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Conjugative Transfer of *Streptomyces* Plasmid pSN22

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pSN22, an 11 kb multicopy conjugative plasmid from *Streptomyces nigrifaciens*, promotes chromosome recombination in *Streptomyces lividans*. Five genes have been identified to be involved in plasmid transfer and pock formation: *traB* is essential for plasmid transfer; *traA* for pock formation; *spdA* and *spdB* are concerned with pock size; and *traR*, which corresponds to a *kor* gene in a *kil-kor* system, encodes a repressor of *traR* itself and the *traA-traB-spdB* (*tra*) operon. Studies on the interaction of TraR with promoter regions suggest that the negative regulation of transfer-related genes by TraR is achieved by two mechanisms, *i.e.* promoter hiding and roadblock. The predicted ATPase activity and the membrane localization of TraB suggest that the protein plays a direct role in ATP-driven DNA translocation. TraB is also thought to be involved in intra- and intermycelial transfers of pSN22.

INTRODUCTION

Conjugation in *Streptomyces*[1] was first revealed when prototrophic recombinants were recovered from mixed cultures of pairs of auxotrophic *Streptomyces coelicolor* derivatives. Sermonti and Casciano (1963) postulated a fertility factor, R (*R*ecombination), to account for the generation of recombinants and described an analogy between R^+ and R^- *S. coelicolor* strains and F^+ and F^- cultures of *Escherichia coli*[2]. Thus such plasmids were invoked as fertility factors and SCP1 was the first physically isolated by Schrempf *et al.* (1975)[3]. The fertility mediated by *Streptomyces* conjugative plasmids is referred to as *c*hromosome *m*obilizing *a*bility (Cma)[4], which is expressed as the ratio between the number of recombinants and the number of both parents. *Streptomyces* plasmids promote chromosomal recombination at frequencies of 10^{-6} (for pJV1)[5], 10^{-5} (for pSN 22)[6], 10^{-4} (SCP2*)[7], and 10^{-3} (for pIJ101)[8], higher than or as high as those promoted by *E. coli* F plasmid (10^{-6})[9] or Hfr (10^{-3})[9]. The Cma mechanism of chromosome-integrating plasmids, such as pSAM2[10,11] and SCP1[12,13] can be explained with an analogy to that of Hfr. However, because non-integrating *Streptomyces* plasmids, such as pIJ101, pSN22, and SCP2*, also show a high frequency of Cma, the mechanism of DNA transfer in *Streptomyces* is fascinating.

In the last 10 years, knowledge on *Streptomyces* conjugative transfer has been accumulating. Conjugation in *Streptomyces* produces pocks which are zones of transient inhibition of growth of recipients. Kieser *et al.* (1982) localized the transfer and spread regions, involved in plasmid transfer and pock formation, respectively, in pIJ101, a multi-copy, broad host range plasmid from *Streptomyces lividans*[8]. Subsequently, Kendall and Cohen (1987) identified the *tra* locus responsible for plasmid transfer, and the *spdA* and *spdB* loci for pock formation[14]. Kataoka *et al.* (1991) proposed that the conjugative transfer of pSN22 proceeds in two steps: intermycelial plasmid transfer between donor and recipient mycelia, and intramycelial plasmid spread within the recipient[6].

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They also showed that the two phenotypes, plasmid transfer and pock formation, can be separated, and they investigated plasmid transfer in great detail. Tai and Cohen (1994) used the term transmission to mean the combined effects of both inter- and intramycelial transfers[15].

Because of a small number of transfer-related genes and a high frequency of DNA transfer including plasmid and chromosome, *Streptomyces* plasmids offer a good system for studying conjugation. The involvement of a small number of genes implies that *Streptomyces* plasmids have a simple conjugative system which might be an origin of other conjugative system, or that it is an uniquely different system.

In this review, we summarize our recent results on the conjugative transfer of a *Streptomyces* plasmid pSN22, particularly on the regulation of transfer-related genes and the structure and function of the main transfer protein, TraB. We also discuss the possible mechanism of DNA transfer by pSN22.

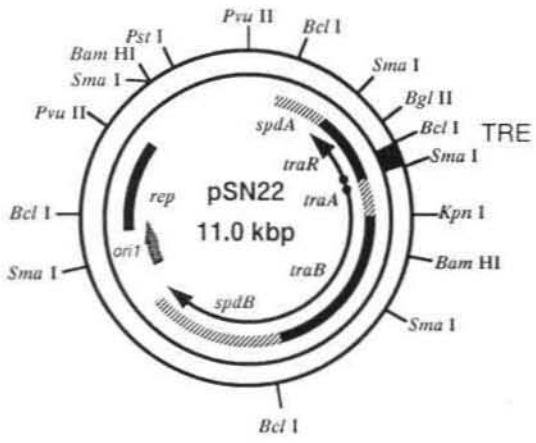


Fig. 1. Map of pSN22. The five genes, *spdA*, *traR*, *traA*, *traB*, and *spdB*, are involved in plasmid transfer, pock formation, and fertility[6]. The Rep protein introduces a strand specific nick at *ori1* to start rolling circle replication[49]. Arrows represent transcriptional units and directions of *traR* and the *tra* operon. The *Bcl*I-*Sma*I region is called TRE (*t*ransfer-gene *r*egulating *e*lement) that contains *traR* and the *tra* operon promoters[22].

The transfer-related genes of pSN22

pSN22, originally isolated from *Streptomyces nigrifaciens*, has been studied in *S. lividans*, a microorganism with a well characterized genetic background. The five genes, *traA*, *traB*, *traR*, *spdA* and *spdB*, on pSN22 shown in Fig. 1, are involved in plasmid transfer, pock formation, and fertility. Kataoka *et al.* (1991) showed that the intermycelial transfer of pSN22 is mediated by *traB*[6]. Mutations in *traB* abolished plasmid transfer, pock formation, and fertility[6]. The *traA*, *spdB* and *spdA* genes are involved in intramycelial transfer within recipient mycelia[6]. Mutations in *spdA* or *spdB* caused a marked reduction in the diameter of pocks[6]. Mutations in *traA* abolished pock formation but not plasmid transfer or Cma, indicating that the two phenomena are separable[6]. The *traA* phenotype is unique to pSN22 and has not been identified in other *Streptomyces* plasmids. Inter- and intramycelial transfers are measured by our assay[6], in which plasmid (Tsr^R) -harboring strains are streaked in a line on a lawn of a plasmid-free *S. lividans* TK24 (Str^R) and, after sporulation, the spores are replicated to a selective medium containing thiostrepton and streptomycin. Inter- and intramycelial transfers are evaluated from the presence and width of colonies on the selective medium.

S. lividans is not transformed successfully with plasmids which have a functional *traB* without *traR*, implying that pSN22 has a *kil-kor* system[6,16], first identified on RK2, a broad host range, Gram-negative conjugative plasmid[17,18]. The *kil-kor* system has also been identified on the well studied *Streptomyces* plasmids pIJ 101[14], pSAM2 from *Streptomyces ambofaciens*[19], and pJV1, a very similar plasmid to pSN22, from *Streptomyces phaeochromo-*

genes[5].

A *kil-kor* system on pSN22

pSN22 derivatives which have mutations or deletions in the *traR* gene cannot be introduced into *S. lividans* by transformation, although they contain the complete replication region of pSN22[6,16]. Those plasmids can, however, be introduced into *S. lividans* TK21 containing a *traR*$^+$ plasmid, indicating that the *traR* gene product acts in *trans*[16]. The *traR* phenotype is the result of expression of the *kil* function caused by loss of the *kor* (*kil* - overide) function. The *kil* gene on pSN22 is *traB*, and *traA* has a non-lethal but negative effect on cell growth[16].

Promoter-probing experiments with the *whiE* reporter gene[20] revealed that a 550 bp *Bgl*II-*Sma*I DNA fragment has promoter activity in both directions[16]. It contains *traR* and the *traA-traB-spdB* (*tra*) operon promoters[16]. Promoter activity and transcriptional level of both are reduced by the presence of *traR*, which encodes a 27 kDa protein with a helix-turn-helix DNA binding motif in its N-terminal region, suggesting that TraR is a repressor that binds to the promoter regions of *traR* itself and the *tra* operon to regulate negatively the expression of both[16,21]. The *kil-kor* system on pSN22 is due to the regulation by TraR of the *tra* operon that comprises a *kil* gene, *traB*.

TraR protein with deletion of at its C-terminal 6 amino acid residues reduces the *traR* and *tra* promoter activities to approximately 20%, but the expression of the intact TraR protein represses them completely[16,22]. A similar observation has been reported for the KorB protein of pIJ101. Stein *et al.* (1989) reported that the KorB derivative, which has a 13 amino acid deletion of the C-terminus, does not repress the *kilB* promoter completely[23]. The C-terminal regions of KorB of pIJ101 and TraR of pSN22 are important for the regulatory function of these two proteins.

Regulation of the transfer genes of pSN22

The expression of the *tra* operon is necessary for intramycelial transfer. However, unregulated expression of *traB* and *traA* results in toxicity of the host cells, as mentioned above. The TraR interaction with the promoter regions of the *tra* operon results in a molecular switch that allows the *tra* operon to express for an adequate amount of time. The question is how does an efficient intramycelial plasmid transfer control the toxicity? To resolve this, we studied the

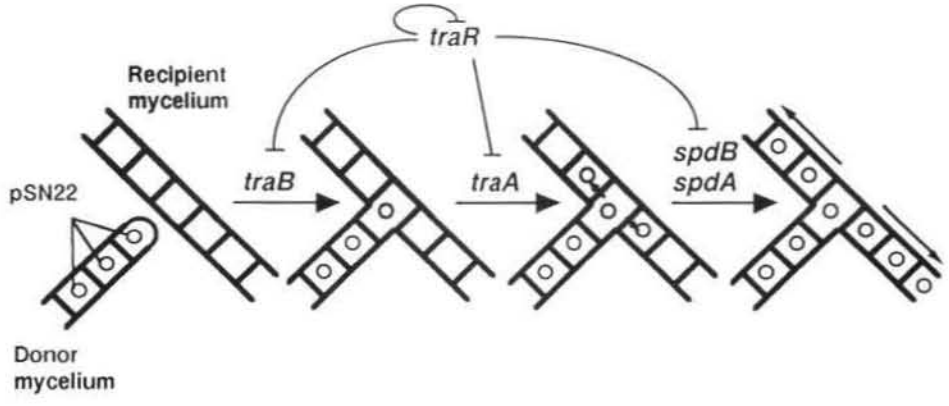


Fig. 2. Conjugative transfer of pSN22.

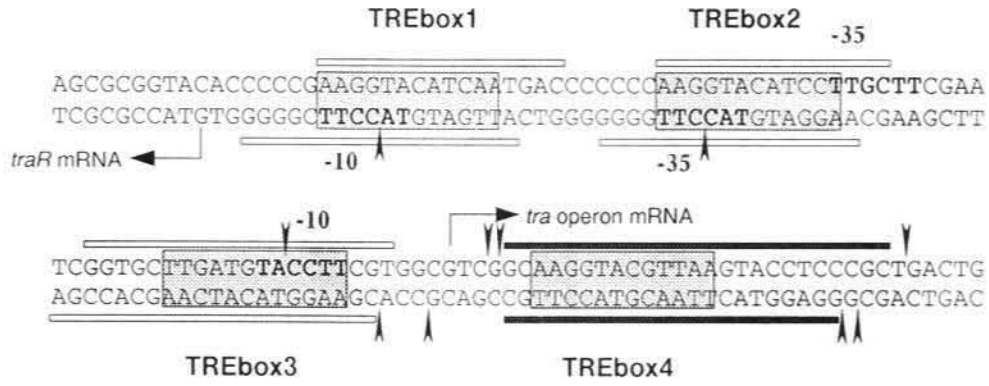


Fig. 3. Double-stranded DNA sequences of the promoter regions. The -10 and -35 sequences are indicated in bold letter. Horizontal arrows represent the transcriptional start sites and directions of *traR* and the *tra* operon mRNA. The four TREbox sequences are shaded. Black and white bars indicate regions strongly and weakly protected in DNase I footprinting[22]. The arrowheads indicate DNase I-hypersensitive sites[22].

interaction of TraR with promoter regions in detail[22].

The *traR* and the *tra* operon promoters are in the 250-bp *Bcl*I-*Sma*I region, called TRE (*t*ransfer-gene *r*egulating *e*lement)[22], of pSN22. The TRE region is responsible for the expression of four of the five transfer-related genes. TraR is a key protein which binds to TRE and regulates the expression of those genes. DNase I footprinting analysis showed that the binding site of TraR has four repeated sequences of 12 nucleotides (TREbox1-4) in TRE and TraR protected the TREbox4 site stronger than the other three sites (Fig. 3). Furthermore, only the TraR interaction at the TREbox4 site is detected by gel-retardation analysis. Footprinting and gel-retardation analyses suggest that the binding affinity of TraR is high at the TREbox4 site and low at the other three sites.

Deletion of the TREbox4 site does not cause the derepression of a reporter gene, indicating that the TraR interaction at TREbox1-3, thought to be weak, is sufficient for the repression[22]. The interaction between the TREbox4 site and the DNA binding motif in a deleted TraR, however, also acts negatively on gene expression in a derepressed state[22].

pESS551, a derivative of pESS500, contains a truncated fragment in which the TREbox4 sequence has been replaced with a 6-bp *Xho*I recognition sequence[22]. The intramycelial transfer efficiency of pESS551 is reduced, suggesting that disruption of the transcriptional regulation of the *tra* operon by the deletion of TREbox4 affects the step after intermycelial transfer[22]. TREbox4 is not essential for plasmid transfer but it is important for an acute regulation which contributes to an efficient plasmid transfer.

We proposed two mechanisms for the negative gene regulation by TraR (Fig. 4): promoter hiding[24] involving the TREbox1-3 sites and roadblock[25] involving TREbox4. The TREbox1, TREbox2, and TREbox3 regions overlap with the -10 region of the *traR* promoter, the -35 regions of both the *traR* and *tra* promoters, and the -10 region of the *tra* promoter, respectively (Fig. 3). TraR prevents initiation of transcription from both promoters by hiding the recognition sequence from RNA polymerase[24]. The binding of TraR with the TREbox4 site is thought to inhibit transcription by blocking chain elongation by RNA polymerase[25]. Expression of the *tra* operon necessary for intra-

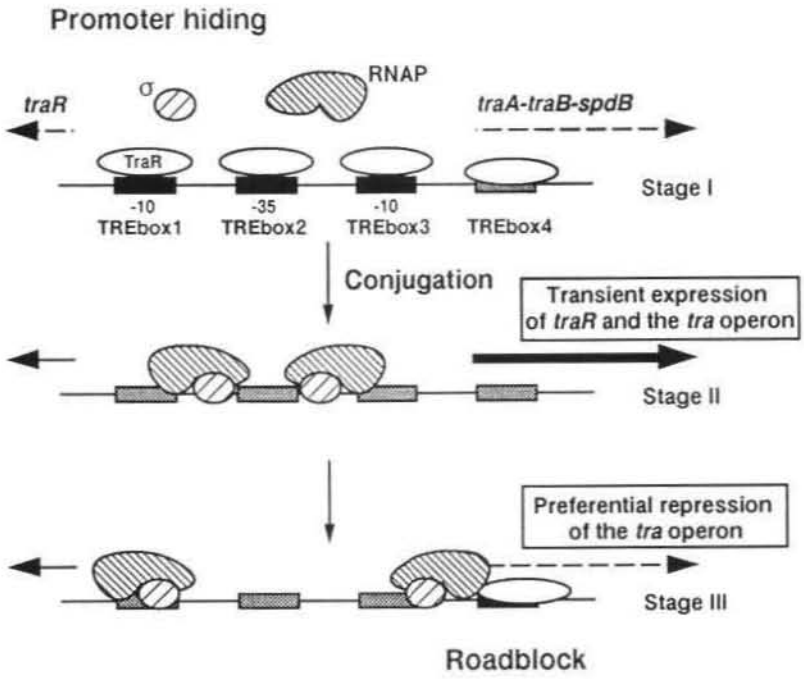


Fig. 4. Putative model of expression and regulation by TraR of transfer-related genes[22]. The thickness of arrows indicates the strength of transcription. RNAP and s indicate RNA polymerase and s70-like sigma factor, respectively. In stage I, TraR interacts with all of the four TREbox sites and represses the expression of *traR* itself and the *tra* operon by promoter hiding before conjugation. In stage II, upon conjugation, dilution of TraR causes the dissociation of TraR from DNA and leads to a transient derepression of both. In stage III, an increased level of TraR leads to a preferential repression of the *tra* operon by roadblock.

mycelial transfer would be expected to result from the dissociation of TraR from TRE, probably caused by dilution of TraR during conjugation, thus resulting in loss of equilibrium between TraR and TRE[16,26,27]. Hence an increase in TraR concentration will make TraR bind to TREbox4 and repress the *tra* operon preferentially. The preferential repression of the *tra* operon by roadblock must therefore be important in regulating the toxicity of TraB and TraA.

TraB, the single essential protein for the intermycelial transfer of pSN22

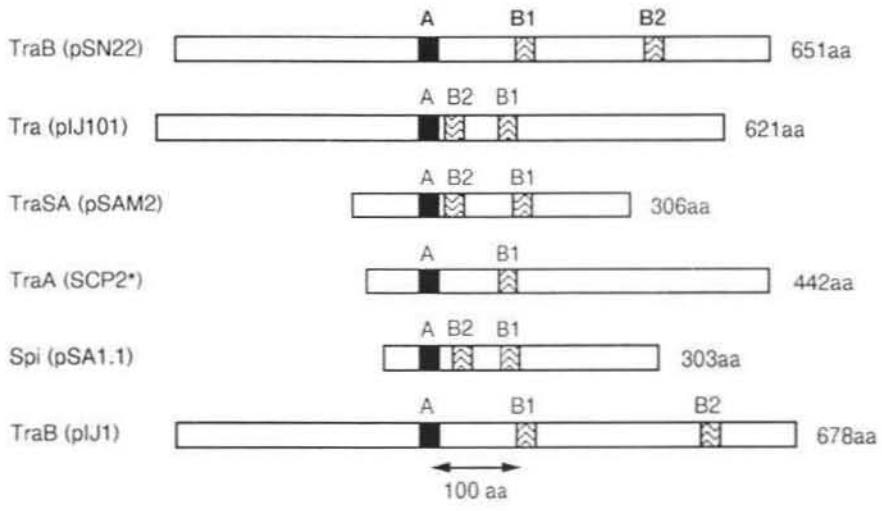
Intermycelial transfer is mediated by a single gene on plasmids pSN22 (*traB*)[21], pIJ101 (*tra*)[28], and pSAM2 (*traSA*)[19]. All of the genes correspond to a *kil* gene in a *kil-kor* system, but the relationship between the lethal activity and function in DNA transfer of these proteins encoded by these genes is still unknown. The Walker type-A and type-B motifs[29,30] are conserved among the above three proteins as well as TraA of SCP2*[7], Spi of pSA1.1[31,32], and TraB of pJV1[5]. The Spi (*sp*orulation-*i*nhibitory) protein has the function of inhibiting sporulation[31] and is involved in pSA1.1 transfer[32]. These motifs are often found in proteins which mediate DNA transport across the membrane in a wide variety of bacteria[33].

We determined the involvement of the putative Walker type-A and type-B NTP-binding sequences of TraB in plasmid transfer and fertility by site-directed mutagenesis[34]. TraB has

one type-A and two type-B like sequences, designated as A (270-GMTGSGKT-), B1 (380-LTWFEEAA-), and B2 (536-QMLLDDDG-). Site-directed mutagenesis experiments revealed that an intact A sequence and multiple acidic residues of the B1 sequence are essential for plasmid transfer and fertility[34].

Fig. 5 shows the location of Walker type-A and type-B motifs in TraB (pSN22), Tra, TraSA, TraA, Spi, and TraB (pJV1). A spacing of about 100 amino acids between type-A and one of type-B candidates is conserved among them. This alignment and the results of site-directed mutagenesis of TraB support the

A



B

Proteins	Motif A	Motif B1	Motif B2	References
TraB (pSN22)	270-GMTGSGKT-	380-LTWFEEAA-	536-QMLLDDDG-	21
Tra (pIJ101)	290-GTSGSGKS-	376-TVFIDEGA-	316-LVVVDPKR-	28
TraSA (pSAM2)	78-GATESGKS-	180-VVLVDEVA-	102-LVGIDCKQ-	19
TraA (SCP2*)	62-GAPGSGKS-	150-ILTMDEIN-		7
Spi (pSA1.1)	43-GANHSGKS-	134-VVLVDEVA-	77-LVGIDCKR-	31. 32
TraB (pIJ1)	272-GMTGSGKT-	383-LAWFEEAA-	574-AALVDEED-	5
	-GxxxxGKS-T	hhhhDEAD	hhhhDEAD	

Fig. 5. Positions and sequences of the nucleotide binding motifs in the main transfer proteins of *Streptomyces* plasmids pSN22, pIJ101, pSAM2, SCP2*, pSA1.1, and pJV1[34]. **A.** The Walker type-A and putative type-B motifs are indicated by black and wavy boxes, respectively. **B.** Protein sequences. Amino acid residues that match the consensus sequences are indicated in bold letters. The consensus sequences shown at the bottom were used to search for matching patterns in the sequences[29,30]. The most highly conserved residues are indicated in bold letters.

hypothesis that the very poorly conserved B1 sequence, located about 100 residues downstream of the type-A sequence, of *Streptomyces* transfer proteins is functionally important. From the structural aspect, the regions containing the 100 amino acids are expected to have a similar secondary or three-dimensional structure.

A hydropathy plot suggested that TraB may contain hydrophobic domains potentially spanning the membrane. A derivative of TraB with a human c-Myc peptide tag[35] at its N-terminus was constructed to facilitate sensitive immunological detection[34]. The resulting plasmid, pESS500mB, expressing the c-Myc tagged TraB instead of the original TraB was introduced into *S. lividans* TK21 by transformation. The retention of its transfer ability was confirmed by the transfer assay. The transformant was subjected to sub cellular fractionation. The c-Myc tagged TraB protein was detected by Western blotting in the total lysate and the membrane fraction, but not in the cytoplasmic or cell-surface fractions[34]. It is noteworthy that the blot had a very low background, thus c-Myc tagging may be generally useful for detecting *Streptomyces* proteins.

The transfer ability of pESS500mB is retained but the efficiency is reduced to a level of the *spdB* phenotype, suggesting that TraB is involved in intramycelial transfer as well as intermycelial transfer and that the N-terminal modification of TraB might inhibit the SpdB function[34]. The *spdB* locus consists of four ORFs, encoding proteins which show a high hydropathy plot, implying that they are localized in the cytoplasmic membrane, just like TraB. It is possible that TraB and SpdBs interact in the membrane. The interaction may be needed for intramycelial transfer, but not for intermycelial transfer, because plasmids with mutations in *spdB* still transfer between mycelia. The TraB protein may be a multi-functional protein which mediates intermycelial transfer by itself and intramycelial transfer by cooperating with SpdBs (Fig. 6).

The membrane localization and the essential NTP-binding motifs suggest that TraB plays a

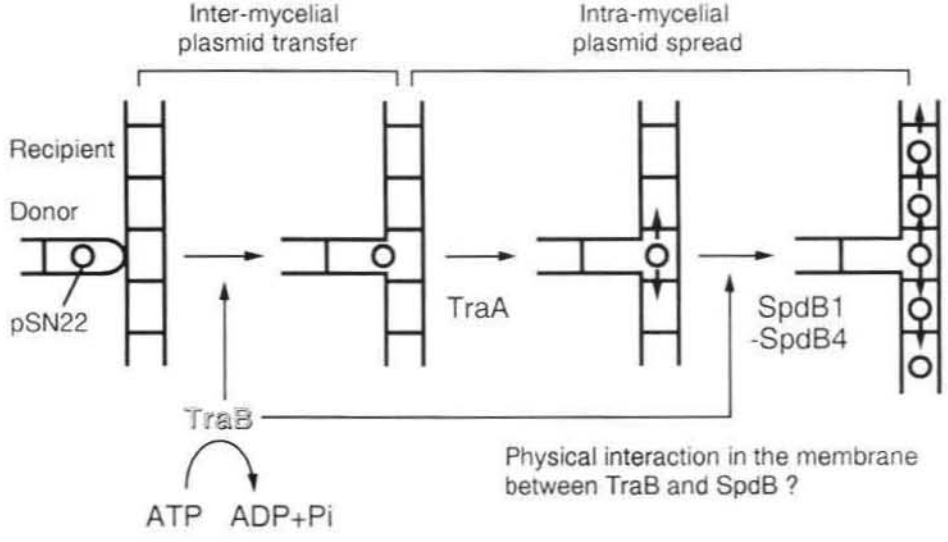


Fig. 6. Putative roles of TraB in conjugative transfer of pSN22.

direct role in ATP-driven DNA translocation.

Mechanism of conjugative transfer in *Streptomyces*

We showed that TraB is likely to be a membrane protein which uses energy from ATP hydrolysis to transfer DNA between mycelia. Proteins with the same characteristic and involved in DNA transfer are divided into two groups: one group, thought to be helicases, is represented by TraI of F[36,37], R100[38]; and the other group, presumed to form a transfer apparatus, comprises TraD[39] of F, TrbB[40] of RK2, and VirB4[41-43] and VirB11[42,44] of Ti. There are several structural and functional similarities among the natural DNA translocation processes, such as bacterial conjugation[45], T-DNA transfer from bacteria to plant cells[46], and genetic transformation in *Bacillus*[47], suggesting that they follow a common mechanism for DNA translocation across the membrane[33]. The common mechanism is achieved by "conjugative replication"[48], which originates at a nicking site, such as *oriT* or T-border, and helicase (or relaxase) produces continuous strands via rolling circle type mechanism. pSN22 replicates by a rolling-circle mechanism, in which the Rep protein introduces a strand-specific nick at *ori1* to initiate plus strand replication[49]. Thus, helicase and *oriT* could be replaced potentially with the Rep protein and *ori1* in the case of pSN22, respectively. Excluding a possibility that TraB is not a helicase, it is implied that TraB has a similar function rather to the second group above.

Recently Xiao *et al.* (1994) suggested a plasmid co-integrating mechanism to explain the mobilization of non-transmissible pIJ101 derivatives by SCP2*[50]. In this model, co-integrates between SCP2* and the pIJ101 derivatives are mobilized via SCP2*-specific pathway. At least an identical sequence of 112 bp is needed for the generation of co-integrates. The mechanism proposed by Xiao *et al.* is similar to the generally proposed DNA transfer model which involves a DNA translocation pathway, but it seems to be difficult to explain the high efficiency of Cma.

Kataoka *et al.* (1991)[6] proposed a cell fusion model as the mechanism of pSN22 transfer. This model explains the absence of integration of pSN22 into chromosome, an enhancement of Cma by pSN22, and co-transfer of non-transmissible plasmids by pSN22. In this model, cell fusion mobilizes plasmid and chromosome directly into recipient cells without DNA translocation apparatus. This model can explain easily a high efficiency of Cma by non-integrating pSN22. In the cell fusion model, TraB is supposed to utilize ATP for enhancing cell fusion. The fusing activity might explain the Kil phenotype of TraB, because unregulated event of membrane fusion would kill the host cell. However, our study of co-transfer of non-transmissible plasmids involves plasmids containing an identical sequence of more than 500 bp[6], long enough to generate co-integrates between pSN22 and its non-transmissible derivatives and we cannot exclude the possibility that unstable co-integrates were generated, though they were not detected by Southern analysis.

Hitherto, evidence supporting DNA translocation pathways or cell fusion as the mechanism of pSN22 is not available. However, the decrease in TraR concentration during conjugation can be explained by either model. The decrease in TraR concentration is important for the following step of intramycelial transfer. More detailed study of TraB will reveal the mechanism of DNA transfer by pSN22 and that it is likely to be an unique aspect of *Streptomyces* conjugation.

Conclusion

The main transfer proteins of *Streptomyces* conjugative circular plasmids show common and different features. Most of them are the single essential protein for transfer, have the

NTP-binding motifs, and have lethal or inhibitory activity on the host in the absence of its repressor. Therefore, these proteins can be regarded as *Streptomyces* Tra protein family. In spite of their functional similarity, those proteins have different sizes and sequences, less than 30% similarity, except for TraSA and Spi (52%), and TraB of pSN22 and TraB of pJV1 (65%). From the point of molecular evolution, it is interesting to consider why *Streptomyces* Tra proteins have kept similar functions without strict regulation on their primary structures.

Recently a gene encoding a different type of *Streptomyces* Tra protein has been reported. pBL1, a conjugative linear plasmid from *Streptomyces bamberigiensis,* has two main transfer proteins encoded by ORF2 and ORF3, neither of which has the NTP-binding motif[51]. It would be expected that pBL1 might have a DNA transport mechanism different from that of other *Streptomyces* circular plasmids.

We have reviewed the recent progress on DNA transfer mediated by *Streptomyces* conjugative plasmids. Essential genes for conjugation, the structure of the gene products, and the regulation mechanism have been identified. However, the mechanism of conjugative transfer remains unknown. Further studies are required to elucidate the still poorly understood transfer of both linear and circular *Streptomyces* plasmids.

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