

УДК. 238.218.60

A GENE CLONING SYSTEM FOR *STREPTOMYCES* *CYANOGENUS* S136

A. Luzhetskyy*, M. Fedoryshyn*, D. Hoffmeister**, A. Bechthold**,
V. Fedorenko*

*Ivan Franko National University of L'viv,
Hrushevskiy st. 4, L'viv 79005, Ukraine,
e-mail: genetic@franko.lviv.ua,

**Albert-Ludwigs-University Freiburg, Pharmazeutische Biologie,
Stefan-Meier-Strasse 19, Freiburg D-79104, Germany,
e-mail: bechthold@uni-freiburg.de

To introduce DNA into *Streptomyces cyanogenus* S136, we explored an intergeneric conjugation method. Plasmids pSET152, pSOK101, pSOK201, and pCHZ101 were transferred from *Escherichia coli* ET12567(pUB307) with different frequencies. The status of plasmids in the recipient strain and the stability of their inheritance were investigated. Hybridization analyses of several exconjugants indicated that pSET152 inserted into two *attB* sites on the chromosome. The insertion of pSET152 had no effect on landomycin A production. pSET152 was stably inherited even under non-selective conditions. A series of non-replicative plasmids harboring different insertions from the landomycin gene cluster were constructed to study the efficiency of homologous recombination in *S. cyanogenus* S136.

Key words: streptomycetes, angucycline antibiotics, conjugation.

Streptomyces cyanogenus S136 is the producer landomycin A, B, and D, which belong to the angucycline-group of antibiotics. Landomycins are benz[α]anthraquinone-type compounds decorated with different phenol-glycosidically linked oligosaccharide chains. In landomycin A, the deoxysugar moiety is presented by an unusual hexasaccharide consisting of four D-olivose and two L-rhodinose (Fig. 1). [12]. The angucyclins are, together with anthracyclines, the largest group of compounds in the class of aromatic polyketide antibiotics. Some angucyclins express strong antitumor activity [13]. Landomycin A was found to inhibit the uptake of [³H] thymidine into DNA in murine smooth muscle cells and to block cell cycle progression as well [6]. The genes coding for landomycin A biosynthesis were cloned and sequenced [12]. It was shown that heterologous expression of glycosyltransferases from the *lan*-cluster led to new angucyclic glycosides in mutants of *S. fradiae* Tu2717, and in *S. argillaceus* [10]. In the light of combinatorial biosynthesis *S. cyanogenus* S136 is also attractive as recipient strain, that could

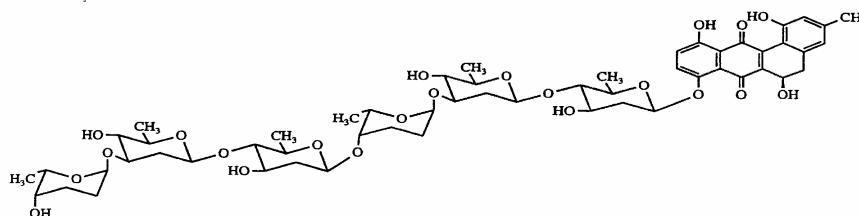


FIG. 1. Structure of landomycin A.

be used to generate new landomycin derivatives by gene inactivation and heterologous gene expression.

In this study we aimed at evaluating ways of introducing recombinant plasmids into *S. cyanogenus* S136, examining effects of integrative and self-replicative plasmids on landomycins production, studying their inheritance stability under selective and non-selective conditions, and investigating the efficiency of homologous recombination between cloned fragments of *lan*-cluster and chromosome in the strain.

We used strain *S. cyanogenus* S136 received from the DSMZ collection (Braunschweig, Germany). *Escherichia coli* ET12567(pUB307), a methylation defective strain (dam-13::Tn9, dcm-6, hsdM), was kindly provided by C.P. Smith from Manchester University (Manchester, UK). Plasmid pUB307 is a derivative of RP1, which contains the *tra* operon required for mobilisation of conjugative plasmids [2]. We used plasmids pSET152, pSOK101 and pCHZ101 for conjugative transfer into *S. cyanogenus* S136. All plasmids contain an *oriT* fragment from *RK2*, *E. coli* replication functions from pUC118, and the *aac(3)IV* gene conferring resistance to apramycin (Am^r) used for selection in both *Streptomyces* and *E. coli*. pSET152 contains a fragment of actinophage ϕ C31 DNA with the attachment site (*attP*) and the *int* gene coding for an integrase [5]. pSOK101 and pCHZ101 carry streptomycetes replication functions from pIJ101 and pHZ1351, respectively, and also the *tsr* gene for thiostrepton resistance [14]. We used the non-replicative conjugative vector pOJmel for development of recombinant plasmids containing genes of the *lan*-cluster. As source for genes of the landomycin cluster we used cosmid H2-26. Isolation of plasmid and chromosomal DNAs from *E. coli* and *Streptomyces* was performed as described [3, 8]. Other DNA manipulations, such as restriction, electrophoresis, ligation of DNA fragments, and DNA-DNA hybridization were carried out as in [8]. PCR was carried out using Perkin Elmer GeneAmp 2400 thermal cycler. The conditions were as described [15] using oligonucleotide primers GT4F (5'-GAACGAACGAAGG-AGCCCGCCG-3') and GT4R (5'-CGGCGGTCCTTCCTCCTGACGG-3'). For analysis of landomycin A production, *S. cyanogenus* S136 strains were grown in SG medium for 48 h, and the antibiotic was extracted with ethyl acetate. The extract was evaporated to achieve a dry state, and the dry powder was dissolved in methanol. TLC analysis was carried out on silica gel plates (silica gel 60 F254, Merck) with chloroform/methanol (9:1) as solvent. 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 colonies number.

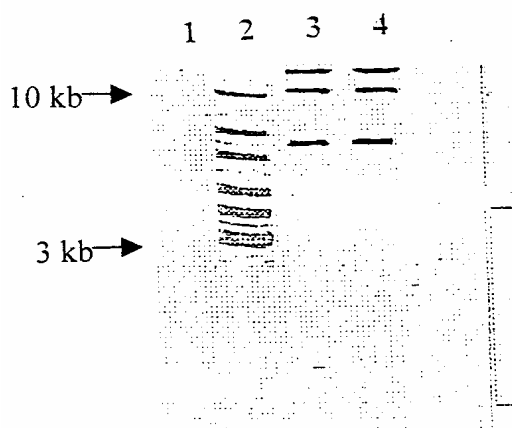


FIG. 2. Southern blot hybridization of total DNA from the *S. cyanogenus* S136 (lane 1) (negative control) and *S. cyanogenus* S136 (pSET152) (lanes 3, 4) strains probed with *oriT* from pSET152. The DNA was digested with *Bam*HI. DNA ladder is on the lane 2.

The donor for conjugation between *E. coli* ET12567(pUB307) and *S. cyanogenus* S136 was prepared as described in [1], and the recipient was prepared as described in [12]. For mating oatmeal medium was used [5].

The process of conjugation is superior to other methods in that it can circumvent restriction barriers in streptomycetes. We carried out conjugal matings between *E. coli* ET12567(pUB307) harboring pSET152, pSOK101, and pCHZ101 and *S. cyanogenus* S136. We have used mycelium of *S. cyanogenus* S136 as recipient in the mating with *E. coli*. Plasmids pSET152, pSOK101, and pCHZ101 were successfully transferred with frequencies 10^{-4} - 10^{-5} per recipient. The frequencies of exconjugants appearance with different plasmids showed that size of plasmid did not correlate with the conjugation efficiency.

pSET152 does not contain replication functions for streptomycetes and is maintained in the cell due to the integration into the host chromosome. Plasmid integration is ensured by DNA fragment of bacteriophage ϕ C31 carrying the *int* gene for integrase and the *attP* site [9]. To confirm the integration of plasmid pSET152 into the *S. cyanogenus* S136 chromosome, we conducted DNA-DNA hybridization experiments. A 0.8-kb *Pst*I fragment of pSET152 carrying the *oriT* site of the vector was used as a probe (*oriT* probe; Fig. 2). Figure 2 shows results of hybridization of the DIG-labeled probe with *Bam*HI fragments of total DNA from two independent *S. cyanogenus* S136 exconjugants. The probe hybridized with three fragments of total DNA (approximately 5.5, 10.5, 12 kb in size). *Bam*HI in the pSET152 plasmid is a unique site located beyond the boundaries of the vector used as a probe. Thus, we can ascertain that DNA of the exconjugants contains at least three pSET152 copies. The number of plasmid copies with the

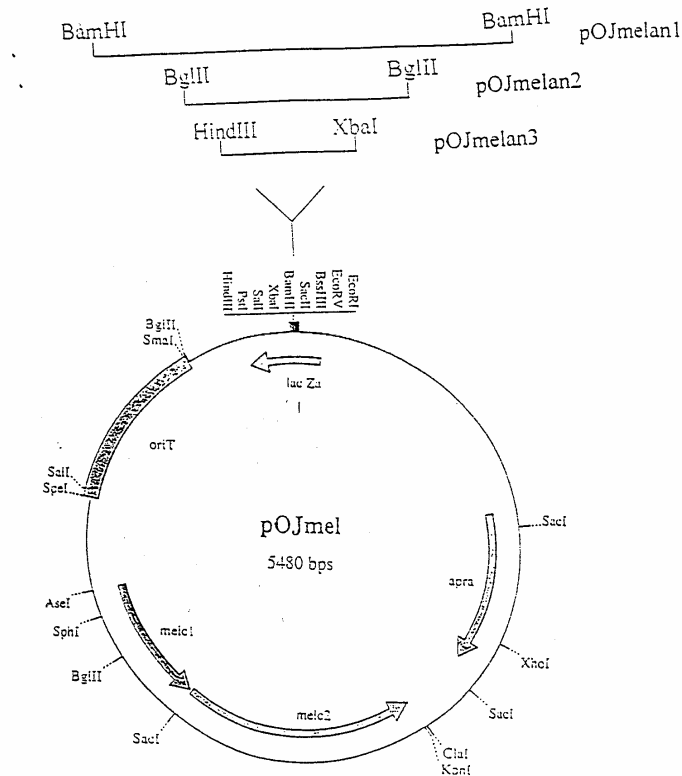


FIG. 3. Scheme of construction of recombinant plasmids pOJmelan1, pOJmelan2, pOJmelan3.

attP site of the $\phi C31$ phage integrated into the chromosome of the strain depends on the number of *attB* sites in the chromosome and differs in different actinomycete species. For example, $\phi C31$ has a unique integration site in *S. coelicolor* and *S. ambofaciens* and two sites in *Saccharopolyspora spinosa* and *S. globisporus* 1912 [2, 9]. Moreover, as shown in some cases, secondary integrations of plasmids may occur via homologous recombination into plasmids, which were initially included into the chromosome by site-specific recombination [2, 5].

The hybridizing band coincides in size with the linearized plasmid pSET152 (Fig. 2). As we have shown previously this hybridization pattern may be explained by a secondary integration of plasmid pSET152, its excision, and plasmid maintenance in an autonomous state. The presence of non-replicative plasmids carrying the *attP-int* fragment from $\phi C31$ in an autonomous state was also shown for other species belonging to the Actinomycetales reflecting the permanent insertion-excision process [8, 10, 11]. To verify whether pSET152 exists in *S. cyanogenus* S136 cells in an autonomous state,

we transformed *E. coli* DH5 α with total DNA isolated from three *S. cyanogenus* S136 exconjugants. In each case, we obtained *E. coli* clones resistant to apramycin. Restriction mapping of isolated plasmid DNA from these clones confirmed that they are identical to pSET152. From these data we concluded that *S. cyanogenus* S136 contains two *attB* sites and that plasmid pSET152 can exist in *S. cyanogenus* S136 both in an integrated form or as an autonomous plasmid.

The stability of plasmids pSET152, pSOK101, pCHZ101 inheritance with respect to the Am^r markers was analysed. Plasmid pSET152 was inherited every time under non-selective conditions. In contrast, pSOK101 and pCHZ101 were not detectable after three passages under non-selective conditions.

A comparative analysis of morphological traits was conducted in the original strain *S. cyanogenus* S136 and its exconjugants carrying plasmids pSET152, pSOK101, and pCHZ101. We discovered that presence of all plasmids had no effects on growth of strain, size of colonies, and pigmentation. No influence on landomycin production was observed.

All these data indicate that integrative plasmids are very useful for the development of stable recombinant *S. cyanogenus* S136 strains.

In order to investigate the effectivity of homologous recombination between cloned fragments of the *lan*-cluster and the chromosome we constructed three non-replicative plasmids which carry fragments with different sizes. H2-26 was digested with *Bam*HI and a 7 kb fragment was ligated into pOJmel to create pOJmelan1. After restriction of H2-26 with *Bgl*II a 2.7 kb fragment was ligated into pOJmel digested with *Bam*HI to yield pOJmelan2. And plasmid pOJmelan3 was constructed by cloning a 1 kb PCR fragment containing a gene of the landomycin cluster into the *Hind*III and *Xba*I sites of pOJmel to yield pOJmelan3. All three plasmids were used to transform *E. coli* ET12567 (pUB307). Recombinant strains were used for mating with *S. cyanogenus* S136. Frequencies of exconjugants were 45-50 c/p (colonies per plate) (pOJmelan1), 5-7 c/p (pOJmelan2), and 2-4 c/p (pOJmelan3). These data indicate that the size of the fragments is very important for effectivity of the process but the data also show that fragments with a size of 1 kb are still large enough for performing knock out experiments.

To date, the *E. coli* – *Streptomyces* conjugations often used to transfer recombinant DNAs into streptomycetes, especially into little-studied strains and into strains in which the introducing by means of PEG-dependent protoplast transformation occurs with a low efficiency [10, 12]. The method we used in this work solved the problem of introducing DNA into *S. cyanogenus* S136. We showed that plasmid pSET152 is useful to develop recombinant *S. cyanogenus* S136 strains. With this conjugation protocol in hand we are now able to study landomycin A biosynthesis in *S. cyanogenus* S136 in more details and we hope to generate new landomycin derivatives by knock out experiments, gene replacement and heterologous gene expression.

Acknowledgements

We are grateful to Prof. Dr. S. Zotchev for providing pSOK101, pCHZ101, pSOK201 and Dr. Y. Demydchuk for pOJmel. This work was supported by a grant of the INTAS for young scientist (YSF-00186) to A.L.

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СТВОРЕННЯ СИСТЕМИ КЛОНУВАННЯ ГЕНІВ ДЛЯ *STREPTOMYCES CYANOGENUS* S136

А. Лужецький*, М. Федоришин*, Д. Хофмейстер**, А. Бехтольд**,
В. Федоренко*

*Львівський національний університет імені Івана Франка,
вул. Грушевського 4, м. Львів, 79005 Україна,
e-mail: genetic@franko.lviv.ua,

**Albert-Ludwigs-University of Freiburg, Pharmazeutische Biologie,
Stefan-Meier-Strasse 19, Freiburg D-79104, Germany,
e-mail: bechthold@uni-freiburg.de

Метод мікродової кон'югації було використано для введення рекомбінантних ДНК в штам *Streptomyces cyanogenus* S136. Плазмиди pSET152, pSOK101, pSOK201, pCHZ101 були перенесені з донорного штаму *Escherichia coli* ET12567 (pUB307). Показано, що інтеграція pSET152 в хромосому *S. cyanogenus* S136 не має негативного ефекту на біосинтез ландоміцину А. З допомогою гібридизаційного аналізу виявлено, що плазміда pSET152 інтегрується в два *attB* сайти хромосоми *S. cyanogenus* S136. Плазміда pSET152 успадковувалась з частотою 100% в не-селективних умовах. Було сконструйовано серію нереплікативних плазмід з різни-ми за розміром вставками з *lan*-кластеру. Показано ефективність проходження го-мологічної рекомбінації між клонованими фрагментами і відповідними ділянками хромосоми штаму *S. cyanogenus* S136.

Ключові слова: Streptomyces, ангуциклінові антибіотики, кон'югація.

Стаття надійшла до редколегії 28.05.2002
Прийнята до друку 20.06.2002