The conjugative plasmid pSG5 from *Streptomyces ghanaensis* DSM 2932 differs in its transfer functions from other *Streptomyces* rolling-circle-type plasmids

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The *Streptomyces ghanaensis* plasmid pSG5 is self-transmissible but does not form the growth-retardation zones (pocks) normally characteristic of the *Streptomyces* plasmid-transfer process. The complete nucleotide sequence of pSG5 was determined on both strands. pSG5 is 12208 bp in length and has a GC content of 68 mol%. Characterization of the open reading frames by insertion and deletion analysis revealed that only a single gene, *traB*, is involved in the transfer of pSG5. The deduced amino acid sequence of TraB is similar to the SpoIIIE protein that is responsible for chromosome translocation during prespore formation of *Bacillus subtilis*. In contrast to the *tra* genes of the other *Streptomyces* plasmids, the pSG5 *traB* does not represent a kill function. Although pSG5 transfer is not associated with pock formation, pSG5 was shown to possess putative *spd* genes that are responsible for the pock phenotype of other *Streptomyces* plasmids. However, promoter-probe experiments revealed that the *spd* genes of pSG5 are not transcribed, thus explaining the deficiency in pock formation.

Keywords: conjugation, *Streptomyces* plasmids, rolling-circle-replication, pock formation

INTRODUCTION

Conjugative gene transfer in bacteria is a very conserved process of acquiring new traits. The same mechanistic principle is realized in phylogenetically different bacteria, allowing horizontal gene transfer even between distantly related organisms. The central steps in conjugation are: generation of cell-to-cell contact (Manning & Achtmann, 1979), initiation of conjugative DNA metabolism (Kingsman & Willetts, 1978; Pansegrau *et al.*, 1990), formation of a mating pore (Haase *et al.*, 1995) and transfer of a single-stranded plasmid molecule (Gross & Caro, 1966).

Conjugation depends on the presence of conjugative plasmids that carry all the genes necessary to synthesize the whole transfer apparatus. The transfer regions of

conjugative plasmids from Gram-negative bacteria are located on rather large DNA fragments which comprise, in the case of the F-plasmid, about 33 kb and encode 36 genes (Frost *et al.*, 1994). More than half these genes are responsible for synthesis of the so-called sex pili that mediate the cell-to-cell contact. Such pili have not been found in Gram-positive bacteria, where mating pairs can be formed by the production of aggregating substances (Clewett, 1993).

Conjugative DNA metabolism of plasmids from Gram-negative and most Gram-positive bacteria is initiated by a sequence-specific endonuclease (relaxase) that nicks the conjugative plasmid at a specific sequence (*oriT*), then forms a relaxosome (Pansegrau *et al.*, 1990) and initiates replication and transfer of a single-stranded molecule by a rolling-circle mechanism (Wilkins & Lanka, 1993; Climo *et al.*, 1996). The single-stranded molecule is transferred to the recipient cell by the action of several plasmid-encoded proteins (Frost *et al.*, 1994). Some of these are hydrophobic proteins, possessing similarity to the secretion apparatus of pathogenicity factors (Weiss *et al.*, 1993) that become integrated into

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Abbreviations: HTH, helix–turn–helix; RCR, rolling-circle replication.

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the membrane of the donor, probably forming a pore to the recipient cytoplasm.

Conjugative plasmid transfer in *Streptomyces* is a very efficient process. In a typical mating experiment, up to 100% of the recipients obtain a plasmid and up to 0.1% acquire chromosomal markers (Kieser *et al.*, 1982; Bibb & Hopwood, 1981). The mechanism of plasmid transfer in *Streptomyces* is completely different from that of plasmids from other bacteria.

The transfer region of a conjugative *Streptomyces* plasmid, with a size of about 5–6 kb (Hopwood & Kieser, 1993), is much smaller than the transfer regions of other plasmids. For the best-studied plasmid, pIJ101 (Kieser *et al.*, 1982), it was shown that a single protein (Tra) together with a short sequence (*clt*) is sufficient to promote plasmid transfer (Pettis & Cohen, 1994). Apart from a conserved nucleotide-binding site, the deduced amino acid sequence of Tra shows no similarity to other proteins involved in conjugative transfer but was reported to be similar to the sporulation protein SpoIIIE of *Bacillus subtilis* (Wu *et al.*, 1995) and the cell-division protein FtsK from *Escherichia coli* (Begg *et al.*, 1995).

In all *Streptomyces* plasmids that replicate via the rolling-circle-replication (RCR) mechanism, the transfer gene *tra* is part of a Kil/Kor function. *tra* is regulated at the transcriptional level by a divergently transcribed gene *korA* (*traR*), encoding a transcriptional regulator of the GntR family (Stein *et al.*, 1989; Kataoka *et al.*, 1994b). Unregulated expression of *tra* is lethal to the host cell. Therefore, *tra* cannot be cloned without *korA*. In addition to the transcriptional control of *tra* expression, Tra seems also to be post-translationally regulated (Pettis & Cohen, 1996).

Furthermore, *Streptomyces* plasmid-transfer is a macroscopically visible event and associated with the formation of pocks. Pocks represent growth-retardation zones where aerial mycelium formation is temporally inhibited, and indicate the spreading of the transferred plasmid within the recipient mycelium (Bibb *et al.*, 1977; Kieser *et al.*, 1982). Pock formation requires the activity of *spd* genes, encoding small hydrophobic proteins. Inactivation of a *spd* gene dramatically reduces the pock size (Kieser *et al.*, 1982).

Plasmid pSG5 is a multi-copy RCR plasmid from *Streptomyces ghanaensis* DSM 2932 (Muth *et al.*, 1988, 1995). Due to its naturally temperature-sensitive replication, derivatives of pSG5 have found wide application as suicide vectors for transposon delivery (Solenberg & Baltz, 1994; Volff & Altenbuchner, 1997) and gene disruption and replacement experiments (Muth *et al.*, 1989, 1997; Yu & Hopwood, 1996).

pSG5 is conjugative, but does not form pocks during the plasmid transfer. The entire pSG5 is transferred to a recipient at a rate of 10^{-1} , which is typical for *Streptomyces* plasmids. Derivatives containing only the minimal replicon are still transferred at a rate of about 10^{-4} . The nucleotide sequence of the pSG5 minimal replicon, encoding a single gene, *rep*, has recently been published

(Muth *et al.*, 1995). Here we report the complete nucleotide sequence of pSG5 and the functional characterization of the pSG5 transfer functions.

METHODS

Strains and plasmids. The *E. coli* strain used for subcloning was XL-1 Blue (Bullock *et al.*, 1987). *Streptomyces lividans* strains were TK23 and TK64 (Hopwood *et al.*, 1983). Unless indicated otherwise, antibiotics were used in the following concentrations ($\mu\text{g ml}^{-1}$): kanamycin 50, spectinomycin 50, streptomycin 40, thiostrepton (kindly provided by Dr Lucania, Squibb & Sons, Princeton, NJ, USA) 25. Plasmid pJG4 was generated by ligating *EcoRI*-digested pUC18 into *EcoRI*-digested pGM11 (Wohlleben & Muth, 1993). To construct pJG5, the 4973 bp *SphI* fragment of pSG5 which encodes the *traB* gene was inserted into the *SphI* site of pUC18 and the resulting plasmid was fused with pGM11 via the *EcoRI* sites. The plasmid pGM19 was constructed by ligating an *aphII*-*tsr* cassette into the single *StuI* site of plasmid pSG5. The 478 bp *NcoI* fragment containing the intergenic region between *traR* and *spdB3* was filled in with Klenow polymerase, subcloned into the *SmaI* site of pUC18 and inserted as a *EcoRI*-*HindIII* fragment into pIJ487 and pIJ486, resulting in pIJ487-*traR* and pIJ486-*spdB3*, respectively. The *traB* promoter region was cloned as a filled-in 460 bp *SgrAI*-*MluI* fragment into *SmaI*-digested pUC18 and subsequently as an *EcoRI*-*HindIII* fragment into pIJ487 (pIJ487-*traB*). To investigate regulation of the *traB* promoter by TraR, the 1810 bp *Bam*HI fragment containing *traR* was subcloned into pUC18 and the resulting plasmid was subsequently fused via *EcoRI* with pIJ487-*traB*, resulting in pJG2. As a control, pIJ487-*traB* was inserted into the single *EcoRI* site of pUC18, yielding pJG1.

Sequence analysis. The nucleotide sequence was determined for both strands on an ALF Express (Pharmacia) sequencer using the Sequenase kit (Pharmacia). Subclones were obtained using various restriction enzymes and the Exonuclease III nested deletion kit (Pharmacia). Gaps in the sequence were closed using oligonucleotide primers and internal labelling. Computer programs used for sequence analysis were FASTA (Pearson & Lipman, 1988), BLAST (Altschul *et al.*, 1990), the Staden package (Staden & McLachlan, 1982), TMpredict (Hofmann & Stoffel, 1993) and CLUSTAL W (Thompson *et al.*, 1994).

Analysis of the mutant phenotype by Tn5 mutagenesis. The Tn5 insertions obtained by mutagenizing the bifunctional shuttle plasmid pSW344E (Muth *et al.*, 1988) were localized by restriction mapping and sequence analysis using pSG5-specific primers. Selected plasmids, carrying Tn5 insertions within the respective genes, were digested with *EcoRI* and religated to remove the 5.4 kb pSLE41 (Muth *et al.*, 1988) part of pSW344E. The resulting plasmids, which comprise the total pSG5, carrying the Tn5 insertion without any further *E. coli* sequences, were used as donors in mating experiments to identify genes affecting conjugal transfer of pSG5.

Promoter-probe experiments. The putative promoter regions were subcloned into the promoter-probe plasmids pIJ486 and pIJ487 (Ward *et al.*, 1986). The cloned pSG5 fragments are summarized in Table 2. To estimate promoter activity, about 10^4 spores were spotted onto the wells of a 24-well microtitre plate containing LB agar supplemented with 0, 25, 50, 100, 200, 400, 600 or 800 $\mu\text{g kanamycin ml}^{-1}$ and incubated at 30 °C. Resistance was determined as the kanamycin concentration that allowed growth as a dense lawn.

Genetic crosses. About 10^7 spores of the plasmid-containing donor *S. lividans* TK23 (*spc-1*) were plated together with the same amount of plasmid-free TK64 (*pro str-6*) spores on SM agar. After 4–5 d incubation at 30 °C, spores were harvested and serial dilutions were plated on selective agar to estimate the transfer frequencies (measured as transconjugants/recipients).

RESULTS

Transfer characteristics of pSG5

Plasmids pGM19 and pSG5-34, which contain the whole of pSG5, were transferred in *S. lividans* to a plasmid-free recipient with a rate of about 10^{-1} (Table 1). The transfer rate of 10^{-4} , which was previously reported for pSG5 derivatives (Muth *et al.*, 1995), was found to be due to the unstable replication of the bifunctional pSW344E. In particular, when pSW344E carried a Tn5 insertion, the plasmid was lost at high frequencies. pSG5 derivatives, such as pGM9 (Muth *et al.*, 1989), carrying exclusively the minimal replicon which only encodes the *rep* gene and the plus origin, were still transferred with a frequency of 10^{-4} .

In contrast to all other conjugative *Streptomyces* plasmids, the transfer of pSG5 was not associated with the formation of clearly visible pocks, which normally indicate transfer and spreading of the plasmids in the recipient mycelium.

Sequence analysis of pSG5

The complete nucleotide sequence of pSG5 was determined on both strands. According to the sequence, pSG5 has a size of 12208 bp and a GC content of 68 mol%. Eleven open reading frames (ORFs) were identified with the codon probability program of the Staden package (Staden & McLachlan, 1982) and a *Strepto-*

myces codon table (Wright & Bibb, 1992). Although there was no clear sequence identity, putative functions were proposed for most genes by sequence similarity to other *Streptomyces* RCR plasmid-encoded proteins (Fig. 1).

Regulatory proteins. *traR* encodes a transcriptional repressor of the GntR family (Haydon & Guest, 1991), showing the highest similarity (56.4% identity, 236 aa) to KorA of pIJ101. A helix–turn–helix (HTH) motif (aa 30–72) is present in its N-terminus. The putative ATG start codon is preceded (7 bp) by a likely ribosome-binding site (RBS), GGAGG. At 11 bp downstream of the TGA stop codon, there is a hairpin structure (-44.3 kJ mol $^{-1}$) with 14 bp in the stem and 7 bp in the loop, which could act as a transcriptional terminator. No *tre* boxes (Kataoka *et al.*, 1994b), or other repeats that could act as binding sites for the TraR protein, as described for the corresponding region of pSN22 and pJV1 (Servin-Gonzales *et al.*, 1995), were found in front of the pSG5 *traR* gene.

The *prg* gene (putative regulatory gene) is preceded by a likely RBS (GGAGG, 6 bp upstream of ATG) and encodes a putative regulatory protein of 265 aa. Prg possesses an HTH motif at the C-terminus with similarities to the HTH motifs of the regulatory proteins FnrN of *Rhizobium leguminosarum* (Hernando *et al.*, 1995), HutC protein of *Pseudomonas putida* (Allison & Phillips, 1990) and ORFL of the linear pSCL1 plasmid of *Streptomyces clavuligerus* (Wu & Roy, 1993). Prg showed no sequence similarity to the KorB regulator of pIJ101 or to the SpdA proteins of pSN22 and pJV1.

Putative spread (*spd*) genes. The ORF *spdB3* encodes a protein of 151 aa with two predicted transmembrane helices (aa 81–102 and 112–130). The C-terminal half of SpdB3 can be aligned (45.9% identity) with SpdB3 of pSN22, a 70 aa protein involved in pock formation. A

Table 1. Transfer rates of pSG5 derivatives carrying Tn5 insertions

Name	Inactivated gene	Insertion/deletion	Transfer rate*
pGM19	ORF106	Insertion of <i>tsr</i> and <i>aphII</i> in <i>StuI</i> site	10^{-1}
pGM9	<i>prg</i> , <i>traR</i> , <i>traB</i> , ORF193/141, <i>spdB3</i> /ORF80/ <i>spdB2</i>	Deleted	10^{-4}
pSW344E†	–	Insertion of pSLE41 in <i>EcoRI</i> site	10^{-4}
pGM12	<i>prg</i>	Replaced by <i>aphII</i>	10^{-1}
pSG5-1	<i>spdB2</i>	Tn5 insertion	10^{-2}
pSG5-22	<i>traB</i>	Tn5 insertion	10^{-5}
pSG5-29b	<i>traR</i>	Tn5 insertion	10^{-2}
pSG5-34	<i>prg</i>	Tn5 insertion	10^{-1}
pSG5-41	<i>spdB2</i>	Tn5 insertion	10^{-3}
pSG5-17	ORF193	Tn5 insertion	10^{-2}

* The transfer frequencies represent the mean values of at least four independent crosses. The data differed from experiment to experiment by less than a factor of 10.

† pSW344E turned out to be highly unstable.

TraB_pSG5 :	.MTEIQQTPTPTSTAPTATLATVTPTVRPADDTEEAALDARQLADYAPAPEMPDKTDLDAFDADP :	73
Tra_pIJ101 :	 :	-
SpoIIIE_B. :	MAKKKKRSRKKQKQLNIKYELNGCTAISIIALQLGVCGQFIYFRFFGEWFLCLGLLGVSLFWKK :	75
TraB_pSG5 :	LLAWWKS.PEGSAFYGCFYRARGCWRWR.....QNTERCQFQGRRAHEWVIEVWQ :	138
Tra_pIJ101 :	EAQAPFF..ARADIE.....DPMAL.....P...ALAQ.....NSST :	45
SpoIIIE_B. :	KTRRRTTRRKGLCLICISLLLSHVQLNLTHGSIESASVVRNWLFMLMDNSASPDLGGMISLFL :	150
TraB_pSG5 :	AAHTAHVL.....REAA.....RF.....IGKKEEMKAEQAAEVALHK :	194
Tra_pIJ101 :	MAKS.....QTTAASKN.....SVWRARTKAARTHRDARTL :	93
SpoIIIE_B. :	..FLFASIGSQMAIVMILIGMILVTGQLQEILKWMSPIGRFKEQWFFIDMFTSNMSSKTLPS :	225
TraB_pSG5 :	..R...NENNRG.....NYGPRAGGGYLATGLLGAA...TFGGAFGRSPYEEADW :	253
Tra_pIJ101 :	..YPATLAAAH.....AAVAGCGSALGWDAGAWP...LANAAVIGRRKVAV :	155
SpoIIIE_B. :	KQKPARAKQQAPEPPDEEGDYETVSP...SE...ISSFSDRNEESPVKEKRAEPKSKPQDQPETGDET :	300
TraB_pSG5 :	..WRRLGDDG.....MSAP...AKKAVIG.....SDET...IQM...LDARGAWSVVDPP :	308
Tra_pIJ101 :	..EAEAE.....RVER...WHE.....RGLGTITQTGLVLTTRRG :	207
SpoIIIE_B. :	..PPPTLENKDYEMPSDLA...KHTG...KKNIYENARKLERTFSFGAKVQHGGVTKYEYYPV :	375
TraB_pSG5 :	..PAKAAARSV...VEAQSVSK...R...YVS...DLPFE...KR...PLAIEGATF...RIS...PV :	382
Tra_pIJ101 :	..RWPPAFKAKHE...RL...DLRMEIA...SHD...RAITL....RSAD...IDTGWTPGAP....VT :	272
SpoIIIE_B. :	..V...IINLS...DLA...DIR...EAPI...AIG...PNAEVAMV...LEVLSEK...NDRPD...VLIG...R :	447
TraB_pSG5 :	..RGLHQS...VEES...GGGEP...AAGT...LAA...DP...IWAAG...GGGLPFEP...CEEFTGA :	450
Tra_pIJ101 :	..PQVPG...R...T...WTRA...AEGSEYD...H...R...AI...HRARTAIS...DVL :	342
SpoIIIE_B. :	..A...E...NMPH...A...VCV...GTILR...PHE...M...LNVNGIPH...LAPVT...P :	522
TraB_pSG5 :	..P.EAFYFMQAAADK...AAKKL...KKD...ESANKHP...LRK...VWVDE...FYTD...V...K...T :	516
Tra_pIJ101 :	..VTDELVE...EHEREL...PQD...QIPE...N...PRTVFID...VVA...AKKTRAKG...K...PDPDW...M :	410
SpoIIIE_B. :	..KK...ASA...KKV...NE...E...F...H...T...N...EGYNDYIKRANNE...KQEP...PYI...TVDELADLMMVAS...DE :	594
TraB_pSG5 :	..KGRN...VSRG...TFC...GS...TS...DL...I...WRC...PEAD...K...AS...SKNISE :	588
Tra_pIJ101 :	..EN...TAR...E...W...AKGG...PQ...Q...YA...A...SGE...V...FED...T...H...H...LP :	483
SpoIIIE_B. :	..DS...R...SQ...H...I...TGV...N...PSI...IFS...QTD...DM...G...LLGRG.... :	661
TraB_pSG5 :	..RAG...WAB...TN...LRAD...NE...E...AADLAKAGTMRP...PL...Q...EGDD...R...LA...V :	660
Tra_pIJ101 :	..PVA...RS...GPKVQ...HPN...TRAPAD...IA...P...PWR...Q...SPARSA...A...AP...K...TAP...P...P...KA :	557
SpoIIIE_B. :	..DM...FLPV...ANK...R...QA...L...EK...HV...TQOK.....QY...E...P...ET...T...THS :	720
TraB_pSG5 :	..FAAS...A...GEELD...WLP...GAV...A...KDT...LDVTA...EK...GA...VRT...D...K...A...A...SV...Y...VEATITGRWG :	735
Tra_pIJ101 :	..PT...K...AA...TGAT...AD...T...TG...NKG...SV...Q...G...VL...SE...SLSVVTVQ...VE...S..... :	621
SpoIIIE_B. :	..LY...ELT...MQTA...S...QRRFR...Y...A...M...GVV...P...PREV...S...EYDELSS... :	787
TraB_pSG5 :	LTA : 738	
Tra_pIJ101 :	... :	-
SpoIIIE_B. :	... :	-

Fig. 2. Alignment of the pSG5 TraB protein with SpoIIIE of *B. subtilis* and the Tra protein of pIJ101. The pSG5 TraB protein was aligned with the Tra protein of pIJ101 and the SpoIIIE protein of *B. subtilis*. Identical and conservative residues are shown in black.

stop codon of *spdB3*, indicating translational coupling of these two genes. The deduced protein showed no similarity to other proteins. ORF80 encodes a very hydrophobic protein that contains a predicted trans-

The ATG start codon of ORF80 overlaps with the TGA

membrane helix (aa 4–24). The next ORF, *spdB2*, is probably also translationally coupled to *spdB3* and ORF80, since its start codon overlaps the termination codon of ORF80. The deduced SpdB2 protein consists of 404 aa. Its N-terminus possesses similarity to the SpdB2 protein of pJV1 (37.4% identity, 91 aa overlap) and to the TraI protein of pMEA300 (22.8% identity, 372 aa overlap). Furthermore, it has some similarity to the Tola protein (38.8%, 85 aa overlap) of *E. coli*, which is involved in the uptake of colicin and single-stranded DNA during phage infection. SpdB2 contains four transmembrane helices (96–112, 126–145, 162–180, 188–204) and a possible fifth one with lower significance (279–297). A structure prediction with TMpredict (Hofmann & Stoffel, 1993) revealed very similar structures for SpdB2 of pSG5, TraI of pMEA300 and SpdB of pIJ101, which had no sequence similarity. The SpdB2 protein of pSG5 has several characteristic repetitive sequences (₄REEKRAD and ₃₅AEEKRAD, ₄₂AEAKA and ₄₈REAKA and ₅₃AEAKR, ₆₉AKKAA and ₇₃AKKAA, and many more less conserved ones) in its N-terminus. Similar repeats are also found in the corresponding SpdB2 proteins of pJV1 and pSN22, the TraI protein of pMEA300, and the Tola protein of *E. coli*.

Transfer genes. The deduced TraB protein showed the highest similarity to the SpoIIIE/FtsK family of proteins. It contains a putative nucleotide-binding site (motif A, GEPGAGKS; motif B, hhhhDEFA; Walker *et al.*, 1982) and possesses a predicted transmembrane helix between aa 220 and 240. The whole TraB protein from pSG5 can be aligned (Fig. 2) with the SpoIIIE homologous proteins of various Gram-positive and Gram-negative bacteria with the C-terminal half showing the highest similarity (*B. subtilis*, 23.2% identity, 328 aa; *Mycobacterium tuberculosis*, 21.6%, 334 aa; *E. coli*, 21.1%, 218 aa). The similarities to *Streptomyces* Tra proteins were: 27.3% identity, 205 aa overlap, to Spi from pSA1.1; 30.7% (192 aa) to TraSA from pSAM2; 20.9% (239 aa) to TraB from pSN22; 27.1% (192 aa) to TraB from pJV1; 21.8% (524 aa) to Tra from pIJ101; and 22.5% (581 aa) to the TraJ protein of pMEA300.

ORFs without known function. ORFs 193/141 show translational coupling. They encode proteins of 193 and 141 aa residues, respectively. ORF106, encoding a protein of 106 residues, is transcribed in the opposite direction. ORF73 is located between *spdB2* and *tra*. All these ORFs have a codon usage typical for *Streptomyces* proteins but do not possess any striking features or similarity to proteins deposited in databases.

Gene organization of the transfer region

All the genes, except *traR* (and a small ORF), were transcribed in the same direction as *rep* (Muth *et al.*, 1995). The gene organization of pSG5 differs from that of all other *Streptomyces* RCR plasmids. In contrast to pIJ101, pSN22, pJV1 and pSAM2, which all carry the *spd* genes downstream of *tra* forming one transcription unit, the putative *spd* genes of pSG5 are located upstream of *traB* (Fig. 1).

Fig. 3. Retarded sporulation of *S. lividans* conferred by unregulated expression of the pSG5 *traB* gene. *S. lividans* TK23/JG4 (control, left) and *S. lividans* TK23/JG5 (carrying *traB*, right) were grown on SM agar for 24, 48, 72 and 96 h, respectively. After 2 d on SM agar *S. lividans* carrying the control plasmid had produced aerial mycelium and spores, but no aerial mycelium formation was observed with *S. lividans* carrying pJG5. After 4 d incubation both strains had sporulated.

traB is the main transfer gene

Selected pSG5 derivatives carrying deletions or Tn5 insertions were analysed with regard to their transfer properties to identify genes that are involved in the conjugal transfer of pSG5 (Table 1). While inactivation of *prg* (pGM12, pSG5-34), *traR* (pSG5-20, pSG5-29b), *spdB2* (pSG5-41, pSG5-1) and ORF193/141 (pSG5-17) had little or no effect on the transfer of the respective derivative, a Tn5 insertion within *traB* reduced transfer by a factor of 10000 to a rate of 10^{-5} . This is the same order of magnitude as obtained with pGM9, carrying only the pSG5 minimal replicon without any transfer functions (Muth *et al.*, 1995).

The *tra* gene of pSG5 is not a kill function

To analyse whether the *traB* gene of pSG5 constitutes a kill function, as described for the corresponding genes of other *Streptomyces* RCR plasmids (Kendall & Cohen, 1987), the 4973 bp *SphI* fragment (Fig. 1) containing the complete *traB* gene (but not the *traR* or *prg* gene) of pSG5 was cloned into a bifunctional pGM11 derivative (pJG5). The resulting plasmid could be introduced into *S. lividans* without interfering with viability. However, on soya/mannitol (SM) agar, sporulation of the *traB*-carrying strain was retarded. *S. lividans* carrying the control plasmid pJG4 began to form aerial mycelium and to sporulate after 1 d; sporulation was complete after 3 d. However, *S. lividans* carrying pJG5 started to develop aerial mycelium only after 3 d. Complete sporulation of *S. lividans* carrying pJG5 was observed after 4–5 d incubation (Fig. 3).

Table 2. Kanamycin resistance mediated by the putative promoter regions of pSG5 genes

Plasmid*	Cloned fragment	Kanamycin resistance ($\mu\text{g ml}^{-1}$)
pIJ487-traB	<i>SgrAI</i> ₅₃₉₈ – <i>MluI</i> ₅₈₅₈	600
pIJ487-traR	<i>NcoI</i> ₃₆₆₁ – <i>NcoI</i> ₃₁₃₈	400
pIJ486-spdB3	<i>NcoI</i> ₃₁₃₈ – <i>NcoI</i> ₃₆₆₁	< 25
pIJ487-rep	<i>Sall</i> ₈₅₅₆ – <i>PmaCI</i> ₈₉₇₄	100
pIJ487-ORF193	<i>BamHI</i> ₇₆₁₃ – <i>BamHI</i> ₈₃₃₇	50
pIJ487-prg	<i>EcoRI</i> ₁ – <i>EcoRV</i> ₆₉₉	200
pJG1	<i>SgrAI</i> ₅₃₉₈ – <i>MluI</i> ₅₈₅₈	600
pJG2	<i>SgrAI</i> ₅₃₉₈ – <i>MluI</i> ₅₈₅₈ , <i>BamHI</i> ₃₆₇₃ – <i>BamHI</i> ₁₈₆₃	< 15

* All fragments were cloned into the promoter-probe plasmids pIJ486 or pIJ487 (Ward *et al.*, 1986). Plasmids pJG1 and pJG2 were bifunctional plasmids, carrying in addition pUC18.

The putative *spd* genes of pSG5 are not transcribed

To identify promoter activity, the upstream regions of the identified ORFs were subcloned into the promoter-probe plasmids pIJ487 and pIJ486. Promoter strength was measured as resistance level against kanamycin (Table 2). The upstream regions of ORF193, *rep*, *prg* and *traB* mediated resistances of 50, 100, 200 and 600 $\mu\text{g ml}^{-1}$, respectively (Table 2). However, the 478 bp *NcoI* fragment, which contained the intergenic region between *traR* and *spdB3*, showed promoter activity (400 $\mu\text{g ml}^{-1}$) only when inserted into pIJ487 (direction of *traR* transcription). The same fragment inserted into pIJ486 (direction of *spdB3* transcription) revealed no promoter activity, thus indicating that the probably translationally coupled *spdB3*/ORF80/*spdB2* genes of pSG5 are not expressed.

The *traB* promoter is regulated by TraR

With the exception of the integrative pSAM2 plasmid, the main transfer gene *traA/B* of the *Streptomyces* RCR plasmids is regulated by a divergently transcribed regulatory gene *traR* (*korA*). In all cases the promoter regions of *traR* and *traA/B* overlap. Since the corresponding genes of pSG5 show a different organization and are not located next to each other, we examined whether TraR influences *traB* expression. Unregulated expression of the *traB* promoter in the promoter-probe plasmids pIJ487-traB and pJG1 mediated a kanamycin resistance of 600 $\mu\text{g ml}^{-1}$. If the TraR-encoding fragment was inserted into pJG1 (pJG2), the resistance level dropped to < 15 $\mu\text{g ml}^{-1}$, demonstrating repression of the *traB* promoter by TraR (Table 2).

DISCUSSION

Several *Streptomyces* RCR plasmids have been sequenced in their entirety (Kendall & Cohen, 1988; Hagege *et al.*, 1993; Kataoka *et al.*, 1994a; Servin-Gonzales *et al.*, 1995). Homology in the replication-initiator protein assigns these plasmids to the pC194 family (Muth *et al.*, 1995). In these plasmids, all genes involved in the conjugative transfer form one operon

which is regulated by a divergently transcribed regulatory gene. The *spd* genes, involved in pock formation by an unknown mechanism, are located downstream of the main transfer gene in pIJ101 (Kendall & Cohen, 1987), pSN22 (Kataoka *et al.*, 1994a), pJV1 (Servin-Gonzales *et al.*, 1995) and pSAM2 (Hagege *et al.*, 1993). Although no pocks are formed during the transfer of pSG5 derivatives, sequence analysis of pSG5 revealed the presence of three genes exhibiting some sequence similarity to *Streptomyces* Spd proteins and possessing the structural characteristics of *spd* genes. They encode very hydrophobic proteins, possess several predicted transmembrane helices and are organized within one operon with the genes most likely translationally coupled.

The SpdB2 protein of pSG5 carries several repetitive sequences. Such repetitive sequences are also present in the SpdB2 proteins of pSN22 (RERER 5 $\times$) and pJV1 (RERQE 5 $\times$), and in the TraI protein (RREA 6 $\times$) which is involved in transfer and pock formation of plasmid pMEA300 (Vrijbloed *et al.*, 1995). The TolA protein of *E. coli* was also shown to possess repetitive sequences (KAAAD 5 $\times$, KAAAE 6 $\times$, KAEA 4 $\times$). These repetitive sequences might enable TolA to form an amphipathic helix spanning the membrane (Levengood *et al.*, 1991).

The TolA protein was shown to interact with the other Tol proteins TolQ and TolR, probably forming a channel through the outer and inner membrane for the uptake of colicins and single-stranded phage DNA (Click & Webster, 1998). The similarity of SpdB2 to the TolA protein allows speculation on the function of the Spd proteins. The *Streptomyces* SpdB2 protein might interact with the other Spd proteins in a similar manner to support translocation of the plasmid, resulting in the spreading of the plasmids into the mycelial compartments.

Whereas the *spd* genes of the other *Streptomyces* RCR plasmids lie in each case downstream of the main transfer gene *tra* (Kendall & Cohen, 1987; Hagege *et al.*, 1993; Kataoka *et al.*, 1994a; Servin-Gonzales, 1995), the putative *spd* genes of pSG5 show a different location.

They lie upstream of *tra* and its promoter, thus separating *tra* from the divergently transcribed regulatory gene *traR*. Therefore, they are not cotranscribed with *tra*. In addition, the upstream region of *spdB3* did not reveal any promoter activity in the direction of *spdB3*, whereas strong activity was found for the upstream regions of *rep*, *traB*, *traR*, ORF193 and *prg* (Table 2). The lack of any promoter activity upstream of the putative *spd* operon explains the absence of pock formation during transfer of pSG5. An interesting question is whether insertion of a promoter in front of the pSG5 *spd* genes renders pSG5 pock-forming.

Functions of the pSG5-encoded genes were investigated by analysing the phenotype of various Tn5 insertions in pSG5 which were obtained during the identification of the pSG5 minimal replicon (Muth *et al.*, 1988). Since the Tn5 insertions had negative effects on the stability of the mutagenized bifunctional plasmids, the *E. coli* part (pSLE41) of pSW344E (Muth *et al.*, 1988) was deleted by *EcoRI* restriction. The resulting plasmids, which comprised the original pSG5 plasmid carrying a Tn5 insertion, were more stable than the rather large (24 kb) pSW344::Tn5 plasmids.

Only inactivation of *traB* significantly affected self-transmissibility of pSG5. The Tn5 insertion within *traB* reduced conjugative transfer of the pSG5 derivative to 10^{-5} , a rate also obtained with the minimal replicon of pSG5 containing only *rep* (Muth *et al.*, 1995). This is in contrast to other plasmids, where inactivation or deletion of *tra* results in transfer rates of 10^{-7} or less (Kieser *et al.*, 1982; Hagege *et al.*, 1993; Kataoka *et al.*, 1994a; Servin-Gonzales *et al.*, 1995).

The TraB protein of pSG5 as well as the Tra proteins of the other *Streptomyces* RCR plasmids showed the highest similarities to the proteins of the SpoIIIE/FtsK family (Begg *et al.*, 1995; Wu *et al.*, 1995). They are of about the same size, have a similar protein structure with predicted N-terminal transmembrane helices, and have been located within the membrane (Tra of pIJ101: Pettis & Cohen, 1996; and TraB of pSN22: Kosono *et al.*, 1996) and membrane septum (SpoIIIE: Wu & Errington, 1997), respectively. They have conserved sequence motifs in the C-terminus (Begg *et al.*, 1995) with an ATP-binding site, which was shown to be essential for the function of TraB from pSN22 (Kosono *et al.*, 1996) and SpoIIIE (Wu & Errington, 1997). These similarities suggest that the Tra protein has a similar role in the plasmid transfer as that reported for SpoIIIE in the translocation of the chromosome from the mother cell to the forespore. Several SpoIIIE monomers were shown to integrate into the septum with their N-termini, forming a ring-like structure. The C-terminus of SpoIIIE, which was localized in the cytoplasm, probably interacts with the chromosome of *B. subtilis* to support its translocation into the forespore (Wu *et al.*, 1995; Wu & Errington, 1997).

The similarity of the SpoIIIE/FtsK protein to the *Streptomyces* Tra protein could also explain how the transfer of the pSG5 minimal replicon could proceed

without a Tra protein. Under certain conditions, the chromosomal SpoIIIE homologue of *S. lividans* might be able to replace (with low efficiency) the plasmid-encoded Tra protein and hence translocate the pSG5 derivative to the recipient. Alternatively, the pSG5 minimal replicon could be mobilized by an unidentified, probably integrated plasmid, carrying transfer functions.

In contrast to the *tra* genes of the other *Streptomyces* RCR plasmids (Kendall & Cohen, 1987; Hagege *et al.*, 1993; Kataoka *et al.*, 1994a; Servin-Gonzales *et al.*, 1995), the *traB* gene of pSG5 does not represent a kill function. This corresponds to the observation that a Tn5 insertion in *traR* of pSG5 did not result in cell death. The reason for the toxic effect of *tra* overexpression on *Streptomyces* is unclear. The incorporation of large amounts of Tra into the membrane may be responsible for the lethal effect, possibly by interfering with membrane integrity. This is supported by the fact that the N-terminal half of the pIJ101 *tra* gene (Kieser *et al.*, 1982; Kendall & Cohen, 1988), which encodes the hydrophobic, most likely membrane-associated part of Tra, is sufficient for killing. Nevertheless, expression of pSG5 *traB* was shown to affect differentiation. Sporulation of *S. lividans* carrying the *traB*-encoding plasmid pJG5 was temporarily retarded, but not completely inhibited. An effect of a *Streptomyces* RCR plasmid gene on differentiation has been previously reported for Spi from the *Streptomyces azureus* plasmid pSA1.1 (Tomura *et al.*, 1993). The deletion of the small 40 bp *Bam*HI fragment during the construction of the pSA1.1 derivatives (Tomura *et al.*, 1993) might have inactivated the TraR homologue of pSA1.1, leading to overexpression of *spi*, resulting in the inhibition of sporulation. Since *spi* was also shown to be involved in conjugal transfer and pock formation, and since sequence analysis of the *spi* gene revealed similarity to the *B. subtilis* SpoIIIE protein, the Spi protein of pSA1.1 most probably represents the Tra homologue. Thus, the *tra* homologue (*spi*) of plasmid pSA1.1 conferred a similar phenotype as the *tra* gene of pSG5. Sporulation was (temporarily) inhibited, but the host was not killed.

In all *Streptomyces* RCR plasmids, transcription of the main *tra* gene is regulated by TraR/KorA, a negative regulator of the GntR family (Haydon & Guest, 1991). With the exception of the integrative plasmid pSAM2 from *Streptomyces ambifaciens*, where *korSA* is transcribed in the same direction as *rep* (Hagege *et al.*, 1993), the *traR* gene is in all cases divergently transcribed to the *tra* gene. TraR was shown to bind to the intergenic region between *traR* and *tra*, which contains the overlapping promoters (Stein *et al.*, 1989; Kataoka *et al.*, 1994b). In pSN22 TraR binds to so-called *tre* boxes, which comprise characteristic 12 bp repeats (Kataoka *et al.*, 1994b). Such repeats have been also found in the intergenic region of the pJV1 *traR* and *tra* genes. In pSG5 the *traR* and *traB* genes are not located side by side. Neither the putative promoter regions of *traR* nor the promoter region of *traB* contain sequences with similarity to *tre* boxes or other striking repeats. Nevertheless, *traB* is negatively regulated by the TraR protein.

The presence of *traR* clearly repressed transcription of the *traB* promoter.

Since none of the characteristic functions of other bacterial transfer systems, such as a system for establishing cell-to-cell contact (Manning & Achtmann, 1979; Clewell, 1993), a specific nicking enzyme (Ilyina & Koonin, 1992; Pansegrau *et al.*, 1990), or an *oriT* as a replication origin for the synthesis of a single-stranded plasmid molecule (Wilkins & Lanka, 1993; Climo *et al.*, 1996), could be found on a *Streptomyces* RCR plasmid, *Streptomyces* seem to have evolved a unique system completely different from the other bacterial transfer systems. The similarity of the main transfer protein Tra to the *B. subtilis* SpoIIIE protein suggests that conjugative plasmid transfer in *Streptomyces* resembles more the translocation system of the chromosome during cell division and sporulation.

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