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EXPERIMENTAL ARTICLES

A Gene Cloning System for the Aranciamycin Producer Strain *Streptomyces echinatus* Lv 22[1]

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Abstract—*Streptomyces echinatus* Lv 22 (=DSM 40730) produces an anthracycline antibiotic aranciamycin. Development of DNA transfer using conjugation from *Escherichia coli* into this strain is described. Various replicative plasmids (pKC1139, pKC1218E, pSOK1O1, pCHZ101) as well as actinophage (φC31- and VWB-based vectors pSET152 and pVWB, respectively, were transferred from *E. coli* ET12567 (pUB307) into the *S. echinatus* at a frequency ranging from 2.4×10^{-3} to 1.6×10^{-4}. The transconjugants did not differ from wild type in their ability to produce aranciamycin and morphological features. There is one *attB* site for pSET152 and pVWB integrative plasmids in the *S. echinatus* chromosome. Developed DNA transfer system was used for expression of heterologous regulatory genes in *S. echinatus* cells. Expression of *relA* gene of ppGpp synthetase increased antibiotic production in *S. echinatus*. The *absA2* gene of *S. ghanaensis* appears to play a negative role in the control of aranciamycin biosynthesis. Additional copies of *absA2* leads to inhibition of aranciamycin's production. *absB* and *afsS* had no effect on aranciamycin biosynthesis as well as on the morphological features of *S. echinatus*. Obtained results indicate efficiency of the developed system for gene cloning in *S. echinatus*.

Keywords: *Streptomyces*, antibiotics, conjugation, regulation

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The use of recombinant DNA technology for *Streptomyces* was advanced greatly during the last years [1–3]. Many streptomycetes, including *Streptomyces coelicolor* A3(2), *S. nogalater* IMET43360, *S. globisporus* 1912, possess a potent methyl-specific restriction system which can present an effective barrier to the introduction of heterologous DNA [1, 2]. It was shown that a widely used technique, such as PEG-dependent protoplast transformation, requires the procedure of preparing protoplasts and usually should be optimized for every strain individually. Since *Escherichia coli–Sreptomyces* intergeneric conjugation was first reported by Mazodier et al. (1989), and developed by Flett et al. [1], this method has been used successfully with a number of streptomycete strains. This procedure allows bypassing a restriction barrier of streptomycetes using a specific *E. coli* donor strains [1, 2]. Nowadays a variety of vectors which can be transferred from *E. coli* to *Streptomyces* by conjugation have been developed [2–4]. The vectors contain the *oriT* sequence from the IncP-group plasmid RK2. The unique feature of IncP plasmids is the coordinate regulation of their replication and transfer functions by three global regulators: KorA, KorB and TrbA [2].

Aranciamycin, an anthracycline antibiotic, produced by *S. echinatus*, was found to be an inhibitor of *Clostridium histolyticum* collagenase [5, 6]. In addition it possesses high antitumor activity. Expression of the aranciamycin biosynthetic gene cluster in *S. diastatochromogenes* Tü 6028 resulted in production of four novel compounds, aranciamycins E, F, G, and H with different "decorations" in the tetracyclic backbone. Analysis of the biological activities of the aranciamycins against human tumor cell lines—MCF-7 and MATU—showed that novel aranciamycins E and G were the most active derivatives, indicating that hydroxylation of the C_1 position of molecule appears to coincide with increased antitumor activity of aranciamycins [5].

Better understanding of regulation of aranciamycin biosynthesis could play a significant role in improvement of industrial strains. The productivity of this antibiotic, as well as many important polyketides, often remains relatively low and improving production of these compounds has been of great interest to the industry. Genetic manipulations of aranciamycin producer require an efficient procedure for DNA transfer into *S. echinatus*.

In this report we provide evidences that intergeneric conjugation from *E. coli* is an efficient way for

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Table 1. Strains and plasmids

Bacterial strain or plasmid	Description	Source or reference
E. coli DH5α	*supE44* Δ*lacU169* (φ*80lacZ*Δ*M15*) *hsdR17 recAlendA1gyrA96 thi-1 relA1*	MBI Fermentas
E. coli ET12567 (pUB307)	*dam-13*::Tn9(Cm^r) *dcm-6 hsdM*	Microbial culture collection of antibiotic producers, Lviv, Ukraine
S. echinatus Lv22 (=DSM 40730)	Producer of aranciamycin	Microbial culture collection of antibiotic producers, Lviv, Ukraine
pSOK101	*E. coli*/*Streptomyces* shuttle vector with pIJ101 replicon; *aac(3)IV*	[4]
pKC1139	*E. coli*/*Streptomyces* shuttle vector with pSG5 replicon; *aac(3)IV*	[7]
pCHZ101	*E. coli*/*Streptomyces* shuttle vector with pHZ1351 replicon; *aac(3)IV*	[4]
pSET152	*E. coli*/*Streptomyces* shuttle vector with φCT1 *attP*/*int* integrative system, *aac(3)IV*	[3]
pVWB	*E. coli*/*Streptomyces* shuttle vector with (φCT31 *attP*/*int* integrative system, *aac(3)IV*	Microbial culture collection of antibiotic producers, Lviv, Ukraine
pKC1218E	*E. coli*/*Streptomyces* shuttle expression vector with PermE and SCP2 replicon, *aac(3)IV*	[7]
pKCabsA2	pKC1139, with cloned 3.0 kb fragment of *S. ghanaensis* chromosome containing *absA2*, *aac(3)IV*	Dr. Rabyk, Ivan Franko National University of Lviv, Ukraine
pIJ8647	pOJ260, with cloned N-terminal 0.8 kb fragment of *relA* gene under *ipAp* promoter, *aac(3)IV*	Prof. Bibb, John Innes Centre, Norwich, UK
pKCEafsS	pKC1218E, with cloned 2.9 kb PstI-fragment under *Erm*Ep, *S. eryihraea*, *aac(3)IV*	Dr. Horbal, Ivan Franko National University of Lviv, Ukraine
pKCabsB	pKC1218E derivative with 1.0 kb fragment containing *absB* gene, *aac(3)IV*	T. Gren, Ivan Franko National University of Lviv, Ukraine

delivery of the plasmid DNA into this strain. The studied plasmids permit successful gene expression in *S. echinatus*. Also we report an efficient heterologeus expression of plejotropic regulatory genes into aranciamycin producer strain.

MATERIALS AND METHODS

Strains and plasmids. *Streptomyces echinatus* Lv 22 was used as aranciamycin producer (Table 1). *Escherichia coli* ET12567 (pUB307) was used as donor strain in intergeneric matings. Replicative vectors pKC1139, pKC1218E, pCHZ101 and pSOK101 contain pSG5, SCP2, pHZ1351 and pIJ101 origins of replication, respectively, *oriT* of RK2 and apramycin-resistance gene [7]. Integrative vectors pSET152, pVWB contain *int*/*attP* fragments of actinophages φC31 and VWB, respectively, *oriT* of RK2 and apramycin-resistance gene [7]. *Sarcina lutea* was used in bioassays as an aranciamycin-sensitive strain.

Media and culture conditions. Oatmeal medium, corn medium and minimal medium [7] were used to obtain spores of *S. echinatus* Lv 22 as well as to plate intergeneric matings. For the isolation of total DNA, *S. echinatus* strains were grown in SG medium [7] for 3 days in the presence of apramycin (25 μg/mL) if needed. For aranciamycin production the strains were grown in SG medium for 6 days *Streptomyces* strains were incubated at 28°C unless otherwise stated. *S. lutea* was grown on LB agar. *E. coli* was grown according to [7]. Oatmeal medium was used for aranciamycin production on solid medium.

Intergeneric *E. coli*–*S. echinatus* conjugation. Donor *E. coli* ET12567 (pUB307) strain was grown on LB agar supplemented with apramycin (50 μg/mL) and kanamycin (50 μg/mL) for 12 h at 37°C. *S. echinatus* spore suspension (harvested from 7-days-old lawn), according to a standard protocol, was heat-treated for 10 min at 50°C [7]. Donor and recipient cells were mixed in a 1 : 1 ratio and spread on oatmeal medium. The plates were incubated (12 h, 28°C) and then covered with 1 mL of water containing 1.5 mg of apramycin and 1.5 mg of nalidixic acid. Transconjugants were counted after 5 days. The frequency of transconjugant occurrence was calculated as a ratio of the number of transconjugants to the titer of recipient strain spores.

Purification of aranciamycin and determination of antibacterial activity. Cultures were centrifuged for 10 min at room temperature. The supernatant was discarded and pellet was extracted with chloroform. The resulting extract was evaporated and re-extracted twice again. The final extract was dissolved in 0.2 mL of chloroform. Aranciamycin was subjected to TLC on silica gel aluminum TLC plates (mobile phase chloroform–methanol–ethanol–water, 120 : 25 : 6 : 4.5). After the run TLC plates were dried and overlaid with 0.7% soft agar containing *S. lutea* (10^9 CFU). The plates were incubated (12 h, 28°C) and visualized with visible light. For semiquantitative determination of aranciamycin production antibiotic extracts from *S. echinatus* strains (incubated in SG medium for 5 days) were stacked onto the lawn of freshly plated *S. lutea*. The productivity of the strains was calculated as the ratio of the diameter of inhibition zone to the diameter of the disc paper with incorporated antibiotic. The mean values of productivity were found from at least triplicates.

DNA manipulations. Molecular biology procedures were done according to standard protocols [7]. DIG DNA Labeling and Detection Kit (Roche) was used to generate DIG-labeled DNA probes for Southern analysis. Isolations of genomic DNA from *Streptomycetes* and plasmid DNA from *E. coli* were carried out using standard protocols [7]. Restriction enzymes and molecular biology reagents were used according to the recommendations of suppliers (MBI Fermentas).

RESULTS

Development of a gene transfer system for *S. echinatus* Lv 22. We tested the possibility of intergeneric *E. coli–S. echinatus* conjugation using common *Streptomyces* plasmids. Particularly, implicative plasmids pKC1139, pKC1218E, pCHZ101 and pSOK101 (pSG5, SCP2, pHZ1351 and pIJ101 replicons, respectively) and integrative plasmids pSET152 (φC31 *attP/int*) and pVWB (VWB *attP/int*) were used. Corn and oatmeal media (see Materials and Methods) were tested for plating of conjugal matings, and the latter turned out to give the best results. All aforementioned plasmids were successfully transferred into *S. echinatus*. The frequency of $pKC1139^+$, $pKC1218E^+$, $pCHZ101^+$, $pSOK101^+$, $pSET152^+$ and $pVWB^+$ transconjugants occurrence was 10^{-3}–10^{-4} (Table 2), with respect to the number of recipient spores. All transconjugants did not differ in sporulation or morphology of the transconjugants when comparing to the initial strain. Transconjugants of all *Streptomyces* strains containing pKC1139, pKC1218E, pSOK101, pCHZ101, pSET152 and VWB plasmids were passaged in the absence of selection in TSB media for 10 culture doublings, then plated on oatmeal media with and without apramycin. Ail transconjugants containing integrated plasmids formed colonies on oatmeal agar plus 50 μg apramycin/mL at efficiencies of

Table 2. Frequency of transconjugants formation[a]

Plasmid	Frequency[b]
pSET152	2.4×10^{-3}
pVWB	4.1×10^{-3}
pKC1139	1.6×10^{-4}
pKC1218E	2.7×10^{-3}
pSOK101	3.6×10^{-3}
pCHZ101	3.2×10^{-3}

[a] Counted on oatmeal medium after incubation for 5 days at 28°C. [b] Average from triplicates at average spore titer of 6×10^9.

90–100% relative to plating efficiencies on agar lacking apramycin. The stability of inheritance of replicative plasmids with respect to apramycin marker was lower than 25–35% for all strains, but all plasmids were stably maintained when grown in selective conditions. High stability of inheritance of integrative plasmids is due to their integration into chromosome using φC31 and VWB *attP/int* systems. This is potentially very attractive for the development of genetically stable recombinant strains.

We investigated the patterns of integration of pSET152 and pVWB into *S. echinatus* genome by Southern analysis. For this purpose *Bam*HI digests of genomic DNA isolated from independent clones of $pSET152^+$ and $pVWB^+$ transconjugants were probed with DIG-labeled *oriT* fragments of pSET152 and pVWB. Identical patterns of hybridization, observed for either of the transconjugants, indicate that each of the plasmids has only one integration site (Figs. 1 and 2). Our data show that all studied plasmids are suitable for gene transfer into *S. echinatus*.

Appearance of *S. echinatus* transconjugants after mating with *E. coli*. To investigate the time dependence of the transconjugants appearance the time inter-

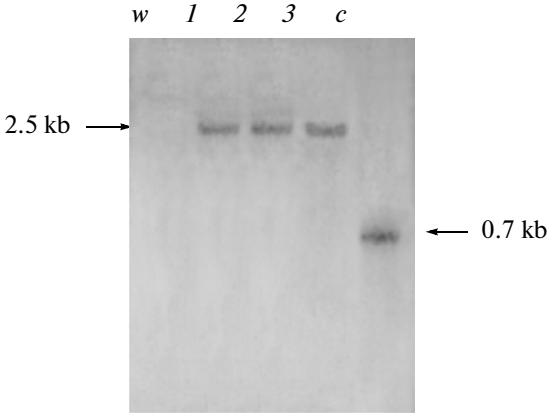

Fig. 1. Southern blot analysis of *Bam*HI digests of genomic DNA of the wild-type *S. echinatus* Lv 22 and its $pSET152^+$ transconjugants; (*1–3*)—independent transconjugants, W—wild-type; C—DIG-labeled probe of *oriT* fragment.

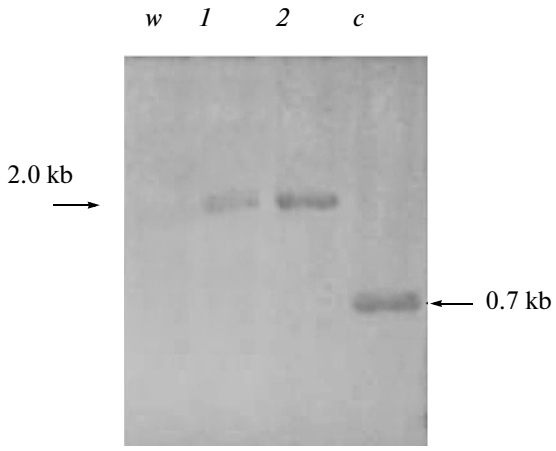

Fig. 2. Southern blot analysis of *Bam*HI digests of genomic DNA of the wild-type *S. echinatus* Lv 22 and its pVWB$^+$ transconjugants; (*1*, *2*)—independent transconjugants, W—wild-type; C—DIG-labeled probe of *oriT* fragment.

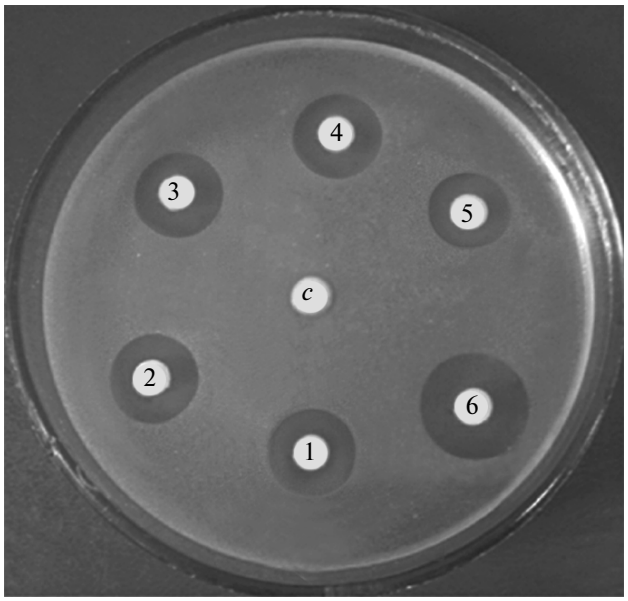

Fig. 3. Growth inhibition zones of the *S. lutea* test culture caused by the antibiotic extracts obtained from *S. echinatus* strains Lv 22 (1, 2)$^+$, *afsS*$^+$ (3), *absB*$^+$ (4), *absA2*$^+$ (5), *relA*$^+$ (6); C—negative control (methanol, used as a solvent); antibiotics were extracted from equal amounts of wet biomass; introduction of empty vectors had no effect on aranciamycin production (pKC1218, pKC1139 and pOJ260) (data not shown).

rupted mating between *E. coli* ET12567 (pUB307) and *S. echinatus* were performed. The conjugal process was interrupted by addition of apramycin and nalidixic acid. Exconjugants appeared after 8 h and the highest number of colonies appeared after 12 h of mating. As recipient spores had been treated for 10 min at 50°C, we can exclude any possibility of mycelium growth.

Introduction of pleiotropic regulators effects on aranciamycin biosynthesis of *S. echinatus*. An improved gene transfer system was used for delivery of pleiotropic regulatory genes in *S. echinatus* cells. For expression, genes *relA, absA2, afsS* and *absB* were used to improve aranciamycin production in *S. echinatus*. Genes *afsS, absA2* and *absB* originate from *S. ghanaensis* ATCC 14672 and cloned under their own promoters (Table 1). Gene *relA* was amplified from *S. coelicolor* genome [8]. In plasmid pIJ8647 this gene is located under the control of the *tipA* promoter.

Plasmid pKCabsA2 (Table 1), carrying *absA2* under control of its own promoter was introduced into *S. echinatus*. While the parental strain and transconjugants accumulated equal amounts of biomass over the course of fermentation, methanol extracts of the *S. echinatus* Lv 22 displayed higher antibiotic activity, as was evidenced from antibiotic disc diffusion assays. This indicates that heterologous expression of the *absA2* gene plays an inhibitory role in the control of aranciamycin biosynthesis. The extracts obtained from the strains were analyzed by TLC. Additional copies of the gene caused a decrease in aranciamycin's derivatives production. Expression of *absA2* had no effect on the morphological features of transconjugants. These results are consistent with the conclusions from *absA2* disruption experiments in *S. ghanaensis* ATCC 14672 and validate the negative role of *absA2* in the regulation of antibiotic biosynthesis.

Additional copies of *absB* and *afsS* had no effect on aranciamycin biosynthesis as well as on the morphological features (size of colonies, their pigmentation) of *S. echinatus*. Evidentially their expression requires additional regulators of higher levels or nutritional factors.

It has been shown for some streptomycetes that expression of additional copies of *relA* gene can lead to antibiotic overproduction [8, 9], while other streptomycetes overproduce antibiotics in the absence of *relA* [10]. In our case *relA* appears to stimulate aranciamycin biosynthesis (Fig. 3). Heterologous expression of *relA* gene has shown the best results concerning the improvement of aranciamycin production levels. This fact can be explained by the probable existence of a homologous gene in the *S. echinatus* genome related to *S. coelicolor relA* and its close involvement in regulation of antibiotic production. Exact mechanisms of these processes remain unclear, but it is evidently that pleiotropic regulatory genes can be used as tools for the improvement of aranciamycin production. Therefore, the data obtained will be of great practical interest for future attempts at engineering novel antitumor agents via combinatorial biosynthesis.

DISCUSSION

The remarkable ability of conjugation to mediate plasmid transfer between taxonomically and geneti-

cally unrelated bacterial hosts facilitates gene sharing within broad microorganisms. Conjugation commonly crosses species and genus boundaries [2] and can extend across biological domains. Because of the existence of general mechanisms such as exclusion, which constrain the conjugative transfer of plasmids, not all strains or species within a community are equally efficient as recipients. In this case the procedure of plasmid delivery should be optimized for every strain individually. As it was shown [2, 3] the IncPα system appears to be the most effective in intergeneric conjugation between *E. coli* and *Streptomyces* primary due to its Mpf system. In our work implicative plasmids pKC1139, pKC1218E, pSOK101, pCHZ101 and integrative plasmids pSET152 and pVWB, based on IncPα system, were successfully transferred from *E. coli* ET12567 (pUB307) into the *S. echinatus*. Our data correlates with conjugative transfer of abovementioned plasmids from *E. coli* ET 12567 (pUB307) to a wide range of streptomycete strains [2, 3, 11]. Moreover, integrative plasmids were stably inherited with a 100% frequency after five passages under nonselective conditions and did not effect aranciamycin production. Stability of inheritance of integrative plasmids is due to their integration into the chromosome using φC31 and VWB *attP/int* systems. This fact, as well as high frequency of conjugative transfer of implicative plasmids, is potentially very attractive for the development of genetically stable *S. echinatus* strains.

The expression of secondary metabolic gene clusters is controlled by many different families of regulatory proteins, some of which are found only in actinomycetes [12–14]. In this regard the application of regulatory genes for the activation of aranciamycin production has a remarkable impact on the improvement of antibiotic production. For delivery of regulatory genes we applicated a method of intergeneric conjugation from *E. coli* ET 12567 (pUB307), developed in this study.

The results described in this work establish a positive role of *relA* in regulation of aranciamycin production. The ribosome-associated ppGpp synthetase (RelA) is required for antibiotic production under condition of nitrogen limitation in *S. coelicolor* A3(2) and for cephamycin C production in *S. clavuligerus* [12]. Inactivation experiments indicated a direct role for ppGpp in activating the transcription of antibiotic biosynthetic genes. In agreement with the findings of [12] we have demonstrated that expression of *relA* had a remarkable impact in increasing of aranciamycin production, clearly demonstrating its role as a positive regulator element. This fact can be explained by the probable existence of a homologous gene in the *S. echinatus* genome related to *S. coelicolor relA* and its close involvement in regulation of aranciamycin production.

We demonstrated that heterologeous expression of the *absA2* gene plays an inhibitory role in the control of arancimycin biosynthesis. Our data correlates with overexpression of *absA2* in *S. ghanaensis* and *S. coelicolor* [15]. This regulator negatively affects moenomycin and actinorhodin biosynthesis, respectively. In contrast, *absA2* deletion had no effect on moenomycin production. The possible explanation of our results is that *absA2* is essential for regulation of aranciamycin production, at least under our laboratory conditions. Identification and further inactivation of *absA2* homolog in *S. echinatus* chromosome could be an essential strategy for generation of strains with overproduction of aranciamycin and to a better understanding of the complex regulatory networks involved in the production of secondary metabolites produced by *S. echinatus*.

Expression of *absB* and *afsS* [16, 18] under control of their promoters had no effect on aranciamycin biosynthesis as well as on the morphological features of *S. echinatus*. It is possible that our laboratory conditions are not appropriate for aranciamycin biosynthesis or *absB* and *afsS* genes expression naturally has little or no connection to its regulation.

Successive transfer of conjugative plasmids to *S. echinatus* makes it a very promising and powerful tool for genetic manipulations. It might be applied to study genetic regulation of aranciamycin biosynthesis. Our results imply some aspects of regulatory network for aranciamycin production in *S. echinatus*. More studies are needed for the elucidation of the regulatory aspects of biosynthesis of aranciamycin, which will eventually lead to new ways to construct recombinant strains that overproduce this antibiotic.

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