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Short Communication

Characterization of the mobilization determinants of pAN12, a small replicon from *Rhodococcus erythropolis* AN12

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Abstract

Bacteria belonging to the Gram-positive actinomycete species, *Rhodococcus erythropolis*, are diverse not only in terms of metabolic potentials but the plasmids they encode. It was shown previously that the *R. erythropolis* AN12 genome harbors a 6.3 kb cryptic plasmid called pAN12, which is a member of the pIJ101 family of plasmids. Here we show that pAN12 is conjugatively mobilizable into other rhodococcal strains. A series of plasmid deletion constructs were tested for loss of mobility to identify the pAN12 *cis*-acting conjugation requirement. In this way, an approximately 700 bp region was found to be required for plasmid transmission. A small 61 bp element within this region confers mobility to an otherwise non-mobilizable plasmid. Unlike pIJ101, which encodes all necessary factors for transfer, pAN12 mobility is dependent on the presence of an AN12 megaplasmid, pREA400.

Keywords: Cryptic plasmid; *Rhodococcus*; Plasmid mobilization; FtsK; SpoIIIE; Origin of transfer; Megaplasmid

1. Introduction

Rhodococcus sp. bacteria are Gram-positive actinomycetes ubiquitous in many terrestrial and marine environments. This ecological versatility can partially be attributed to plasmid-encoded operons. In particular, megaplasmids-extrachromosomal elements ranging in size from 50 kb to greater than 1 Mb found in these rhodococci—often encode advantageous properties, such as the degradation of aromatic compounds (Konig et al., 2004) and virulence factors required for plant pathogenesis (Crespi et al., 1992). Besides megaplasmids, rhodococci may also harbor plasmids of smaller sizes. Plasmids are traditionally classified according to type of replication they undergo. Three general mechanisms of plasmid replication have been characterized; theta, strand displacement, and rolling circle (del Solar et al., 1998). A number of smaller plasmids proposed to replicate via either the theta type (De Mot et al., 1997; Hirasawa et al., 2001; Kulakov et al., 1997; Lessard et al., 2004) or the rolling circle type (Kostichka et al., 2003; Nakashima and Tamura, 2004) of replication have been discovered in rhodococci.

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These *Rhodococcus* plasmids are cryptic, and have been studied mainly for use as *Escherichia coli–Rhodococcus* shuttle vectors. Several of these plasmids have proven to be useful for metabolic engineering purposes (Hirasawa et al., 2001; Kostichka et al., 2003).

Many species of *Rhodococcus* can exchange plasmids via conjugation. It has been proposed that mechanisms similar to those demonstrated for Gram-negative bacterial plasmids function in Gram-positive plasmid conjugation (Grohmann et al., 2003). These processes are initiated with proteins called relaxases or nickases that act in *trans* upon plasmid sequences required in *cis* called origins of transfer (*ori*T), within which reside specific cleavage sequences called *nic* sites (Lanka and Wilkins, 1995). It is assumed for these plasmids that a single-stranded DNA intermediate is transferred from donor to recipient cells. Interestingly, this is apparently not the case for a subset of plasmids found in mycelium-forming actinomycetes. For the pIJ101 conjugative plasmid of *Streptomyces lividans*, the transfer of double-stranded DNA (Possoz et al., 2001) is mediated by a single plasmid-borne and membrane-bound gene product called Tra (Pettis and Cohen, 1996, 2000, 2001). Tra shares protein similarity with the Gram-negative FtsK ATPase, which functions in DNA segregation during cell division (Aussel et al., 2002; Pettis and Cohen, 2000).

Though the exchange of genetic information has been documented for rhodococci (formerly classified as *Nocardia erythropolis* and *Nocardia canicruria*) as early as the 1960s (Adams and Bradley, 1963; Adams, 1964; Brownell and Kelly, 1969), few reports exist that describe determinants governing plasmid conjugation for these actinomycetes. It is unknown whether the mechanisms in rhodococci are more analogous to single-stranded DNA transfer typified by Gram-negative systems, or more closely resemble those observed for the *Streptomyces* pIJ101. Further understanding of the mechanisms of *Rhodococcus* plasmid conjugation will also facilitate its genetic analysis and manipulation. Since direct rhodococci transformations can prove difficult due to low transformation efficiency and/or DNA rearrangements, conjugation can be a powerful tool to introduce DNA in the context of a plasmid that is stably maintained by both the donor and recipient strains. Recently, the first conjugative small (<10 kb) *Rhodococcus* plasmid, pB264, was described in *Rhodococcus* sp. B264-1 (Lessard et al., 2004). In this study, a 700 bp region required in *cis* for pB264 transfer was defined. The presence of seven inverted repeats and one direct repeat within this region suggests that one or more of these elements are substrates for the plasmid transfer mechanism. It is unknown for pB264 what protein factors are required in *trans* for its transfer, although it has been proposed that those factors may be encoded by genes that reside on B264-1 megaplasmids (Lessard et al., 2004). In addition, data from our laboratory and others suggest that relaxase-mediated plasmid conjugation mechanisms appear to function in *R. erythropolis* AN12 (Yang et al., 2006), *R. erythropolis* PR4 (Sekine et al., 2006), and *Rhodococcus equi* ATCC33701 (Takai et al., 2000).

The goal for this study was to identify conjugal determinants of a small circular plasmid, pAN12, found in *R. erythropolis* strain, AN12. AN12 was first isolated and so named for its ability to use the aromatic compound, aniline, as a carbon source (Bramucci et al., 2002). The isolation of its cryptic plasmid, pAN12, and characterization of two plasmid-borne genes, *rep* and *div* (involved in plasmid replication and segregation during cell division, respectively), was recently reported (Kostichka et al., 2003). pAN12 was assigned to the pIJ101/pJV1 family of rolling-circle plasmids based on conservations of Rep protein motifs and sequence similarities of the plasmid replication origins. Although the related streptomycete plasmids pIJ101 and pJV1 have both been found to be conjugative (Kieser et al., 1982; Servin-Gonzalez et al., 1995), it was not known prior to this study whether pAN12 is transmissible, and if so, what are the determinants for its transfer.

In this report, we provide the first evidence of pAN12 mobility and describe the identification of a minimal 61 bp *cis*-acting element on pAN12 necessary for its mobilization. Furthermore, we show that pAN12 does not encode all necessary factors for its own transmission. In fact, data suggest that the presence of the recently identified AN12 megaplasmid, pREA400, in the donor strain is required to mobilize pAN12 and derivative plasmids.

2. Materials and methods

2.1. Bacterial strains and culturing conditions

Bacterial strains used in the present study are summarized in Table 1. Both *Rhodococcus erythropolis* AN12 and

Table 1
Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer name	Description or sequence	Source or reference
Strains		
DH5α	*Escherichia coli*; *supE*44 Δ*lacU*169 (ϕ80 *lacZ*ΔM15) *recA*1 *endA*1 *hsdR*17 *thi*-1 *gyrA*96 *relA*1	Invitrogen (Carlsbad, CA)
EC100D *pir*-116	*E. coli*; F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80d*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara*, *leu*)7697 *gal*U *gal*K λ^- *rps*L *nup*G *pir*-116(DHFR)	Epicentre (Madison, WI)
TOP10	*E. coli*; F-*mcrA*Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ*ΔM15 Δ*lacX*74 *recA*1 *deoR araD*139 Δ(*ara-leu*)7697 *galU galK rpsL* (Sm^R) *endA*1 *nupG*	Invitrogen
AN12	Environmental isolate of *Rhodococcus erythropolis* with three megaplasmids (pREA400, pREA250, and pREA100)	Kostichka et al. (2003)
SQ1	Environmental isolate of *R. erythropolis*; Rf^R Sm^R	ATCC4277-1
DDO319	environmental isolate of *Rhodococcus ruber*	Gift from E.I. DuPont de Nemours
AN12PL	AN12 derivative that was spontaneously cured of the smallest megaplasmid, pREA100	Yang et al. (2006)
AN12PL (pREA400::EZTn)	*R. erythropolis* AN12PL mutant recovered from transformation with EZTn transposome; (pREA400::EZTn); Km^R; also known as AN12PL-1F6	Yang et al. (2006)
AN12 (pREA250::EZTn)	*R. erythropolis* AN12 mutant recovered from transformation with EZTn transposome; (pREA250::EZTn); Km^R; also known as AN12-5A6	Yang et al. (2006)
JY524	*R. erythropolis* SQ1 derived transconjugant recovered from mating with AN12PL-1F6; (pREA400::EZTn); Km^R Rf^R Sm^R	Yang et al. (2006)
JY650	*R. erythropolis* AN12 (pAL319); Gm^R	This study
JY660	*R. erythropolis* AN12 (pAL281); Gm^R	This study
JY671	*R. erythropolis* SQ1 (pAL319); Gm^R	This study
JY700	*R. erythropolis* AN12 (pAL321); Gm^R	This study
JY709	*R. erythropolis* SQ1 (pAL321); Gm^R	This study
JY720	*R. erythropolis* AN12 (pJY29); Gm^R	This study
JY735	*R. erythropolis* AN12 (pJY30); Gm^R	This study
JY737	*R. erythropolis* AN12 (pJY31); Gm^R	This study
JY750	*R. erythropolis* AN12 (pJY33); Gm^R	This study
JY813	*R. erythropolis* AN12(pJY35); Gm^R	This study
JY825	*R. erythropolis* AN12 *traA*::pJY37; Km^R	Yang et al. (2006)
JY855	JY825(pAL321); Gm^R Km^R	This study
Plasmids		
pREA400	Endogenous AN12 megaplasmid	Yang et al. (2006)
pREA400::EZTn	AN12 pREA400 megaplasmid with a transposon insertion (EZ:Tn <R6Kγ/Kan-2>) in its *pemK* locus	Yang et al. (2006)
pREA250	Endogenous AN12 megaplasmid	Yang et al. (2006)
pREA250::EZTn	AN12 pREA250 megaplasmid with a transposon insertion (EZ:Tn <R6Kγ/Kan-2>) whose precise location was not mapped	Yang et al. (2006)
pREA100	Endogenous AN12 megaplasmid	Yang et al. (2006)
pAN12	Endogenous AN12 cryptic plasmid	Kostichka et al. (2003)
pAL281	*E. coli*-rhodococci shuttle vector; Gm^R	Lessard et al. (2004)
pAL319	pAL281 derivative carrying the intact pAN12 plasmid ligated at the *Pst*I site	This study
pAL321	pAL319 derivative with the pAN12 *rep* region deleted from 3413 to 4634 bp	This study
pJY29	Derivative of pAL321 carrying a deletion from 758 to 1819 bp between *Xmn*I sites	This study
pJY30	Derivative of pAL321 carrying a deletion from 1253 to 3413 bp between *Cla*I sites	This study
pJY31	Derivative of pAL321 carrying a deletion from 2559 to 4435 bp between *Sal*I sites	This study
pJY33	Derivative of pAL281 constructed by ligating the 740 bp from *Xmn*I and *Sal*I sites of pAL321 to unique *Eco*RV and *Xho*I sites of pAL281, respectively	This study
pJY35	Derivative of pAL281 constructed by ligating annealed complementary primers JYP573 and JYP574 into *Eco*RI and *Xho*I	This study

(*continued on next page*)

Table 1 (*continued*)

Strain, plasmid, or primer name	Description or sequence	Source or reference
Primers		
JYP516	Forward primer to amplify AN12 chromosomal sequences; 5′ GCTACTCATGCCTGCATTCTC 3′	This study
JYP517	Reverse primer to amplify AN12 chromosomal sequences; 5′ AGCGAAAGCGAGTCCGAATAG 3′	This study
JYP558	Forward primer to amplify pAN12 sequences; 5′ CTGATCTGCTGGTCAGTGCGG 3′	This study
JYP559	Forward primer to amplify pAN12 sequences; 5′ CGACCTTGGTGCACTAGTCGG 3′	This study
JYP573	Forward primer to clone the pAN12 *clt*-like region; includes *Eco*RI linker; PAGE purified 5′ Phos/ AATTCCGTGGGCAGGTTTCGGCGTGAGGCGAGTTTTTCTCCT GCCTCATGTGCAACCTCCTCAAA 3′	This study
JYP574	Reverse primer to clone the pAN12 *clt*-like region; includes *Xho*I linker; PAGE purified 5′ Phos/ TCGATTTGAGAAGGTTGCACATGAGGCAGGAGAAAAACTCG CCTCACGCCGAAACCTGCCCACGG 3′	This study

SQ1, as well as strains of *Escherichia coli*, were grown in LB liquid media or on LB plates with 2% agar (Sambrook and Russell, 2001) supplemented with the following antibiotics purchased from Sigma-Aldrich (St. Louis, MO) as appropriate; gentamicin (Gm, 10 μg/ml), kanamycin (Km, 100 μg/ml), rifampicin (Rf, 20 μg/ml), and streptomycin (Sm, 150 μg/ml). *R. erythropolis* AN12 and SQ1 cells were cultivated at 30 °C, while *Escherichia coli* and *R. ruber* DDO319 were cultivated at 37 °C. All liquid culture flasks were shaken on an orbital shaker at 120 rpm, and small (<5 ml) volumes of liquid cultures were agitated using a roller drum. Frozen stocks of each strain were prepared by mixing equal volumes of saturated liquid cultures of bacteria and sterile 40% glycerol, then storing cells at −80 °C until use. All experiments were conducted with colonies that had been cultured for fewer than ten days from the initial frozen stock inoculum.

2.2. Electrocompetent AN12 cells

AN12 electrocompetent cells were prepared essentially as previously described (Kostichka et al., 2003), except cells were grown in NBYE/0.05% Tween-80 media in a 1L baffled flask with shaking until OD_{600} of about 0.5 was reached.

2.3. Rhodococcus conjugation and plasmid conjugation efficiency

Matings using 100 μl of overnight *Rhodococcus* cultures were carried out on solid LB agar surfaces as previously described (Lessard et al., 2004). The plasmid conjugation efficiencies were determined by plating appropriate serial dilutions of the mating cell resuspensions (consisting of 1:1 mixtures of donor cells and recipient cells) onto selective media, and plating control recipient cell dilutions to obtain recipient viable counts on non-selective media. Mating efficiencies were calculated as transconjugant CFU per recipient viable cell counts.

2.4. DNA manipulation and plasmid constructions

Plasmids used in the present study, as well as a brief description of the cloning strategies used for the construction of each, are summarized in Table 1. All DNA modifying enzymes and DNA size ladders were purchased from New England Biolabs (Beverly, MA) and used according to manufacturer's instructions. All PCR primers used in this study were purchased from Integrated DNA Technologies (Coralville, IA). *Rhodococcus* genomic DNA was prepared as previously described (Lessard et al., 2004). *Rhodococcus* plasmid DNA was isolated using the procedures previously described (Kostichka et al., 2003).

2.5. Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFG) electrophoresis was carried out as previously described (Yang et al., 2006).

2.6. Southern blot analysis

For standard hybridizations, 2–5 μg genomic DNA was digested with appropriate restriction enzymes, then separated on agarose gels. Gels were subjected to depurination with 0.25 M HCl for 40 min, denaturation with 0.5 N NaOH for 30 min, and neutralization with Tris–Cl for 30 min. DNA was then transferred to positively-charged nylon membranes (Roche Diagnostics Corp., Indianapolis, IN) using 20× SSC as the transfer buffer for 24 h. DIG-11 dUTP labeled probes were generated and hybridizations were carried out using reagents in the DIG-High Prime DNA Labeling and Detection Starter Kit per

manufacturer's instruction. Hybridizing species were detected using Kodak Biomax X-AR or Biomax Light scientific imaging films.

2.7. Rhodococcus erythropolis colony PCR

Approximately 100 μg of cells were collected using a pipet tip, then resuspended in a 50 μl PCR using all of the reagents and protocols from the HotStarTaq Polymerase Kit (Qiagen Sciences, Valencia, CA). DNA was amplified in a PTC-200 Cycler (Bio-Rad, Waltham, MA) using cycling parameters of an initial heating step of 15 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C, ending with an extension cycle of 10 min at 72 °C. Annealing temperatures and extension times were adjusted to optimize performance of each primer pair.

3. Results and discussion

3.1. Discovery of pAN12 transmission, and definition of a cis-acting region required for transmission

It was shown previously that *Rhodococcus erythropolis* AN12 harbors a 6.3 kb cryptic plasmid called pAN12 (Kostichka et al., 2003). Recently, we reported on two novel conjugative *R. erythropolis* AN12 megaplasmids, pREA400 and pREA250 (Yang et al., 2006) that can be maintained in the recipient strain, *R. erythropolis* SQ1. In these experiments, SQ1 derived transconjugants could be distinguished from AN12 donor cells since SQ1 is naturally resistant to rifampicin (Rf) and streptomycin (Sm) antibiotics (Lessard et al., 2004). In the course of characterizing pREA400 conjugation by pulsed-field gel (PFG) electrophoresis, we noticed the co-transfer of a smaller replicon, which we suspected was pAN12 (Fig. 1A). Two additional lines of evidence confirmed that indeed, pAN12 can be mobilized. First, the plasmid can be isolated from wild type AN12 and from SQ1(pREA400::EZTn) transconjugants, but not from the wild type SQ1 strain. Second, the extracted plasmids from both wild type AN12 and SQ1(pREA400::EZTn) yielded the same restriction enzyme digests patterns, the band sizes of which could be predicted using the published pAN12 sequence (Genbank Accession No. AY178757).

A series of pAN12-derived plasmids were then constructed to further define a *cis*-acting region required for its mobilization. To monitor its mobilization, pAN12 was fused with a plasmid (pAL281) encoding gentamicin resistance (Gm^R) and a broad host-range NG2 *rep* region (Fig. 1B). pAL281 is capable of replication in both *E. coli* and *Rhodococcus* SQ1 (Lessard et al., 2004) as well as AN12 (this work). To verify that pAL281 is not mobilizable by itself, AN12(pAL281), also known as JY660, was mated to SQ1, and transconjugants selected using triple (Rf Sm Gm) drug selection. We failed to recover any transconjugants (Table 2), and interpret this data to mean that pAL281 cannot be mobilized detectably from AN12 to SQ1. Thus, pAL281 should be useful to analyze what sequences from pAN12 are involved in conjugative transfer.

The transfer of the resulting pAN12-pAL281 fusion plasmid, pAL319, could be monitored based on the gain of Gm^R in recipient cells. Because pAL319 might be unstable due to the presence of two functional rhodococcal plasmid replication systems, a second plasmid, pAL321, was created that deletes the pAN12 *rep* ORF. Representative plasmid maps of pAN12, pAL281, pAL319, and pAL321 are shown in Fig. 1B. Intact pAL319 plasmid can be established and recovered from some AN12 transformants. However, the pAL319 fusion plasmid had been rearranged in 2 out of 12 transformants, and the endogenous pAN12 plasmid had been lost in 8 out of 12 transformants (data not shown), suggesting some level of incompatibility between pAL319 and pAN12. In contrast, pAL321 is stably maintained by AN12 along with the endogenous pAN12.

AN12 stably carrying either pAL319 or pAL321 were mated to SQ1. The appearance of roughly equal numbers of triply (Rf Sm Gm) resistant colonies (data not shown) strongly suggested that AN12 can mobilize both pAL319 and pAL321 at comparable frequencies, and that the pAN12 *rep* region appears to be dispensable in *cis* for plasmid transfer. The stable maintenance of pAL321 in SQ1 was confirmed with a Southern blot using the entire pAL319 plasmid as a probe template and total genomic DNA samples from AN12, donor JY700, recipient SQ1, and transconjugant JY709 (Fig. 1C). The endogenous pAN12 was linearized with either the *Eco*RI and *Bgl*II double enzyme digest, or the *Pst*I single enzyme digest, and was also detected using the pAL319 probe. Hybridizing bands of the expected sizes from each sample were observed, strongly suggesting that the pAL321 plasmid is indeed mobilizable, and that the SQ1 derived transconjugant JY709 stably maintains pAL321. Interestingly, the endogenous pAN12 plasmid co-mobilized with pAL321 in all cases examined. Furthermore, the

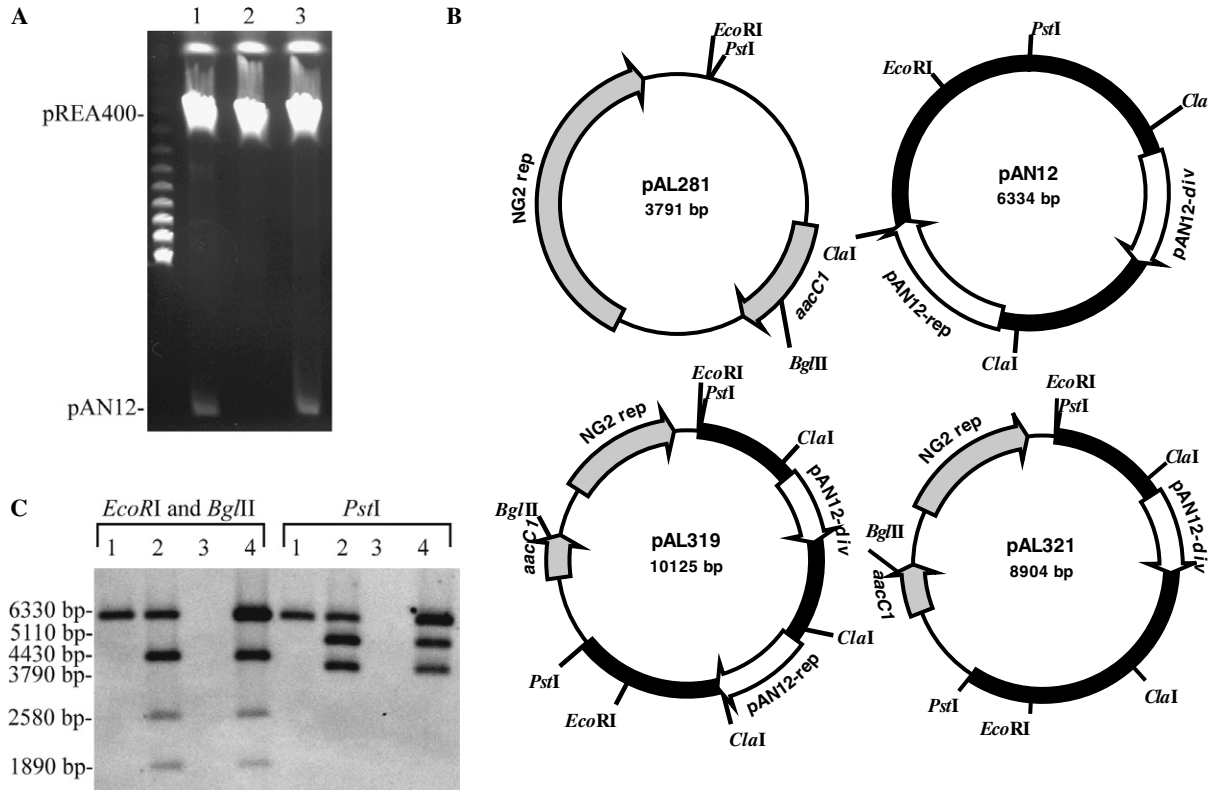

Fig. 1. The *R. erythropolis* AN12 plasmid, pAN12, and its derivatives, pAL319 and pAL321, can be transmitted to and maintained by SQ1. (A) PFG analysis of replicons of the donor strain *R. erythropolis* AN12PL(pREA400::EZTn) (1), the recipient strain SQ1 (2), and the SQ1 derived transconjugant strain JY524 (3). The wild-type SQ1 strain harbors its own megaplasmid of ca. 400 kb, however this megaplasmid is not related to the AN12 pREA400 megaplasmid (data not shown). Replicons (pREA400 and pAN12) gained by *R. erythropolis* SQ1 following conjugation are labeled. Although we only selected for transfer of megaplasmid pREA400::EZTn by virtue of the Km^R marker it carries, pAN12 was transferred as well. (B) Maps of relevant plasmid constructs. The endogenous pAN12 plasmid was fused to pAL281 at an unique *Pst*I site, resulting in the plasmid pAL319. The pAL321 plasmid was constructed by digesting pAL319 with *Cla*I to remove the pAN12 *rep* ORF then re-ligating the major product. *Eco*RI and *Bgl*II sites important for subsequent analysis are also indicated. (C) Southern blot analysis of pAL321 mobilization and maintenance. Genomic DNA prepared from AN12 (1), pAL321 donor strain JY700 (2), SQ1 (3), and transconjugant strain JY709 (4) were digested with the indicated enzymes and transferred onto a positively charged nylon membrane. Treatment of pAL321 with *Eco*RI and *Bgl*II should produce 4427, 2575, and 1890 bp products. Treatment of pAL321 with *Pst*I should produce 5109 and 3787 bp products. Linearized endogenous pAN12 is expected to migrate at 6334 bp. A digoxygenin (DIG) labeled Southern probe was prepared using the entire pAL319 plasmid as template. Approximate sizes of the annealing fragments are indicated on the left.

Table 2
Summary of conjugation efficiencies of pAN12 derived plasmids to SQ1

Strain (plasmid)	No. trials	Weighted average mobilization efficiency[a]	Standard deviation
JY660 (pAL281)	4	0[b]	0[b]
JY700 (pAL321)	7	1.5×10^{-5}	4.0×10^{-7}
JY720 (pJY29)	6	2.0×10^{-6}	9.8×10^{-7}
JY735 (pJY30)	6	1.4×10^{-9}	3.5×10^{-10}
JY737 (pJY31)	5	1.4×10^{-6}	3.3×10^{-7}
JY750 (pJY33)	4	4.6×10^{-7}	2.3×10^{-8}
JY813 (pJY35)	4	2.4×10^{-7}	8.5×10^{-8}

[a] Conjugation efficiencies were calculated by dividing the number of transconjugant CFUs observed on selective plates by the number of recipient CFUs observed on permissive plates.

[b] The designation of "0" is defined as less than 1 event per 2.5×10^9 recipient CFU, the maximum number of recipient CFU in the average experiment.

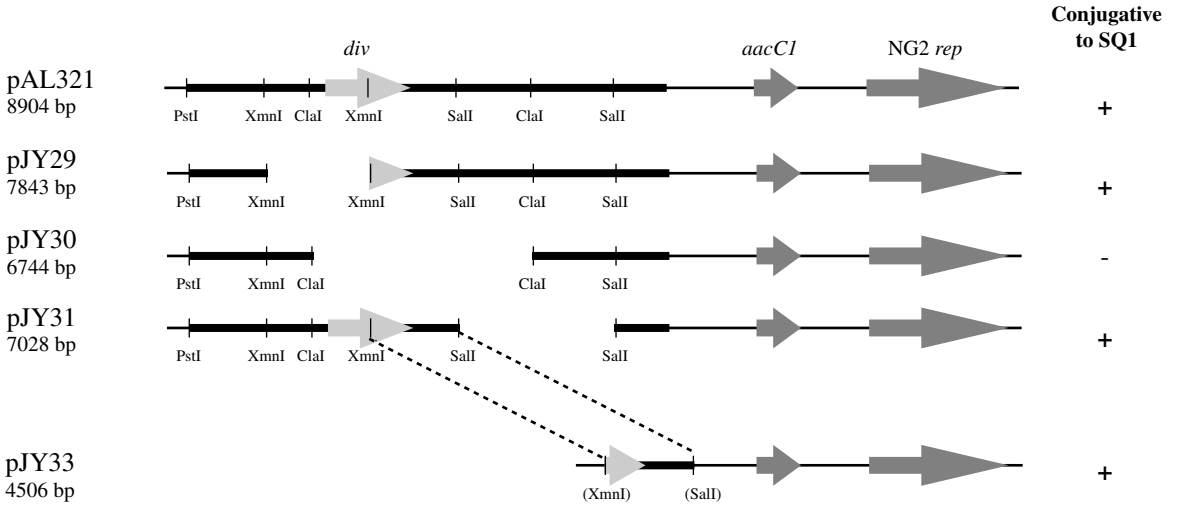

Fig. 2. Diagram of the pAN12 constructs, and summary of transmissibility to SQ1. pAL281 plasmid sequences are represented as thin horizontal lines, and pAN12 sequences are represented as thick horizontal lines. Relevant restriction enzyme recognition sites are noted with thin black vertical lines. pAL281 ORFs, *aacC1* (encoding Gm^R) and *rep* (encoding plasmid replication function) are indicated with dark gray arrows. The pAN12 ORF *div* is indicated with a light gray arrow. pJY29 through pJY31 are direct deletions of pAL321, and sequences deleted are indicated with gaps. pJY33 was constructed by fusing the indicated *Xmn*I–*Sal*I fragment to pAL281 digested with *Eco*RV and *Xho*I. Whether a plasmid can be mobilized (+) or not (−) to SQ1 is indicated on the right.

pAL321 mobilization frequency of 1.5×10^{-5} events per recipient cell (Table 2) is comparable to frequencies observed for *R. erythropolis* AN12 megaplasmids pREA400 and pREA250 (ca. 5×10^{-4} per recipient cell).

Using AN12(pAL321) as a positive control, and AN12(pAL281) as a negative control, AN12 carrying a series of pAL321 deletion plasmids were assayed for mobilization to SQ1 (Fig. 2). In each of these cases, plasmids were re-isolated from transconjugants to verify the transfer to and their subsequent maintenance by SQ1 without rearrangements or recombinations. The mobilization frequency for each pAL321 derivative plasmid was also determined (Table 2). The mobilization frequency for pJY30 (1.4×10^{-9} per recipient cell) is much lower than pAL321, strongly suggesting that sequences deleted between these *Cla*I sites are important for pAN12 mobility. Unlike pJY30, other deletion plasmids (pJY29 and pJY31) transferred to SQ1 with frequencies similar to pAL321. Taken together, data suggest that the 746 bp region between *Xmn*I and *Sal*I sites of pAN12 (from nt. 4,034 to 4,770, Genbank Accession No. AY178757) is necessary in *cis* for pAN12 mobilization. Indeed, when this sequence was fused with the pAL281 backbone, it is sufficient to confer mobility to the resulting plasmid, pJY33, confirming this hypothesis (Table 2).

3.2. Identification of a small cis-acting transfer element on pAN12s

pAN12 belongs to the pIJ101/pJV1 family of rolling-circle plasmids (Kostichka et al., 2003). Protein sequence analysis suggests that not only are the replication proteins and origins of replications similar, the Div and Tra proteins of pAN12 and pIJ101 share core motifs with the FtsK/SpoIIIE family of DNA segregation proteins (Errington et al., 2001). Intriguingly, data suggest that during sporulation, *Bacillus* subtilis SpoIIIE acts upon a double-stranded (ds) DNA substrate (Adams and Wake, 1980; Lewis et al., 1994; Sargent, 1980). Relatedly, a dsDNA transfer mechanism has also been proposed for *Streptomyces* plasmids pIJ101 (Ducote and Pettis, 2006) and pSAM2 (Possoz et al., 2001) which encode SpoIIIE-like proteins. As pAN12 also encodes a SpoIIIE-like protein, it is possible that pAN12 utilizes its Div protein for mobility, and transfers as dsDNA during *Rhodococcus* conjugation. The mobilization of the pAL321 deletion plasmid, pJY29, in which the Div protein function was removed (Table 2), might argue against this proposal. However, it is important to note the endogenous pAN12 plasmid is stably maintained with this plasmid in the donor cell, and its intact Div protein may act in *trans* for the mobilization of pJY29. As we have not yet found conditions in which pAN12 could be cured or

separated from pAL321, the potential role of pAN12 encoded Div protein (or Div-like factors encoded elsewhere in the genome) that act in *trans* for plasmid transfer remains to be addressed.

A small 61 bp element (Fig. 3A) within the *Xmn*I-*Sal*I region of pAN12 was tested for a *cis*-acting role in plasmid mobility to further refine contributing sequences. This small element was introduced in the form of oligonucleotides (JYP573 and JYP574), which were annealed and ligated into pAL281. The resulting plasmid, pJY35, was used to transform AN12. Gentamicin resistant transformants were analyzed for the presence of the intact plasmid by re-isolating the plasmid and sequencing. One confirmed AN12(pJY35) strain, JY813, was mated to SQ1, as were the AN12(pAL321) strain, JY700, and the AN12(pAL281) strain, JY660, as positive and negative controls, respectively (Fig. 3B). Importantly, the addition of this pAN12 element confers mobility to the otherwise non-mobile pAL281. We note that pJY35 is not transferred with the same efficiency as pAL321 (1×10^{-7} versus 1×10^{-5}, respectively). This, combined with the fact that the *Cla*I deletion plasmid pJY30 retains a low level of transferability (1.4×10^{-9}), suggests that sequence outside of the pAN12 *Cla*I sites contributes to its transfer. Indeed the *Enterococcus* plasmid, pAD1, appears to harbor two origins of transfer (Francia et al., 2001). Nevertheless, this experiment demonstrates that the 61 bp pAN12 element supplied the necessary *cis*-acting function to direct the transfer of the otherwise non-mobile pAL281 from AN12 to SQ1, an assay which has been used to define other plasmids' transfer origins.

Recently, a minimal 54 bp sequence (*clt*) in pIJ101 was found to be essential for its conjugation (Ducote et al., 2000). We looked within the 61 bp pAN12 element and asked whether it is similar to the pIJ101 *clt*. Indeed, the pAN12 element shares weak nucleotide identity (50%) to the pJI101 *clt* (not shown). Other related *Streptomyces* plasmids, such as pJV1 and pSN22, exhibit little identity with pIJ101 *clt* (Kataoka et al., 1994; Servin-Gonzalez, 1996). The work with *Streptomyces* pIJ101 has shown that its *clt* region consists of 3 direct repeats of G(A/C)AAC, as well as one imperfect inverted repeat (IR) that are important to direct conjugation (Ducote et al., 2000). Although we were unable to detect the same direct repeats, we were able to locate an imperfect IR in this 61 bp pAN12 element (Fig. 3A). The significance of the imperfect IR in pAN12 remains to be tested. Interestingly, the localization of this 61bp *cis*-acting transfer element on pAN12 downstream of the *div* locus is consistent with the localizations of *clt* regions downstream of genes encoding Ftsk-like products of conjugative *Streptomycete* plasmids pJV1 (Franco et al., 2003) and pSVH1(Reuther et al., 2006). A very similar sequence (96% sequence identity) is also present in the closely related *R. erythropolis* plasmid, pRE8424, the mobility of which has been not tested (Nakashima and Tamura, 2004). These data are

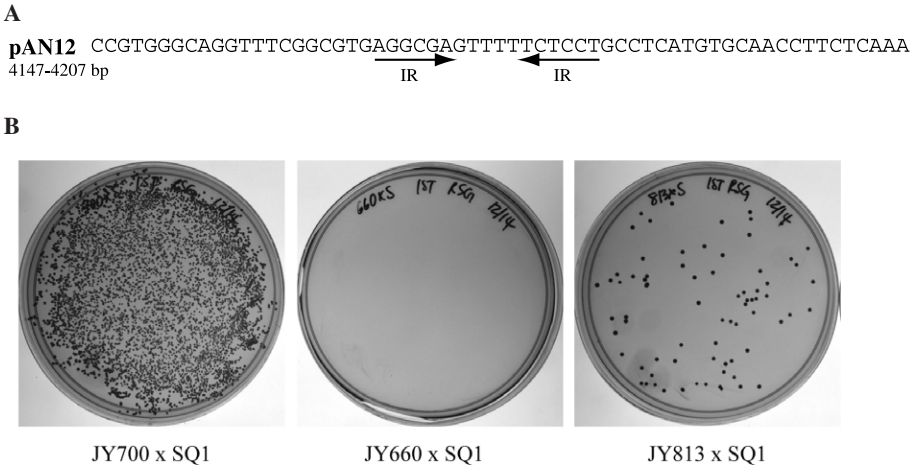

Fig. 3. Identification of the minimal pAN12 *cis*-acting transfer element and demonstration of its function in *Rhodococcus* plasmid conjugation. (A) The 61 bp element from nt. 4147 to 4207 of pAN12 (Genbank Accession No. AY178757) tested for plasmid mobilization. Imperfect inverted repeats (IR) are indicated with arrows. (B) pAL281 with the pAN12 *cis*-acting transfer element is transmissible. A 1:4 dilution of each mating cell suspension (donor:recipient) were plated onto LB agar supplemented with Rf, Sm, and Km. Plates were incubated for 96 h at 30 °C prior to being photographed.

consistent with a model in which the function of these actinomycetes plasmids' *cis*-acting transfer elements is conserved.

3.3. Co-mobilization of AN12 megaplasmids and pAN12 during conjugation

Co-mobilization of pAN12 with pREA400:Tn prompted us to begin this investigation of the basis of pAN12 conjugation. Interestingly, the co-mobilization of pAN12 appeared to be less frequent when AN12-5A6 (which carries the megaplasmid pREA250::EZTn) was mated to SQ1. We sought to determine an approximate frequency of pAN12 co-mobilization to explore its potential dependence on other plasmids for mobilization. Because PFG electrophoresis is a time-consuming process, a *Rhodococcus erythropolis* colony PCR assay was developed to examine larger sample numbers of transconjugants.

Colony PCR primers (JYP558 and JYP559) generated a PCR product when AN12 cells were used in the reaction, and did not generate a product when wild-type SQ1 cells were used. In addition, positive control primers (JYP516 and JYP517) which amplify chromosomal sequences common to both AN12 and SQ1, gave expected products in both reactions (data not shown). This demonstrated that colony PCR is a viable technique to assay for presence of specific elements in both of these *R. erythropolis* strains. A total of 72 SQ1(pREA400::EZTn) and 75 SQ1(pREA250::EZTn) transconjugants were then tested for the presence of pAN12 using the primers JYP558 and JYP559. We found that pAN12 was co-mobilized in 34% of SQ1 (pREA400::EZTn) transconjugants examined (three independent mating experiments, one standard deviation of 19%), and only 7% of SQ1 (pREA250::EZTn) transconjugants examined (three independent mating experiments, one standard deviation of 2%), suggesting that pAN12 co-transfer is common and that its frequency may depend on which megaplasmid is transferred.

3.4. Involvement of the pREA400 megaplasmid in pAL321 transfer

One possible explanation for the more frequent co-transfer of pAN12 with pREA400 than pREA250 could be that the larger AN12 megaplasmid is somehow involved in pAN12 transfer. This involvement was not expected for pAN12, as it is quite well documented that pIJ101 encodes all necessary *cis*- and *trans*-acting factors required for its conjugation, in the forms of the *clt* and Tra proteins, respectively. It was not possible to directly assay AN12 strains for the *trans*-acting requirements residing on megaplasmid(s) as no method currently exist to cure specific megaplasmids in *Rhodococcus*. Instead, SQ1 strains were generated that stably maintained pAL321 along with either the pREA400::EZTn or the pREA250::EZTn megaplasmid. These SQ1 derivative strains are useful as donors as they made it possible to test individual AN12 megaplasmids separately.

Another issue to overcome was that AN12 could not be used as the recipient in the planned experiment when SQ1 strains are used as donors, as no selection remains to distinguish AN12-derived transconjugants from SQ1 donors. For this reason, the *Rhodococcus ruber* strain, DDO319, was used as the ultimate recipient since its growth at 37 °C could be exploited as a selection criterion. Neither SQ1 nor AN12 grow above 32 °C. Maintenance of pAL321 by DDO319 was not a concern since the NG2 replicon (supplied in the pAL281 backbone of pAL321) functions at 37 °C (Lessard et al., 2004). To eliminate the possibility that pAL281 backbone could confer non-pAN12 related transfer functions, an experiment was done in which AN12(pAL281) was mated to DDO319, and transfer of the gentamicin resistance marker used to monitor pAL281 transfer. No transconjugants were ever detected in these experiments, indicating that the pAL281 backbone would not be involved in the transfer of the pAL321 fusion plasmid between AN12 and DDO319.

The following experiment, depicted in Fig. 4, indicates that pREA400 is indeed involved in the transfer of pAN12 and its derivative, pAL321. Either AN12PL (pREA400::EZTn) or AN12(pREA250::EZTn) donors were mated to SQ1(pAL321) recipient cells. Twelve separate transconjugants that were quadruply (Rf, Sm, Gm, and Km) resistant from each of these matings were then isolated and used as donors in individual matings with DDO319. Twelve SQ1(pAL321) isolates that did not gain either of the tagged megaplasmids served as negative control. AN12(pAL321) strain, JY700, was always used as a positive control donor in these experiments. Only the transfers of pAL321, and not the tagged megaplasmids, were selected for in these subsequent matings to DDO319. We found that eleven out of twelve SQ1(pREA400:: EZTn + pAL321) donors gave rise

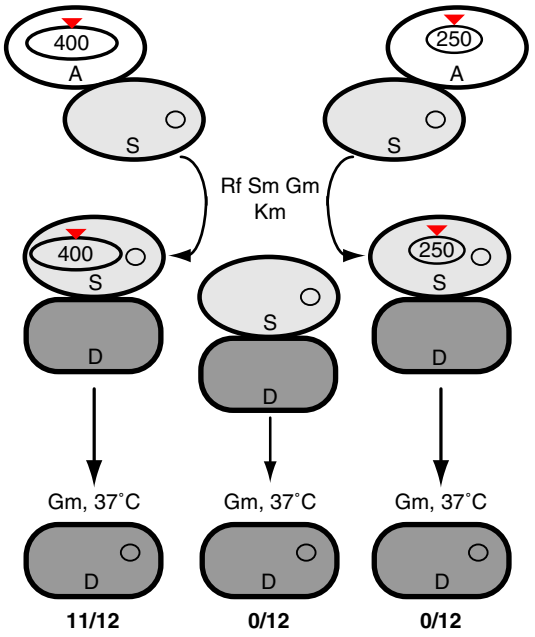

Fig. 4. Involvement of the *R. erythropolis* AN12 megaplasmid, pREA400, in pAL321 conjugation. *R. erythropolis* strains are represented as large ovals whereas the *Rhodococcus ruber* strains, DDO319, are represented as dark rounded rectangles (D). AN12 derivatives (A, white oval) have been distinguished from SQ1 derivatives (S, light gray oval) by the label and shading. Transposon-tagged megaplasmids are indicated as smaller ovals with inverted triangles. The numbers inside the ovals distinguish between pREA400::EZTn (400) and pREA250::EZTn (250). The pAN12-derived plasmids, pAL321 (Gm^R), have been indicated as small, unlabelled circles. Selections (antibiotics and/or temperature) used at each mating step have been indicated. The numbers of successful matings using individual SQ1 donors of the indicated plasmid composition that produced viable *R. ruber* DDO319(pAL321) transconjugants after selections, versus the total number of such matings, are expressed as fractions, respectively.

to DDO319 (pAL321) transconjugants. In contrast, none of the twelve SQ1(pREA250:: EZTn+ pAL321) donors and none of the twelve SQ1(pAL321) negative control donors tested gave rise to DDO319(pAL321) transconjugants. These results implicate pREA400 encoded factor(s) in pAN12 mobility. Interestingly, this factor(s) is not the *traA* encoded relaxase/helicase that we found to be indispensable for pREA400 conjugation (Yang et al., 2006), as the AN12 mutant *traA* strain efficiently transferred pAL321 to both *R. erythropolis* SQ1 and *R. ruber* DDO319 (data not shown). Future efforts directed at sequencing pREA400 should offer candidate genes to test for involvement in directing pAN12 mobilization.

There may be two different modes of plasmid conjugation in *Rhodococcus erythropolis* AN12. Protein sequence analysis of the megaplasmid-encoded TraA relaxase (Yang et al., 2006) indicates that it is most similar to the relaxases required for the "classical" single-stranded DNA (ssDNA) plasmid transfer, whereas the pAN12 61 bp *cis*-acting determinant and encoded Div protein are most similar to features of *Streptomyces* plasmids that undergo dsDNA plasmid transfer (Possoz et al., 2001). Interestingly, this is not the first actinomycete identified to date with the coding capacity for both the classical single-stranded and *Streptomyces*-like plasmid transfer systems. Plasmids have been isolated from *Bifidobacteria* with either ORFs encoding a putative relaxase (Park et al., 1999) or a putative FtsK/SpoIIIE septal DNA translocator protein (O'Riordan and Fitzgerald, 1999). Future functional studies of both AN12 *div* and *traA* gene products should shed light on mechanisms underlying actinomycete plasmid conjugation.

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