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New Phenomenon

Two distinct conjugal transfer systems on *Streptomyces* plasmid pZL1

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The mechanism of conjugal transfer of plasmids in Gram-negative and unicellular Gram-positive bacteria is commonly via a type IV secretion system (T4SS) [1]. The genes encoding the T4S proteins are usually arranged in a single operon or a few operons. In Gram-negative *Agrobacterium tumefaciens*, the T4SS is encoded by the *virB* and *virD* operons (11 and 5 genes, respectively) [2]. *Streptomyces* are multicellular mycelial Gram-positive bacteria that form unicellular spores. There are fundamental differences in the mechanisms of conjugal transfer between *Streptomyces* plasmids and those of Gram-negative and unicellular Gram-positive bacteria. Conjugal transfer of *Streptomyces* plasmids requires a *tra* gene encoding an FtsK/SpoIIIE-family DNA translocator and a few adjacent genes [3]. No bacterial T4SS has previously been found on *Streptomyces* conjugative plasmids. We reported here the co-existence of both a T4SS-like and an FtsK/SpoIIIE-family DNA translocator on a 128-kb *Streptomyces* plasmid, pZL1.

Using a method of recombinational cloning [4], an ~130-kb pZL1/pQX17 co-integrated plasmid (pZQ96) was cloned in *Escherichia coli*. Shotgun sequencing and assembling of the complete nucleotide sequence of pZQ96 showed that pZL1 is comprised of 127,862 bp, with 70.5% G+C content. Of 127 open reading frames predicted by 'FramePlot 4.0 beta' (http://nocardia.nih.go.jp/fp4/), 41 resemble the genes with known function and 86 are hypothetical or unknown genes. Surprisingly, four (i.e. *pZL1.38*, *pZL1.40*, *pZL1.41*, and *pZL1.43c*) of the nine clustered genes (*pZL1.35–pZL1.43c*) encoded VirB6, VirB4, VirD4, and TraA, respectively, resembling those of the T4SS components of bacterial conjugative plasmids. TraA (pZL1) is a large protein (1880 amino acids) containing multiple domains including relaxase and helicase, resembling those of the TraI protein (1756 amino acids) of the *E. coli* F plasmid [identity: 90/281 (32%); expectation value: 2×10^{-20}]. In another pZL1 locus, a gene (*pZL1.95c*) encoding an FtsK/SpoIIIE-family translocator resembles the major transfer protein TraB of *Streptomyces* plasmid pSVH1 [identity: 118/388 (30%); expectation value: 2×10^{-27}]. These results suggested that two distinct conjugal transfer genes co-existed in plasmid pZL1.

To investigate whether the cluster of nine genes (*pZL1.35–pZL1.43c*) might mediate plasmid conjugal transfer, various fragments were cloned in pQC578 [5], which contained a replication origin of *Streptomyces* plasmid pSLA2 and a selection marker *tsr* (thiostrepton resistance) gene. The resulting plasmids (**Supplementary Table S1**) were introduced by being transformed [6] into *Streptomyces lividans* ZX7 [7]. These transformants were used as donors. *Streptomyces lividans* ZX7 containing a chromosome-integrated pSET152 [8] carrying an *aac(3)IV* gene, conferring apramycin resistance, was used as a recipient. The donor and recipient strains were co-cultured [9] and spores were plated on the medium containing thiostrepton and/or apramycin. As shown in **Fig. 1**, plasmid pZLQ201 carrying the intact *pZL1.35–pZL1.43c* gene cluster could conjugally transfer in *S. lividans* at a frequency of $(3.5 \pm 1.6) \times 10^{-2}$. Deletions of the six genes (i.e. *pZL1.35–pZL1.40*) in plasmid pZQ205 stimulated about five times of transfer frequency. However, deletions of *traA* (pZLQ206) or *virD4* (pZLQ208,) almost abolished transfer of the plasmids. In comparison to pZLQ201, deletion of a small gene, *pZL1.42* (pZLQ207), significantly decreased the transfer frequency (~100 times). Thus, in the T4SS-like locus on pZL1, only three genes (*virD4*, *pZL1.42*, and *traA*) were essential for plasmid conjugal transfer in *S. lividans*.

To investigate if *traB*-like *pZL1.95c* and adjacent genes could mediate plasmid conjugal transfer in *S. lividans*, various fragments were cloned in pQC578. *Streptomyces lividans* containing the resulting plasmids were used as donors (**Supplementary Table S1**), while the same recipient strain as above was used. As shown in **Fig. 2**, plasmids pZLQ101 and pZLQ102 containing a cluster of eight genes (*pZL1.94c–pZL1.101*) were required for plasmid transfer at high frequencies ($\sim 5 \times 10^{-1}$) in *S. lividans.* Deletion of the *pZL1.95c* gene (designated *traB*) in pZLQ105 almost abolished the transfer of plasmid ($\sim 3 \times 10^{-6}$). Deletion of *pZL1.94c* in pZLQ104 decreased transfer frequencies ($\sim 8 \times 10^{-3}$).

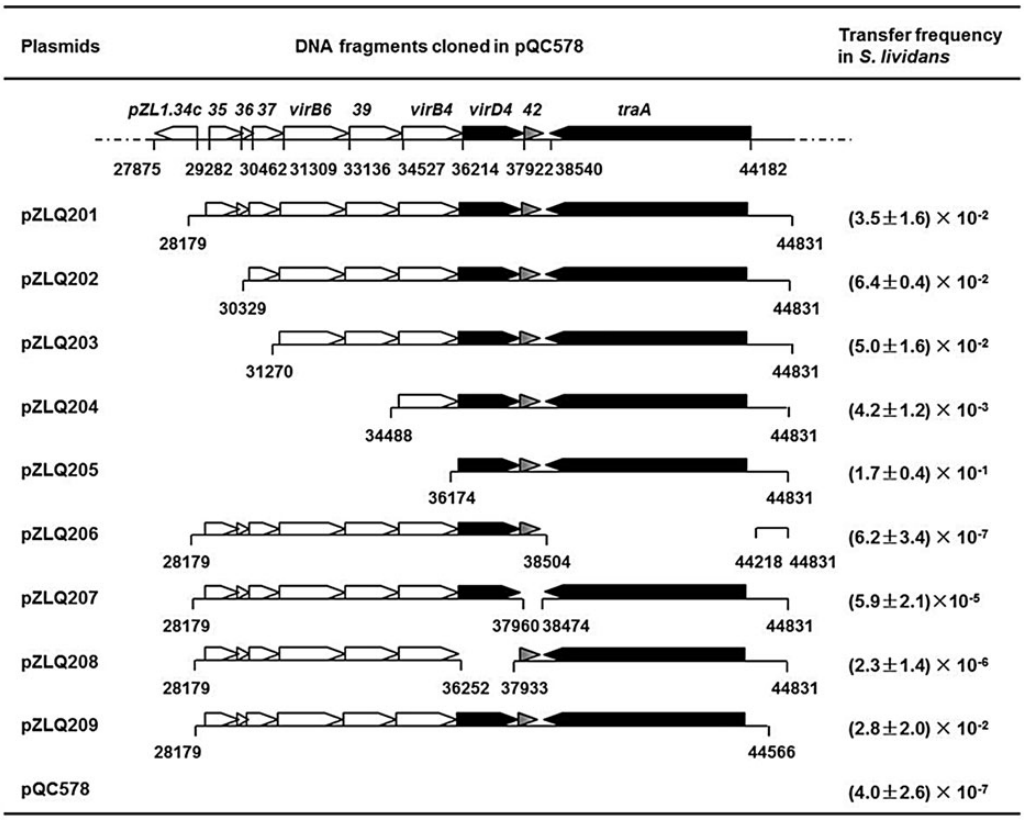

Figure 1. Identification of a T4SS-like locus on pZL1 for conjugal transfer in *Streptomyces* Plasmids were constructed in *E. coli* (**Supplementary Table S1**) and introduced into *S. lividans* [6]. Mating experiments between donor and recipient strains were performed [9]. Transfer frequencies of plasmids in *S. lividans* are shown. The frequencies given represent the mean values of three independent experiments and standard deviations are given. Relevant genes are indicated by open arrowheads and conjugal transfer-related genes by filled arrowheads.

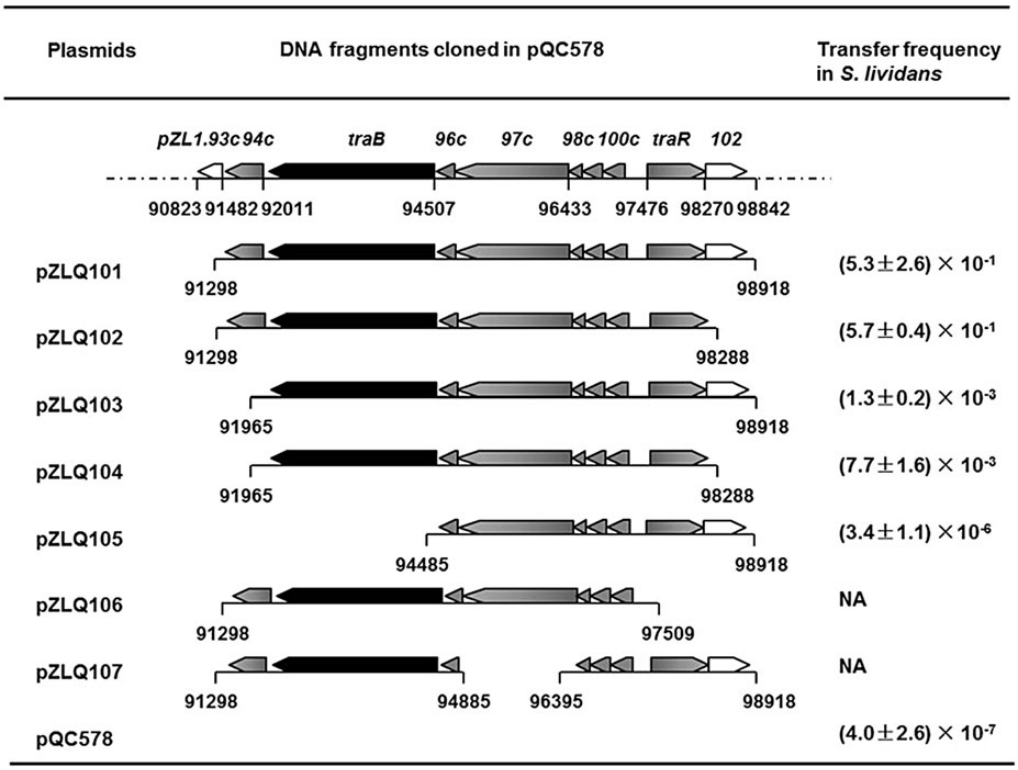

Figure 2. Identification of a *traB* (*FtsK/SpoIIIE*-like gene) locus on pZL1 for conjugal transfer in *Streptomyces* Genes are indicated as in **Fig. 1**. NA, not available.

Interestingly, deletion of *pZL1.101* (*traR*) or *pZL1.97c* (pZLQ106 or pZLQ107, respectively) could not transform in *S. lividans*, showing lethal phenotypes. These results indicated that *traB* is a major transfer gene.

To study if the plasmid containing the two distinct transfer systems could efficiently transfer in *S. lividans*, we cloned the *traB* and adjacent genes (*pZL1.94c–pZL1.102*) in pZLQ201 containing the functional T4S-like genes to obtain plasmid pZLQ1021 (**Supplementary Table S1**). pZLQ1021 could conjugally transfer at a very high frequency ($\sim 8 \times 10^{-1}$) in *S. lividans*, suggesting that co-existence of the FtsK/SpoIIIE-family translocator and the T4S-like system of pZL1 in the plasmid are functional during conjugative transfer.

In summary, our results showed that unlike other previously described *Streptomyces* plasmids, the 128-kb large plasmid pZL1 contains both a T4SS-like and an FtsK/SpoIIIE-family DNA translocator for conjugal transfer in *Streptomyces*. The T4SS-like locus on pZL1 contains a cluster of nine genes (*pZL1.35–pZL1.43c*), six (*pZL1.35–pZL1.40*) of them encoding five membrane proteins (including VirB4 and VirB6) and one hypothetical protein. The T4S translocation pore of *A. tumefaciens* consists of the core complex proteins (VirB7, VirB9, and VirB10), the inner-membrane pore proteins (VirB4, VirB6, and VirB8) and the pilus-associated proteins (VirB2, VirB3, VirB5, and possibly VirB1) [10]. Thus, in contrast to that of the Gram-negative bacterial T4S transmembrane pore, a simplified T4S-like transmembrane pore might be formed by these pZL1 proteins. Our results showed that only three genes (*virD4*, *pZL1.42*, and *traA*) are essential for plasmid transfer in *S. lividans*, and the other six genes (including *virB4* and *virB6*) can be deleted without affecting the transfer frequency. No *virB4* and *virB6* homologs of plasmid pZL1 are encoded on the chromosome of *S. lividans* (NZ_GG657756.1). These results suggested that an alternative chromosome-encoded transmembrane pore may also be used for transfer of the T4S-like locus.

When the pZL1 TraA protein or its relaxase domain was over-expressed in *E. coli* and the resulting strain were incubated with plasmid pZLQ201 DNA *in vitro*, no specific nicking activity was detected (data not shown). Using the *Sal*I restriction and modification system (*SalIR/SalIM*), Possoz *et al*. [11] showed that double-stranded DNA probably acts as an intermediate during the transfer of *Streptomyces* plasmid pSAM2. However, by cloning the *SalIR*/*SalIM* gene in a chromosome-integrating plasmid pSET152 to yield pZLQ1561K, we found that *S. lividans* containing pZLQ1561K also restricted conjugal transfer of plasmid from *E. coli* into this recipient (decreasing *ca*. 100 times in contrast to the recipient *S. lividans* containing pSET152). These results suggested that more sensitive and reliable assays would be employed for the determination of transfer intermediate of the T4S of pZL1.

Supplementary Data

Supplementary Data is available at *ABBS* online.

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