APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2006, p. 4839–4844 Vol. 72, No. 7
0099-2240/06/$08.00+0 doi:10.1128/AEM.00167-06

Excisable Cassettes: New Tools for Functional Analysis of *Streptomyces* Genomes

Alain Raynal,* Fatma Karray, Karine Tuphile, Emmanuelle Darbon-Rongère, and Jean-Luc Pernodet

Laboratoire Microbiologie Moléculaire des Actinomycètes, Institut de Génétique et Microbiologie, UMR CNRS 8621, Bât. 400, Université Paris-Sud, F-91405 Orsay, France

Received 23 January 2006/Accepted 17 April 2006

The functional analysis of microbial genomes often requires gene inactivation. We constructed a set of cassettes consisting of single antibiotic resistance genes flanked by the *attL* and *attR* sites resulting from site-specific integration of the *Streptomyces* pSAM2 element. These cassettes can easily be used to inactivate genes by in-frame deletion in *Streptomyces* by a three-step strategy. In the first step, in *Escherichia coli*, the cassette is inserted into a cloned copy of the gene to be inactivated. In the second step, the gene is replaced by homologous recombination in *Streptomyces*, allowing substitution of the wild-type target gene with its inactivated counterpart. In the third step, the cassette can be removed by expression of the pSAM2 genes *xis* and *int*. The resulting strains are marker-free and contain an "*attB*-like" sequence of 33, 34, or 35 bp with no stop codon if the cassette is correctly chosen. Thus, a gene can be disrupted by creating an in-frame deletion, avoiding polar effects if downstream genes are cotranscribed with the target gene. A set of cassettes was constructed to contain a hygromycin or gentamicin resistance gene flanked by the *attL* and *attR* sites. The initial constructions carrying convenient cloning sites allow the insertion of any other marker gene. We tested insertion and excision by inserting a cassette into *orf3*, the third gene of an operon involved in spiramycin biosynthesis. We verified that the cassette exerted a polar effect on the transcription of downstream genes but that, after excision, complementation with *orf3* alone restored spiramycin production.

Advances in genetic engineering for both prokaryotic and eukaryotic organisms have led to great improvements in their behavioral traits, with tremendous potential benefits for health, industrial, environmental, and agricultural applications. *Streptomyces* spp. are both academically and industrially important bacteria. The gene functions in these bacteria, particularly those of the gene sets involved in secondary metabolite production, have been studied through gene inactivation by targeted disruption.

In a previous work, we constructed gene cassettes carrying antibiotic resistance markers that were selectable in both *Escherichia coli* and *Streptomyces*. These were used to inactivate genes of interest through insertional mutagenesis (2). These cassettes carry heterologous genes that confer antibiotic resistance. If these markers are widely used to select genetic transformants, they may have an unintended detrimental environmental impact. Besides this concern, there are other reasons for removing and/or recycling selective markers, especially when several modifications are required. If every genetic modification in a given strain results in an antibiotic marker being retained in the modified organism, and if the combination of several mutations needs to be analyzed, it becomes progressively more difficult to find new antibiotic resistance genes to use. Moreover, this situation prevents a plethora of selectable markers from being used in subsequent rounds of gene modification in the same host. Also, the insertion of a resistance cassette into a gene that is part of an operon may have a polar effect on the expression of downstream genes.

This work aimed to set up a system that allows the cassette to be removed, leaving only a short sequence with no polar effect. For the gene to be inactivated under these conditions, the insertion of the cassette must be combined with the deletion of part or all of the gene. After excision, it should be possible to obtain a gene deletion where the original reading frame is maintained. Such a mutation is not expected to be polar.

pSAM2 is an 11-kb integrative element from *Streptomyces ambofaciens* (15). It possesses a site-specific recombination system very similar to that of temperate phages (3). It also has functions common to *Streptomyces* plasmids, such as replication, transfer, pock formation, and mobilization of chromosomal markers (24). The *repSA*, *xis*, and *int* genes, which encode the replicase, the excisionase, and the integrase, respectively, are organized as an operon that is activated by the *pra* gene product (21). The integrase can promote intermolecular recombination between the attachment sites *attP* and *attB*, leading to the formation of *attL* and *attR*, which flank the integrated sequence. The expression of both *int* and *xis* leads to excision via intramolecular recombination between *attL* and *attR*. The *att* sequences required for site-specific recombination have been studied in detail and precisely defined (17, 18). Our work aimed to produce an *attL*-antibiotic resistance gene-*attR* cassette that carries blunt end restriction sites at both extremities. This allows easy insertion into the cloned target gene that is to be inactivated. In a second step, this construction can be integrated via a double crossover to replace the wild-type gene with its disrupted counterpart in the *Streptomyces* genome. The third step consists of removing the cassette

* Corresponding author. Mailing address: Laboratoire Microbiologie Moléculaire des Actinomycètes, Institut de Génétique et Microbiologie, UMR CNRS 8621, Bât. 400, Université Paris-Sud, F-91405 Orsay, France. Phone: 33 1 69 15 46 75. Fax: 33 1 69 15 45 44. E-mail: alain.raynal@igmors.u-psud.fr.

TABLE 1. Plasmid characteristics and construction

Plasmid	Resistance	Construction and/or properties	Reference
KS+ΔH3	Ap	pBluescript KS (+) deleted for HindIII site in MCS	This paper
pOSV501	Ap	CASS1 in KS+ΔH3 digested with EcoRV	This paper
pOSV502	Ap	CASS2 in KS+ΔH3 digested with EcoRV	This paper
pOSV503	Ap	CASS3 in KS+ΔH3 digested with EcoRV	This paper
*att1*ΩAb^r+/−[a]	Ap Ab	pOSV501 + Ω*Ab^r* in HindIII site	This paper
*att2*ΩAb^r+/−[a]	Ap Ab	pOSV502 + Ω*Ab^r* in HindIII site	This paper
*att3*ΩAb^r+/−[a]	Ap Ab	pOSV503 + Ω*Ab^r* in HindIII site	This paper
pWHM3	Ap Ts	Replicative shuttle vector for *E. coli* and *Streptomyces*	26
pWHM3Hyg	Ap Hm	pWHM3, *tsr* replaced by *hyg*	This paper
pOSV510	Ap Hm	*prap-aph* in pWHM3Hyg	This paper
pSET152	Gn	Integrative plasmid in *Streptomyces*	1
pOSV511	Gn Hm	*att1*Ω*hyg* in EcoRV site of pSET152	This paper
pOSint3	Ap	*trcp-xis-int* in pTRC99A	18
pOSV507	Ap Ts	*ermE*p-xis-int* in pIJ903	This paper
pOSV508	Ap Ts	*trcp-xis-int* in pWHM3	This paper
pOS49.99	Ap	*orf1-2-3-4* in pUC19	Unpublished
pOSV512	Ap Hm	*orf1-2-orf3*::*att1*Ω*hyg-orf4* in pOS49.99	This paper
pOJ260	Gn	Integrative plasmid in *Streptomyces*	1
pOSV513	Gn Hm Ap	*orf1-2-orf3*::*att1*Ω*hyg-orf4* in pOJ260	This paper
pOS49.52	Ap Ts	*ermE*p-orf3* in pIJ903	Unpublished
pOS527	Ap Ts	*prap* in pIJ487	22

[a] Ab^r, antibiotic resistance gene *aac* or *hyg*; +, resistance gene transcribed in the *attL*-to-*attR* orientation; −, resistance gene transcribed in the opposite orientation.

via a site-specific excision event promoted by the expression of the *xis* and *int* genes in *trans*. Polar effects were avoided for a disrupted gene belonging to an operon by constructing different cassettes that leave a 33-, 34-, or 35-bp "*attB*-like" sequence after excision.

MATERIALS AND METHODS

Strains and media. *E. coli* strain DH5α (8) was used for cloning experiments and plasmid DNA propagation. *Streptomyces* strains were routinely cultivated on Hickey-Tresner medium at 30°C as described previously (15). *Streptomyces lividans* strain TK24 and *Streptomyces ambofaciens* ATCC 23877 were used for transformation experiments. Transformants carrying the *hyg*, *tsr*, or *aac* resistance gene were selected with 150 to 200 μg hygromycin (Hm) ml^{-1}, 25 μg thiostrepton (Ts) ml^{-1}, or 50 μg geneticin (Gn) ml^{-1}, respectively, in R2YE medium (10). Transformants harboring the *aac* gene can also be selected with apramycin or gentamicin. *Streptomyces ambofaciens* ATCC 23877 produces the macrolide antibiotic spiramycin (16). Bacteria containing plasmids were routinely grown on LB medium supplemented with 100 μg ampicillin (Ap) ml^{-1} and 20 μg Gn ml^{-1}, and bacterial cultures in liquid LB medium were supplemented with 30 μg Ap ml^{-1} and 10 μg Gn ml^{-1}.

Plasmids, antibiotic resistance genes, and oligonucleotides. All restriction endonuclease digestions, ligation reactions, DNA modifications, and PCR amplifications were carried out according to standard protocols (19). *E. coli* and *Streptomyces* were transformed according to standard protocols (11, 19). The plasmid pGEM-T Easy (Promega) was used for cloning of PCR products, and pBluescript (Stratagene) was used for the cloning experiments. All of the plasmids used in this study are listed in Table 1. KS+DH3, a pBluescript KS (+) derivative deleted for the HindIII restriction site, was constructed to clone the antibiotic resistance genes.

The genes encoding Hm resistance and Gn resistance were provided by Ω*hyg* and Ω*aac* cassettes (2), respectively, derived from the Ω interposon as HindIII-HindIII fragments. A HindIII fragment containing only the *aac* gene was obtained by PCR amplification using the primers KF42 (5′AAGCTTGTACGGC CCACAGAATGATGTCAC3′) and KF43 (5′AAGCTTCGACTACCTTGGTG ATCTCGCCTT3′) (HindIII sites are underlined), with an Ω*aac* cassette containing a plasmid as the DNA template. The resulting PCR product was inserted into pGEM-T Easy. A HindIII fragment carrying the *aac* gene was obtained from the resulting plasmid. Initial *attL-attR* cassettes were obtained after two successive PCR steps using the following primers: Cas1R (5′CATGC CGGTCGGGATATCGCGCGCTTCGTTCG3′), Cas2R (5′CATGCCGGTCG GGATATCGGCGCGCTTCGTTCG3′), Cas3R (5′CATGCCGGTCGGGATA TCGCGCGCGCTTCGTTCG3′), CasR (5′AGATCTGTTAACAAGCTTCTC GAGGGATCCCTGTCAGTCATGCGGG3′), CasL (5′GGATCCCTCGAGA AGCTTGTTAACAGATCTCCCGGCTCGTCGGAC3′), Cas1/2L (5′CCCGG GGATCTGGATATCTACCTCTTCGTCCC3′), and Cas3L (5′CCCGGGGAT CGTGATATCTGCCTCTTCGTCCC3′).

Spiramycin production assays. For spiramycin production, *S. ambofaciens* was grown in MP5 liquid medium at 27°C (14). For bioassays, the supernatant of the culture was applied to Whatman AA paper discs. The discs were laid on plates containing *Micrococcus luteus*, and the plates were first incubated at 4°C for 2 hours to allow antibiotic diffusion and then incubated at 37°C. The growth inhibition area was measured and compared to standards obtained using spiramycin, as described previously (14).

Construction of *S. ambofaciens* ATCC 23877 derivative devoid of pSAM2. Since we were using the site-specific recombination system from pSAM2 to carry out excision in *S. ambofaciens*, we preferred to work with an *S. ambofaciens* strain devoid of pSAM2 to avoid all kinds of interference due to the integrated copy of pSAM2. Therefore, experiments were undertaken to obtain an *S. ambofaciens* strain cured of the integrated copy of pSAM2. It has been reported that the production and regeneration of bacterial protoplasts can promote the loss of plasmids (5). Since the pSAM2 functions are well characterized, we designed a reporter system allowing positive selection for the loss of pSAM2.

The *pra* gene has been described as an activator of pSAM2 replication, and its inactivation leads to the disappearance of free pSAM2 (22). KorSA has been identified as a central transcriptional repressor (23) that binds to the *pra* gene promoter, thus repressing *pra* gene expression. The rationale behind this experiment is that derepression of the *pra* promoter should be observed in the absence of the KorSA repressor, indicating the loss of pSAM2. We used pOS527 to obtain a fragment carrying the *pra* promoter fused to the promoterless *aph* gene (22), which was inserted into the unstable replicative vector pWHM3hyg, a pWHM3 (26) derivative in which the *tsr* gene (conferring Ts resistance) is replaced by *hyg* (conferring Hm resistance). The resulting plasmid, pOSV510, was used to transform protoplasts of the *S. ambofaciens* ATCC 23877 strain. Selection with neomycin of clones expressing the *aph* reporter gene allowed *S. ambofaciens* strains devoid of pSAM2 to be isolated. The absence of pSAM2 was checked by Southern blotting (data not shown), and one of the obtained clones was named OSC2. The OSC2 strain was not affected in growth, sporulation, or spiramycin production.

Nucleotide sequence accession numbers. The following sequences were deposited in the EMBL database with the indicated accession numbers: *att1*Ω*aac*+, AM238621; *att2*Ω*aac*+, AM238622; *att3*Ω*aac*+, AM238623; *att1*Ω*hyg*+, AM238624; *att2*Ω*hyg*+, AM238625; *att3*Ω*hyg*+, AM238626; *att1*Ω*aac*−, AM238627; *att2*Ω*aac*−, AM238628; *att3*Ω*aac*−, AM238629; *att1*Ω*hyg*−, AM238630; *att2*Ω*hyg*−, AM238631; and *att3*Ω*hyg*−, AM238632.

RESULTS

Construction of *attR*-antibiotic resistance-*attL* cassettes. The pSAM2 chromosomal *attB* site shares a 58-bp identity segment with *attP* that extends from the region encoding the anticodon loop to the 3′ end of the $tRNA^{Pro}$ gene. Site-specific recombination that leads to integration or excision, promoted by the pSAM2 Int and Xis proteins, has been described for *E. coli* (18). It has also been shown that a 26-bp *att* sequence (*attB26*) centered on the region encoding the anticodon stem-loop of the tRNA and not completely included in the identity segment retains the entire functionality of *attB* (18). The minimal *attP* site has also been defined (17). Therefore, after site-specific intermolecular recombination between *attP* and *attB26*, it is possible to obtain what may be considered the minimal *attL* and *attR* sites. This experiment aimed to clone these minimal *attL* and *attR* sites as a single fragment, with restriction sites at the junction between *attL* and *attR* allowing the insertion of antibiotic resistance markers. When required, intramolecular site-specific recombination between these *attL* and *attR* sequences will excise the entire sequence between them, including the resistance marker, reconstituting the minimal *attB* site. We were able to add 1 or 2 bp to the 26-bp minimal *attB* site to construct a set of three different cassettes, leaving sequences of *n*, *n* + 1, and *n* + 2 bp after excision. Thus, depending on the length of the insertion/deletion within the coding sequence of the target gene, it is possible to choose one of the cassettes to maintain the original reading frame after excision, thus avoiding polar mutations.

Construction was undertaken starting from plasmid pOSCo26, a cointegrate resulting from site-specific recombination between pSAM2 *attP* and the minimal *attB*26 site (16). In the first step (Fig. 1a), *attR* and *attL* were amplified as individual fragments from the cointegrate molecules. The CasR and CasL primers carried a 30-nucleotide common sequence, with an inverted orientation in one of the primers. This allowed both to associate with the *attR*/*attL* molecules obtained from the first PCR and to create restriction sites that allow the antibiotic resistance genes to be inserted between *attR* and *attL* in a further step. In a second PCR step (Fig. 1a), the PCR products from the first step were used as templates for amplification, using different couples of external primers that provided EcoRV restriction sites. This allowed a set of cassettes to be generated, named CASS1, CASS2, and CASS3, containing both *attR* and *attL* and having respective sizes of 485, 486, and 487 bp (Fig. 1b). These cassettes were further inserted as EcoRV-EcoRV fragments into the plasmid KS+DH3, leading to the plasmids pOSV501, pOSV502, and pOSV503, respectively. In the next step, an antibiotic resistance cassette was inserted between the *attL* and *attR* sites in these plasmids (Fig. 1c). Both Ω*hyg* and Ω*aac* cassettes (2), carrying Hm and Gn resistance genes, respectively, were inserted as HindIII fragments into pOSV501, pOSV502, and pOSV503. Since there were three types of sequence left after excision (*att1*, 33 bp; *att2*, 34 bp; and *att3*, 35 bp) (Fig. 1d), two possible orientations of the marker genes (+ denotes a resistance gene transcribed in the *attL*-to-*attR* orientation, and − denotes a resistance gene transcribed in the other orientation), and two different fragments carrying the resistance genes (Ω*hyg* and Ω*aac*), this led to a set of 12 plasmids containing the cassettes *att1*Ω*hyg*+/−, *att2*Ω*hyg*+/−, *att3*Ω*hyg*+/−, *att1*Ω*aac*+/−, *att2*Ω*aac*+/−, and *att3*Ω*aac*+/− (Table 1). The derivatives of plasmid KS+DH3 containing these cassettes are designated by the letter "p" followed by the name of the cassette.

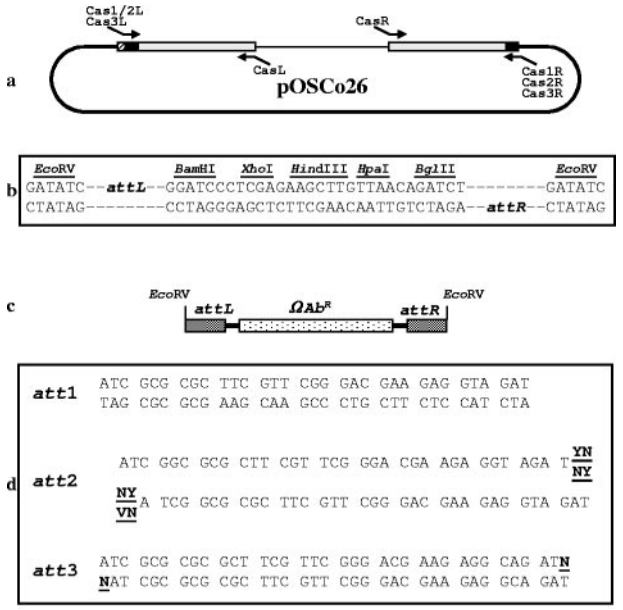

FIG. 1. Construction and characteristics of excisable cassettes. (a) *attL* and *attR* were amplified individually by PCRs using the CasR and CasL primer families. (b) The CasL-CasR central primers share a 30-nucleotide inverted repeat sequence that allows *attL1*, *-2*, and *-3* to be associated with *attR1*, *-2*, and *-3*, respectively, followed by PCR amplification using external CasL and CasR primers carrying an EcoRV restriction site sequence. The second PCR step generates the cassettes, CASS1, -2, and -3 (c), carrying a multicloning site in the central part, allowing antibiotic resistance cassette cloning. (d) Representation of the 33-, 34-, and 35-bp sequences remaining after excision, with respect to the reading frame and the cassette cloning orientation. In *att2* and *att3*, the bold characters indicate nucleotides coming from the disrupted gene. There are some constraints in the possible nucleotide that can be used to avoid a stop codon in the sequence remaining after excision.

Construction of *xis-int*-expressing plasmids for *Streptomyces*. In pSAM2, the *int* and *xis* genes are located downstream from the *repSA* gene that encodes the replicase, and the three genes are cotranscribed. Moreover, *pra* expression is required for their efficient transcription (21). Therefore, it was necessary to place these genes under the control of a heterologous promoter to obtain a simple construction expressing *xis* and *int*. A first plasmid, pOSV507, was constructed in which the *xis* and *int* genes are transcribed from the *ermE** promoter and carried by the low-copy-number vector pIJ903. Another plasmid, pOSint3, expressing *xis* and *int* under the control of the *E. coli trc* promoter, was constructed in a previous study (18). Since the *E. coli trc* promoter is functional in *Streptomyces*, a fragment carrying *trcp-xis-int* isolated from pOSint3 was inserted into plasmid pWHM3 to obtain pOSV508. pWHM3 is an *E. coli*/*Streptomyces* shuttle vector that is not very stable in *Streptomyces* in the absence of selective pressure. In this case, the instability was an advantage as it allowed easy curing of the plasmid.

Functionality of excision in *E. coli* and *Streptomyces*. The excisable cassettes can be isolated as blunt-ended EcoRV fragments from the plasmids that carry them and easily inserted into any gene, as insertion of the cassette can be selected by the appropriate antibiotic in both *E. coli* and *Streptomyces*. Excision of the cassette can be achieved by expressing the *xis* and *int* genes and can be screened for by the loss of antibiotic resistance. The signatures left by the cassettes after excision are shown in Fig. 1d with respect to the reading frame and the cassette cloning orientation in the disrupted gene. Some constraints exist for using the cassette that leaves the *att2* or *att3* sequence.

We investigated the excision event by inserting the *att1Ωhyg* cassette, isolated as an EcoRV fragment, into pSET152 digested by EcoRV, yielding pOSV511. The vector pSET152, which can replicate in *E. coli*, carries a Gn resistance marker (Gn^r) and the FC31 attachment site, which allows its integration into the chromosomes of several *Streptomyces* species (1). pOSV511 was introduced into a strain of *E. coli* containing pOSint3 (18) and therefore expressing the *int* and *xis* genes. Among the transformants selected as Gn^r colonies, >90% were Hm^s, showing very efficient excision of the cassette in *E. coli*.

We tested the excision efficiency in *Streptomyces* by introducing pOSV511 into *S. lividans* strain TK24 by protoplast transformation. Integrative transformants were selected as Gn^r Hm^r colonies. Southern blotting with three different transformants showed that the fragment containing *att1Ωhyg*+ had been integrated into the *S. lividans* chromosome.

We introduced the plasmid pOSV507 expressing the *xis* and *int* genes from pSAM2 into *S. lividans* carrying integrated pOSV511 to promote the excision of the cassette (data not shown; see below). The transformants were picked and grown on plates with no Hm selection. After two rounds of sporulation under these conditions, we tested the isolated clones for their Hm resistance. Hm^s clones were readily obtained. Southern blotting with total DNA extracted from two of these Hm^s clones confirmed excision of the cassette (data not shown). The regions containing the sequence left after excision were cloned by marker rescue and sequenced; in both cases, we found the expected 33-nucleotide sequences flanked by the two EcoRV cloning sites (data not shown). All of these results clearly showed that the excision of the cassette was efficient in *S. lividans* as well as in *E. coli* and was site specific, leaving the expected *attB*-like sequence after excision.

Example of the use of the cassettes: insertion of a cassette into a target gene. We showed how these cassettes could be used by choosing to inactivate a gene belonging to an operon located in the spiramycin biosynthetic cluster of *Streptomyces ambofaciens*. Seven genes, *orf1* to *orf7*, encoding enzymes involved in various steps of spiramycin biosynthesis form an operon (N. Oestreicher et al., unpublished). It was shown that inserting the *Ωhyg* cassette into *orf3*, the third gene of this operon, abolished spiramycin production. However, insertion of this cassette caused a polar effect on transcription of the downstream genes, *orf4* to *orf7*. After several gene replacement steps, it was possible to obtain an in-frame deletion in *orf3*; this deletion still abolished spiramycin biosynthesis but did not cause a polar effect (N. Oestreicher et al., unpublished). We repeated the *orf3* inactivation, using an excisable cassette to test whether there was no polar effect after excision.

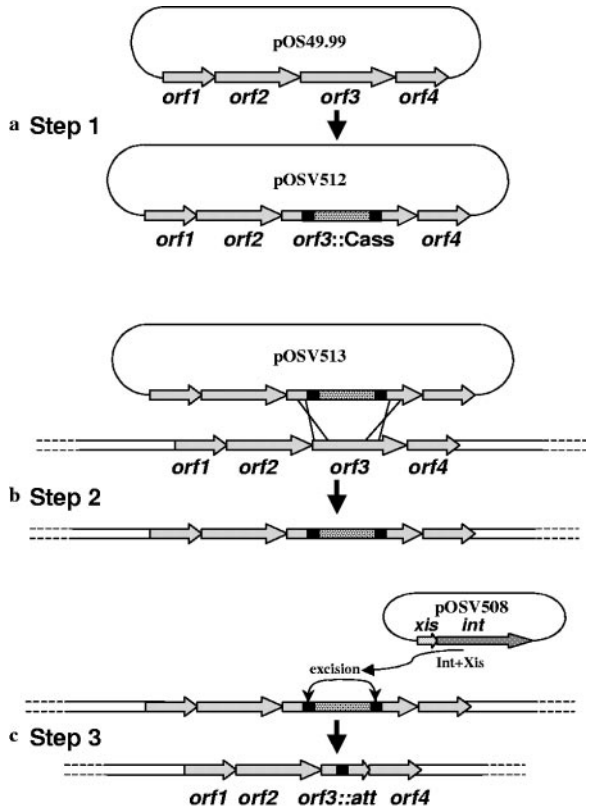

FIG. 2. Schematic representation of the different steps for generating unmarked mutant strains. (a) Step 1, cloning of the gene of interest, followed by its disruption by insertion of the chosen cassette. (b) Step 2, replacement of the wild-type copy of the target gene by the disrupted gene via a double recombination event. (c) Step 3, excision of the cassette at the chromosomal locus after transitional expression of Xis and Int.

A 4.5-kb EcoRI-BamHI fragment containing *orf1* to *orf4* of this operon was inserted into pUC19, yielding pOS49.99. In this fragment, *orf3* was disrupted by inserting the *att1Ωhyg* cassette, isolated as an EcoRV fragment. This fragment was inserted into pOS49.99 after PmlI/Asp718I digestion, followed by filling in of the two protruding ends, giving the plasmid pOSV512. The insertion of the cassette was accompanied by deletion of 270 bp of the *orf3* coding sequence. A fragment carrying *orf1-2-orf3::att1Ωhyg-orf4* was inserted into pOJ260, a vector unable to replicate in *Streptomyces* (1), leading to pOSV513. This first step is represented schematically in Fig. 2a.

Gene replacement and cassette excision in *S. ambofaciens*. Protoplasts of the OSC2 strain were transformed with pOSV513 DNA denatured by alkali treatment according to the method of Oh and Chater (13). Transformants were selected as Hm^r colonies and were further grown on medium containing Gn to screen for Hm^r Gn^s colonies in which gene replacement might have occurred. Total DNA was extracted from several of these clones and analyzed by Southern blotting. Two transformants in which *orf3* had been replaced by *orf3::att1Ωhyg* were

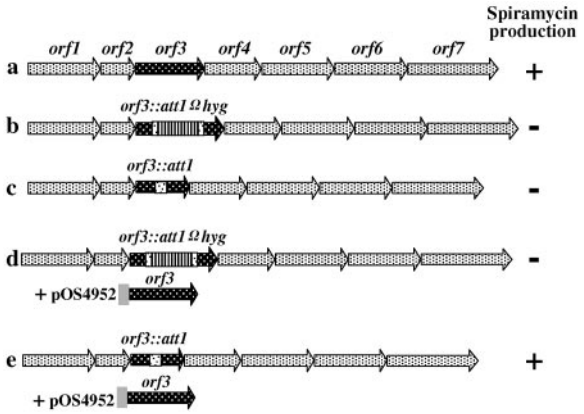

FIG. 3. Representation of the different structures at the *orf1-orf7* operon in different strains of *S. ambofaciens*. (a) *S. ambofaciens* OSC2, an ATCC 23877 derivative devoid of pSAM2. (b) Same strain after inactivation of *orf3* by insertion of *att1*Ω*hyg*− (*orf3*::*att1*Ω*hyg*−). (c) Same strain after excision of the cassette (*orf3*::*att1*). (d) Construct b with *orf3* carried by plasmid pOS49.52. (e) Construct c with *orf3* carried by plasmid pOS49.52. Spiramycin production is indicated for all strains.

selected for further analysis. Both clones were unable to produce spiramycin (Fig. 2b and 3b).

Excision was carried out using the plasmid pOSV508, in which *xis* and *int* are transcribed from the *E. coli trc* promoter. Excision with pOSV508 was more efficient than that with pOSV507, and pOSV508 was less stable in *Streptomyces* than pSOV507, allowing a rapid loss of the *xis-int*-expressing plasmid. We picked 35 transformants grown on solid medium without antibiotic, which were then grown for 4 days until sporulation. After being replica plated on media with Hm, with Ts, and without antibiotics, all transformants appeared to be sensitive to Hm and Ts, suggesting both efficient excision and a very rapid loss of plasmid pOSV508. Excision of the cassette was confirmed by Southern blotting and PCR on total extracted DNA. After excision of the cassette, a residual 33-bp sequence was left. Since insertion of the cassette had been accompanied by a deletion of 270 bp, the reading frame was not changed in the inactivated *orf3*::*att1* copy.

Absence of polar effect after cassette excision. We demonstrated the absence of a polar effect after excision by studying the restoration of spiramycin production by *orf3* expression in the *orf3*::*att1*Ω*hyg* strain (before excision) (Fig. 3d) and in the *orf3*::*att1* strain (after excision) (Fig. 3e). A DNA fragment carrying the *Streptomyces ermE** promoter upstream from the entire *orf3* coding sequence was inserted into vector pIJ903, yielding pOS49.52. This plasmid did not restore spiramycin production in the *orf3*::*att1*Ω*hyg* strain, indicating a polar effect due to disruption of the expression of the downstream genes, whose products are necessary for spiramycin biosynthesis (Fig. 3d).

Further experiments were carried out on two independent clones from which the cassette had been excised. The two clones were transformed with pOS49.52. Transformants were selected on R2YE medium containing Ts and further allowed to sporulate on Hickey-Tresner medium containing Ts. Three independent transformants were assayed for the ability to produce spiramycin in liquid medium. All produced spiramycin at the same level as the OSC2 strain (Fig. 3e), demonstrating the absence of a polar effect due to the *orf3*::*att1* mutation.

DISCUSSION

This work aimed to design excisable cassettes that could be used to easily obtain nonpolar in-frame deletions in a gene of interest. For this purpose, we used the integration/excision properties of pSAM2, an integrative conjugative element from *Streptomyces ambofaciens*. The integration, promoted by the pSAM2 integrase, occurs by intermolecular site-specific recombination between the *attP* site of the element and the chromosomal *attB* site, generating *attL* and *attR* sites. Excision occurs by intramolecular site-specific recombination between *attL* and *attR* and requires the expression of both the integrase and the excisionase.

In this work, we have described a set of cassettes that contain antibiotic resistance genes inserted between the *attL* and *attR* sites (*attL*-antibiotic resistance-*attR*) and a set of vectors that express the Int and Xis proteins from pSAM2. A cassette can be removed from the vector as a blunt-ended fragment (EcoRV-EcoRV) and inserted into any cloned gene. The combination of cassette insertion with a deletion of the desired size in the target gene guarantees inactivation of the gene after excision of the cassette. The presence of the cassette could be selected for by the acquisition of resistance in both *E. coli* and *Streptomyces*.

This system is useful for inactivating genes of interest and for obtaining stable mutants. Because this system ultimately relies on a double crossover taking place between the cloned, mutagenized gene and its wild-type counterpart in the bacterial chromosome, this procedure will only work efficiently in those instances where the cassette is flanked on each side by at least 200 to 300 bp of DNA identical to the targeted gene (9, 11). The removal of the antibiotic resistance genes allows the markers to be recycled for successive rounds of gene inactivation. Also, if the gene is part of an operon, insertion of a cassette, especially those containing the T4 transcriptional terminators, will introduce a polar effect on the expression of downstream genes. However, if the right cassette is used, in accordance with the size of the insertion/deletion introduced in the coding sequence of the target gene, the original reading frame will be restored after excision, ensuring the unaffected expression of downstream genes.

These cassettes were originally designed to be inserted into the target gene by cloning, hence leaving a 33-, 34-, or 35-bp sequence. Although the use of only one of the cassettes is reported here, several others have been used successfully, leaving scars of the expected sizes (our unpublished results). The PCR targeting method is now widely used for studies with *Streptomyces* (6, 7). The cassettes described here have also been inserted successfully into target genes by PCR targeting and lambda Red-mediated recombination (our unpublished results), using previously described *E. coli* strains expressing *red* and *gam* (4, 27). Amplification of the cassettes containing *hyg* is sometimes not very efficient, probably due to the presence of the *hyg* gene terminator, although the PCR conditions needed to overcome this problem have been described (25). Thus, the cassettes constructed by Gust et al. (6, 7) could be

used to easily generate unmarked, nonpolar, in-frame deletions in *Streptomyces*. By expressing the FLP recombinase in *E. coli*, the central part of the cassette could be removed, leaving an 81-bp sequence. The resulting construction can then be introduced into *Streptomyces*, with clones resulting from a single crossover being selected and then screened for double crossovers replacing the copy of the gene disrupted by the cassette with a copy containing the in-frame deletion. The cassettes described here offer a simple alternative to this method. They may also be used to generate unmarked frameshift mutations if required.

These cassettes, or derivatives of them, could be used in a wide range of bacteria. The selection of these cassettes should be possible in different organisms by using, for example, resistance markers such as the *aac-4* gene, which has been expressed from its own promoter in a wide range of bacteria, including *E. coli* and *Streptomyces*. Alternatively, the multicloning site (BamHI, XhoI, HindIII, HpaI, and BglII) present in the initial plasmids (pOSV501, -502, and -503) allows the easy cloning of other selectable markers if needed. We have clearly shown that excision is very efficient both in *E. coli* and in *Streptomyces* and requires only the expression of *int* and *xis*. The expression of these two genes may be obtained easily by using suitable constructions in various bacteria. Therefore, excision could be functional in many different hosts and heterologous environments. The pSAM2 integrase has been shown to work in *Mycobacteria* (12, 20). The use of excisable cassettes allowing the removal of antibiotic resistance markers could increase the acceptance of genetically engineered organisms in biotechnological applications.

ACKNOWLEDGMENTS

We thank M. Guérineau for providing help, suggestions, and comments, A. Friedmann for excellent technical assistance, and B. Al-Dabbagh for a critical reading of the manuscript.

This work was partly supported by Sanofi-Aventis Pharma. F.K. received a fellowship from CNRS (BDI).

REFERENCES

1. **Bierman, M., R. Logan, K. O'Brien, E. T. Seno, R. N. Rao, and B. E. Schoner.** 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. Gene **116:**43–49.
2. **Blondelet-Rouault, M. H., J. Weiser, A. Lebrihi, P. Branny, and J. L. Pernodet.** 1997. Antibiotic resistance gene cassettes derived from the omega interposon for use in *E. coli* and *Streptomyces*. Gene **190:**315–317.
3. **Boccard, F., T. Smokvina, J. L. Pernodet, A. Friedmann, and M. Guerineau.** 1989. The integrated conjugative plasmid pSAM2 of *Streptomyces ambofaciens* is related to temperate bacteriophages. EMBO J. **8:**973–980.
4. **Chaveroche, M. K., J. M. Ghigo, and C. d'Enfert.** 2000. A rapid method for efficient gene replacement in the filamentous fungus *Aspergillus nidulans*. Nucleic Acids Res. **28:**E97.
5. **Furumai, T., K. Takeda, and M. Okanishi.** 1982. Function of plasmids in the production of aureothricin. I. Elimination of plasmids and alteration of phenotypes caused by protoplast regeneration in *Streptomyces kasugaensis*. J. Antibiot. (Tokyo) **35:**1367–1373.
6. **Gust, B., G. L. Challis, K. Fowler, T. Kieser, and K. F. Chater.** 2003. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc. Natl. Acad. Sci. USA **100:**1541–1546.
7. **Gust, B., G. Chandra, D. Jakimowicz, T. Yuqing, C. J. Bruton, and K. F. Chater.** 2004. Lambda red-mediated genetic manipulation of antibiotic-producing *Streptomyces*. Adv. Appl. Microbiol. **54:**107–128.
8. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
9. **Hilleman, D., A. Puhler, and W. Wohlleben.** 1991. Gene disruption and gene replacement in *Streptomyces* via single stranded DNA transformation of integration vectors. Nucleic Acids Res. **19:**727.
10. **Hopwood, D. A., D. J. Lydiate, F. Malpartida, and H. M. Wright.** 1985. Conjugative sex plasmids of *Streptomyces*. Basic Life Sci. **30:**615–634.
11. **Kieser, T., M. J. Bibb, M. J. Buttner, K. F. Chater, and D. A. Hopwood.** 2000. Practical *Strepromyces* genetics. John Innes Foundation, Norwich, United Kingdom.
12. **Martin, C., P. Mazodier, M. V. Mediola, B. Gicquel, T. Smokvina, C. J. Thompson, and J. Davies.** 1991. Site-specific integration of the *Streptomyces* plasmid pSAM2 in *Mycobacterium smegmatis*. Mol. Microbiol. **5:**2499–2502.
13. **Oh, S. H., and K. F. Chater.** 1997. Denaturation of circular or linear DNA facilitates targeted integrative transformation of *Streptomyces coelicolor* A3(2): possible relevance to other organisms. J. Bacteriol. **179:**122–127.
14. **Pernodet, J. L., M. T. Alegre, M. H. Blondelet-Rouault, and M. Guerineau.** 1993. Resistance to spiramycin in *Streptomyces ambofaciens*, the producer organism, involves at least two different mechanisms. J. Gen. Microbiol. **139:**1003–1011.
15. **Pernodet, J. L., J. M. Simonet, and M. Guerineau.** 1984. Plasmids in different strains of *Streptomyces ambofaciens*: free and integrated form of plasmid pSAM2. Mol. Gen. Genet. **198:**35–41.
16. **Pinnert-Sindico, S.** 1954. Une nouvelle espèce de *Streptomyces* productrice d'antibiotiques: *Streptomyces ambofaciens* n. sp. caractères culturaux. Ann. Inst. Pasteur (Paris) **87:**702–707.
17. **Raynal, A., A. Friedmann, K. Tuphile, M. Guerineau, and J. L. Pernodet.** 2002. Characterization of the attP site of the integrative element pSAM2 from *Streptomyces ambofaciens*. Microbiology **148:**61–67.
18. **Raynal, A., K. Tuphile, C. Gerbaud, T. Luther, M. Guerineau, and J. L. Pernodet.** 1998. Structure of the chromosomal insertion site for pSAM2: functional analysis in *Escherichia coli*. Mol. Microbiol. **28:**333–342.
19. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
20. **Seoane, A., J. Navas, and J. M. Garcia Lobo.** 1997. Targets for pSAM2 integrase-mediated site-specific integration in the *Mycobacterium smegmatis* chromosome. Microbiology **143:**3375–3380.
21. **Sezonov, G., A. M. Duchene, A. Friedmann, M. Guerineau, and J. L. Pernodet.** 1998. Replicase, excisionase, and integrase genes of the *Streptomyces* element pSAM2 constitute an operon positively regulated by the *pra* gene. J. Bacteriol. **180:**3056–3061.
22. **Sezonov, G., J. Hagege, J. L. Pernodet, A. Friedmann, and M. Guerineau.** 1995. Characterization of pra, a gene for replication control in pSAM2, the integrating element of *Streptomyces ambofaciens*. Mol. Microbiol. **17:**533–544.
23. **Sezonov, G., C. Possoz, A. Friedmann, J. L. Pernodet, and M. Guerineau.** 2000. KorSA from the *Streptomyces* integrative element pSAM2 is a central transcriptional repressor: target genes and binding sites. J. Bacteriol. **182:**1243–1250.
24. **Smokvina, T., F. Boccard, J. L. Pernodet, A. Friedmann, and M. Guerineau.** 1991. Functional analysis of the *Streptomyces ambofaciens* element pSAM2. Plasmid **25:**40–52.
25. **Stratigopoulos, G., and E. Cundliffe.** 2002. Expression analysis of the tylosin-biosynthetic gene cluster: pivotal regulatory role of the tylQ product. Chem. Biol. **9:**71–78.
26. **Vara, J., M. Lewandowska-Skarbek, Y. G. Wang, S. Donadio, and C. R. Hutchinson.** 1989. Cloning of genes governing the deoxysugar portion of the erythromycin biosynthesis pathway in *Saccharopolyspora erythraea* (*Streptomyces erythreus*). J. Bacteriol. **171:**5872–5881.
27. **Yu, D., H. M. Ellis, E. C. Lee, N. A. Jenkins, N. G. Copeland, and D. L. Court.** 2000. An efficient recombination system for chromosome engineering in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **97:**5978–5983.