Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Biotechnology 120 (2005) 146–161
Journal of BIOTECHNOLOGY
www.elsevier.com/locate/jbiotec

Introduction of DNA into Actinomycetes by bacterial conjugation from *E. coli*—An evaluation of various transfer systems

Franca Blaesing [a], Agnes Mühlenweg [b], Silke Vierling [b], Günter Ziegelin [a], Stefan Pelzer [b], Erich Lanka [a,*]

[a] *Max-Planck-Institut für Molekulare Genetik, Ihnestrasse 63-73, D-14195 Berlin, Germany*
[b] *Combinature Biopharm AG, Robert-Rössle-Str. 10, D-13125 Berlin, Germany*

Received 24 March 2005; received in revised form 19 May 2005; accepted 10 June 2005

Abstract

Gene transfer is a basic requirement for optimizing bioactive natural substances produced by an increasing number of industrially used microorganisms. We have analyzed quantitatively horizontal gene transfer from *Escherichia coli* to Actinomycetes. The efficiencies of DNA transfer of four different systems were compared that consist of conjugative and mobilizable plasmids with a broad-host range. Three novel binary vector set-ups were constructed based on: (i) the IncQ group of mobilizable plasmids (RSF1010), (ii) IncQ-like pTF-FC2 and (iii) pSB102 that belongs to a new class of broad-host-range plasmids. The established system based on the IncPα group of conjugative plasmids served as the reference. For all plasmids constructed, we confirmed the functional integrity of the selected transfer machineries by intrageneric matings between *E. coli* strains. We demonstrate that the transfer systems introduced in this study are efficient in mediating gene transfer from *E. coli* to Actinomycetes and are possible alternatives for gene transfer into Actinomycetes for which the IncPα-based transfer system is not applicable. The use of plasmids that integrate into the recipients' chromosomes compared to that of plasmids replicating autonomously is shown to allow the access to a wider range of hosts.

Keywords: Novel conjugative binary vector systems; Intergeneric transfer; *Streptomyces*/Actinomycetales

1. Introduction

Bacterial conjugation is a powerful tool for gene transfer into various bacteria including the genus *Streptomyces*, the most important industrially used producers of antibiotics as cephalosporin, chloramphenicol, kanamycin and tetracycline and other pharmacologically relevant secondary metabolites, such as the antitumor agents bleomycin, doxorubicin and mitomycin or the immunosuppressive agent rapamycin (Kieser et al., 2000; Demain, 1999). The technique allows: (i) the identification and functional analysis of biosynthetic

* Corresponding author. Tel.: +49 30 8413 1696; fax: +49 30 8413 1130.
E-mail address: lanka@molgen.mpg.de (E. Lanka).

0168-1656/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2005.06.023

pathways for pharmaceutically interesting metabolites and (ii) the targeted manipulation of the biosynthesis by pathway engineering of homologous and heterologous genes, a strategy, which is called combinatorial biosynthesis (for an example, see Rix et al., 2002).

Trieu-Cuot et al. (1987) were the first to report conjugative transfer of a shuttle plasmid between Gram-negative *Escherichia coli* strains and a variety of Gram-positive bacteria. The initial protocol for intergeneric transfer of plasmids from *E. coli* to *Streptomyces* spp. was developed by Mazodier et al. (1989). Since then, this method has been used successfully with a number of different *Streptomyces* strains (Bierman et al., 1992; Tabacov et al., 1994; Matsushima and Baltz, 1996; Fouces et al., 2000) and other Actinomycetes as *Amycolatopsis* (Stegmann et al., 2001), *Actinoplanes* (Heinzelmann et al., 2003), *Nonomuraea* (Stinchi et al., 2003), *Saccharopolyspora* (Matsushima et al., 1994), *Actinomadura*, *Micromonospora*, *Nocardia* and *Rhodococcus* (Voeykova et al., 1998), but also with strains of the amino acid producing genus *Corynebacterium* (Schäfer et al., 1990; Kirchner and Tauch, 2003). Moreover, first Jaoua et al. (1992) and later Molnar et al. (2000) and Kopp et al. (2004) adapted this system for the conjugative transfer of DNA from *E. coli* to the Gram-negative Myxobacteria like *Sorangium cellulosum*, which recently gained attention as a valuable source of important natural products including epothilone.

A series of cloning vectors for the conjugative transfer of DNA from *E. coli* to *Streptomyces* spp. was constructed by Bierman et al. (1992), comprising plasmid or cosmid vectors: (i) which do not replicate in *Streptomyces* spp., (ii) which can replicate autonomously or (iii) which can integrate site-specifically at the bacteriophage ΦC31 attachment site. Several other vectors which can be transferred from *E. coli* to Actinomycetes have been presented (Smokvina et al., 1990; Motamedi et al., 1995; Voeykova et al., 1998). However, all conjugation systems developed so far are based on the IncPα (RP4) transfer system. The system is known to be efficient, mediating effective gene transfer not only between different bacterial species but also into other cell types such as yeast (Bates et al., 1998) and mammalian cells (Waters, 2001). The development of additional conjugation systems with different transfer functions might still render bacterial conjugation an even more versatile tool.

In this study, we introduce alternative conjugation systems, which might facilitate the transfer of plasmids into Actinomycetes strains as yet not accessible to that technique. We also examine limitations in the use of plasmid vectors which integrate site-specifically into the host chromosome in comparison to autonomously replicating plasmids. Four different *Streptomyces* strains and one related nocardioform Actinomycete were selected for the evaluation of transfer efficiencies. In the following, all strains used will be referred to as *Streptomyces* spp., unless noted otherwise.

2. Materials and methods

2.1. Bacterial strains and media

E. coli strain SCS1 [F^-, *endA1*, *hsdR17*(r_K^-, m_K^+), *supE44*, *thi-1*, λ^-, *recA1*, *gyrA96*, *relA1*; Stratagene] was used for all cloning procedures. *E. coli* strain DH5α [F^-, Φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*), U169, *recA1*, *endA1*, *hsdR17*(r_K^-, m_K^+), *phoA*, *supE44*, λ^-, *thi-1*, *gyrA96*, *relA1*; Hanahan, 1983] was used as the donor in intrageneric conjugations and *E. coli* strain GM2163 [F^-, *ara-14*, *leuB6*, *fhuA31*, *lacY1*, *tsx78*, *glnV44*, *galK2*, *galT22*, *mcrA*, *dcm-6*, *hisG4*, *rfbD1*, *rpsL136* (Sm^r), *dam13*::*Tn9* (Cm^r), *xylA5*, *mtl-1*, *thi-1*, *mcrB1*, *hsdR2*; New England Biolabs] as the recipient in intrageneric conjugations and as the donor strain in intergeneric conjugations. *E. coli* strains HB101 [F^-, Δ(*mcrC-mrr*), *hsdS20*, *recA13*, *ara-14*, *proA2*, *lacY1*, λ^-, *galK2*, *rpsL20* (Sm^r), *xyl-5*, *mtl-1*, *supE44*; Boyer and Roulland-Dussoix, 1969] and the nalidixic acid-resistant derivative HB101Nx^r were additionally applied for intrageneric matings. Recipients in intergeneric conjugations were the following *Streptomyces* strains: *S. aureofaciens* Tü13 (*S. ambofaciens* DSM 40697); *S. coelicolor* A3(2)M145 (SCP1-, SCP2-; Kieser et al., 2000); *S. lividans* 66 TK24 (*str-6*, SLP2-, SLP3-; Kieser et al., 2000); *S. diastatochromogenes* Tü6028 (Paululat et al., 1999) and the nocardioform Actinomycete *Amycolatopsis japonicum* MG417-CF17 (*A. japonica* DSM 44213; Stegmann et al., 2001).

E. coli cells were grown in YT medium (Miller, 1972) or LB broth (Sambrook et al., 1989) buffered with 25 mM 3-(*N*-morpholino)propanesulfonic acid (sodium salt, pH 8.0) and supplemented with

25 μg thiamine-hydrochloride ml^{-1} and glucose (0.1%, w/v). If appropriate, antibiotics were used at the following concentrations: tetracycline hydrochloride (Tc), 10 μg ml^{-1}; kanamycin sulfate (Km), 30 μg ml^{-1}; chloramphenicol (Cm), 10 μg ml^{-1}; apramycin sulfate (Am), 25 μg ml^{-1}; nalidixic acid, sodium salt (Nx), 30 μg ml^{-1}. The final concentration of $HgCl_2$ for selective growth of plasmid pSB102 was 20 μg ml^{-1}.

MS medium (Hobbs et al., 1989) containing 10 mM $MgCl_2$ was used for plating of *E. coli*/*Streptomyces* spp. in intergeneric conjugation experiments. Exconjugants were further cultured on selective HA medium (4 g Bacto yeast extract, 10 g malt extract, 4 g glucose-monohydrate l^{-1}; pH 7.3) with antibiotics added in the following concentrations: phosphomycin disodium salt (Pm), 400 μg ml^{-1}, and Am, 25 μg ml^{-1} (selection of pSET vectors), or Km, 50 μg ml^{-1} (selection of pUWLoriTaph and RSF1010K). *Streptomyces* cultures for DNA isolation were grown in CRM Medium (10 g Bacto yeast extract, 20 g tryptone soy broth, 103 g sucrose l^{-1} and 20 ml 2.5 M $MgCl_2$ solution to be added to the sterilized medium) containing 0.5% (w/v) glycine, 400 μg ml^{-1} Pm and the appropriate selective antibiotics (see HA medium).

2.2. Plasmids and oligonucleotides

Plasmids used in this study are listed in Table 1. Oligonucleotides used for molecular cloning, PCR analysis or nucleotide sequencing are listed in Table 2.

2.3. DNA techniques

Standard molecular cloning techniques were performed as described (Sambrook et al., 1989). PCR fragments were generated using the following polymerases under conditions recommended by the manufacturer: Deep $Vent_R$ DNA polymerase (New England Biolabs), ProofStart DNA polymerase (Qiagen) or *Taq* DNA polymerase (Qiagen). Constructions were verified by DNA sequence analysis.

2.4. Intrageneric conjugation procedure

For quantitative filter matings defined amounts of donor cells (0.5 ml, 1×10^8 cells ml^{-1}) and GM2163 recipient cells (5 ml, 3–4 $\times 10^8$ cells ml^{-1}) were mixed and collected onto a cellulose nitrate filter (0.45 μm pore size, 25 mm diameter; Sartorius). The filter was incubated for 1 h at 30 °C on a YT agar plate without selection. Cells were resuspended, and aliquots of serial 10-fold dilutions were plated on YT agar plates with 10 μg chloramphenicol ml^{-1} for selection of GM2163 plus the appropriate antibiotic for selection of the plasmid to be transferred. The transfer frequency is the number of exconjugants per donor cell.

2.5. Intergeneric conjugation procedure

Conjugation of mobilizable plasmids from *E. coli* into different *Streptomyces* strains was essentially done as described (Flett et al., 1997; Kieser et al., 2000). *E. coli* donor cells were grown to an OD_{600} of 0.5. Cultures were inoculated from single colonies rather than diluted from overnight cultures because of extremely long lag phases of stationary phase donor cells derived from liquid culture. Cells were harvested by centrifugation, washed twice with LB to remove residual antibiotics and resuspended 10-fold concentrated in LB. *Streptomyces* spore suspensions stored at −80 °C were used as recipients. For each mating experiment, 10^8 *Streptomyces* spores were added to 500 μl 2× YT broth, heat shocked for 10 min at 50 °C to activate germination and cooled to room temperature. An aliquot of 500 μl of *E. coli* cells was added and the mixture centrifuged for 2 min at 1000 × *g*. A defined volume of 900 μl of the supernatant was removed and the bacterial pellet was resuspended in the remaining volume of approximately 100 μl. Serial 10-fold dilutions of the mixture were spread on MS agar containing 10 mM $MgCl_2$. The plates were incubated for 16–20 h at 29 °C and then overlaid with 3 ml soft nutrient agar containing 1 mg Pm for counterselection against *E. coli* and the appropriate antibiotic for exconjugant selection (1 mg Am and 0.75 mg Km, respectively). Incubation was continued for 2–5 days until exconjugants appeared. The transfer frequency is the number of exconjugants per recipient spore. Exconjugants were further cultured on selective HA medium and analyzed by specific PCR or plasmid DNA isolation (see below).

2.6. Isolation of DNA from exconjugants

Genomic *Streptomyces* DNA was isolated using the Nucleospin Tissue Kit (Macherey & Nagel) according to the product manual for Gram-positive strains.

Table 1
Plasmids used in this study

Plasmid	Description[a]	Reference or source
Cloning vectors		
pBR329	pMB1 replicon, Ap^r, Cm^r, Tc^r	Covarrubias and Bolivar (1982)
pBluescript II SK-	ColE1 origin, f1 (−) origin, *lacZ*, MCS, Ap^r	Stratagene
pJF119EH	pMB1 replicon, P*tac*/*lacI*^{q}, Ap^r	Fürste et al. (1986)
pGZ119EH	ColD replicon, P*tac*/*lacI*^{q}, Cm^r	Lessl et al. (1992)
pGZ219EH	pJF119EHΔ[*Bsp*HI–*Nru*I 2.23-kb fragment]Ω[RP4 *tetA*, nucleotide positions 13,921–15,273; RSF1010D1 *Pvu*II–*Pst*I, 5.7-kb fragment; adaptor *Nsi*I–*Nru*I]; RSF1010D1 replicon, P*tac*/*lacI*^{q}, Tc^r	G. Ziegelin, unpublished
pGZ1119EH	pGZ119EHΔ[*Hin*dIII–*Sca*I]Ω[pGZ219 *Hin*dIII–*Xho*I 1.85-kb fragment]; ColD replicon, P*tac*/*lacI*^{q}, Tc^r	G. Ziegelin, unpublished
pUWl201	pUC18 origin, pIJ101 origin, P*ermE**, Ap^r, Th^r	Doumith et al. (2000)
pGM9	pSG5 replicon, Km^r, Th^r	Muth et al. (1989)
IncPα plasmids[b]		
pUB307	RP4ΔTn*A*[c], IncPα replicon Km^r, Tc^r	Bennett et al. (1977)
pUB307dK	pUB307Δ[RP4 *aphA* nucleotide positions 38,860–38,996]; IncPα replicon, Tc^r	This work
pML123	pGZ119EHΔ[*Eco*RI–*Bam*HI]Ω[*Eco*RI–*Xmn*I adaptor; RP4 *Xmn*I–*Not*I, nucleotide positions 18,841–30,042]; (*trbB* to *trbM*)$^+$, ColD replicon, Cm^r	Lessl et al. (1993)
pML100	pJF119EHΩ[RP4 *Eco*47III–*Eco*47III, nucleotide positions 45,871–48,933]; (*traF*, *traG*)$^+$, pMB1 replicon, Ap^r	Lessl et al. (1993)
pMS124	pML123Δ[*Bam*HI–*Hin*dIII]Ω[*Bam*HI–*Sac*I adaptor, pML100 *Sac*I–*Hin*dIII, 3.1-kb fragment]; (*trbB* to *trbM*)$^+$, (*traF*, *traG*)$^+$, ColD replicon, Cm^r	This work
IncQ plasmids[d]		
RSF1010	IncQ replicon, Sm^r, Su^r	Guerry et al. (1974); Scholz et al. (1989)
RSF1010K	RSF1010 replicon, Km^r	Lessl et al. (1993)
pFB219EH	RSF1010Δ[*Ssp*I–*Xmn*I 3.04-kb fragment]Ω[pGZ219 *Nsi*I–*Xho*I 3.44-kb fragment/T4]; RSF1010 replicon, P*tac*/*lacI*^{q}, Tc^r	This work
pFB1124	pFB219Δ[*Eco*RI–*Hin*dIII]Ω[pMS124 *Eco*RI–*Hin*dIII, 14.4-kb fragment]; (*trbB* to *trbM*, *traF*, *traG*)$^+$, RSF1010 replicon, P*tac*/*lacI*^{q}, Tc^r	This work
pTF-FC2 plasmids[e]		
pDER412	12.4 kb pTF-FC2 in pBR325 [*Pst*I]Δ[*Xho*I/*Sal*I]; pTF-FC2 replicon, Cm^r	Rawlings et al. (1984)
pFB319EH	pDER412Δ[*Eco*RI–*Pst*I]Ω[pGZ219 *Nsi*I–*Xho*I 3.44-kb fragment]; pTF-FC2 replicon, P*tac*/*lacI*^{q}, Tc^r	This work
pFB1224	pFB319Δ[*Eco*RI–*Hin*dIII]Ω[pMS124 *Eco*RI–*Hin*dIII, 14.4-kb fragment]; (*trbB* to *trbM*, *traF*, *traG*)$^+$, pTF-FC2 replicon, P*tac*/*lacI*^{q}, Tc^r	This work
pSB102 plasmids[f]		
pSB102	Conjugative broad-host-range Hg^r plasmid	Schneiker et al. (2001)
pFB714	pGZ1119Ω[pSB102 *Xba*I–*Xba*I, nucleotide positions 4495–35,997]; Tra$^+$, ColD replicon, Tc^r	This work
Integrative *oriT* plasmids		
pSET152	pUC replicon, Am^r, *lac*Zα, MCS, *int*$^{\Phi C31}$, IncPα *oriT*	Bierman et al. (1992)
pSEToriT$^-$	pSET152Δ[*oriT*, *Pst*I–*Pst*I, nucleotide positions 2798–3600]; pUC replicon, Am^r, *lac*Zα, MCS, *int*$^{\Phi C31}$	This work
pSET101	pSEToriT$^-$Ω[RSF1010 *oriT*]; pUC replicon, Am^r, *lac*Zα, MCS, *int*$^{\Phi C31}$, RSF1010 *oriT*	This work
pSET201	pSEToriT$^-$Ω[pTF-FC2 *oriT*]; pUC replicon, Am^r, *lac*Zα, MCS, *int*$^{\Phi C31}$, pTF-FC2 *oriT*	This work
pSET102	pSEToriT$^-$Ω[pSB102 *oriT*, nucleotide positions 11,064–11,494]; pUC replicon, Am^r, *lac*Zα, MCS, *int*$^{\Phi C31}$, pSB102 *oriT*	This work

Table 1 (*Continued*)

Plasmid	Description[a]	Reference or source
Non-integrative *oriT* plasmids		
pMS713-1n	pBR329Ω[pSB102 *oriT*, nucleotide positions 11,064–11,494]; pMB1 replicon, Ap^r, Cm^r	This work
pOJ436	pUC replicon, Am^r, $(cos)3^{\lambda}$, $int^{\Phi C31}$, IncPα *oriT*	Bierman et al. (1992)
pUWLoriTaph	pUWL201Ω[pOJ436 *Pst*I 0.76-kb *oriT* fragment; pGM9 1.85-kb *aphII* cassette]; pUC18 origin, pIJ101 origin, P*ermE*, IncPα *oriT*, Th^r, Km^r	This work

[a] Selective markers: Am, apramycin; Ap, ampicillin; Cm, chloramphenicol; Hg, mercury; Km, kanamycin; Sm, streptomycin; Su, sulfonamide; Tc, tetracycline; Th, thiostrepton.
[b] IncPα-derived helper plasmids and plasmids used for their construction.
[c] Sequence data for the Tn*A* deletion in pUB307: CGTAATG-5463—Δ nucleotide positions 5464–12,044—12,045-GGA; coordinates refer to the sequence of RP4 (EMBL nucleotide sequence database, accession no. L27758).
[d] IncQ-derived helper plasmids and plasmids used for their construction.
[e] pTF-FC2-derived helper plasmids and plasmids used for their construction.
[f] pSB102-derived helper plasmids.

An aliquot of 2 ml of Actinomycetes culture was used. Cells were harvested by centrifugation and washed once with 500 μl of TE/10% sucrose and stored at −20 °C for further processing.

Plasmid DNA was isolated from *Streptomyces* cells by alkaline lysis essentially as described (Birnboim and Doly, 1979; Sambrook et al., 1989) with the following modifications: cells were washed and stored until further processing as described for genomic DNA isolation. The resuspension buffer (50 mM glucose; 25 mM Tris–HCl; 10 mM EDTA; pH 8.0) contained lysozyme (4 mg ml^{-1}). The cells were incubated for 30 min at 37 °C before adding the alkaline buffer (0.4 M NaOH; 2%, w/v, SDS). Incubation at room temperature was for 10 min. Three molar potassium acetate, pH 5.2, was used as neutralization buffer. The DNA pellet was resuspended in 50 μl TE buffer (10 mM Tris–HCl; 1 mM EDTA; pH 8.0).

Table 2
Oligonucleotides used in this study

Sequence[a]	Description
5′-**C**GGGAGGGCGCACTTACCGGTTTCTCTTCGAGAAACTGG**GCATG**-3′	44mer; *oriT* RSF1010 cloning
5′-**C**CCAGTTTCTCGAAGAGAAACCGGTAAGTGCGCCCTCCC**GCATG**-3′	44mer; *oriT* RSF1010 cloning
5′-TTACTGAT**GCATGC**GCGCCGAATGGGCAGGC-3′	31mer; *oriT* pTF-FC2 cloning
5′-TTACTGAT**GCATGC**GCGCCTTTTATGCTTGTTGCAAAGTG-3′	40mer; *oriT* pTF-FC2 cloning
5′-TTATGCAT**AAGCTT**GCTGGGGCCGGTGTCAAGG-3′	33mer; pBR/*oriT*102 cloning
5′-TTACTGAT**AAGCTT**CAAGCGCCTCCAATCCAATTAAGGGGAC-3′	42mer; pBR/*oriT*102 cloning
5′-CTCCATT**GCATGC**TCCTTAGCTCCTGATGTT-3′	31mer; pSET102 cloning
5′-TCCATA**GCATGC**GCCTGACTGCGT-3′	24mer; pSET102 cloning
5′-TGACGCTCAATGCCGTTTGGC-3′	21mer; pSET analytical PCR and sequencing primer
5′-CAGTTGGGGCACTACTCCC-3′	19mer; pSET analytical PCR and sequencing primer
5′-GTGCGTCACGACTGGGTC-3′	18mer; pUB307 sequencing primer
5′-TTAATTCATGAACTAGTCATGTATTCGGCTGCAACTTTGTCA-3′	42mer; RP4/tetA cloning
5′-CTCGAGGCAT**AGGCCT**ATCGTTTCCACG-3′	28mer; RP4/tetA cloning
5′-CCCC**ATGCAT TCG**-3′	13mer; *Nsi*I/*Nru*I adaptor
5′-**CGA ATGCAT**GGGGTGCA-3′	17mer; *Nsi*I/*Nru*I adaptor
5′-**GATCC**GGCGCGCC**GAGCT**-3′	20mer; *Bam*HI–*Sac*I adaptor
5′-**C**GGCGCGCC**G**-3′	10mer; *Bam*HI–*Sac*I adaptor
5′-TTGGTCG**CATATG**TCGAACCCCAGAGT-3′	27mer; pGM9/*aphII* cloning
5′-GGCATG**CATATG**TCACGCTGCCGCAA-3′	26mer; pGM9/*aphII* cloning

[a] Nucleotides belonging to restriction sites introduced for cloning purposes are in bold.

3. Results

3.1. Selection of transfer systems and molecular cloning of essential Tra components

pUB307/pSET152 (Flett et al., 1997) served as a reference system for the evaluation of Tra systems used in this study. Plasmid pUB307, a RP4 derivative lacking Tn*A* (Bennett et al., 1977; Table 1), had to be modified such that it could be used with co-resident Km^r plasmids RSF1010K and pUWLoriTaph (see below). pUB307, linearized at a unique *Hin*dIII site in the *aphA* gene, was used for transformation of *E. coli* to generate small deletions of the plasmid, yielding the Km^s plasmid pUB307dK. The plasmid lacks 137 bp, destroying the *aphA* reading frame (nucleotide positions 38,860–38,996 of plasmid RP4).

Potential alternatives for the IncPα Tra system to be used for conjugative DNA transfer into *Streptomyces* and related Actinomycetes have to be especially efficient in semi-solid surface matings, since conjugative transfer in *Streptomyces* takes place only on solid media under prolonged physical contact of the parental strains (Hopwood and Kieser, 1993). Three systems were selected for this study: the mobilization (Mob) system of IncQ plasmid RSF1010, the Mob system of IncQ-like plasmid pTF-FC2 and the Tra system of pSB102, a plasmid recently isolated from the microbial population of the alfalfa rhizosphere (Schneiker et al., 2001). The selection was done due to the following considerations: RSF1010 and pTF-FC2 are both broad-host-range plasmids. They are known to be highly mobilizable and possess well-characterized Mob functions (reviewed in Rawlings and Tietze, 2001). In contrast, pSB102 belongs to an as yet undescribed group of broad-host-range plasmids. The putative mating pair formation (Mpf) and DNA transfer and replication (Dtr) genes form a compact cluster, which makes the system appropriate for the cloning procedures required.

Although not self-transmissible, RSF1010 is mobilized at high frequency to a large number of hosts in the presence of conjugative helper plasmids, such as RP4 of the *E. coli* IncPα and R751 of the IncPβ group. Transfer of the plasmid has been reported for a wide range of Gram-negative bacteria (Frey and Bagdasarian, 1989; Haring and Scherzinger, 1989) and several Gram-positive bacteria including *S. lividans* and *Mycobacterium smegmatis* (Gormley and Davies, 1991), as well as plant (Buchanan-Wollaston et al., 1987) and animal cells (Yoshida et al., 1997). Likewise, pTF-FC2 has been mobilized at high frequency between *E. coli* strains by the co-resident IncPα plasmid RP4 (Rawlings and Woods, 1985), from *E. coli* to *Pseudomonas fluorescens* (Herrera et al., 1994) and from *Agrobacterium tumefaciens* to plant cells (Dube and Thompson, 2003; Dube et al., 2004).

Conjugative transfer of pSB102 was demonstrated for *Sinorhizobium meliloti* L331, *Pseudomonas* sp. B131 and *E. coli* HBR101 with *S. meliloti* FP2 as the donor strain (Schneiker et al., 2001). While the mobilization functions of IncQ and IncQ-like plasmids have been extensively studied (for a review, see Rawlings and Tietze, 2001), the location and extension of the transfer region of pSB102 so far only has been predicted on the basis of sequence similarities to those of other systems (Schneiker et al., 2001).

For conjugation experiments, binary vector systems were constructed with the subset of Tra systems. *oriT* sequences as the only *cis*-acting elements necessary for plasmid transfer were positioned on one plasmid, genes for transfer proteins required for the formation of the relaxosome and subsequent translocation of the DNA to the recipient cell together are provided on co-resident helper plasmids. The physical structure of the conjugative helper plasmids together with an outline of the cloning strategy is given in Fig. 1. pFB1124 and pFB1224 carry the mobilization and replication functions of plasmids RSF1010 and pTF-FC2, respectively, and the RP4 Tra2 region including the genes *traF* and *traG*, which are required for mobilization of the non-conjugative plasmids RSF1010 and pTF-FC2. pFB714 carries the putative Tra region of plasmid pSB102, which is located on a 31.5-kb *Xba*I fragment. The physical structure of the *oriT* plasmids is given in Fig. 2. They represent two different types. Derivatives of pSET152 (Bierman et al., 1992) do not replicate in *Streptomyces* spp. and related Actinomycetes but allow for site-specific integration in the host chromosome at the bacteriophage ΦC31 attachment site (Fig. 2a). These plasmids, pSET152 (RP4 *oriT*), pSET101n/u (RSF1010 *oriT* in n and u orientations, respectively), pSET201 (pTF-FC2 *oriT*) and pSET102 (pSB102 *oriT*), will be referred to as integrative *oriT* plasmids. A derivative of plasmid pUWL201 (Doumith et al., 2000) carrying the IncPα *oriT* of plasmid pOJ436 (Bierman et al., 1992) contains the repli-

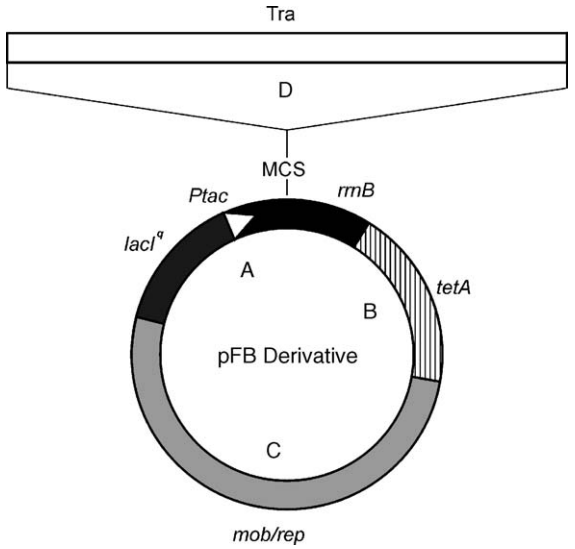

Fig. 1. Physical structure of conjugative helper plasmids. Plasmid pJF119EH (Fürste et al., 1986) was the basis for the construction of the conjugative helper plasmids used in this study. The plasmid carries the *lacI*^{q} gene, the *tac* promotor, a multi-cloning site and the *rrnBt1/t2* terminator (A). The ampicillin resistance gene of pJF119EH was replaced by the tetracycline resistance gene of plasmid RP4 (*tetA* with residual promoter, RP4 nucleotide positions 13,921–15,273; B). For the construction of RSF1010 and pTF-FC2 helper plasmids, the pMB1 replicon was replaced by the corresponding *mob/rep* regions (Scholz et al., 1989; Rohrer and Rawlings, 1992; C). For the construction of the pSB102 helper plasmid, ColD, derived from plasmid pGZ119 (Lessl et al., 1992), was used as the replicon (C). The RP4 Tra2 region and the genes *traF* and *traG*, required for mobilization of the non-conjugative plasmids RSF1010 and pTF-FC2, were inserted at the pJF119EH multi-cloning site, yielding plasmids pFB1124 and pFB1224, respectively (D). The putative Tra region of pSB102 is located on a 31.5-kb *Xba*I fragment. The fragment, inserted into the pJF119EH multi-cloning site, yielded plasmid pFB714 (D). Genetic maps of the vector plasmids and conjugative helper plasmids with essential restriction sites are provided as supplementary data.

cation functions of the *Streptomyces* broad-host-range multi-copy plasmid pIJ101 (Kieser et al., 1982) allowing for autonomous replication in the host (Fig. 2b). This plasmid, pUWLoriTaph, will be referred to as non-integrative *oriT* plasmid. Detailed descriptions of the cloning procedures of both the helper and the *oriT* plasmids are given in Table 1 and included as supplementary data. An overview of the new constructs together with an outline of the experimental procedure is given in Fig. 3.

3.2. Tra constructs are functional in intrageneric matings between two E. coli strains

Plasmids pFB1124 (Tra system IncQ/IncPα Mpf; Table 1; Fig. 3), pFB1224 (Tra system pTF-FC2/IncPα Mpf; Table 1; Fig. 3) and pFB714 (pSB102 Tra system; Table 1; Fig. 3) were constructed as self-transmissible plasmids with the ability to mobilize co-residing plasmids carrying the homologous *oriT* sequence. The advantage of self-transmissible helper plasmid is to offer the possibility of triparental matings without the need for preceding DNA isolation. Those matings include methylation proficient *E. coli* strains, such as DH5α which are used for the conservation of cosmid libraries, and the methylation deficient GM2163 preferably used for conjugation with Actinomycetes.

The presence of functional Tra elements in all constructed plasmids was confirmed by intrageneric matings between *E. coli* strains.

In a semi-quantitative approach, we first tested the capacity for conjugative transfer of the helper plasmids themselves. Ten-fold serial dilutions of plasmid-harboring HB101 cells were spotted onto a lawn of nalidixic acid-resistant HB101Nx cells and selected for tetracycline/nalidixic acid resistance. Tc^{r}/Nx^{r} colonies were obtained with each of the plasmids with dilutions as far as 10^{-5}, indicating the presence of functional Tra systems in each of the constructs (data not shown). The mating experiments show that the combination of the IncQ- or IncQ-like Mob functions with the IncP Mpf functions, including the genes *traF* and *traG*, on a single plasmid renders the plasmid self-transmissible. In addition, we demonstrated with these experiments that the 31.5-kb *Xba*I fragment of pSB102, carrying the predicted Tra functions, is sufficient to confer the ability of conjugative transfer to a recombinant plasmid. This is the first approach to determine the extension of the pSB102 Tra region.

Quantitative filter mating experiments with DH5α as donor and GM2163 as recipient strain were carried out to assess the frequencies of mobilization of the recombinant *oriT* plasmids by their respective conjugative helper plasmids (Table 3). pUB307dK/pSET152 served as the reference system. With regard to the intergeneric matings between *E. coli* and *Streptomyces* spp., we included pUB307dK-mediated mobilization of plasmids RSF1010K and pUWLoriTaph in our anal-

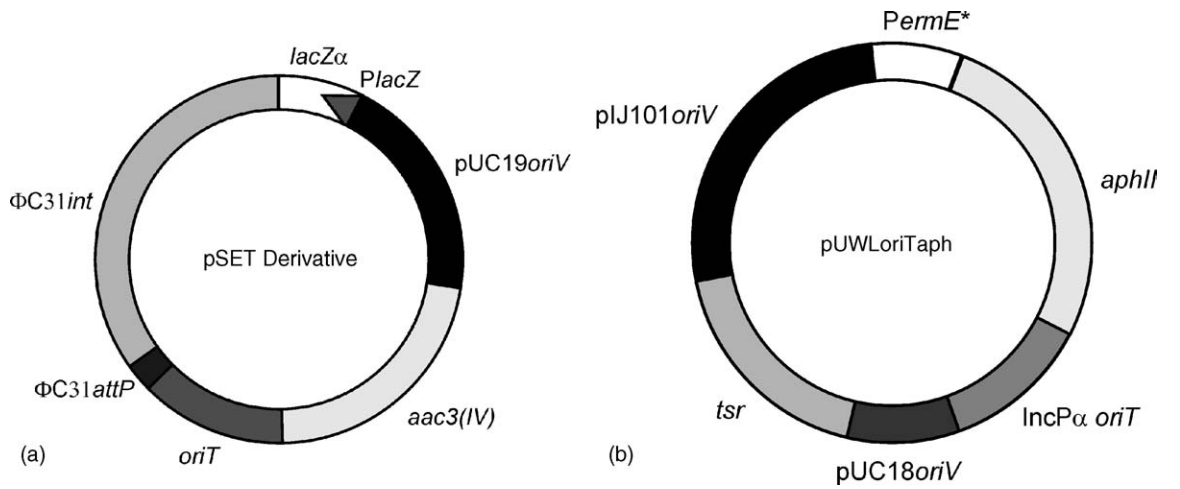

Fig. 2. Physical structure of *oriT* plasmids. (a) *oriT* plasmids integrating into the Actinomycetes host chromosome by site-specific recombination. pSET152 (Bierman et al., 1992) provided the basis for each of the integrative *oriT* plasmids. The plasmids encode the LacZα peptide with a multi-cloning site and the replicon of plasmid pUC19, the attachment site [*attP*] and integrase functions [*int*] of the temperate phage ΦC31 for site-specific integration into the recipient Actinomycete chromosome and the apramycin resistance marker [*aac3*(*IV*)], which can be used for selection in both *E. coli* and Actinomycetes. The 0.8-kb IncPα *oriT* fragment of plasmid pSET152 was replaced by the following *oriT* sequences, which were inserted into the single *Sph*I site (pSET152 nucleotide position 3606). Insertion of the 38 bp RSF1010 *oriT* (Brasch and Meyer, 1987) yielded plasmid pSET101. Insertion of the 138 bp pTF-FC2 *oriT* (Rohrer and Rawlings, 1992) yielded plasmid pSET201. The putative pSB102 *oriT* is located on a 2.36-kb *Hin*dIII fragment (pSB102 positions 9863–12,225; EMBL nucleotide sequence database, accession no. AJ304453). Part of the fragment was amplified (pSB102 positions 11,064–11,494) for molecular cloning of the putative *oriT* region, yielding plasmid pSET102. pSET101 was obtained with the RSF1010 sequence in both orientations (pSET101n and u, respectively), while pSET201 contained the pTF-FC2 *oriT* sequence exclusively in the u orientation. A genetic map of the plasmid pSEToriT$^-$ with essential restriction sites is provided as supplementary data. (b) *oriT* plasmid autonomously replicating in the *Streptomyces* host. Plasmid pUWL201 (Doumith et al., 2000) was the basis for the construction of an *oriT* plasmid autonomously replicating in *Streptomyces* spp. pUWL201 contributes the replication functions of the *Streptomyces* broad-host-range multi-copy plasmid pIJ101 (Kieser et al., 1982), the *tsr* gene conferring resistance to thiostrepton in *Streptomyces*, the promoter P*ermE** (Bibb et al., 1994) and the pUC18 origin for replication in *E. coli*. Insertion of the 0.8-kb *Pst*I fragment of plasmid pOJ436 (Bierman et al., 1992) containing the IncPα *oriT* generated pUWLoriT. Subsequent insertion of the *aphII* gene of plasmid pGM9 (Muth et al., 1989) into the *bla* gene of pUWL201 yielded Ap^s/Km^r plasmid pUWLoriTaph.

ysis. Employing IncPα plasmid pUB307dK as helper, transfer efficiencies approached one per donor cell with each of the *oriT* plasmids. This is in accordance with the data for IncPα-mediated plasmid transfer reported by other authors (e.g. Lessl et al., 1993). Transfer frequencies obtained with plasmids pFB1124, pFB1224 and pFB714 were about 6–25-fold lower compared to those obtained with pUB307dK. pFB714 was the most efficient of the three, yielding transfer frequencies with an average of 1.4×10^{-1}. In contrast, employing the original plasmid pSB102, the number of exconjugants obtained was about 150-fold lower compared to that obtained with pFB714 harboring the isolated pSB102 Tra region (Table 3). This might result from replication deficiencies of the environmental plasmid pSB102 in the *E. coli* host, since retarded growth of the DH5α (pSB102/pSET102) donor cells was observed. Mobilization experiments with both pSB102 and pFB714 confirm the postulated location of the pSB102 *oriT* sequence. Control experiments with pSEToriT$^-$ yielded transfer frequencies near the detection limit with the exception of experiments carried out with pSB102 as helper plasmid. The latter exhibited reproducible higher transfer frequencies with an average of 4×10^{-6}. Recombination events taking place during the long growth period might be the reason for this observation.

3.3. Selected Tra systems are suitable for intergeneric conjugative transfer from E. coli to Streptomyces spp.

Subsequently, RSF1010, pTF-FC2 and pSB102 Tra systems were compared among one another and

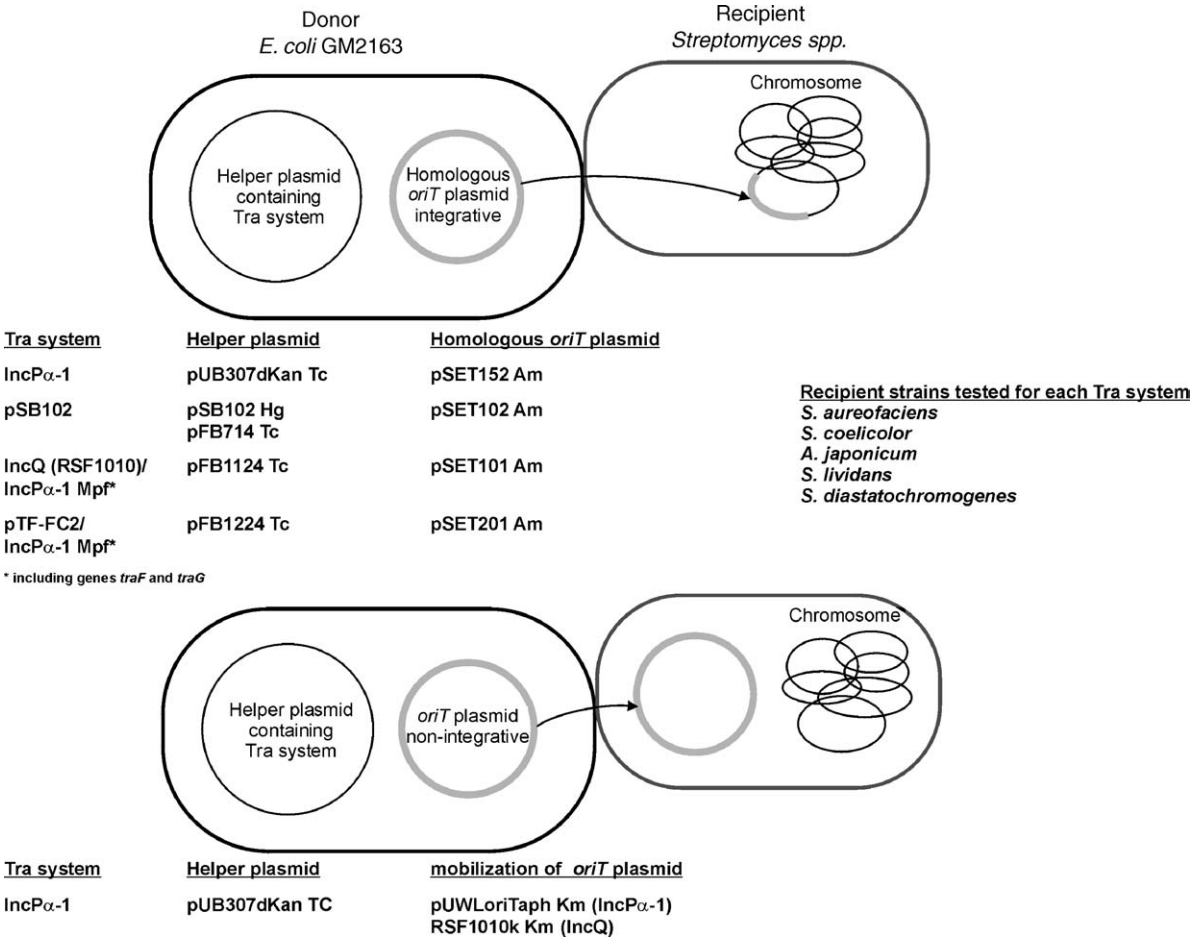

Fig. 3. Mobilization systems from *E. coli* to *Streptomyces* spp. and experimental set-up.

with the IncPα system pUB307dK/pSET152 in intergeneric conjugation experiments between *E. coli* strain GM2163 as the donor and five different recipient strains (Fig. 3). *Streptomyces* strains selected were the following: *S. lividans* and *S. coelicolor* were selected as genetically well characterized strains often used in standard experiments. Additionally, *S. diastatochromogenes*, a polyketomycin producing strain (Paululat et al., 1999), was chosen as a potentially convenient recipient strain for combinatorial biosynthesis, and *S. aureofaciens*, a producer of congocidin and spiramycin. The fifth recipient strain selected was *A. japonicum*, a nocardioform Actinomycete producing the biodegradable ethylenediaminetetraacetic acid (EDTA) isomer ethylenediaminedisuccinic acid (EDDS). The selected strains carry the ΦC31*attB* site for site-specific integration of the mobilized pSET derivatives, which lack a replicon to be autonomously maintained in these hosts. To assess whether the need of the mobilized plasmids to integrate into the host chromosome proves a limiting factor for the formation of exconjugants, we included in our studies conjugation experiments with two plasmids able to replicate in *Streptomyces* spp., pUWLoriTaph and RSF1010K. pUWLoriTaph contains the replication functions of the *Streptomyces* broad-host-range multicopy plasmid pIJ101 (Kieser et al., 1982). RSF1010 was shown to be transferred to and stably maintained in *S. lividans* and *M. smegmatis* (Gormley and Davies, 1991). For more convenient selection, we used the Km^r derivative RSF1010K (Table 1) instead of the original

Table 3
Intrageneric plasmid mobilization

Tra system	Plasmids in donor strain DH5α	Transfer frequency[a]
IncPα	pUB307dK, pSET152	8.5×10^{-1}
	pUB307dK, pUWLoriTaph	5.3×10^{-1}
	pUB307dK, pSEToriT$^-$	9.2×10^{-7}
pSB102	pSB102, pSET102	9.0×10^{-4}
	pFB714, pSET102	1.4×10^{-1}
	pSB102, pSEToriT$^-$	4.2×10^{-6}
	pFB714, pSEToriT$^-$	$<1 \times 10^{-7}$
IncPα/IncQ	pFB1124, pSET101u	3.5×10^{-2}
	pFB1124, pSET101n	5.5×10^{-2}
	pUB307dK, RSF1010K	>1
	pFB1124, pSEToriT$^-$	6.5×10^{-7}
IncPα/pTF-FC2	pFB1224, pSET201u	5.2×10^{-2}
	pFB1224, pSEToriT$^-$	2.9×10^{-6}

[a] Number of exconjugants per donor cell. The recipient strain was GM2163 Cm^r. The frequencies are average values for two independent experiments. Transfer frequencies for a given Tra system were always in the same order of magnitude.

RSF1010 plasmid encoding resistances against streptomycin and sulfonamide.

Control experiments using pSEToriT$^-$ were performed with all Tra systems to exclude possible spontaneous resistance.

Table 4 summarizes the frequencies of exconjugant formation per recipient spore. Exconjugants were obtained with all Tra systems and all *Streptomyces* strains employed. However, conjugation experiments employing *A. japonicum* as recipient strain yielded transfer frequencies near the detection limit and are therefore left out in the following evaluation of the Tra systems. The actual transfer of pSET DNA into *A. japonicum* could be confirmed, though, for the IncPα and pSB102 system but not for the IncPα/IncQ system and the IncPα/pTF-FC2 system (see below and Fig. 4).

Control experiments with pSEToriT$^-$ were negative with all tested Tra systems with the exception of one single *Streptomyces* colony appearing with helper plasmids pFB714, pFB1124 and pFB1224 and *S. livdans* as recipient.

The most efficient Tra system for the recipient strains used in this study proved to be the established IncPα system (pUB307dK/pSET152) with transfer frequencies between approximately 5×10^{-3} and 1×10^{-4} (Table 4). Highest transfer frequencies were obtained with *S. lividans* as recipient. No transfer frequency could be calculated for pUB307dK/pSET152 with *S. aureofaciens* as recipient strain. Exconjugant colonies were extremely small and formed a lawn on the agar plate even at high dilutions.

Table 4
Transfer frequencies for plasmid mobilization from *E. coli* to *Streptomyces* spp.[a]

Tra system	Plasmids in *E. coli* donor strain GM2163	Recipient strain				
		S. aureofaciens	*S. coelicolor*	*A. japonicum*	*S. lividans*	*S. diastatochromogenes*
IncPα	pUB307dK, pSET152	N.A.[b]	2.1×10^{-3}	$<1 \times 10^{-7}$	5.3×10^{-3}	1.6×10^{-4}
	pUB307dK, pUWLoriTaph	5.9×10^{-5}	4.8×10^{-4}	6.0×10^{-7}	1.5×10^{-3}	4.7×10^{-7}
	pUB307, pSEToriT$^-$	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$
pSB102	pSB102, pSET102	5.4×10^{-4}	9.7×10^{-6}	1.5×10^{-7}	4.8×10^{-5}	2.9×10^{-6}
	pFB714, pSET102	4.5×10^{-5}	1.9×10^{-5}	$<1 \times 10^{-7}$	6.6×10^{-5}	2.1×10^{-6}
	pSB102, pSEToriT$^-$	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$	N.D.[b]	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$
	pFB714, pSEToriT$^-$	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$	N.D.[b]	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$
IncPα/IncQ	pFB1124, pSET101u	2.8×10^{-5}	6.4×10^{-6}	$<1 \times 10^{-7}$	1.2×10^{-5}	8.0×10^{-7}
	pFB1124, pSET101n	2.2×10^{-6}	1.1×10^{-6}	$<1 \times 10^{-7}$	4.0×10^{-6}	3.0×10^{-7}
	pUB307dK, RSF1010K	$<1 \times 10^{-8}$	3.4×10^{-5}	1.0×10^{-7}	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$
	pFB1124, pSEToriT$^-$	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$	1×10^{-8}	$<1 \times 10^{-8}$
IncPα/pTF-FC2	pFB1224, pSET201u	2.7×10^{-5}	3.6×10^{-6}	$<1 \times 10^{-7}$	1.8×10^{-5}	2.8×10^{-6}
	pFB1224, pSEToriT$^-$	1×10^{-8}	$<1 \times 10^{-8}$	$<1 \times 10^{-8}$	1×10^{-8}	$<1 \times 10^{-8}$

[a] Number of exconjugants per recipient spore. With the exception of negative control experiments, the transfer frequencies were determined from at least two independent experiments. For each Tra system applied to a given receptor strain, the transfer frequencies vary at the most by factor 10.

[b] N.A., not applicable; N.D., not determined.

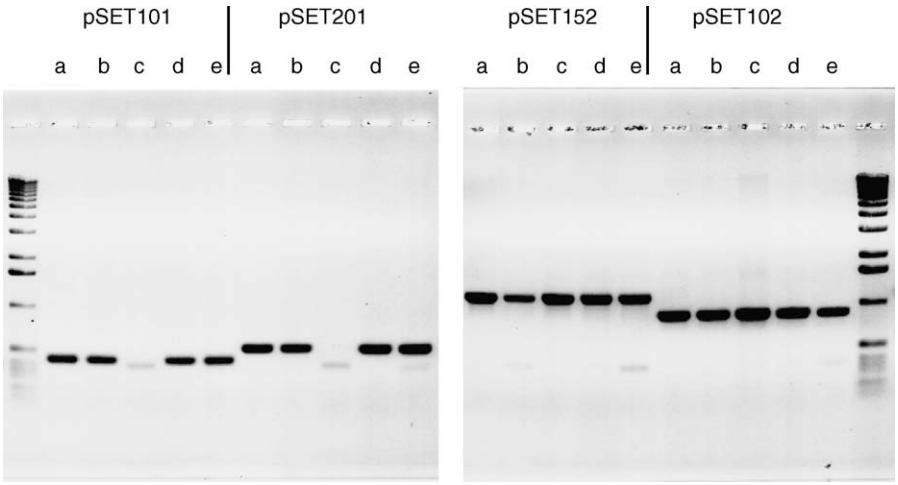

Fig. 4. PCR analysis of exconjugants. PCR reactions were carried out in 100 μl with 2 μl of genomic Actinomycetes DNA, 50 pmol primers each, 200 μM dNTPs and 2 U *Taq* polymerase in PCR buffer (10 mM Tris–HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$). The template was denatured for 3 min at 95 °C. Amplification was for 30 cycles with 1 min 95 °C, 1 min 58 °C and 1 min 72 °C and additional 5 min for the final synthesis step. Ten microliters of each PCR reaction was loaded onto 0.8% agarose gels and subjected to electrophoresis in TAE buffer (40 mM Tris–HCl, pH 7.9; 5 mM sodium acetate; 1 mM EDTA). Primers used map to both sides of the *Sph*I site in pSEToriT$^-$ and yield a PCR product of 390 bp (not shown). *oriT* specific products display bands of 390 bp plus the size of the corresponding *oriT* sequence. Exconjugants: (a) *S. aureofaciens*; (b) *S. coelicolor*; (c) *A. japonicum*; (d) *S. lividans*; (e) *S. diastatochromogenes*. Molecular size standard: 1 kb ladder (Invitrogen).

Mobilization of the non-integrative plasmid pUWLoriTaph by pUB307dK yielded transfer frequencies between 1×10^{-3} and 5×10^{-7} (Table 4), which is 2–300-fold lower for the corresponding recipient strains. The integration of the plasmid into the host chromosome seems therefore not a limiting factor for the formation of exconjugants.

The IncPα/IncQ system (pFB1124/pSET101) yielded transfer frequencies between 3×10^{-5} and 3×10^{-7}, which is 2–2000 times lower for a given recipient strain compared with the IncPα system (Table 4). Highest transfer frequencies were obtained with *S. lividans* as recipient, but also with *S. aureofaciens*. In contrast to the exconjugants obtained with the IncPα Tra system, *S. aureofaciens* exconjugants obtained with the IncPα/IncQ Tra system were large and well growing. Plasmid RSF1010K could be mobilized into and replicated in *S. coelicolor* and *A. japonicum*, but no exconjugants were obtained with *S. lividans*, *S. aureofaciens* and *S. diastatochromogenes*.

The IncPα/pTF-FC2 Tra system yielded transfer frequencies similar to those obtained with the IncPα/IncQ Tra system (3×10^{-5} to 3×10^{-6}; Table 4). Transfer frequencies obtained with *S. aureofaciens* were highest, and exconjugants also here formed large colonies.

Of the three alternative transfer systems, the pSB102 system was the most efficient in intergeneric conjugation experiments with all *Streptomyces* strains yielding transfer frequencies between 5×10^{-4} and 2×10^{-6} (Table 4). Transfer frequencies were similar with both the original pSB102 plasmid as helper plasmid and plasmid pFB714 containing the pSB102 Tra region. As in intrageneric conjugation experiments, *E. coli* donor cells carrying pSB102 together with the corresponding *oriT* plasmid were growing rather slowly. The pSB102 system was especially effective in conjugation experiments with *S. aureofaciens* as recipient strain. *S. aureofaciens* exconjugants displayed good growth as in the experiments using the IncPα/IncQ or IncPα/pTF-FC2 Tra system.

Taken together, the Tra systems assayed proved to be suitable for intergeneric conjugation between *E. coli* and *Streptomyces* spp. in the following gradation: pUB307dK > pSB102 > pTF-FC2 ≥ RSF1010, with integrative > non-integrative. The three alternative

systems offer the advantage that some recipient strains as *S. aureofaciens* seem to be more susceptible than with the IncPα system.

3.4. Confirmation of plasmid transfer

Samples of exconjugants were picked and cultivated on selective HA agar plates to confirm the conjugative transfer of the pSET derivatives and their stable maintenance in the *Streptomyces* hosts. Phosphomycin was added to counterselect the sensitive *E. coli* donor.

For a further verification of transfer of the integrative *oriT* plasmids into the five different recipient strains, genomic DNA was isolated and the presence of integrated pSET DNA was confirmed by PCR analysis using primers mapping at the *Sph*I site (see Table 2). PCR products of the size specific for the corresponding *oriT* sequences located at this site were obtained with all samples with the exception of RSF1010 and pTF-FC2 *oriT* (plasmids pSET101 and pSET201, respectively) in *A. japonicum* (Fig. 4). The weak band visible in the latter two samples is not *oriT* specific but a PCR by-product also contained in some of the other samples (Fig. 4). We cannot exclude that the colonies obtained are spontaneous resistants. However, true exconjugants were also obtained with *A. japonicum* as shown for the conjugation experiments with pSET152 and pSET102. The three colonies obtained in control experiments with pSEToriT$^-$ did not yield a PCR product (data not shown).

Likewise, the conjugative transfer and stable maintenance of non-integrative plasmids pUWLoriTaph and RSF1010K was confirmed by cultivating the exconjugants on selective HA agar plates with simultaneous counterselection against the *E. coli* donor. The plasmids were then isolated from stable exconjugants and their plasmid content analyzed by agarose gel electrophoresis (data not shown). Plasmid pUWLoriTaph was stably maintained in *S. coelicolor*, *S. lividans* and *S. diastatochromogenes*, but not in *S. aureofaciens* and *A. japonicum*. *S. aureofaciens* exconjugants did not grow on HA medium containing kanamycin and phospomycin, indicating the loss of the plasmid. *A. japonicum* exconjugants were stable under the selective conditions applied, but pUWLoriTaph plasmid DNA was not obtained. Likewise, RSF1010K plasmid DNA was isolated from *S. coelicolor*, but not from *A. japonicum*. This might indicate that the plasmid isolation protocol used is not applicable for this strain, that the copy number of the plasmid in *A. japonicum* is reduced, or else, that spontaneous chromosomal mutations led to the formation of Km^r *A. japonicum* colonies.

4. Discussion

This study is the first systematic analysis of various transfer systems with different *Streptomyces* recipient strains. Together with the established IncPα system, three additional systems were assayed for conjugative transfer of DNA from *E. coli* to four *Streptomyces* strains and one *Amycolatopsis* strain; two of these transfer systems have not been applied with *Streptomyces* before.

We present for the first time the experimental application of the pSB102 transfer system that has not been experimentally characterized so far. The proposed transfer region of pSB102, predicted on the basis of sequence similarity to those of other plasmids (Schneiker et al., 2001), was isolated by molecular cloning. The conjugative ability of the construct was experimentally confirmed. Likewise, the putative *oriT* sequence, located on a 2.3 kb *Hin*dIII fragment, was isolated and delimited to a functional region of about 450 bp. pSB102 represents a new class of plasmids unrelated to the known plasmid incompatibility groups. The putative Tra region consists of a compact cluster of genes, covering about one half of the plasmid. The putative Mpf genes, designated *traA* to *traM*, show the highest similarity to the *virB* gene cluster of the type IV secretion systems of *Brucella suis* (O'Callaghan et al., 1999) and *Brucella abortus* (Sieira et al., 2000). Thus, using pSB102, we employed membrane and cell surface components with specificities and affinities possibly differing from those of the IncPα system, which is the basis for all transfer systems so far developed for conjugation between distantly related bacterial species. The transfer was efficient with frequencies ranging approximately between 5×10^{-4} and 2×10^{-6}, similar to those reported for the IncPα transfer systems. We conclude that the Mpf system of pSB102 was able to mediate the contact between *E. coli* donor and *Streptomyces* recipient cells that is necessary for conjugative DNA transfer. pSB102 might also prove to be a valuable tool for genetic manipulation of other industrially and medically important Actinomycetes for which up

to now no transformation procedure or conjugation system is available.

This study also demonstrates for the first time conjugative transfer of a pTF-FC2-derived plasmid into *Streptomyces* spp. The promiscuous properties of the IncQ-like mobilizable plasmid pTF-FC2, originally isolated from the biomining bacterium *Acidithiobacillus ferrooxidans* (previously: *Thiobacillus ferrooxidans*; Rawlings et al., 1984), are underlined with the observation that it can be mobilized effectively also into Gram-positive bacteria. IncQ plasmid RSF1010 has already been reported to be capable of conjugative transfer to and stable maintenance in *S. lividans* and *M. smegmatis* (Gormley and Davies, 1991). Using pUB307dK as helper plasmid, we confirmed the conjugative transfer of a RSF1010 derivative from *E. coli* to *Streptomyces* spp. However, evidence for mobilization of RSF1010K was obtained with *S. coelicolor*, but not with *S. lividans*. This discrepancy to the observations of Gormley and Davies might be due to properties of the wildtype RSF1010 compared with the RSF1010K derivative used in our study or from experimental conditions applied.

The need for a plasmid to replicate and to express the resistance gene to be stably maintained in the new host can be circumvented by employing an integrative variant. A pSET152 derivative carrying the RSF1010 minimal *oriT* was successfully mobilized to all *Streptomyces* strains, indicating that the RSF1010 mobilization functions themselves are appropriate for use with *Streptomyces* spp. To develop new transfer systems for the genetic engineering of Actinomycetes, the results obtained with the IncQ transfer system suggest a decision in favor of integrative rather than non-integrative plasmids, since they seem to allow the access to a wider range of hosts. We addressed this question additionally by a direct comparison of the mobilization frequencies of the integrative IncPα plasmid pSET152 with those of the non-integrative IncPα plasmid pUWLoriTaph employing pUB307dK as helper. Transfer frequencies obtained were reproducibly higher when employing pSET152 than with pUWLoriTaph. pSET152 is likely to have integrated into the chromosome, although episomal forms of the plasmid have been reported for *Nonomuraea* (Stinchi et al., 2003). From our experiments, we conclude that the necessity to integrate into the chromosome is not a limiting factor for the formation of exconjugants. The advantages of integrative *oriT* plasmids can consequently be summarized as follows: their use is more versatile since replication and stable maintenance of plasmids obviously is a greater burden for the cell than to provide the functions required for plasmid integration. At the same time, the application of integrative plasmids does not affect the transfer frequencies adversely. The disadvantage of using integrative *oriT* plasmids is the necessity for the recipient strains to contain the ΦC31 *attB* site in the chromosome. The phage ΦC31 integrative system, however, was shown to function in a multitude of *Streptomyces* spp. and other strains of different genera of the order *Actinomycetales* (Voeykova et al., 1998), even in strains not susceptible for a ΦC31 infection.

Using the IncPα transfer system, we obtained transfer frequencies of 5×10^{-3} to 1×10^{-4}, which is in accordance with the results reported by other authors. However, no standardized protocol for the conjugative transfer between *E. coli* and Gram-positive bacteria is available up till now. Different modifications of the transfer system and conjugation conditions are used, affecting the frequency of exconjugant formation. Also, the methods to calculate transfer frequencies vary significantly. Using *E. coli* strain S17-1 carrying an RP4 derivative on the chromosome (Simon et al., 1983) and conjugative *E. coli–Streptomyces* shuttle plasmids, Mazodier et al. (1989) obtained transfer frequencies of about 10^{-4} of the viable *S. lividans* spores. Bierman et al. (1992) reported exconjugant frequencies of 10–15% of the initial viable recipient population using *E. coli* S17-1 (pSET152) as donor and *S. fradiae* germinated spores or vegetative mycelia as recipients. Flett et al. (1997) employed pSET152 derivatives with different insertions of chromosomal *Streptomyces* DNA fragments. The number of exconjugants obtained (10^{-6} to 10^{-1}) varied depending on the insert, the donor strain (S17-1 or methylation defective ET12567 (pUB307)) and the recipient strain (*S. lividans* or methyl DNA-restricting *S. coelicolor*). Since the methylation-specific restriction system of *S. coelicolor* was shown to drastically reduce the number of exconjugants obtained from intergeneric matings with the methylation proficient *E. coli* donor, we used the methylation deficient *E. coli* strain GM2163 in our experiments.

Each of the DNA transfer systems assayed in this study was shown to be applicable for conjugation between *E. coli* and *Streptomyces* spp. However, none

of the four systems yielded transfer frequencies above the level of control experiments when *A. japonicum* was used as recipient strain. Recently, the conjugation system pUB307/pSET152 has been employed for this strain (Stegmann et al., 2001). The authors report a titer of 2.4×10^{-5} exconjugants per recipient. We could not confirm these results under our conditions. Since molecular genetics is not well established for *Amycolatopsis*, the appropriate conjugation parameters for this strain still have to be worked out.

The three newly applied systems pSB102, pTF-FC2/IncPα Mpf and RSF1010/IncPα Mpf did not yield a quantitative improvement in comparison to the established IncPα system pUB307/pSET152. However, they provide valuable alternatives for conjugative DNA transfer into *Streptomyces* strains poorly or not accessible by the IncPα system as shown with the experiments carried out with *S. aureofaciens* as recipient strain. The results obtained using this strain with the pSB102, but also with the IncPα/IncQ and the IncPα/pTF-FC2 Tra system imply that not only the Mpf components of a given transfer system is of relevance for the conjugation process but also the functions provided by the mobilization genes and/or the interplay of Mpf and Dtr components.

Taken together, the transfer systems presented in this study provide the basis for the development of new conjugative systems apart from the mere modification of the established ones exclusively based on conjugative plasmid RP4. Especially pSB102 might offer the access to a wider range of bacterial species since cell surface and membrane components with possibly different affinities and/or specificities from those of the IncPα system are involved. In future experiments, there will be the choice between different Tra systems in the effort to find optimal conditions for conjugative DNA transfer into individual representatives of the order *Actinomycetales*.

Acknowledgements

We thank Doug Rawlings for kindly providing plasmid pDER412 and Susanne Schneiker for plasmid pSB102. Plasmid pUWL201 was kindly provided by Udo Wehmeier and the *A. japonicum* strain MG417-CF17 by Efthimia Stegmann. The expert technical assistance of Marianne Schlicht is greatly appreciated. E.L., F.B. and G.Z. thank Hans Lehrach for generous support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2005.06.023.

References

Bates, S., Cashmore, A.M., Wilkins, B.M., 1998. IncP plasmids are unusually effective in mediating conjugation of *Escherichia coli* and *Saccharomyces cerevisiae*: involvement of the tra2 mating system. J. Bacteriol. 180, 6538–6543.

Bennett, P.M., Grinsted, J., Richmond, M.H., 1977. Transposition of TnA does not generate deletions. Mol. Gen. Genet. 154, 205–211.

Bibb, M.J., White, J., Ward, J.M., Janssen, G.R., 1994. The mRNA for the 23S rRNA methylase encoded by the *ermE* gene of *Saccharopolyspora erythraea* is translated in the absence of a conventional ribosome-binding site. Mol. Microbiol. 14, 533–545.

Bierman, M., Logan, R., O'Brien, K., Seno, E.T., Nagaraja Rao, R., Schoner, B.E., 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. Gene 116, 43–49.

Birnboim, H.D., Doly, J., 1979. A rapid alcaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7, 1513–1523.

Boyer, H.W., Roulland-Dussoix, D., 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. 41, 459–472.

Brasch, M.A., Meyer, R.J., 1987. A 38 base-pair segment of DNA is required in *cis* for conjugative mobilization of broad host-range plasmid R1162. J. Mol. Biol. 198, 361–369.

Buchanan-Wollaston, V., Passiatore, J.E., Cannon, F., 1987. The *mob* and *oriT* mobilization functions of a bacterial plasmid promote its transfer to plants. Nature 328, 172–175.

Covarrubias, L., Bolivar, F., 1982. Construction and characterization of new cloning vehicles. VI. Plasmid pBR329, a new derivative of pBR328 lacking the 482-base-pair inverted duplication. Gene 17, 79–89.

Demain, A.L., 1999. Pharmaceutically active secondary metabolites of microorganisms. Appl. Microbiol. Biotechnol. 52, 455–463.

Doumith, M., Weingarten, P., Wehmeier, U.F., Salah-Bey, K., Benhamou, B., Capdevila, C., Michel, J.-M., Piepersberg, W., Raynal, M.-C., 2000. Analysis of genes involved in 6-deoxyhexose biosynthesis and transfer in *Saccharopolyspora erythraea*. Mol. Gen. Genet. 264, 477–485.

Dube, T., Thompson, J.A., 2003. Conjugal transfer of plasmid pTF-FC2 from *Agrobacterium* to plant cells in the absence of T-DNA borders. Plasmid 50, 1–11.

Dube, T., Kovalchuk, I., Hohn, B., Thompson, J.A., 2004. *Agrobacterium tumefaciens*-mediated transformation of plants by the

pTF-FC2 plasmid is efficient and strictly dependent on the MobA protein. Plant Mol. Biol. 55, 531–539.

Flett, F., Mersinias, V., Smith, C.P., 1997. High efficiency intergeneric conjugal transfer of plasmid DNA from *Escherichia coli* to methyl DNA-restricting streptomycetes. FEMS Microbiol. Lett. 155, 223–229.

Fouces, R., Rodriguez, M., Mellado, E., Diez, B., Barredo, J.L., 2000. Conjugation and transformation of *Streptomyces* species by tylosin resistance. FEMS Microbiol. Lett. 186, 319–325.

Frey, J., Bagdasarian, M., 1989. The biology of IncQ plasmids. In: Thomas, C.M. (Ed.), Promiscuous Plasmids of Gram-Negative Bacteria. Academic Press Ltd., London, UK, pp. 79–93.

Fürste, J.P., Pansegrau, W., Frank, R., Blöcker, H., Scholz, P., Bagdasarian, M., Lanka, E., 1986. Molecular cloning of the RP4 primase region in a multi-host-range *tac*P expression vector. Gene 48, 119–131.

Gormley, E., Davies, J., 1991. Transfer of plasmid RSF1010 by conjugation from *Escherichia coli* to *Streptomyces lividans* and *Mycobacterium smegmatis*. J. Bacteriol. 173, 6705–6708.

Guerry, P., van Embden, J., Falkow, S., 1974. Molecular nature of two nonconjugative plasmids carrying drug resistance genes. J. Bacteriol. 117, 619–630.

Hanahan, D., 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. 166, 557–580.

Haring, V., Scherzinger, E., 1989. Replication proteins of the IncQ plasmid RSF1010. In: Thomas, C.M. (Ed.), Promiscuous Plasmids of Gram-Negative Bacteria. Academic Press Ltd., London, UK, pp. 95–124.

Heinzelmann, E., Berger, S., Puk, O., Reichenstein, B., Wohlleben, W., Schwartz, D., 2003. A glutamate mutase is involved in the biosynthesis of the lipopeptide antibiotic friulimicin in *Actinoplanes friuliensis*. Antimicrob. Agents Chemother. 47, 447–457.

Herrera, G., Snyman, S.J., Thomson, J.A., 1994. Construction of a bioinsecticidal strain of *Pseudomonas fluorescens* active against the sugarcane borer, *Eldana saccharina*. Appl. Environ. Microbiol. 60, 682–890.

Hobbs, G., Frazer, C.M., Gardner, D.C.J., Cullum, J.A., Oliver, S.G., 1989. Dispersed growth of *Streptomyces* in liquid culture. Appl. Microbiol. Biotechnol. 31, 272–277.

Hopwood, D.A., Kieser, T., 1993. Conjugative plasmids of *Streptomyces*. In: Clewell, D.B. (Ed.), Bacterial Conjugation. Plenum Press, New York, pp. 293–311.

Jaoua, S., Neff, S., Schupp, T., 1992. Transfer of mobilizable plasmids to *Sorangium cellulosum* and evidence for their integration into the chromosome. Plasmid 28, 157–165.

Kieser, T., Hopwood, D.A., Wright, H.M., Thompson, C.J., 1982. pIJ101, a multicopy broad host range *Streptomyces* plasmid: functional analysis and development of DNA cloning vectors. Mol. Gen. Genet. 185, 223–228.

Kieser, T., Bibb, M.J., Buttner, M.J., Chater, K.F., Hopwood, D.A., 2000. Practical *Streptomyces* Genetics. John Innes Foundation, Norwich, UK.

Kirchner, O., Tauch, A., 2003. Tools for genetic engineering in the amino acid-producing bacterium *Corynebacterium glutamicum*. J. Biotechnol. 104, 287–299.

Kopp, M., Irschik, H., Gross, F., Perlova, O., Sandmann, A., Gerth, K., Müller, R., 2004. Critical variations of conjugational DNA transfer into secondary metabolite multiproducing *Sorangium cellulosum* strains So ce12 and So ce56: development of a mariner-based transposon mutagenesis system. J. Biotechnol. 107, 29–40.

Lessl, M., Balzer, D., Lurz, R., Waters, V.L., Guiney, D.G., Lanka, E., 1992. Dissection of IncP conjugative plasmid transfer: definition of the transfer region Tra2 by mobilization of the Tra1 region in *trans*. J. Bacteriol. 174, 2493–2500.

Lessl, M., Balzer, D., Weyrauch, K., Lanka, E., 1993. The mating pair formation system of plasmid RP4 defined by RSF1010 mobilization and donor-specific phage propagation. J. Bacteriol. 175, 6415–6425.

Matsushima, P., Broughton, M.C., Turner, J.R., Baltz, R.H., 1994. Conjugal transfer of cosmid DNA from *Escherichia coli* to *Saccharopolyspora spinosa*: effects of chromosomal insertions on macrolide A83543 production. Gene 146, 39–45.

Matsushima, P., Baltz, R.H., 1996. A gene cloning system for *Streptomyces toyocaensis*. Microbiology 142, 261–267.

Mazodier, P., Petter, R., Thompson, C., 1989. Intergeneric conjugation between *Escherichia coli* and *Streptomyces* species. J. Bacteriol. 171, 3583–3585.

Miller, J.H., 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Molnar, I., Schupp, T., Ono, M., Zirkle, R., Milnamow, M., Nowak-Thompson, B., Engel, N., Toupet, C., Stratmann, A., Cyr, D.D., Gorlach, J., Mayo, J.M., Hu, A., Goff, S., Schmid, J., Ligon, J.M., 2000. The biosynthetic gene cluster for the microtubule-stabilizing agents epothilones A and B from *Sorangium cellulosum* So ce90. Chem. Biol. 7, 97–109.

Motamedi, H., Shafiee, A., Cai, S.-J., 1995. Integrative vectors for heterologous gene expression in *Streptomyces* spp. Gene 160, 25–31.

Muth, G., Nußbaumer, B., Wohlleben, W., Pühler, A., 1989. A vector system with temperature-sensitive replication for gene disruption and mutational cloning in streptomycetes. Mol. Gen. Genet. 219, 341–348.

O'Callaghan, D., Cazevieille, C., Allardet-Servent, A., Boschiroli, M.L., Bourg, G., Foulongne, V., Frutos, P., Kulakov, Y., Ramoz, M., 1999. A homologue of the *Agrobacterium tumefaciens* VirB and *Bordetella pertussis* Ptl type IV secretion systems is essential for intracellular survival of *Brucella suis*. Mol. Microbiol. 33, 1210–1220.

Paululat, T., Zeeck, A., Gutterer, J.M., Fiedler, H.P., 1999. Biosynthesis of polyketomycin produced by *Streptomyces diastatochromogenes* Tü 6028. J. Antibiot. 52, 96–101.

Rawlings, D.E., Woods, D.R., 1985. Mobilization of *Thiobacillus ferrooxidans* plasmids among *Escherichia coli* strains. Appl. Environ. Microbiol. 49, 1323–1325.

Rawlings, D.E., Tietze, E., 2001. Comparative biology of IncQ and IncQ-like plasmids. Microbiol. Mol. Biol. Rev. 65, 481–496.

Rawlings, D.E., Pretorius, I.M., Woods, D.R., 1984. Expression of a *Thiobacillus ferrooxidans* origin of replication in *Escherichia coli*. J. Bacteriol. 158, 737–738.

Rix, U., Fischer, C., Remsing, L.L., Rohr, J., 2002. Modification of post-PKS tailoring steps through combinatorial biosynthesis. Nat. Prod. Rep. 19, 542–580.

Rohrer, J., Rawlings, D.E., 1992. Sequence analysis and characterization of the mobilization region of a broad-host-range plasmid, pTF-FC2, isolated from *Thiobacillus ferrooxidans*. J. Bacteriol. 174, 6230–6237.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schäfer, A., Kalinowski, J., Simon, R., Seep-Feldhaus, A.H., Pühler, A., 1990. High-frequency conjugal plasmid transfer from gram-negative *Escherichia coli* to various Gram-positive coryneform bacteria. J. Bacteriol. 172, 1663–1666.

Schneiker, S., Keller, M., Dröge, M., Lanka, E., Pühler, A., Selbitschka, W., 2001. The genetic organization and evolution of the broad host range mercury resistance plasmid pSB102 isolated from a microbial population residing in the rhizosphere of alfalfa. Nucleic Acids Res. 29, 5169–5181.

Scholz, P., Haring, V., Wittmann-Liebold, B., Ashman, K., Bagdasarian, M., Scherzinger, E., 1989. Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. Gene 75, 271–288.

Sieira, R., Comerci, D.J., Sanchez, D.O., Ugalde, R.A., 2000. A homologue of an operon required for DNA transfer in *Agrobacterum* is required in *Brucella abortus* for virulence and intracellular multiplication. J. Bacteriol. 182, 4849–4855.

Simon, R., Priefer, U., Pühler, A., 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram negative bacteria. BioTechnology 1, 784–791.

Smokvina, T., Mazodier, P., Boccard, F., Thompson, C.J., Guerineau, M., 1990. Construction of a series of pSAM2-based integrative vectors for use in Actinomycetes. Gene 94, 53–59.

Stegmann, E., Pelzer, S., Wilken, K., Wohlleben, W., 2001. Development of three different gene cloning systems for genetic investigation of the new species *Amycolatopsis japonicum* MG417-CF17, the ethylenediaminedisuccinic acid producer. J. Biotechnol. 92, 195–204.

Stinchi, S., Azimonti, S., Donadio, S., Sosio, M., 2003. A gene transfer system for the glycopeptide producer *Nonomuraea* sp. ATCC39727. FEMS Microbiol. Lett. 225, 53–57.

Tabacov, V.Y., Voeykova, T.A., Tokmakova, I.L., Bolotin, A.P., Vavilova, E.Y., Lomovskaya, N.D., 1994. Intergeneric conjugation of *Escherichia coli* and *Streptomyces* as a means for the transfer of conjugative plasmids into producers of the antibiotics chlortetracycline and bialaphos. Russ. J. Genet. 30, 49–53.

Trieu-Cuot, P., Carlier, C., Martin, P., Courvalin, P., 1987. Plasmid transfer by conjugation from *Escherichia coli* to Gram positive bacteria. FEMS Microbiol. Lett. 48, 289–294.

Voeykova, T., Emelyanova, L., Tabakov, V., Mkrtumyan, N., 1998. Transfer of plasmid pTO1 from *Escherichia coli* to various representatives of the order Actinomycetales by intergeneric conjugation. FEMS Microbiol. Lett. 162, 47–52.

Waters, V.L., 2001. Conjugation between bacterial and mammalian cells. Nat. Genet. 29, 375–376.

Yoshida, K., Takegami, T., Katoh, A., Nishikawa, M., Nishida, T., 1997. Construction of a novel conjugative plasmid harboring a GFP reporter gene and its introduction into animal cells by transfection and trans-kingdom conjugation. Nucleic Acids Symp. Ser. 37, 157–158.