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 λcos site to promote packaging of vectors containing 35–45-kb DNA fragments into λ particles. pTYM18 and pTYM19 contain kanamycin and thiostrepton resistance genes, respectively, and have multiple cloning sites including EcoRI and HindIII 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 rebC strain, which is defective in rebeccamycin production because of a rebC deletion, was restored to rebeccamycin production by complementation 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 EcoRI and one HindIII sites1). 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 Streptomyces2). Some examples of heterologous expression of secondary metabolite biosynthesis genes with pKC505 have been reported3,4). It seems that DNA cloning by chromosomal integration is more stable than plasmids. The pOJ436 vector is a chromosomal integration vector5), and its application has been reported6).

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

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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 clones7). The stability and utility of pKU402 for construction of genomic libraries has been confirmed by genome analysis of Streptomyces avermitilis8,9).

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 EcoRI and HindIII sites in a lacZα 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 transconjugation10). Growth conditions and manipulations of E. coli were as described by SAMBROOK and RUSSELL11), and those in Streptomyces were as described by KIESER et al.13).

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 KpnI 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 accession No. A00659), have the following sequences; intN: 5'-CGCGGATCCGGTGCGAGCAATCGCCC-3' and intC: 5'-GGCGGATCCCTTCCGCTGCCCAGGAAGCC-3', in which the BamHI sites are attached at the 5' end for cloning into the pKF19 BamHI 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 EcoRI and two HindIII sites in the int gene, site directed mutagenesis 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 Smal-PstI fragment containing the oriT gene was prepared from pPM80310), made blunt-ended with Klenow fragment and cloned into the HinII site of pUC19 to generate pUCori. The tsr gene fragment was prepared from pIJ702 by digesting with BclI and subcloned into the BamHI site of pUCori to construct pUCoriT to construct pUCoriTsr. pKFint-kai was digested with BamHI, and the resulting larger fragment was made blunt-ended with Klenow fragment and cloned into the Smal site of pUCori-tsr to yield pUC-TIO. The construction scheme is shown in Fig. 2. pUC-TIO was digested with XbaI and filled-in with NTPs and Klenow fragment and religated to inactivate the XbaI site to generate pTIO.

Construction of pTYMrebAD

A 6.3-kb BglII-BamHI fragment containing rebO, rebD and N-terminal region of rebC was excised from pREB112) and cloned into the BamHI site of pREB512) 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 EcoRI-HindIII fragment and ligated to pTYM19 digested with EcoRI-HindIII, resulting in
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 obtained 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 organisms7). 1.75-kb BamHI

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**Fig. 1.** Restriction map of plasmids pTOYAMAcos, pTYM18, and pTYM19.
fragment containing streptomycin/spectinomycin-resistance gene (aad3") was used as a stuffed fragment at the unique BamHI cloning site. A cassette plasmid pTIO which contains the actinomycete replication module was digested with PstI and KpnI, and the resulting larger fragment was purified and both ends were filled with Klenow fragment, followed by cloning into the Ndel 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, EcoRI and HindIII 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 BamHI and converted to a blunt-ended molecule with Klenow fragment, and the resulting larger fragment was ligated to the SspI 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 SmaI and SalI and allows blue/white screening in an E. coli 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 monoxygenase in rebeccamycin biosynthesis gene cluster and produces 7-deoxo-7-hydroxy-4’-demethylrebeccamycin instead of rebeccamycin. 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.
effective for use in *L. aerocolonigenes* ATCC39243 as well as *Streptomyces*. Actinophage $\phi$C31 has the ability to infect many streptomycetes and lysogenises about two-third of these streptomycetes, due to the site-specific recombination between $attP$ of $\phi$C31 and the chromosome $attC$ by $\phi$C31 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.

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