VOL. 57 NO. 6, JUNE 2004 THE JOURNAL OF ANTIBIOTICS pp. 383 — 389

Generation of *Streptomyces globisporus* SMY622 Strain with Increased Landomycin E Production and It's Initial Characterization

Oleksandr Gromyko[†], Yuriy Rebets[†], Bohdan Ostash[†], Andriy Luzhetskyy[†], Masahiro Fukuhara[††], Andreas Bechthold[†††], Tatsunosuke Nakamura[††] and Victor Fedorenko[†,*]

[†] Department of Genetics and Biotechnology of Ivan Franko National University of L'viv, Grushevskogo st.4, L'viv 79005, Ukraine
[††] Department of Microbiology, Niigata University of Pharmacy, Kami Shinei-cho 5-13-2, Niigata 950-2081, Japan
[†††] Albert-Ludwigs-University of Freiburg, Pharmazeutische Biologie, Stefan-Meier-Strasse 19, 79104 Freiburg, Germany

(Received for publication January 15, 2004)

Landomycin E (LaE) overproducing strain *Streptomyces globisporus* SMY6222 has been developed using UV induced mutagenesis and selection for streptomycin resistance. SMY622 has been shown by HPLC to produce 200-fold higher amounts of LaE when comparing with parental strain. The levels of transcription of regulatory gene *lndI* and oxygenase gene *lndE* are two times higher in the mutant than in the wild type. Gene *rpsL* for ribosomal protein S12 from SMY622 was shown to contain point mutation K43R. Possible reasons for increased LaE synthesis in SMY622 are discussed.

Improvement of production of clinically or industrially valuable antibiotics in bacteria is of great economic importance. The low level production is found in strains producing both natural and so called "hybrid" antibiotics (resulted from heterologous gene expression or gene knockout experiments). Great variety of gene and cellular engineering methods as well as random mutagenesis is used to increase the production of desired compounds.

Landomycins (La) are group of polyketide angucycline antibiotics having interesting spectrum of antitumor activities *in vitro*. However *in vivo* studies showed their high cytotoxicity with regard to normal cells[1)]. Development of novel La derivatives with increased antitumor activities or decreased side effects has immense theoretical and practical interest.

Two La producers are known–*Streptomyces cyanogenus* S136 (principal product–LaA) and *S. globisporus* 1912[1)] (produces LaE-trisaccharide intermediate to LaA, never found in *S. cyanogenus* culture broths; Fig. 1A). Gene clusters for LaE (*lnd*) and LaA (*lan*) biosynthesis have been cloned[2,3)]. Genes controlling first steps of LaE polyketide framework synthesis were studied through gene disruption techniques. Wild type *S. globisporus* 1912 produces LaE at very low level, and disruption of cyclase genes involved in LaE synthesis resulted in trace production of respective intermediates[4)]. Further, *S. globisporus* strain was constructed impaired in last deoxysugar attachment and C11-hydroxylation[5)]. Monoglycosylated La identified are supposed to be attractive targets for "structure-activity relationships" studies. Although they are produced very poorly, that hinders their scrutiny. All these facts have prompted us to develop LaE superproducer that can be used as more convenient host for future experiments on combinatorial biosynthesis of novel La.

We used UV-induced mutagenesis of *S. globisporus* spores to look for streptomycin resistant (Sm^r) mutants. Obtained Sm^r mutants were screened for increased LaE production. The selection of the overproducing mutants resistant to aminoglycosides is well established for actinorhodin producers *S. coelicolor* A3(2) and *S. lividans*[6)], salinomycin and erythromycin producers *S. albus*[7)] and *Saccharopolyspora erythraea*[8)], respectively, and the reasons for increased antibiotic production are thoroughly studied. Here we report the generation of the

* Corresponding author: v_fedorenko@franko.lviv.ua

Table 1. Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	Source or reference
S. globisporus 1912	wild type LaE producer	Prof. B. Matselukh, Institute of Microbiology and Virology, NAS of Ukraine
S. globisporus SMY622	LaE overproducer	This work
E. coli DH5α	*supE44 ΔlacU169(φ80lacZΔM15) hsdR17 recA1endA1gyrA96 thi-1 relA1*	MBI Fermentas
E. coli ET12567 (pUB307)	*dam-13*::Tn9 (Cm^r) *dcm-6 hsdM*; contains RK2-based conjugative plasmid pUB307 (Km^r)	C.P.Smith, UMIST Manchester, UK
pUC18	*E. coli* cloning vector; Ap^r	MBI Fermentas
pSET152	*E. coli-Streptomyces* conjugative vector; Am^r	[10]
pSI2-9	pSET152 that contains *lndI* gene	[9]
pIJ8660	φC31-based, promoter probe vector using enhanced green fluorescent protein (EGFP) gene as a reporter; Am^r	[10]
pIJ8660H	pIJ8660 with 0.47 kb *Eco*RI-*Bam*HI fragment, containing *lndI* promoter, inserted upstream of EGFP gene.	[17]
pIJ8660E	pIJ8660 with 0.57 kb *Eco*RV-*Eco*RI fragment, containing *lndE* promoter, inserted upstream of EGFP gene.	[17]
pT7rpsL1	pT7blue vector with *S.globisporus* 1912 *rpsL* gene PCR fragment	This work
pT7rpsL2	pT7blue vector with *S.globisporus* SMY622 *rpsL* gene PCR fragment	This work

strain *S. globisporus* SMY622 producing 200-fold higher quantities of LaE in comparison with parental strain 1912. The results of initial experiments aiming at understanding of the molecular mechanisms of LaE overproduction are presented.

Materials and Methods

Bacterial Strains and Plasmids Used

These are listed in the Table 1.

Culture Conditions for *S. globisporus* and *E. coli* Strains

S. globisporus strains were grown at 30°C. Oatmeal medium (OM; oatmeal 3%, agar 1.8%, pH 9.0 before autoclaving) was used to obtain *S. globisporus* spores and to plate *E. coli* - *S. globisporus* matings. For fermentations, *S. globisporus* strains were grown for 3 days in SG medium[9]. For total and plasmid DNA preparation, EGFP production analysis *S. globisporus* cultures grown in TSB medium[10] were used. *E. coli* strains were grown on LA or LB[11]. Where it was necessary, 100 μg/ml of ampicillin, 50 μg/ml of kanamycin, 25 μg/ml of chloramphenicol and apramycin, various quantitites of streptomycin (Sm) were added to the media.

Mutation

Spores from 5 days old *S. globisporus* 1912 culture were suspended in saline and treated with UV rays in dose leading to approx. 1% survival of the spore population. Irradiated spores were spread on OM plates supplemented with 2.5 μg/ml of Sm (Growth of parental strain was inhibited completely at 0.5 μg/ml of Sm). Induced Sm resistant (Sm^r) mutants that grew on 2.5 μg/ml of Sm were replica plated onto OM plates supplemented with 100, 200, 300, 500, 1000 μg/ml of Sm in order to determine their actual

Sm resistance level.

Analysis of LaE Production and Resistance to Antibiotics

Pure sample of LaE was kindly provided by Prof. J. ROHR (College of Pharmacy, Kentucky, USA). TLC and HPLC of La has been done essentially as described[9]. The total protein in the specimens taken from fermentation media was determined by Bradford[11]. The amount of LaE produced by the strains were calculated using standard calibrating HPLC and expressed with regard to the same amount of total protein. The resistance of *S. globisporus* strains to antibiotics was determined *via* titration of spores on OM plates with various concentrations of antibiotics or with the help of discs with antibiotics (Fereyn, Russia).

DNA Manipulations

Genomic DNA from *S. globisporus* strains and plasmid DNA from *E. coli* was isolated using standard protocols[10]. T4-DNA ligase, *Pfu*I and *Taq* thermopolymerases and restriction enzymes were used as recommended by the suppliers. Other DNA manipulations were performed following standard procedures as specified by manufacturers (MBI Fermentas, NEB, Invitrogen, Boehringer Mannheim, Pharmacia). Nucleotide sequences were determined on a Beckman Coulter CEQ2000XL sequencer and analyzed using DNASIS software (version 2.1, Hitachi Software Engineering) and BLAST programs[12]. *E. coli* transformation and intergeneric matings *E. coli*-*Streptomyces* were performed as described[11,13]. In case of SMY622 strain mycelia instead of spores were used in the matings.

Microscopy of *S. globisporus* Strains Expressing EGFP Gene

Sample preparation and EGFP production analysis was done as described[14]. Here we used Fluoroview confocal system (Olympus) with an Olympus OL BX50 microscope and 488 nm argon laser.

Cloning of *rpsL* Gene Internal Fragment from *S. globisporus* 1912 and SMY622

Primers to conservative regions of *S. lividans rpsL* gene[15] were designed and used in PCR to amplify 0.32 kb DNA fragments from both 1912 and SMY622 genomic DNA as templates. SFXhoI (5′-GAAGGGCCGGCAGGACAAGCTCGAGAAG; site for *Xho*I is underlined) is complementary to the region of *rpsL* starting from 10th amino acid, and SRXbaI (5′-CGGGCCTGCTTGCGGTCTAGACAGCCTG; site for *Xba*I is underlined)-to the end of *rpsL* (from 115th aa). BioRad thermal cycler iCycler was used at the following conditions: 3 minutes of incubation at 96°C; 30 cycles of 96°C for 1 minute, 65°C for 1 minute, 72°C for 1 minute; and a final step at 72°C for 10 minutes. After finishing 1u of *Taq* polymerase was added and mixture was incubated at 72°C for 10 minutes to generate terminal adenine nucleotides for cloning into T-vector. PCR products were purified using GFX Gel Band Purification kit (Amersham Biosciences) and cloned into pT7Blue T-vector. Recombinant plasmids were mapped with *Xho*I and *Xba*I restriction endonucleases. Five independent clones harboring *rpsL* from each strain were sequenced to minimize the mistakes of PCR and sequencing. Within these two groups of plasmids the sequences of *rpsL* were identical and two plasmids were selected referred to as pT7rpsL1 (*rpsL* from wild type 1912) and pT7rpsL2 (from SMY622).

Results and Discussion

Wild type strain *S. globisporus* 1912 produces up to 2.8±0.4 mg/liter of LaE under our fermentation conditions. Attempts to develop more optimal fermentation process leading to significant increase in LaE synthesis were unsuccessful.

Spontaneous Sm^r mutants appeared at frequency $(4.1\pm0.6)\times10^{-8}$, when Sm concentration was 2.5 μg/ml, and they did not produce significantly higher amounts of LaE. UV-induced mutagenesis of *S. globisporus* 1912 has resulted in appearance of Sm^r mutants at frequency $(6.0\pm1.0)\times10^{-4}$. In total, 2710 Sm^r clones were obtained. Preliminary selection based on degree of LaE accumulation in OM agar (visible as dark blue pigmentation) showed that approx. 6% (162 clones) possessed higher level of LaE production than parental strain. Analytical TLC showed that among 162 clones, 114 produced LaE roughly 5～10-fold, 45 produced 10～50-fold, 3 produced more than 50-fold. One clone from the latter group marked as SMY622 was taken for detailed analysis.

S. globisporus strain showed low frequency of spontaneous Sm^r mutants appearance, in comparison with what is described about Sm^r mutants of *S. coelicolor*, *S. lividans*, *S. albus*[7,15], *Saccharopolyspora erythraea*[8], *S. kanamyceticus*[16]. Thus we looked for LaE overproducers among UV induced mutants. This approach proved to have higher efficiency over screening of spontaneous Sm^r mutants.

LaE Production and Morphology of SMY622

SMY622 has been shown to produce roughly 200-fold

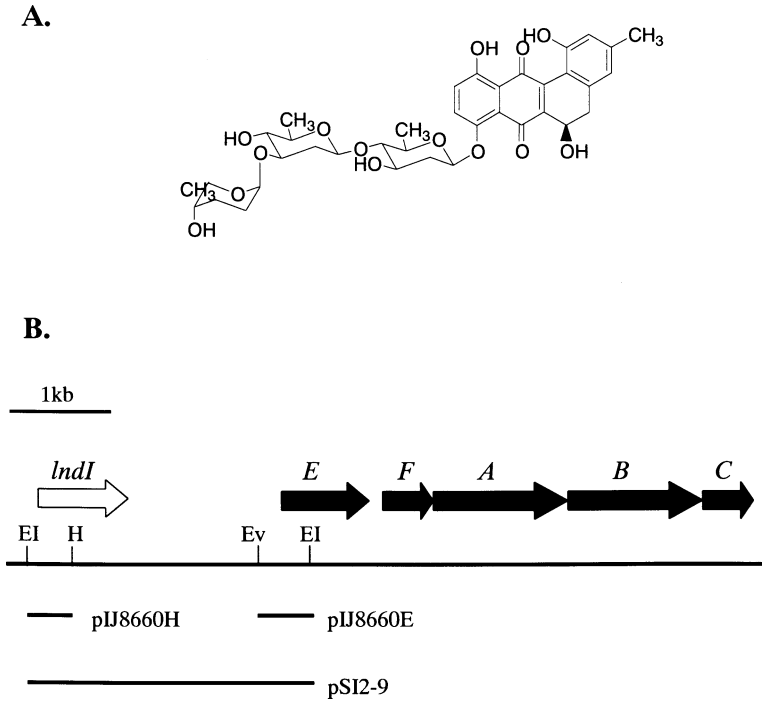
quantities of LaE (560 mg/liter) when comparing with parental strain. While wild type produce spores abundantly, SMY622 fails to sporulate on various replete agar media and Hopwood minimal medium. Unlike initial strain, SMY622 mycelium can be easily scraped out from agar plates, and septa of it's substrate mycelium are shorter than in wild type (as we could judge from confocal microscopy of liquid cultures). Mutations of Sm resistance are shown to have pleiotropic effects on morphology of the mutants of *S. albus*, *Saccharopolyspora erythraea*[7,8], however, the presence of other mutations scattered in SMY622 genome can not be ruled out. We tested the level of SMY622 resistance to Sm and it appeared to be resistant to as much as 1000 μg of Sm per ml of medium. SMY622 has essentially the same as 1912 strain resistance pattern to aminoglycosides (kanamycin, gentamycin), polyketides (erythromycin, oleandomycin, tetracycline), β-lactams (ampicillin, benzylpenicillin, carbenicillin) and rifampicin.

The dramatic increase of LaE production in SMY622 can be partially accounted for very low basal level of LaE synthesis in 1912 strain. Certain mutations within gene *rpsL* for ribosomal protein S12 lead to Sm^r phenotype and overproduction of secondary metabolites in *S. lividans* and *S. coelicolor*. These mutations are believed to have global positive effect on ribosome stability, translational accuracy and cellular metabolism[6]. As evident from HPLC, the production of other unrelated with LaE pigments and polyketides is increased in SMY622 (data not shown). This hints at possible analogies in molecular mechanisms mediating LaE and actinorhodin overproduction in respective mutants.

Transcription of LaE Biosynthetic Genes *lndI* and *lndE* in SMY622

The increased LaE production should be rooted in enhanced expression of LaE biosynthetic and, possibly, regulatory genes. To verify this assumption, we tested the expression of EGFP gene from promoter region of LaE pathway specific regulatory gene *lndI*. In the same manner we have checked the expression of oxygenase gene *lndE*, which is localized upstream of the rest structural *lnd* genes (Fig. 1B). Recombinant plasmids pIJ8660H and pIJ8660E[17]

Fig. 1.



A. Structure of landomycin E (LaE).

B. LaE biosynthetic gene (*lnd*) cluster fragment.

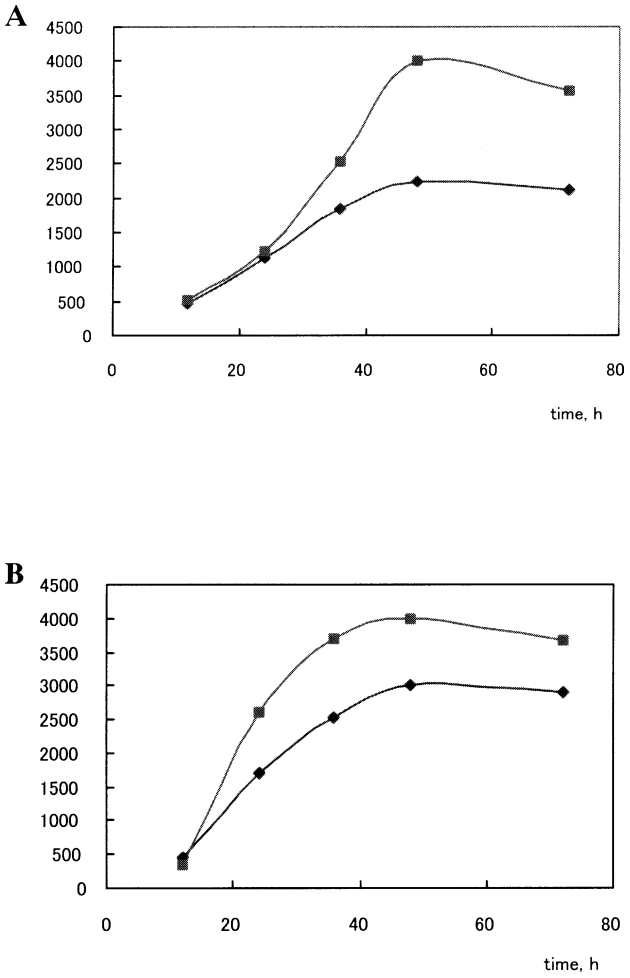
Gene functions: *lndI*-transcriptional activator of structural *lnd* genes, *lndE*-oxygenase, *lndF*-third/fourth ring cyclase, *lndABC*-minimal PKS. Below the cluster sublones are shown used in promoter probing (pIJ8660H, pIJ8660E) and *lndI* overexpression (pSI2-9) experiments.

harboring *lndI* and *lndE* promoters regions fused to EGFP gene, respectively, were transferred into SMY622 and 1912 strains. The measurements of green fluorescence intensities were done each 12 hours of culture growth up to 72 hours. It was found that during first 48 hours of growth the average level of P_{lndI}-EGFP and P_{lndE}-EGFP expression is 2 and 1.5 times higher, respectively, in SMY622 than in 1912 (Fig. 2). We wondered whether it is possible to increase further *lndI* transcription in mutant and wild type strains and thus to enhance LaE production. Additional *lndI* copies on plasmid pSI2-9 have been introduced into SMY622 and 1912. pSI2-9 is φC31-based integrative plasmid existing in at least three copies per *S. globisporus* genome[9,13]. LaE production in SMY622 and 1912 increased in 1.3 and 11 times, respectively, after introduction of pSI2-9. Thus, for SMY622, the top level of LaE production so reached was approx. 0.72 g per 1 liter of fermentation medium. In this strain increased *lndI* copy number caused only little enhancement of LaE biosynthesis. Probably, there is some maximal possible level of LaE production for given strain, which can not be improved by simple changes in *lndI* gene dosage. This hints at existence of yet unknown feedback regulatory mechanism linking LaE synthesis and *lnd* genes expression and preventing the cells from overproduction of potentially toxic compound. This mechanism could function through binding of antibiotic with *lndI* promoter or LndI, by analogy to the situation described in doxorubicin biosynthesis[18]. Summarizing the experimental data presented here, we suggest that increased expression of regulatory gene *lndI* in SMY622 is one of the reasons for it's LaE overproduction phenotype.

Analysis of *rpsL* Gene from *S. globisporus* SMY622

There is strong evidence that Sm resistance accompanied by enhanced antibiotic production frequently results from

Fig. 2. Average EGFP expression profile from *lndI* (A) and *lndE* (B) promoters in SMY622 (■) and 1912 (◆) measured by confocal microscopy scanning of respective strains cultures in different time-points of growth.



mutations in the *rpsL* gene for ribosomal protein S12[15]. We therefore sequenced and compared the *rpsL* genes from wild type and SMY622 mutant. Sequenced fragments showed end-to-end identity with *S. lividans* and *S. coelicolor rpsL* genes. SMY622 has been shown to contain single mutation within *rpsL* gene fragment where the altered nucleotide (from A to G) was found at the position 128. This results in an alteration of Lys-43 to Arg.

K43R mutation was isolated and characterized in *S. coelicolor* and *S. lividans*. Although it confers these strains to high level of resistance to Sm (200 μg/ml), it does not appear to activate actinorhodin production in *S. lividans* TK21 or *S. coelicolor*[15]. Nevertheless, K43R substitution shows restrictive phenotype (increased translational accuracy)[6]. Translational machinery has highly conservative nature not only within genus *Streptomyces* but also between different taxons of higher order. Thus it seems to be unlikely, that K43R mutation would result in phenotype different from that ones described for other studied streptomycetes. We believe that identified mutation contributes at least partially to Sm^r phenotype of SMY622, but the reason for LaE overproduction lies outside of *rpsL* gene.

UV mutagenesis used in our selection scheme, can induce several mutations in SMY622 affecting it's Sm resistance and cellular metabolism at the levels of transcription and translation. Another evidence for presence of unknown mutations in SMY622 is it's exceptionally high level of Sm resistance. *S. globisporus* mutants producing 5～50 fold quantities of LaE were resistant up to 300 μg/ml of Sm, whereas SMY622-to 1 mg/ml. This line of reasoning allows us to suggest tentatively, that SMY622 carries a special set of mutations positively affecting *lndI* transcription in particular and other yet not examined regulatory mechanisms. In any case, studied LaE overproducer does not possess mutations within *rpsL* gene, commonly found in actinorhodin overproducing *S. coelicolor* and *S. lividans* strains[6,7,19]. This makes interesting further in-depth studying of SMY622 strain as a potential source of discovery of novel useful mutations for improvement of antibiotic production.

In conclusion, the utility of Sm^r mutants selection has been demonstrated for LaE overproducers development. Further LaE synthesis improvement can be achieved on combining random Sm^r mutants selection and *lndI* regulatory gene manipulations. Initial investigations addressed the question about mutations nature leading to LaE increased synthesis, which most likely are distinct from that described for *S. lividans* and *S. coelicolor* Sm^r mutants.

Acknowledgements

This work was supported by Western Ukrainian BioMedical Research Center grant (2003～2004, to O.G.) and the grant Bg-117b from the Ministry of Education and Science of Ukraine (to V.F.). We are grateful to Prof. M. J. BIBB, Dr. J. SUN and Prof. K. F. CHATER (JIC, Norwich, UK) for gift of pIJ8660 plasmid, Dr. M. MIYAMOTO (Niigata University of Pharmacy, Niigata, Japan) for his help in DNA sequencing and Dr. M. NASHIMOTO and Dr. H. URAKAMI (Niigata University of Pharmacy, Niigata, Japan) for their advices.

Reference

1) KROHN, K. & J. ROHR: Angucyclines: total syntheses, new structures and biosynthetic studies of an emerging new class of antibiotics. Top. In Curr. Chem. 188: 127～195, 1997
2) FEDORENKO, V.; L. BASILIA, K. PANKEVYCH, L. DUBITSKA, B. OSTASH, A. LUZHETSKYY, O. GROMYKO & H. KRUGEL: Genetic control of antitumor antibiotics-polyketides by actinomycetes. Bull. Inst. Agr. Microbiol. (Ukr.) 8: 27～31, 2000
3) WESTRICH, L.; S. DOMANN, B. FAUST, D. BEDFORD, D. A. HOPWOOD & A. BECHTHOLD: Cloning and characterization of a gene cluster from *Streptomyces cyanogenus* S136 probably involved in landomycin A biosynthesis. FEMS Microbiol. Lett. 170: 381～387, 1999
4) OSTASH, B.; Y. REBETS, V. YUSKEVICH, A. LUZHETSKYY, V. TKACHENKO & V. FEDORENKO: Targeted disruption of *Streptomyces globisporus lndF* and *lndL* cyclase genes involved in landomycin E biosynthesis. Folia Microbiol (Praha) 48: 484～488, 2003
5) OSTASH, B.; U. RIX, L. L. REMSING, T. LIU, F. LOMBO, A. LUZHETSKYY, O. GROMYKO, C. WANG, A. F. BRANA, C. MENDEZ, J. A. SALAS, V. FEDORENKO & J. ROHR: Generation of new landomycins by combinatorial biosynthetic manipulation of the IndGT4 gene of the landomycin E cluster in *S. globisporus*. Chem. & Biol. 11: 547～555, 2004
6) OKAMOTO-HOSOYA, Y.; T. HOSAKA & K. OCHI: An aberrant protein synthesis is linked with antibiotic overproduction in *rpsL* mutants of *Streptomyces coelicolor* A3(2). Microbiology 149: 3299～3309, 2003
7) NORIMASA, T.; T. HOSAKA, J. XU, H. HU, N. OTAKE & K. OCHI: Innovative approach for improvement of an antibiotic-overproducing industrial strain of *Streptomyces albus*. Appl. Environ. Microbiol. 69: 6412～6417, 2003
8) ZAVOROTNAIA, S.; V. A. FEDORENKO & V. N. DANILENKO: Genetic instability of the feature of streptomycin resistance in *Streptomyces erythraeus*. Antibiot. Khimioter. (Russ) 35: 18～21, 1990
9) REBETS, Y.; B. OSTASH, A. LUZHETSKYY, D. HOFFMEISTER, A. BRANA, C. MENDEZ, J.A. SALAS, A. BECHTHOLD & V. FEDORENKO: Production of landomycins in strains *Streptomyces globisporus* 1912 and *S. cyanogenus* S136 is regulated by genes encoding putative transcriptional activators. FEMS Microbiol. Lett. 222: 149～153, 2003
10) KIESER, T.; M. J. BIBB, M. J. BUTTNER, K. F. CHATER & D. A. HOPWOOD: Practical *Streptomyces* genetics. John

Innes Foundation, Norwich, United Kingdom, 2000
11) SAMBROOK, J. & D. W. RUSSELL: Molecular cloning, a laboratory manual. 3rd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y, 2001
12) ALTSCHUL, S. F.; T. L. MADDEN, A. A. SCHAFFER, J. ZHANG, Z. ZHANG, W. MILLER & D. J. LIPMAN: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389～3402, 1997
13) LUZHETSKII, A. N.; B. O. OSTASH & V. O. FEDORENKO: Intergeneric conjugation *Escherichia coli-Streptomyces globisporus* 1912 using integrative plasmid pSET152 and its derivatives. Russian Journal of Genetics. 37: 1123～1129, 2001
14) KYUNG, Y.-S.; W.-S. HU & D. H. SHERMAN: Analysis of temporal and spatial expression of the CcaR regulatory element in the cephamycin C biosynthetic pathway using green fluorescent protein. Mol. Microbiol. 40: 530～541, 2001
15) SHIMA, J.; A. HESKETH, S. OKAMOTO, S. KAWAMOTO & K. OCHI: Induction of actinorhodin production by *rpsL* (encoding ribosomal protein S12) mutations that confer streptomycin resistance in *Streptomyces lividans* and *Streptomyces coelicolor* A3(2). J. Bacteriol. 178: 7276～7284, 1977
16) DEMIDCHUK, I. A.; L. M. GOLETS & V. A. FEDORENKO: Characteristics of *Streptomyces kanamyceticus* mutants resistant to aminoglycoside antibiotics. Antibiot. Khimioter. (Russ) 41: 15～20, 1996
17) REBETS, Y.; B. OSTASH, A. LUZHETSKYY, S. KUSHNIR, M. FUKUHARA, A. BECHTHOLD, T. NAKAMURA & V. FEDORENKO: DNA binding activity of LndI protein and temporal expression of it's gene that upregulates landomycin E production in *Streptomyces globisporus* 1912. J. Bacteriol. in press
18) HUTCHINSON, C. R.: Biosynthetic studies of daunorubicin and tetracenomycin C. Chem. Rev. 97: 2525～2535, 1997
19) HU, H. & K. OCHI: Novel approach for improving the productivity of antibiotic producing strains by inducing combined resistant mutations. Appl. Environ. Microbiol. 67: 1885～1892, 2001