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Gene Replacement through Homologous Recombination in *Mycobacterium intracellulare*

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Mycobacterium intracellulare **is a slow-growing pathogenic mycobacterium closely related to *Mycobacterium avium*. In contrast to *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG, *M. intracellulare* has received little attention as a model species for studies of mycobacterial molecular biology and genetics. This study shows that *M. intracellulare* 1403 (ATCC 35761) can be transformed by electroporation with high frequencies (up to 10^6 transformants per μg of DNA), using plasmids pYT937 and pMH94 as replicative and integrative vectors, respectively. We also describe an experimental system that we used to study DNA recombination in *M. intracellulare*. First, an integrative plasmid was introduced into *M. intracellulare* 1403. A nonreplicative, nonintegrative plasmid having homology with the integrated plasmid was then introduced, and the resultant recombinants were analyzed to distinguish between events of homologous and illegitimate recombination. No illegitimate recombination occurred; in all recombinants, a single crossover between homologous regions of the two plasmids was noted. During subsequent growth of a recombinant clone, a spontaneous deletion occurred that resulted in a gene replacement on the chromosome of *M. intracellulare* 1403. The ability to construct site-specific mutations in *M. intracellulare* will provide novel insights into the biology of slow-growing mycobacteria.**

16S rRNA sequencing shows that the genus *Mycobacterium* can be divided into two large subgroups: the fast-growing and the slow-growing mycobacteria, the latter group containing the overt mycobacterial pathogens (24). Developments in the fields of mycobacterial molecular biology and genetics have been very important for studies of slow-growing mycobacteria. Electroporation enables high-frequency transformation with replicative and integrative vectors. These are shuttle vectors that facilitate the use of *Escherichia coli* and *Mycobacterium smegmatis* (a fast-growing mycobacterium) for characterization of mycobacterial genes and gene products (10). A major obstacle for mycobacterial geneticists is illegitimate recombination of DNA; this occurs with a high frequency in *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG and has prevented the construction of site-specific mutants in these species (1, 15). To date, all of the phenotypic mutants of slow-growing mycobacteria that have been studied either are spontaneous mutants or result from chemical mutagenesis. An explanation remains to be found as to why, in some mycobacteria, illegitimate recombination events outnumber those of homologous recombination. Although the RecA protein of the slow-growing mycobacteria has been the subject of extensive and detailed studies (6, 7), little is known about the molecular mechanisms of DNA recombination in these species.

Mycobacterium intracellulare is a slow-growing mycobacterium closely related to *Mycobacterium avium*; these two species constitute what is known as the *M. avium* complex (MAC) (12). Members of MAC are ubiquitous and can act as opportunistic pathogens of humans and other animals. In particular, *M. avium* has become an important agent of disseminated infection in immunocompromised individuals, especially patients with AIDS (28). Molecular studies of MAC are sparse. Apart from the basic problems encountered when working with all mycobacteria (severe clumping and long generation times), additional factors exist that may have discouraged genetic work with MAC. These include a high intrinsic resistance to many antibiotics (14) and reports of DNA restriction-modification systems (4). Many MAC isolates carry plasmids that have been exploited for epidemiological studies (20), and a MAC insertion sequence (IS*901*) has been cloned and characterized (16). However, neither insertion sequence elements nor plasmids have been used for genetic manipulations of MAC.

This study was pursued with the aim of developing genetic techniques for *M. intracellulare* such that this species could be used as a model for studies of slow-growing mycobacteria in general and virulence factors of MAC in particular. We here demonstrate that, in contrast to previous observations with *M. tuberculosis* and BCG, homologous DNA recombination events in *M. intracellulare* are of a frequency that allows for directed gene replacements. We report how, through two rounds of homologous recombination, the first site-directed mutagenesis of a slow-growing mycobacterium was achieved.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. intracellulare* (TMC [Trudeau Mycobacterial Collection] 1403, ATCC 35761) was grown on Middlebrook 7H10 agar and in 7H9 liquid medium supplemented with oleic acid dextrose complex (OADC). *E. coli* K-12 strain HB101 (3), used as a host strain for plasmids throughout the study, was grown in Luria broth or on Luria agar. Bacteria were grown at 37°C. Antibiotics used for selection were kanamycin (20 μg/ml) and gentamicin (5 μg/ml). Liquid cultures of *E. coli* were grown in a shaking incubator, whereas mycobacterial liquid cultures were grown statically (shaken every 1 to 2 days).

Plasmids. pYUB12 (23), pEP2 (22), pYT937 (8), and pMH94 (18) have been previously described. These vectors all carry the gene for kanamycin resistance (Km^r) from Tn*903* (21). pMH94Δ*int* is a *Sal*I deletion derivative of pMH94

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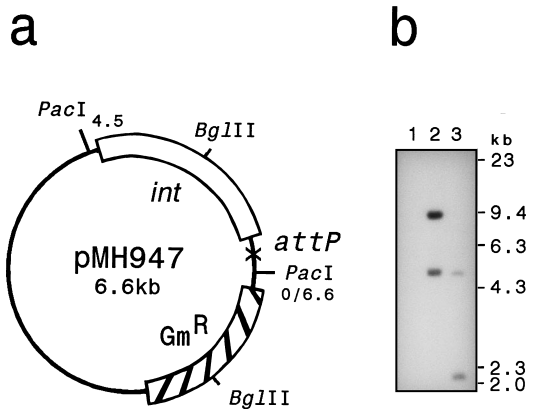

FIG. 1. (a) Schematic drawing of plasmid pMH947. *int*, integrase gene of *M. smegmatis* bacteriophage L5; *attP*, attachment site of bacteriophage L5; Gm^r, *aacC1* gene; *Pac*I and *Bgl*II, restriction sites of the respective endonucleases. (b) Southern hybridization with *Bgl*II-digested DNA from *M. intracellulare* 1403 (lane 1), 1403-947 (lane 2), and pMH947 (lane 3). The 1.7-kb fragment of pMH947 carrying *aacC1* (Gm^r) was used as a probe.

lacking the integrase (*int*) gene and the phage attachment (*attP*) site. pMH947 was constructed by cloning a 1.7-kb *Eco*RI fragment carrying the *aacC1* gene for gentamicin resistance (Gm^r) (27) from pPC110 (kindly provided by S. Lory and D. Simpson) into the *Eco*RI site of pMH94 and deleting the 1-kb *Hin*dIII fragment carrying a Km^r cassette. In addition, two *Pac*I linkers were introduced into the remaining single sites for *Hin*dIII and *Xba*I, and a *Bcl*I linker was introduced into the single *Sma*I site (at position 0.02) (Fig. 1a). pMH952 was obtained from pMH94Δ*int* by introducing the Gm^r cassette of pPC110 (*Eco*RI cloning), removing the Km^r cassette (*Hin*dIII deletion), and introducing a 1-kb *Bam*HI fragment carrying the Tn*903* gene for Km^r from pUC4K (25) into a *Bgl*II site in the *aacC1* gene.

Electroporation. Cultures of *M. intracellulare* 1403 (50 to 100 ml; optical density at 600 nm of 0.1 to 0.3) were harvested, washed twice in cold 10% glycerol (30 and 1 ml), and resuspended in 0.2 to 0.5 ml of 10% glycerol; 100 μl of the bacterial suspension was used for electroporation in a Bio-Rad GenePulser apparatus (2.5 kV, 200 Ω, 25 mF). After pulsing, the bacteria were transferred to 1 ml of 7H9 medium and grown statically for 16 to 20 h before plating on selective agar. To disperse mycobacterial cells for viable count determinations and plating, cultures were bath sonicated twice for 30 s each time, using a Tekman sonic disruptor (setting 60). Following sonication, dilutions were done in 0.9% NaCl supplemented with 0.1% Tween 80.

Mycobacterial DNA preparations. Total DNA was purified from 100-ml cultures of *M. intracellulare* (optical density at 600 nm of 0.2 to 0.6) as previously described (6) except that phenol-chloroform extractions were substituted with chloroform extractions.

DNA probes and hybridizations. Digested DNA was separated on 0.7% agarose gels and transferred to Hybond-N filters. DNA was cross-linked by UV exposure for 3 min on a standard UV illuminator. Prehybridizations were performed in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS)–5 mM EDTA–5× Denhardt's solution–100 μg of sonicated herring sperm DNA per ml at 63°C for 12 to 18 h. Hybridizations were performed under the same conditions for 18 to 24 h. The filters were probed with the Gm^r 1.7-kb *Eco*RI fragment of pMH947 (and reprobed with *Hin*dIII-digested λ DNA for size estimates of hybridizing fragments). Filters were washed in 2× SSC–0.1% SDS and 0.1× SSC–0.1% SDS (twice for 15 min each time), dried, and exposed.

RESULTS

Transformation of *M. intracellulare* 1403 by electroporation. MAC isolates available at our laboratory were screened for antibiotic resistance. *M. intracellulare* 1403 was relatively susceptible to the antibiotics kanamycin and gentamicin, which indicated that it would be an appropriate choice for molecular genetic studies. A protocol for electroporation of *E. coli* was used to determine if *M. intracellulare* 1403 could be transformed with the mycobacterial replicative vectors pYUB12 (23), pEP2 (22), and pYT937 (8) and the integrative vector pMH94 (18). The results are summarized in Table 1. No transformants were obtained with plasmid pEP2. After 9 days of incubation of kanamycin selective plates, no colonies were seen from cultures that had been electroporated with plasmid pYUB12. However, after further incubation (>14 days), large numbers of colonies started to emerge (>10^4/μg of plasmid DNA). Compared with *M. intracellulare* 1403, these colonies grew extremely slowly; they also differed distinctly from 1403 in colony morphology, being flat, rough, and white as opposed to domed, smooth, and yellowish. When restreaked on kanamycin selective agar, colonies of the flat, white type produced only up to two colonies that were flat, rough, white, and slow growing. No growth was observed in 7H9 liquid medium supplemented with kanamycin. When flat, white colonies were restreaked on nonselective agar, large numbers of colonies, which grew with the same phenotype as *M. intracellulare* 1403, were obtained. These observations indicated that the colonies resulted from abortive transformants (analogous to abortive transductants [19]), i.e., that pYUB12 was introduced into separate cells but no replication occurred.

pYT937 (8), which carries the origin of replication of plasmid pMSC262 from *Mycobacterium scrofulaceum*, produced ~6×10^4 Km^r transformants per μg of plasmid DNA when used to transform *M. intracellulare* 1403 (Table 1). These clones had the colony morphology of *M. intracellulare* 1403, though the colonies were smaller than those of 1403 grown on nonselective agar. Aliquots of total DNA prepared from two representative clones were used for electroporation of *M. intracellulare* 1403, and Km^r transformants with a small colony size were obtained. This finding confirmed that pYT937 replicated in *M. intracellulare* 1403. However, when the stability of the plasmid in cells grown in nonselective media was investigated, pYT937 was found to be highly unstable. A steady decline of Km^r colonies of about 1 log unit/week was noted (data not shown).

pMH94 carries the *int* gene and the *attP* site of the temperate bacteriophage L5 of *M. smegmatis* (18). As a prophage, L5 integrates at a bacterial attachment site (*attB*) in a tRNA^{Glu} gene; this sequence is conserved in *M. smegmatis*, *M. tuberculosis*, and BCG, and pMH94 will integrate at this site in all of these species (18). Electroporation of *M. intracellulare* 1403 with pMH94 gave 10^4 to 10^6 Km^r transformants per μg of plasmid DNA (Table 1). The transformants were indistinguishable from *M. intracellulare* 1403 in colony morphology and growth rate. No transformants were obtained with the plasmid derivative pMH94Δ*int*, which lacks *int* as well as *attP*. *M. intracellulare* 1403 was also transformed with the pMH94 plasmid derivative pMH947 (Fig. 1a), and a Gm^r clone (1403-947) was purified. From this clone, total DNA was prepared, and in a Southern hybridization, a *Bgl*II digest was probed with a fragment of pMH947 that carries the *aacC1* (Gm^r) gene. *Bgl*II-restricted pMH947 was used as a control (Fig. 1b). pMH947 carries two *Bgl*II sites, of which one is located within the *aacC1*

TABLE 1. Transformation of *M. intracellulare* 1403 by electroporation

Transforming DNA (1 μg/sample)	No. of transformants/μg of plasmid DNA[a]
None	<5
pYUB12	<5
pEP2	<5
pYT937	6×10^4
pMH94	10^4–10^6
pMH94Δ*int*	<5

[a] 7H10 plates with 20 μg of kanamycin per ml were used for selection. Plates were read after 10 to 14 days of incubation.

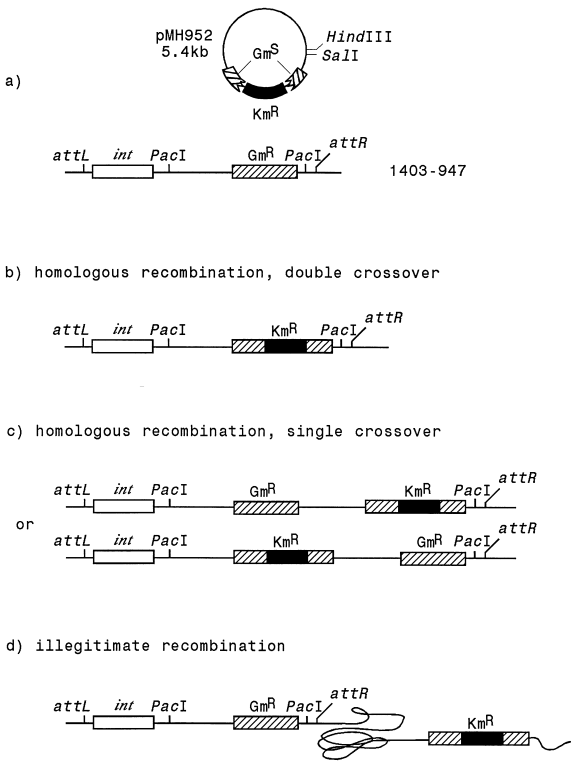

FIG. 2. Schematic representation of the study of DNA recombination in *M. intracellulare* 1403. (a) Schematic drawing of plasmid pMH952 and *M. intracellulare* 1403-947. The straight line represents the chromosome of a 1403 derivative in which plasmid pMH947 has integrated at *attB* (1403-947). *Sal*I denotes the position of a *Sal*I restriction site in plasmid pMH952; this site is a breakpoint of DNA homology between pMH952 and pMH947 (see Fig. 1a), Km^R, gene for kanamycin resistance; Gm^S, disrupted gene for gentamicin resistance; Gm^R, functional gene for gentamicin resistance; *attL* and *attR*, left and right sites of attachment (phage L5); *Pac*I and *Hin*dIII, positions of sites for the restriction endonucleases *Pac*I and *Hin*dIII, respectively. By selecting for Km^r clones following electroporation of 1403-947 with pMH952, several genotypic variants could potentially be recovered. (b) Km^r Gm^s clones resulting from a double-crossover event between homologous sequences of the chromosome and pMH952. (c) Km^r Gm^r clones resulting from a single-crossover event between homologous sequences of the chromosome and pMH952. pMH947 and pMH952 sequences are in a direct repeat. Depending on which side of the Km^r cassette the recombination takes place, two variant genotypes are possible. (d) Km^r Gm^r clones resulting from illegitimate recombination. The *attR-attL* region is identical to that of 1403-947, and pMH952 has been inserted elsewhere in the genome.

gene. The probe hybridized to two fragments of pMH947 (2.0 and 4.5 kb) and two fragments of 1403-947 (4.5 and ~8 kb). The large hybridizing fragment of pMH947 corresponded to the small hybridizing fragment of 1403-947. The small hybridizing fragment of pMH947 (carrying the *attP* site) was absent in 1403-947, where instead a large fragment (~8 kb) hybridized. These results confirmed that pMH947 had integrated on the chromosome of *M. intracellulare* 1403 and suggested that the integration resulted from a site-specific recombination between the *attP* site of the plasmid and an *attB* site on the genome of *M. intracellulare* 1403.

DNA recombination in *M. intracellulare* 1403. By introducing pMH94 and pMH947 into *M. intracellulare* 1403, we obtained clones with chromosomal genetic selectable markers (1403-94 and 1403-947, respectively). One of these, 1403-947, was used to study DNA recombination in *M. intracellulare* as outlined in Fig. 2. We constructed a plasmid, pMH952, that was homologous to pMH947 over a region of ~4.5 kb but in which the Gm^r cassette was disrupted by the introduction of a 1-kb Km^r cassette (Fig. 2a). A second breakpoint of homology was located at the single *Sal*I site of pMH952, and the plasmid was also devoid of *Pac*I, *Bcl*I, and *Bgl*II sites. pMH952 could neither replicate nor undergo site-specific integration in *M. intracellulare* 1403. Any Km^r clone isolated from a 1403-947 culture electroporated with pMH952 could result from either a double crossover of homologous recombination (Fig. 2b), a single crossover of homologous recombination (Fig. 2c), or an event of illegitimate recombination (Fig. 2d).

pMH952 was introduced into 1403-947 by electroporation, and Km^r clones were selected. After 7 to 9 days of incubation, Km^r clones were recovered both from cultures that had been electroporated with pMH952 and from control cultures to which no DNA had been added, suggesting that these clones were spontaneous Km^r mutants. After further incubation (>10 days), additional Km^r clones emerged from cultures transformed with pMH952. The number of Km^r clones obtained varied extensively between experiments, ranging from 0 to 500 in eight separate electroporations. The number of colonies on control plates (no DNA) ranged from 0 to 15.

Fifty of the Km^r clones arising after >10 days of incubation were tested by patching on gentamicin plates. All were Gm^r; i.e., no double-crossover event of homologous recombination had occurred (Fig. 2b). Total DNA was prepared from 15 additional clones and digested with *Pac*I. Southern hybridization was performed on this digested DNA, using a fragment of pMH947 carrying the *aacC1* (Gm^r) gene as a probe (Fig. 3). A single fragment of ~4.5 kb from 1403-947 hybridized (lane 2). This fragment was absent in all 1403-947-952 clones, which instead showed a hybridizing fragment of ~9.9 kb (lanes 3 to 17). This result confirmed that the entire plasmid pMH952 had been inserted through a single-crossover event of homologous recombination with the integrated pMH947 (Fig. 2c) and that the recombination had occurred in the region between the two *Pac*I sites shown in Fig. 2a. Further hybridizations revealed that in these clones the recombination had occurred on the side of the Km^r cassette with the longer region of homology (data not shown). Screening of 20 early clones by Southern hybridization demonstrated that a majority (18 of 20) did not hybridize when probed with a fragment carrying the Km^r cassette, confirming that they were spontaneous Km^r mutants. However, the remaining two clones hybridized; in a subsequent hybridization, a single-crossover event of homologous recombination between the integrated and the nonreplicative plasmids was confirmed for these clones (data not shown).

RecA-mediated homologous recombination is known to be

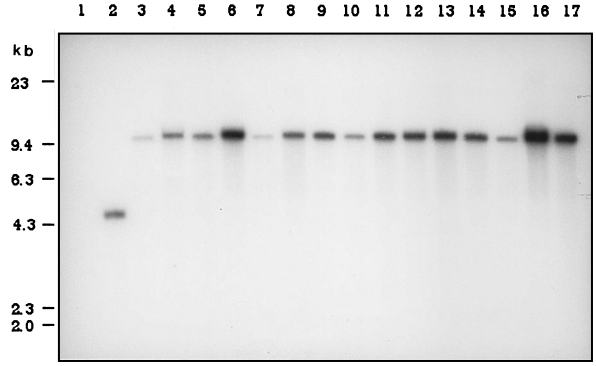

FIG. 3. Southern hybridization with *Pac*I-digested total DNA from *M. intracellulare* 1403 (lane 1), 1403-947 (lane 2), and 1403-947-952 clones 1 to 15 (lanes 3 to 17). The 1.7-kb fragment of pMH947 carrying *aacC1* (Gm^r) was used as a probe.

TABLE 2. Recombination during growth of 1403-947-952 clone 9 in nonselective medium[a]

Time (day)	No. of colonies tested	No. of colonies		
		Km^r Gm^s	Km^s Gm^r	Km^s Gm^s
3	672	0	0	0
21	784	0	18	20
28	224	0	6	4
35	224	0	8	8
42[b]	1,008	1		

[a] 1403-947-952 clone 9 was grown in 4 ml of nonselective medium and shaken daily. Every third day, 4 drops of culture was transferred to a tube with fresh medium. At time points noted, 1 ml of culture was removed, bath sonicated twice for 30 s each time, diluted, and spread on nonselective medium; 7 to 9 days later, separate colonies were patched onto kanamycin selective, gentamicin selective, and nonselective agar plates. Results from three separate cultures were combined.

[b] The sample was spread on kanamycin selective plates, and separate colonies were patched on gentamicin-kanamycin and kanamycin selective agar.

most efficient at regions where the DNA is single stranded, e.g., at DNA single- and double-strand breaks (26). To determine if the introduction of linear DNA would result in a higher number of recombinants or affect what type of recombination occurred, *M. intracellulare* 1403-947 was transformed with *Hin*dIII-linearized pMH952 (Fig. 2a). In five separate experiments, 0 to 50 Km^r clones were obtained. Sixteen late clones were investigated by Southern hybridization of *Bgl*II-digested DNA; all showed the same genotype as 1403-947-952 clones 1 to 15, and no evidence of illegitimate recombination was obtained.

Allelic exchange by homologous recombination in *M. intracellulare* 1403. We hypothesized that during growth of an *M. intracellulare* 1403-947-952 clone, a second event of homologous recombination would occur. This recombination would excise a nonreplicative plasmid that would be lost during cell division. The resulting clone either would be a re-creation of 1403-947 (i.e., exclusion of pMH952) and be Km^s Gm^r or would carry an *aacC1* gene disrupted by a Km^r cassette and therefore be Km^r Gm^s (Fig. 2b). 1403-947-952 clone 9 was grown continuously in liquid medium without antibiotic selection (Table 2). At various time points, samples were taken from the culture, sonicated (to disperse bacteria), and plated. The colonies that resulted were tested for resistance to kanamycin and gentamicin by patching on selective agar. After 21 days of culture, Km^s Gm^r clones were recovered; the relative number of such clones increased during culturing (Table 2). The first Km^r Gm^s clone was isolated only after 42 days of growth and extensive screening of colonies. Representatives of each class, 1403-947-952 clone 9B (Km^s Gm^r) and 1403-947-952 clone 9M (Km^r Gm^s), were purified, and their genotypes were determined by Southern hybridization of *Bgl*II-digested total DNA, using the *aacC1* (Gm^r) gene as a probe (Fig. 4). The numbers and sizes of hybridizing fragments from the various clones confirmed that their genotypes were as schematically presented in Fig. 5. Clone 9B (lane 4) was identical to 1403-947 (lane 2), while clone 9M (lane 5) had a single high-molecular-weight hybridizing fragment, demonstrating that this clone resulted from a gene replacement.

In addition to Km^s Gm^r and Km^r Gm^s clones, Km^s Gm^s clones were recovered during the plate screening (Table 2). To determine whether the Km^s Gm^s clones resulted from large deletions in the $tRNA^{Glu}$ region or from specific *attR-attL* crossovers, Km^s Gm^s clones recovered from four different cultures were purified and tested for the ability to be transformed with pMH947. All four clones gave Km^r transformants with frequencies comparable to that of *M. intracellulare* 1403 (data not shown), which strongly suggests that the Km^s Gm^s clones resulted from *attR-attL* deletions. The L5 *int* system has been reported to be relatively, though not absolutely, stable in *M. smegmatis* (18).

DISCUSSION

M. intracellulare has received little attention as a model species for studies of slow-growing mycobacteria. The presence of DNA restriction-modification in MAC (4) suggests that it could be difficult to establish introduced vectors in these species. However, this study (and preliminary trials with *M. avium* and a second isolate of *M. intracellulare*) show that restriction-modification systems do not prevent molecular genetic studies of MAC. *M. intracellulare* 1403 has several features that make it an isolate well suited for genetic experimentation. Whereas many isolates of MAC have a high intrinsic resistance to antibiotics used for selection of mycobacterial vectors, *M. intracellulare* 1403 was found to be relatively susceptible to both kanamycin and gentamicin. Colonial variation is a common trait of MAC, and some colonial variants are difficult to work with on agar. *M. intracellulare* 1403 shows a stable colony morphology and grows as smooth, domed, chromogenic colonies that are easy to count and patch. Using a standard protocol for electroporation, high frequencies of transformants of *M. intracellulare* 1403 were obtained.

Our genetic studies show that homologous recombination of DNA is far more common than illegitimate recombination in *M. intracellulare*, in contrast to previous findings of high frequencies of illegitimate recombination in two other slow-growing mycobacteria, *M. tuberculosis* and BCG (1, 15). Illegitimate recombination has prevented the use of gene replacement strategies for directed mutagenesis of the *M. tuberculosis* and the BCG genomes. The reason for this difference in DNA recombination between different species of slow-growing mycobacteria is not known. There is at least one difference in the DNA recombination machineries of *M. tuberculosis* and *M. bovis* versus *M. intracellulare*. The former two species have a RecA carrying a protein intron; this intron is not present in the *M. intracellulare* analog (6, 7).

Electroporation is an efficient method for transformation of mycobacteria, and both replicative and integrative vectors have been described (10). The replicon of pAL5000 (a plasmid from *Mycobacterium fortuitum* [17]) is commonly used in mycobacterial genetics (10). We made several attempts to propagate pYUB12 (23), a plasmid carrying the pAL5000 origin of replication, in *M. intracellulare* 1403; all attempts were unsuccessful. However, after an extended incubation of selective plates,

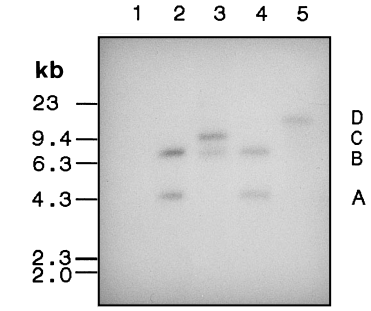

FIG. 4. Southern hybridization with *Bgl*II-digested total DNA from *M. intracellulare* 1403 (lane 1), 1403-947 (lane 2), 1403-947-952 clone 9 (lane 3), 1403-947-952 clone 9 derivative B (lane 4), and 1403-947-952 clone 9 derivative M (lane 5). The 1.7-kb fragment of pMH947 carrying *aacC1* (Gm^r) was used as a probe. Hybridizing fragments labeled A to D are indicated in Fig. 5.

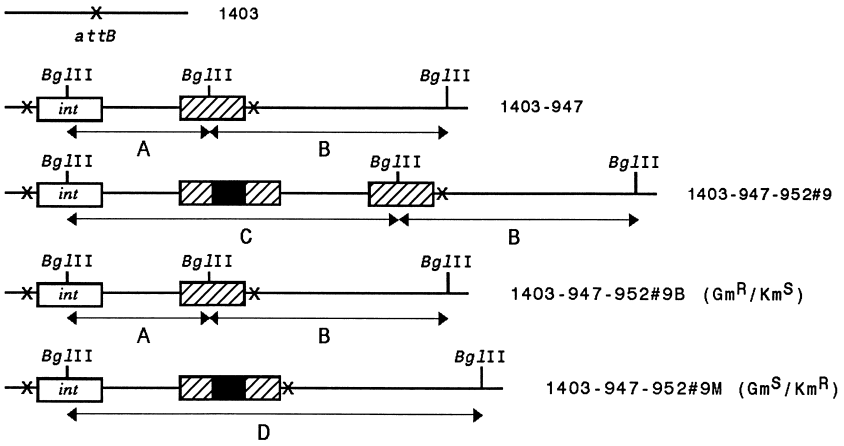

FIG. 5. Schematic presentation of the genotypes of clones used for Fig. 4. The straight line represents the chromosome of *M. intracellulare*. *int*, integrase gene of *M. smegmatis* bacteriophage L5 (open box); ×, attachment site of bacteriophage L5; hatched box, *aacC1* gene of pMH947; solid box, Km^r cassette of pUC4K; *Bgl*II, position of a *Bgl*II restriction site.

large numbers of false clones with an altered colonial morphology were obtained. These clones were most likely abortive transformants in which a single cell in each colony carried the plasmid. The colonies would consist of daughter cells (to the single cell) that survived and grew until the enzyme that provided antibiotic resistance was diluted out or, with time, degraded (analogous to abortive transduction [19]).

Plasmid pYT937 (8) could be introduced into *M. intracellulare* 1403 (Table 1). However, 1403/pYT937 clones grew notably more slowly than *M. intracellulare* 1403, and a rapid loss of the plasmid was noted when clones were grown in nonselective media. Thus, pYT937 may not be useful for gene cloning in *M. intracellulare*. Its instability makes it a candidate for the construction of a transposon delivery vector in *M. intracellulare* 1403. The only vector currently described for transposon mutagenesis in mycobacteria is a temperature-sensitive pAL5000 derivative (9), which is unlikely to work in *M. intracellulare* 1403, considering our results with pYUB12 (see above).

The integrative plasmid pMH94 integrates at a tRNA^{Glu} gene of *M. smegmatis*, *M. tuberculosis*, and BCG (18) and gave a high frequency of transformants following electroporation of *M. intracellulare* 1403. The *int-attP* cassette was required for integration, which indicates that the integration site is a corresponding *attB* sequence at the tRNA^{Glu} gene of *M. intracellulare*. pMH94 was used for the construction of the integrative cloning vector pMH947. The rationale behind constructing pMH947 was to obtain a cloning vector with gentamicin selection such that a cloned and integrated fragment could easily be recovered from the *M. intracellulare* genome (Fig. 1a). The *Bcl*I site of pMH947 would allow for cloning of *Sau*3A-*Bam*HI-*Bgl*II-digested DNA; the plasmid also has single sites for *Kpn*I and *Sac*I. Two *Pac*I sites flank the L5 *int-attP* cassette. *Pac*I has the recognition sequence TTAATTAA, which makes it an extraordinarily rare cutter in the 65 to 70% GC-rich DNA of mycobacteria, and the chance of a *Pac*I site within the insert of a recombinant plasmid is very low. *Pac*I digestion of chromosomal DNA would result in all but the *int-attP* cassette of an integrated plasmid being excised, and after religation, the resulting plasmid could be introduced into *E. coli* for characterization. The *int-attP* cassette could be reintroduced by *Pac*I cloning to allow for a second round of shuttling of DNA between mycobacteria and *E. coli*. Gentamicin selection has the advantage that pMH947 recombinant clones could be subjected to further genetic manipulation using vectors and transposons with selection for kanamycin resistance.

Apart from being a versatile cloning vector, pMH947 provided an *M. intracellulare* 1403 derivative, 1403-947, with a chromosomal genetic selectable marker. This marker was used as a target for allelic replacement in 1403-947 (Fig. 2). In our hands, the frequencies of DNA transformation and/or homologous recombination of *M. intracellulare* 1403 were not enough for the recovery of a single-step double-crossover recombinant. Instead, two separate steps were needed. First, a single-crossover event occurred between homologous regions of the chromosome and our nonreplicative plasmid. The second step was a spontaneous deletion by a second crossover event between regions of homologous DNA, whereby either the original genotype was regained or a gene replacement derivative resulted.

We found that the number of clones obtained that had undergone the first crossover event was highly variable between experiments. This may be a reflection of varying transformation frequencies. (The fact that no stable replicative vector has been reported for *M. intracellulare* makes it impossible to determine the transformation frequency of *M. intracellulare* 1403-947.) It is possible that the cells are more prone to DNA recombination at a certain stage of growth; thus, the point of harvest may be important for maximizing the yield of recombinants, as has been suggested for BCG (1). The majority of the recombinant clones were recovered after extended incubation (10 to 20 days). This observation suggests that it is critical to select for recombinants by using a bacteriostatic level of the antibiotic to allow time for cells transformed with the plasmid to undergo DNA recombination.

Once a recombinant clone (in which the nonreplicative plasmid had recombined at a homologous site on the chromosome) had been obtained and confirmed, growth under nonselective conditions was undertaken and mutants resulting from spontaneous deletions were recovered. Mycobacteria are highly hydrophobic by nature and will form clumps even when grown in media containing detergents. Any genetic experiment that includes a screening step for a low-frequency event is therefore absolutely dependent on a good dispersion protocol so that colonies studied are descendants of single bacterial cells. Evidently, our present procedure for dispersion (bath sonication) was adequate for the studies presented here. It is difficult to estimate the generation time of recombinant clones in our liquid cultures, and we have not examined the relative number of single cells in the dispersed cultures. Therefore, we have not attempted to report any frequencies for the deletion events.

The screening procedure with colony patching is time-consuming and labor-intensive. It could, in theory, be minimized if the mutagenesis was performed with a large-size fragment in which the mutation to be introduced (preferentially a point mutation or a small deletion rather than a large insertion) had a central location. The screening could be avoided if a *pyrF* mutant of *M. intracellulare* were to be constructed so that 5-fluoro-orotic acid selection could be used for the second step as described for gene exchange in *M. smegmatis* (11) and attempted for BCG (1).

The gene-exchange protocol outlined in this report is technically very simple. In theory, it could be used for knockout mutagenesis, site-specific gene manipulation, or targeted introduction of a reporter gene (e.g., *lac* [2], *lux* [13], or *xylE* [5]) in any cloned gene of *M. intracellulare*. The mutated gene would be delivered on a nonreplicative plasmid that could be supercoiled or linearized. A pUC-based plasmid was used for this study, but any plasmid lacking homologies to the mycobacterial genome would be appropriate. Antibiotic resistance genes on the gene delivery plasmid would allow for easy monitoring of the progress. If one is attempting to mutagenize an essential gene, the first step would produce a partial diploid with a wild-type allele and a mutant allele in tandem. As the excision of the wild-type allele would be lethal, a fully biased recovery of the original genotype in the screening would strongly suggest that the target gene is essential for cell viability.

In summary, we have shown that *M. intracellulare* 1403 can be transformed by electroporation with high frequency by using replicating and integrating vectors. We have demonstrated homologous recombination of DNA in *M. intracellulare* 1403 and shown how this can be used to achieve gene replacement. The possibility of constructing site-specific mutants of slow-growing mycobacteria adds yet another novel tool to the growing list available for genetic manipulation of a group of important and very interesting bacteria.

ADDENDUM

Gene replacement by homologous recombination in BCG has been recently reported (20a).

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