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Method to integrate multiple plasmids into the mycobacterial chromosome

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ABSTRACT

In order to create a system in which two independent plasmids can be integrated into a mycobacterial chromosome, a mycobacterial plasmid was constructed containing the phage attachment site *attP* from the mycobacteriophage *L5* genome and additionally containing the bacterial attachment site, *attB*. This plasmid will integrate into the mycobacterial chromosome via recombination of the plasmid-borne *attP* site with the chromosomal *attB* site in the presence of a mycobacterial vector carrying the *L5* integrase (*int*) gene. The integrated plasmid has a plasmid-borne *attB* site that is preserved and will accept the integration of additional mycobacterial plasmids containing the *L5 attP* site. This system should be useful in the construction of novel mycobacterial strains. In particular, this system provides a method by which several recombinant antigens or reporter constructs can be sequentially inserted into a mycobacterial strain and subsequently tested.

INTRODUCTION

Integration-proficient vectors have previously been developed for use in mycobacteria (1). Mycobacteriophage *L5* contains a phage attachment site, *attP*, that integrates into the bacterial attachment site, *attB*, by site-specific recombination via the phage integrase, *int*, and the mycobacterial integration host factor *mIHF* (2–9). Vectors containing no mycobacterial origin of replication and having *attP* and the integrase gene of *D29* or *L5* readily integrate in single copy into the mycobacterial chromosome (10). Situations may arise where it would be useful to integrate a plasmid into a bacterial chromosome and at a later time have the option to integrate an additional plasmid into the same locus of the chromosome. We describe the development of a system by which the bacterial attachment site, *attB*, is placed on a mycobacterial plasmid containing *attP* and is integrated into the *Mycobacterium smegmatis* chromosome. The integration, via site-specific recombination of *attP* into the chromosomal *attB*, destroys the attachment sites by creating *attL* and *attR* but results in the persistence of a functional plasmid-borne *attB* within the integrated plasmid. This site can be used in subsequent studies as the acceptor of additional integrating plasmids containing an *attP* site and provides a method to sequentially integrate two plasmids within the same locus of the chromosome.

MATERIALS AND METHODS

Bacterial strains and plasmids

Mycobacterium smegmetis strain mc(2)155 and *Escherichia coli* strain SH288 were used for all experiments (11). The L5 *attP* and *integrase*-containing plasmid *pBS20* was used as the acceptor for the *attB* site. *pBS20* was cut with EcoRI and XbaI. Eighty-seven base pair primers, *BS47* and *BS48*, were created that contain the L5 *attB* site. When these primers are hybridized they create overhangs for XbaI and EcoRI. The primers were kinased, hybridized and ligated into the XbaI–EcoRI cut *pBS20* resulting in *pBS29*. *pMH94* was a kind gift from Graham Hatfull (1). *pBS11* was created by removing the *aph*-containing HindIII fragment of *pMH94* and inserting the hygromycin-resistance-containing PmeI–XbaI fragment from pJG1004 into the SmaI–XbaI sites. *pBS33* and *pBS37* were created by cutting out the integrase-containing PstI fragment of *pBS11* and *pBS29* respectively. *PBluescriptint* was obtained as a kind gift from Burkhard Springer (12).

Assays

Stability of the plasmids was tested by growing *M.smegmatis* containing either *pBS29* and *pBS11* or *pBS33* and *pBS37* in triplicate in 7H9 growth media for 20 generations in the absence of any antibiotic selection. The bacteria were then diluted into fresh 7H9 media and plated onto 7H10 agar plates to obtain single colonies. One hundred individual colonies each were then patched onto 7H10 agar containing hygromycin or 7H10 agar containing kanamycin. Colonies that did not grow had become sensitive to antibiotic.

Southern blot analysis

Chromosomal DNA was purified from *M.smegmatis* alone or containing *pBS37* or *pBS37* and *pBS33*. The chromosomal DNA was cut with MluI or PmlI and electrophoresed on a 0.7% agarose gel. The DNA was transferred to nitrocellulose

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and was then bound to a ^{32}P-radiolabeled *aph* gene fragment. The bound *aph* was then removed and the chromosomal DNA was rebound to a ^{32}P-radiolabeled *hygromycin* gene fragment.

RESULTS

In order to create a system by which multiple plasmids can be integrated into the mycobacterial chromosome, we sought to place a bacterial attachment site, *attB*, onto the kanamycin-resistant integrating plasmid *pBS20*, creating *pBS29*. Two complementary 87 base pair primers were created that contain the *attB* sequence and hybridized to one another (6). The primers were hybridized, kinased and ligated into *pBS20* cut with EcoRI/XbaI. The wild-type *attB* sequence was unable to be recovered as it was consistently rearranged in the *E.coli* acceptor cells. A mutated version of *attB* was obtained (Fig. 1) that can support site-specific recombination with *attP*. We hypothesize that the wild-type *attB* sequence of *M.smegmatis* is toxic for *E.coli* due to the presence of the mycobacterial *tRNAgly* within the *attB* site (3). The mutations in the altered *attB* site were within the sequence for *tRNAgly* (6).

pBS29 was used to transform *M.smegmatis*. The plasmid *attP* could either recombine with the plasmid-borne *attB*, resulting in a vector incapable of integrating into the mycobacterial chromosome thus being lost from the cell, or could recombine with the chromosomal *attB* site, resulting in integration of the plasmid into the chromosome and preservation of a functional plasmid-borne *attB* (Fig. 2). *pBS29* had a 10× lower transformation efficiency than parental *pBS20* (5×10^3 c.f.u./μg versus 5×10^4 c.f.u./μg, respectively). This lower transformation efficiency was probably due to the above described competition between recombination of the *attP* site with the plasmid *attB* or chromosomal *attB* sites. The hygromycin-resistant *pBS11*, which contains the L5 *attP* and L5 *int* was then used to transform *M.smegmatis* carrying *pBS29* integrated into the chromosome (Fig. 2). Transformation efficiency of *pBS11* into *M.smegmatis* carrying *pBS29* was only 5-fold less than into wild-type *M.smegmatis* (1×10^4 c.f.u./μg versus 5×10^4 c.f.u./μg, respectively), indicating the mutant *attB* serves well as an acceptor for *attP* integration. As a control, *pBS11* and *pNBV1* were transformed separately into *M.smegmatis* carrying *pBS20*. While *pNBV1*, an extrachromosomal plasmid carrying the hygromycin gene, had a transformation efficiency of 1×10^4 c.f.u./μg, transformation with *pBS11* resulted in no detectable transformants. Therefore the transformation efficiency was <1 c.f.u./0.125 μg of DNA. Thus *pBS11* has a low rate of spontaneous integration in the absence of a functional *attB* site. All transformations were performed in triplicate.

The stability of the vectors was investigated. *Mycobacterium smegmatis* containing both *pBS29* and *pBS11* was grown in 7H9 media without antibiotic selection for 20 generations and was then plated onto 7H10 plates without antibiotics. Bacteria were patched onto 7H10 plates containing kanamycin or 7H10 plates containing hygromycin to test for maintenance of the plasmids as evidenced by antibiotic resistance. Plasmids *pBS29* and *pBS11* were unstable in *M.smegmatis* as 28% of the bacteria became kanamycin sensitive and 8% became hygromycin sensitive. The bacteria that had become sensitive to kanamycin were analyzed for the presence of the kanamycin gene within the chromosome. Twenty colonies were tested by PCR using primers which amplify the *aph* gene. None of the colonies that were kanamycin sensitive had the *aph* gene amplify while those that were kanamycin resistant had the *aph* gene amplify (data not shown), indicating that the *aph*-containing *pBS29* had been lost from the chromosome and that sensitivity to kanamycin was not due to a mutation in *aph*.

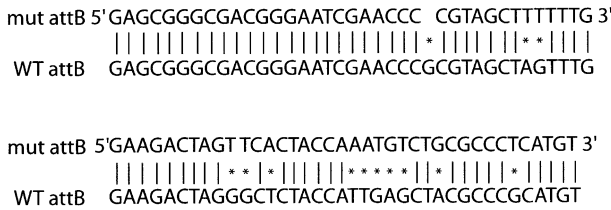

Figure 1. An *attB* site was cloned into *pBS20* creating *pBS29*. A mutated version of the *attB* site was obtained. Sequence runs 5′ to 3′ left to right, top to bottom. Vertical bars denote bases that are identical. An asterix denotes bases that have been changed.

It has recently been found that the *L5* integrase can act to excise integrated vectors at a low rate and contribute to plasmid instability (12–14). As first demonstrated by Lewis and Hatfull, placement of the integrase gene on a separate unlinked non-replicating plasmid could induce integration of plasmids carrying only the *attP* site while preventing low-level plasmid loss. To accomplish this *int* and *attP* can be placed on separate vectors. *AttP* can be placed on an integrating vector while *int* on a plasmid without a mycobacterial origin of replication. Co-transformation leads to extremely stable integration of the *attP* integration vector with subsequent loss of the *int*-containing vector (3,14). We removed the *int*-containing PstI fragments of *pBS29* and *pBS11* creating *pBS37* and *pBS33* respectively. *pBS37* was subsequently co-transformed into *M.smegmatis* with a vector devoid of a mycobacterial origin of replication and containing the L5 integrase (*pBluescriptint*). The transformants were obtained and subsequently used for transformation with *pBS33* and *pBluescriptint*. The resultant bacteria were tested for the stability of *pBS33* and *pBS37* in *M.smegmatis* by growth for 20 generations in 7H9 in the absence of antibiotic selection. In contrast to the previous work with *pBS29* and *pBS11*, *pBS33* and *pBS37* were stably maintained with no detectable (<1%) loss of kanamycin or hygromycin resistance.

Further Southern blot analysis revealed that *pBS33* is integrated into the same locus as *pBS37* (Fig. 3). Chromosomal DNA was purified from *M.smegmatis* containing no plasmid, *pBS37*, or *pBS33* and *pBS37*, and then cut with the restriction enzymes MluI or PmlI which do not cut within *pBS33* or *pBS37*. The cut chromosomal DNA was electrophoresed on a 0.7% agarose gel, transferred to nitrocellulose, and bound to ^{32}P-labeled DNA containing *aph* or *hyg*. *Mycobacterium smegmatis* does not contain the genes *aph* or *hyg* and hence does not bind the radiolabeled DNA fragments (Fig. 3). MluI and PmlI cut chromosomal DNA from *M.smegmatis* containing *pBS37* had a fragment of ~8.5 and 8.0 kb respectively that bound to the ^{32}P-labeled *aph* fragment (Fig. 3). MluI and PmlI cut chromosomal DNA from *M.smegmatis* containing both *pBS37* and *pBS33* had chromosomal fragments that bound to both ^{32}P-labeled *aph* and *hyg*

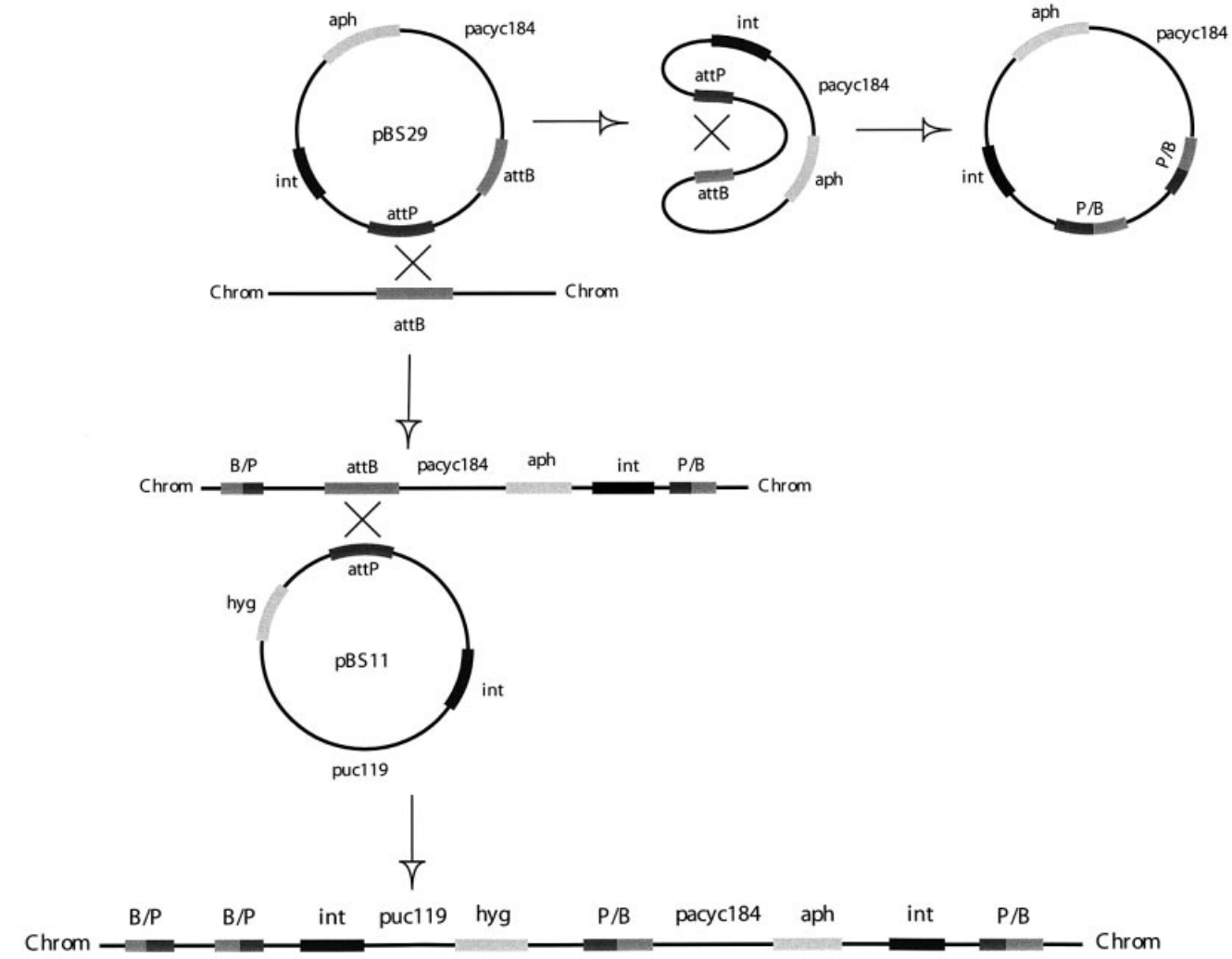

Figure 2. Schematic representation of the site-specific recombination of the *attP* site of *pBS37* with either the *M.smegmatis* chromosomal *attB* site with retention of the plasmid-borne *attB* site, or the site-specific recombination with the plasmid-borne *attB* site with the concomitant loss of the plasmid from the bacterium. Once *pBS29* is integrated into the mycobacterial chromosome, *pBS11* can undergo site-specific recombination of its *attP* site with the *attB* site of *pBS29*.

that were significantly higher in molecular weight presumably because they contained the integrated *pBS33* (Fig. 3).

Transformation frequencies of *M.smegmatis* with *pBS37* and subsequently *pBS33* are low due to the obligate use of the *int* on a second vector thus requiring two plasmids to enter the bacterium at once. This procedure, however, results in the extremely stable sequential integration of *pBS37* and *pBS33* into the mycobacterial chromosome.

DISCUSSION

It may be desirable to create recombinant mycobacterial strains that overexpress various antigens. In some cases it may be desirable to express more than one antigen. We have created a method to stably integrate more than one plasmid into the *Mycobacterium tuberculosis* chromosome. The first plasmid, containing an *attP* and an additional *attB*, was integrated into the chromosomal *attB* site with the aid of an *int* gene on a non-replicating vector. An additional plasmid containing an *attP* site was then integrated into the chromosome using the integrated plasmid-borne *attB* site and the *int* gene on a non-replicating vector. This system would make it feasible to construct and compare a pair of mycobacterial strains expressing one and two antigens, or one and two reporter constructs. We propose that this new method will be useful to researchers and aid in vaccine development.

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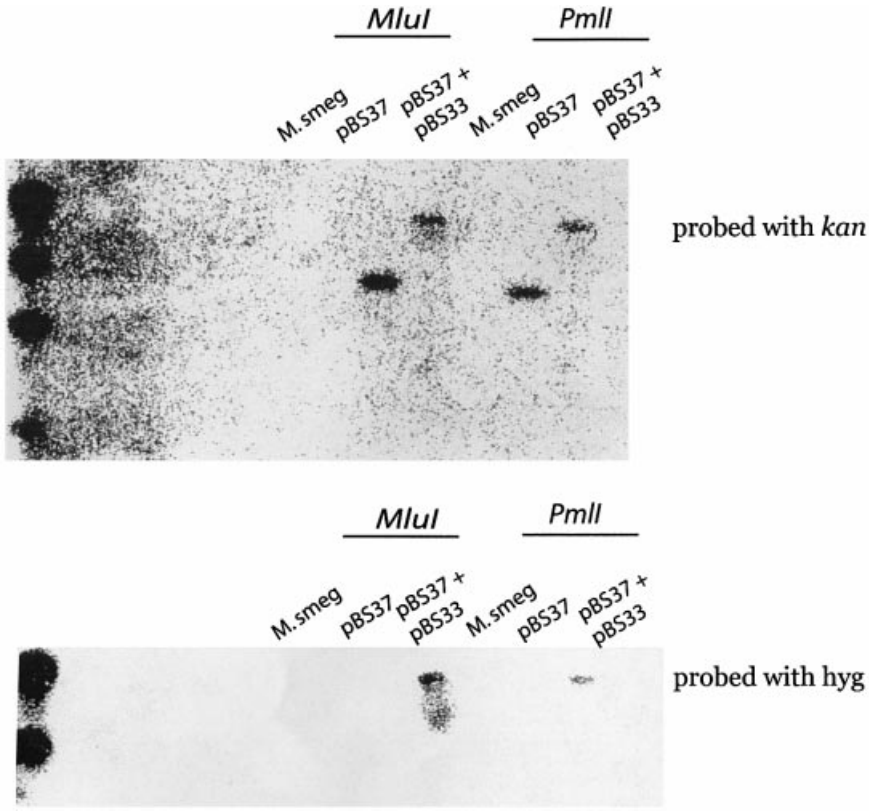

Figure 3. *Mycobacterium smegmatis* chromosomal DNA was cut with MluI or PmlI and run on a 0.7% agarose gel and transferred to nitrocellulose. The chromosomal DNA was then bound to ^{32}P-labeled *kan* or *hyg* gene fragments.

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