An efficient gene transfer system for the pimaricin producer *Streptomyces natalensis*

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Keywords

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Abstract

Streptomyces natalensis produces the antifungal polyene macrolide pimaricin. Genetic manipulation of its biosynthetic genes has been hampered by the lack of efficient gene transfer systems. We have developed a gene transfer system based on intergeneric conjugation from *Escherichia coli*. Using this approach, we managed to attain transformation efficiencies of 1×10^{-4} exconjugants per recipient when using self-replicating vectors such as pHZ1358. The use of integrative vectors such as pSET152 or pSOK804 resulted in significantly lower efficiencies. Site-specific integration or the use of self-replicating plasmids did not affect pimaricin production or the essential functions of *S. natalensis*. Use of DNA methylation proficient *E. coli* donor strains resulted in no transformants, indicating the presence of methyl-specific restriction systems in *S. natalensis*. This methodology will enable easier manipulation of the genes responsible for pimaricin biosynthesis, and could prove valuable for the generation of new designer polyene macrolides with better antifungal activity and pharmacological properties. As an example of the validity of the method, we describe the introduction of Supercos-1-derived cosmid vectors into *S. natalensis* in order to promote gene replacements by double crossover recombination.

Introduction

Pimaricin is a 26-member tetraene macrolide antifungal antibiotic produced by *Streptomyces natalensis*, which is widely used for the treatment of fungal keratitis, and also in the food industry prevent mold contamination of cheese and other nonsterile foods (i.e. cured meat, sausages, ham, etc.). Being a polyene, its antifungal activity lies in its interaction with membrane sterols, thus causing the alteration of membrane structure and leading to the leakage of cellular materials (Aparicio *et al.*, 2004). As other macrocyclic polyketides, pimaricin is synthesized by the action of so-called type I modular polyketide synthases (Aparicio *et al.*, 2003). We have previously sequenced the pimaricin biosynthetic gene cluster completely and demonstrated its identity by gene disruption experiments (Aparicio *et al.*, 1999, 2000). The gene cluster encodes 13 polyketide synthase modules within five multifunctional enzymes, and 12 additional proteins that presumably govern modification of the polyketide skeleton, export and regulation of gene expression (Aparicio *et al.*, 2000; Mendes *et al.*, 2001, 2005; Antón *et al.*, 2004).

Although some pimaricin derivatives have been obtained by genetic engineering (Mendes *et al.*, 2001), the lack of an efficient gene transfer system for the pimaricin-producing *S. natalensis* strain has slowed down the rational modification of the pimaricin biosynthetic pathway in order to produce less toxic and more soluble and active pimaricins. So far, only phage-mediated gene transfer has allowed the introduction of recombinant DNA into this strain; other commonly used methodologies for DNA delivery into *Streptomyces* strains such as protoplast transformation, conjugation or electroporation (Kieser *et al.*, 2000) have been completely unsuccessful. The phage technology, however, is inefficient, time consuming, and has limitations on the size of the DNA inserts that the phage can accommodate. Intergeneric transfer of plasmid DNA from *Escherichia coli* to *Streptomyces* spp. was first reported by Mazodier *et al.* (1989); since then effective conjugation protocols have been established for a

number of *Streptomyces* strains, in which the plasmid can self-replicate or integrate into the chromosome by site-specific or homologous recombination (Smokvina *et al.*, 1990; Bierman *et al.*, 1992; Matsushima *et al.*, 1994; Kieser *et al.*, 2000). Here we report the development of an efficient gene transfer system based on conjugation from *E. coli* to *S. natalensis* ATCC 27448. This procedure will enable easier genetic manipulation of this industrially important strain in order to generate novel structural analogues.

Materials and methods

Bacterial strains, cloning vectors and cultivation

Escherichia coli strain DH5α was used as the general cloning host. Two strains of *E. coli* were used as donors in intergeneric conjugations: XL1-Blue [pUZ8002] and ET12567 [pUZ8002]. ET12567 is a methylation defective strain (*dam*-13::Tn9, *dcm*-6, *hsdM*) (MacNeil *et al.*, 1992). Plasmid pUZ8002, a nontransmissible *oriT* mobilizing plasmid (Paget *et al.*, 1999), was transferred into both strains by transformation. *Escherichia coli* BW25113 was used as the host for Red recombination (Datsenko & Wanner, 2000) and to propagate plasmid pIJ790 (Gust *et al.*, 2003). *Streptomyces natalensis* ATCC 27448, the wild-type pimaricin producer, was used as recipient. The *oriT*-containing plasmids used in this study are listed in Table 1.

Sporulation of *S. natalensis* ATCC 27448 was achieved in TBO medium at 30 °C (Aparicio *et al.*, 1999). For pimaricin production, the strain was grown in YEME medium (Kieser *et al.*, 2000) without sucrose. Standard conditions for culture of *S. natalensis* were as described (Antón *et al.*, 2004). *Candida utilis* (syn. *Pichia jadinii*) CECT 1061 was used for pimaricin bioassay experiments. *Escherichia coli* strains were grown in Luria–Bertani (LB) broth and agar supplemented with antibiotics when required. Apramycin (10 mg mL^{-1}), chloramphenicol (50 mg mL^{-1}), ampicillin (100 mg mL^{-1}) and kanamycin (100 mg mL^{-1}) were added to growth media as required.

Table 1. oriT vectors used in this study

Plasmid	Relevant characteristics	Reference
pHZ1358	Tsr^R, Ap^R, ColE1/pIJ101 replicons	Sun *et al.* (2002)
pSET152	Am^R, pUC18 replicon, ΦC31 *attP*	Bierman *et al.* (1992)
pSOK201	Neo^R, Am^R, ColE1/pSG5 replicons	Zotchev *et al.* (2000)
pSOK804	Am^R, ColE1 replicon, ΨWB *attP*	Sekurova *et al.* (2004)

Am^R, apramycin resistant; Ap^R, ampicillin resistant; Neo^R, neomycin resistant; Tsr^R, thiostrepton resistant.

Conjugation procedure

Intergeneric conjugation between *E. coli* and *S. natalensis* was performed as described (Flett *et al.*, 1997) with some modifications. A culture of the donor *E. coli* [pUZ8002] was grown in LB to an $OD_{600_{nm}}$ of 0.5–0.6 at 37 °C, washed twice, and resuspended in 0.1 volumes of LB. Aliquots of an *S. natalensis* spore suspension stored at −20 °C in the presence of 0.025% triton X-100 (volume in volume, v/v) and 30% glycerol (v/v) were used as recipients. Viable spores (from 10^4 to 10^9 per conjugation) were washed with 2 × TY (Sambrook & Russell, 2001), resuspended in the same medium (500 µL) and incubated at various temperatures for different time periods to activate germination (see Results). *Escherichia coli* (500 µL) was added to the treated spores, mixed thoroughly, and the mixture was pelleted by centrifugation. The pellet was resuspended in the residual liquid and spread on dried MS (Hobbs *et al.*, 1989) or TBO (Aparicio *et al.*, 1999) agar plates supplemented with 10 mM $MgCl_2$. Plates were incubated at 30 °C for 16–18 h, and then overlaid with 1 mL of water containing 500 µg nalidixic acid and the antibiotic used to select for the *oriT*-containing plasmid. Plates were incubated further for 7–10 days, until exconjugants appeared. Putative exconjugants were screened by Southern hybridization.

Viable cells of the donor culture were determined by spreading samples on LB agar supplemented with the appropriate antibiotics. The number of viable spores was determined on TBO plates.

Other genetic procedures

Standard genetic techniques with *E. coli* and *in vitro* DNA manipulations were as described by Sambrook and Russell (2001). Isolation of *Streptomyces* total DNA was performed as described previously (Kieser *et al.*, 2000). Southern hybridization was carried out with probes labeled with digoxigenin using the digoxigenin DNA labeling kit (Roche Biochemicals, Basel, Switzerland). The gene disruption cassette *aac(3)IV* (Am^R) and the *oriT* were

jointly amplified from pIJ773 (Gust *et al.*, 2003) using the primers (a) 5′-TGGGGGAGTGTCCGACCTGCCGCATACGCTGGAGTCATGattccggggatccgtcgacc-3′ and (b) 5′-TGCTGTGGCCGGCCACCGGCCCTGGCCGCGGCCGGCCTAtgtaggctggagctgcttc-3′ [the primers have 39 nucleotides of identity with *S. natalensis* DNA (uppercase), and 20 or 19 nucleotides of identity with pIJ773 DNA (lowercase)]. Priming sites are separated by 1489 bp in *S. natalensis* chromosome. The primers used to analyze the knockout were (F) 5′-CAGCGACGCACTGTCGTACAT-3′ and (R) 5′-CTACGGCTCGAACTTGTAGCC-3′. Priming sites are separated by 642 bp in the chromosome. Target *S. natalensis* DNA sequence is deposited in the GenBank database under the accession number AM176576.

Assay of pimaricin production

To assay pimaricin in culture broths, 0.5 mL of culture was extracted with 4 mL of butanol, and the organic phase was diluted in water-saturated butanol to bring the absorbance at 319 nm in the range of 0.1–0.4 U. Control solutions of pure pimaricin (DSM, Delft, the Netherlands) were used as control. To confirm the identity of pimaricin, a UV–visible absorption spectrum (absorption peaks at 319, 304, 291 and 281 nm) was routinely determined in a Hitachi U-2001 spectrophotometer (Tokyo, Japan). The fungicidal activity of pimaricin was tested by bioassay using *C. utilis* CECT 1061 as test organism. Quantitative determination of pimaricin was performed with a Waters 600 HPLC (Milford, MA) with a diode array ultraviolet detector set at 304 nm, fitted with a μ-Bondapack RP-C18 column (10 μm; 3.9 × 300 mm). Elution was performed with a gradient (1 mL min^{-1}) of 100% methanol (methanol concentration: 50% 0–3 min, up to 90% 3–12 min, 90% 12–20 min, down to 50% 20–25 min, 50% 25–30 min). Retention time for pimaricin was 14.5 min.

Results and discussion

Streptomyces natalensis spores are more sensitive to heat shock than those of other *Streptomyces*

One of the critical parameters affecting intergeneric conjugation is the temperature used for the heat treatment of the spores in order to induce germination. The heat shock usually applied to *Streptomyces* species is at 50 °C during 10 min (Flett *et al.*, 1997; Voeykova *et al.*, 1998; Kieser *et al.*, 2000; Paranthaman & Dharmalingam, 2003). This heat treatment is used because spore germination is thought to be necessary for an efficient conjugation (Mazodier *et al.*, 1989), and because the heat may reduce temporarily the activity of restriction enzymes that could attack the incoming DNA (Bailey & Winstanley, 1986; Engel, 1987). To determine the appropriate temperature for spore germination, the heat tolerance of *Streptomyces natalensis* spores was assessed. As shown in Fig. 1, a dramatic loss in spore viability was observed at 50 °C. While spores treated at or above 50 °C lost 90% viability after a 10 min incubation, more than 90% remained viable at 45 °C.

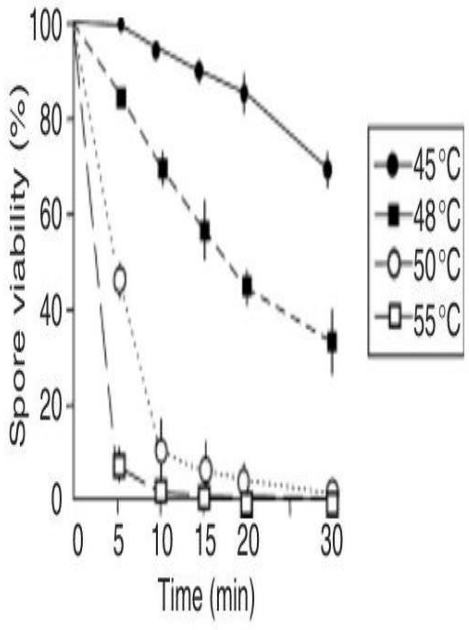

Fig. 1. Effect of temperature on spore viability of *Streptomyces natalensis*. Spores were held at various temperatures (45–55 °C) for the time periods indicated. Spores were then plated out onto TBO medium and incubated for 7 days at 30 °C before counting colonies. Values are the mean of three independent experiments.

Spore sensitivity to heat was reflected in pHZ1358 transfer efficiencies, as monitored by enumeration of exconjugants, when we used *Escherichia coli* ET12567 [pUZ8002] as donor. Transconjugation frequency after heat shock of the spores (5×10^8) at 45, 48 and 50 °C during 10 min was dependent on the temperature of the heat shock. The highest number of exconjugants (3.3×10^{-5} per recipient) was obtained when using a 45 °C heat shock, reaching levels 10-fold higher than those obtained at 50 °C. The true nature of exconjugants was verified by Southern blot hybridization (not shown). No exconjugants were obtained when spores were not subjected to the heat shock treatment. Similarly, all our attempts to achieve intergeneric conjugal transfer between *E. coli* and *S. natalensis* mycelium were unsuccessful. We hereafter used a 45 °C heat shock for 10 min to induce germination of *S. natalensis* spores before conjugation.

Other factors affecting conjugal transfer

In order to establish an optimal *E. coli* donor cell number for a given number of spores, we set up 20 series of matings with two different numbers of donor cells, which differed by a factor of 10. Viable spore concentrations ranged from 2×10^6 to 1.5×10^9, and the number of donor cells ranged from 5×10^4 to 1×10^7. As a result, the number of exconjugant colonies was always found to increase with the number of donor cells used in each series. These values ranged from two- to sixfold. In total, the experiments covered a range of recipient-to-donor ratios from 0.36 to 1800 (Fig. 2). In contrast to what occurs in *S. coelicolor* and *S. nodosus*, where the number of donor cells per recipient does not affect the efficiency of DNA transfer (Flett *et al.*, 1997; Nikodinovic *et al.*, 2003), in *S. natalensis* there is an optimal donor/recipient ratio for conjugal transfer when there is an excess of donor cells (Fig. 2). Similar results have been observed in the intergeneric conjugal transfer of plasmid DNA from *E. coli* to the actinomycete *Kitasatospora setae*, where an excess of donor cells is required to achieve the highest transconjugation frequency (Choi *et al.*, 2004).

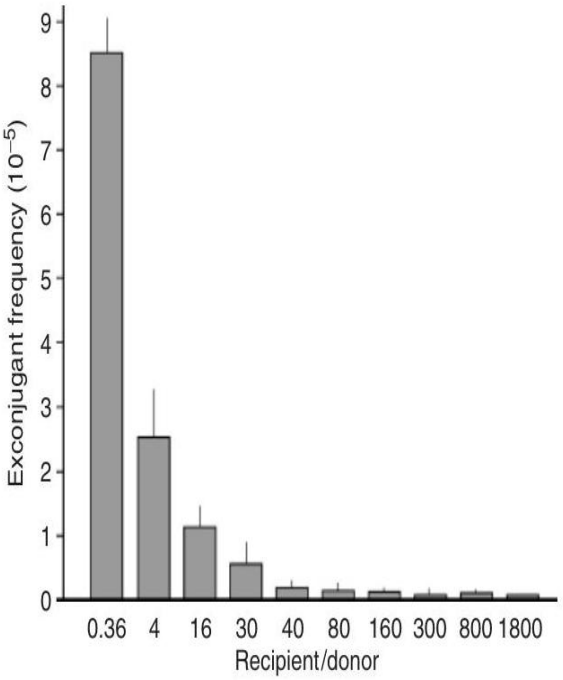

Fig. 2. Effect of the viable recipient spores/donor cells ratio on transconjugation efficiency. Plasmid pHZ1358 was transferred from *Escherichia coli* ET12567 [pUZ8002] to *Streptomyces natalensis* after a 10 min heat shock of the spores at 45 °C. Values are presented as exconjugant frequency per recipient. Data are the average of three independent experiments.

All these conjugation experiments were performed using MS agar plates containing 10 mM $MgCl_2$. We also tested TBO medium supplemented with the same concentration of $MgCl_2$ because it yields good growth and spore formation, but conjugation frequencies were always found to be 10–100 times lower than in MS medium. We therefore continued to use modified MS medium.

All previous exconjugants were obtained using the methylation defective strain *E. coli* ET12567 [pUZ8002] as donor. When we used the methylation proficient strain XL1-Blue [pUZ8002] instead, we obtained no exconjugants (Table 2).

Table 2. Comparison of *Escherichia coli* strains ET12567 and XL1-Blue as donors in intergeneric conjugation experiments with *Streptomyces natalensis*

Donor	Number of recipient spores	Exconjugant frequency
ET12567 [pUZ8002]	2×10^6	8.55×10^{-5}
	1×10^7	4.1×10^{-5}
	1×10^8	5.0×10^{-6}
XL1-Blue [pUZ8002]	2×10^6	$< 2.2 \times 10^{-6}$
	1×10^7	$< 1.3 \times 10^{-8}$
	1×10^8	$< 1.3 \times 10^{-8}$

The same plasmid (pHZ1358) and number of donor cells (4×10^6) were used in each experiment. Values are presented as exconjugant frequency per recipient. Data are the average of three independent experiments.

This result suggests that methylation-specific restriction systems operate in *S. natalensis*, and that transfer of DNA by conjugation from a methylation proficient strain does not evade *S. natalensis* restriction barriers. The presence of methyl-specific restriction systems has been described in other *Streptomyces* strains such as *S. bambergiensis*, *S. avermitilis* or *S. coelicolor*, and their involvement in restriction of ssDNA has been well documented (MacNeil, 1988; Kieser & Hopwood, 1991; Zotchev *et al.*, 1995; Oh & Chater, 1997).

Self-replicating vs. integrative vectors

The use of different *oriT*-containing plasmids yielded different transconjugation frequencies. We used three different plasmids and compared their conjugation efficiency with that of pHZ1358: one of them, pSOK201, was able to replicate autonomously into *Streptomyces* species as occurs with pHZ1358, and the other two integrate at the *attB* site of *Streptomyces* sp. chromosome, pSET152 and pSOK804 (Table 1). All of them were first introduced into the donor strain ET12567 [pUZ8002] by transformation. The number of donor cells was set at 6×10^6, and the number of viable spores at 1×10^6 in all conjugation experiments.

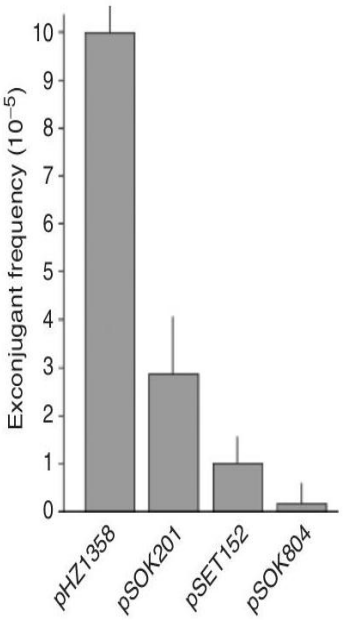

Fig. 3. Comparison of transconjugation frequencies obtained when using different *OriT*-containing plasmids. Plasmids were transferred from *Escherichia coli* ET12567 [pUZ8002] to *Streptomyces natalensis* after a 10 min heat shock of the spores at 45 °C. The same number of donor cells (6×10^6) and recipient spores (1×10^6) was used in each experiment. Values are presented as exconjugant frequency per recipient. Data are the average of three independent experiments.

As shown in Fig. 3, best transconjugation frequencies were obtained when we used pHZ1358; this represents a 3.4-fold higher frequency than that obtained with pSOK201. It is likely that the different origins of replication of both vectors could be responsible for such differences, e.g. one replicon being more stable than the other in the recipient *S. natalensis*. The first one derives from *S. lividans* plasmid pIJ101 (Kendall & Cohen, 1988), whereas the second one has been constructed from *S. ghanaensis* plasmid pSG5 (Muth *et al.*, 1995). Similar results have been observed in *S. nodosus* where transconjugation frequencies when using pSOK101, a pIJ101 replicon-containing plasmid, were substantially higher than those obtained with pSOK201 (Nikodinovic *et al.*, 2003). It is therefore conceivable that the generation and use of vectors containing the replication functions of the *S. natalensis* autonomously replicating plasmid pSNA1 (Mendes *et al.*, 2000) could yield higher transconjugation efficiencies.

Frequencies obtained with integrative vectors were significantly lower (Fig. 3) as expected, given that they require integration into the *S. natalensis* chromosome in order to be maintained. Again, both plasmids were not transferred with equal efficiencies, pSET152 being sixfold more efficient than pSOK804. Both vectors use different 'integration functions': those of the first one derive from ΦC31, whereas the second one uses the functions derived from the *S. venezuelae* phage ΨWB (Kieser *et al.*, 2000), and in the latter case have been described as less efficient than those of ΦC31 (Van Mellaert *et al.*, 1998). The difference in efficiency observed could thus be attributed to the different efficiencies of integration functions. Besides, it could also be possible that both vectors do not integrate at the same region of *S. natalensis* chromosome. In *S. lividans*, phages ΦC31 and ΨWB seem to recombine at different regions (Van Mellaert *et al.*, 1998). If this were the case in *S. natalensis*, the different integration points could also explain the different efficiencies observed. The true nature of exconjugants was verified by Southern blot hybridization in all cases (not shown).

Site-specific integration or the use of self-replicating plasmids did not affect pimaricin production or the essential functions of *Streptomyces natalensis*

Certain plasmids introduced by conjugation into some *Streptomyces* strains have shown a negative effect on morphogenesis or antibiotic production (Voeykova *et al.*, 1998). In order to determine whether the presence of integrative or self-replicating plasmids into *S. natalensis* had affected essential functions, the spores of exconjugants were serially diluted and plated on minimal medium. All exconjugants grew well in the minimal medium, which indicates that genes involved in amino acid biosynthesis were not affected. Other cultural and morphological properties of the exconjugants were also unaffected by the presence of the plasmids studied. Furthermore, when we analysed pimaricin production, no alteration was observed in exconjugants when compared with the nontransformed control strain (not shown). Also, no pigment production was observed. More studies will be necessary to get a deeper insight into the effect of the different replicons and integration functions in other aspects of secondary metabolism.

Validation of conjugation technology, a practical case

In order to demonstrate the utility of the technology described here to enable an easier manipulation of *S. natalensis* genes, we decided to use the recently described Red/ET recombination methodology adapted to *Streptomyces* (Gust *et al.*, 2003). This methodology is based on the discovery that allelic exchanges on the *E. coli* chromosome can be achieved by recombination with a selectable marker flanked by only short stretches of nucleotides homologous to the desired region of the chromosome, when either Redα/Redβ of phage λ or RecE/RecT from phage Rac is present in the targeted strain (Zhang *et al.*, 1998, 2000; Muyrers *et al.*, 1999; Datsenko & Wanner, 2000). We used λ-Red to promote recombination in *E. coli* between a PCR-amplified apramycin resistance cassette (selectable in *E. coli* and *S. natalensis*) including the origin of transfer (*oriT*) from RK2, and *S. natalensis* DNA on a cosmid (FA1) from an *S. natalensis* Supercos1 cosmid library (Aparicio *et al.*, 1999). The gene disruption cassette *apra* (Am^R) and the *oriT* were jointly amplified from pIJ773 (Gust *et al.*, 2003) using the primers indicated in the Methods section, and introduced by electroporation into *E. coli* BW25113/pIJ790 containing cosmid FA1. Cells were then grown at 30 °C in the presence of 10 mM arabinose to induce λ-Red genes present on pIJ790, and recombinants Am^R, Km^R were selected. Isolated mutant cosmids were verified by restriction analysis and introduced into the donor strain ET12567 [pUZ8002] by electroporation. Then the mutant cosmid, in which the DNA fragment comprised between the two priming sites was replaced by the Am^R *oriT* cassette, was transferred to *S. natalensis* by conjugation. As the cosmid cannot replicate autonomously in *Streptomyces* it can only be maintained if it integrates by homologous recombination, but given the long regions of sequence identity present in it, recombination is efficiently promoted. The number of donor cells was set at 1×10^8, and the number of viable spores ranged between 1×10^6 and 1×10^9. Conjugation was only achieved when 10^9 spores were used as recipients. Efficiencies of 7×10^{-7} exconjugants per recipient were obtained. Most of these exconjugants were Am^R and Km^R and presumably contained the entire cosmid integrated into the chromosome by a single crossover event. Double-crossover mutants were screened by apramycin resistance and kanamycin sensitivity (Fig. 4). These (about 2%) were verified by both PCR and Southern blot analysis. Figure 4

shows the PCR analysis and Southern blot of a randomly chosen exconjugant DNA. PCR was performed using primers F and R (see Methods). An amplification band of 642 bp is observed from the wild-type genomic DNA but not from the mutant as expected. The Southern blot was probed with the *apra* probe, indicating that the gene disruption cassette is present in the mutant strain. Both results demonstrate that gene replacement took place.

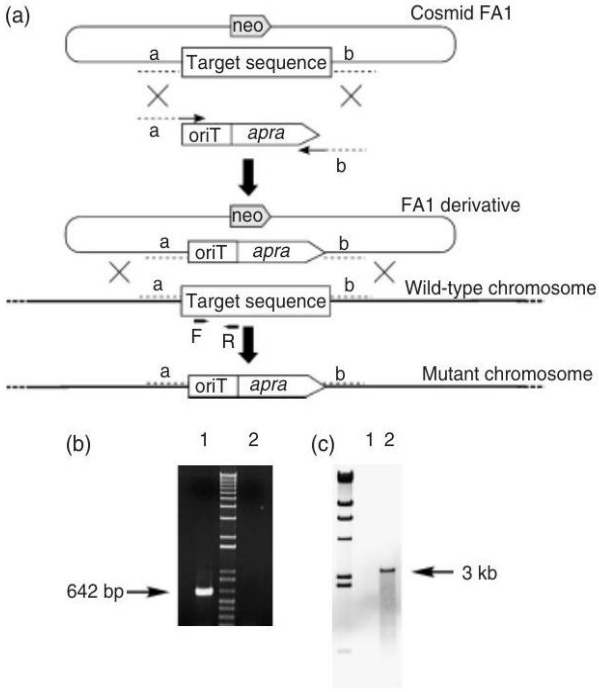

Fig. 4. Strategy used for the generation of *Streptomyces natalensis* mutants. (a) Dotted lines a and b indicate the 39 nucleotides of the primers that are identical to the regions flanking the *S. natalensis* target DNA sequence. The Am^R*oriT* cassette was amplified from pIJ773 and introduced into *Escherichia coli* BW25113/pIJ790 (expressing the λ-Red recombination functions) containing the Supercos-1 cosmid containing the cloned target sequence. Recombinant cosmids were transferred to *S. natalensis* by conjugation, and double-crossover mutants were screened by apramycin resistance and kanamycin sensitivity. PCR primers F and R used for analyzing the knockout are indicated by thick lines. (b) Agarose gel analysis of PCR products. Primers F and R amplified the expected 642 bp DNA band from the wild-type genomic DNA (lane 1) but not from the mutant (lane 2). Size markers are the 1 kb Plus DNA Ladder (Invitrogen, Carlsbad, CA). (c) Southern analysis confirming the integration of the gene disruption cassette into the mutant's chromosome. Hybridization pattern of the *Pvu*II digested chromosomal DNA of the wild-type *S. natalensis* (lane 1) and the mutant (lane 2) probed with the *apra* fragment. Size markers are digoxigenin–labelled λDNA digested with *Hin*dIII.

The transformation frequencies reported in this study are comparable to intergeneric conjugation efficiencies reported for other *Streptomyces* species (Voeykova *et al.*, 1998; Nikodinovic *et al.*, 2003). This methodology will enable an easier manipulation of the genes responsible for pimaricin biosynthesis, and will become in the future the method of choice for gene manipulation of this industrially important bacterium.

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