The site-specific recombination locus of mycobacteriophage Ms6 determines DNA integration at the tRNA^{Ala} gene of *Mycobacterium* spp.

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Genetic determinants of the temperate mycobacteriophage Ms6 required for chromosomal integration were identified. DNA sequence analysis of an *attP*-containing fragment revealed an ORF encoding a protein of 372 amino acid residues with a C-terminus similar to other conserved C-terminal regions typical of the phage integrase family. Comparison of the sequences of *attP*, *attB* and bacteria–prophage junctions *attL* and *attR* showed a 26 bp common core sequence, where recombination takes place, near the 5' end of the integrase gene. Nucleotide sequence analysis of the *attB* chromosomal region showed that the core site overlaps the 3' end of the tRNA^{Ala} gene. An integration-proficient plasmid vector was constructed and efficiently inserted at the tRNA^{Ala} gene of *Mycobacterium smegmatis*, *Mycobacterium vaccae*, *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* H37Ra. It was demonstrated that Ms6 and D29 integrative systems can be used in conjunction for inserting genes at multiple loci. The site-specific integration system of mycobacteriophage Ms6 is a new tool for mycobacterial genetic analysis and is poorly related to those of the L5 bacteriophage family.

Keywords: *Mycobacterium* phage, site-specific recombination, tRNA^{Ala}, integrative vectors

INTRODUCTION

Many temperate bacteriophages integrate their DNA into the host chromosome by a site-specific recombination mechanism following the Campbell model (Campbell, 1962). The recombination event is catalysed by a phage-encoded recombinase and involves a common core sequence present in both phage (*attP*) and bacterial (*attB*) genomes. The integrase family of recombinases was identified in temperate coliphages and contains proteins that share four absolutely conserved residues: two arginines, one histidine and one tyrosine (Abremski & Hoess, 1992; Argos *et al.*, 1986). Recombinases belonging to the integrase family were found in temperate bacteriophages of both Gram-negative (Argos *et al.*, 1986; Leong *et al.*, 1986) and Gram-positive (Lee & Iandolo, 1986, 1988; Lee *et al.*,

1991; Lillehaug & Birkeland, 1993; Rausch & Lehmann, 1991) bacteria. In actinomycetes, integration of plasmids, such as pSAM2 (Boccard *et al.*, 1989), pSE211 (Brown *et al.*, 1990), pSE101 (Brown *et al.*, 1994) and SLP1 (Brasch *et al.*, 1993), is also mediated by site-specific recombinases. Recently, a different type of phage-encoded enzymes, resolvase-like proteins, were shown to mediate site-specific integration in *Lactococcus lactis* (Christiansen *et al.*, 1996) and *Streptomyces* strains (Matsuura *et al.*, 1996).

Integration into the bacterial genome by site-specific recombination can occur in genes (Christiansen *et al.*, 1994; Lee & Iandolo, 1986; Rausch & Lehmann, 1991), in intergenic regions (Lee & Iandolo, 1988; Rausch & Lehmann, 1991) or in tRNA genes (Campbell, 1992; Reiter *et al.*, 1989). Therefore, the identification of the attachment site in the host genome of a new integrative genetic element provides a genetic marker for chromosomal mapping (Canard & Cole, 1990) and may help in the identification of new genes.

The GenBank accession number for the sequence reported in this paper is AF030986.

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

Strain, plasmid, phages or primer	Description	Reference or source
Bacterial strains		
<i>Escherichia coli</i>		
XL1-Blue MRA	Designed to prepare λ EMBL4 vector	Stratagene
XL1-Blue MRA(P2)	Designed to select λ EMBL4 recombinant clones	Stratagene
Top10 F'	Eukaryotic TA cloning kit	Invitrogen
JM109	Host for M13mp vectors, <i>recA</i>	Yanisch-Perron <i>et al.</i> (1985)
<i>Mycobacterium</i> spp.		
<i>M. smegmatis</i> mc ² 155	High-transformation-efficiency mutant	Snapper <i>et al.</i> (1990)
<i>M. bovis</i> BCG	Vaccine strain 1173 P2	Pasteur Institute collection
<i>M. tuberculosis</i> H37Ra	Non-virulent strain	Pasteur Institute collection
Plasmids		
pCR3	Vector for cloning PCR products	Invitrogen
pAV8800	4.8 kb <i>Bgl</i> II <i>Ms6 attP-int</i> fragment in pUC19, Km ^r	This study
pAV1	1818 bp <i>Ms6</i> integrative cassette in pCR3	This study
pRR3	<i>E. coli-Mycobacterium</i> shuttle vector	Ranes <i>et al.</i> (1990)
pRM16	1.6 kb <i>Pma</i> CI- <i>Sma</i> I D29 <i>attP-int</i> fragment in pBK-CMV (Stratagene), Km ^r	This study
pAV Ω	273 bp <i>attP-Ms6</i> in pUC18, Strp ^r (Ω gene)	This study
Phages		
Ms6	Temperate phage from <i>M. smegmatis</i>	Portugal <i>et al.</i> (1989)
λ EMBL4	Genomic replacement vector	Stratagene
M13mp18/19	Single-stranded sequencing vector	Pharmacia
Primers (5'-3')		
C2	GATCCGGGGCCTCGTACTCG	This study
PRPAV10	CAACCCGACGGTGTTCGG	This study
P1	CGCTGTTGGTGGGCCTGATCG	This study
M1	ACACGCCGGATGAATGACC	This study

In mycobacteria, the only well-documented site-specific integration system is that of temperate mycobacteriophage L5. Integration of L5 DNA occurs in the tRNA^{Gly} gene of *Mycobacterium smegmatis* by recombination within a 43 bp sequence present in both phage and mycobacterial genomes (Lee *et al.*, 1991). The development of integrative vectors derived from the site-specific recombination of bacteriophage L5 allowed complementation experiments for genetic analysis in mycobacteria to be performed (Dussurget *et al.*, 1996; Pascopella *et al.*, 1994) and the construction of stable recombinant *Mycobacterium bovis* BCG strains for multivalent vaccination (Stover *et al.*, 1991).

To improve our understanding of lysogeny in mycobacteria, we studied the site-specific integration of Ms6 bacteriophage DNA into the mycobacterial chromosome. We have previously described some properties of mycobacteriophages Ms6, including the ability to form stable lysogens in strains of *M. smegmatis* (Portugal *et al.*, 1989; Anes *et al.*, 1992). In this paper we report the site-specific integration system of mycobacteriophage Ms6, and demonstrate that this system can direct the insertion of heterologous DNA into the tRNA^{Ala} gene of *M. smegmatis*. An Ms6-derived integrative plasmid

vector was constructed and inserted by electroporation into *Mycobacterium vaccae*, *M. bovis* BCG and *Mycobacterium tuberculosis* H37Ra genomes. The transformants were stably maintained after several generations. Stable recombinant *M. bovis* BCG vaccines expressing multiple antigens can be produced using integrative expression vectors based on the integration functions of mycobacteriophage Ms6.

METHODS

Bacteriophages, bacterial strains and plasmids. The bacteriophages, bacterial strains and plasmids used in this study are listed in Table 1. Growth conditions were as previously described (Anes *et al.*, 1992). *M. bovis* BCG and *M. tuberculosis* were grown in Middlebrook 7H9 medium, supplemented with an albumin, dextrose and catalase enrichment from Difco.

DNA manipulation and sequencing. DNA manipulations, phage and bacterial chromosomal DNA extractions, transformation, electrophoresis, restriction endonuclease digestion, Southern blotting and hybridizations were carried out using standard techniques (Sambrook *et al.*, 1989).

The 4.8 kb *Bgl*II fragment from purified Ms6 phage particles was sheared by sonication (Deininger, 1983) and inserted into

M13 sequencing vectors (Yanisch-Perron *et al.*, 1985). Single-stranded DNA was prepared from randomly chosen clones and sequenced using the Deaza G/A ⁷⁷Sequencing Mixes (Pharmacia). Sequences from over 150 clones were determined and aligned with the ASSEMBLY program of PCGene (IntelliGenetics) to form a contiguous sequence. Towards the end of the project, oligonucleotide primers were used to complete the DNA sequence on both strands, and a small number of clones were constructed using restriction enzyme cleavage of the 4.8 kb *Bgl*II fragment of the Ms6 DNA. ORFs and other features were identified using the PCGene (IntelliGenetics) programs.

Isolation of *attR*, *attL* and *attB* DNA. The chromosomal DNA of an *M. smegmatis* strain containing the integrative plasmid pAV8800 (see Table 1) was digested with *Eco*RI, extracted with phenol/chloroform and precipitated with ethanol. *Eco*RI restriction fragments (10 µg) were ligated to 1 µg λEMBL4 arms previously digested with *Eco*RI and treated with calf intestine alkaline phosphatase (Stratagene) in a 2 µl final volume. Then, 1 µl of the ligation mix was packaged *in vitro* using Gigapack II XL packaging extract (Stratagene), as recommended by the suppliers. Recombinant phages containing the DNA junctions *attR* and *attL* between pAV8800 and the chromosome were isolated by screening the λ library with the radiolabelled *attP* sequence. Two hybrid *Sal*I fragments (0.9 and 1.2 kb) were subcloned into the M13mp18 and M13mp19 vectors and sequenced (Sanger *et al.*, 1977). The oligonucleotides P1 and M1 (Table 1) were designed from the nucleotide sequences of *attL* and *attR* DNAs and used to amplify a 496 bp fragment (*attB*) from the chromosomal DNA of a non-lysogenic *M. smegmatis* strain.

PCR amplification was carried out with 100 ng chromosomal DNA, 10 pmol primers (shown in Table 1), 250 µM dNTPs, 2 mM MgSO₄ and 2 U *Taq* DNA polymerase (Gibco) in a final volume of 50 µl. Reactions were carried out for 30 cycles as follows: denaturation at 95 °C for 3 min (except in the first cycle: 5 min at 95 °C), primer annealing at 65 °C for 2.25 min and primer extension at 70 °C for 1 min.

Construction of the integrative plasmid pAV1. The integrative cassette of Ms6 was amplified by PCR of 10 ng phage DNA, using primers C2 and PRPAV10 (Table 1). PCR amplification was carried out in 100 µl 1×Gibco buffer containing 250 µM dNTPs, 2 mM MgSO₄, 10 pmol each primer and 2 U *Taq* DNA polymerase (Gibco). Thirty cycles were performed as follows on a Hybaid Omnigene thermal cycler: 1 min at 95 °C for denaturation (except 5 min at 95 °C for the first cycle), 30 s for primer annealing at 55 °C and 1.5 min at 70 °C for strand synthesis. A final cycle for 5 min at 72 °C was performed to incorporate T protruding ends. The 1818 bp PCR product was purified by phenol/chloroform extraction, precipitated with ethanol and ligated into the linearized vector pCR3 of the Eukaryotic TA cloning kit (Invitrogen).

RESULTS

Localization of the Ms6 *attP* site

To locate the phage attachment site (*attP*) on the Ms6 genome, a comparison of Southern blots of lysogenic genomic DNAs and the DNA extracted from mycobacteriophage was carried out. This revealed a new 5.8 kb *Bgl*II band in lysogens, representing the hybrid phage–host junction that was absent from the phage

Table 2. Transformation efficiency of Ms6 derived integrative vectors

Strain	Transformants (µg DNA) ⁻¹		
	pAV8800	pAV1	pRR3
<i>M. smegmatis</i>	1.3 × 10 ⁴	1.0 × 10 ⁵	> 10 ⁵
<i>M. vaccae</i>	1.2 × 10 ⁴	ND	8.0 × 10 ⁴
<i>M. bovis</i> BCG	4.0	3.0 × 10 ³	4.0 × 10 ³
<i>M. tuberculosis</i> H37Ra	ND	3.0 × 10 ³	4.0 × 10 ³

Fig. 1. Southern-blot analysis of pAV8800 transformants (lanes 1–3) with a putative restriction fragment harbouring the Ms6 *attP* region as probe (4.8 kb *Bgl*II or 0.8 kb *Sal*I fragment). The junction fragments correspond to both 12 and 6.6 kb *Eco*RI fragments in the electrophoretic profile of all transformants. The 8.8 kb *Eco*RI fragment in lane 4 is linear pAV8800 DNA.

DNA digest. This suggested that the *attP* site was located in the 4.8 kb *Bgl*II fragment (results not shown). Taking into consideration that, in the majority of the prokaryotic site-specific integration systems, the integrase (*int*) gene is close to the *attP* site, the 4.8 kb *Bgl*II fragment was cloned into the pUC19-derived plasmid pZM3, and the resulting recombinant plasmid (pAV8800) was electroporated into *M. smegmatis* mc²155. Kanamycin-resistant transformants were obtained with a transformation efficiency of 5.3 × 10⁴ per microgram of DNA (Table 2). Southern-hybridization analysis of transformant DNA probed with pAV8800 showed the same profile for all transformants, two hybrid bands (Fig. 1) that were not present in the pAV8800 digest, confirming the insertion of pAV8800 at a specific site of the bacterial genome. These data demonstrate that the 4.8 kb *Bgl*II fragment contains the complete site-specific integration locus of bacteriophage Ms6.

The localization of the *attP* site within the 4.8 kb *Bgl*II fragment was further checked by Southern-hybridization analysis using three different regions of this fragment as probes (0.8 kb *Sal*I, 0.4 kb *Sal*I and 2.4 kb *Ssp*I–*Hind*III restriction fragments) (Fig. 2a). The hybridization patterns probed with the 0.8 kb *Sal*I,

Fig. 2. Organization and nucleotide sequence of the Ms6 integrative region. (a) Restriction map of the 4.8 kb *Bgl*II fragment. The location and translational direction of the *int* gene are represented as an arrow on the right of the *attP* site. C2 and PRPAV10 are primers designed for PCR amplification of the *attP-int* region. (b) Amino acid sequence comparison of the C-terminal regions of integrases of the Int family and Ms6 Int protein. Perfectly conserved amino acids are indicated by asterisks and highly conserved amino acids by dots.

2.4 kb *Ssp*I–*Hind*III or 4.8 kb *Bgl*II fragments were the same (Fig. 1). This shows that the *attP* site is in the 0.8 kb *Sal*I fragment at the left end of the 4.8 kb *Bgl*II fragment (Fig. 2a).

Nucleotide sequence and genetic organization of the integration region

To identify the Ms6 site-specific integration locus and knowing that the *attP* site was located in the 0.8 kb *Bgl*II–*Sal*I fragment, we sequenced the 2.2 kb *Bgl*II–*Ssp*I fragment (Fig. 2a). The base composition of this region was 63 mol% G + C, which is practically identical to other mycobacterial genetic elements. A 1119 bp ORF starting with an ATG initiation codon at position 697 and ending in a stop codon at position 1815 was identified. The deduced protein sequence of 372 amino acid residues was aligned with other integrases (Fig. 2b). Although integrases have an overall low level of amino acid sequence similarity, their C-terminal regions can be aligned in two domains associated with the recognition of specific DNA sequences. The alignment revealed the presence of an arginine residue in the middle of the first domain, as well as histidine, arginine and tyrosine residues in the second domain, a characteristic shared by all members of the integrase family (Abremski & Hoess, 1992; Argos *et al.*, 1986). This observation shows that this ORF encodes the integrase of bacteriophage Ms6. A threonine residue in the first domain is also present in all the integrase sequences shown in Fig. 2(b), but is absent

Table 3. Percentage amino acid identity between different mycobacterial integrative systems

Data were obtained using the PALIGN program (structure–genetic matrix, using default settings), included in the PCGene (IntelliGenetics) software package. The GenBank accession numbers of the sequences analysed are: AF030986 (Ms6); X70352 (D29); M65194 (L5); X63702 (FRAT1); X14899 (pSAM2). The full-length amino acid sequences of the Int proteins were used in these alignments.

	Ms6	D29	L5	FRAT1	pSAM2
Ms6	–	14.11	13.95	13.21	17.20
D29	14.11	–	83.18	99.10	20.42
L5	13.95	83.18	–	82.88	18.60
FRAT1	13.21	99.10	82.88	–	20.72
pSAM2	17.20	20.42	18.60	20.72	–

from some λ Int-related proteins. Table 3 shows the amino acid identity between Ms6 and other mycobacterial Int-related proteins. The Int proteins of mycobacteriophages L5, D29 and FRAT1 are highly related, with identity reaching 99.1% between FRAT1 and D29. In contrast, the Ms6 integrase amino acid sequence is only poorly related to those of other mycobacteriophage Int proteins.

The *attP-int* region of Ms6 is delimited by two inverted repeat sequences. At the 3' terminus of the *int* gene lies

Fig. 3. Comparison of the DNA sequences of the attachment sites. The 26 bp common core sequence is indicated. The position of the tRNA^{Ala} gene is represented by an arrow below the attB sequence.

a structure resembling a rho-independent transcriptional terminator that can form a hairpin secondary structure, followed by a T-rich sequence. A second inverted repeat is located at position 480 (GenBank accession number AF030986). This repeat can form a 10 bp stem-loop, but no T-rich sequence is present close to it, indicating that it is not part of a rho-independent terminator.

Identification of the phage and bacterial attachment sites

The common core site where the recombination between the phage DNA and the bacterial genome occurs was determined from the alignment of four DNA fragments: the right and left host-phage DNA junctions (attR and attL) between the integrative vector pAV8800 and the bacterial genome; the bacteriophage attP-int region; and the attachment site in the bacterial genome, attB (Fig. 3). To identify the host-phage DNA junctions, a λ EMBL4 library was constructed from the genome of an *M. smegmatis*/pAV8800 transformant (Table 1). A recombinant phage containing the complete pAV8800 sequence, inserted into an EcoRI bacterial restriction fragment, was analysed by Southern hybridization using the attP sequence as a probe. Two SalI restriction fragments (0.9 kb and 1.2 kb) containing the putative attR and attL sites were identified and sequenced. The region that contains the attachment site in the bacterial genome was directly amplified by PCR from *M. smegmatis* using the specific oligonucleotides P1 and M1, designed from the right and left junction sequences (see Methods). Alignment of these sequences revealed a 26 bp common core site where the site-specific recombination between the phage DNA and the bacterial genome occurs (Fig. 3).

The chromosomal attB site is located in a tRNA^{Ala} gene

In the *M. smegmatis* genome, the common core region lies at the 3' end of a sequence that shows 83.8% identity with the tRNA^{Ala} of *Micrococcus luteus* and 76% with the tRNA^{Ala} of *Escherichia coli*. Moreover, this target sequence possesses a characteristic base pair (G3-U70) in the amino acid acceptor stem of the tRNA structure found in all tRNA^{Ala} species studied to date (Fig. 4) (Hou & Schimmel, 1989). These observations indicate that Ms6 DNA is inserted at the 3' end of the *M. smegmatis* tRNA^{Ala} gene.

Fig. 4. Alanine tRNA encoded by the *M. smegmatis* attB region. The folded structure of the alanine tRNA is shown. Asterisks indicate the altered bases in the tRNA^{Ala} of *M. tuberculosis*.

The common core sequence is also found in the cosmid cSCY22D7 of the gene library of *M. tuberculosis* H37Rv (supplied by Dr Stewart Cole, Institut Pasteur, Paris, France), sequenced by the Sanger Center (Cambridge, UK). Sequence analysis of the homologous segment in the *M. tuberculosis* genome indicates that both tRNA genes are present in a similar organization. However, there are two altered bases that lie within the D loop (a T to C change in *M. tuberculosis*) and the TΨGC loop (G instead of A), as shown in Fig. 4. Nevertheless, this G to A change within the core region does not appear to have a significant effect on the efficiency of integration, as determined by transformation frequencies (Table 2).

Construction of an integrative vector

To demonstrate that attP and the int gene are sufficient to direct site-specific integration into the mycobacterial chromosome, a fragment of 1818 bp containing the attP-int region was produced by PCR using primers C2 and PRPAV10 (Table 1) and cloned into the plasmid vector pCR3 (Invitrogen) (Fig. 2a). The recombinant plasmid pAV1 can replicate extrachromosomally in *E. coli* but not in mycobacterial cells because it does not contain a mycobacterial origin of replication. We

Fig. 5. Southern-hybridization analysis of *M. smegmatis* pAV1 transformants. Three independently isolated pAV1 transformants (lanes 1–3), chromosomal DNA from *M. smegmatis* (lane 4) and pAV1 (lane 5) were digested with *Bam*HI and probed with the 0.8 kb *Sal*I *attP* fragment (a) or with the *M. smegmatis attB* region (b).

searched for *in vivo* integration of pAV1 into the *M. smegmatis*, *M. vaccae*, *M. bovis* BCG and *M. tuberculosis* H37Ra genomes using electroporation and selection of kanamycin-resistant transformants. The number of transformants per microgram of DNA was 1.0×10^5 and 3.0×10^3 in *M. smegmatis* and *M. bovis* BCG/*M. tuberculosis*, respectively (Table 2). These transformation efficiencies are similar to those obtained with pRR3 (Ranes *et al.*, 1990), a shuttle plasmid that replicates extrachromosomally in *Mycobacterium* spp. and *E. coli*. Disruption of the ORF present in the Ms6 integrative region destroys the ability to transform mycobacteria. Deletions and insertions performed on the 3' end of the *int* gene lead to non-integrative activity (data not shown).

Southern hybridization analysis confirmed that the Ms6-derived integrative plasmid (pAV1) was inserted into the same site (*attB*) in all transformants examined (Fig. 5). Chromosomal DNA from three independent kanamycin-resistant transformants and from pAV1 was cut with *Bam*HI and hybridized with a radiolabelled *attP* fragment. This probe did not hybridize with chromosomal DNA from *M. smegmatis* (Fig. 5a, lane 4), while two hybridizing bands of 0.6 and 14 kb were seen in the chromosomal digests of all the kanamycin-resistant transformants (Fig. 5a, lanes 1–3). The 0.8 kb *Bam*HI restriction fragment from pAV1, containing the *attP* region, is intact in lane 5. In Fig. 5(b), hybridization with the *attB* probe (an *M. smegmatis* 0.5 kb DNA fragment obtained by PCR amplification and cloned into pCR3) revealed a 13.8 kb fragment in *M. smegmatis* DNA (lane

4) that is absent in all transformants. Instead, in these digests the two hybrid fragments (0.6 and 14 kb) were detected (lanes 1–3). The sizes of the two hybridizing bands were consistent with the predicted integration of one copy of pAV1 into the *M. smegmatis* genome at the attachment site (*attB*) present in the 13.8 kb *Bam*HI fragment (Fig. 5b, lane 4). The 6 kb restriction band in Fig. 5(b) corresponds to common sequences present in both pCR3 and pAV1 plasmids. The 3 kb fragment found in all *M. smegmatis* DNA digests might correspond to another region that contains sequences with high homology with this probe.

pAV1 was also efficiently integrated, by site-specific integration, in *M. bovis* BCG (Fig. 6a and b) and *M. tuberculosis* H37Ra (Fig. 6c) genomes. In all *M. bovis* BCG transformants, two hybrid *Bam*HI restriction fragments of 2.4 and 8.0 kb were detected using the Ms6 *attP* region as a probe (Fig. 6a, lanes 2–5). The same pattern of hybridization was obtained in *M. tuberculosis* pAV1 transformants (results not shown). A schematic representation of pAV1 site-specific integration in the *M. tuberculosis* genome is presented in Fig. 7.

In slow-growing mycobacteria (*M. bovis* BCG and *M. tuberculosis*), the plasmid-integration target site hybridizes with the *M. smegmatis attB* sequence (Fig. 6c, lane 2). However, sequences that are linked to the *tRNA*^{Ala} gene in *M. tuberculosis* are different from those of *M. smegmatis*. As a consequence, the 8.0 kb *Bam*HI hybrid band cannot be detected by probing with the 0.5 kb *attB* region of *M. smegmatis*. With this probe, only the 2.4 kb *Bam*HI hybrid band, which includes the *tRNA*^{Ala} gene, is revealed (Fig. 6c, lanes 3–5). A 7.9 kb cross-hybridizing band to *M. smegmatis attB*, common to all *M. bovis* BCG and *M. tuberculosis* patterns (Fig. 6b and c), is distinct from the 8.0 kb hybrid band.

It can be concluded that the pAV1 integration site in the host genome is conserved in fast- and slow-growing mycobacteria and that it overlaps the last 25 bases of the *tRNA*^{Ala} gene, remaining outside this gene by a single base, cytosine (Fig. 4).

The *M. smegmatis* transformants retained the integrative plasmid for long periods of time. All the transformants tested retained kanamycin resistance after 150 generations of growth in a nonselective medium.

It is notable that the transformation efficiency and the stability of the transformants diminish when the entire 4.8 kb *Bgl*II fragment is used for transformation (pAV8800). This instability was most apparent in *M. bovis* BCG transformants that could not be amplified. These observations suggest that an excisionase activity is encoded in this large integrative fragment.

To demonstrate that the ORF of this integrative cassette is absolutely required for site-specific integration and encodes an integrase, a nonsense mutation within the *int* gene was constructed by site-directed mutagenesis. It was observed that all of the mutants thus generated completely lost the ability to integrate (data not shown).

Fig. 6. Southern hybridization of *Bam*HI-digested chromosomal DNAs of *M. bovis* BCG and *M. tuberculosis* pAV1 transformants. pAV1 (lane 1), four independent *M. bovis* BCG transformants (lanes 2–5) and *M. bovis* BCG genomic DNA (lane 6) were probed with the 0.8 kb *Sal*I *attP* fragment (a) or with *M. smegmatis* *attB* DNA (b). Three *M. tuberculosis* pAV1 transformants (lanes 3–5) and *M. tuberculosis* H37Ra genomic DNA (lane 2) were also probed with *M. smegmatis* *attB* DNA (c).

Fig. 7. Schematic representation of the pAV1 site-specific integration into the *tRNA^{Ala}* gene of the *M. tuberculosis* genome. The 2.4 and 8.0 kb *Bam*HI fragments are the phage–host DNA junction restriction fragments.

Double recombination by Ms6- and D29-derived site-specific integration vectors

Since Ms6 and L5 prophages integrate at different specific loci in the mycobacterial genome, it is conceivable that complex recombinants can be made using integration-proficient vectors derived from both bacteriophages.

The *attP*–*int* region of mycobacteriophage D29, a member of the L5 bacteriophage family (Table 3), was

cloned into the pBK-CMV (Stratagene) plasmid vector, which contains the kanamycin-resistance gene *aph*; the resulting plasmid was designated pRM16. The Ms6 integration region was introduced in a pUC18-derivative plasmid containing the Ω gene, which confers resistance to streptomycin (pAV Ω). pRM16 and pAV Ω were both introduced into *M. smegmatis* by electroporation and double-recombinants were selected on plates with kanamycin and streptomycin. To confirm that, in the double-recombinants, the two plasmids were integrated at two distinct loci in the same bacterial genome, DNA from three kanamycin-resistant, three streptomycin-resistant and three double kanamycin- and streptomycin-resistant bacterial clones was isolated, digested with *Sal*I, and a comparative Southern hybridization analysis was performed using two probes, *attB*–Ms6 and *attP*–D29 (Fig. 8). The *M. smegmatis* *attB*–Ms6 region was located on a 1.0 kb *Sal*I fragment (Fig. 8a, lane 2). This fragment is not present in any of the streptomycin-resistant clones (lanes 3–5), which contain two new *Sal*I fragments of 6.2 and 5.6 kb, indicating that pAV Ω was integrated into the bacterial genome at the *attB*–Ms6 region.

Linear pRM16 (which contains the 6.4 kb *Sal*I fragment of Fig. 8c, lane 12) was disrupted to give two bands of 2.5 and 6.2 kb in all kanamycin-resistant transformants (Fig. 8, lanes 9–11). It is notable that in *M. smegmatis* and in pAV Ω transformants (Fig. 8, lanes 2–5), a 2.0 kb *Sal*I fragment is detected. This band does not appear in pRM16 transformants, suggesting that it contains the *attB*–D29 region. These results show that pRM16 and pAV Ω are integrated at two different genomic targets. The two types of integration patterns described above were found in each of the three independent double-recombinant clones (Fig. 8b, lanes 6–8), demonstrating that integrated pRM16 and pAV Ω coexist in the same genomic DNA.

Fig. 8. Southern hybridization of genomic DNA from *M. smegmatis* transformants digested with *Sall* and probed with: (a) 0.5 kb *attB*-Ms6 fragment; (b) 0.5 kb *attB*-Ms6 fragment and 0.5 kb *KpnI*-*PvuII* *attP*-D29 fragment; (c) 0.5 kb *KpnI*-*PvuII* *attP*-D29 fragment. Lanes: 1 and 13, DNA size standards; 2, untransformed *M. smegmatis*; 3-5, pAV Ω transformants; 6-8, pAV Ω and pRM16 double-transformants; 9-11, pRM16 transformants; 12, linear pRM16.

DISCUSSION

In this study we have characterized the genetic elements involved in the site-specific integration event between bacteriophage Ms6 DNA and the mycobacterial genome. Although the genetic organization of the Ms6 integration region is similar to other bacteriophages, we have demonstrated that Ms6 does not belong to the L5 mycobacteriophage family.

The bacteriophage attachment site has a high A+T content and is rich in direct and inverted repeats. These structures are also observed in *attP* sites of other temperate bacteriophages and may be the target sites for phage- and bacterial-encoded proteins such as integrase, excisionase (Weisberg & Landy, 1983) or the equivalent of *E. coli* integration host factor (Craig & Nash, 1984) and the FIS protein (Thompson *et al.*, 1987). Amino acid sequence alignments showed that Ms6 Int is weakly related with the mycobacteriophage L5 (Lee *et al.*, 1991), D29 (Peña *et al.*, 1997) and FRAT1 (Haeseleer *et al.*, 1992) Int proteins. The common core region where recombination occurs between Ms6 DNA and the mycobacterial genome is 26 bp. This sequence overlaps the 3' end of the tRNA^{Ala} gene and seems to be conserved in fast- and slow-growing mycobacteria. Furthermore, the core sequence does not show any homology to the common core sequence of bacteriophage L5, which is longer, with 43 nucleotide residues.

We demonstrated that Ms6 integrase directs the insertion at the 3' end of the tRNA^{Ala} gene in both fast- and slow-growing mycobacteria. tRNA genes are also used as insertion targets by other actinomycete genetic elements, including integrative plasmids (Omer & Cohen, 1986; Madon *et al.*, 1987; Mazodier *et al.*, 1990) and temperate bacteriophages (Lee *et al.*, 1991). The majority of actinomycete tRNA genes associated with

the *attB* site do not contain the 3' terminal CCA sequence located at position 74-76 in the structure proposed by Sprinzl *et al.* (1989). These tRNAs belong to class II-type genes which require a post-transcriptional modification by a nucleotidyltransferase to acquire a CCA 3' end. This is also true for tRNA^{Gly} and tRNA^{Ala} genes that contain the *attB* sites for the temperate mycobacteriophages L5 and Ms6, respectively.

In many genetic elements that mediate integration into tRNA genes, the common core sequence extends from the anticodon loop to the 3' end (Reiter *et al.*, 1989). For example, in bacteriophages HP1, P22 and L5, the points of strand exchange are separated by 7 bp and these regions overlap domains corresponding to the anticodon loops of the tRNA^{Leu}, tRNA^{Thr} and tRNA^{Gly} genes, respectively (Hauser & Socca, 1992; Peña *et al.*, 1996; Smith-Mungo *et al.*, 1994). In a few examples, such as phage P4 (Pierson & Kahn, 1987) and ϕ R73 (Sun *et al.*, 1991), the common core sequence is shorter, extending from the T Ψ CG loop to the 3' end. In the Ms6 *attP* site we found that two short inverted sequences are separated by 7 bp at the 5' end of the common core sequence. This structure overlaps the T Ψ CG loop of the tRNA^{Ala} gene, suggesting that the points of strand exchange lie on this stem-loop.

pAV1, an Ms6-derived integrative-proficient vector, mediates the insertion of foreign DNA into the tRNA^{Ala} gene of *M. smegmatis*, *M. vaccae*, *M. bovis* BCG and *M. tuberculosis*. The alignment of tRNA^{Ala} genes of *M. smegmatis* and *M. tuberculosis* showed that this sequence is highly conserved and we believe that this integrative vector could also be used in other mycobacterial species.

We found a high stability in all transformants generated with this method. This feature is very useful for the

construction of multivalent vaccines based on *M. bovis* BCG (Stover *et al.*, 1991). The use of *M. bovis* BCG as an antigen-delivery system requires stable expression of the foreign DNA during the propagation in the organism.

Site-specific integration vectors are also suited for the isolation of mycobacterial virulence determinants, through the stable insertion of large DNA fragments from virulent mycobacteria into avirulent strains and the search for pathogenic recombinants in animal models (Pascopella *et al.*, 1994). In this study we have demonstrated that complex recombinants can be made using integration-proficient vectors derived from Ms6 and D29 bacteriophages. This strategy may make the search for virulence determinants easier since two separate regions of the *M. tuberculosis* genome can be inserted into two different target sites on the bacterial chromosome.

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