VOL. 56 NO. 11, NOV. 2003 THE JOURNAL OF ANTIBIOTICS pp. 950 — 956

pTOYAMAcos, pTYM18, and pTYM19, Actinomycete-*Escherichia coli* Integrating Vectors for Heterologous Gene Expression

HIROYASU ONAKA*, SHIN-ICHI TANIGUCHI, HARUO IKEDA†, YASUHIRO IGARASHI and TAMOTSU FURUMAI

Biotechnology Research Center, Toyama Prefectural University,
Kosugi, Toyama 939-0398, Japan
† Kitasato Institute for Life Sciences & Graduate School of Infection Control, Kitasato University,
1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan

(Received for publication June 12, 2003)

A novel shuttle integration cosmid vector (pTOYAMAcos), based on pKU402, and shuttle integration vectors (pTYM18 and pTYM19) were constructed for the cloning of actinomycete DNA and its heterologous expression. These vectors contain *oriT* of an IncP transmissible plasmid in order to transfer genes by conjugation from *Escherichia coli* to actinomycetes, and they also contain *int* derived from actinophage ϕC31 in order to integrate site-specifically into the chromosomal DNA. pTOYAMAcos contains the λ*cos* site to promote packaging of vectors containing 35~45-kb DNA fragments into λ particles. pTYM18 and pTYM19 contain kanamaycin and thiostrepton resistance genes, respectively, and have multiple cloning sites including *Eco*RI and *Hin*dIII sites, which are available for blue/white screening in *E. coli*. To demonstrate the utility of these vectors, we expressed the entire gene cluster for rebeccamycin biosynthesis from *Lechevalieria aerocolonigenes* using pTOYAMAcos and detected rebeccamycin production in transformed *S. lividans*. In addition, we demonstrated the utility of pTYM19 in a gene-disruption complementation test. *L. aerocolonigenes ΔrebC* strain, which is defective in rebeccamycin production because of a *rebC* deletion, was restored to rebeccamycin production by complemention by *rebC* cloned in pTYM19.

The analysis of biosynthesis genes for secondary metabolites of actinomycetes is sometimes difficult due to the lack of suitable host-vector systems. The establishment of host-vector systems has been limited by the differences in the DNA restriction systems. The studies of secondary metabolite biosynthesis genes have been conducted by using heterologous biosynthesis gene expression in *Streptomyces lividans*, which has an established host-vector system.

Although a number of actinomycete-*E. coli* shuttle vectors are available today. The number of integrating plasmids suitable for various uses are limited. In "*Practical Streptomyces Genetics*" edited by KIESER *et al.*, only nine integrating plasmids are described and all of them have fewer unique multiple cloning sites than pUC19, because the ϕC31 *int* gene, which is essential gene for site-specific recombination with chromosome *attC* site, has two *Eco*RI and one *Hin*dIII sites[1]. An integrating vector which contains multiple cloning sites like pUC19 would be useful for manipulating and studying actinomycete genes.

When analyzing biosynthesis genes, it is convenient to deal with an entire cluster of biosynthesis genes. Some shuttle cosmid vectors are available for this purpose. The pKC505 vector is one of a family of cosmids which contains the cos site for λ packaging of clones with large fragment inserts and the SCP2* ori for replication in *Streptomyces*[2]. Some examples of heterologous expression of secondary metabolite biosynthesis genes with pKC505 have been reported[3,4]. It seems that DNA cloning by chromosomal integration is more stable than plasmids. The pOJ436 vector is a chromosomal integration vector[5], and its application has been reported[6].

An important point in cosmid library construction is how to minimize chimeric clones in which two or more DNA inserts are ligated into one vector. Generally, this problem can be avoided by preparing the fragments to be inserted

* Corresponding author: onaka@pu-toyama.ac.jp

more than 40-kb in size. Size fractionation of DNA fragments is conventionally carried out for the preparation of such fragments, but it consists of two or more steps and is complicated. The pKU402 cosmid vector makes the construction of genomic libraries easier, because it contains inverted repeat sequences flanking the multiple cloning sites. If vector-vector ligation occurs, this sequence forms a palindrome structure resulting in instability of the replication of vector. Therefore, in constructing libraries with pKU402, the partial digested chromosomal DNA can be dephosphorylated with alkaline phosphatase to reduce the generation of chimeric clones[7]. The stability and utility of pKU402 for construction of genomic libraries has been comfirmed by genome analysis of *Streptomyces avermitilis*[8,9].

In this paper, we report on the development of a heterologous expression system by constructing a new cosmid vector, pTOYAMAcos, based on pKU402. Large DNA fragments inserted into pTOYAMAcos were transferred to the actinomycete host by transconjugation from *E. coli*, and the recombinant vector subsequently integrated into the actinomycete chromosome. Moreover we discuss the construction of integrating plasmids, pTYM18 and pTYM19, which contain multiple cloning sites including both *Eco*RI and *Hin*dIII sites in a *lacZ*α gene fragment for ease of selection of inserts on X-Gal containing plates. These vectors are very useful for gene expression and complementation analysis.

Materials and Methods

Bacterial Strains, Plasmids and Growth Condition

Streptomyces lividans TK23 served as a heterologous expression host. *Escherichia coli* DH-5α served as a host for plasmid subcloning in pUC19 and its derivatives. *E. coli* XL1-Blue MR was used for the cosmid libraries. *E. coli* S17-1 was used for transconjugation[10]. Growth conditions and manipulations of *E. coli* were as described by SAMBROOK and RUSSELL[11]. The production medium for *S. lividans* and *L. aerocolonigenes* was Medium G134[12]. The seed medium for *S. lividans* or *L. aerocolonigenes* was V-22 medium[13]. Bennett's glucose agar, nutrient agar and mannitol soya flour agar[1] were used for transconjugation.

General Recombinant DNA Techniques

Restriction endonucleases, T4 DNA ligase, and *Taq* polymerase were purchased from New England Biolabs. PCR was carried out with PTC-200 DNA Engine (MJ research, MA, USA). DNA manipulations in *E. coli* were as described by SAMBROOK and RUSSELL[11], and those in *Streptomyces* were as described by KIESER *et al.*[1].

Construction of pTIO

pTIO is a cassette vector which contained a thiostrepton-resistance gene (*tsr*), a ϕC31 integration gene (*int*) and the RK2 origin of transfer (*oriT*). The ϕC31 *int* gene was amplified by PCR using primers and 3.9-kb *Kpn*I fragment of ϕC31Δc1, followed by cloning into pKF19 (Takara Shuzo Co. Ltd.) to give pKFint. The primers, which were designed on the basis of the upstream region of the promoter and *C*-terminal DNA sequence of ϕC31 *int* gene (GenBank acsession No. AJ006589), have the following sequences; intN: 5′-CGCGGATCCCCCGTGCCGGAGCAATCGCCC-3′ and intC: 5′-GGCGGATCCTTCCCGCTGCCCCAGGAAGCC-3′, in which the *Bam*HI sites are attached at the 5′ end for cloning into the pKF19 *Bam*HI site (underline indicated). PCR was carried out at 94°C for 1 minute, 43°C for 1 minute, 72°C for 1 minute for a total of 30 cycles. To inactivate one *Eco*RI and two *Hin*dIII sites in the *int* gene, site directed mutagenensis was carried out with Mutan-Super Express Km kit (Takara Shuzo) with pKFint. The primers were intEcoRI-1: 5′-GCGCGAGAACTCGAGCGCAG-3′, intHindIII-1: 5′-CAACAAGCTCGCGCACTCGA-3′, intHindIII-2: 5′-CCCGAAGCTCCCCCTTGACC-3′, in which points mutations are underlined. After mutagenesis, the DNA sequence was confirmed to have the desired sequences in the resulting plasmid designated pKFint-kai, and the mutated *int* gene is designated *int**. A *Sma*I-*Pst*I fragment containing the *oriT* gene was prepared from pPM803[10], made blunt-ended with Klenow fragment and cloned into the *Hinc*II site of pUC19 to generate pUCori. The *tsr* gene fragment was prepared from pIJ702 by digesting with *Bcl*I and subcloned into the *Bam*HI site of pUCoriT to construct pUCoritsr. pKFint-kai was digested with *Bam*HI, and the resulting larger fragment was made blunt-ended with Klenow fragment and cloned into the *Sma*I site of pUCori-tsr to yield pUC-TIO. The construction scheme is shown in Fig. 2. pUC-TIO was digested with *Xba*I and filled-in with NTPs and Klenow fragment and re-ligated to inactivate the *Xba*I site to generate pTIO.

Construction of pTYMrebAD

A 6.3-kb *Bgl*II-*Bam*HI fragment containing *rebO*, *rebD* and *N*-terminal region of *rebC* was excised from pREB1[12] and cloned into the *Bam*HI site of pREB5[12] which contains *C*-terminal region of *rebC* and *rebP*. Then, a 9.7-kb fragment containing *rebO*, *rebD*, *rebC*, and *rebP* was excised as an *Eco*RI-*Hin*dIII fragment and ligated to pTYM19 digested with *Eco*RI-*Hin*dIII, resulting in

pTYMrebAD.

HPLC and LC/MS Analysis of the Products

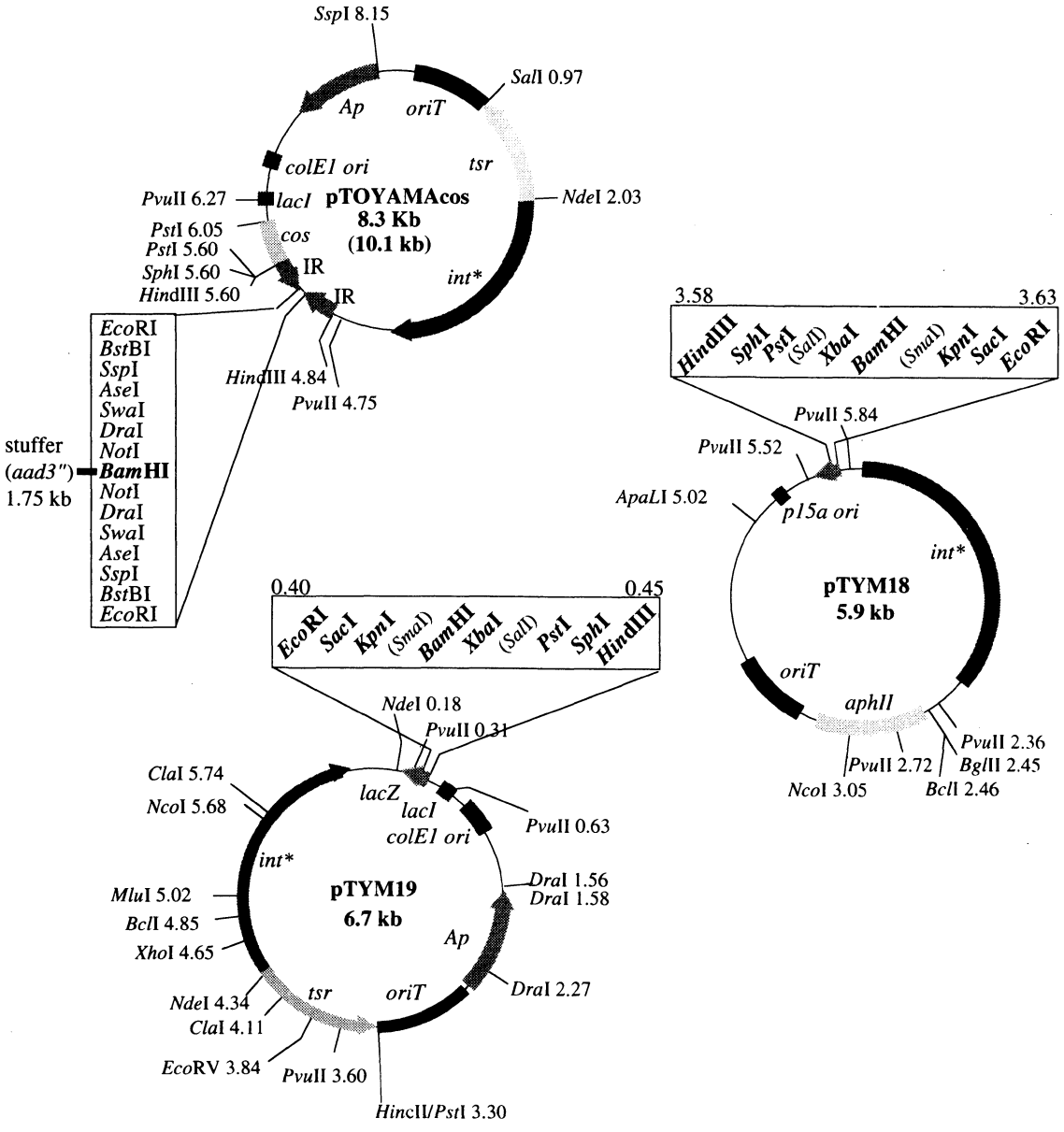
HPLC analysis was performed on a HP1090 system with a diode array detector (Hewlett Packard) using a C18 Rainin microsorb column (3 μm, 100×4.6 mm, i.d.; Rainin Instrument Co. MA, USA). The HPLC conditions and sample preparation were as described by ONAKA *et al.*[13]. LC-MS spectra were obtained on an API165 mass spectrometer (Applied Biosystems). UV-visible spectra were obtains using a HP1090 system.

Results and Discussion

Construction of pTOYAMAcos, pTYM18, and pTYM19

pTOYAMAcos is based on the pKU402 cosmid vector and has an actinomycete replication module which consists of an integration gene from ϕC31 actinophage (*int*), a thiostrepton resistance marker (*tsr*) and an RK2 (IncP) origin of transfer (*oriT*) (Fig. 1). The pKU402 has rare-cutter restriction endonuclease cloning sites suitable for cloning DNA from GC-rich organisms[7]. 1.75-kb *Bam*HI

Fig. 1. Restriction map of plasmids pTOYAMAcos, pTYM18, and pTYM19.



The unique restriction endonuclease sites available for cloning are indicated by bold type. *Ap*: ampicillin resistance gene. *aphII*: kanamycin resistance gene. IR: inverted repeat sequence.

fragment containing streptomycin/spectinomycin-resistance gene (*aad3″*) was used as a stuffed fragment at the unique *Bam*HI cloning site. A cassette plasmid pTIO which contains the actinomycete replication module was digested with *Pst*I and *Kpn*I, and the resulting larger fragment was purified and both ends were filled with Klenow fragment, followed by cloning into the *Nde*I site, which was filled with Klenow fragment, of pKU402 to yield pTOYAMAcos.

pTYM18 is a versatile shuttle integrating vector containing useful multiple cloning sites for multi-purpose gene cloning (Fig. 1). In order to acquire multiple cloning sites in pTYM18, we inactivated the recognition sites for selected restriction endonuclease in the replication module. For actinomycete DNA cloning, *Eco*RI and *Hin*dIII sites have been used frequently, and therefore we inactivated the recognition sites in *int* by site-directed mutagenesis to give *int**. The plasmid pKFint-kai which contains *int** was digested with *Bam*HI and converted to a blunt-ended molecule with Klenow fragment, and the resulting larger fragment was ligated to the *Nhe*I site located at the outside of the multiple cloning site in pK18mob such that it could be cleaved by either enzyme. pK18mob contains the p15a replicon, kanamycin resistance of Tn5, inducible *lacZα*, *oriT*, and pUC18 multiple cloning sites[14]. As a result, the pTYM18 vector can be selected with kanamycin in both *E. coli* and actinomycete and it retains the multiple cloning sites of pK18mob except for *Sma*I and *Sal*I and also allows blue/white screening in an *E. coli* host.

pTYM19 is also a shuttle integrating vector containing useful multiple cloning sites (Fig. 1). pTIO was digested with *Kpn*I and *Pst*I, and the resulting larger fragment was converted to a blunt-ended molecule and ligated to the *Ssp*I site that is located at the outside of the multiple cloning sites of pUC19 (Fig. 2). The pTYM19 can be selected with thiostrepton in actinomycete and with ampicillin in *E. coli*. pTYM19 retains the multiple cloning sites of pUC19 except for *Sma*I and *Sal*I and allows blue/white screening in an *E. coli* host.

Heterologous Expression of Rebeccamycin Biosynthetic Genes with pTOYAMAcos

We demonstrated expression of heterologous biosynthesis genes for rebeccamycin in *S. lividans* using the pTOYAMAcos vector. Rebeccamycin is an indolocarbazole compound produced by *L. aerocolonigenes* ATCC39243. The biosynthesis gene cluster of rebeccamycin was cloned and the function of each gene was characterized[12]. The rebeccamycin biosynthesis gene cluster consists of 10 genes spanning 17 kilobases in *L. aerocolonigenes*. A genomic library (100,000 clones) of this strain was prepared by subcloning *Sau*3AI partial digested and dephosphorylated chromosomal DNA into the *Bam*HI site of pTOYAMAcos. The clones containing the rebeccamycin biosynthesis genes were screened by colony hybridization with probes designed for *rebH* and *rebG*, which are located the both end of the cluster on the chromosome. One of the cosmid clones, designated pTYMCreb, was selected by this screening. pTYMCreb was transformed into *S. lividans* TK23 and the transformants obtained were cultured in rebeccamycin production medium. After 11-days, the mycelium was collected and extracted with *n*-butanol. HPLC analysis of this *n*-butanol extract revealed the production of rebeccamycin (Fig. 3A) which was not produced by the wild-type TK23 strain (Fig. 3B). The peak at 14.0 minutes exhibited a UV-visible spectrum characteristic to rebeccamycin and showed the molecular ion $[M+H]^+$ at *m/z* 571 on LC/MS analysis. Therefore we demonstrated that pTOYAMAcos can work to clone and transfer genes into a heterologous host.

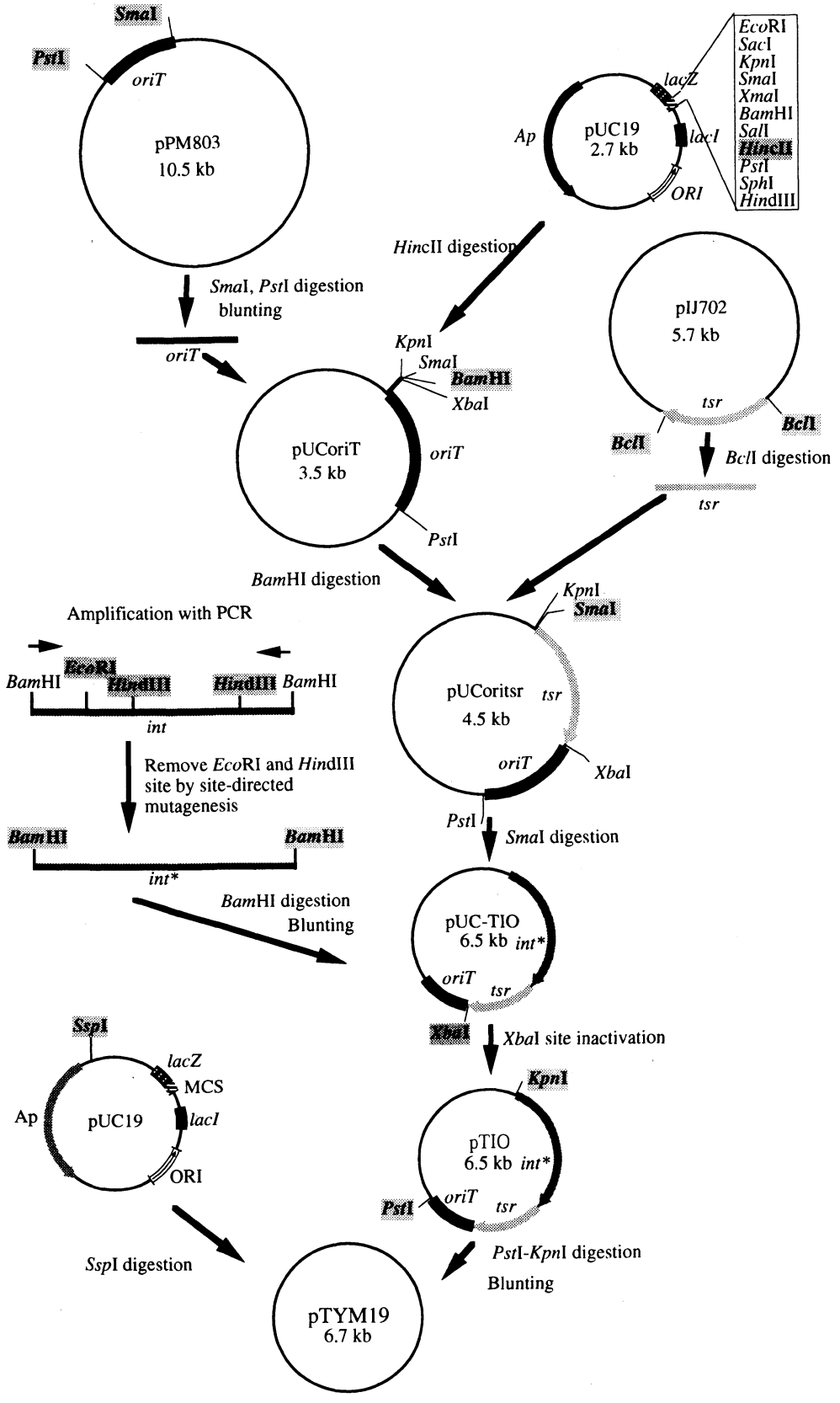
Complementation Experiments of Rebeccamycin Biosynthesis Gene Disruptants with pTYM19

To prove that pTYM19 can work in *L. aerocolonigenes*, we conducted complementation experiments in *L. aerocolonigenes ΔrebC* transformed with pTYM19 derivatives. *L. aerocolonigenes ΔrebC* is a deletion mutant of *rebC* which encodes monooxygenase in rebeccamycin biosynthesis gene cluster and produces 7-deoxo-7-hydroxy-4′-demethylrebeccamycin instead of rebeccamycin[12]. The pTYMrebAD (*rebO*, *rebD*, *rebC*, and *rebP* cloned into pTYM19) was constructed for this complementation experiment. These four genes are translationally coupled and the promoter upstream of *rebO* is necessary for *rebC* transcription. We introduced pTYMrebAD into *L. aerocolonigenes ΔrebC* and cultured the mutant in a liquid medium. The fermentation broth of *L. aerocolonigenes ΔrebC* harboring pTYM19 and *L. aerocolonigenes ΔrebC* harboring pTYMrebAD was extracted with *n*-butanol and the metabolites were analyzed by HPLC. HPLC of *n*-butanol extract from *L. aerocolonigenes ΔrebC* harboring pTYMrebAD revealed the production of rebeccamycin (Fig. 3C) whereas the control *L. aerocolonigenes ΔrebC* harboring pTYM19 strain did not produce it (Fig. 3D).

Conclusion Remarks

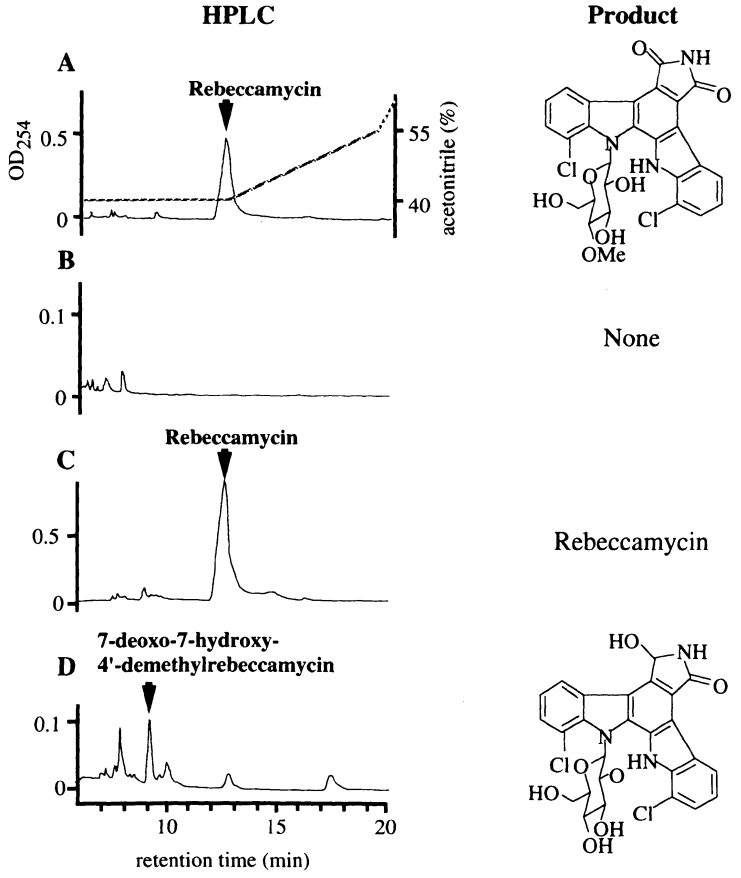
Three small integrating vectors, pTOYAMAcos, pTYM18, and pTYM19, were constructed and proved

Fig. 2. Construction map of a plasmid, pTYM19.



Restriction endonuclease sites digested in each construction stage are shown by shading. MCS: multiple cloning sites.

Fig. 3. HPLC analysis of the product.



S. lividans TK23 harboring pTYMCreb (A), *S. lividans* TK23 harboring pTOYAMAcos (B), *L. aerocolonigenes ΔrebC* harboring pTYMrebAD (C), and *L. aerocolonigenes ΔrebC* harboring pTYM19 (D). The elution was done with a linear gradient as indicated on the right-hand scale in A.

effective for use in *L. aerocolonigenes* ATCC39243 as well as *Streptomyces*. Actinophage ϕC31 has the ability to infect many streptomycetes and lysogenises about two-third of these streptomycetes, due to the site-specific recombination between *attP* of ϕC31 and the chromosome *attC* by ϕC31 *int* gene product[1]. A number of actinomycete species can be utilized as hosts for these vectors. The size of pTOYAMAcos is 8.33-kb, which to the best of our knowledge, is the smallest size integrating cosmid vector for actinomycetes, and 35~45-kb DNA fragments could be cloned in this vector. pTOYAMAcos facilitates cosmid library construction because contains inverted repeat sequences. In pTYM18 and pTYM19, their extensive cloning sites and availability to the blue/white screening can facilitate actinomycete gene cloning.

Acknowledgement

We thank Y. IKEDA at Toyama Prefectural University for collecting of LC/MS spectra. This work was supported in part by a Grant-in-Aid for Scientific Research to H.O. from the Japan Society for the Promotion of Science.

References

1) KIESER, T.; M. J. BIBB, M. J. BUTTNER, K. F. CHATER & D. A. HOPWOOD: *Practical Streptomyces genetics*. (The John Innes Foundation, Norwich, UK), 2000
2) RICHARDSON, M. A.; S. KUHSTOSS, P. SOLENBERG, N. A. SCHAUS & R. N. RAO: A new shuttle cosmid vector, pKC505, for streptomycetes: its use in the cloning of three different spiramycin-resistance genes from a *Streptomyces ambofaciens* library. Gene 61: 231~241, 1987
3) LACALLE, R. A.; J. A. TERCERO & A. JIMENEZ: Cloning of the complete biosynthetic gene cluster for an

aminonucleoside antibiotic, puromycin, and its regulated expression in heterologous hosts. EMBO J. 11: 785~792, 1992

4) BORMANN, C.; V. MOHRLE & C. BRUNTNER: Cloning and heterologous expression of the entire set of structural genes for nikkomycin synthesis from *Streptomyces tendae* Tu901 in *Streptomyces lividans*. J. Bacteriol. 178: 1216~1218, 1996
5) BIERMAN, M.; R. LOGAN, K. O'BRIEN, E. T. SENO, R. N. RAO & B. E. SCHONER: Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. Gene 116: 43~49, 1992
6) MATSUSHIMA, P.; M. C. BROUGHTON, J. R. TURNER & R. H. BALTZ: Conjugal transfer of cosmid DNA from *Escherichia coli* to *Saccharopolyspora spinosa*: effects of chromosomal insertions on macrolide A83543 production. Gene 146: 39~45, 1994
7) PANG, C. H.; M. SHIIYAMA, H. IKEDA, H. TANAKA & S. ŌMURA: Cosmid vector for cloning and analysis of *Streptomyces* DNA. Actinomycetol. 8: 21~25, 1994
8) IKEDA, H.; T. NONOMIYA, M. USAMI, T. OHTA & S. ŌMURA: Organization of the biosynthetic gene cluster for the polyketide anthelmintic macrolide avermectin in *Streptomyces avermitilis*. Proc. Natl. Acad. Sci. USA 96: 9505~9514, 1999
9) IKEDA, H.; J. ISHIKAWA, A. HANAMOTO, M. SHINOSE, H. KIKUCHI, T. SHIBA, Y. SAKAKI, M. HATTORI & S. ŌMURA: Complete genome sequence and comparative analysis of the industrial microorganism *Streptomyces avermitilis*. Nat. Biotechnol. 21: 526~531, 2003
10) MAZODIER, P.; R. PETTER & C. THOMPSON: Intergeneric conjugation between *Escherichia coli* and *Streptomyces* species. J. Bacteriol. 171: 3583~3585, 1989
11) SAMBROOK, J. & D. W. RUSSELL: Molecular cloning, a laboratory manual, third edition. Cold spring harbor laboratory press, NY, USA, 2001
12) ONAKA, H.; S. TANIGUCHI, Y. IGARASHI & T. FURUMAI: Characterization of the biosynthetic gene cluster of rebeccamycin from *Lechevalieria aerocolonigenes* ATCC 39243. Biosci. Biotechnol. Biochem. 67: 127~138, 2003
13) ONAKA, H.; H. TABATA, Y. IGARASHI, Y. SATO & T. FURUMAI: Goadsporin, a chemical substance which promotes secondary metabolism and morphogenesis in streptomycetes. I. Purification and characterization. J. Antibiotics 54: 1036~1044, 2001
14) SCHAFER, A.; A. TAUCH, W. JAGER, J. KALINOWSKI, G. THIERBACH & A. PUHLER: Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene 145: 69~73, 1994