Targets for pSAM2 integrase-mediated site-specific integration in the *Mycobacterium smegmatis* chromosome

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An improved integrative cassette from plasmid pSAM2 has been constructed containing plasmid *int* and *attP* genes but excluding the *xis* gene, which should result in increased stability by suppression of the excision reaction. This cassette was included in both suicide and thermosensitive plasmids and used for integration in *Mycobacterium smegmatis*. Suicide plasmids containing this cassette integrated at a single site (*attB1*) in the *M. smegmatis* chromosome. The sequence of the *attB1* site has been determined and was identified as a putative tRNA^{Pro} gene. Thermosensitive plasmids containing the cassette integrated both at the same *attB1* site and at other different sites, often giving rise to simultaneous integration at two sites. A second integration site (*attB2*) has been sequenced, which was located in the region encoding 16S rRNA of one of the two *rrn* operons of *M. smegmatis*.

Keywords: *Mycobacterium smegmatis*, plasmid pSAM2, integrase, site-specific recombination

INTRODUCTION

Genetic manipulation is a key procedure for the study of mycobacterial biology and for the engineering of the genus to produce either new vaccines or other attenuated strains. Homologous recombination in mycobacteria is a process which is not fully understood, and seems to work better in fast-growing than in slow-growing pathogenic species. Its use for genetic analysis of this genus is also hampered by the high rates of illegitimate recombination (McFadden, 1996). Site-specific recombination in mycobacteria has not been well studied either. However, this kind of RecA-independent process is simpler than homologous recombination and does not usually require extensive sequence homology between the recombining partners. A single recombinase is sufficient with the assistance of some accessory proteins that are ubiquitous in bacteria. Site-specific recombination may therefore provide a useful approach to the genetic manipulation of mycobacteria.

Mycobacteriophages such as L5 have integrases similar to λ -integrase which have been successfully used for site-specific gene integration in mycobacteria (Lee *et al.*,

1991). Integrative plasmids from *Streptomyces* are capable of autonomous replication but they can also integrate into the bacterial chromosome through a site-specific recombination event (Pernodet *et al.*, 1984). The integrative functions of plasmid pSAM2 have been well characterized (Boccard *et al.*, 1989a; Kuhstoss *et al.*, 1991; Smokvina *et al.*, 1991). A gene that encodes an integrase belonging to the λ -integrase family has been located in pSAM2 (Boccard *et al.*, 1989b). Two identical 58 bp *att* sequences have been found in the plasmid (*attP*) and in the *Streptomyces ambofaciens* chromosome (*attB*). The *attB* site overlaps a putative gene encoding a tRNA^{Pro}, conserved in many actinomycetes, including mycobacteria (Mazodier *et al.*, 1990). It is not known whether the complete tRNA gene is needed for integration or whether a part of it could be sufficient. The use of the integrative functions of plasmid pSAM2 has already been exploited for *Streptomyces* vector construction (Smokvina *et al.*, 1990; Kuhstoss *et al.*, 1991). In addition, the presence of a sequence similar to pSAM2 *attB* in the chromosome of mycobacteria suggests the use of this plasmid for the construction of mycobacterial integrative vectors (Eiglmeier *et al.*, 1991). Integration mediated by the pSAM2 integrase has been demonstrated in *Mycobacterium smegmatis* (Martín *et al.*, 1991) and shown to be site specific. Here, with the aim of improving both the stability and frequency of the integration of pSAM2-based plasmids

The GenBank accession numbers of the sequences reported in this paper are AF004309 (*attR1*), AF004310 (*attL1*), AF004311 (*attB1*), AF004312 (*attR2*), AF004313 (*attL2*), AF004314 (*attB2*) and AF004315 (*attP*).

into the *M. smegmatis* chromosome, we have developed new integrative plasmids and have analysed their integration sites in the *M. smegmatis* chromosome.

METHODS

Plasmid constructions. A 1.4 kb *MscI*–*Bam*HI DNA fragment from the plasmid pTSN39 (Martin *et al.*, 1991) containing the *int* gene and the *attP* site of pSAM2 was ligated to the *Eco*RI (Klenow filled)/*Bam*HI sites of the vector pUC18. The resulting plasmid was called pLAS3. The kanamycin-resistance gene from Tn903 obtained from Pharmacia (Kanblock) was inserted as a *Pst*I fragment to the *Pst*I site of pLAS3. This 5.4 kb plasmid was called pLAS4 and was used as a suicide integrative vector for *M. smegmatis*. An *Int*[−] derivative of pLAS4 was obtained by introducing a frameshift mutation into its *int* gene by filling the *Bgl*II site as described by Boccard *et al.* (1989b). This *Int*[−] plasmid was called pLAS4b.

A thermosensitive *Mycobacterium*–*Escherichia coli* shuttle plasmid, carrying the *int*–*attP* integrative cassette from pLAS4, was also constructed. The thermosensitive plasmid pCG63 (Guilhot *et al.*, 1992) was linearized with *Bam*HI (Klenow filled)/*Sph*I and then ligated to a 1.6 kb *Pvu*II–*Sph*I fragment from pLAS3 containing the pSAM2 integrative cassette. The resulting plasmid was called pLAS633. An *Int*[−] derivative of pLAS633, pLAS633b, was constructed as described above for pLAS4. A restriction and genetic map of these plasmids is shown in Fig. 1.

Isolation of integration events. Purified DNA (0.1 µg) of either pLAS4 or pLAS4b was used to electrotransform competent *M. smegmatis* mc²155 cells. Transformants growing at 37 °C on plates containing kanamycin at 50 µg ml^{−1} should carry the plasmid integrated in the chromosome. Integration of the thermosensitive plasmid pLAS633 was done as described by Guilhot *et al.* (1994) with slight modifications. Briefly, pLAS633 was electrotransformed into competent *M. smegmatis* mc²155 cells and stable transformants were selected on plates containing kanamycin at 30 °C. Independent trans-

formants were analysed for the presence of the plasmid and grown in 5 ml kanamycin-supplemented 7H9 medium for 48 h at 30 °C. Dilutions were plated on kanamycin-containing plates, which were incubated for 24 h at 30 °C and then shifted to 42 °C until colonies developed. Colonies obtained from these plates should carry pLAS633 integrated in the chromosome.

Isolation of chromosomal DNA and Southern blot hybridization. Total DNA from mycobacteria was purified basically as described by Ausubel *et al.* (1990). Chromosomal DNA to be used as a sequencing template was further purified in CsCl gradients. Total DNA was separated in agarose gels after treatment with restriction endonucleases and blotted on nylon membranes. Probes were labelled with digoxigenin and hybridization detected by a luminescent assay using the DIG DNA labelling and detection kit (Boehringer Mannheim).

DNA sequencing. Plasmid and chromosomal DNA sequences were determined with the *Taq* polymerase based *fmol* cycle-sequencing system (Promega), using the primers indicated in the text. Samples of 1.5 pmol primer end-labelled with [γ -³²P]ATP and polynucleotide kinase were mixed with either 6 µg chromosomal DNA or 100 ng plasmid DNA. The thermocycling programme included a denaturing step at 95 °C for 2 min, and then 30 cycles each at 95 °C for 30 s, 50 °C for 30 s and 70 °C for 1 min. The sequences obtained were compared to those in GenBank using the BLAST server at the National Centre for Biotechnology Information.

RESULTS

Plasmid integration in the chromosome of *M. smegmatis*

The suicide plasmid pLAS4 was introduced by electroporation into *M. smegmatis* mc²155. The number of colonies obtained was around 150 per µg plasmid DNA. Since this plasmid was unable to replicate in mycobacteria, we assumed that kanamycin-resistant trans-

Fig. 1. Restriction and genetic maps of the plasmids pLAS4 and pLAS633.

Fig. 2. Hybridization analysis of pLAS4 integration in the *M. smegmatis* chromosome. The complete pLAS4 was labelled and used as a probe. Sizes of the fragments were 2.6, 1.5 and 1.3 kb. (See Fig. 1 for the location of the restriction sites in pLAS4.) The 1.5 kb fragment contained the *attP* site. This fragment did not appear in the colonies carrying the plasmid integrated, suggesting that integration occurred through the *attP* site. The six clones analysed produced the same hybridization pattern (lanes 2–7), indicating that there was a single integration site in the chromosome. Lane 1, plasmid pLAS4 digested with *PvuII* and *XbaI*.

Fig. 3. Hybridization analysis of pLAS633 integration in the *M. smegmatis* chromosome. The probe was a 1.3 kb fragment containing the pSAM2 *int* gene and *attP* site that hybridized with a 2.1 kb *PstI* DNA band from pLAS633 (see Fig. 1). This band was absent in all the colonies analysed, demonstrating the absence of free replicating plasmid. Two or four hybridization bands were observed instead, suggesting the presence of one or two integrated copies of the plasmid. Five different hybridization patterns were obtained, corresponding to four different integration sites. The sample in lane 8 shows a single integration at a site that we arbitrarily called site no. 1. Samples in lanes 1, 2 and 3 were identical, and contained a single copy of pLAS633 but in a different integration site, site no. 2. Samples in lanes 4 and 9 carry two copies of the plasmid, probably one at site no. 1 and one at site no. 2. Samples in lanes 5, 6 and 7 were identical and they probably carry two plasmid copies, one at site no. 1 and the other at a new site, site no. 3. Lane 10, total DNA from *M. smegmatis* mc²155 (pLAS633) (unintegrated form) digested with *PstI*.

formants arose as a result of IntpSAM2-mediated integration in the chromosome. This was confirmed by the lack of kanamycin-resistant transformants when the same technique was repeated with the plasmid pLAS4b, an Int⁻ derivative of pLAS4.

To improve the integration frequency we performed a similar experiment with the plasmid pLAS633, containing a thermosensitive mycobacterial replicon. The plasmid was introduced into *M. smegmatis* mc²155, and kanamycin-resistant transformants were selected at 30 °C, a temperature allowing pLAS633 replication. Colonies able to grow in the presence of kanamycin at 42 °C were selected as described in Methods. With this procedure we routinely obtained around 2000 kanamycin-resistant colonies per ml culture. Experiments similar to those described above were performed with plasmid pLAS633b, an Int⁻ derivative of pLAS633. In this case, however, no kanamycin-resistant colonies could be obtained after the shift to 42 °C.

Hybridization analysis of the integration sites

The colonies obtained in the above experiments were checked for the absence of free replicating plasmids by electrophoretic analysis of plasmid lysates and by hybridization of total DNA, with negative results. Total DNA from six colonies obtained from independent experiments that should carry pLAS4 integrated into the chromosome was digested with *PvuII* and *XbaI*, blotted and hybridized with pLAS4. The results, shown in Fig. 2, indicated that the six colonies analysed were identical.

In addition, we found that all colonies contained two of the pLAS4 restriction fragments but the third fragment, containing the *attP* site, was not present and was replaced by two new fragments formed as a result of the integration event. These results demonstrated that the plasmid was integrated into the chromosome and that integration probably occurred as a result of IntpSAM2-mediated site-specific recombination.

In a similar way, total DNA from nine colonies obtained in the integration experiment with pLAS633 was digested with *PstI* and hybridized against a 1.3 kb *EcoRI*–*Bam*HI fragment from pLAS3, containing the *int*–*attP* region (identical to pLAS633). The probe hybridized to a 2.1 kb *PstI* fragment of pLAS633. This fragment did not hybridize in any of the recombinant clones, suggesting that the plasmid was indeed integrated into the chromosome. Nevertheless, the nine colonies analysed

Fig. 4. Southern hybridization analysis of pLAS633 integration with the whole plasmid as a probe to show that the complete plasmid was integrated into the *M. smegmatis* chromosome. DNAs were digested with *Sma*I and hybridized with the whole plasmid pLAS633. Lane 1, single integration at site no. 1; lane 2, single integration at site no. 2; lane 3, purified pLAS633. Plasmid pLAS633 produced five fragments upon digestion with *Sma*I, of 4.3, 2.6, 1.4, 1.0 and 0.97 kb. The two smaller fragments were not resolved in this gel, and appeared as a double band. The *attP* site is in the 4.3 kb fragment. The *rep* genes are in the 2.6 and the 0.97 kb fragments (see Fig. 1).

were not identical. At least four different hybridization patterns could be observed, indicating that more than one integration site was being used in the chromosome. In some cases only two hybridization bands were present, suggesting a single integrative event, while in other cases there were four hybridizing bands, suggesting the presence of two copies of the plasmid at different chromosomal positions (Fig. 3).

To show that the complete plasmid was integrated in the chromosome of these colonies, chromosomal DNA from two recombinants was digested with *Sma*I and probed with pLAS633. The results (Fig. 4) indicated that the whole plasmid was integrated into the chromosome. Analysis of fragment sizes indicated again that the plasmid was probably integrated through the *attP* site.

DNA sequence of the integration sites

The nucleotide sequence of pLAS4 around the *attP* site was verified using as sequencing primer the oligonucleotide TAGTCACGCAGATAGACAC, mapping at the 3'

Fig. 5. Nucleotide sequences at the plasmid chromosome junctions of integrations at the *attB1* and *attB2* sites. The top of the figure shows a model illustrating the recombination event and describing the location of the sequences. The first sequence shown is pSAM2 *attP*. The sequencing primers are white on a black background. Plasmid sequences are indicated with lower case letters and chromosomal sequences are in upper case. The 43 bp sequence common to *attP* and *attB1* is in bold. The sequences at *attR* and *attL* sites and the *attB* sequences deduced from them are in boxes.

side of *attP*, according to the sequence reported for pSAM2 *attP* by Boccard *et al.* (1989b). The second chain was sequenced with the oligonucleotides AGTGTCTA-ACTGCGTGACAA and AGAAGCACACTGGCCCAA, deduced from the sequence obtained with the first primer (Fig. 5). The same primers were used to determine the sequence of the *attL* and *attR* sequences in the clones carrying the integrated plasmids.

Chromosomal fragments containing the integrated plasmid pLAS4 were cloned taking advantage of the absence of *Kpn*I restriction sites in this plasmid. Chromosomal DNA was digested with *Kpn*I, and the fragments were self-ligated and used to transform *E. coli* cells to kanamycin resistance. With this method a 7 kb plasmid was obtained, and was used to sequence the pLAS4-

chromosome junctions using the primers described above. The sequences obtained corresponding to the *attR1* and *attL1* sites are shown in Fig. 5.

The sequence at the *attB1* site was deduced from the *attL1* and *attR1* sequences on the basis of the previously described integration mechanism of pSAM2. The sequence was compared to DNA sequences in GenBank. A region of *attB1* was found to be identical to the tRNA^{Pro} from *S. ambofaciens* and similar to other bacterial tRNA^{Pro} genes. In addition, the analysis of *attB1* with the program tRNAsearch from the PCGENE package revealed that the region between position 87 and position 160 (74 bases) of *attB1* could be a tRNA^{Pro} with the anticodon CCG.

In contrast, integration sites of pLAS633 were determined by direct sequencing of chromosomal DNA, due to the lack of restriction enzymes appropriate for the self-ligation approach. Chromosomal DNA was purified from the colonies analysed in Fig. 3. An examination of the sequences revealed that integration in colony 8 had occurred in the previously described *attB1* site. However, integration in colonies 1, 2 and 3 occurred at a new site which was identical in all three cases. The *attR2* and *attL2* sequences obtained from this site, and the sequence *attB2* deduced from them, are shown in Fig. 5. Comparison with sequences in GenBank revealed that the sequence *attB2* was identical to a region of the *M. smegmatis* gene encoding the 16S rRNA (Rogall *et al.*, 1990). Sequencing of DNA from colonies 4, 5, 6, 7 and 9 produced mixed sequencing ladders, confirming that they carried at least two plasmid copies in the chromosome and precluding the identification of other integration sites.

Stability of the integrated plasmids

Loss of the integrated plasmids was investigated through the loss of the kanamycin-resistance marker after growth in a nonselective medium. Three clones carrying each of the integrated plasmids were grown for 56 generations at either 37 °C (pLAS4) or 42 °C (pLAS633) and then plated on an antibiotic-free medium. Two hundred colonies from each clone were replicated onto kanamycin plates. All the replicas grew in the presence of kanamycin, indicating that integration was stable, and plasmid loss was lower than 0.5% after serial passage in a drug-free medium.

DISCUSSION

In this study we have demonstrated that IntpSAM2-mediated site-specific recombination was active in *M. smegmatis* if we introduced a plasmid carrying an integrative cassette containing only the integrase gene and the *attP* site; *attB* sequences were present in the *M. smegmatis* chromosome, and any accessory protein necessary for the recombination process was provided by the host.

A different integrative behaviour was observed depending on the type of plasmid used to harbour the integrative

cassette. Single insertions at an unique chromosomal location were observed for suicide integrating plasmids. This integration site coincided with the previously described pSAM2 *attB*. Double insertions involving at least three different integration sites were found when the integrative cassette was placed in a temperature-sensitive plasmid. This difference probably resulted from the time that integrase had to react in the mycobacterial cells. Non-replicating plasmids are expected to be diluted within the population, if not lost, as a result of degradation by the restriction systems of the host. As a result, the level of expression attained by the integrase will be high enough to produce only single, highly specific integrative events at the *attB1* site. However, if thermosensitive plasmids carrying the integrative cassette were allowed to grow for some time at the permissive temperature, the integrase had a longer time for expression and to act in the cells, producing multiple integrative events involving more than one integration site. We have sequenced one of the additional sites and found it in one of the two rRNA operons present in the *M. smegmatis* chromosome. This site was located precisely in the middle of the 16S rRNA gene, and was called *attB2*. Integration at the canonical *attB1* site was presumably favoured by the existence of 43 bp of sequence in common between *attB1* and *attP* sites. Although only three common base pairs were observed between the sites *attP* and *attB2*, these could be enough to facilitate recombination at the *attB2* site as previously demonstrated for integration of the bacteriophage ϕ C31 (Kuhstoss & Rao, 1991).

The existence of 43 bp of homology between the *attB1* and *attP* sites prevented determination of the exact location of the crossing-over in the IntpSAM2 recombination reaction at *attB1*. However, the observation of recombination at *attB2*, with only 3 bp of sequence homology with *attP*, allowed us to localize the crossing-over site to these 3 bp, located in the 5' end of *attP*. If it is assumed that the same site was used in the recombination at *attB1*, then the crossing-over site in *attB1* could also be precisely located. This position was coincident with the first nucleotide of the anticodon loop of the tRNA as described for other site-specific recombination systems that use tRNA genes as targets. The mycobacteriophage L5 integrates in the chromosome of *M. smegmatis* through a tRNA^{Gly} gene adjacent to a tRNA^{Pro} gene (Lee *et al.*, 1991). Interestingly, this gene was slightly different from the tRNA^{Pro} gene used for pSAM2 integration.

Plasmid integration at the 16S RNA gene of *M. smegmatis* should result in some inactivation of one of the two rRNA operons present in this species (Bercovier *et al.*, 1986). However, no evident differences in growth rate were observed between clones carrying plasmid pLAS633 integrated at *attB2* and clones with insertions at *attB1*.

Integrations of suicide and thermosensitive plasmids were both stable. Excision of pSAM2 has been observed in *Streptomyces lividans* (Simonet *et al.*, 1987) and *S. ambofaciens* (Kuhstoss *et al.*, 1989). The increased

stability of both pLAS4 and pLAS633 integrations in the *M. smegmatis* chromosome can probably be explained by the absence of the pSAM2 *xis* gene in these plasmids.

The results reported here represent a new useful tool for the integration of genes into the mycobacterial chromosome and provide further insight into the understanding of the IntpSAM2 mechanism of site-specific recombination.

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