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Replication and conjugative transfer of
the *Streptomyces* plasmid pSN22

1994

Masakazu Kataoka



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**Replication and conjugative transfer of
the *Streptomyces* plasmid pSN22**

(放線菌プラスミドpSN22の複製および接合伝達)

1994

Masakazu Kataoka

Replication and conjugative transfer of the streptomycin plasmid pSN22

(学位論文) 学位論文

1994

Masakazu Kataoka

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*This dissertation is dedicated
to
my mother Kazue Kataoka
and
the late Professor Hisaharu Taguchi.*

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INTRODUCTION

Bacterial cells often harbor other replicons, bacteriophages and plasmids in addition to the chromosome. These extra chromosomal cytoplasmic elements exist as parasitic factors in bacterial cells. Plasmid is identified as the genetic elements multiplying independently of the host chromosome and being maintained stably through generations in the host.

The word "plasmid" is initially proposed by Lederberg (1952) as a generic name for replicons in the cytoplasm. Nowadays a plasmid is defined as a factor able to multiply in the bacterial cytoplasm. Phages, in addition, produce a coat that protects their DNA outside the cells. The biology of plasmids has been studied since the discovery of the existence of sex in the bacterial community mediated by the F factor of *Escherichia coli* (reviewed by Willets and Skurray 1987). The investigations on plasmids have dramatically increased after the discovery of R factors by Japanese scientists (reviewed by Watanabe 1963). The relationship between plasmids and drug resistance plays a significant role in the spread of bacterial disease, e.g. by MRSA (methicilin resistant *Staphylococcus aureus*) which exhibit multi-drug resistance. The spread of drug resistance is quite often in hospitals. Following the development of recombinant DNA technique, the study of plasmid became a powerful tool indispensable in the field of genetic engineering.

Plasmid studies have been focused mainly on two functions, replication and self-transmission. Plasmid biology is also thought to be an interesting issue for the investigation of bacterial evolution and of genetic information currency. Self-transmission of plasmids is regarded to play an important role in increasing the genetic diversity in mono-sexual bacteria (reviewed by Amabile-Cuevas and Chicurel 1992) because the dynamic nature of genetic material is the basis of evolutionary processes: novel DNA sequences are continually being produced by mutation and recombination. Through the stabilization and transmission of these genetic products, evolutionary directions are established. Vertical transmission, the transfer of genes from parents to offspring, was generally thought to be the main means of propagating new genes. However, it is becoming increasingly apparent that another form of transmission, horizontal transfer, contributes greatly to evolutionary processes in a wide

variety of organisms. Horizontal gene transfer refers to the mobilization of genetic information from one organism to another. Such exchange is a well-established phenomenon in the prokaryotic kingdom. Mobilization of genetic information primarily involves extra chromosomal elements, many of them encode systems for their own transfer. A consequence of such mobilization is the existence of a pool of genetic information that is accessible to virtually every bacterial cell. Indeed, it has been proposed that the prokaryotic community may be viewed as a single, heterogenetic, multicellular organism, containing replicons that are in continuous movement from one group of cells to another (Sonia 1991). This thesis describes the genetical and functional analyses of a conjugative *Streptomyces* plasmid which is proposed to mediate gene flux.

Streptomycetes are Gram-positive bacteria undergoing a complex life cycle of morphological differentiation and have occupied an important position in micro biological industries due to their capability of producing many kinds of antibiotics and useful enzymes. The door to the molecular genetics in *Streptomyces* was opened by Okanishi et al (1974) who developed a method of protoplast regeneration and Bibb et al (1978) who first introduced plasmid DNA by transformation into *Streptomyces* protoplast. Plasmids in *Streptomyces* have been studied mainly for the purpose of developing cloning vectors for the genetic manipulation of these organisms (reviewed by Hopwood et al 1987). The study of the biology of *Streptomyces* plasmids has been focused mainly on plasmid transfer (pock-formation), replication, site-specific integration of plasmids into the host chromosome and on the relationship of plasmids with antibiotic production. Most *Streptomyces* plasmids are highly transmissible and are able to form "pocks", the formation of which is a unique feature of *Streptomyces* plasmid and is almost always associated with plasmid transfer. Pocks are macroscopically visible circular zones of retarded growth that develop around colonies of individual plasmid-harboring spores seeded in a dense lawn of plasmid-free spores. Pock-formation depends on a plasmid transfer functions; a plasmid mutated at a locus for self-transmission cannot form pocks. The fact that all self-transmissible plasmids so far investigated can mobilize chromosomal markers, as well as themselves, from donor to

recipient indicates that pock-forming plasmids should play a major role in gene flux among *Streptomyces* strains.

Plasmid transfer between *Streptomyces* cells is supposed to be accomplished in two steps, intermycelial transfer and intramycelial transfer (Kieser et al. 1982). The loss of so called "spread" function reduces pock size and is thought to be an expression of intramycelial plasmid transfer. Very different from the large transfer operons of plasmids in other species like F in *E. coli*, the number of genes for the self-transmission of *Streptomyces* plasmids is thought to be small.

The replication of two of *Streptomyces* plasmid has been investigated. DNA fragments about 2 kbp of pIJ101 and SCP2* contain all the function for autonomous replication. As shown in the review by Gruss and Ehrlich (1989), the replication mechanism for some plasmids of Gram-positive bacterium, *Staphylococcus aureus*, is well characterized. These plasmids replicate via a rolling circle mechanism with single-stranded intermediates. All of these plasmids have three functions involved in their complete replication. The Rep protein and a nicking site which is recognized by Rep are essential for autonomous replication, the nicking site also function as a replication origin (*ori1*). The investigation of the Rep protein of pT181 indicated that it has a topoisomerase-like activity. The other region, designated minus origin (M-O) or *ori2* which is an origin of second strand synthesis has been identified. If this region is deleted, single-stranded intermediates accumulate and the stability of plasmid is decreased. In *Streptomyces*, the replication mechanism of pIJ101 has been investigated in detail. pIJ101 replicate via a rolling circle mechanism, just like other Gram-positive bacterial plasmids. A region which confers both strong incompatibility (*sti*) between pIJ101 derivatives and the conversion of single strand to double strand form, similar to the M-O of *S. aureus* plasmids, was also identified.

Site-specific recombination functions for the integration of plasmids into the host chromosome were observed in some *Streptomyces* plasmids, however pSN22 does not have this function. The integration functions were studied in detail in SLP1.2 and pSAM2. Two proteins encoded by *int* and *xis* genes, and the recombination sites designated as *attP* and *attC* are involved. For the integration of plasmids into the host chromosome, the integrase

encoded by *int* is required. For the excision of plasmids from chromosome, both integrase and excision enzyme (encoded by *xis*) are required. In the case of pSAM2, the integrase is similar to the integrase of temperate bacteriophages. So this integration system is thought to be derived from a bacteriophage (Boccard et al. 1989).

The understanding of plasmid functions is thought to be a good source of information not only for biotechnology but also for the understanding the flow of genetic information required for the survival of bacteria. In this thesis, pSN22 originally isolated from *Streptomyces nigrifaciens* was analyzed genetically. pSN22 is a self-transmissible, high copy number, broad host range plasmid.

In Chapter I, mutational analysis on the function of self-transmission and pock-formation of pSN22 is presented. The purpose of this investigation was to characterize pSN22, to understand how pSN22 transfers in *Streptomyces*, and to identify the functions relating to plasmid transfer.

In Chapter II, the *kil-kor* system of pSN22, and the regulation of transfer related genes are discussed. The study in this chapter was designed to understand the lethal phenotype of *traR*⁻ mutations described in Chapter I, and to study the regulation of transfer genes.

In Chapter III, the essential region for autonomous replication and rolling circle replication mechanism of pSN22 were studied. It was the intent of the research in this chapter to characterize the replication mechanism, which is another way to transfer of genetic information of pSN22 and may contribute to plasmid transfer.

In chapter IV, the complete nucleotide sequence of pSN22 is shown, and the probable molecular functions of pSN22 are presented. The sequence was determined mainly as a preparation for future work, which aims to investigate the molecular functions of gene products encoded by pSN22.

Chapter I

Genes involved in self transmission of a *Streptomyces* plasmid, pSN22

INTRODUCTION

Self-transmissible (conjugative) plasmids are found widely in Gram-negative (Bukhari et al. 1977, Cavalli et al. 1953, Hayes 1953, Ippen and Minkley 1986) and Gram-positive bacteria (Chapman and Carlton 1985, Clewell 1981, Thomas and Archer 1989). In *Streptomyces* strains, several self-transmissible plasmids such as SCP2 (Bibb et al. 1981), pIJ101 (Kieser et al. 1982) and pSK2 (Akagawa et al. 1984) have been isolated, but except for pIJ101 (Kendall and Cohen 1987 1988, Kieser et al. 1982, Stein et al. 1989, 1990) and SCP2* (Bibb and Hopwood 1981, Lydiate et al. 1985) there are few genetic and functional analyses concerned with pock-formation and self-transmission. pIJ101 has been especially well characterized and used for the development of many cloning vectors. Other plasmids, such as SLP1 (Grant et al. 1989, Omer and Cohen 1984), pSG5 (Muth et al. 1988), SCP1 (Kinashi et al. 1987, Wright and Hopwood 1976) and pSAM2 (Boccard et al. 1989, Perrondet et al. 1984), have been studied in lesser detail. Most self-transmissible plasmids isolated from *Streptomyces* form "pocks" when a few cells of a plasmid-harboring strain (donor strain) are seeded in a confluent lawn of a plasmid-free strain. Pocks are circular zones in the confluent lawn where differentiation is retarded (Bibb et al. 1977, Hopwood et al. 1973). Kieser and his colleague distinguished two functions, "transfer" and "spread", encoded on pIJ101. The DNA sequence determined by Kendall and Cohen suggested that four pIJ101 genes, *tra*, *spdA*, *spdB* and *kilB* are involved in plasmid transfer and pock-formation. Hopwood et al. (1985) suggested that the plasmid transfer of SCP2* and pIJ101 is accomplished through two steps: the intermycelial transfer of a conjugative plasmid directed by transfer function(s) and the intramycelial transfer mediated by spread function(s). The process of self-transmission of *Streptomyces* plasmids seems to be genetically much simpler than that of conjugative plasmids of Gram-negative bacteria, where a large number of transfer

genes are required for the synthesis of sex pili (Willet and Skurry 1987).

In this chapter, I describe the structural and functional analyses of the self-transmission of pSN22 from *S. nigrifaciens*. Among five genes within a 7 kilobase pairs (kbp) fragment of pSN22, two were involved in plasmid transfer and/or pock-formation, and another two influenced pock size. The fifth gene was similar to the *kor* gene of pIJ101. The concept of plasmid transfer and the biological function of *Streptomyces* plasmids are also discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids *Streptomyces nigrifaciens* SN22 harboring pSN22 was isolated from Thai soil. *S. lividans* 1326 (Lomorskaya et al. 1972), TK21 (SLP2⁻ SLP3⁻) and TK24 (*str6* SLP2⁻ SLP3⁻) were used as *Streptomyces* hosts for transformation or recipient strains in the plasmid transfer assay. An auxotrophic derivative of TK24, strain TKC244 (*thr-1 str-6* SLP2⁻ SLP3⁻), was isolated after UV mutagenesis. Other *Streptomyces* strains used for testing the host range of pock formation were obtained from American Type Culture Collection (ATCC), Rockville Md., and Japan Collection of Microorganism (JCM), Wako, Saitama, Japan. Strain JM109 (*recA1 endA1 gryA96 thi hsdR17 supE44 relA1 Δ[lac- proAB][F' traD36 proAB lacI9 ZΔM15]*) (Vieria and Messing 1985) and strain GM33 (*dam*) (Maniatis 1973) were used as hosts for *Escherichia coli* plasmids.

The *Streptomyces* plasmids pIJ101, pIJ364 (Kieser et al. 1982) and pIJ6 (Thompson et al. 1982), and the *E. coli* plasmids pUC12, pUC19 (Yanisch and Messing 1985) and pACYC177 (Chang and Cohen 1978) were used in various cloning experiments. *S. lividans* 1326 and TK24, and the pIJ-series of plasmids were kindly provided by D. A. Hopwood (The John Innes Institute, Norwich, England) and strain TK21 was from H. Ogawara (Meiji College of Pharmacy, Tokyo, Japan).

Media and bacterial growth *Streptomyces* strains were grown at 30°C in YEME medium, R2YE medium, MM agar medium (Hopwood et al. 1985) or yeast extract malt

extract agar medium (Difco). For the preparation of *Streptomyces* spores and for the assay of plasmid transfer, a modified R2YE medium with 0.1 % instead of 0.5 % yeast extract was used. *E. coli* was grown at 37°C in LB broth or on LB agar medium. Antibiotics were used at the following concentrations; ampicillin (Meiji Seika) 50 µg/ml, kanamycin (Meiji Seika) 25 µg/ml, thiostrepton (thiopeptide, Fujisawa) 50 µg/ml, streptomycin (Meiji Seika) 20 µg/ml, and viomycin (Pfizer) 40 µg/ml.

DNA manipulation and transformation DNA isolation by alkaline lysis from *E. coli* and *Streptomyces* strains was according to the methods of Birnboim et al. (1979) and Kieser (1984), respectively. *In vitro* DNA manipulation were carried out according to the methods described by Maniatis et al. (1982). DNA fragments generated by digestion with restriction enzymes were separated by electrophoresis on a low melting point agarose gel (Weislander 1979). Transformations of *E. coli* and *S. lividans* were performed following the protocols of Maniatis et al. (1982) and Hopwood et al. (1985), respectively. Enzymes for DNA manipulations and oligonucleotide linkers were purchased from Takara Shuzo or Toyobo.

Genetic crosses Pock-formation and plasmid transfer were tested under two conditions. For condition A, mainly used for the assay of chromosomal recombination, similar numbers of spores of a plasmid donor and a recipient strain were spread together on R2YE medium. After 5 days incubation, spores were harvested and plated out at appropriate dilutions on selective media. Crossing condition B was used for the pock-forming assay, and was as condition A except that 10² to 10³ spores of a donor strain were spread together with 10⁶ to 10⁷ spores of a recipient strain. After sporulation, the morphology of the pocks (zones of growth-inhibition of recipient cells) was observed, or the frequency of antibiotic resistant progeny was determined as for condition A. Qualitative analysis of plasmid transfer efficiency was made by replica plating: spores of a donor strain were streaked in a line on a lawn of spores of a recipient strain. After sporulation, plates were replicated to selective media. The efficiency of plasmid transfer was estimated from the width of cell-growth along the line after 5 days incubation.

RESULTS

Isolation and physical mapping of a self-transmissible plasmid, pSN22
Streptomyces nigrifaciens SN22 contains covalently closed circular plasmid DNA, designated as pSN22, which was isolated by alkaline lysis and ethidium bromide-CsCl gradient centrifugation. The molecular size of pSN22 was estimated to be 11.0 kbp and its physical map is shown in Fig. I-1. The copy number of pSN22 estimated densitometrically from the staining intensity of the DNA bands of total DNA on an ethidium bromide stained agarose gel appeared to be high (60 - 180 per chromosome); pIJ364 (100 - 300 per chromosome) was used as control.

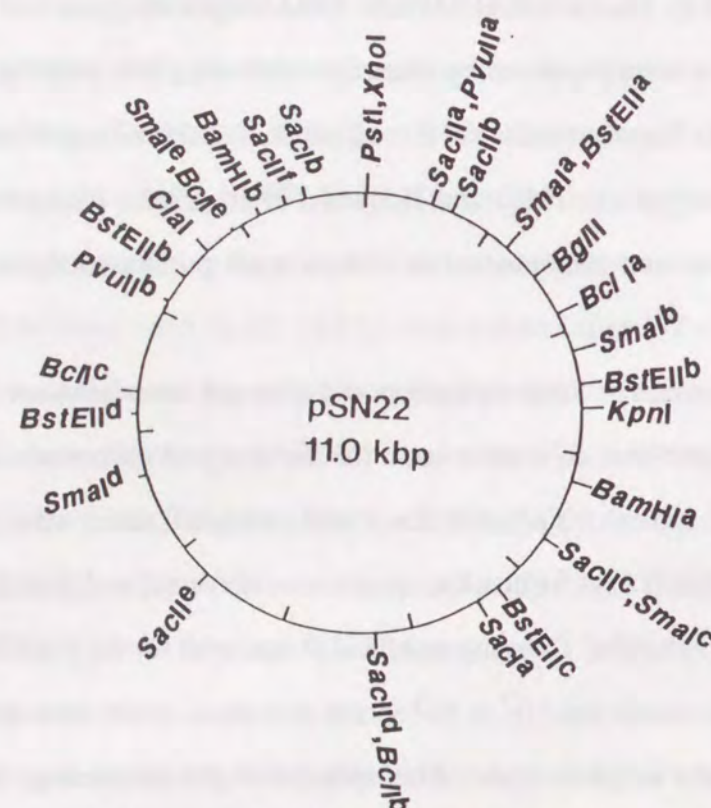


Figure I-1. Restriction map of pSN22. *EcoRI*, *HindIII* and *DraI* did not cut the plasmid. Where a restriction endonuclease has more than one site, they are distinguished by alphabetical superscripts.

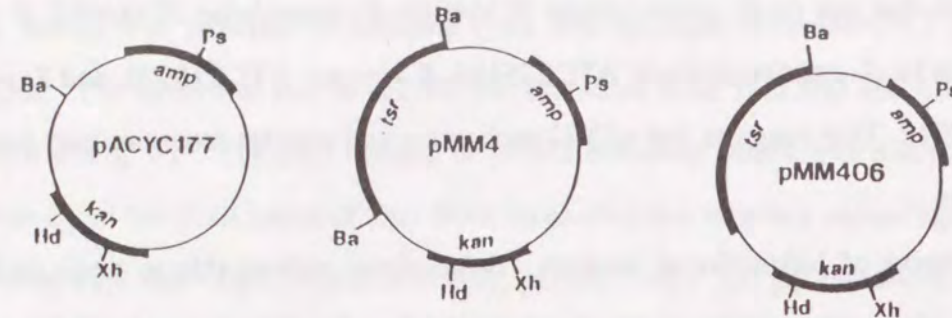
pSN22 seemed to be self-transmissible, because it could be transferred from *S. nigrifaciens* SN22 to *S. lividans* 1326 by mixed culture. pSN22 transferred by conjugation or transformation was maintained stably in *S. lividans*. *S. lividans* TK21 transformed with a pSN22 derivative, pMT911 (Fig. I-2C), also formed pocks on 7 out of 11 *Streptomyces* and

2 *Actinomadula* strains, which were classified into diverse species by numerical taxonomy (Locci 1989), under crossing condition B; pocks were observed on *S. albogiseolus* ATCC23875, *S. azureus* ATCC14921, *S. corchorusii* JCM4467, *S. glaucescens* ATCC23662, *S. halstedii* JCM4584, and *Actinomadula luteofluorescens* JCM4203 and JCM4491, but not on *S. griseoviridis* JCM4643, *S. lavendulae* JCM4055, *S. parvulus* ATCC12434, *S. pristinaespiralis* ATCC25486, *S. rimosus* ATCC10970, and *S. venezuelae* ATCC10712. This suggests that pSN22 replication and transfer functions may have a wide host range.

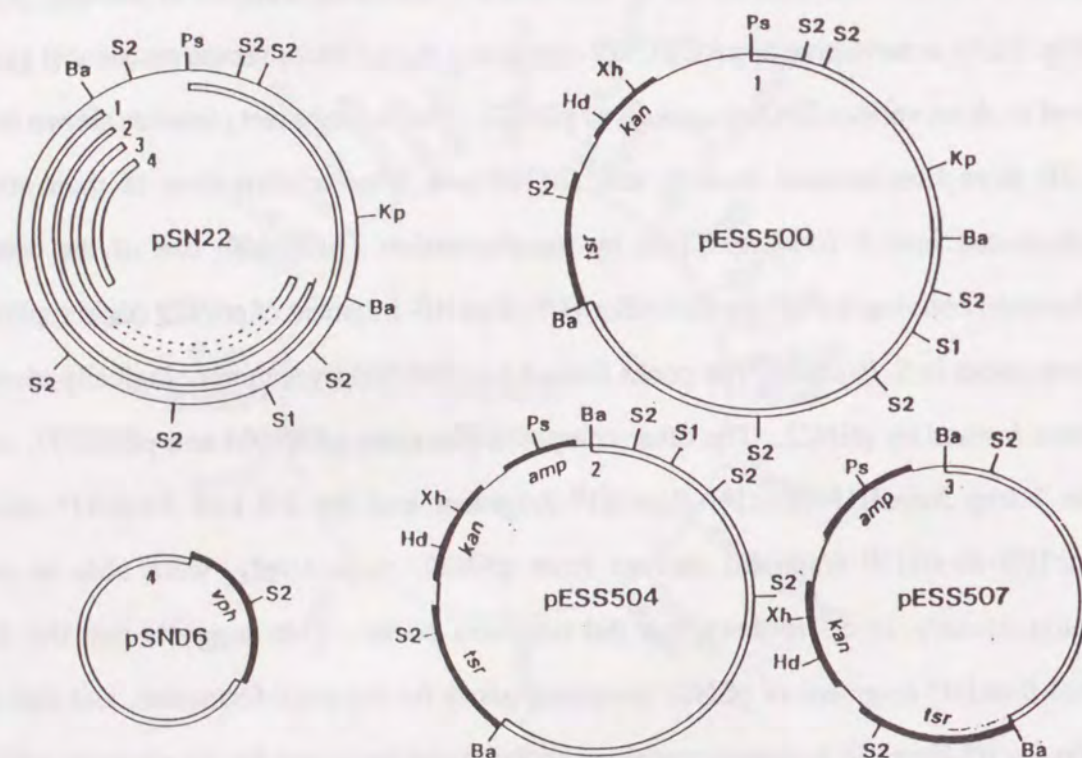
Development of bifunctional vectors Bifunctional vectors able to replicate in *E. coli* and *S. lividans* were constructed to facilitate the functional analysis of pSN22. pMM406 (Fig. I-2A), a derivative of pACYC177 containing the *tsr* (thiostrepton resistance) gene, was used to clone various DNA fragments of pSN22. The recombinant plasmids shown in Figure I-2B were first isolated from *E. coli* JM109 and, after confirmation of their structure, introduced into *S. lividans* TK21 by transformation. pESS500, one of the composite plasmids, carrying the 10 kbp *PstI*-(*Bam*HI^a)-*Bam*HI^b fragment of pSN22 could replicate and form pocks in *S. lividans*. The pocks formed by pESS500 were morphologically identical to those formed by pSN22. The other composite plasmids, pESS504 and pESS507, carrying the 7 kbp *Bam*HI^a-(*Sac*I^a)-*Bam*HI^b fragment and the 3.5 kbp *Bam*HI^a- Δ *Sac*II^c-*Sac*II^e)-*Bam*HI^b fragment derived from pSN22, respectively, were able to replicate autonomously in *S. lividans*, but did not form pocks. This suggests that the 3.2 kbp *PstI*-*Bam*HI^a fragment of pSN22 contained genes for the pock-formation, and that the 3.5 kbp *Sac*II^e-*Bam*HI^b fragment contained all functions necessary for the plasmid replication. Southern hybridization (data not shown) indicated that the latter fragment was very similar in its DNA sequence to the essential region of pIJ101. The region required for the pock-formation, however, did not hybridize with the transfer region of pIJ101.

pESS500, pESS504 and pESS507 were structurally stable in *E. coli* (kanamycin selection) and in *S. lividans* (thiostrepton selection). After one cycle of sporulation under nonselective condition, pESS500, pESS504 and pESS507 were lost at frequencies of 5, 54, and 78 %, respectively. Remarkably, pSND6 (Fig. I-2B), which contained no *E. coli*

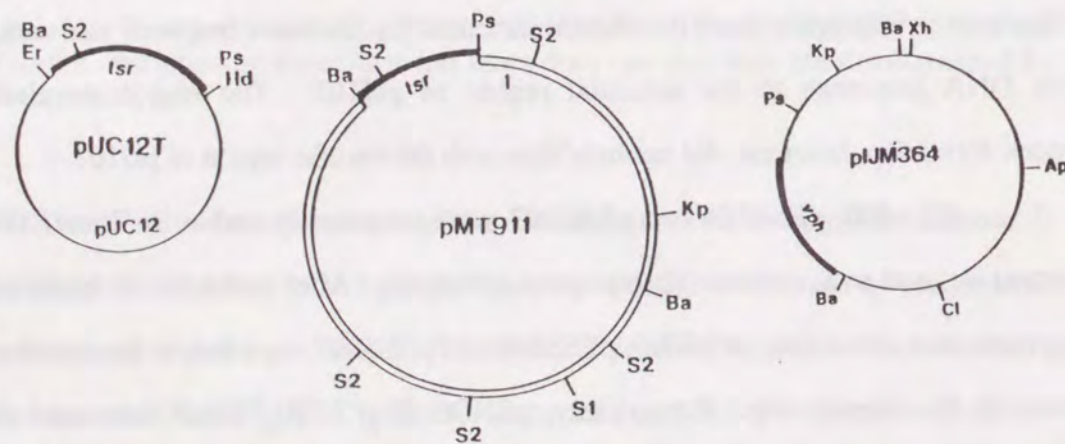
A



B



C



A

B

C

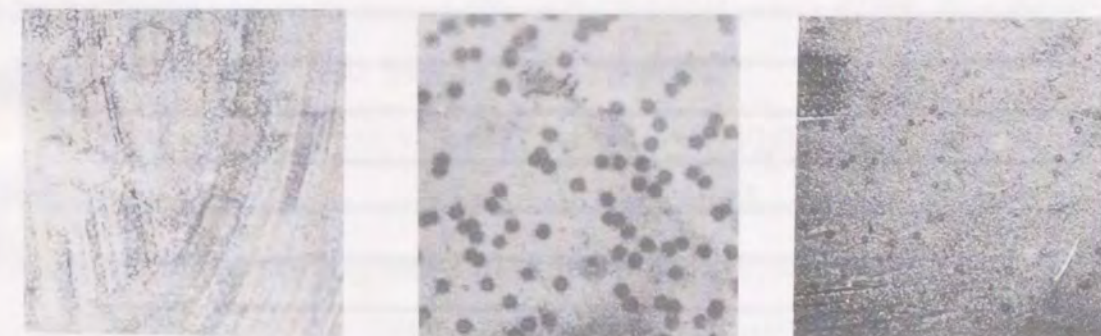


Figure I-3. Morphology of pocks formed by pMT911 (wild type; A), pMT921 (*spdA*⁻; B) and pMT926 (*spdB*⁻; C) plasmids. An appropriate number of spores of strain TK21 carrying the different plasmids was mixed with a large excess of spores of strain TK21 (plasmid free). The photographs were taken at the same magnification, and the bar indicates 5 mm.

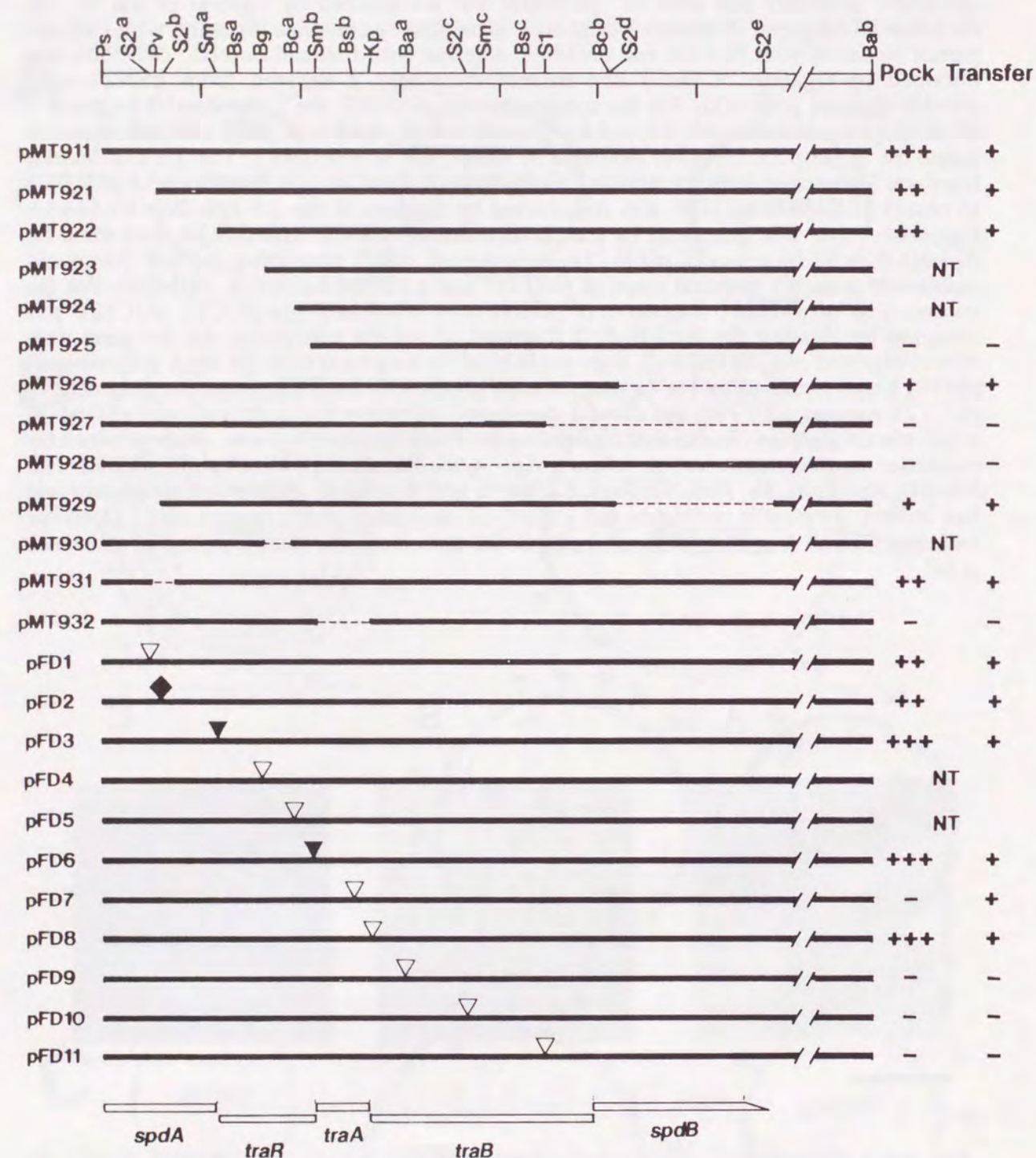


Figure I-4. Mutational analysis of the loci involved in plasmid transfer and pock-formation. The pock sizes formed by mutant plasmids are represented by +++, ++ and + for normal, small and tiny pocks, respectively, as described in the text and Fig. I-3. The plasmid transfer ability (transfer) is shown by + (positive) and - (negative). The derivatives marked with NT could not be introduced into *S. lividans* by transformation. Parts of pESS500 or pMT911 were deleted by digestion with appropriate restriction endonucleases followed by the addition of appropriate linker adapters. The parts marked with dashed lines show deleted regions. Insertions and small deletions were introduced in pESS500. In pFD3 and pFD6, an 8 base *XhoI* linker d(5'-CCTCGAGG-3') (Takara Shuzo) was introduced (filled triangle), and the T4 polymerase-treated *PstI*-*BamHI* fragment containing the *vph* gene was inserted in pFD2 (filled square). In other pFD plasmids, potential frame disruptions were introduced by digestion with restriction endonucleases, removal of the cohesive ends with T4 polymerase and blunt-end ligation (blank triangle). Triangles and the square show the sites of these mutations. The top open bar shows the restriction map of the *PstI*-*BamHI*^b fragment of pSN22. The bottom open bars show putative loci for the genes related to the pock-formation and plasmid transfer. Each gene is described in the text. Abbreviations for the restriction endonucleases are as follows: Bg; *BglII*, Bs; *BstEII*, Pv; *PvuII* and Sm; *SmaI*, and others are the same as in Fig. I-2.

Mutational analysis of the genes concerned with pock-formation and plasmid transfer For the genetic analysis of pock-formation and plasmid transfer, deletions and insertions were introduced *in vitro* into the 5.3 kbp *PstI*-*SacII*^e region of pESS500 (Fig. I-2). Mutations were grouped into five phenotypes designated as SpdA⁻ (*spd*, plasmid spread), SpdB⁻, TraA⁻ (*tra*, plasmid transfer), TraB⁻, and Kil. *S. lividans* harboring *spdA*⁻ or *spdB*⁻ plasmids formed small or tiny pocks in a plasmid-free recipient strain (Fig. I-3). *traA*⁻ plasmids were transmissible, but lost the ability to form pocks, while *traB*⁻ plasmids were deficient in both plasmid transfer and pock-formation. The last group of mutations showing the Kil phenotype were similar to the Kil mutation of pJ101, since Kil plasmids could not be introduced into *S. lividans* by transformation.

Deletion mutants, pMT923, pMT924 and pMT930 (Fig. I-4), were derived from pESS500. All other deletion mutants shown in Fig. I-4 were constructed from pMT911 (Fig. I-2C), in which the DNA fragment derived from pACYC177 was removed from pESS500 to exclude the effect of *E. coli* DNA on plasmid stability. pMT921, pMT922 and pMT931, which lack the *PstI*-*PvuII*^a, *PstI*-*SmaI*^a and *SacII*^a-*SacII*^b fragments of pSN22, respectively, showed the SpdA⁻ phenotype. pMT923, pMT924 and pMT930, lacking the *PstI*-*BglII*, *PstI*-*BclII*^a and *BglII*-*BclII*^a fragments, respectively, gave no thiostrepton-resistant *S. lividans* transformant (*kil* phenotype). pMT927 and pMT928, lacking the *SacI*^a-*SacII*^e and *BamHI*^a-*SacI*^a fragments from pMT911, respectively, showed the TraB⁻ phenotype. pMT926, having a deletion of the *SacII*^d-*SacII*^e fragment, showed the SpdB⁻ phenotype. The

double mutant (pMT929), lacking the *PstI*-*PvuII*^a and *SacII*^d-*SacII*^e fragments, formed the same tiny pocks as pMT926 (*spdB*⁻). The *traA*⁻ phenotype could not be obtained by deletion. Though pMT925 and pMT932 with the *PstI*-*SmaI*^b and *SmaI*^b-*KpnI* fragments, respectively, deleted, also showed the TraB⁻ phenotype like pMT927 and pMT928, they might have lost the *cis* elements, e.g. promotor(s) or ribosome binding site(s), affecting the expression of the TraB⁻ phenotype.

Potential frame-disruptions were introduced in the *PstI*-*BclI*^b fragment of pESS500 by insertion of *XhoI* linkers or viomycin-resistant fragments, and by deletion of some bases at restriction sites. The results are shown in Fig. I-4. Frame-shifts at the *SacII*^a and *PvuII*^a sites (pFD1 and pFD2) introduced *spdA*⁻ mutations, and those at the *BglII* and *BclI*^a sites (pFD4 and pFD5) generated *Kil* mutations. The mutations at the *BamHI*^a (pFD9), *SacII*^c (pFD10) and *SacI*^a sites (pFD11) abolished both pock-formation and plasmid transfer. The frame-disruption at the *BstEII*^b site (pFD7) caused the loss of pock-formation, but the mutated plasmid was still transmissible, though the efficiency of plasmid transfer was about one hundredth that of the parent plasmid, pMT911 (Table I-1). This TraA⁻ phenotype seemed to be a phenotype not found among the mutants of pIJ101 or other *Streptomyces* conjugative plasmids.

These results suggest that the pSN22 transfer region is divided functionally into at least five loci, as summarized in Fig. I-4. The genes corresponding to the SpdA⁻, SpdB⁻, TraA⁻ and TraB⁻ phenotypes were designated as *spdA*, *spdB*, *traA* and *traB*, respectively. As the *Kil* phenotype of pSN22 was considered to be similar to that of pIJ101, the corresponding gene, *traR*, was postulated to code for a regulatory factor involved in the expression of transfer genes (Kendall and Cohen 1987, Stein et al. 1989). The genes, *traA*, *traB* and *traR*, were considered to lie within the *SmaI*^b-*KpnI*, and *KpnI*-*SacII*^c and *SmaI*^a-*SmaI*^b fragments, respectively, since the potential reading frame disruptions at the *SmaI*^a (pFD3), *SmaI*^b (pFD6) and *KpnI* (pFD8) sites affected neither pock-formation nor plasmid transfer. The *spdA*⁻ and *spdB*⁻ plasmids were transmissible, but the mutations affected pock size. The results suggest that the genes are needed for efficient plasmid transfer within the mycelial recipient in a similar fashion to the *spd* gene of pIJ101. The *spdA* gene was located on the *PstI*-*SmaI*^a fragment;

while one end of the *spdB* locus seemed to be close to the *BclI*^b site, the other end was not defined. The functions and alignments of the pSN22 genes required for the plasmid transfer and pock-formation, except for *traA*, seem to be very similar to those of pIJ101.

Table I-1. Plasmid transfer efficiencies of pSN22 derivatives

| Plasmid | Genotype of plasmid | Crossing condition | Number of colonies growing on | | Efficiency of plasmid transfer B/(A-B) |
|---------|----------------------------------------------|--------------------|-------------------------------|-----------------------|----------------------------------------|
| | | | Tsr (A) | Tsr, Str (B) | |
| pMT911 | wild | B | 1.2 x 10 ⁸ | 9.0 x 10 ⁷ | 3 |
| pMT921 | <i>spdA</i> ⁻ | B | 9.3 x 10 ⁸ | 6.0 x 10 ⁸ | 1.8 |
| pMT926 | <i>spdB</i> ⁻ | B | 1.6 x 10 ⁹ | 1.9 x 10 ⁸ | 3.8 x 10 ⁻¹ |
| pFD7 | <i>traA</i> ⁻ | B | 1.0 x 10 ⁴ | 5.0 x 10 ² | 5.2 x 10 ⁻² |
| pESS507 | Δ (<i>spdA traR traA traB spdB</i>) | A | 1.9 x 10 ⁸ | 3.2 x 10 ¹ | 1.7 x 10 ⁻⁷ |
| pFD10 | <i>traB</i> ⁻ | A | 1.4 x 10 ⁸ | 6.0 x 10 ¹ | 4.3 x 10 ⁻⁷ |
| pFD11 | <i>traB</i> ⁻ | A | 4.0 x 10 ⁶ | < 1 | < 2.5 x 10 ⁻⁷ |

The efficiency of plasmid transfer was determined by plating spores of TK21 harboring various Tsr^r pSN22 derivatives with TK24 (Str^r) on R2YE medium under crossing condition A or B (see Materials and Methods). The phenotypes of spores generated in these crosses were tested by spreading appropriate dilutions on YM medium containing thiostrepton (A), or thiostrepton and streptomycin (B). The efficiency of plasmid transfer, calculated as B/(A-B), is the ratio of the number of recipients which have received a plasmid from a donor to the number of donors.

Estimation of the efficiency of plasmid transfer For the quantitative determination of the efficiency of plasmid transfer, spores of a donor strain harboring pMT911 (*tsr*) or its mutant derivatives (*tsr*) were mixed with the spores of recipient strain TK24 (*str-6*) according to the crossing conditions A or B (see Materials and Methods), and incubated for 5 days. The spores harvested from the cross were spread at appropriate dilutions on selective media containing thiostrepton, or both streptomycin and thiostrepton. The efficiency of plasmid transfer was calculated as the ratio of Str^r Tsr^r (streptomycin- and thiostrepton-resistant) colonies per donor colony (Tsr^r). Table I-1 shows that the pMT911

(wild type) was transferred with an efficiency of about 3.0, whereas pESS507 ($\Delta[spdA traR traA traB spdB]$), pFD10 ($traB^-$) and pFD11 ($traB^-$) were transferred with efficiencies of less than 4.3×10^{-7} , which is thought to be similar to the frequency of conjugation between plasmid-free cells. The result indicated that the *traB* gene is essential for plasmid transfer.

The plasmid transfer efficiency was not only affected by plasmid transfer from donor cells to recipient cells, but also by plasmid spread in recipient cells. The transfer efficiency of the *spdA*⁻ plasmid (pMT921; $\Delta spdA$) was almost the same as that of the wild type plasmid, though the pock-size of pMT921 was significantly smaller than that of the wild type plasmid (Fig. I-3). The *spdB*⁻ mutation (pMT926; $\Delta spdB$), which resulted in tiny pocks (Fig. I-3), decreased the transfer efficiency to one-tenth (3.8×10^{-1}) of that of the wild type plasmid. The transfer efficiencies of the *spdA*⁻ and *spdB*⁻ mutants were also tested qualitatively by the replica method (see Materials and Methods). Figure I-5 shows that *spdA*⁻ plasmids were transferred and spread more effectively in the recipient mycelia than *spdB*⁻ plasmids, but less effectively than the wild type plasmid. These results suggested that the *spdA* and *spdB* genes both contributed to plasmid spread in the recipient mycelia, like the *spd* gene of pIJ101.

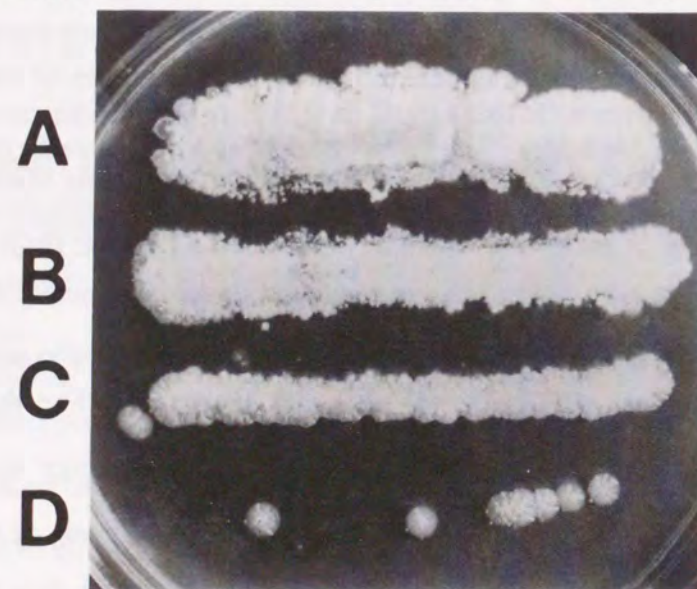


Figure I-5. Spreading patterns of mutated plasmids derived from pSN22 in recipient mycelial cells. Loopfuls of spore suspensions (10^5 /ml) of strain TK21 containing pMT911 (wild-type: A), pMT921 (*spdA*⁻: B), pMT926 (*spdB*⁻: C), and pFD7 (*traA*⁻: D), respectively, were streaked in lines (about 1 mm width) on a confluent lawn of spores of recipient strain TK24. After a 5 day incubation, the resulting spores were replica plated to R2YE medium containing thiostrepton and streptomycin.

The *traA*⁻ plasmid, pFD7, formed no pocks, but was transmissible at a low efficiency (5.0×10^{-2}). The replica method confirmed that the *traA* plasmid was transmissible, but no plasmid spread in the recipient mycelia was apparent.

Co-transfer of a non-transmissible plasmid along with pSN22 derivatives As pSN22 and pMT911 were transferred to recipient cells at a high frequency, we tested whether pSN22 derivatives could mobilize a non-transmissible plasmid.

Table I-2. Mobilization of non-transmissible plasmids mediated by the pSN22 derivatives

| Cross ^a | Plasmids | Selected phenotype | Number of colonies | Efficiency of plasmid transfer |
|--------------------|------------------|--------------------------------------------------------|--------------------|--------------------------------|
| 1 | pESS500, pIJM364 | Tsr ^r | 8.5×10^6 | 1.6 |
| | | Tsr ^r , Str ^r | 5.2×10^6 | |
| | | Vio ^r | 3.8×10^7 | 2.0×10^{-2} |
| | | Vio ^r , Str ^r | 7.2×10^5 | |
| | | Tsr ^r , Vio ^r | 1.9×10^6 | 5.5×10^{-2} |
| | | Tsr ^r , Vio ^r , Str ^r | 1.0×10^5 | |
| 2 | pMT911, pSND6 | Tsr ^r | 7.4×10^6 | 4.3 |
| | | Tsr ^r , Str ^r | 6.0×10^6 | |
| | | Vio ^r | 2.0×10^6 | 2.7×10^{-3} |
| | | Vio ^r , Str ^r | 5.3×10^3 | |
| | | Tsr ^r , Vio ^r | 1.8×10^6 | 2.8×10^{-3} |
| | | Tsr ^r , Vio ^r , Str ^r | 5.0×10^3 | |

The efficiency of plasmid transfer was measured by spreading the spores of a donor strain, TK21, harboring a self-transmissible derivative of pSN22 (*tsr*) and a non-transmissible plasmid (*vph*), and spores of a recipient, strain TK24 (*str-6*), together on R2YE agar medium. The phenotypes of the generated spores were tested by spreading at appropriate dilutions on YEME medium containing combinations of thiostrepton, streptomycin and viomycin.
a: Crosses were carried out under crossing condition A (see Materials and Methods).
b: The efficiency of plasmid transfer was determined as in Table I-1.

A non-transmissible derivative of pIJ364, pIJM364 (*vph* Δ [*tra spd*]; Fig. I-2C), and a non-transmissible pSN22 derivative, pSND6 (*vph* Δ [*spdA traR traA traB spdB*]; Fig. I-2C), were used for the co-transfer tests with pSN22 derivatives pESS500 and pMT911, which have a complete set of the genes needed for plasmid transfer. The donor strain, TK21, harboring pIJM364 (or pSND6) and pESS500 (or pMT911) was mixed with the recipient strain TK24 (*str-6*) under crossing condition B and cultivated on R2YE medium without antibiotics. The spores generated were then spread on selective media containing thiostrepton, thiostrepton and streptomycin, viomycin, viomycin and streptomycin, or thiostrepton, viomycin and streptomycin. Since *Str^r Vph^r* colonies were obtained at a high frequency, almost the same as that of *Str^r Tsr^r Vph^r* colonies, the non-transmissible plasmids, pIJM364 and pSND6, were considered to be mobilized along with pESS500 or pMT911, respectively, and coexisted in strain TK24 with these transmissible plasmids (Table I-2).

The plasmid transfer efficiencies of pIJM364 and pSND6 without the coexistence of transmissible plasmids were less than 1×10^{-7} . The co-transfer efficiency was, however, lower than the plasmid-transfer efficiency of pESS500 or pMT911, and almost identical to the efficiency of the *traA* mutant, which lacked the spread function (Table I-1). This suggested that the spread of the non-transmissible plasmids in the recipient mycelia was not supported by *spdA* and *spdB* genes encoded on the transmissible plasmid, and that the *spdA* and *spdB* genes might only be effective in *cis*, although the gene products essential for plasmid transfer functioned in *trans*. It is noteworthy that the two plasmids co-existed independently in the host strain without recombination in the host, since the result of Southern hybridization using the 1.3 kbp *PstI-BamHI vph* fragment as a probe showed no presence of a co-integrated plasmid or a modified plasmids in the co-transferred cells (data not shown).

Enhancement of the inter-chromosomal recombination by pSN22 derivatives Spores of strain TK21 (prototroph) harboring various thiostrepton-resistant pSN22 derivatives were mixed with an excess of the recipient spores of strain TKC244 (*thr-1 str-6*). Spores generated from this cross were plated onto selective media, i.e., a modified R2YE medium supplemented with thiostrepton and streptomycin, and on minimal medium with streptomycin (Table I-3). The absolute number of *Tsr^r*, *Str^r* spores which are TKC244

carrying a transferred plasmid was affected by the mutations in *spdB* and *traA* similar to the results shown in Table I-1, whereas the absolute number of chromosomal recombinants (*Thr⁺*, *Str^r*) was not. It is noteworthy that even the *traA* mutation, which reduced plasmid transfer remarkably, did not reduce chromosomal recombination. On the other hand, the *traB* mutation abolished chromosomal recombination, and recombination between the plasmid-free strains was scarcely detectable, suggesting that the self-transmissible plasmids enhance chromosomal recombination through the *traB* function. The fact that the ratios of the plasmid-transferred recipients to the recombinant colonies were reduced by the *spdA*, *spdB* and *traA* mutations accords well with the plasmid spread abilities tested by replica assay (Fig. I-5), indicating that the plasmids may be spread in recipient mycelia by the *spdA* and *spdB* functions, and also by the *traA* function.

Table I-3. Inter-chromosomal recombination and plasmid transfer

| Plasmid | Genotype | Number of colonies ^a growing on | | | Plasmid-transferred colonies/ recombinant colonies ^b |
|------------|-------------------------|--------------------------------------------|-------------------|-------------------|--------------------------------------------------------------------|
| | | Tsr | Tsr, Str, Thr | Str | |
| pMT911 | wild | 3.7×10^7 | 3.2×10^7 | 2.6×10^2 | 1.2×10^5 |
| pMT921 | <i>spdA⁻</i> | 4.9×10^7 | 3.4×10^7 | 1.3×10^3 | 2.6×10^4 |
| pMT926 | <i>spdB⁻</i> | 9.0×10^6 | 3.0×10^6 | 5.7×10^3 | 5.2×10^2 |
| pFD7 | <i>traA⁻</i> | 5.1×10^6 | 6.1×10^4 | 5.6×10^2 | 1.1×10^2 |
| pFD11 | <i>traB⁻</i> | 3.0×10^6 | 8 | < 1 | - |
| No plasmid | - | - | - | < 1 | - |

a: Spores of a donor, strain TK21, carrying a pSN22 derivative (*tsr*) were spread on the lawn of spores of a recipient, strain TKC244, under crossing condition A. The phenotypes of generated spores were tested by spreading on YEME media containing thiostrepton, and thiostrepton and streptomycin, and MM medium supplemented with streptomycin.

b: The number of recipient colonies which received a plasmid (growing on Str+Tsr+Thr but not on Tsr alone) divided by the number of chromosomal recombinant colonies growing on Str.

DISCUSSION

We have isolated an 11.0 kbp self-transmissible and pock-forming plasmid, pSN22, from *S. nigrifaciens*. The plasmid was maintained stably in *S. lividans* TK21. The mutational analysis of pSN22 in *S. lividans* TK21 indicated that at least five genes are involved in plasmid transfer of pSN22. The *spdA*⁻ and *spdB*⁻ mutations reduced the pock size (Fig. I-3). The *spdB*⁻ phenotype of pSN22 was similar to the Spd⁻ phenotype of pIJ101, whereas no phenotype of pIJ101 like *spdA*⁻ of pSN22 has been reported. The replica assay (Fig. I-5) also showed that the extent of plasmid spread by *spdA*⁻ and *spdB*⁻ plasmids transferred in recipient mycelia was reduced similarly to their pock size. The Spd⁻ phenotypes were considered to be caused by reduced intramycelial plasmid-transfer. These results suggested that intramycelial transfer of pSN22 may be accomplished through at least two steps.

The *traB*⁻ mutations abolished both pock-formation and plasmid transfer. On the other hand, the mutation of *traA* abolished pock-formation, but reduced plasmid transfer by a factor of 5.2×10^{-2} which is well above the background level observed with *traB* mutants (Table I-1). The result of the replica assay (Fig. I-5) shows that the *traA*⁻ mutation affected the initial step of intracellular plasmid transfer, but not the conjugation frequency of the donor and recipient cells, since the chromosomal recombination was not affected by the *traA*⁻ mutation (Table I-2). This indicates that intermycelial transfer of pSN22 is independent of pock-formation. Weber et al. (1979) observed high-frequency chromosomal recombination in crosses of mutants of *S. erythreus*. They could not, however, detect any pock-formation, leaving the possibility that a conjugative plasmid unable to form pocks similar to our *traA*⁻ mutant plasmid pFD7 or a gene like *traB* without *traA* encoded in the chromosome may contribute in high-frequency chromosomal recombination. The Kil phenotype caused by the mutation in *traR* was defined by the inability to transform *S. lividans*. The inviable mutations are probably analogous to the *kor* mutations of pIJ101 and RK2 (Figurski et al. 1982). Similar mutations were observed in pSK2 isolated from *S. kasugaensis* (H. Akagawa, personal communication), indicating that they may be a general feature of pock-forming plasmids in *Streptomyces*.

To summarize the results, I propose a putative model of pock-formation and plasmid transfer (Fig. I-6). The co-transfer of non-transmissible plasmids and the enhancement of chromosomal recombination mediated by the transmissible plasmids suggest strongly that plasmid transfer accompanies the cytoplasmic mixing through cell fusion, which may contribute to increase genetical diversity among *Streptomyces* strains. The *traB* gene mediates the first step of plasmid transfer by enhancing cell fusions where donor and recipient cells meet. Following cell fusion, the *traA* gene promotes the initial step of intramycelial plasmid transfer, since the plasmid mutated in *traA* could be transferred to, but not spread within the recipient mycelia. If the pock-formation is a result of growth delay of recipient cells, as suggested by Bibb et al. (1977) and Hopwood et al. (1973), the gene might also be responsible for growth inhibition of the recipient cells. Plasmid within the recipient mycelia seems to spread further under the control of the *spdA* and *spdB* genes. The *traR* gene, which is considered to be related to the *kil* phenotype, might be involved in the regulation of *traA* and *traB* genes.

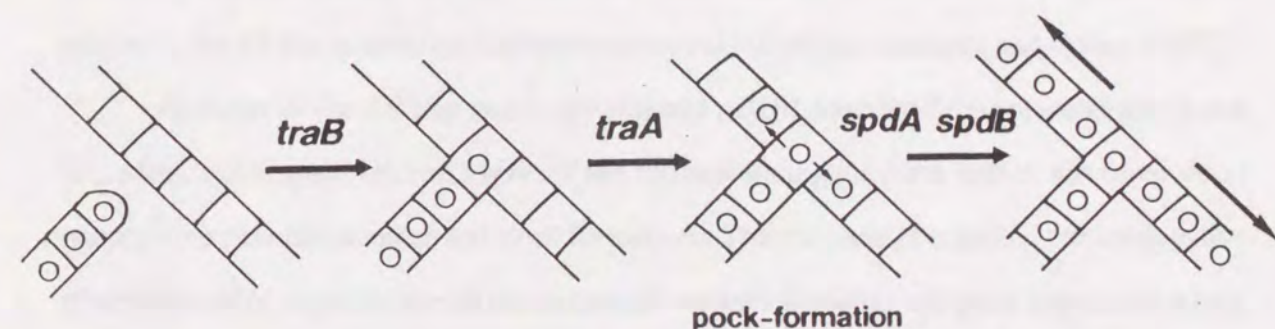


Figure I-6. Putative model for the functions of the genes involved in plasmid transfer. The mycelia of donor and recipient strains grow independently, and the *traB* gene seems to stimulate the contact and fusion of donor and recipient mycelia, required for plasmid transfer. The *traA* gene might be required for initial plasmid transfer within recipient mycelia, while *spdA* and *spdB* genes are considered to be needed for the further spread of the plasmid molecules in recipient mycelia, in which compartments separated by septa are illustrated putatively to be uni-chromosomal.

SUMMARY

A 11 kbp multi-copy plasmid, pSN22, was isolated from *Streptomyces nigrifaciens* SN22. pSN22 is self-transmissible (conjugative), maintained stably in *S. lividans* TK21, and forms pocks in a wide range of *Streptomyces* strains. Mutational analyses showed that a fragment of pSN22 contained five genes involved in plasmid transfer and pock-formation. *traB* was essential for plasmid transfer. *traA* was required for pock-formation, but not for plasmid transfer. Mutation of *spdA* or *spdB* decreased the pock size. The fifth gene, *traR*, could be deleted together with other genes to give non-transmissible plasmids, but plasmids with insertions/deletions only within *traR* became non-viable. *traR* is probably needed to counterbalance the lethal effects of another plasmid gene. The plasmid transfer of pSN22 promoted the co-transfer of non-transmissible plasmids and enhanced the chromosome recombination between the host and recipient strains, suggesting that plasmid transfer accompanied cytoplasmic mixing.

Chapter II

Regulation and function of the genes involved in pock-formation and inviable phenotype of a *Streptomyces* plasmid pSN22

INTRODUCTION

Chapter I described identification of five genes on pSN22 which are involved in plasmid transfer and pock formation. Three of them, *traA*, *traB* and *traR*, are essential for pock formation. Plasmids mutated at the *traR* locus could not be introduced into *S. lividans* by transformation.

This phenomenon, a mutation on a plasmid out side of essential region introduced inviability to the plasmid or its host, was first reported for an Inc P group plasmid, RK2, in *E. coli* (Figurski et al. 1982). The concept of the inviability is that the existence of both potentially lethal gene and supressor gene on lethality. The supressor gene called *kor* (*kil* override) is essential for counterbalance of the lethal gene called *kil*. In *Streptomyces* plasmids, the *kil-kor* interaction has been reported about pIJ101 (Kendall and Cohen 1987).

Analysis of the 8.9 kbp multicopy plasmid pIJ101 identified four plasmid genes, *tra* (transfer), *spdA*, *spdB* (plasmid spread) and the repressor gene *korA*, which are involved in conjugative plasmid transfer and pock formation. The *tra* gene is essential for conjugative plasmid transfer, mobilization of chromosomal markers (fertility) and pock formation, which is the result of extensive plasmid transfer. *korA*, which may be supplied either in *cis* or in *trans*, cannot be inactivated if the *kilA* function, which maps to the amino terminus of the *tra* gene, is present in the same cells. It is not known if the lethal *kilA* function resides on a protein or an RNA.

In the case of pSN22, *traR* could not be inactivated in plasmids which expressed *traA* and *traB*. This lethal effect is thought to be corresponding to the *kil-kor* interaction for RK2 and pIJ101. In deletion analyses in Chapter I, pMT925 which had a deletion of the *traR* locus but not *traA* and *traB* was viable and I discussed that the phenotype was due to a polar effect.

This suggests that the *kil-kor* interaction of pSN22 is originated by disorder of the transcriptional regulation of transfer relating genes like to pIJ101.

In this chapter, I describe a further analysis of the *traR*, *traA* and *traB* region of pSN22 with focusing on the *kil-kor* interaction. I also present the transcriptional regulation of transfer-related gene of pSN22.

MATERIALS AND METHODS

Bacterial strains and plasmids *S. lividans* strains and *E. coli* strains used in this chapter were described in chapter I. *Streptomyces* plasmids, pMT5 and pMT6, used in this chapter were derivatives of pSN22 (Fig. II-1), and a promoter-probe plasmid, pARC1 (Horinouchi and Beppu 1985), a gift from T. Beppu (University of Tokyo). *E. coli* plasmids, pUC12, pUC19 and pBluescript II SK(-) (Stratagene) were used for subcloning or in vitro synthesis of RNA probes.

Media and cultivations Media and cultivations of *S. lividans* and *E. coli* were same as chapter I. Cell growth of *S. lividans* in YEME medium was monitored by measuring optical density at 660nm (OD₆₆₀). Cells from exponentially growing culture were used to inoculate into fresh YEME medium to give an initial OD₆₆₀ of 0.05.

DNA isolation, manipulation and transformation Plasmid isolation from *E. coli* and *in vitro* manipulations of DNA were according to the method of Sambrook et al. Plasmid isolation from *S. lividans* followed the method of Kieser (1984). DNA fragments digested with restriction enzymes were separated on agarose by electrophoresis and extracted by Gene clean II (Bio 101). Other procedures of DNA recombination used in this chapter were the same as Chapter I.

Promoter activity *In vivo* promoter activity was tested with the promoter-probe plasmid, pARC1, which develops a brown pigment when a promoter fragment is inserted into the *Bam*HI site upstream of a part of *whiG* genes (Davis and Chater 1990) originated from *S. coelicolor*. Transformants harboring pARC1 with a promoter-insert were recognized by the production of brown pigment on R2YE medium containing thiopeptine. The activity of the

inserted promoter was estimated by measuring the intensity of pigment color produced in liquid YEME medium containing thiopeptine; the culture broth was mixed with an equal volume of acetone, and after centrifugation the optical density of the acetone extract was measured at 400nm.

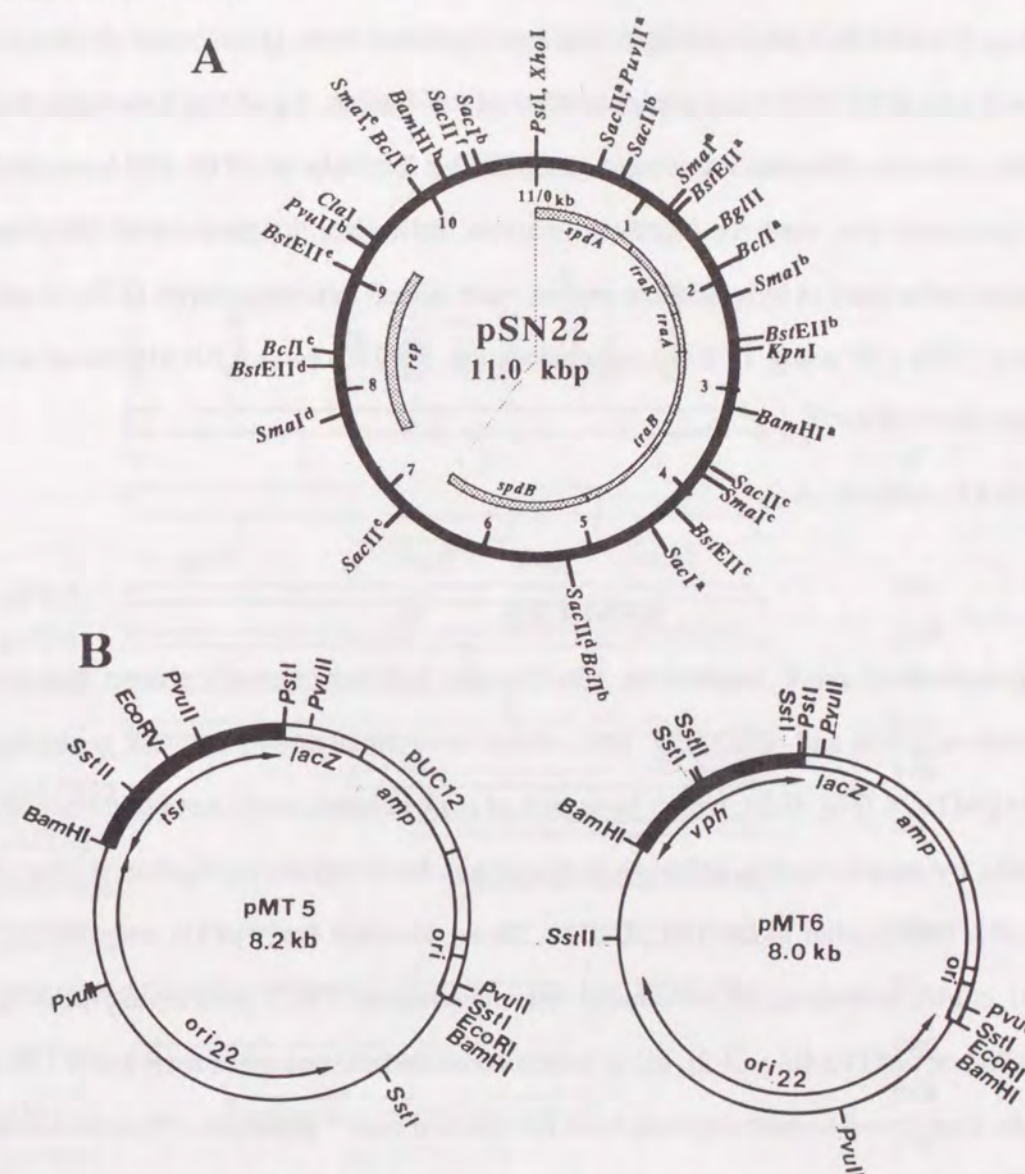


Figure II-1. Physical maps of *Streptomyces* plasmids used in this chapter. A, physical and functional map of pSN22. The rep region is essential for plasmid replication, and other regions are related to plasmid transfer and pock formation (see text). B, shuttle vectors, pMT5 and pMT6 which replicate with the origin of pSN22 in *S. lividans* and with that of pUC12 in *E. coli*, respectively. For the constructions of these plasmids the 1.6 kbp *Bst*EII (flushed by T4 polymerase)-*Bam*HI fragment of pIJ6 (Thompson et al. 1980) containing the *tsr* (thiostrepton resistance) gene or the 1.4 kbp *Pst*I-*Bam*HI fragment of pIJ364 (Kieser et al. 1982) containing the *vph* (viomycin resistance) gene were ligated with the *Hinc*II-*Bam*HI and *Pst*I-*Bam*HI fragments of pUC12. Then the 4 kbp *Bam*HI^a- Δ (*Sac*II^c-*Sac*II^e)-*Bam*HI^b fragment of the pSN22 derivative pESS507 (Chapter I) was inserted into the *Bam*HI site of the above plasmid to give pMT5 or pMT6.

RNA isolation and Northern blotting RNA was prepared from *S. lividans* as follows. Strains were either cultivated on a nitrocellulose filter (Millipore HA 0.45 μ m) which was placed on R2YE medium and harvested by scraping with a spatula, or in YEME medium and harvested by filtration. Cells were resuspended in PSE buffer (10 % phenol, 2 % SDS, 0.1 M EDTA, 50 mM Tris-HCl, pH 8.0) and homogenized with glass beads (5 mm in diameter) for 3 min at 4°C on a Vortex mixer. After centrifugation, 1 g of CsCl was added to 1 ml of lysate. The sample was centrifuged at 120,000 *g* for 20 hr at 20°C. RNA isolated from those gradients was used for Northern analysis, following the method of Thomas. DNA sequences to be used as hybridization probes were cloned into pBluescript II SK(-) and labeled with α -[³²P]-CTP using T7 RNA polymerase (ca. 5×10^7 cpm/ μ g RNA) (Studier and Moffatt 1986).

RESULTS

Suppression of *traR* mutations In Chapter I, I have already shown that the pSN22 derivatives pFD4 and pFD5 (Fig. II-2), which have mutations in the *traR* gene, and pMT923 and pMT924 (Fig. II-2), which have part of *traR* deleted, could not be introduced into *S. lividans* by transformation although they contain the complete replication region of pSN22. *traR* is thus similar to *korA* of pLJ101. Those plasmids (only pFD4 and pMT924, were tested) could, however, be introduced into *S. lividans* TK21 containing pSN22, pMT61, pMT62 or pMT63 (Fig. II-2), all of which contained the complete *traR* gene. The *traR* plasmids were found to exist separate from the resident *traR*⁺ plasmids. This indicated that the *traR* gene product acted in *trans*. The smallest DNA fragment providing *traR* function was the 0.9 kbp *Sma*I fragment presented in pMT63. The *traR* plasmids could not be introduced into *S. lividans* containing pLJ101, indicating that neither *korA* nor *korB* can replace *traR* of pSN22.

Localization of the lethal gene of pSN22 To limit the region determining the inviability of *traR* plasmid, various DNA fragments from the *tra* region of pSN22 were

equipped with *Pst*I linkers, d(5'-GCTGCAGC-3'), and cloned into *Pst*I-digested and dephosphorylated pMT5 (Fig. II-1). The constructs were first isolated from *E. coli* JM109 and then used to transform *S. lividans* TK21. pMT515 (Fig. II-3) which carried the complete *traA* and *traB* loci on a 3 kbp *Bcl*I fragment, but lacks *traR*, failed to give transformants. the plasmids pMT511, pMT512, pMT513 and pMT514 (Fig. II-3) which lack *traR* and part or all of *traB* could be introduced into TK21 with low transformation efficiency of 3-10 % compared to the efficiency obtained with *traR*⁺ plasmids. The resulting colonies were unusually small.

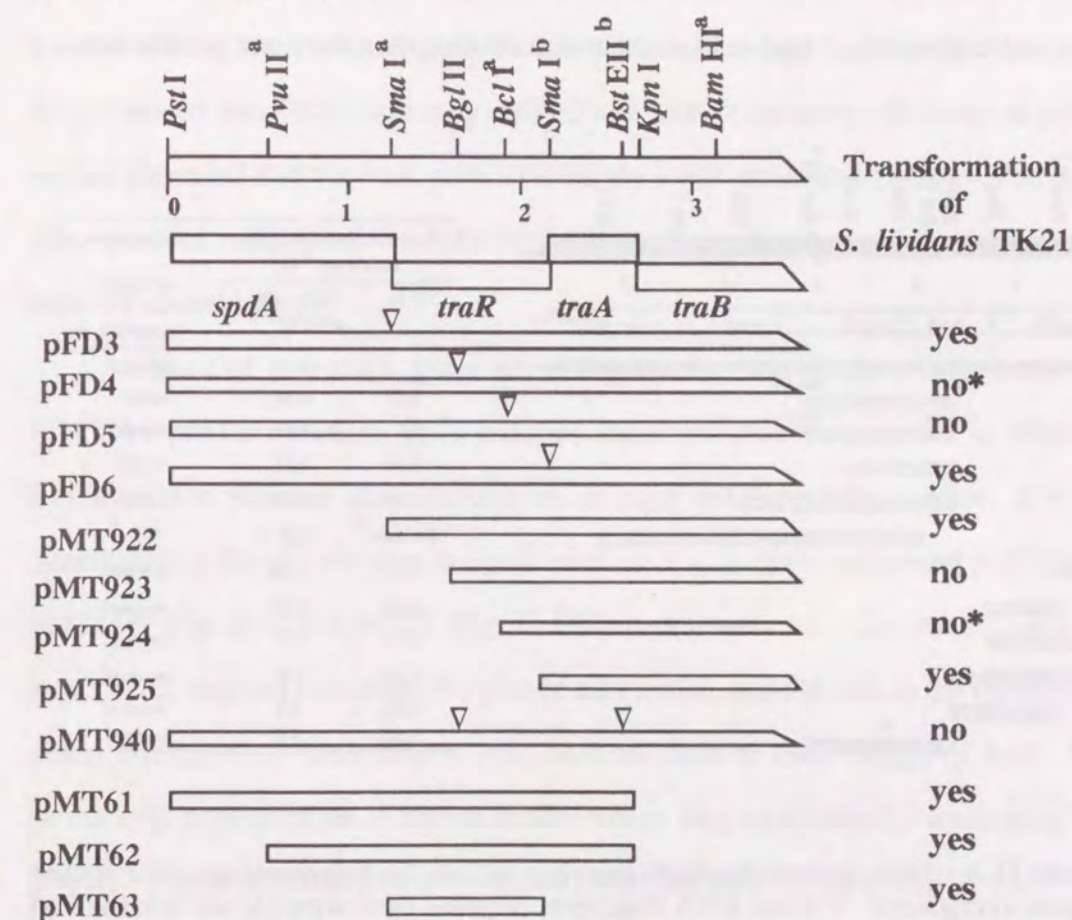


Figure II-2. Restriction map of the pSN22 *tra* region and structures of mutant plasmids which were tested for their ability to transform *S. lividans*. The *tra* genes were defined by mutational analyses (Chapter I). Bars indicate DNA which is present in the named plasmids. The pFD plasmids carried the whole *tra* region, but had 4 or 8 base insertions potentially causing frame-shift mutations at the indicated position (reverse triangle). The pMT922-pMT925 plasmids had a part of the *tra* regions deleted. pMT940 has 4 and 5 bp insertions at the *Bgl*III and *Bst*EII sites, respectively. For the construction of pMT61, pMT62 and pMT63, the indicated DNA fragments of the *tra* region were subcloned into pUC19, cut out with *Hind*III and *Eco*RI, and ligated with the *Hind*III-*Eco*RI fragment of pMT6 containing the replication region of pSN22. Transformation ability of those plasmids was tested using host strain TK21. * pFD4 and pMT924 gave thiostrepton resistant transformants of TK21 harboring viomycin-resistant pMT61, pMT62 or pMT63.

pMT925 (Fig. II-2) which lacks the complete *traR* locus (*Sma*I^a-*Sma*I^b fragment), but contains *traA* and *traB*, could be introduced into TK21 by transformation in the absence of *traR*. As described below, however, the *traA* and *traB* genes in pMT925 may not be expressed because their promoter was deleted. pMT940 (Fig. II-2) which contains inserts in both *traR* and *traA* could not be introduced into TK21 by transformation, presumably because it contains a functional *traR* gene. The plasmids pMT516-pMT520 (Fig. II-3) could be introduced into TK21 with the same frequency as the parental plasmid pMT5.

These results indicate that, in the absence of the regulatory gene *traR*, expression of *traB* is lethal and expression of *traA* reduces the transformation frequency and growth rate.

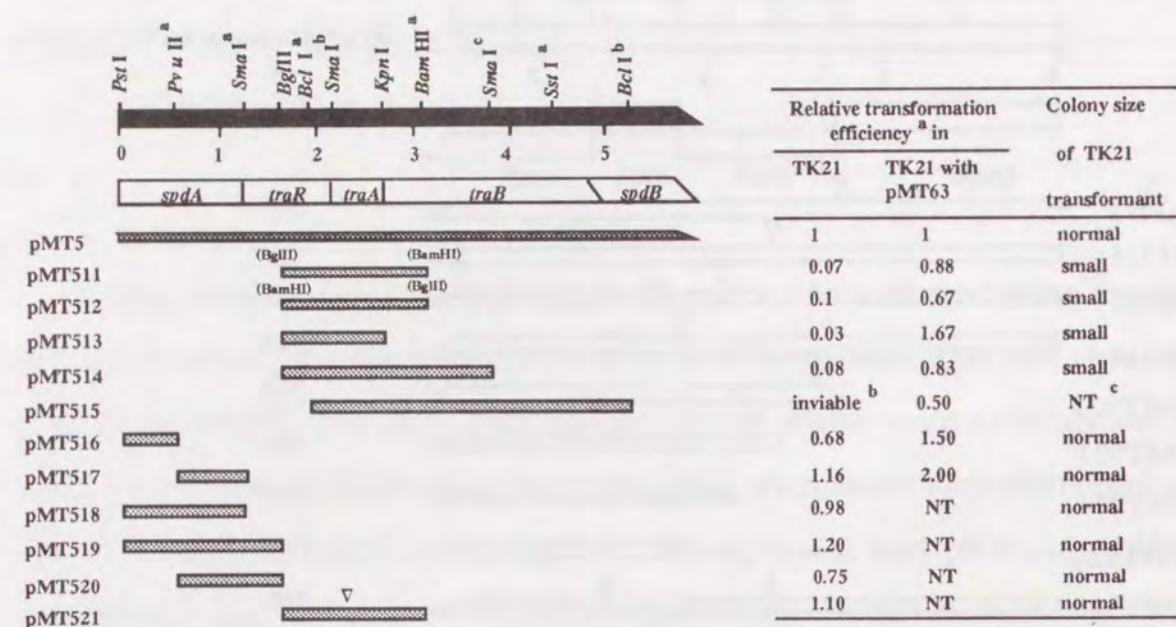


Figure II-3. Structures of plasmids carrying various *tra* fragments used for testing *traR*-*traA* gene interactions. Various DNA fragments (stippled bars) were cloned into the *Pst*I site of pMT5 (Fig. II-1) followed by the addition of *Pst*I linkers. In pMT511 and pMT512, the *Bgl*II-*Bam*HI^a fragment was cloned in different orientations, respectively. pMT521 derived from pMT511 had a potential frame shift at the *Bst*EII site (reverse triangle) by a 5 base insertion. a; *S. lividans* TK21 in the absence or presence of the *traR*⁺ plasmid pMT63 was transformed with DNA of the respective plasmids isolated from *E. coli*. Transformation efficiency was calculated as an average of three transformation using 0.01, 0.1 and 1 μ g of plasmid DNA and the same preparation of protoplasts. The transformation efficiency of pMT5 was taken as 1. Absolute transformation efficiency of TK21 with pMT5 was $3.6 \times 10^4/\mu$ g DNA, and that of TK21 harboring pMT63 was $1.8 \times 10^4/\mu$ g DNA. b; inviable phenotype, and c; not tested.

Effect of the *traA* gene on the transformation The *traA*⁺ *traR*⁻ *traB*⁻ plasmids, pMT511, pMT512, pMT513 and pMT514, isolated from *E. coli* JM109 gave a 10 to 30-fold lower transformation frequency than *traR*⁺ plasmids (Fig. II-3). When, however, transformations were carried out with a host strain, TK21, harboring the *traR*⁺ plasmid pMT63 (Fig. II-2), the transformation efficiencies obtained with the above plasmids (pMT512-pMT514) was like that of *traR*⁺ plasmid. pMT521 which is pMT511 with a 5 base insertion in the *traA* locus showed normal transformation efficiency and also normal growth rate. pMT515 (*traR*⁻ *traA*⁺ *traB*⁺) could not be introduced into TK21 by transformation, even when high DNA concentrations (5 μ g) were used. It could, however, be introduced into TK21 harboring pMT63 with almost the same efficiency as pMT5. These results indicated that the *traA* gene affected the transformation efficiency, and that the *traR* gene-product suppressed the inhibition of transformation caused by *traA* as well as the lethality caused by *traB*.

Effect of the *traA* gene on cell growth The *traR*⁻ *traA*⁺ *traB*⁻ plasmids- (pMT511-pMT514) which gave reduced transformation frequencies in plasmid-free *S. lividans* also showed slow growth on an agar medium. To confirm this phenotype quantitatively, the growth rates in liquid medium of *S. lividans* containing pMT511, pMT512, pMT518 (Fig. II-3) and pMT5 (Fig. II-1B), were measured. The strains were inoculated into YEME medium containing 5 μ g/ml of thiopeptine, and the cells in the logarithmic growth phase ($OD_{660}=0.5$) were diluted into fresh medium to final OD_{660} of 0.05. Figure II-4 shows cell growth of the different strains which was monitored by measuring the optical density. The growth rate of transformants harboring pMT511 or pMT512 (*traA*⁺ *traR*⁻) was about half of those harboring pMT5 or pMT518 (*traA*⁻ *traR*⁻). The strains containing pMT511 and pMT512 which differ only in the orientation of *traA* consistently grew with different growth rates as shown in Fig. II-4.

S. lividans containing both pMT511 and pMT63 (*traR*⁺) grew with the same rate as *S. lividans* harboring pMT5. This result suggested that the *traR* gene product was able to suppress both the growth inhibition and the decrease of transformation efficiency caused by

traA in the absence of *traR*. Growth inhibition by the *traA* gene may be important for pock-formation since *traA* plasmids were self-transmissible, but did not form pocks (Chapter I).

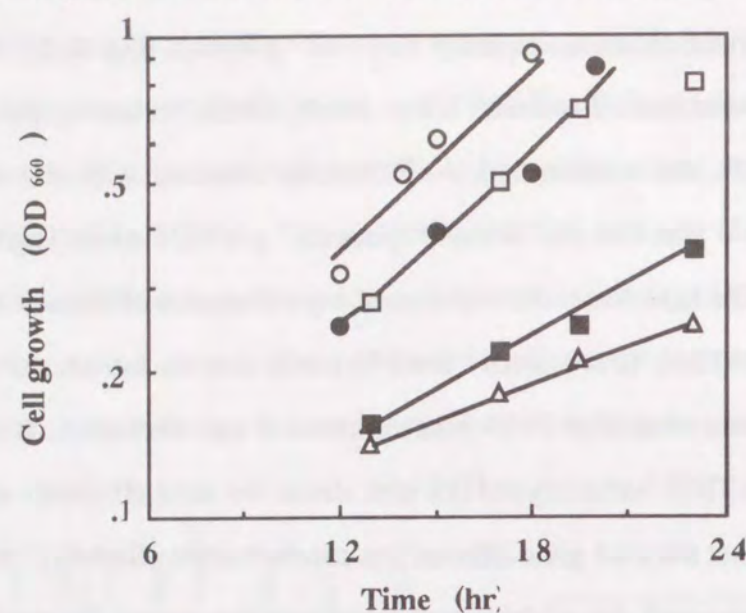


Figure II-4. Cell growth of the $\Delta traR^- traA^+$ plasmids with/without the *traR*⁺ plasmid, pMT63. Transformants with various plasmids are shown with the following symbols: filled circle; pMT5, blanked circle; pMT518, filled square; pMT511, blanked triangle; pMT512, and blanked square; pMT511 and pMT63.

Promoters of the *tra* region which are repressed by the *traR* function

The promoter activities in the *tra* region were investigated by using the promoter-probe vector pARC1. Various fragments from the *Pst*I-*Sac*II^e region of pSN22 (see Fig. II-1) were cloned into the unique *Bam*HI site of pARC1, after tailing with *Bam*HI linkers, d(5'-CGGATCCG-3'). We detected promoter activity in both orientations from the *Bgl*III-*Sma*I^b fragment cloned in pPT1 and pPT2 (Fig. II-5). The promoter reading towards *traR* (detected by pPT2) was designated P-*traR*, and the promoter which was reading towards *traA* (detected by pPT1) was called P-*traA*. The activity of P-*traA* monitored by brown-pigment production in liquid YEME medium was 4 times higher than that of P-*traR* (Fig. II-6). A third promoter, P-*spdA*, which might be associated with the *spdA* gene was detected in the *Pst*I-*Pvu*II^a fragment in the same orientation as P-*traA*, but the activity was lower than that of P-*traA* (data

not shown). No other promoter activity was detected with fragments from the *traB* and *spdB* regions.

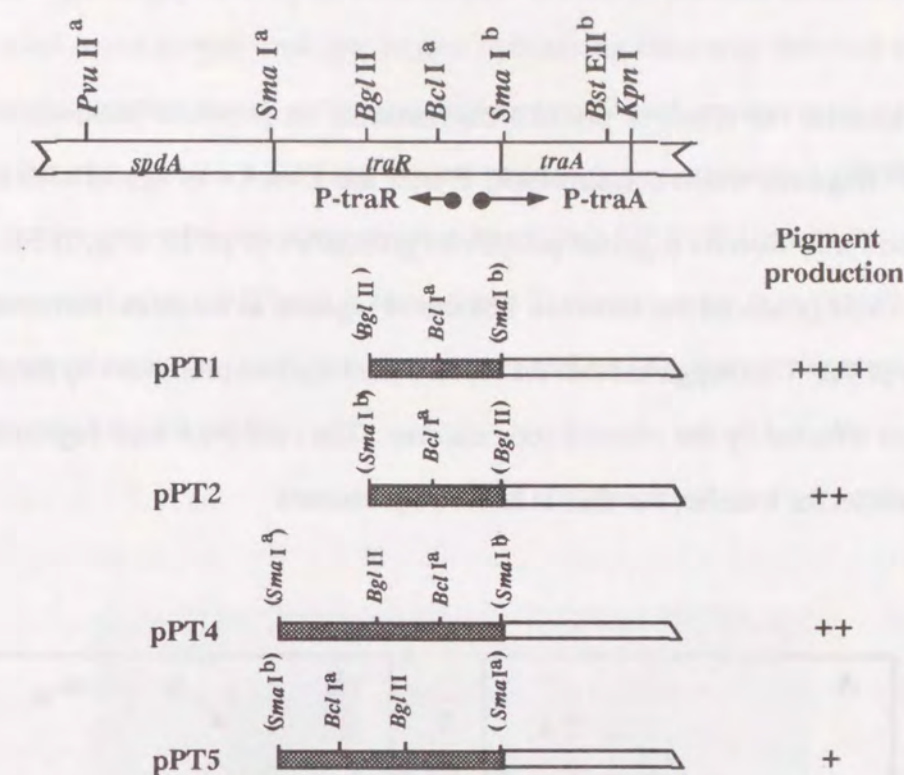


Figure II-5. pARC1 derivatives used to test promoter activity. The *Bgl*III-*Sma*I^b and *Sma*I^a-*Sma*I^b fragments (shown with shaded lines) in the *traR* locus were cloned into *Bam*HI site of pARC1. Open bars indicate the brown-pigment producing gene(s) of pARC1. P-*traA* and P-*traR* are putative promoters for the *traA* and *traR* genes. Pigment-production was tested on a solid medium.

Repression of P-*traA* and P-*traR* by the *traR* gene Since, as mentioned above, the *traR* gene seemed to affect the *traA* function, the activities of P-*traA* and P-*traR* were tested in the presence of the *traR* gene. Strain TK21 harboring the pARC1 derivatives pPT1 or pPT2 together with pMT63 (*traR*⁺, high copy number) were constructed by conjugating pPT1 or pPT2 into TK21 containing pMT63. The promoter activities in those strains were estimated by the intensity of pigment production (Fig. II-6). pMT63 reduced pigment production from both pPT1 and pPT2 by about five-fold, but did not affect the growth of the strains. pMT62, which provides *traR* and *traA* at high copy number (Fig. II-2), gave an even stronger reduction of pigment production, indicating that *traA* might enhance the repressor function of *traR*, or that *traR* might not fall entirely within the *Sma*I^a-*Sma*I^b

fragment in pMT63. Northern analysis confirmed that the different level of pigment production correlated with the amount of mRNA from the pARC1 pigment genes (data not shown).

To elucidate the effect of plasmid copy-number on promoter intensity, the 0.9-kb *Sma*I^a-*Sma*I^b fragment which contains *traR*, P-*traR* and P-*traA* was ligated with the help of *Bam*HI linkers into *Bam*HI digested pARC1 to give pPT4 or pPT5 (Fig. II-5). The two plasmids in TK21 produced the same low amount of pigment as the strain harboring pMT63 and pPT1 or pPT2. This suggested that the repression of the two promoters by the *traR* gene-product is not affected by the plasmid copy number. The *traR*-*traA*-*traB* region of pSN22, which is essential for transfer, has thus at least two promoters.

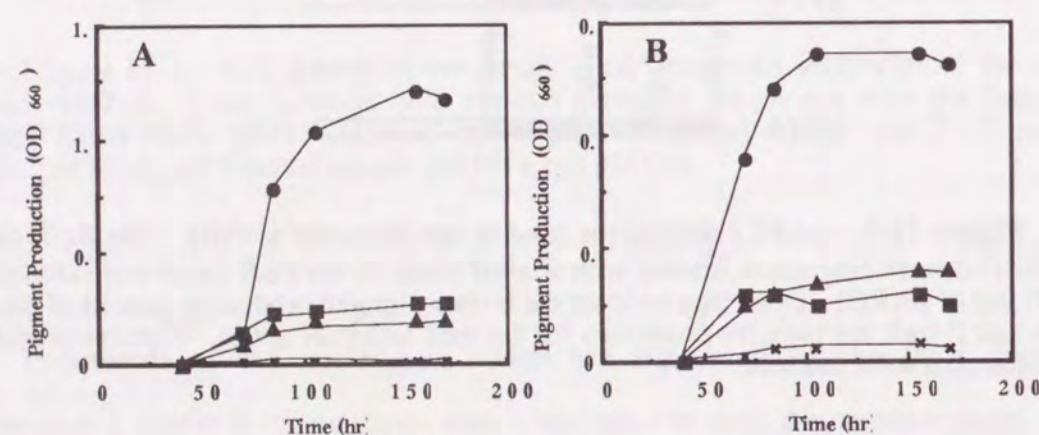


Figure II-6. Pigment productions in liquid medium through P-*traA* (A) and P-*traB* (B). A: Pigment production by strain TK21 harboring pPT1 (circle), pPT1 and pMT63 (square), pPT4 (triangle) and pPT1 and pMT63 (x). B: Pigment production by TK21 harboring pPT2 (circle), pPT2 and pMT63 (square), pPT5 (triangle) and pPT2 and pMT63 (x).

Detection of mRNA originating from P-*traA* and P-*traR* Total RNA was isolated from TK21 and from TK21 harboring pMT911 (Chapter I) which carried the whole set of the genes for plasmid transfer and pock-formation. Ten μ g of total RNA were separated on an agarose gel, transferred onto a nylon membrane and hybridized with specific ribo-probes (see Materials and Methods). A probe corresponding to the *Sma*I^a-*Bgl*III fragment which covered a part of the *traR* gene hybridized to a 1.0 kb RNA (Fig. II-7, lanes 5

and 6), which may be a transcriptional product of the *traR* gene. A minor signal was also detected at the 0.8 kb position. When hybridized with the labelled *Sma*I^b-*Bam*HI^a fragment covering *traA* and a part of *traB*, the largest hybridizing band was detected at the 5.2 kb position, suggesting that the transcriptional product from P-*traA* covered *traA*, *traB* and *spdB*. Many smaller signals, which may correspond to degraded or attenuated mRNA, were also detected. It is noteworthy that the hybridization signals for an RNA sample isolated from mycelia on a solid medium (Fig. II-7, lanes 6 and 9) were stronger than those isolated from vegetative mycelia grown in a liquid medium (lanes 5 and 8), though the same amounts of RNA were used for each sample.

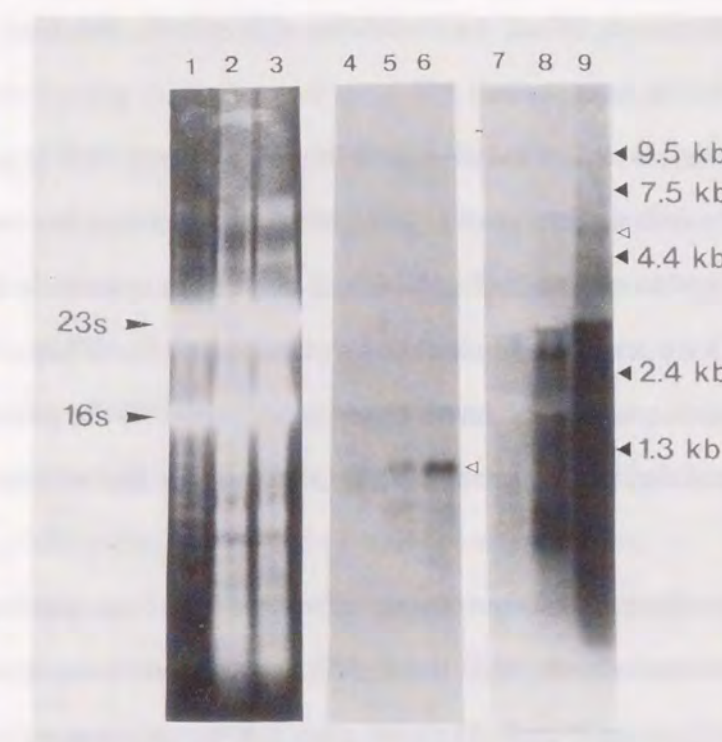


Figure II-7. Northern blot analyses of the transfer region of pSN22. Lanes 1, 2, and 3: RNA pattern stained directly. Lanes 4, 5, and 6: Northern hybridization using the ribo-probe covering the *Sma*I^b-*Bgl*III region. Lanes 7, 8, and 9: Northern hybridization using the probe covering the *Sma*I^b-*Bam*HI^a region. Lanes 1, 4 and 7: RNA extracted from strain TK21 on R2YE agar medium, lanes 2, 5 and 8: RNA from TK21 harboring pMT911 cultivated in YEME liquid medium, lanes 3, 6 and 9: RNA from TK21 harboring pMT911 on R2YE. Positions of mRNA detected were indicated with (blanked square). Molecular sizes of RNA on an agarose gel electrophoresis were estimated by using a size standard (filled triangle) (0.29-9.5 kb RNA ladder; Bethesda Research laboratories).

DISCUSSION

I have indicated the functions of five genes that are involved in plasmid transfer and pock formation by the *Streptomyces* plasmid, pSN22; the *traB* gene is essential for plasmid transfer and *traA* for pock formation, *traR* is a regulatory gene, and both *spdA* and *spdB* affect pocks size. In this chapter, I have focused on the function of the *traR* gene in relation to the regulation of *traA* and *traB* gene-expression. *traR* mutations were lethal (showed Kil phenotype), though the *traR* product could complement in trans (Fig. II-2). Deletion of *traB* (e.g., in pMT511, Fig. II-3), however, restored viability of *traR* mutations, suggesting that the *traB* gene is the inviability determinant. As shown in Fig. II-6A, the *traR* gene-product most probably regulated the expression of the gene(s) downstream of P-*traA*, which is the promoter of the *traA-traB-spdB* operon. These facts indicate that the excessive expression of the *traB* gene in *traR* mutants causes the inviability of host cells, resulting in the loss of transformation ability of the *traR*⁻ plasmid.

The excessive expression of the *traA* gene in the absence of *traR* (e.g., in pMT511; $\Delta traR traA^+ \Delta traB$) caused the inhibition of cell growth, which could be complemented in trans by *traR* on a co-resident plasmid (Figs. II-3 and 4). Previous results indicated that the *traA* gene is essential for pock formation but not for plasmid transfer. These results strongly suggest that the appropriate amount of the *traA* gene product inhibits the growth of recipient cells during plasmid transfer, making growth inhibition zones of recipient cells, the so-called pocks.

A promoter activity, P-*traR*, detected upstream of the *traR* locus was repressed by the *traR* gene-product in *cis* and in *trans* (Figs. II-5 and 6); the *traR* gene is considered to be auto-regulated as well as regulating P-*traA*. The *traR* gene and two promoters for the *traR* and *traA-traB-spdB* operon are contained within the *Sma*I^a-*Sma*I^b fragment. These characteristics seem to be similar to those of the cI repressor of lambda phage (Ptashne 1986) as well as the *korA* and *korB* promoters of pIJ101 (Stein et al. 1989).

P-*traA* and P-*traR* in pPT1 and pPT2, respectively, were repressed more severely by pMT62 carrying the *traR-traA* fragment than by pMT63 carrying the *traR* fragment (Figs. II-5

and 6). The different levels in promoter activity suggest that pMT63 has an incomplete *traR* gene or that the *traR* gene product acts cooperatively with the *traA* gene product. The mRNA specific for the *traR* and *traA-traB-spdB* genes were expressed at very low level under normal conditions, since I could detect mRNA by Northern hybridization only using highly radioactive ribo-probes. It seemed, however, that the expression was stronger in agar-grown mycelia than in liquid-grown culture. This is not surprising since conjugative plasmid transfer occurs on solid but not in liquid medium.

The arrangement and regulation of the *tra* genes of pSN22 are similar to those of pIJ101. The *traR* function of pSN22 is most probably the same as that of *korA* in pIJ101 since both genes are functioning as the auto-repressors and as repressors of their *tra* operons; the *traA-traB-spdB* operon in pSN22 and the *tra-spdA-spdB*-66aa (amino acid) ORF operon in pIJ101. *tra* mutations in both plasmids also show the Kil phenotype (Fig. II-2). Our data, however, show clearly that the *traB* gene is a determinant of Kil phenotype, and that overexpression of *traB* may cause the inviability of host cells, while in pIJ101 the *kilA* locus was considered to be include in the *tra* locus. I have not found the second *kil-kor* system analogous to *kilB/korB* genes of pIJ101. The function corresponding to *traA* of pSN22 was not observed in pIJ101. It is, however, possible to presume that the *kilB* gene has a similar function to that of pSN22 *traA*; the *kilB* mutations of pIJ101 are defective partially in their pocking reaction (Kendall and Cohen 1987), whereas the pSN22 *traA* mutations are defective completely in pock formation though they could be transmissible.

These results suggest a putative scheme for plasmid transfer and pock-formation as follows: the *traB* product expressed at the basal level stimulates the intracellular fusion of plasmid-harboring and plasmid-less cells, which causes a dilution of the *traR* gene-products causing derepression of the *traA* promoter. Overproduction of the *traA* and *traB* gene-products inhibits the growth of plasmid-receiving cells, resulting in pock formation. After some time, the *traR* gene-product will reach its normal level, and suppress the expression of the *traA* and *traB* genes. The difference between P-*traA* and P-*traR* activities may contribute to the differential process of plasmid transfer and pock formation.

SUMMARY

pSN22 is a 11 kbp multicopy plasmid from *Streptomyces nigrifaciens* which is being studied in *Streptomyces lividans*. A segment of about 7 kbp of pSN22 contains five genes involved in conjugation. Three of them, *traA*, *traB* and *traR* are essential for plasmid transfer and for the mobilization of chromosomal markers (fertility), while the remaining two genes, *spdA* and *spdB*, merely enhance the efficiency of plasmid transfer, resulting in the formation of larger pocks. *In vivo* promoter-probing experiments identified a 550 bp *Bgl*III-*Sma*I DNA fragment with promoter activity in both orientations; northern hybridization identified corresponding divergent transcripts of 1 and 5.2 kb for *traR* and the *traA-traB-spdB* operon, respectively. The *traR* gene product repressed its own transcription and also the transcription of the *traA-traB-spdB* operon. Plasmids containing a functional *traB* gene could not "survive" without *traR* being present in the same cell either in *cis* or in *trans*, presumably because unregulated expression of *traB* is lethal to the host. Plasmids with a functional *traA* gene but without *traR* gave a low transformation efficiency and inhibited the growth of host cells.

Chapter III

Replication of pSN22 through single stranded intermediates

INTRODUCTION

Numerous plasmids of Gram-positive bacteria replicate via single-stranded (ss) intermediates probably by rolling circle replication like ssDNA phages of *Escherichia coli* (Bass and Jansz 1988, Boe et al. 1989, Gruss and Ehrlich 1989). Extensive studies on the staphylococcal plasmids, pT181 and pC194, have identified and characterized the replication genes (Gros et al. 1987, Khan et al. 1988, Koepsel et al. 1985). Plasmid replication by the rolling circle mechanism requires a plus origin (*ori1*), a replication protein (Rep) and a minus origin (*ori2*). The Rep protein is thought to introduce a strand-specific nick at *ori1* and to terminate replication by ligating the single-stranded DNA of the displaced strand to form strand-specific circular DNA molecules (Koepsel et al. 1985). Plasmids lacking *ori2* accumulate ssDNA, but remain viable. Minus origins are orientation and host-dependent (Boe et al. 1989, Viret and Alonso 1988, del Solar et al. 1987, Gruss et al. 1987).

Streptomyces strains, Gram-positive but G+C rich bacteria, also harbor various plasmids. The minimal replication region of pIJ101 (Kieser et al. 1982), pBT1 (Nakanishi et al. 1986), SCP2* (Larson and Hershberger 1986, Lydiate et al. 1985), and pSL1 (Shindo et al. 1987), are about 2-kb in size. DNA sequence and functional analyses of pIJ101 revealed a putative Rep protein and a non-coding region probably containing an origin of replication in the 2.1 kb minimal replicon region (Kendall and Cohen 1988, Kieser et al. 1982). pIJ101 probably replicates by a rolling circle mechanism. Minimal replicon plasmids lacking the minus origin accumulate substantial amounts of ssDNA intermediates (Deng et al. 1988, Pigac et al. 1988). A minus origin required for efficient conversion of ssDNA intermediates to ds plasmids was located outside the minimal replication region, overlapping with the *sti* locus, which is involved in strong incompatibility (Deng et al. 1988).

The wide-host-range conjugative multi-copy plasmid, pSN22, was originally isolated

from *Streptomyces nigrifaciens*. It is stably maintained in plasmid-free *S. lividans* strains. Chapter I described the functions of pSN22 involved in plasmid transfer and pock formation. This chapter describes the 1.9 kbp minimal replication region of pSN22 containing *cis* and *trans*-acting elements, and a 500 bp region outside the minimal replicon that acts as a preferential starting-point for the synthesis of the complementary strand.

MATERIALS AND METHODS

Strains and plasmids *S. lividans* TK21, and *Escherichia coli* JM109, GM33 and XL-1 blue (Stratagene) were used as hosts. The *Streptomyces-E. coli* shuttle plasmids, pESS500, pESS504, and pESS507 (Chapter I) are derivatives of pSN22. *E. coli* plasmids, pUC12, pUC18, pUC19, and pBluescript SK⁻ were used for subcloning and construction of various derivatives of pSN22.

Media and cultivation *S. lividans* was cultivated in YEME medium or on R2YE medium (Hopwood et al. 1985) at 30°C. *E. coli* was grown in LB medium (Sambrook et al. 1991) or LB agar at 37°C. Transformants of *S. lividans* and *E. coli* were selected on the media supplemented with the following antibiotics if needed: 40 µg/ml ampicillin (Meiji Seika Kaisha), 25 µg/ml thiopeptine (kindly provided by Fujisawa Pharmacy), 40 µg/ml viomycin (kindly provided by Pfizer).

Plasmid isolation, DNA manipulation, and transformation Plasmid isolations from *S. lividans* were carried out in accordance with the methods of Kieser (Kieser 1984). Bacterial handlings and *in vitro* DNA manipulation were carried out by the method of protocols for *S. lividans* (Hopwood et al. 1985) and *E. coli* (Sambrook et al. 1991), respectively. Enzymes for DNA manipulation were purchased from Takara Shuzo or Toyobo Co. Ltd..

Southern blotting analysis and detection of single-stranded DNA Southern blot hybridization was performed by the method of Southern (1975). Single-stranded DNA was detected by the method of nondenaturing DNA transfer (te Riele et al. 1986). Probe DNA was labelled with [α -³²P]dCTP by using the random primed DNA labelling kit

(Boehringer Mannheim), or by the end-labelling method (Maxam and Gilbert 1980) with T4 polynucleotide kinase for quantitative analysis. The radioactivity of hybridized probe DNA was measured with a RI imaging system (Ambis system Inc).

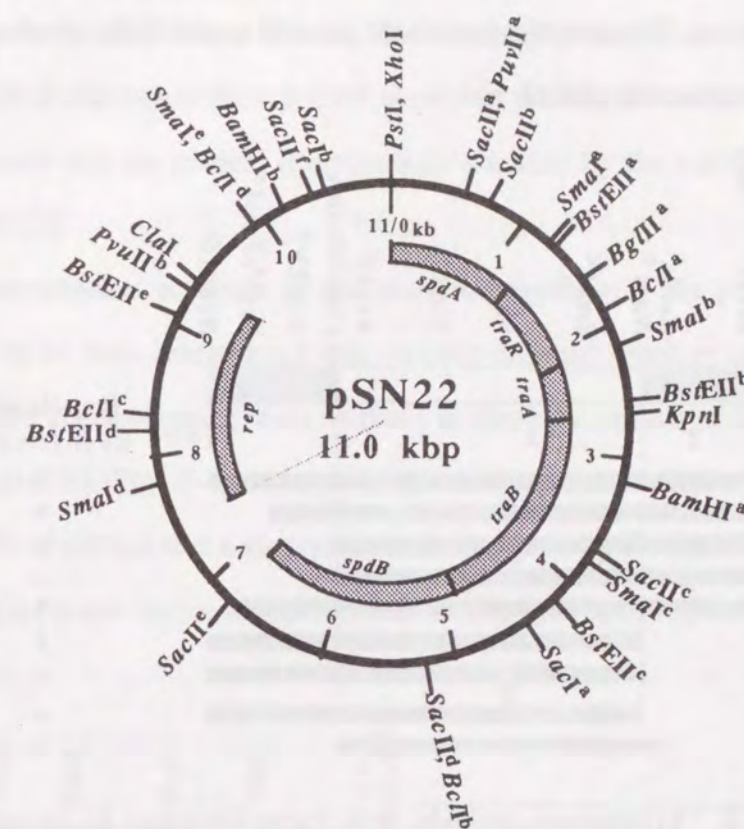


Figure III-1. Restriction map of pSN22. Where a restriction endonuclease cuts at more than one site, they are distinguished by alphabetical superscripts. The region marked as *rep* is essential for DNA replication, and those labelled *tra* and *spd* are involved in plasmid transfer and pock formation, respectively.

RESULTS

The pSN22 minimal replication region In Chapter I, I described the localization of the region needed for the autonomous replication of pSN22 within a 3.3 kbp *SacII*^e-*BamHI*^b fragment of pSN22 (Fig. III-1), which was shown by Southern hybridization to be similar to the replication region of pIJ101. The extent of the minimal replication region was narrowed to 1.9 kbp using *Bal31* nuclease (Fig. III-2). On the left side, deletions from *SacII*^e towards *NaeI*^b did not affect the transformation ability (see pMT213), but further deletion about 100 bp

past *NaeI*^b in pMT212 made the plasmid unable to be introduced into *S. lividans* by transformation. To the right, deletion from *Bam*HI^b towards *Eco*RV^a had no effect (pMT223), whereas further deletion about 50 bp past the *Pvu*II^b site caused the loss of transformation ability (pMT224). These results suggested that the 1.9 kbp *NaeI*^b-*Eco*RV^a region was essential for replication. The smallest functional plasmid is pMT216, which contains the 2.1 kbp *Bal*I-*Eco*RV^a fragment of pSN22.

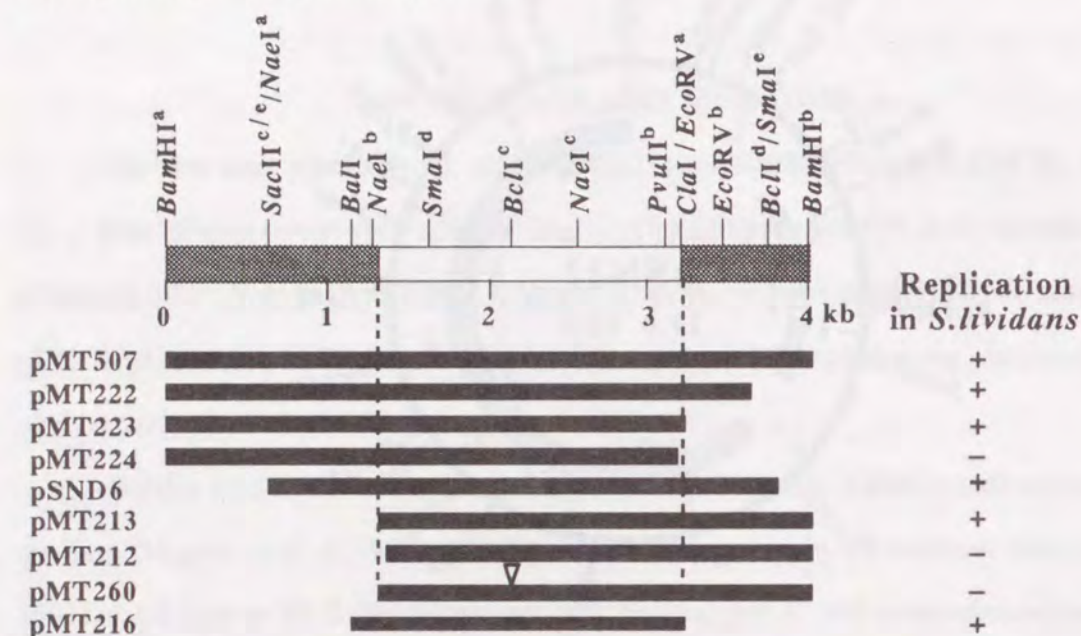


Figure III-2. Mutational analysis of a locus involved in plasmid replication. Replication ability was tested by transformation of *S. lividans* TK21. At the top the restriction map of the *Bam*HI^a-Δ(*Sac*II^c-*Sac*II^e)-*Bam*HI^b fragment of pSN22 is shown. The superscripts for the restriction enzyme sites are same as in Fig. 1, except for *NaeI* and *Eco*RV. Derivative plasmids, except for pSND6 which was obtained independently (Fig. I-2), were constructed as follows: the *Bam*HI^a-Δ(*Sac*II^c-*Sac*II^e)-*Bam*HI^b fragment of pESS507 (Fig. I-2) was subcloned into pUC19. The resulting plasmids were digested with *Eco*RI or *Hind*III, and then treated with *Bal*31. The DNA fragments were then treated with T4 DNA polymerase to give blunt ends, digested with *Eco*RI or *Hind*III, purified from agarose gel, and subcloned into pUC19 digested with *Eco*RI and *Sma*I, or *Hind*III and *Sma*I. Plasmid DNA was isolated from ampicillin resistant *E. coli* transformants, digested with *Eco*RI and *Hind*III. The fragment originated from pSN22 was ligated to a 1.7 kbp *Eco*RI-*Hind*III fragment of pUT12T (Fig. I-2) containing the thiostrepton resistance gene. The ligation mixture was used to transform *S. lividans* TK21 protoplasts directly. pMT260 has a 4 bp insertion at the *Bcl*I^c site of pMT213. pMT216 contains the *Bal*I-*Eco*RV fragment of pSN22 ligated to the 1.6 kbp *tsr* fragment.

All of these constructs contained no *E. coli* DNA fragment to exclude the effect of foreign DNA sequence of low GC content on plasmid stability in *S. lividans*. The inability of replication of pMT212 and pMT223 was reconfirmed by construction of shuttle plasmids containing the assayed fragment, *tsr* fragment and pUC18. It is noteworthy that all derivatives having the

transformation ability were maintained with 100 % stability in *S. lividans* during one round of cell growth from spores to spores in nonselective condition.

The DNA sequence of pSN22 has been determined (show Chapter IV). The analysis of 1.9 kbp minimal replicon predicts the existence of an ORF designated as *rep* ORF encoding a protein of 451 amino acid residues. The location of *rep* ORF was shown in Fig. III-3. A 4 bp insertion at the *Bcl*I site within the *rep* ORF abolished the transformation ability (PMT260). This result indicate that the protein (Rep protein) encoded by the *rep* ORF is essential for replication of pSN22.

Complementation analysis of the minimal replicon Six plasmids, pMTE201-pMT206 (Fig. III-3) were constructed that contain different parts of the pSN22 minimal replication region. These plasmids were used to transform protoplasts of *S. lividans* TK21 and TK21/pMT63. pMT63 (Fig. II-1) contains the minimal replication region (*Bam*HI^a-Δ[*Sac*II^c-*Sac*II^e]-*Bam*HI^b) of pSN22 and a viomycin resistance gene (*vph*). pMTE202 containing the *Bal*I-*Eco*RV region could replicate both in TK21 and in TK21/pMT63 (as pMT216, shown in Fig. III-2).

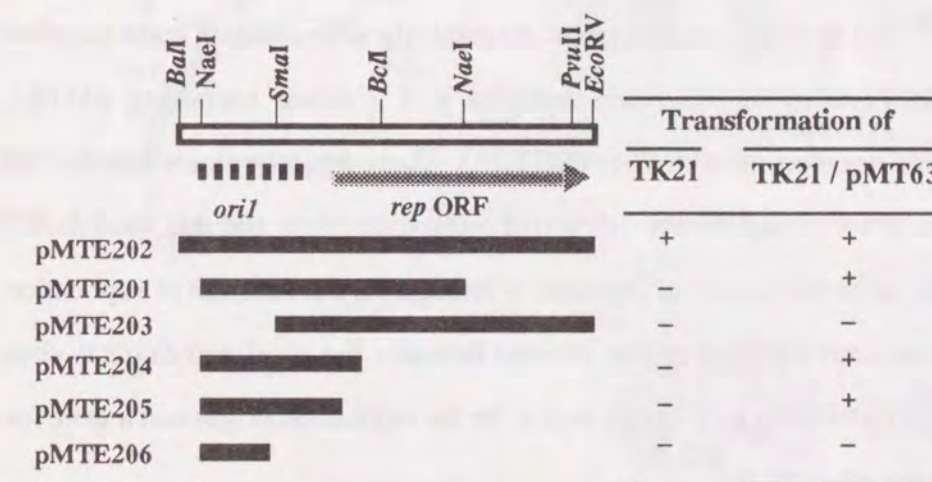


Figure III-3. Complementation analysis. The replication ability of the plasmids were tested by protoplast transformation using *S. lividans* TK21 and TK21 harboring pMT63 as host strains. The three plasmids pMTE201 - pMTE206 were constructed as follows: The *NaeI*-*NaeI*, *Bal*I-*Eco*RV, and *Sma*I-*Sma*I, *NaeI*-*Hinc*II, *NaeI*-*Bst*EII and *NaeI*-*Sma*I fragments were cloned into the *Sma*I-digested pUC19 (the ends were made blunt if necessary by treatment with T4 polymerase). 1.7 kbp thiostrepton resistant gene from pUC12T, which was digested with *Bam*HI and *Pst*I, was cloned into the resulting plasmid digested with the same enzymes. Each plasmid carried the fragment marked with solid lines. The *lacZ* gene of pUC19 reads leftward in all six plasmids. Shaded arrow indicates Rep ORF from sequencing analysis. Dashed line indicates the putative replication origin.

pMTE201 carrying the 1.3 kbp *NaeI* fragment could replicate in TK21/pMT63 but not in TK21. Autonomous replication of pMTE201 in the *Streptomyces* transformant was confirmed by re-transformation of *E. coli* for direct selection of pMT201. Plasmid DNAs from the transformant of TK21/pMT63 with pMTE201 was used to transform *E. coli* JM109. pMTE202, which carries an *E. coli* replicon and the *bla* gene, was recovered without structural change from the ampicillin-resistant transformants of *E. coli*, but pMT63 was not detected since it has no *E. coli* replicon. From a few *Streptomyces* transformant (less than 1 % of the transformants) large-size plasmids which were co-integrates between pMTE201 and pMT63 were recovered. The result indicated that pMTE201 replicated autonomously with a help of the putative *trans*-acting element (Rep protein) specified by pMT63.

pMTE203 (Fig. III-3) did not give thiostrepton-resistant transformants of TK21 or TK21/pMT63, indicating that the deleted 360 bp *NaeI-SmaI* region was required in *cis* for plasmid replication. This suggested that the replication origin (*ori1*, see below) of pSN22 might be in this region. For the determination of *ori1* locus more precisely, we assayed replication ability of pMTE204, pMTE205 and pMTE206 containing an 1.1 kbp *NaeI-HincII*, 750 bp *NaeI-BstEII* and 360 bp *NaeI-SmaI* fragment, respectively with a help of *trans* supplied Rep protein. pMTE204 and pMTE205 could replicate in *S. lividans* harboring pMT63, whereas pMTE206 could not replicate as well as pMTE203. These results indicate that the 360 bp *NaeI-SmaI* fragment is not enough for the function of replication origin and that *NaeI-BstEII* fragment which located upstream region of *rep* ORF is enough for the function of replication origin. The 1.9 kbp minimal replicon region located between the *NaeI* and *EcoRV* sites contains two components which are a *cis*-acting region for the replication origin and a gene for a *trans*-acting Rep protein (Fig. III-3).

Single-stranded DNA accumulation by pSN22 and its derivatives

Southern blot analysis indicated that a small amount of single-stranded form of a wild type pSN22 was present in total DNA isolated from TK21/pSN22 (Fig. III-4-B): a weak signal was detected at the same migration distance as that of heat-denatured DNA of pSN22, and the signal was titrated by the treatment of total DNA with S1 nuclease (data not shown). This suggested that single-stranded molecules were generated as intermediates during pSN22 replication. The

pSN22 derivatives, pESS507 (see Fig. III-5) and pMT213 (Fig. III-2), which carried only the region essential for replication, accumulated more ssDNA molecules (Fig. III-4-C,D) than wild-type pSN22 or another derivative as pMT311 (Fig. III-4-B,C). This suggested that the minimal replicon plasmid lacked a specific DNA sequence designated as *ori2* for the initiation of second-strand synthesis. Another possibility is that another plasmid gene not present in the minimal replication region might be required for efficient conversion of ss to dsDNA.

The accumulation of ssDNA molecules was examined quantitatively. Total DNA from the strains TK21 harboring pSN22, pMT311, pESS504, and pMT313 (Fig. III-5) was isolated. After electrophoresis, the DNA was denatured, transferred onto a nylon membrane, and hybridized with the *BclII*^c-*BclII*^d DNA fragment end-labeled with ³²P. The radioactivity of bands was measured with an RI imaging system. Table III-1 shows that the minimal-replicon plasmids pESS507 and pMT313 accumulated the same amount of ss and dsDNA, while for pSN22 and pMT311 the relative amount of ssDNA was only 30 % of dsDNA.

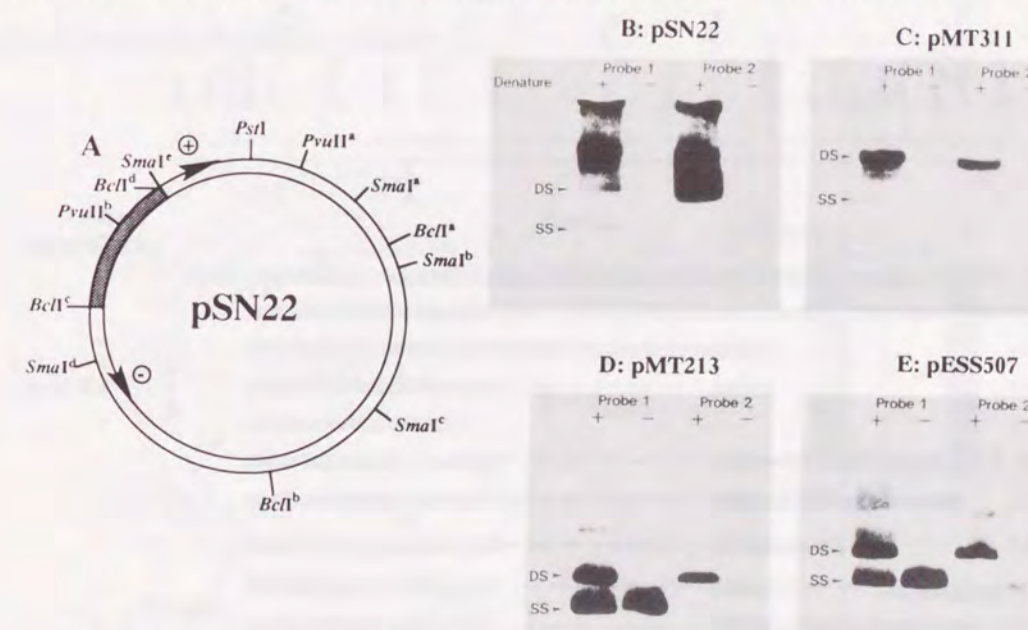


Figure III-4. Southern blot analysis of pSN22 and its derivatives using strand-specific probes. A: restriction map of pSN22. The shaded *BclII*^c-*BclII*^d fragment was cloned in both orientations into the *Bam*HI site of pBluescript SK⁻ for the preparation of ssDNA probes complementary to the clockwise (+)(probe 1) and anti-clockwise (-)(probe 2) strands, respectively. ssDNA of each derivative was prepared using the helper phage R408 according to the protocol from the supplier (Stratagene). The [³²P]dCTP-labelled strand-specific probes were prepared by the primer extension method using Klenow fragment. The arrows indicate the 5' to 3' direction of the labelled probe. B: Southern blots of total DNA of TK21/pSN22, C: TK21/pMT311 (Fig. III-5), D: TK21/pMT213 (Fig. III-2), and E: pESS507. Total DNA was prepared under neutral pH condition and transferred to the membranes with the (+) or without prior denaturation (-). Arrows marked with DS and SS show the positions of closed circular dsDNA and ssDNA molecules, respectively.

Table III-1. Ratio of single-stranded intermediate to double-stranded plasmids of pSN22 derivative

| Plasmid | <i>ori2</i> region | Ratio of radioactivity of ssDNA to dsDNA |
|---------|--------------------|------------------------------------------|
| pSN22 | + | 0.29 |
| pESS507 | - | 0.99 |
| pMT311 | + | 0.32 |
| pMT313 | - | 1.24 |

Total DNAs were extracted under the neutral pH condition, electrophoresed on an 1% agarose gel, transferred to a nylon membrane (with prior denaturation) and hybridized with the ³²P-labelled probe of the *Bcl*I^c-*Bcl*I^d fragment (see Fig. III-2). The radioactivity in the bands was measured using a RI imaging system. The molecular ratio was calculated as the amount of radioactivity in the ssDNA band divided by a half the radioactivity in the dsDNA band.

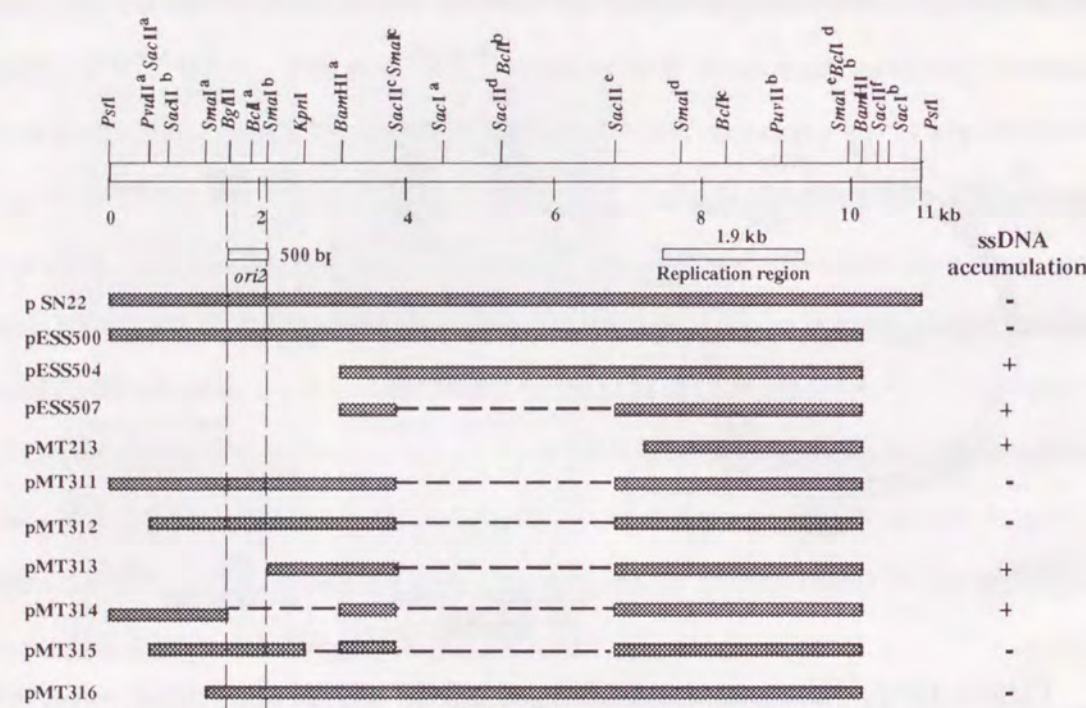


Figure III-5. Search for a locus involved in the ssDNA accumulation. The restriction map of pSN22 is shown at the top. The superscripts of restriction enzymes are the same as in Fig. 1. ssDNA accumulation was tested by Southern blotting as described in Fig. III-4. The thick lines indicate DNA fragments present on the pSN22 derivatives. Dotted lines indicate deletions. pESS500, pESS504, and pESS507 are the shuttle plasmids replicating in *S. lividans* and in *E. coli*. The construction of pMT213 was described in Fig. III-2. pMT311, pMT312, and pMT313 were constructed from pMT507 (Fig. III-2) by inserting *Pst*I-*Bam*HI^a, *Pvu*II^a-*Bam*HI^a, and *Sma*I^b-*Bam*HI^a fragments, respectively. pMT314 and pMT315 are deletion derivatives of pMT311. pMT316 was constructed similar to pMT507 (Fig. III-2).

The strand specificity of the single-stranded intermediates was tested by Southern blot analysis using strand-specific probes. Probe 1 complementary to the clockwise strand (marked as plus strand in Fig. III-4-A) hybridized strongly with ss and dsDNA of pESS507 and pMT213, but only faintly with ssDNA of pSN22. Probe 2 specific for an anti-clockwise (minus) strand, however, gave no significant signal corresponding to ssDNA, though it hybridized as well as probe 1 to dsDNA. This indicated clearly that the rolling circle replication proceeded in the clockwise direction around pSN22.

Origin of second-strand synthesis To determine the region required for the initiation of complementary strand synthesis, several derivatives of pSN22 were constructed. As shown in Fig. III-5, lack of the 500 bp *Bgl*II-*Sma*I^b fragment (*ori2*) caused the accumulation of ssDNA: for example, TK21 harboring pMT314, in which the region was deleted, accumulated ssDNA molecules significantly, but pMT315 that contains this region did not. It is noteworthy that *ori2* is within the region of the divergent *traR* and *traA* promoters in the transfer region of pSN22 (Chapter II).

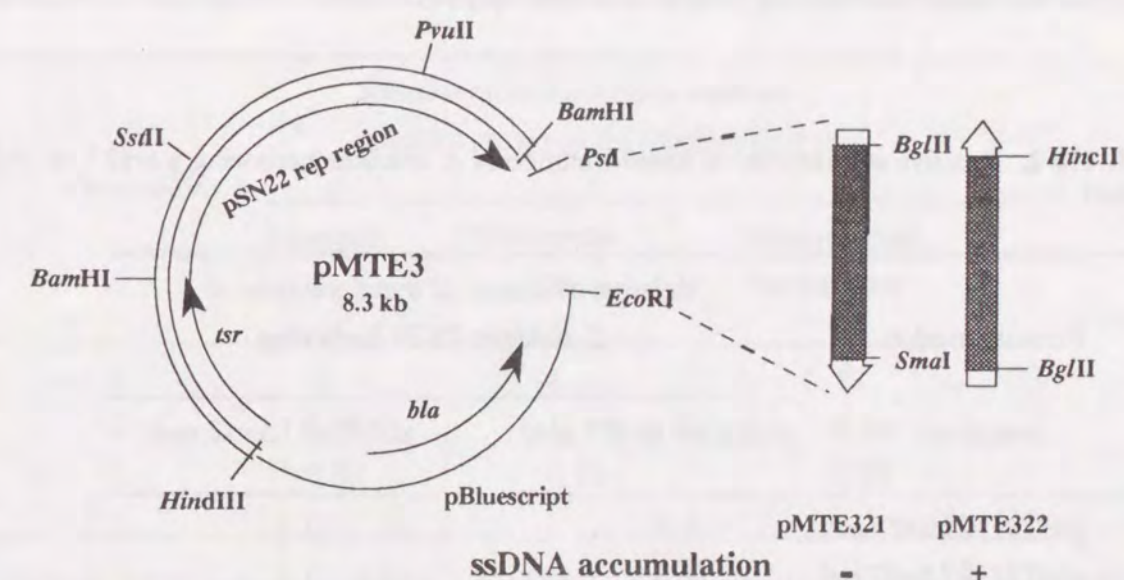


Figure III-6. Effect of orientation of the *ori2* fragment. The ssDNA accumulation was tested by Southern blotting as described in Fig. III-4. The 500 bp *Bgl*II-*Sma*I^b fragment of pSN22 was cloned in different orientations in pUC18 digested with both *Bam*HI and *Sma*I or *Bam*HI and *Hinc*II. The *Pst*I-*Eco*RI fragments generated by digesting these plasmids were subcloned on pMTE3 which consisted of the minimal replicon from pESS507, the *tsr* fragment from pUC12T, and pBluescript SK⁻. The resulting plasmids, pMTE321 and pMTE322, carried the *ori2* region (marked with shade) in original and reverse directions with the pUC18 and pUC19 linker fragments (boxed line), respectively.

The effect of orientation of the *ori2* fragment on ssDNA accumulation was tested. Two plasmids, pMTE321 and pMTE322, were constructed that carry the 500 bp *Bgl*III-*Sma*I^b (*ori2*) fragment in the original and reverse orientation to the *rep* region, respectively (Fig. III-6). pMTE321 replicated without accumulation of ssDNA molecules, but pMTE322, which has *ori2* in the reverse orientation, accumulated a significant amount of ssDNA. This is consistent with our hypothesis that the *ori2* region functions as a initiation site for second strand synthesis.

Effect of the *ori2* region on plasmid stability and incompatibility Deng, Kieser, and Hopwood (1988) identified a *cis*-acting region, *sti*, on pIJ101. The *sti* region functions as *ori2*, and also confers the strong incompatibility phenotype: *sti*⁻ pIJ101 derivatives accumulate ssDNA molecules, and cannot co-exist with *sti*⁺ derivatives of pIJ101. The loss of the *ori2* region of pSN22, however, did not change the incompatibility phenotype of the plasmid. The *ori2*⁻ derivative pMT313 (Δ *ori2* *tsr*) could be introduced into strain TK21/pMT450 (*ori2*⁺ *vph*) by protoplast transformation (Table III-2). Also pMT911 (*ori2*⁺ *tsr*) was compatible with TK21/pSND6 (Δ *ori2* *vph*). This indicated clearly that the *ori2* region of pSN22 was not involved in any strong incompatibility phenotype.

Table III-2. Relative efficiencies of transformation of *S. lividans* harboring a *ori2*⁺ or *ori2*⁻ plasmid

| Plasmid used to transform | Relative efficiency of transformation of <i>S. lividans</i> TK21 harboring | |
|-------------------------------------------------------------|----------------------------------------------------------------------------|--------------------------------------------------------|
| | pMT450 ^a (<i>ori2</i> ⁺ <i>vph</i>) | pSND6 ^b (Δ <i>ori2</i> <i>vph</i>) |
| pMT911 ^c (<i>ori2</i> ⁺ <i>tsr</i>) | 1.0 | 1.1 |
| pMT313 ^d (Δ <i>ori2</i> <i>tsr</i>) | 2.3 | 1.1 |

Efficiencies were normalized independently in case of the transformation of *S. lividans* harboring pMT450 or pSND6.

^a pMT450 was almost same as pMT911 except containing the *vph* gene instead of the *tsr* gene and having mutation at the *traB* locus.

^b pSND6 carried only the minimal replicon (Fig. III-2).

^c pMT911 carried the same fragment as pESS500 (Fig. III-5).

^d see Fig. III-5.

We also examined the stability of co-existing *ori2*⁺ and *ori2*⁻ plasmids. TK21 harboring pMT911 (*ori2*⁺ *tsr*) and pSND6 (Δ *ori2* *vph*) was grown on R2YE medium (no antibiotics). The thiostrepton and viomycin resistances were retained during three spore generations (Table III-3). The co-existing plasmids were thus maintained stably with no significant directional bias of plasmid segregation. A previous study (Chapter I and II) indicated that co-integrate plasmids between pSN22 derivatives do not occur frequently. The segregation of plasmids without bias was thought to a typical pattern of the random selection model for plasmid replication and partition of a relaxed type plasmid (Novick 1987). The result also suggested that the *ori2* region was not involved in preferential inheritance of *ori2*⁺ plasmids that are in competition with minimal replicon *ori2*⁻ plasmids.

Table III-3. Segregation of co-existing pSND6 (Δ *ori2* *vph*) and pMT911 (*ori2*⁺ *tsr*)

| Generation | Relative ratio of antibiotic-resistant colonies grown on the medium containing | | |
|------------|--------------------------------------------------------------------------------|--------------|---------------------------|
| | Viomycin | Thiostrepton | Viomycin and Thiostrepton |
| 1 | 1 | 1 | 1 |
| 2 | 0.94 | 1 | 0.94 |
| 3 | 0.83 | 0.93 | 0.75 |

S. lividans TK21 harboring pSND6 and pMT911 (see Table III-2) was cultivated on R2YE medium in absence of antibiotics. The generated spores were spread, with appropriate dilutions, on R2YE medium containing viomycin and/or thiopeptine, and onto the medium without antibiotics. Colonies were counted, and the ratio of the numbers of antibiotic-resistant colonies to that of colonies obtained on the non-selective medium was calculated. The process was repeated three times (three generations from spores to spores).

DISCUSSION

I identified two regions of pSN22 that are involved in plasmid replication. The 1.9 kb *rep* minimal replication region is sufficient for the stable inheritance. It consists of one *cis*-acting element, probably a replication origin (*ori1*), and a *trans*-acting element which may encode a replication protein (Fig. III-3). In *S. lividans* strains containing pSN22 a small amount of strand-specific (clockwise in Figs. III-1 and 7) single-stranded plasmid DNA could be detected. The proportion of ss : ds plasmid DNA is increased about four-fold in *S. lividans* strains containing minimal replication plasmids. This is consistent with the hypothesis that pSN22, like many plasmids of Gram-positive bacteria (reviewed by Gruss and Ehrlich 1989), replicates by a rolling circle mechanism that requires a specific replication protein that nicks the ds plasmid DNA at the plus origin (*ori1*) and produces a strand-specific single-stranded replication intermediate. For the conversion of ssDNA to dsDNA, a minus origin (*ori2*) is required that can be anywhere on the plasmid as long as it is in the correct orientation relative to *ori1*. I identified a 500 bp DNA fragment in the transfer region of pSN22 that reduces the amount of ss plasmid DNA that is accumulated when cloned in the correct orientation into minimal replicon plasmids. This fragment has thus the characteristics of a minus origin (Fig. III-7). The *ori2* sequence, however, was only one of at least two initiation sites for second strand synthesis, since ds plasmid DNA was synthesized in the absence of the *ori2* region (Table III-1).

What is *ori2*? Different from Minus-origins of *S. aureus* plasmids, *ori2* deficient plasmids were not affected its stability. This fact is thought to indicate that *ori2* is a preferential binding or recognition site for a primase on single-stranded intermediate. The ssDNA conversion to dsDNA of a *ori2* deficient plasmid occurred not so low frequency, and the difference of the ssDNA accumulation among various *ori2*⁻ plasmids could not be observed. These facts are proposed to suggest the existence of random priming function(s) in *S. lividans*.

pSN22 resembles, in its mode of replication, the multi-copy *Streptomyces* plasmids pIJ101 (Deng et al. 1988). This resemblance extends to the DNA sequence of the *rep* region that hybridized strongly with the *rep* region of pIJ101 (Chapter I). DNA sequence analysis

showed 92 % identity in the *rep* ORF region of the two plasmids and the predicted replication proteins show 95 % amino acid identity (data is shown in Chapter IV). Also the second-strand origin of the two plasmids seem to be located in similar positions in the transfer regions which otherwise seem to be quite different from each other.

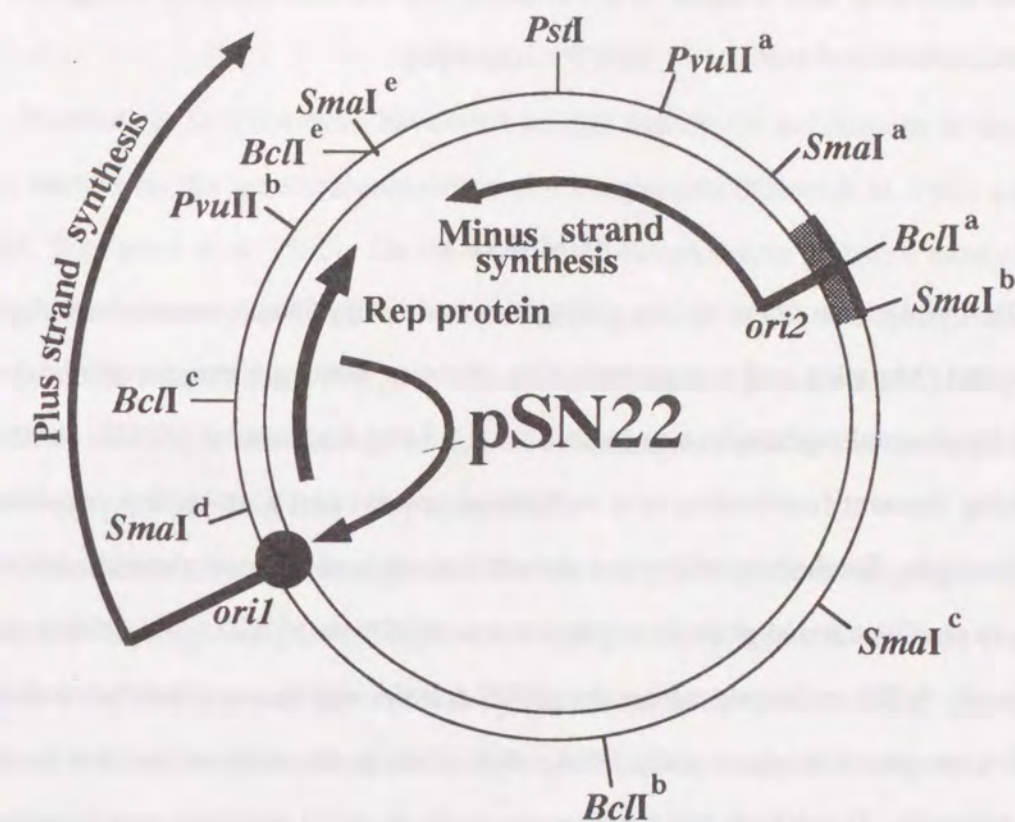


Figure III-7. Model for the replication of pSN22.

There is, however, an important difference between the *ori2* regions of pIJ101 and pSN22. In pIJ101 this region has been called *sti* for strong incompatibility that prevents the co-existence of *sti*⁺ and *sti*⁻ plasmids in the same mycelium. No such incompatibility has been observed between pSN22 derivatives that differ in the presence of *ori2*. It is thus possible that *sti* and the second-strand origin of pIJ101 are two separate functions.

Because of using shuttle vectors, we made all experiments in *S. lividans* which had only weak restriction barriers. Though pSN22 was originated from *S. nigrifaciens*, the result suggested the existence of the host factor(s) of *S. lividans* which interacted with *ori2* of pSN22. If pSN22 and pIJ101 are general replication type plasmids which have three factors, a

Rep protein, a replication origin and a minus origin, the host factor(s) interacting with *ori2* may have wide distribution in *Streptomyces*. In this view point, *ori2* is thought to be active in many kinds of *Streptomyces* including *S. nigrifaciens*. From the DNA sequence comparison between the regions having minus-origin activity of pSN22 (*ori2*) and pIJ101 (*sti*) indicated no significant similarity (see Chapter IV), I consider that the host factor(s) recognizes three-dimensional structure of *ori2*.

SUMMARY

DNA replication of the 11 kbp conjugative multi-copy *Streptomyces* plasmid, pSN22, was analyzed. Mutation and complementation analyses indicated that the minimal region essential for plasmid replication was located on a 1.9 kbp fragment of pSN22, containing a *trans*-acting element functioning as a replication protein and a *cis*-acting sequence as a replication origin. Southern hybridization showed that minimal replicon plasmids accumulated much more single-stranded plasmid molecules than wild-type pSN22. Only one strand was accumulated. A 500 bp fragment from the pSN22 transfer region was identified that reduced the relative amount of single-stranded DNA, when added in the native orientation to minimal replicon plasmids. This 500 bp DNA sequence may be an origin of second strand synthesis. It had no effect on the efficiency of co-transformation, plasmid incompatibility, or stability. The results indicate that pSN22 replicates via single-stranded intermediates by a rolling circle mechanism.

Chapter IV

Complete nucleotide sequence of pSN22

INTRODUCTION

Plasmids in *Streptomyces* have been studied mainly for the purpose of developing cloning vectors for the genetic manipulation of the organisms (Kieser et al. 1982, Lydiate et al. 1985, Thompson et al. 1982). On the other hand, *Streptomyces* plasmids have also been investigated for their replication (Deng et al. 1988, Pigac et al. 1988), site-specific integration and excision (Boccard et al. 1989), pock-formation (Bibb and Hopwood 1981, Hopwood et al. 1985, Kieser et al. 1982, Kataoka et al. 1991, see Chapter I and II) and the relationship to antibiotic production (Wright and Hopwood 1986).

I have described the investigation of a multi-copy, broad host range, pock-forming plasmid, pSN22, originating from *Streptomyces nigrifaciens* (Chapter I). Genetic analysis of pSN22 revealed that plasmid transfer was epistatic over pock formation, and that there are five genes involved in plasmid transfer and pock-formation. One of these genes, *traB*, has been supposed to catalyze inter-mycelial plasmid transfer like the *tra* gene of pIJ101 (Kendall and Cohen 1987 1988, Kieser et al. 1982). *traA* was identified as a gene essential for pock-formation. *traR*, like *kora* of pIJ101, is a transcriptional regulator for a transfer operon and also for its own expression. *traA* and *traB* without the presence of *traR* inhibit or kill *S. lividans*, respectively. *traR* and *traB* are in a similar relationship as *kora* and *kila* (*tra*) or *korB* and *kilB* of pIJ101. Two loci, *spdA* and *spdB*, downstream of *traR* and *traB*, respectively, are probably involved in plasmid spread in recipient mycelia as was proposed for the *spd* genes of pIJ101.

The replication of pSN22 has also been investigated. The region essential for pSN22 replication was limited in a 3.5 kbp segment. Southern blot analysis indicated that the replication region of pSN22 was highly homologous with that of pIJ101. Similar to other

plasmids in Gram-positive bacteria, pSN22 and pIJ101 probably replicate via single-stranded intermediates.

This chapter describes the complete nucleotide sequence of pSN22, and correlates the open reading frames [ORFs] with the genetic properties identified previously. Since pIJ101 and pSN22 have similar functions, the comparison of their DNA sequences and of their derived amino acid sequences are probably useful for the identification of functionally important features and also for understanding plasmid evolution.

MATERIALS and METHODS

Bacterial strains and plasmids The M13 sequencing vectors, mp18 and mp19, were used throughout this study for preparing single-stranded templates. All DNA fragments used for sequencing were initially cloned into pUC18 or pBluescript SK⁻ for constructing deleted derivatives. The host strains and other plasmids used in this chapter were described in chapter I and III.

Cultivation, transformation, plasmid isolation and DNA manipulation Bacterial cultivation, transformation, plasmid DNA isolation and purification were according to the methods described previous chapters. DNA fragments were isolated from agarose gels using Gene Clean II (BIO 101). Restriction enzymes, Exonuclease III, Mungbean nuclease and T4 DNA polymerase were purchased from Takara Shuzo or Toyobo and were used as specified by the manufactures. Ligation reactions were performed with the DNA ligation kit (Takara).

DNA sequencing M13 clones were sequenced by a modification of the method of Sanger et al. (1977). 7-deaza-deoxyguanosine triphosphate (7-deaza-dGTP) instead of dGTP and the single-strand DNA binding protein (SSB, Stratagene) were used to overcome problems with stable secondary structures due to high G-C content of *Streptomyces* DNA. The sequencing reactions were performed with the 7-deaza-dGTP sequencing kit containing Sequenase II (U. S. Biochemical Corp) according to the protocol of the supplier, except for the addition of SSB: after annealing, 1 µg of SSB was added to the annealing mixture, after the termination reaction, SSB was digested with 1 µg of Proteinase K (Sigma) at 37°C for 30

min, and then the stop solution was added. The sequencing reaction products were resolved on a 6% polyacrylamide-8M urea sequencing gel. The gels were usually run at maximum voltage of 2500 V and maximum current of 25 mA for 2 h or 4.5 h. Over 300 bp of sequences could usually be resolved.

Computer-assisted sequence analysis DNA sequence analysis and Data Base search were performed with Genetix program (SDC soft wear) on a PC-9800 computer (NEC). Open reading frames (ORFs) were searched with a program similar to FRAME (Bibb et al. 1984), which was written by Mikio Nakajima in n-88 basic language for the PC-9800.

RESULTS

DNA sequence of pSN22 and prediction of ORFs pSN22 (Fig. IV-1) was digested with restriction endonucleases to generate following nine fragments of up to 2 kbp, such as *Pst*I-*Bgl*II, *Bgl*II-*Bam*HI^a, *Bam*HI^a-*Sac*I^a, *Sac*I^a-*Sac*II^d, *Sac*II^d-*Sac*II^e, *Sac*II^e-*Sma*I^d, *Sma*I^d-*Pvu*II^b, *Pvu*II^b-*Bam*HI^b and *Bam*HI^b-*Pst*I. The DNA fragments were subcloned into pUC18 or pBluescript SK⁻, and the recombinant plasmids were amplified in JM109. The clones were then used for the construction of nested deletions with Exonuclease III and Mung bean nuclease (Henikoff 1984). After self-ligation, deleted plasmids were introduced into JM109 by transformation. The sizes of the deleted plasmids were checked by agarose gel electrophoresis, after digestion with appropriate restriction endonucleases. Fragments of appropriate sizes (c. 200-bp between deletions) were isolated from the agarose gel and subcloned into M13 vectors for the isolation of ssDNA to be used for sequencing. The sequences around the restriction sites used for the initial subcloning of pSN22 fragments were determined from a second set of overlapping subclones. The sequence of pSN22 was determined for both strands, and each base pair was determined at least twice.

The complete nucleotide sequence of pSN22 is presented in Fig. IV-2. pSN22 is a circular DNA molecule of 10922 bp with 71.76% G+C base pairs. The unique *Pst*I recognition site has been chosen as nucleotide number 1 (nt 1). The potential ORFs were

54

10 20 30 40 50 60 70 80 90
CTGCAGGCCGGTGACTCCAGAGAAGGGTTGAAGCGGAGTTGCGGGGCTAGCCCCCGAGCCTCCATCGTTCGACACGCCCGGCCCTGTCCG

100 110 120 130 140 150 160 170 180
GGACGGGAGGACTTTTCGAGCACCTGGAGAGCACGTGACCAACACGACGAGGACCAGGAGCAGCCGACGCGCCCGCGGACCGTGGCC
orf56 V T N Q Q Q D Q E Q P Q R P A D R G

190 200 210 220 230 240 250 260 270
GCCGCGAGTTCGCGAAGAAGGGAGCCGTGACCGTGCTGCGCGCTCTCGTCTCCGAGCTGCTCGGGCGGTGGTAGCCACCTCCTCACGG
R R E F A K K G A V T V L A A L V S G A A R A V V A H L L T

280 290 300 310 320 330 340 350 360
GAGGTGGCGAGTGACGCGGCTCGGCAGCCGGGCGCGCGGACGAGGACGAGCGGCCCGCGGAAGCCGGGCGCGCGGCGCCG
G G G E

370 380 390 400 410 420 430 440 450
CGCGGGCCGCTTGATGGAGTAGGAAAGTTCTACCGCGCCCACTCGCCACGCCACGACGTGCCCGGTACGGTCTCGTTCATGAGCA
spda M S

460 470 480 490 500 510 520 530 540
CAGAGACACCCACGAACCGGCGACGGCGAGACGCTGTGCGCCTGGTTCGGCCGTGGCCCCGTGCCACCCAGCCGGGGAACCAAGCCGCGG
T E T P T N G D G E T L C A W C G R G P V P P S R G T K P R

550 560 570 580 590 600 610 620 630
CCTACTGCTCGCGCAGCTGCGTCCAGCGAGCCACGAGTTCGCGAAAGCTCCGCAAGAAGCTGCTCGGCGCGTACATGAAGGGCCGGGCGG
A Y C S R S C V Q R A H E S R K L R K K L L G A Y M K G R A

640 650 660 670 680 690 700 710 720
AGGAGGCTGAGCTGCGCGGAGGAAAGTCAGGTGACGATGCAGGAAAGTCACGTGACTTTCCCGGTTCGGCAGGCCCCAGCAAAGTCAGGTG
E E A E L R G G K S R D D A G K S R D F P G R Q A P A K S R

730 740 750 760 770 780 790 800 810
ACTTTCACAAACCCAGGTCAACCTGGGGTTCGCGCTCCCGCGGTCCCGGTGACGTCCGCGCCCGGTTCGAAAGGCGCGCGCCCGTGC
D F P K P Q V N P G V P R P A V P V T S A P R S K G R R P L

820 830 840 850 860 870 880 890 900
TGCCCCCGCGCCGGGCTGACGCGGGAGACGCTCCCGTGTTCGGCGACGACGACGACGCGCCCGCCCTCGATGGCGCGCCGACA
L P P A P G V T R E T L P L F G D D D T Q P G P L D G R A D

910 920 930 940 950 960 970 980 990
CGGAGTGACCACGACGCGCGTACCCCATCGCGGCAGCGCCGCTCCCCATCGACCCGAGACGGGCGGTTCGGTTCGCTTACGACGCCC
T E

1000 1010 1020 1030 1040 1050 1060 1070 1080
AGCCCCGCGACGCGGGGGTACGGGGGGTTCGGGCGACTCCTGGCCCGCCAGACGGCCGACAGAGCCGCCGACCCCCACCCCTCCCGC

1090 1100 1110 1120 1130 1140 1150 1160 1170
CAGCCGTCGGCGACGCGCACAAACGACGATGCCCGCGCGCGGTGACGGCCCGCATGTAAACCGCTCAGGGATGCCGCTGTGGGCGAG
E G M P V R A G P
AGAAAGCCCCCGCGGGTCTCGGACGGGGGCTCAATGGGGAGGTAGGGCGGGGGCTGGGGTCACTCGCCCATGGGCACCCGGGCGCGG

A Y V D E W Y G L A Q E D D H F T V T T V M V A I A S H A P
GGGCGTAGACGTCTTCCAGTAGCCGAGCGCTGTTCTGTCGTGTTGAACGTGACGGTGGTGACCATACGGCGATGGCTGAGTGGGCGG

P T S R L A N L E D Q S A Q R A H A A R Q G K V V E R G T R
GGGCGTCGATCTCAGAGCGTTGAGTTCGTCTCGGACGCTGCCGTGCGTGGGCGGCGCTGTCCCTTACCACTTCGCGACCGGTGC

E S Y L K D F Q K V M R V D E G L E P V A A V T H P P Y V S
GCTCGCTGTAGAGCTTGTGCAACTGCTTACCATCCGTACGTCTCCCCAGCTCGGGGACGGCGGCCACCGTGTGGGGCGGGTAGACGG

V G V S T P K D D Q R F V R I R I V A E D G P E L T L A T C
AGACCGCGACCGACGTTCGGCTTGTCTGCTGCGGGAAGACGCGAATGCGGATCAGCGCTTCGTGCGGGGCTCAGGGTGAGAGCGGTGC

I E P D H V S R T M V R H G S S T E G P A Y R M G N K E M R
AGATCTCGGGATCGTGACGACGACCGGTTCATACGCGGTGGCCGGAGGAGTCTCGCCGGCGCGTAGCGCATCCGTTCTTCTCCATGC

K L R D A G T I V I P N K E V V T G L A G R S S V L G E S K
GCTTCAAGCGGTCCGCGCCAGTGATGACGATGGGGTCTTCTCCACGACCGTACCCAGCGCCCGCGCGAGCTGACCAGGCCCTCGCTCT

L V A L A R S V T K A A V G F Q A R I D A V S P L T D G P A
TCAGCAGCGGCAAGGCCCGGCTCAGGCTCTTGCCCGCCACGCCGAAGTGGGACGAGTGTACGCGAGGACGGACGCGTGTTCGCCCGGT

56

57

spdB3 M

| | | | | | | | | | |
|-----|----------|---------|---------|---------|----------|----------|----------|---------|---------|
| | 7300 | 7310 | 7320 | 7330 | 7340 | 7350 | 7360 | 7370 | 7380 |
| | CACCGGTT | CGTCAAG | ACCAGCG | CGCGCC | ACGCGCG | CGCCTT | CGGCGCG | CGCGCG | CGCGCG |
| | 7390 | 7400 | 7410 | 7420 | 7430 | 7440 | 7450 | 7460 | 7470 |
| | GGCGGAT | CGAAGCC | GCTGCCT | GAAACAC | GGAAGCCC | CGCGGCTT | TCGCTT | CGGCGCG | CGCAAT |
| | 7480 | 7490 | 7500 | 7510 | 7520 | 7530 | 7540 | 7550 | 7560 |
| | ACGGGCG | GCTGCT | CGCGGGG | GAACCT | CCGGCAC | AAAAAGT | GCCAGGAT | CACCCC | AGCAAA |
| | 7570 | 7580 | 7590 | 7600 | 7610 | 7620 | 7630 | 7640 | 7650 |
| | CTGACCG | CTTCT | GACGTC | CGCGCGG | ATACCA | ACCAAGG | ACTCGT | CTGTT | GAAACAG |
| | 7660 | 7670 | 7680 | 7690 | 7700 | 7710 | 7720 | 7730 | 7740 |
| | CCGGCTC | CGCGCGT | CCGTC | CGCGT | GCCACT | GCGGCA | CTGCGAT | CAGCAC | CGCCG |
| | 7750 | 7760 | 7770 | 7780 | 7790 | 7800 | 7810 | 7820 | 7830 |
| | TGCAAGC | AGCGGCG | GAAGCG | CGCTCT | TGCCA | AGATCG | CCCGGA | AGCCG | CGCGT |
| | 7840 | 7850 | 7860 | 7870 | 7880 | 7890 | 7900 | 7910 | 7920 |
| | AAGAAAC | AAACAG | AGTTT | CCCGC | ACCCCT | CACCTG | CGGAA | ACGTG | CGCGGG |
| | 7930 | 7940 | 7950 | 7960 | 7970 | 7980 | 7990 | 8000 | 8010 |
| | GCGCCCG | GAAGGCT | CGCGGT | ACGCGA | AACCGC | CGGACG | CTGTG | GCGGAT | CACCGG |
| | 8020 | 8030 | 8040 | 8050 | 8060 | 8070 | 8080 | 8090 | 8100 |
| | TGGACCC | CGGCTT | CGGCGT | GATCGT | CGCCAG | ACGGGCG | GGAAGT | CCCGT | GCTCCT |
| rep | M | D | P | A | S | G | V | I | V |
| | 8110 | 8120 | 8130 | 8140 | 8150 | 8160 | 8170 | 8180 | 8190 |
| | GCCCGGT | CTGCG | CGCGCA | CAGAT | CCGCG | CACAAG | CGGCG | GAGGAT | CACCG |
| | C | P | V | C | A | A | T | I | R |
| | 8200 | 8210 | 8220 | 8230 | 8240 | 8250 | 8260 | 8270 | 8280 |
| | ACCTGGT | CACCTT | CACCGC | CGCGC | ACGGG | CACACG | GACCGG | CTCAT | GAGCG |
| | Y | L | V | T | F | T | A | R | H |
| | 8290 | 8300 | 8310 | 8320 | 8330 | 8340 | 8350 | 8360 | 8370 |
| | CTCCCCG | CGCGCG | GCGGCT | GCTAC | CAACG | GCTGAT | CACGG | CGCGC | ACATG |
| | A | P | R | R | P | G | A | Y | Q |
| | 8380 | 8390 | 8400 | 8410 | 8420 | 8430 | 8440 | 8450 | 8460 |
| | GCGAAGG | CGATCC | GCGAC | CGGAT | CGGCT | ACGTC | AGTCAT | GCGCG | CGGAC |
| | R | E | G | I | R | D | R | I | G |
| | 8470 | 8480 | 8490 | 8500 | 8510 | 8520 | 8530 | 8540 | 8550 |
| | TCCACGC | AGTCT | GCTGCT | CGCGCG | CGGAC | GCGAGG | GTCCG | CGACCT | TTCGAG |
| | I | H | A | I | V | L | V | G | G |
| | 8560 | 8570 | 8580 | 8590 | 8600 | 8610 | 8620 | 8630 | 8640 |
| | TCGACGA | GTGGCA | AGGCCA | GTGGC | GAGCG | GTGTG | GACCG | TGCCT | GCGCA |
| | L | D | E | W | Q | G | Q | W | R |
| | 8650 | 8660 | 8670 | 8680 | 8690 | 8700 | 8710 | 8720 | 8730 |
| | TTGACTT | CAAGCG | GCTGG | GAGACC | GAAACG | CAGCG | ACCAAC | GACCT | CGCCG |
| | V | D | F | K | R | L | E | T | E |
| | 8740 | 8750 | 8760 | 8770 | 8780 | 8790 | 8800 | 8810 | 8820 |
| | AACTCGC | CGCGC | CGGAC | CTCAAG | ACGCG | GGAACG | CGCGG | GAACG | TCGCG |
| | E | L | A | R | A | D | L | K | T |
| | 8830 | 8840 | 8850 | 8860 | 8870 | 8880 | 8890 | 8900 | 8910 |
| | TGACCGA | GAGCAG | CGCGC | GCGGG | TGCGCT | CGCTG | GGAAT | GGAAC | TGCGC |
| | M | T | E | D | D | A | A | G | V |
| | 8920 | 8930 | 8940 | 8950 | 8960 | 8970 | 8980 | 8990 | 9000 |
| | CCATCGA | ATGGAC | CGCTAC | CTGCGG | CAGAT | GTCTCG | GCGCT | CGACG | CGCGC |
| | A | I | E | W | T | R | Y | L | R |
| | 9010 | 9020 | 9030 | 9040 | 9050 | 9060 | 9070 | 9080 | 9090 |
| | ACGCGCA | CGCGCG | GGAAC | TCCGCG | CGCGG | GTCGCG | GTGAC | CGGAG | CGGAT |
| | | | | | | | | | |

9190 9200 9210 9220 9230 9240 9250 9260 9270
ACGCCGTGGTGGTCTACCTCCGGCGAGGTGCGCCGAGGCGTACGCCGACATGCTCGCCGCCCTCGCCCTGCGCCGAGGAAGCAGCTG
D A V V V L T S G E V A E A Y A D M L A A L A L R R E E A A

9280 9290 9300 9310 9320 9330 9340 9350 9360
CACGCCGCGCGGGAGCAGGACGACGACCAGGACGACGACGCCGACGACGCCAGGAGCGGGCGCCGGCAGCATCGCCGACTGCGGA
A R R R R E Q D D D Q D D D A D D R Q E R A A R H I A R L R

9370 9380 9390 9400 9410 9420 9430 9440 9450
ACTGATATCGATCCGCACTAACTCGCTGCCCGCCCCCTACTCCCGCGCGACCTCTCCGTGACCCGACGGAGAGGTGTCGGCGCGGTG
N

9460 9470 9480 9490 9500 9510 9520 9530 9540
GAGGCTTGCCACAGGCGCGACCTGCGAGGCGAGCGAGGCTTGCCACGGGGCTCCACCTCGGTCCCACCTCGGTCCACCTTC

9550 9560 9570 9580 9590 9600 9610 9620 9630
GGTCCACGCTGGACGCGAGGTTGGAGCAACGCCGAGCCCCCTGCTGAAGCAACCCCGCGGGCGGTCACTGATATCAGTGACCC

9640 9650 9660 9670 9680 9690 9700 9710 9720
ACAACCTCGCTCTGCCTGTGGTTACTGCCTCCGAGGACCGCCATCGGGTCCGCCAGCCACCGCCATACGCCCGCCACGACCGCCATCC

9730 9740 9750 9760 9770 9780 9790 9800 9810
GACCGGAATGCATGGCGGTCCCATGGCGGTGCGATCGGACCCCATGGCGGACCTTGCGGTGCCATGGCGGACCGAGGAGCGAGCAA

9820 9830 9840 9850 9860 9870 9880 9890 9900
GTTATCGCGAGAGCAATGCTCTCGCGGGCGCTCGTTGGGGCGAGCAAGTTATCCGCTTGAGACTCCAGCGGTGCCCGACCGAGGGCGG

9910 9920 9930 9940 9950 9960 9970 9980 9990
TCGGGGTTCCCCGGGAGGGGAACCCCTTTGCTCTACCCCGGTTTGTATCAGCTCGGCTACGCCGACGACCCGCGCGGCGAGCC

10000 10010 10020 10030 10040 10050 10060 10070 10080
GTGCGGAACGGAACCCGCTGCCGATCCCTCGCCCGCGCCCGGCTTCCCGCCCACTCCCTCTCTCTGCTGCTGCGGCTC

10090 10100 10110 10120 10130 10140 10150 10160 10170
GTGGGTGGCTAGAGGGATGTCTGCCAAGCGAAGCCCCGACCATGCGCGGTGACGTGGGACGCGGGAAGCCCGGAACCGGATCCC

10180 10190 10200 10210 10220 10230 10240 10250 10260
CGCAACACCGAGCGCAACCCATGGCGCAACCCATGGCGCAACCCGACGCGCAACCCGACCAAGGAGCGGCGGAACCCGCTACGACACC

10270 10280 10290 10300 10310 10320 10330 10340 10350
CCCTCGACGGGCGAGCGCTCGACTCCCGGTCCGAGCGTCCGCGCGCTTCCGGGCGGCCACGTCCTGGTGCTCGATGTCCCCAGGAGT

10360 10370 10380 10390 10400 10410 10420 10430 10440
GCGTCCGGTCCACTCGCTCGACGGGCGAGCGCGGACTCGCGTCCCGGTGCGCGTCTCTGGTGGTGCTCGGGCGGTGCCGAGG

10450 10460 10470 10480 10490 10500 10510 10520 10530
TGACGGCGCGGGTGTCTATGACGGGAGTCTCCCGTCCCGTCCCGGAGTCCCGCAGCGGCCCTGATCGAGCGGTGCGGCTTGTGCGTT

10540 10550 10560 10570 10580 10590 10600 10610 10620
CGTGAATGCAAGAGGTGTGACCGTTCTTCGCGTGACGCGTGTCTGTCACCTGTGACGCGGTACGGCTTCGCCCGGACTTCGTCG

10630 10640 10650 10660 10670 10680 10690 10700 10710
TGACCGGAGCGGTCACACCTCAGGCATTACGAATGACCTCGGCTGTCGCGCCCTGCGTTGTGCCCTGGGTGCGGTCTGGATTCGGGCC

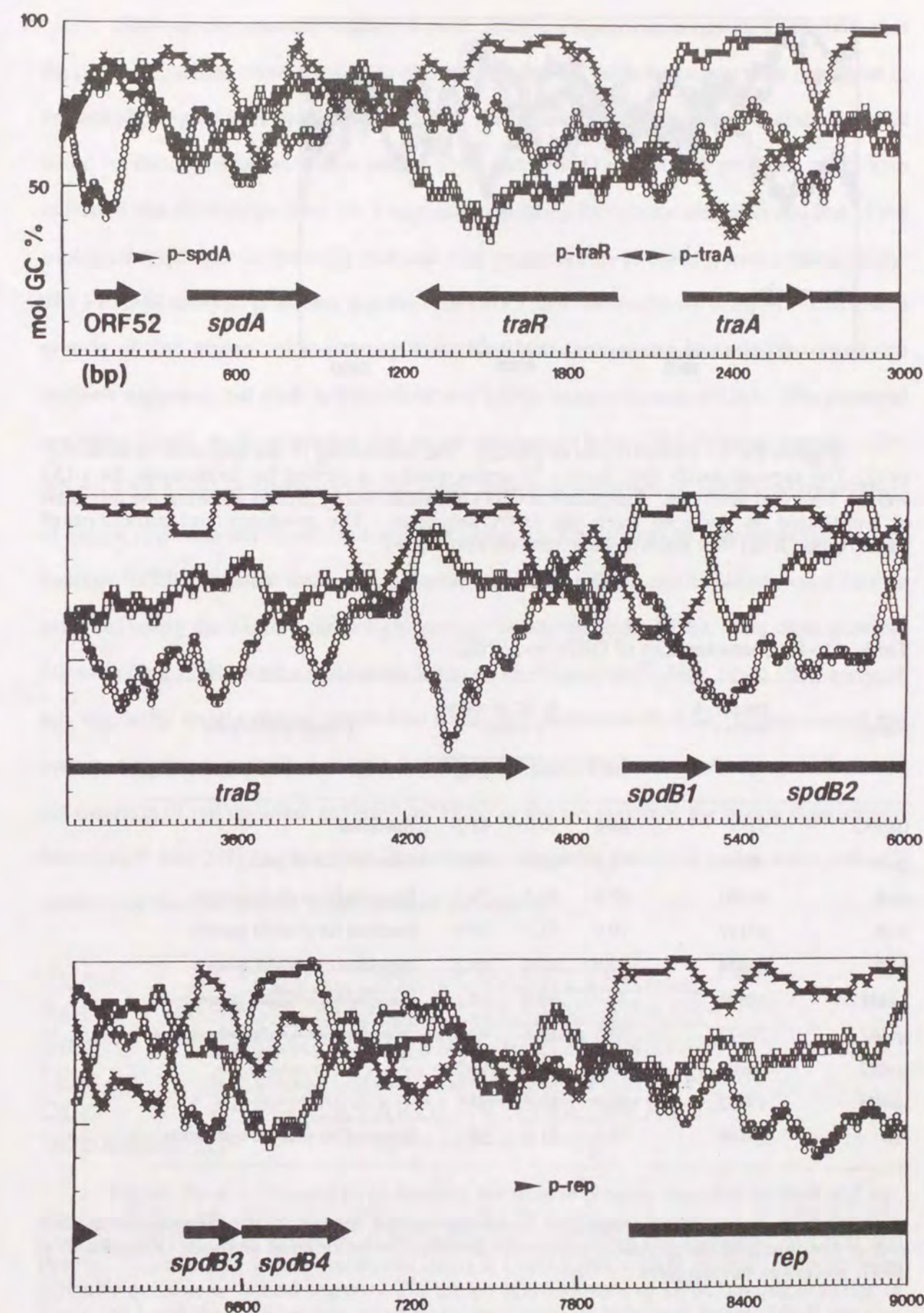
10720 10730 10740 10750 10760 10770 10780 10790 10800
TGAGTCACTGCTGGGTGCGACCGGACGGGTCCCCGGCGCGCCCGTTTCGGCGCGGTGACCTGGTGGACAAGGGGCGGTGCGGTGCCG

10810 10820 10830 10840 10850 10860 10870 10880 10890
TTCCGCGCGCGACTTCTTGTGGGAGCGGTGTCGGCGGGCGCTTCGGATCGGAATGCAAGGGTGTGCGGCTTGTGCTGCTGCCGTACGTC

10900 10910 10920 10930 10940 10950 10960 10970 10980
ATCTCGAAGAGTACGCGGCGGTGCGTACGAGTACGAGGAACGAGCGGGCTCGGCTCGGCTTGGTGCACCCAGGGGCTTTTTCGT

10990
CTGCGGTCTCTGT

Figure IV-2 Complete nucleotide sequence of pSN22. Only the clockwise strand of pSN22 as in Fig. 1 is shown. The sequence starts at the first base of the recognition sequence of the unique *Pst*I site. The amino acid sequences of putative ORFs reading clockwise are shown under the nucleotide sequence, and the amino acid sequence of *traR* reading anti-clockwise is shown above the sequence.



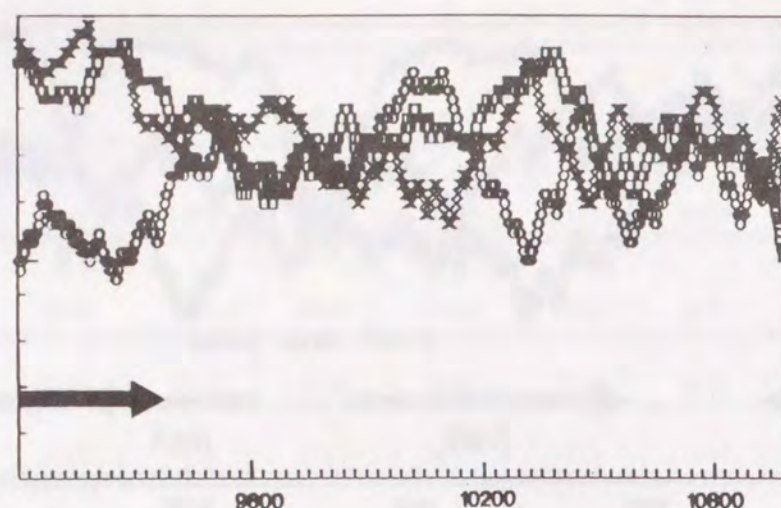


Figure IV-3 FRAME plot of pSN22. The numbering of the sequence is as in Fig. IV-2. The average mol% G+C over a 50-codon window is plotted for 3n (square), 3n-1 (X) and 3n-2 (circle) positions. The putative ORFs are indicated by arrows showing the direction of translation as deduced from the DNA sequence. The positions and directions of transcription from four known promoters are also shown.

Table. IV-1 Characteristics of ORFs on pSN22

| Name | Predicted mol wt | % GC at codon position | | | Genetical character |
|--------------|------------------|------------------------|------|------|-----------------------------------|
| | | 1st | 2nd | 3rd | |
| ORF52 | 5454 | 84.6 | 50.0 | 82.7 | Unknown |
| <i>spdA</i> | 16598 | 71.4 | 61.7 | 79.9 | Increases size of pocks |
| <i>traA</i> | 16390 | 67.1 | 52.3 | 94.2 | Essential for pock-formation |
| <i>traB</i> | 69167 | 70.9 | 53.1 | 93.2 | Essential for plasmid transfer |
| <i>traR</i> | 26604 | 67.9 | 47.6 | 90.2 | Regulator of transfer genes |
| <i>spdB1</i> | 10648 | 61.7 | 59.8 | 97.2 | Essential for efficient plasmid |
| <i>spdB2</i> | 27973 | 74.5 | 53.8 | 90.8 | transfer within recipient mycelia |
| <i>spdB3</i> | 7886 | 70.0 | 48.6 | 94.3 | |
| <i>spdB4</i> | 13167 | 71.9 | 55.5 | 93.0 | |
| <i>rep</i> | 49108 | 75.3 | 51.2 | 92.7 | Essential for plasmid replication |

Each ORF is represented its predicted molecular weight, average G+C composition at each codon position and the genetical character identified by mutational analyses (Kataoka et al 1991, see previous chapters).

ORFs in the transfer region, i) *traR* ORF In a previous chapters, I described that the pSN22 functions corresponding to plasmid transfer and pock-formation were contained in the 6.8 kbp region between *Pst*I and *Sac*II^e. Five genes, *spdA*, *spdB*, *traA*, *traB* and *traR* could be distinguished as shown on the inner circle of Fig. IV-1. A previous study also indicated that the *traR* product was a repressor regulating its own transcription and that of the *traA-traB-spdB* operon, from the *traR* and *traA* promoters (p-*traR* and p-*traA*), respectively. The FRAME analysis predicted that the *traR* ORF reads anticlockwise in Fig. IV-1 to give a protein of 246 amino acids (starting at nt 1235 and terminating at nt 1972). Northern analysis suggested that *traR* is transcribed as a 1.0 kb, mono-cistronic mRNA. The genetical analysis of *traR* made us predict that the protein would have DNA binding activity. The AXXXGXXXXXL/V (Pabo and Sauer 1984) conserved sequence of DNA binding proteins of procaryote was not found, but analysis using the Chou-Fasman algorithm (Chou and Fasman 1978) predicted several α -helix-turn- α -helix (HTH) motifs which were further analyzed using the Dodd-Egan weight matrix (Dodd and Egan 1990). The most possible DNA binding HTH motif was detected between Pro³⁴ and Ala⁵⁵ (Fig. IV-4). The score of this region by weight matrix is 980 with a standard deviation of 2.52. Disruptions of the *traR* reading frame at *Bcl*I^a (nt 1906) or at *Bgl*II (nt 1621) abolished *traR* repressor function, but insertion of a 8 bp linker at *Sma*I^a (nt 1249) or the truncation of the region downstream from *Sma*I^a (nt 1249) had no effect. These results suggested that the 6 amino acids at the C-terminus of the *traR* protein is not essential for activity.

```

TraR   34-PSVADIRAQFGVAAKTVSRALA-
Rep    269-GMTEDDAAGVGSLEWNLARWHE-
Cap    168-ITRQEIGQIVGCSRETIVGRILK-
LacI    4-VTLYDVAEYAGVSYQTVSRVVN-
Cro     14-FGQTKTAKDLGVYQSAINKAIH-
consensus      A---G-----h

```

Figure IV-4 Putative DNA-binding domains in proteins encoded by *traR* and *rep*. Each predicted amino acid sequence was scanned for conserved sequences of DNA binding proteins, and for HTH motifs in its secondary structure using the algorithm of Chou and Fasman. Candidates were re-analyzed using a Dodd-Egan weight matrix. Underlining indicates putative α -helical regions. The amino acid sequence of DNA binding domains of λ cro, lac I and the CAP protein taken from the review by Pabo and Sauer (1984) are also indicated. In the consensus sequence, "h" indicates a hydrophobic amino acid residue.

ii) *traA* ORF Previous studies indicated that the *traA* locus, involved in pock-formation and intramycelial transfer, was located on the 750 bp *BclII*^a-*KpnI* fragment. The FRAME analysis predicted the existence of an ORF starting at nt 2113 and terminating at nt 2670, and capable of encoding a protein of 186 amino acid residues. Insertion of a 8 bp linker at *SmaI*^b (nt 2167) or a small deletion of 4 bp at *KpnI* had no phenotypic effect, however, insertion of 5 bp at *BstEII* (nt 2580) led to a dramatic decrease of the transfer efficiency and abolished pock-forming ability (*traA* phenotype). Thus the *traA* protein was thought to be encoded between nt 2167 and nt 2679. In this region an ORF starting at nt 2206 and terminating at nt 2670, possibly of encoding a protein of 155 amino acids is present. There is a sequence complementary to 3' end of 16s rRNA of *S. lividans* upstream of the potential ATG initiation codon, making it likely that *traA* codes for a protein of 155 amino acid.

iii) *traB* ORF The essential gene for inter-mycelial plasmid transfer, *traB*, has been located within the 2.6 kbp *KpnI* to *SacII*^d fragment. The FRAME analysis predicted the existence of a large ORF starting at nt 2688 and terminating at nt 4640, and encoding a protein of 651 amino acids. The functions of both *traB* of pSN22 and *tra* of pIJ101 are lethal for *S. lividans* when overexpressed in the absence of their repressors, *traR* and *korA*, respectively. They are also both essential for inter-mycelial plasmid transfer. It was thus surprising to find no extensive similarity between the nucleotide or deduced amino acid sequences of the two proteins. The only common feature of both proteins is a type A (P-loop) nucleotide triphosphate (NTP) binding domain (Walker et al. 1982) found in many ATP-requiring enzymes. These sequences are characterized by a GXXXXGKTXXXXhh string (where h represents a hydrophobic amino acid). The typeB sequence, characterized by a string of largely hydrophobic residues, followed within a few amino acids by negatively charged residues, was also found both in *traB* of pSN22 and *tra* of pIJ101 (Fig. IV-5). The NTP binding domain in these proteins thus probably have important functional roles. The hydropathy plot (Kyte and Doolittle 1982) suggested that the putative *traB* protein contains hydrophobic domains long enough to span a membrane. The interaction of the *traB* protein

with the cell membrane is also suggested by its presumed function in inter-mycelial plasmid transfer.

A

| | | |
|-----------------|-----|---------------------------------|
| ATPase α | 164 | -RELIIGDRQT G KTALAIDAI- |
| ATPase β | 145 | -KVGLFGGAGV G KTVNMMELV- |
| RecA | 61 | -IVEIYGPES S GKTTLTLQVI- |
| RecB | 17 | -ERLIEASAGT G KTFTIAALY- |
| UvrD | 24 | -NLLVLGAG S GKTRVLVHRI- |
| AMP kinase | 11 | -IIFVVGGPG S GKTQCEKIV- |
| ComG1 | 139 | -MLIFTGPTG S GKTTTLYSLA- |
| VirB11 | 164 | -TMLLCGPTG S GKTMSKTLI- |
| TraI | 987 | -FTVVQGYAGV G KTTQFRAVM- |
| | | |
| Tra101 | 285 | -RMLIAGTSG S GKSWSTRALL- |
| TraB | 265 | -HLLVMGMTG S GKTEGAVPLL- |
| consensus | | hh-G----GKT-----hh |

B

| | | |
|------------|-----|-----------------------------|
| RecB | 341 | -VAMID E F- |
| UvrD | 171 | -NILV D E F - |
| AMP kinase | 115 | -LLLYV D A- |
| ComG1 | 52 | -MIIL G E I - |
| VirB11 | 54 | -RILL G E M - |
| | | |
| Tra101 | 315 | -RLVV V D P - |
| TraB | 546 | -QML L D D D- |

Figure IV-5 Comparison of the proposed NTP binding site sequence of putative proteins encoded in *traB* locus and those of several NTP binding proteins from *E. coli*. The *tra* protein of pIJ101, ComG1 of *Bacillus subtilis*, TraI of the F plasmid and VirB11 of *Agrobacterium tumefaciens* which are supposed to be involved in conjugative DNA transfer are also aligned. A: Type A motif. Bold letters indicate the conserved residues. B: Type B motif. Conserved negatively charged residues are underlined.

iv) *spd* ORFs Previous genetic analyses suggested that the two loci designated as *spdB* and *spdA* were involved in intra-mycelial plasmid transfer. *spdA* was localized within a *PstI*-*SmaI*^a fragment and *spdB* overlapped with the *SacII*^d-*SacII*^e fragment. The FRAME analysis predicted the existence of two ORFs within the *spdA* locus, one, ORF52 starting

with GTG at nt 126 and terminating at nt 281, possibly encoding a protein of 52 amino acids and the other starting from ATG at nt 444 and terminating at nt 905. No function has been identified for ORF52. The second ORF, however, probably encodes the *spdA* protein because a deletion of the *SacII*^a-*SacII*^b fragment or frame-disruption by insertion of a 8 bp linker at the *PvuII*^a gave a *spdA*⁻ phenotype which was characterized by slightly decreased pock size (Chapter I). *spdA* probably encodes a protein of 154 amino acids. The nucleotide sequence of the *spdA* ORF contains the palindrome sequence, 5'- GGAAAGTCACGTGAC (tttccc)-3', repeated three times (nt 651-665, 672-692, 708-728). These repeated sequences are in the same frame in the deduced amino acid sequence [GKSRD(FP)]. It is noteworthy that there are TCA (Ser) and TTT (Phe) codons which are rarely found in *Streptomyces* DNA. As shown in Fig. IV-3, the G+C mol% of third letter in the codons decreases specifically in the region containing these repeated sequences though that of 1st or 2nd letter did not change. For excluding the possibility of mistakes in DNA sequencing of this region, we sequenced this region at least four times for each strand using independent subclones.

Previous studies indicated that *spdB* of pSN22 had a similar function as *spd* of pIJ101, reducing pock size. The FRAME analysis predicted the existence of four ORFs designated as *spdB1* (nt 4978-5298), *spdB2* (nt 5307-6059), *spdB3* (nt 6387-6596) and *spdB4* (nt 6596-6979), capable of encoding proteins of 107, 251, 70 and 128 amino acids, respectively. In these ORFs, *spdB1* and *spdB4* use GTG triplet as initiation codon. From the result that a deletion from *SacII*^d to *SacII*^e or from *Eco47III*^a(nt 5198) to *Eco47III*^c (nt 6466) gave a *spdB*⁻ phenotype which was characterized as forming tiny pocks and reduced plasmid transfer, the *spdB* products were thought to be essential for effective plasmid spread in recipient mycelia. The functions of *spdB3* and *spdB4* have been not identified. None of four putative proteins showed similarity to known proteins. Only the hydropathy plots indicated that all four putative SpdB proteins have hydrophobic domains in their N-terminus region that might associate with the cytoplasmic membrane.

DNA sequence of the replication region The deletion analysis in chapter I suggested the existence of a 3.5 kbp region required for autonomous replication. The FRAME analysis predicted the existence of an ORF designated *rep*, starting at nt 8010 and

terminating at nt 9362, capable of encoding a protein of 451 amino acid. Insertion of 4 bp into the *BclI*^c (nt 8311) in *rep* abolished autonomous replication, suggesting that the *rep* might encode a protein essential for pSN22 replication. Southern blot analysis indicated that pIJ101 and pSN22 were very similar in their replication region. Sequence comparison showed 92% homology in the nucleotide sequences and 95% homology in the deduced amino acid sequences. The alignment of the putative amino acid sequences is shown in Fig. IV-6.

| | | | | | | | |
|--------|------------|------------|------------|------------|-------------|-------------------|-------------------|
| pSN22 | <i>rep</i> | MDPASGVIVA | QTAAGTSVVL | GLMRCGRWL | CPVCAATIRH | KRAEEITAAY | VEWIKRGGTA |
| pIJ101 | <i>rep</i> | ----- | ----- | ----- | ----- | ----- | ----- |
| | | 61 | YLVTFTHRHG | HTDRLADLMD | ALQGTTRKTAD | APRRPGAYQR | LITGGTWAGR |
| | | | ----- | ----- | -----P- | S----- | RAKDGHRAD |
| | | 121 | REGIRDRIQY | VGMIRATEVT | VGQINGWHPH | IHAIVLVGGR | TEGERSAKQI |
| | | | ----- | ----- | ----- | ----- | VGTFEPSEAA |
| | | | ----- | ----- | ----- | ----- | -A---TG-- |
| | | 181 | LDEWQGWRA | VWTAALRKVN | PQFTPDHRHG | VDFKRLTER | DANDLAIEYIA |
| | | | -----H--S | ----- | -A----- | ----- | KTQDGKAPAL |
| | | 241 | ELARADLKTA | NGGNVAPPFL | LGRIGDLTGG | <u>MTEDDAAGYG</u> | <u>SLEWNLARWH</u> |
| | | | ----- | T----- | ----- | -----S-- | -----R-- |
| | | 301 | AIEWTRYLRQ | MLGLDGGDTE | ADDLDLLAA | DADGGELRAG | VAVTEDGWAH |
| | | | ----- | ----- | ----- | ----- | VTRRALDLAA |
| | | | ----- | ----- | ----- | ----- | -----E- |
| | | 361 | TQAAEGTDGN | TDPAAMGERV | REVLAHADAA | DAVVVLTSGE | VAEAYADMLA |
| | | | -R---K--- | E---V--- | ---L--- | -T---A-- | ---Q----- |
| | | 421 | ARRRREQDDD | QDDADDDRQE | RAARHIARLR | N | |
| | | | ----- | ----- | -----A | SGPTSH | |

Figure IV-6 Comparison of the deduced amino acid sequences of the *rep* protein of pIJ101 and of pSN22. Bars indicates identical amino acid residues. The underlined sequence indicates the putative DNA-binding domain as in Fig. IV-4. Residues represented with bold letter show the amino acid sequence thought to be essential for replication proteins of plasmids or bacteriophages replicating via single-stranded intermediates (see text).

The two *rep* proteins also contain a region matching the consensus sequence, AKYXXKXXD, found at the enzymatically active sites of replication proteins of ϕ x174 (Bass and Jansz 1988) and plasmids in Gram-positive bacteria and bacteriophages replicating with a rolling circle mechanism (Gruss and Ehrlich 1989). It is noteworthy that a fusion protein between Protein A and *rep* had DNA-nicking activity *in vitro* (data not shown). Like the *traR* protein, the *rep* protein is thought to have DNA binding activity. As shown in Figs. IV-4 and 6, we detected a HTH DNA binding domain in the putative *rep* sequence. The

analysis using the Chou-Fasman algorithm predicted the helix-turn-helix structure, however, the weight matrix gave a low score. The DNA sequence upstream of the *rep* ORF revealed the presence of some AT-rich palindromic structures with some of similarity to the corresponding region of pIJ101. No similarity with known nicking sites of other rolling circle plasmids was detectable.

Promoters in pSN22, its location and relationship to identified ORFs Using the promoter-probe plasmid pARC1 (Horinouchi and Beppu 1985), we have identified three fragments having *in vivo* promoter activity. P-*traA* and P-*traR* are between *BclI*^a and *SmaI*^b reading in opposite directions (Kataoka et al. 1991, see Chapter II). In the *spdA* region, a promoter activity was detected in the *NruI* (nt 190)-*NruI* (nt 579) fragment which included the upstream region of *spdA*. All other DNA fragments from the transfer relating region lacked promoter activity when cloned into pARC1. This and the result of Northern blot experiment indicated that *traA*, *traB* and *spdB* form a *tra* operon (Chapter II). In the *rep* region, we identified a promoter activity in the *NaeI* (nt 7417)-*BstEII* (nt 8195) fragment reading in the same direction as *rep*. The locations and directions of the four identified promoters are shown in Fig. IV-1 and 3.

DISCUSSION

The complete nucleotide sequence of the *Streptomyces* plasmid pSN22 from *S.nigrifaciens* have been determined. The FRAME analysis predicted ten ORFs, nine of them have been correlated with specific genetic properties investigated previously.

traR encodes a repressor protein that regulates its own transcription and that of the transfer operon of (*traA-traB-spdB*) similar to the *korA* and *korB* proteins of pIJ101 (Stein et al. 1989, Stein and Cohen 1991). The functional analysis suggested that the *traR* protein binds to DNA, and in support of this, the deduced amino acid sequence of the *traR* protein contains a helix-turn-helix motif common to the DNA binding domains of many DNA related proteins (Pabo and Sauer 1984). Recently we detected specific DNA binding of the *traR* protein synthesized in *E.coli* to the putative target segment.

traB, essential for transfer, probably encodes a large protein that is lethal to the host when overexpressed in the absence of *traR*. The deduced amino acid sequence contains a NTP binding domain common to most ATP-requiring enzymes. This domain has also been detected in the putative *tra* protein of pIJ101 which is essential for plasmid transfer and pock-formation, and in the TraI protein of the F plasmid which is involved in conjugative plasmid transfer and has the activity of DNA helicase I (Bradshaw et al. 1990). NTP binding domains have also been found in the ComG1 protein of *Bacillus subtilis* which is essential for competence of *B.subtilis* (Alabano et al. 1989) and the VirB11 protein from *Agrobacterium tumefaciens* Ti plasmid, which is involved in conjugative DNA transfer, transporting T-DNA from the bacteria to plant cells (Thompson et al. 1988, Ward et al. 1988) as shown in Fig. IV-5. All of these proteins are involved in horizontal DNA transfer. I propose that plasmid transfer in *Streptomyces* is accomplished by inter-mycelial cell fusion, and that the *traB* gene acts principally in this step. *traB* may also act passively in chromosome mobilization by using the hydrolysing energy of nucleotide triphosphates.

I detected four ORFs in the *spdB* locus which is essential for effective plasmid transfer within recipient mycelia. In pIJ101, there are two genes *spdA* and *spdB*. The two presumptive *spd* proteins of pIJ101 and the four presumptive *spdB* proteins of pSN22 have hydrophobic domains in their N-terminal region which might interact with the cytoplasmic membrane. Thus I think that plasmid spread may involve membrane transport, however, I have no idea about the molecular mechanisms.

Both plasmids pSN22 and pIJ101 have very similar *rep* genes, encoding essential proteins for replication. At the nucleotide sequence there is 92% homology and at deduced amino acids sequence there is 95% homology. Obviously the two replicon have a recent common ancestor. Both presumptive *rep* proteins contain a HTH DNA binding motif and a DNA nicking domain conserved among replication proteins of plasmids and bacteriophages replicating via single-stranded intermediates. I thus supposed that the two *rep* proteins have topoisomerase activity like the replication initiation protein of pT181 (Koespel et al. 1985).

It is very interesting that the transfer regions of pIJ101 and pSN22 indicate no homology. Also *traR* and *korA* which have most probably similar repressor functions, can

not replace each other (Kataoka et al. 1991, see Chapter II). I thus suppose that the two replication regions and transfer regions evolved separately. Despite the complete difference in the transfer regions of both plasmids, some of characteristic sequence motifs are present in the deduced amino acid sequences from both plasmids: these are DNA binding domains in *traR* of pSN22 and *korA* of pIJ101, NTP binding domains in *traB* (pSN22) and *tra* (pIJ101), and hydrophobic domains in the four putative *spdB* proteins of pSN22 and the two *spd* proteins of pIJ101. I think that these conserved features between the probably analogous transfer proteins may be useful for the understanding of the mechanism of plasmid transfer in *Streptomyces*.

SUMMARY

The complete nucleotide sequence of the multicopy, self-transmissible and broad host range *Streptomyces* plasmid pSN22, originating from *Streptomyces nigrifaciens*, has been determined. pSN22 is a circular DNA molecule, 10922 base-pair in length and has a G+C content of 71.76%. The computer-assisted analysis identified ten open reading frames (ORFs), eight of them, *traA* (155 amino acids [aa]), *traB* (651 aa), *traR* (246 aa), *spdB1* (107 aa), *spdB2* (251 aa), *spdB3* (70 aa), *spdB4* (128 aa) and *spdA* (154 aa) are involved in plasmid transfer and pock-formation. One ORF, *rep* (451 aa), probably encodes a replication protein that is similar to known replication proteins of rolling circle replicons. The four *spdB* genes have hydrophobic amino termini that might attach to the cytoplasmic membrane. The deduced *rep* proteins of pSN22 and pIJ101 are very similar, suggesting that both are derived from a recent common ancestor. The transfer regions of the two plasmids are, however, very different. The only detectable similarities between presumably analogous proteins are DNA and NTP binding motifs and hydrophobic regions. This suggests that two transfer regions are of separate origins.

Note

The nucleotide sequence data reported in this chapter appears in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the accession number D14281.

OVERALL DISCUSSION

This thesis focused on the basic biology of the 11 kbp multicopy plasmid, pSN22, of *Streptomyces nigrifaciens*. The study of plasmids and bacteriophages has, in the part, added much to the understanding of principal functions of life. pSN22 is a conjugative plasmid. Conjugative *Streptomyces* plasmids can be much smaller than conjugative plasmids of other bacteria, which contain a great many *tra* genes. Therefore they may be good systems for identifying the most essential conjugation functions. Also the replication mechanism of pSN22 was studied to obtain a more complete understanding of the biology of the whole plasmid. Both conjugation and replication are important for the exchange of genetic information between bacteria, and are part of the survival strategy of genes.

A hypothesis was formulated to explain the involvement of genes in plasmid transfer and "pock" formation: Transfer of pSN22 occurs in two steps, intermycelial transfer between recipient and donor strains, followed by intramycelial transfer. *spdA*⁻ and *spdB*⁻ mutations affected the size of pocks, very similar to the *spd*⁻ mutants of pIJ101. From the transfer efficiency data, *spdA* and *spdB* genes are involved in, but not essential for the intramycelial transfer. The fact that the *traA*⁻ plasmid could not form pocks but retained the ability for low-efficiency transmission suggested that the *traA* gene was essential for intramycelial transfer. *traB*⁻ mutants did not transfer at all; *traB* is probably essential for intermycelial transfer. The efficiency of chromosomal recombination mediated by pSN22 transfer was also tested. Only *traB* and its essential regulator, *traR*, seemed to be involved in chromosome mobilization. The frequency of recombinants was not affected by mutations in any other genes. The putative *traB* gene product contains a p-loop NTP binding motif. Transfer genes from only three other *Streptomyces* plasmids pIJ101, pSAM2 (Hagege et al. 1993), SCP2* (Brolle et al. 1993) and pSN22 have been sequenced. *tra* of pIJ101, *traSA* of pSAM2, and *traA* of SCP2* have probably similar function as *traB* of pSN22, but neither the DNA nor the deduced amino acid sequences of these genes are similar, except for the NTP binding motif which can be found as a common feature of all their transfer genes of *Streptomyces* plasmid. In the case of the F plasmid of *E. coli*, *traI* and *traD* have NTP binding motif. The TraI

protein is known as DNA helicase I (Abdel-Monen et al. 1983) and the TraD protein is thought to make a "conjugative pore" for DNA transfer between recipient and donor cells (Panicker and Minkley 1993). In the case of the *Agrobacterium tumefaciens* Ti plasmid, putative NTP binding motif has been found in *virB4* and *virB11* (Thompson et al. 1988). *virB4*, similar to TraD of F, is thought to make a "pore" between the bacterial and plant membranes (Berger and Christie 1993). From the genetic information, *traB* of pSN22 may also act as a mediator between recipient and donor cell, analogous to *traD* or *virB4*.

Bifunctional pSN22 derivative plasmids mutated at the *traR* locus could not be introduced into *S. lividans* by transformation. Similar phenomena, mutations outside the replication region causing inviability of the plasmid or its host, have been reported for RK2 and pIJ101. And these phenomena were designated as *kil-kor* interaction. As in the case of pIJ101, the *kil* phenotype of pSN22 *traR*⁻ mutants corresponded with the disruption of the transcriptional regulation of transfer genes. Also pSAM2 has a *kil-kor* system and it seems likely that they are a general feature of conjugative *Streptomyces* plasmids. In the case of pSN22, the *traR* product acts as a repressor of both *traA* and *traR* promoters. Northern analysis indicated that a 5.7 kb mRNA was transcribed from p-*traA*. *In vitro* promoter probing indicated that there was no promoter activity in the *traA-traB-spdB* region. This indicated that *traR* regulates most transfer genes, similar to *kor* of pIJ101, which regulates its own expression and that of the *tra-spdA-spdB* operon. The presence of *traR* without *traA* resulted in an 80% decrease of the activities of both p-*traA* and p-*traR*. The presence of both *traA* and *traR* reduced both promoter activities beyond the detection limit (below 1%). This suggested that *traA* plays an important role in the regulatory circuit. *traA* is thought to regulate *tra* genes indirectly, because purified *traA* protein expressed in *E. coli* did not bind to the promoter region *in vitro*, while several molecules of TraR expressed in *E. coli* bind multiply to this DNA region *in vitro* (Kosono and Kataoka unpublished).

What is *traA*? The specific growth rate of host strains harboring the plasmids containing *traA* without *traR* and *traB* was about half compared to strains containing *traA* together with *traR*. From this and the result of the genetic analysis, *traA* is thought to be

linked with pock-formation, which is an inhibition of the growth of recipient cells. The fact that *traA* is also involved in transcriptional regulation of the *tra* genes seems paradoxical.

The replication mechanisms of pSN22 was also studied. Like other plasmids from Gram-positive bacteria, pSN22 replicates via single stranded intermediates. The replicon of pSN22 was localized within a 1.9 kbp fragment. DNA sequence analysis in this region revealed the existence of a *rep* gene encoding a replication protein. Southern blot analysis showed that pSN22 and pIJ101 were highly homologous in their replication regions. This was confirmed by the 92% nucleotide sequence similarity and the 95% deduced amino acid sequence similarity between the *rep* genes of the two plasmids. This suggests that the *rep* regions of the two plasmids may have arisen recently from a common ancestor. On the other hand, the transfer-related regions of both plasmids showed no similarity of the nucleotide sequences or the deduced amino acid sequences. This probably means that the replicon and the transfer functions of pSN22 have evolved separately. pSN22, one of the star players of horizontal transfer of genes, may itself be the product of horizontal gene transfer.

The amino acid sequence of the putative Rep protein of pSN22 showed a region that is conserved among Rep proteins of rolling circle replication (RCR) plasmids, and corresponds to the DNA nicking domain of ϕ X174 protein. A MalE-Rep fusion protein expressed in *E. coli* gave DNA nicking activity but, different from the RepC protein of *S. aureus* plasmid pT181, the expressed Rep protein did not show a topoisomerase-like activity (Suzuki and Kataoka, unpublished).

Complementation analysis revealed that an *E. coli* plasmid containing the 500 bp fragment upstream of the *rep* gene can replicate in *S. lividans*, with the help of *trans*-supplied Rep protein from another pSN22 derivative. But the growth rate of transformants was slow, except when the transformants harbored co-integrates between the two plasmids. This indicated that the position of *oriI* or the distance between *oriI* and the *rep* gene affected the efficiency of replication. As in other RCR plasmids, the minus origin (designated as *ori2*) is present on the leading strand of pSN22. Different from the case of *S. aureus* or *B. subtilis* plasmids, deletion of the *ori2* region had no effect on pSN22 stability. The same result was shown in the analysis of pIJ101 replication. In the case of pIJ101, minus strand synthesis

mainly depends on the region designated as *sti* (strong incompatibility). Plasmid without *sti* cannot exist with *sti*⁺ plasmid. The *ori2* deletion of pSN22 did not affect incompatibility. This means that the *sti* function and the minus origin function of pIJ101 may be separate. *ori2*⁻ pSN22 derivatives accumulated about three times as much ssDNA as the wild type plasmid, but no decrease of plasmid copy number or stability were detected. This suggests that second-strand synthesis for the conversion of ssDNA to dsDNA can start at alternative sites.

What kind of reaction is involved in the conversion reaction? Different from the conversion of *B. subtilis* plasmid pUB110, the conversion was not inhibited by rifampicin (Kuno and Kataoka, unpublished). This result means that the priming reaction, which is the initial step of the conversion, is independent of RNA polymerase. *In vitro* priming reactions using a partially purified fraction of *S. lividans* lysate, resulted in a priming activity without rNTP (Suzuki and Kataoka, unpublished). This indicates the existence of a DNA primase in *S. lividans*, and the possibility that the DNA priming activity is one key point for determine the host range of broad host range plasmids such as pSN22 or pIJ101.

I hope that the result of this thesis will be of great help for understanding *Streptomyces* plasmid biology and lead to the development of new strategies for strain improvement with subsequent applications in the field of bioengineering.

CONCLUSION

In this thesis, the whole functions of *Streptomyces nigrifaciens* was analyzed. This study was undertaken with the aim of understanding the biology of a conjugative plasmid as a life system, and to apply the obtained information for the improvement of *Streptomyces* strains with gene cloning techniques.

Chapter I describes the isolation and physical characterization of pSN22, the approximate localization of regions involved in autonomous replication and conjugation, and the detailed genetic analysis of conjugative plasmid transfer and pock-formation. A 7 kb region contains five genes designated as *traA*, *traB*, *traR*, *spdA* and *spdB* which are involved in plasmid transfer. Based on the phenotypical analysis of *in vitro* constructed mutant plasmids, a model was proposed, which explains how plasmids transfer in *Streptomyces*. As shown in Fig. C-1, pSN22 transfer is thought to occur in two steps, inter- and intramycelial transfer. *traB* was thought to be the essential gene for intermycelial transfer, because *traB*⁻ plasmids cannot transfer or form pocks. Transmissible derivatives of pSN22 could mobilize both the chromosome of their host and non-transmissible plasmid which contains only the pSN22 replication region. The efficiency of chromosome mobilization was the same for all mutants plasmids which were still transmissible. This result suggests that intermycelial transfer involves cytoplasmic mixing mediated by *traB*. A plasmid mutated in *traA* cannot form pocks, but residual plasmid transfer was detected, and chromosomal mobilization was same as efficient with the wild type plasmid. These results suggested that *traA* is involved in plasmid spreading within the recipient mycelia. Both of the *spdA*⁻ and *spdB*⁻ mutations were characterized for pock-formation. Since the transfer efficiency was decreased by the *spdB*⁻ mutation, this gene was thought to be involved in the second step of intramycelial transfer. This mutational analysis also indicated the existence of a hierarchy among transfer-related genes of pSN22. *traR* is indispensable because the *traR*⁻ plasmids are lethal for the host or itself. *traB* is essential for intermycelial plasmid transfer and therefore it is epistatic over *traA*, *spdA* and *spdB*. *traA* is essential for

intramycelial plasmid transfer. *spdB* and *spdA* are involved in efficient plasmid spreading after the action of *traA*.

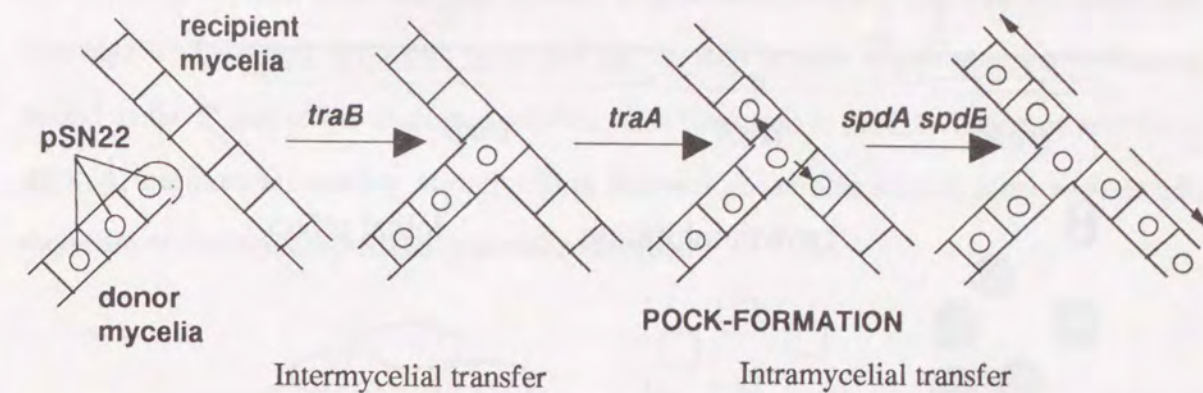


Fig. C-1. A putative model of pSN22 transfer

In Chapter II, the lethality caused by *traR*⁻ mutations was investigated. The results obtained from studies in this chapter are summarized in Fig. C-2. A fragment of pSN22, containing both *traA* and *traB* without *traR*, cloned in shuttle vector, could not be transferred into *S. lividans*. Disruptions of *traA* could not suppress the lethality. These results indicated that *traB* was the lethal function (*kil* gene). A fragment containing *traA* without *traR* inhibited the growth of host cell. This suggested that *traA* plays an essential role in pock-formation which is characterized by growth inhibition of recipient cells. The deletion of the 250 bp *BclI-SmaI* fragment suppressed the inhibitory or lethal effect of *traA* and *traB*. This suppression was thought to be a polar effect. In fact, two promoter activities, p-*traA* and p-*traR*, were detected in the 250 bp region. Analysis using a promoter-probe plasmid suggested that *traA*, *traB* and *spdB* were transcribed as an operon from p-*traA*. Northern analysis using highly radioactive ribo-probes detected a 5.7 kb mRNA which could contain the *traA*, *traB* and *spdB* genes transcribed from p-*traA*, and a 1 kb mRNA transcribed from p-*traR*. The investigation of the interaction of *traR* with p-*traA* or p-*traR* indicated, that both promoters were repressed by the *traR* gene product. These results suggested that the lethal effect of a *traR*⁻ mutation was caused by overexpression of *traB*.

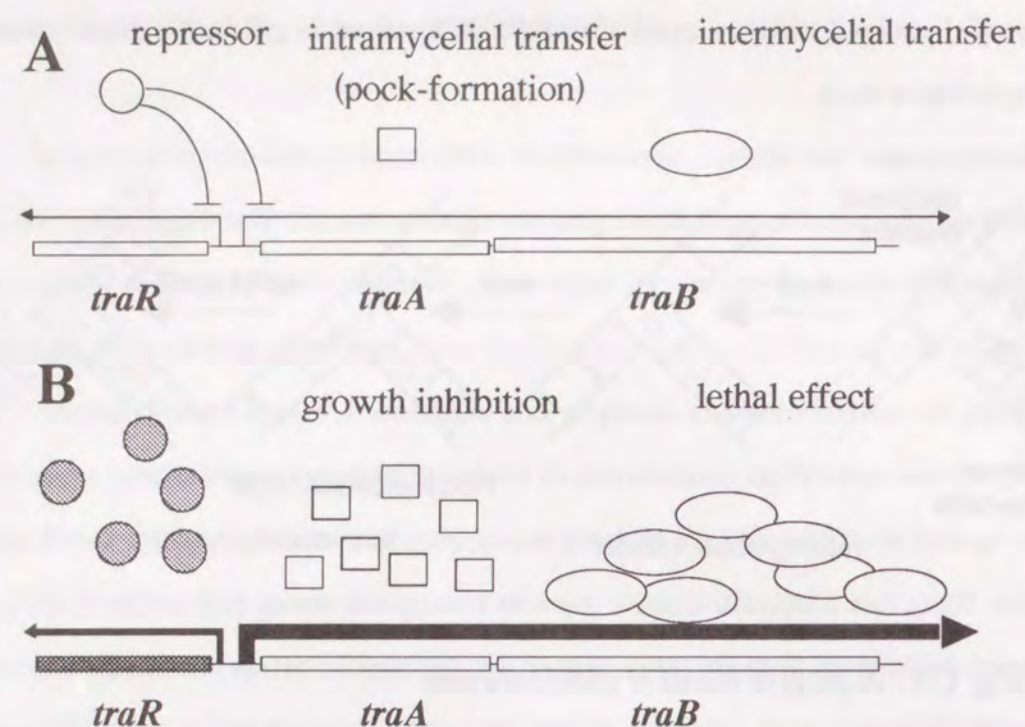


Fig. C-2. Model of the transcriptional regulation of the transfer-related genes involved in *kil-kor* interactions. A; Wild type. B; *traR*⁻ mutant. Arrows indicate the direction and amount of transcripts. square, TraA; oval, TraB; circle, TraR; shaded circle, mutated TraR.

In Chapter III, the replication of pSN22 was investigated. The model of pSN22 replication is shown in Fig. C-3. The essential replication region was localized within a 1.9 kbp DNA fragment which encodes the Rep protein. A Frame-shift mutation in *rep* gene inhibited plasmid replication. The replication region also contains the replication origin, *ori1*, which is *cis*-acting. A 500 bp fragment upstream of *rep*, containing *ori1* had the capability of replicating when the Rep protein was supplied. Like other Gram-positive bacterial plasmids which replicate via a rolling circle mechanism (RCR), pSN22 replicates through single-stranded intermediates. Non-denaturing Southern blot of total DNA from *S. lividans* harboring pSN22 could detect single-stranded mono-circular pSN22. Southern blot analysis also showed that pSN22 derivatives lacking the 250 bp region designated as *ori2*, accumulated larger amount of ssDNA than the wild-type plasmid. *ori2* is located outside the 1.9 kb essential region. Deletion of *ori2* affected neither plasmid stability nor copy number. As in RCR plasmids, the initial step of pSN22 replication is thought to start with

nicking of the leading strand at *ori1*. The nicked strand is then elongated at the 3' terminus by a DNA polymerase of unknown identity. At the end of the cycle, the single-stranded DNA is cleaved at *ori1* and ligated to form a circular molecule. The last two reactions, cleavage and ligation, are again catalyzed by the Rep protein which remains covalently bound to the 5' end of the displaced ssDNA. The final step is the conversion of ssDNA to dsDNA, preferential starting at *ori2*. This conversion can also start at other regions and therefore *ori2* is not essential for plasmid replication.

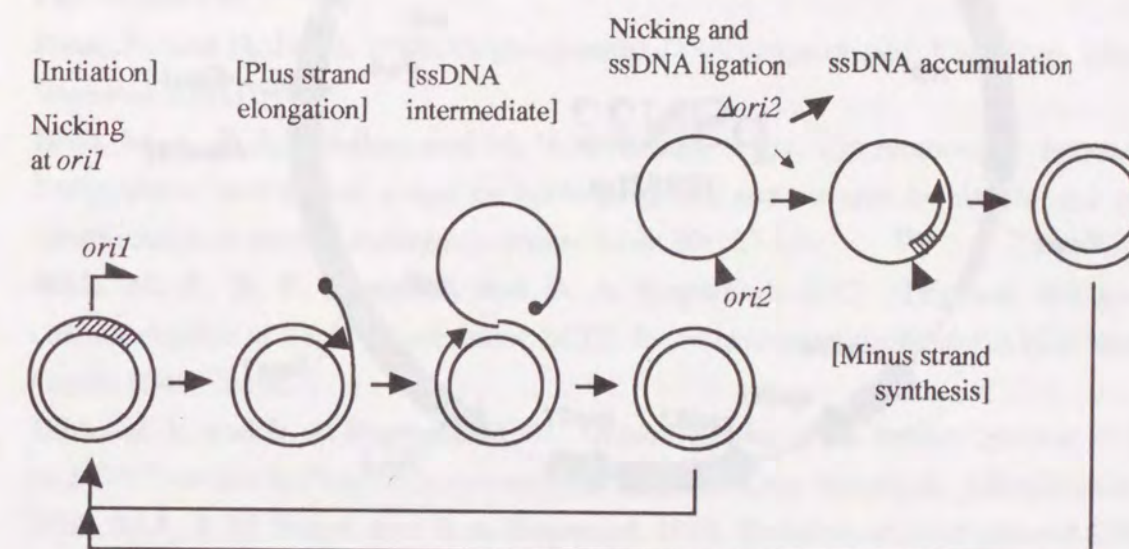


Fig. C-3 Rolling circle replication as proposed for pSN22.

In Chapter IV, the DNA sequence of pSN22 was determined and analyzed to find potentially translated ORFs. The result is shown in Fig. C-4. The putative TraB protein contains a p-loop NTP binding domain. Also TraD and TraI protein of the F plasmid of *E. coli*, VirB4 and VirB11 protein of the Ti plasmid of *Agrobacterium tumefaciens* and *tra* genes of other *Streptomyces* plasmids contain a p-loop motif. The putative Rep protein of pSN22 contains a nicking sequence which is shown to be conserved among Rep proteins of RCR plasmids. pSN22 showed a high degree of DNA and protein sequence similarity with the replication regions of pIJ101. Surprisingly, there was no DNA or amino acid sequence similarity between the transfer regions of pIJ101 and pSN22.

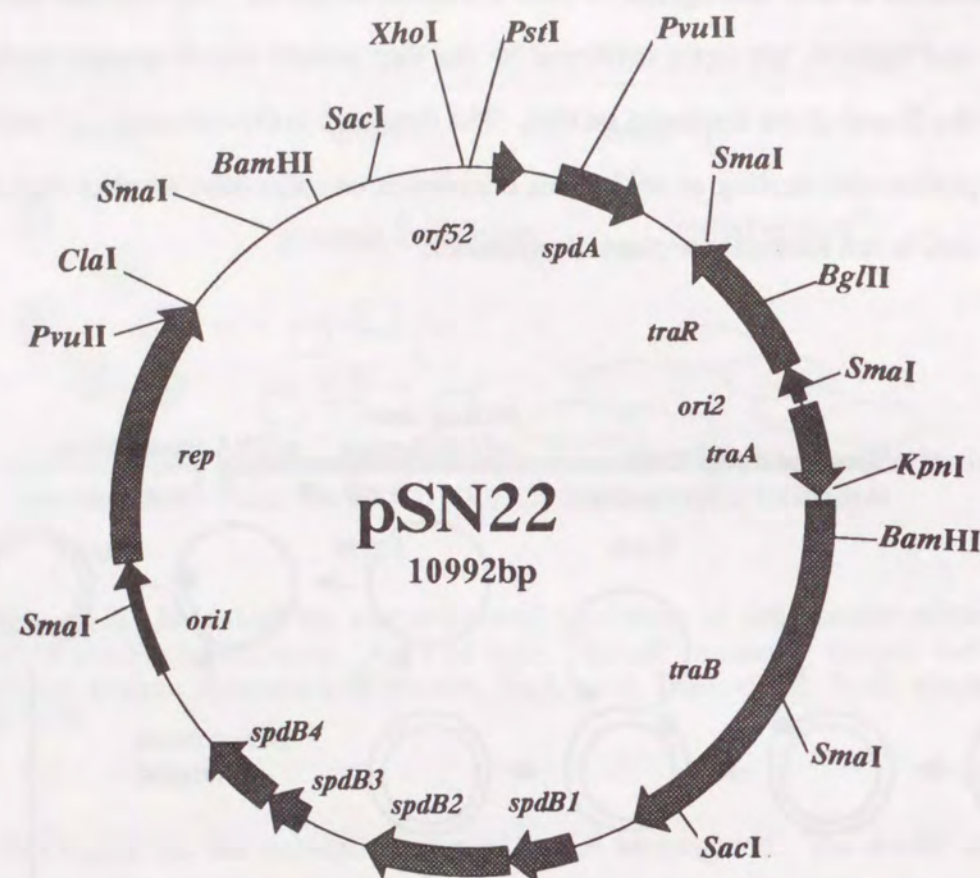


Fig. C-4 pSN22, physical map and ORFs. Shaded arrow, identified ORF; black arrow, *cis* element for replication.

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Publications related to this thesis

Five genes involved in self-transmission of pSN22, a *Streptomyces* plasmid.

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J. Bacteriol. 173:4220-4228. (1991)

Regulation and function of the *Streptomyces* plasmid pSN22 genes involved in pock formation and inviability.

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Complete nucleotide sequence of the *Streptomyces nigrifaciens* plasmid, pSN22; genetic organization and correlation with genetic properties.

M. Kataoka, Y-M. Kiyose, Y. Michisuji, T. Horiguchi, T. Seki, and T. Yoshida.

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