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Characterization of The Essential Gene Components for Conjugal Transfer of *Streptomyces lividans* Linear Plasmid SLP2*

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Abstract Commonly, the interbacterial transfer of circular plasmids is initiated by nicking at an internal sequence, oriT, followed by transferring one strand as single-stranded DNA through a type Ⅳ secretion channel on cell membrane. In contrast, *Streptomyces* conjugative linear plasmids, containing a free 3′-end but a protein-capped 5′-end, can potentially undergo cell-to-cell transfer by transfer of non-nicked DNA. It was reported that circular derivatives of the *Streptomyces lividans* linear plasmid SLP2, as well as the parental linear plasmid itself can transfer efficiently. And the genetic requirements for such transfer was described. Efficient transfer of plasmid requires six co-transcribed SLP2 genes, encoding a Tra-like DNA translocase, cell wall hydrolase, two cell membrane proteins that interact with an ATP binding protein, and a protein of unknown function. Reduced transfer efficiency of plasmid from *Sal* Ⅰ R-/M- to *Sal* Ⅰ R/M hosts argues that transfer of both the circular and linear forms of the plasmid involves double-stranded DNA. These results suggest that conjugal transfer occurs by a similar mechanism for SLP2-derived linear and circular plasmids, and cellular membrane/wall functions in the transfer process.

Key words *Streptomyces lividans*, linear plasmid, conjugal transfer

Conjugative transfer of Gram-negative and uni-cellular Gram-positive bacterial circular plasmids includes two steps: it is initiated by nicking at oriT of the double stranded (ds) plasmid DNA and the single stranded (ss) DNA-protein complex is pumped through a cell membrane channel designated type Ⅳ secretion system[1~4]. *Streptomyces* are multi-cellular mycelia and uni-cellular spore forming Gram-positive bacteria. When a plasmid-containing donor is inoculated onto confluent plasmid-free recipients on solid media, conjugal transfer of plasmid can be macroscopically visible as "pocks" 1～3 mm diameter zones of growth inhibition[5,6]. Many *Streptomyces* plasmids, including autonomous and integrative plasmids, are able to conjugal transfer in species with the efficiency up to 100%, as well as mobilize chromosomal marker at a frequency ranging between 0.1% and 1%[7]. The major plasmid transfer gene tra, encoding a protein containing a domain of Ftsk/Spo Ⅲ E, exists widely among *Streptomyces* plasmids[2]. In plasmid pIJ101, the two plasmid-borne elements——a tra gene and a short *cis*-acting sequence clt——are required for efficient inter-mycelium transfer of plasmid as well as chromosomal markers mobilization[8~11]. The pIJ101-Tra protein, located in cell membrane[11], lacks the ability of nicking at the clt sequence, suggesting that the clt may not act as the oriT[12]. By using *Sal* Ⅰ restriction and modification system (*Sal* Ⅰ R/*Sal* Ⅰ M), Possoz *et al*[13] showed that the ds-DNA probably act as an intermediate during transfer of *Streptomyces* circular plasmid. These results indicate that the components and process of conjugative transfer of *Streptomyces* circular plasmids differ from that of other bacteria, although the mechanisms need to be further

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investigated[2,7].

Unlike most eubacteria, linear plasmids are common in *Streptomyces* species[7,14]. Like the circular plasmids, linear plasmids (*e.g.* SLP2, SCP1, pBL1 and pSLV45) can transfer efficiently in *Streptomyces* species[15~19]. The linear plasmids vary in size between 12 kb[20] and 1 700 kb[21] and their 5′ telomeric ends are linked covalently to terminal proteins[22,23]. Because linear plasmids naturally bear the structure of "open" DNA ends, Chen[24] predicted that the oriT might be at the telomeric termini. Recently, a terminally located gene ttr, encoding a helicase-like protein, was identified for transfer of linear plasmid SLP2[25].

The 50 410 bp linear plasmid SLP2 of *Streptomyces lividans* is conjugative[15,16,25]. Here we report that the SLP2-derived plasmids, both linear and circular, can transfer efficiently in *Streptomyces* and the ds-DNA may act as an intermediate. Six co-transcribed SLP2 genes are identified for efficient transfer of plasmid. Interactions between the ATP binding protein with itself and with the two membrane proteins are detected.

1 Materials and methods

1.1 Materials

E. coli strain DH5α (Life Technologies Inc) and plasmid pSP72 (Promega) were used as cloning host and vector. Plasmids isolation, transformation and PCR amplification followed Sambrook *et al*[26]. *S. lividans* ZX7 and TK23 were the hosts for plasmids transfer[6] and TK20 was the host for harboring plasmid SLP2[16]. Plasmid pIJ4461 provided kindly by Christophe Possoz and Jean-Luc Pernodet, contains the *Sal* Ⅰ restriction and modification genes in an *E. coli* plasmid[13]. *Streptomyces* culture, preparation of protoplasts and transformation followed Kieser *et al*[6]. Conjugation experiments followed Pettis and Cohen[10]. The *Streptomyces* ORFs were predicted by "FramePlot 3.0 beta"[27] (see website: http://watson.nih.go.jp/~jun/cgi-bin/frameplot-3.0b.pl). Sequences similarity of proteins and DNA were searched by blasting at the website (http://www.ncbi.nlm.nih.gov). The Matchmaker GaL4 Two-Hybrid System 3 (Clontech Laboratories, Inc) was employed for yeast two-hybrid assay.

1.2 Reverse tTranscription (RT) -PCR assays for the expression of SLP2 genes

Total RNA of *Streptomyces* TK20 (harboring plasmid SLP2) was prepared by following the procedure[6] with slight modifications. The RNA sample was treated with *DNase* Ⅰ (Promega) to remove trace contaminated DNA, then heated at 95℃ for 5 min, and phenol-extracted. The RNA was precipitated with ethanol, dissolved in diethyl pyrocarbonate-treated water, and quantified by spectrophotometer. One microgram of total RNA was reverse-transcribed into cDNA by using "RevertAid First Strand cDNA Synthesis" kit (MBI Fermentas). Then, 2 μl products were subjected to PCR amplification. PCR conditions were: the template DNA denatured at 94℃ for 3 min, then 94℃ 35 s, 63℃ 50 s, 72℃ 1 min, 30 cycles.

1.3 Yeast two hybrid assay for detecting the interactions of SLP2 proteins

The individual five SLP2 genes (*pQC542.1c~3c*, *pQC542.5c ~6c*) of plasmid transfer were PCR amplified, and cloned into the DNA-binding domain of "Bait" vector pGBKT7 to yield B1～B3/B5～B6, and into the GAL4-activation domain of "Target" vector pGADT7 to yield T1～T3/T5~T6. The pairs of B and T plasmids were co-introduced by transformation into the recipient yeast strain AH109. Interaction of the B-T proteins resulted in the transcription of the reporter genes *ade2* and *his3*, detected by growing cells on nutritional selection medium. Yeast colonies grown on the SC-Leu-Trp-His +3AT were inoculated into the corresponding liquid broth to determine β-galactosidase activity as described in the protocols supplied by Clontech Inc.

2 Results

2.1 Efficient conjugal transfer of SLP2-derived plasmids, both as linear and circular

Previous work showed that SLP2-derived plasmid pQC542 (Figure 1a) which contained two telomeres, *tsr*/*melC* markers and the 22 816 bp fragment (containing SLP2 genes *SLP2.4-23* with a few differences) of SLP2 was able to propagate in a linear mode in *S. lividans* ZX7 (Xu *et al*, unpublished data). When a suspension of ZX7 spores harboring pQC542 linear plasmid was inoculated onto the plate of confluent plasmid-free ZX7 spores, as shown in Figure 1b, a zone of growth inhibition ("pocks") was observed, indicating that linear plasmid pQC542 was conjugative.

To investigate if the SLP2-derived circular plasmids could transfer *via* conjugation, plasmids

pQC587 or pQC588 were constructed by deleting one or two telomeres of pQC542. These plasmids were introduced by transformation into ZX7 (*str*^{r}), and the resulting strains were mated with *S. lividans* TK23 (*spec*^{r}). As shown in Table 1, like their parental plasmid pQC542, both circular plasmids pQC587 and pQC588 were capable of transferring at high frequency in *Streptomyces*, suggested that the genes responsible for conjugative transfer functioned independently of DNA linearization.

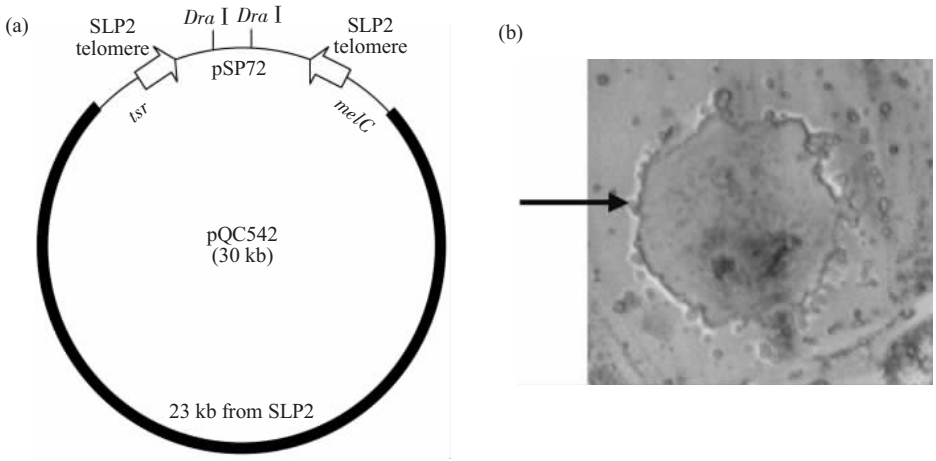

Fig. 1 Identification of SLP2-derived conjugative linear plasmid pQC542

(a) Schematic drawing of plasmid pQC542. The ~ 23 kb fragment of partially *Sau*3AI-treated plasmid SLP2 was inserted at the *Bcl* Ⅰ site of plasmid pQC177 (Qin and Cohen, unpublished) to obtain plasmid pQC542. Telomeres were indicated by pair of filed arrowheads. (b) Conjugal transfer of plasmid into cells indicated by the inhibition of growth. The concentrated spores of ZX7 strain containing pQC542 linear plasmid were diluted 100 times and 5 μl inoculated on the confluent spore patch of ZX7, and grew for 3 days at 30℃. The inhibition zone was indicated.

Table 1 Conjugal transfer of SLP2-derived linear and circular plasmids

Plasmids	Telomeres No.	Donor	Recipient	Transfer frequency[1)]
pQC542	2	ZX7	TK23	6.0×10^{-1}
pQC587	1	ZX7	TK23	4.6×10^{-1}
pQC588	0	ZX7	TK23	1.4×10^{-1}
pQC542	2	ZX7	ZX7 (pQC601)	2.5×10^{-3}
pQC588	0	ZX7	ZX7 (pQC601)	5.0×10^{-4}

[1)]Approximately equivalent numbers of spores of donors and recipients were mixed and plated on CM medium. After incubated at 28℃ for 7 days, spores were harvested and diluted and plated on CM medium containing thiostrepton, spectinomyces and thiostrepton/spectinomyces. Transfer frequency was determined as the ratio of *spec*^{r} to the *thio*^{r}/*spec*^{r} colonies.

2.2 Double stranded DNA probably is an intermediate during transfer of SLP2 linear and circular plasmids

The ds- and ss-DNAs have different sensitivities to restriction endonucleases. The conformation of DNA intermediates during plasmid transferring into a recipient was tested by using a recipient strain with the *Sal* Ⅰ restriction-modification system (*Sal* Ⅰ R/*Sal* Ⅰ M, see[13,28]). Strain ZX7 containing plasmids pQC542 or pQC588 (donors) was mated with ZX7 containing the pIJ101 derived plasmid pQC601 (recipient) carrying the *Sal* Ⅰ R/*Sal* Ⅰ M genes. Compared with conjugation to a recipient lacking the *Sal* Ⅰ R/*Sal* Ⅰ M (*e.g.* TK23, see Table 1), the transfer frequency of linear plasmid pQC542 and circular plasmid pQC588 decreased ~ 200 and 300 times, respectively, suggesting that the ZX7 with pQC601 restricted the transferring of both the non-*Sal* Ⅰ M-modified ds-linear and -circular plasmids.

2.3 Identification of a cluster of co-transcribed SLP2 genes for plasmids transfer

To identify plasmid genes involved in conjugal transfer, pQC542 fragments of various length were cloned into a circular plasmid pQC578, derived from *Streptomyces* linear plasmid pSLA2 lacking the ability of transfer on its own [29]. Strain ZX7 containing the resulting plasmids were mated with strain TK23. As shown in Figure 2, plasmids (e.g. pXQ92 and pXQ102) containing a cluster of at least the six SLP2 genes *pQC542.1c-6c* transferred efficiently in *Streptomyces*. Deletions of the *pQC542.1c* (pXQ99) or of part of *pQC542.6c* (pXQ132) decreased the transfer frequency of plasmids by ~ 10^5 times, while decreased the frequency by ~ 10^3 times for deletion of the *pQC542.4c* (pXQ131). These results suggest that the six genes *pQC542.1c-6c* (corresponding to the five SLP2 genes *SLP2.19-23* in Huang *et al* [25]. except for the one more predicted ORF *pQC542.4c*), respectively, encoding a Tra-like DNA translocase, cell wall hydrolase, unknown protein, two cell membrane proteins and ATP binding protein, were essential for conjugal transfer of plasmid.

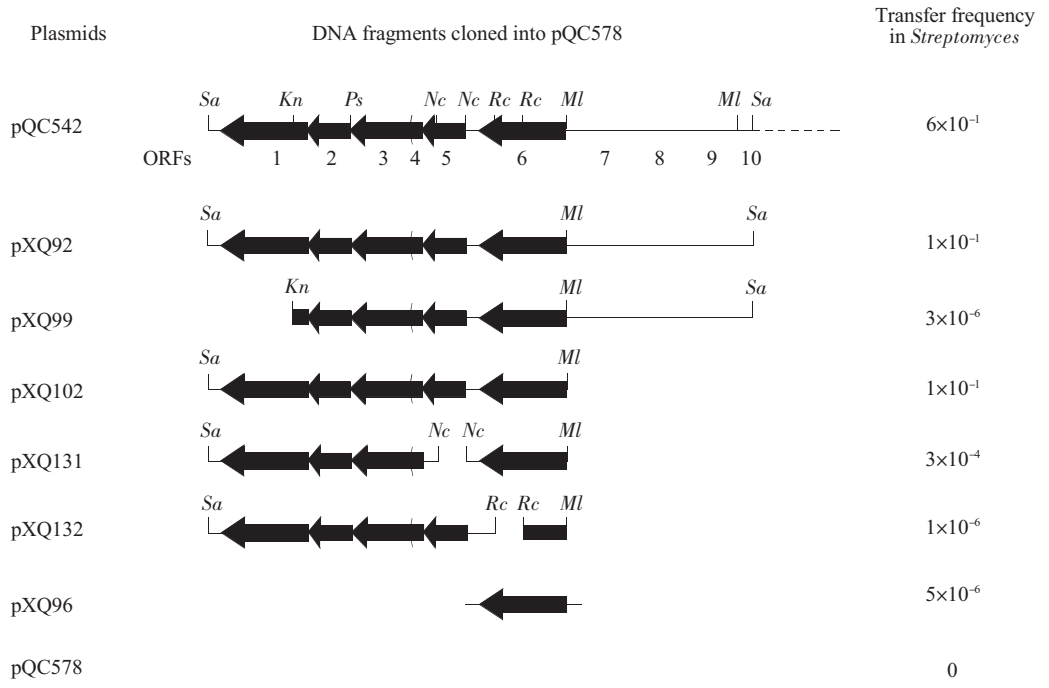

Fig. 2 Identification of the genes of plasmid pQC542 required for conjugal transfer of circular plasmids

Plasmid pQC542 DNA were digested by the enzymes *Sac* Ⅰ, *Sac* Ⅰ/*Kpn* Ⅰ, or *Sac* Ⅰ/*Mlu* Ⅰ, and ligated with pSLA2-derived non-conjugative plasmid pQC578 (29) to yield plasmids pXQ92, pXQ99, or pXQ102, respectively. Plasmid pXQ102 were digested with *Nco* Ⅰ or *Rca* Ⅰ, and self-ligated to obtain plasmids pXQ131 or pXQ132, respectively. The PCR-amplified pQC542.6c was ligated with pQC578 to yield plasmid pXQ96. These plasmids DNA were isolated from *E. coli* and introduced by transformation into ZX7 (strr), and the spores were mated with TK23 (specr). Frequency of plasmids transfer was shown. The plasmid transfer related ORFs and their transcription directions were indicated by bold arrowheads. Abbreviations were: *Sa*, *Sac* Ⅰ; *Kn*, *Kpn* Ⅰ; *Ps*, *Pst* Ⅰ; *Nc*, *Nco* Ⅰ; *Rc*, *Rca* Ⅰ; *Ml*, *Mlu* Ⅰ.

Reversal transcription-PCR (RT-PCR) assay was employed (see **Materials and methods**) to characterize the transcription of the six genes. As shown in Figure 3, all the PCR products were detected on an agarose gel and of the expected size, indicating that the junction between each gene was part of the same transcript and that these genes were co-transcribed in an operon.

2.4 Interactions between the SLP2 ATP-binding protein with itself and with other two SLP2 membrane proteins

To investigate possible interactions of the proteins encoded by the six co-transcribed SLP2 genes, yeast two-hybrid assay was employed. The 5 genes (*pQC542.1c* ～*3c* and *pQC542.5c* ～*6c*) were PCR amplified individually and cloned into the Bait- or

Target-plasmids to yield B1～3/5～6 or T1～3/5～6. These plasmids were introduced into yeast in pairs by co-transformation (see **Materials and methods**). As shown in Table 2, protein-protein interactions, including the SLP2 ATP-binding protein with itself (B1 + T1) and with the two SLP2 membrane proteins (B1 + T2 and B1 + T3), were detected. No interactions of other SLP2 proteins were observed by the assay.

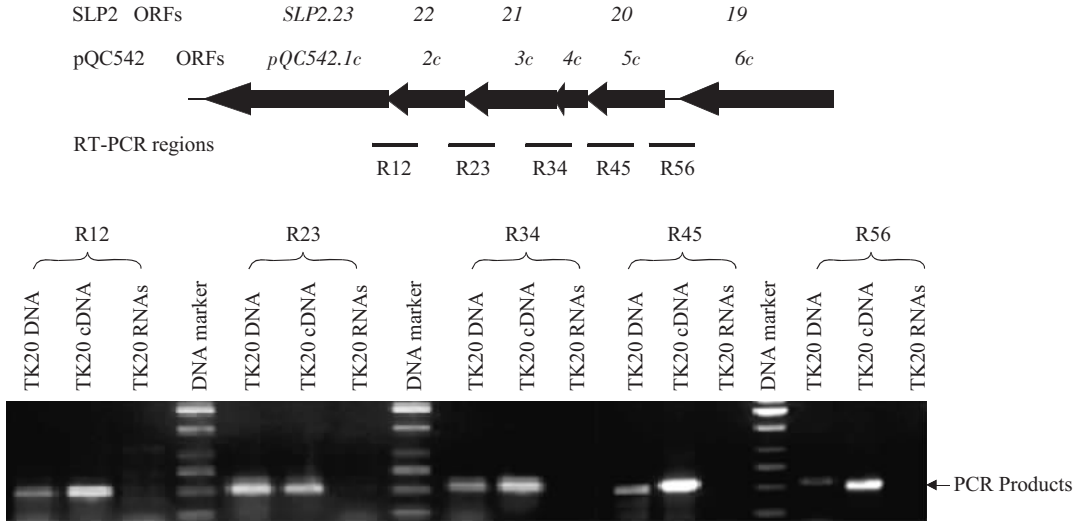

Fig. 3 Detection of transcripts of the 6 SLP2-ORFs by RT-PCR assay

Total RNA of *Streptomyces* TK20 (harboring plasmids SLP2) was prepared and transcribed reversely as cDNA. The TK20DNA, cDNA and RNA were used as templates in PCR amplification, the products were electrophoresed in a 1% agarose gel at 100 V for 2 h. RT-PCR regions on the ORFs of pQC542 or SLP2 were indicated and PCR products on gel were shown.

Table 2 Protein-protein interactions of the SLP2 genes essential for conjugal transfer

Co-transformation	Growth on SC + Leu + Trp	Growth on SC + Leu + Trp + His + 3AT	β-Galactosidase activity/U
pGBKT7-53+ pGADT7-RecT	+	+	22
B1 + T1	+	+	1.0
B1 + T2	+	+	1.1
B1 + T3	+	+	0.5
B1 + T5	+	–	–
B1 + T6	+	–	–
B2 + T2	+	–	–
B2+ T3	+	–	–
B2 + T5	+	–	–
B2 + T6	+	–	–
B3 + T3	+	–	–
B3 + T5	+	–	–
B3 + T6	+	+/–	–
B5 + T5	+	–	–
B5 + T6	+	–	–
B6 + T6	+	–	–

Abbreviations: SD medium, synthetic complete drop-out media. SD/-Leu/-Trp: SD drops out Leucine and Tryptophan. SD/-Leu/-Trp/-His+3 mmol/L 3-AT: SD drops out Leucine, Tryptophan and Histidine and adding 3 mmol/L 3-AT (3-amino-1, 2,4-triazole).

3 Discussion

The linear plasmid SLP2 is naturally conjugative in *Streptomyces*[15,16]. Recently, Huang *et al*.[25] identified a terminally located gene *ttrA* encoding a helicase for conjugal transfer of SLP2. We showed here that a SLP2 derived linear plasmid pQC542 lacking the *ttrA* still transferred efficiently, suggesting that multiple mechanisms of plasmid transfer co-exist on SLP2. Chen [24] speculated that the protein-capped ends of *Streptomyces* linear plasmids might act as the origin of transfer (*oriT*). We find that the SLP2 derived plasmids, both in linear and circular forms, can transfer efficiently, indicating that the DNA ends on linear plasmids are not the sole origin for initiating transfer of plasmid DNA. By using the *Sal* Ⅰ restriction and modification system[13], we find that the ds-DNA is likely to act as an intermediate during transfer of both the linear and circular forms of SLP2, suggesting that common processes mediate conjugal transfer of *Streptomyces* linear and circular plasmids.

Interestingly, four (*SLP2.20-23*) of the 6 SLP2 conjugation related genes are significantly similar to the *S. coelicolor* chromosomal genes (*SCO4127*, *SCO4128*, *SCO4129* and *SCO4132*, see [30]) located in membrane proteins-rich operons, suggesting the chromosomal genes involving in plasmid transfer. The VirB4 ATPase, an essential component of the type Ⅳ system of *A. tumefaciens*, interacts with itself (to dimerization) and with other membrane proteins in the *vir* operon to assemble a pore for transfer of DNA-protein complex [31,32]. We find the SLP2 ATP-binding protein (SLP2.23) interacts with itself and with the other two SLP2 membrane proteins (SLP2.21-22). These results suggest that *Streptomyces* may have a type Ⅳ-like secretion system on cell membrane for plasmid transfer.

Cell wall of Gram-positive and -negative bacteria differ markedly, the former consisting of thick peptidoglycan, while the latter containing a multi-layer structure including periplasm, peptidoglycan, outer membrane and lipopolysaccharide[33]. Conjugal transfer of Gram-negative plasmids from donor to recipient requires sex pili, whereas plasmid transfer between *Streptomyces* requires tight cell-to-cell contact [2,7], suggesting that *Streptomyces* cell wall hydrolases are required for degrading the thick layer of peptidoglycan to make a pore. One of SLP2 transfer related gene *SLP2.23* encodes a protein (Xu *et al*.[25], unpublished data) resembling a transglycosylase (SCO4132) of *S. coelicolor* (expectation value 1×10^{-73}, identify 51%) or cell wall hydrolase of *Mycobacterium tuberculosis* (expectation value 2×10^{-23}, identify 32%), supporting this hypothesis.

Thus, as a summary, we suggest a preliminary model for the conjugative transfer of circular and linear plasmids in *Streptomyces* (Figure 4). The linear or non-nicked circular plasmids are translocated by Spo Ⅲ E/FtsK-like Tra proteins to cell membrane, through a membrane channel consisting of an ATPase (VirB4-like) and membrane proteins, and cross the long pore on the thick layer of peptidoglycan being made by cell wall hydrolases and other proteins.

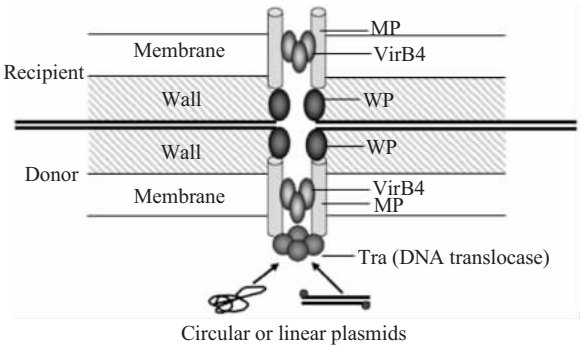

Fig. 4 The postulated conjugal transfer model for circular and linear plasmids in *Streptomyces*

Streptomyces cell membrane and cell wall are indicated by lined and striped regions. Abbreviations: Mp, membrane proteins; Wp, cell wall hydrolase and other wall related proteins; VirB4, the Virb4-like ATPase. See text for additional details.

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变铅青链霉菌内源线性质粒接合转移必需功能区的鉴定 *

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摘要　通常细菌间环型质粒在接合转移过程中，单链质粒 DNA 在质粒内部“oriT”接合转移起始位点发生缺刻. 随后，打开的单链质粒 DNA 通过细胞膜的Ⅳ型分泌系统转移到受体菌中. 但是，链霉菌中的接合型线型质粒带有游离 3′端，5′端与末端蛋白结合，因而不能以细胞 - 细胞间方式转移单链缺刻 DNA. 报道了变铅青链霉菌线型质粒 SLP2 衍生的环型质粒，与 SLP2 一样可以高频高效接合转移，并鉴定了接合转移功能区. 质粒有效的接合转移功能区包含 6 个共转录的基因，分别编码一个 Tra 样的 DNA 转移酶、胞壁水解酶、2 个膜蛋白 (可以与 ATP 结合蛋白相互作用) 和一个功能未知的蛋白质. 从 *Sal* Ⅰ R-/M- 向 *Sal* Ⅰ R/M 宿主转移的质粒频率下降表明，线型和环型的质粒都是以双链的形式转移的. 上述研究结果表明 SLP2 衍生的线型质粒和环型质粒以相似的与细胞膜 / 胞壁功能相关的机理进行接合转移.

关键词　链霉菌，线型质粒，接合转移

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