid pWA1 was digested with *Bgl*II and processed with bacterial alkaline phosphatase to prevent the ligation of the vector itself. *Bam*HI fragment, which carries genes of landomycin E biosynthesis cluster (*lnd*-cluster): *lndA* ( $\beta$ -ketoacylsynthase), *lndB* (chain length determinant), *lndE* (oxygenase), and *lndD* (ketoreductase), was eluted from gel and ligated to pWA1 digested with *Bgl*II. *E.coli* DH5 $\alpha$  was transformed with ligation mix, and transformants were selected on the LA with chloramphenicol. Clones which contain recombinant plasmids were selected as sensitive to neomycin because the incorporation of *lnd*-fragment into pWA1 gene *aphII* was inactivated. Fig2. shows scheme of construction of new recombinant plasmid pWPK. Restriction analysis has confirmed structure of pWPK (Fig3).

After mating *E.coli* DH1 (pUB307, pWPK) with *S.galilaeus* HKI022, exconjugants were obtained with frequency  $3,4 \pm 0,5 \times 10^{-6}$ . Obviously, the decrease in frequency of appearing of *S.galilaeus* HKI022 (pWPK) is due to its larger size in comparison to pWA1. Checking of antibiotic production shows that exconjugants *S.galilaeus* HKI022 (pWPK) synthesizes antibiotic on the level of original strain. Then antibiotics were extracted with ethylacetate and analysed by spectrophotometer in visible light (310nm – 800nm). Maximum of absorption at 430 nm is showed by aclacinomycins, but we determined additional peak of absorption at 528 nm in extract from *S.galilaeus* HKI022 (pWPK). Probably, this compound is hybrid antibiotic, which is produced due to influence of *lnd*-genes' products on the way of aclacinomycins biosynthesis in *S.galilaeus* HKI022.

Results of this work are important for further gene engineering development of *S.galilaeus* HKI022, especially in the case of vectors for genes expression. Perspectivity of using *lnd*-genes for heterologous expression in *S.galilaeus* HKI022 to obtain new hybrid antibiotics was shown.

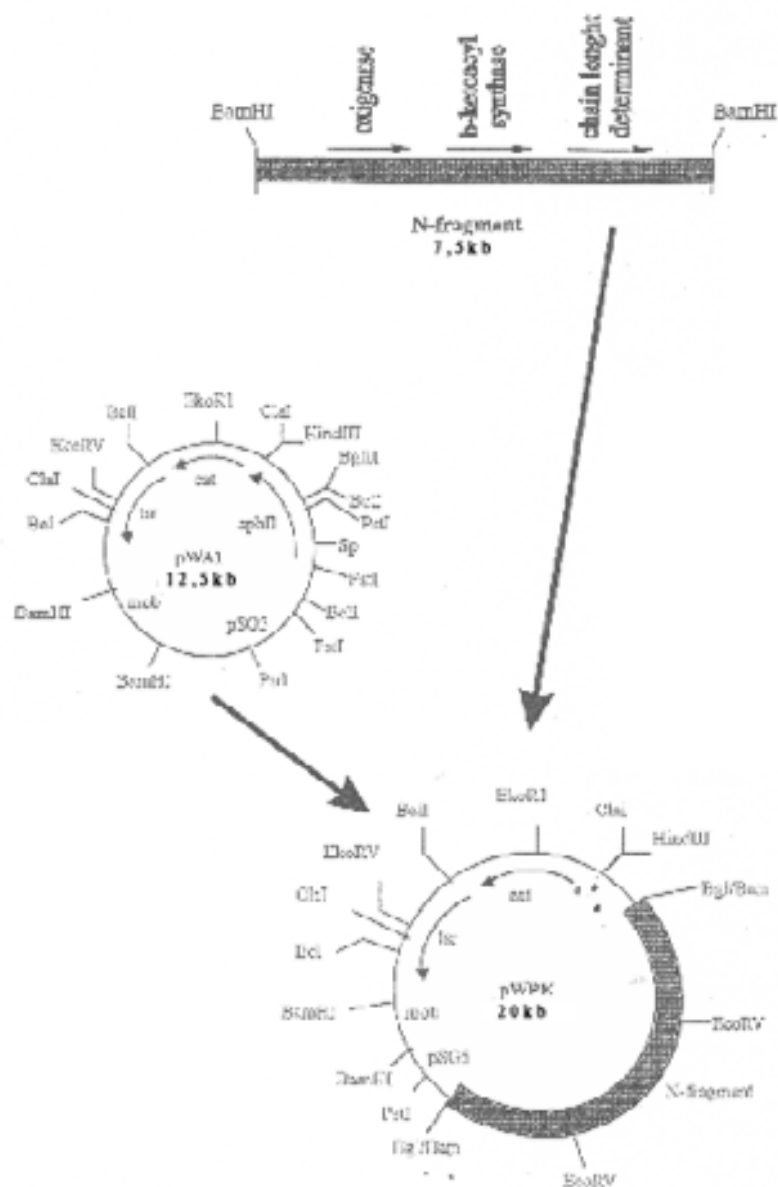


Fig. 2. The scheme of construction of recombinant plasmid pWPK.

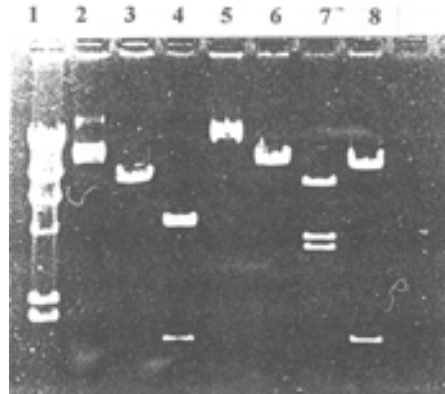


Fig. 3. The restriction analysis of pWPK. Lane 1. DNA of phage  $\lambda$  digested with *Hind*III; lane 2, native plasmid pWA1; lane 3, plasmid pWA1 digested with *Hind*III; lane 4, plasmid pWA1 digested with *Bam*HI; lane 5, native plasmid pWPK; lane 6, plasmid pWPK digested with *Hind*III; lane 7, plasmid pWPK digested with *Eco*RV; lane 8, plasmid pWPK digested with *Bam*HI.

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**ОТРИМАННЯ І ВИВЧЕННЯ ЕКСКОН'ЮГАНТІВ *STREPTOMYCES GALILAEUS* НКІ022, ЯКІ НЕСУТЬ КОН'ЮГАТИВНІ ПЛАЗМІДИ pWA1, pSET152 та pWPK**

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У штам-продуцент аклациноміцину *Streptomyces galilaeus* НКІ 022 виконано кон'югативне перенесення автономної плазмиди pWA1 і інтегративної плазмиди pSET152 з донорних штамів *Escherichia coli* DH1(pUB307) і *E.coli* ET12567(pUB307). Показано, що інтеграція pSET152 в хромосому *S.galilaeus* НКІ 022 має негативний вплив на антибіотичну активність штаму. За допомогою гібридизаційного аналізу виявлено, що в штамі містяться дві копії pSET152. Плазміда pSET152 успадковувалась із частотою 100% за неселективних умов. Рівень успадкування pWA1 складав 83% після сьомого пасажу в неселективних умовах. Сконструйовано автономну плазмиду pWPK, яка несе гени біосинтезу ландоміцину E (*IndA* (β-кетואцилсинтази), *IndB* (фактору контролю довжини), *IndE* (оксигенази), *IndD* (кеторедуктази). Отримано екскон'юганти *S.galilaeus* НКІ 022 (pWPK) з частотою  $3,4 \times 10^{-6}$ . При порівнянні антибіотиків із штамів *S.galilaeus* НКІ 022 (pWA1) і *S.galilaeus* НКІ 022 (pWPK), виявлено, що штам, який містить pWPK, синтезує нову сполуку з максимумом поглинання в області 528 нм.

*Ключові слова:* *Streptomyces*, кон'югація, протипухлинні антибіотики

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