**Mycobacterium bovis** BCG recA Deletion Mutant Shows Increased Susceptibility to DNA-Damaging Agents but Wild-Type Survival in a Mouse Infection Model

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Pathogenic microorganisms possess antioxidant defense mechanisms for protection from reactive oxygen metabolites which are generated during the respiratory burst of phagocytic cells. These defense mechanisms include enzymes such as catalase, which detoxifies reactive oxygen species, and DNA repair systems, which repair damage resulting from oxidative stress. To (i) determine the relative importance of the DNA repair system when oxidative stress is encountered by the *Mycobacterium tuberculosis* complex during infection of the host and to (ii) provide improved mycobacterial hosts as live carriers to express foreign antigens, the recA locus was inactivated by allelic exchange in *Mycobacterium bovis* BCG. The recA mutants are sensitive to DNA-damaging agents and show increased susceptibility to metronidazole, the first lead compound active against the dormant *M. tuberculosis* complex. Surprisingly, the recA genotype does not affect the in vitro dormancy response, nor does the defect in the DNA repair system lead to attenuation as determined in a mouse infection model. The recA mutants will be a valuable tool for further development of BCG as an antigen delivery system to express foreign antigens and as a source of a genetically stable vaccine against tuberculosis.

*Mycobacterium bovis* BCG is a live vaccine against tuberculosis that has been administered to more than one billion people worldwide (6). In addition, BCG is used as a nonspecific immunotherapeutic agent in cancer treatment (38, 41). Although BCG shows a great deal of geographic variability in its ability to protect against lung tuberculosis (17), in most trials, BCG revealed significant protection against early childhood tuberculosis and disseminated manifestations of the disease (6). *M. bovis* BCG has been suggested as an ideal delivery system for expression of foreign antigens due to the long persistence of BCG in the immunized host (1, 21, 23, 33, 48, 58).

Despite its widespread use BCG is known to cause severe infections in immunocompromised individuals (24, 45, 46, 52), indicating that this organism is endowed with residual virulence properties which may be manifested in the absence of an effective immune response. It is thought that the ability of BCG to survive for prolonged periods without causing progressive infection in immunocompetent individuals is an important component of its protective properties (2).

BCG is known to have undergone significant genetic rearrangements, and recent evidence suggests that major recombinational events resulting in duplication of large segments of the chromosome have occurred and are still occurring (3, 16). Thus, if BCG is to be retained as a vaccine in its own right, developed as a carrier for generating novel vaccines and still used as an immunotherapeutic agent, it is essential that a more genetically stable strain be developed.

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Phagocytic cells are able to generate superoxide, hydrogen peroxide, and other reactive oxygen metabolites which are capable of damaging microbial DNA, proteins, and membranes (22). Microorganisms possess multiple defenses against oxidative stress including enzymes such as catalase and DNA repair systems, which repair damage resulting from oxidative stress (47). The enzyme catalase catalyzes the decomposition of toxic hydrogen peroxide to water and oxygen; a correlation between catalase activity and virulence has been observed for the *Mycobacterium tuberculosis* complex (7, 28).

RecA is the regulator of the error-prone DNA repair mechanism (SOS response) and a key element of homologous recombination (53). The RecA of the *M. tuberculosis* complex has an unusual structure, in that it contains a protein-splicing element, termed intein (10). Difficulties in achieving homologous recombination in the *M. tuberculosis* complex have, at least partially, been attributed to this unusual structure (34). However, recent data suggest that the *M. tuberculosis* RecA intein does not interfere with RecA function (18, 39). DNA repair mechanisms in general and RecA function in particular have been shown to be essential for the survival of intracellular pathogens by repairing DNA damage resulting from oxidative stress, e.g., in *Salmonella enterica* serovar Typhimurium (5).

In general, live vaccine strains should possess a RecA− phenotype. (i) Mutant recA strains are genetically more stable than their recA+ counterparts, reducing concerns about major genetic changes resulting in an altered phenotype (25). (ii) RecA acts as an inducible positive regulator of interspecies gene exchange in bacteria (31, 32); a mutant recA allele averts reversion of virulence attenuation by interspecies gene transfer (35). (iii) Mutant recA strains are more sensitive to UV irradiation and other DNA-damaging agents and thus show re-
duced persistence in the environment (35). (iv) Expression of foreign antigens is more stable in a mutant recA background (43). However, if the lack of a functional RecA protein results in a reduced ability to survive in the host, the immunogenicity of, for example, BCG could be compromised.

To generate *M. bovis* BCG *recA* knockout mutants, we wanted to adapt a technique previously established successfully for *Mycobacterium smegmatis* (44). This technique is based on the dominant-negative selectable marker *rpsL*<sup>−</sup>, which confers streptomycin sensitivity to a streptomycin-resistant host, allowing the isolation of allelic replacement mutants from single-crossover recombinants. The successful adoption of this strategy would complement other techniques to generate targeted mutants in the *M. tuberculosis* complex which make use of a combined counterselection provided by *sacB* and a thermosensitive origin of plasmid replication (42). Here we describe the generation of an *M. bovis* BCG *recA* knockout mutant by use of *rpsL*. As a counterselectable marker and report on the characterization of the mutant with respect to DNA repair, in vitro induced dormancy, and survival in vivo.

**MATERIALS AND METHODS**

**DNA manipulations and isolation of plasmids.** Standard techniques were used for DNA manipulation. All initial cloning procedures were performed with *E. coli* XL1-Blue MRF. Plasmids were prepared with a Qiagen plasmid preparation kit according to the manufacturer’s recommendations. Plasmid DNA was dissolved in Tris-EDTA buffer in concentrations of 500 to 1,000 ng/μl.

**Generation of suicide vectors.** For the generation of suicide vector precA::aph-rpsL, the following cloning steps were performed. A 5.2-kb *Apal* fragment from plasmid pEJ126 (10) containing *M. tuberculosis* recA was subcloned into the *PstI* site of plasmid pBluescript KSII (−) (Stratagene), resulting in plasmid pBluescript-recA. From this vector, a 1.3-kbp internal *PstI* fragment was replaced by a 1.3-kbp *apal* cassette isolated as a *PstI* fragment from plasmid pUC4K (Pharmacia), resulting in plasmid precA::aph. A fragment comprising the inactivated *recA* was removed by digestion with EcoRV and SpeI and cloned into pTRIA1::rpsL previously digested with *SacI*, blunt ended, and subsequently digested with *SpeI* (44), resulting in suicide vector precA::aph-rpsL. The cloning procedures were confirmed by DNA sequencing.

**Cultivation of mycobacteria.** When cultivated on solid medium, *M. bovis* BCG was grown on Middlebrook 7H10 agar supplemented with oleic acid-albumin-dextrose (OADC) (Difco) for 3 weeks at 37°C. Tween 80 was added to liquid broth 7H9-OADC to avoid clumping; incubation was performed in a roller bottle for 10 to 20 days. Antibiotics were added to the following concentrations: kanamycin, 25 μg/ml; streptomycin, 25 μg/ml.

**Southern blot analyses.** For Southern blot analyses, 200 ng of genomic DNA was digested with an appropriate restriction enzyme, separated on an agarose gel, and treated according to standard protocols. DNA was transferred to a Hybond-N membrane (Amersham) and cross-linked by UV irradiation. DNA gel, and treated according to standard protocols. DNA was transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore). The membrane was blocked with 10% nonfat milk in TTBS (20 mM Tris [pH 7.5], 0.5 M NaCl buffer containing 0.1% Tween 20) and incubated with a 1:1,000 dilution of a mouse antiserum raised against the purified *M. tuberculosis* RecA protein. A mouse antibody conjugated to horseradish peroxidase (Dako) was used as secondary antibody. After being washed with TTBS, the blot was developed with diaminobenzidine reagent solution as described previously (11).

**Generation of *M. bovis* BCG SMRI.** To generate streptomycin-resistant mutants, *M. bovis* BCG (strain Pasteur) was spread on 7H10-OADC agar containing streptomycin (20 μg/ml). After 4 weeks of incubation, single colonies were picked. The genotypes of the streptomycin-resistant strains were determined by PCR-mediated amplification of *rpsL* and *rpsL*. The sequencing of *rpsL* and *rpsL* PCR products revealed a single A→G transition in *rpsL* codon 88 conferring an amino acid change from lysine to arginine.

**Transformation of *M. bovis* BCG SMRI.** *M. bovis* BCG SMRI was grown in a 2-liter roller bottle containing 400 ml of 7H9-OADC–TWEEN until an optical density (OD) of 0.6 was achieved. One day before harvesting the cells, the antibiotic was added to a final concentration of 1.5% (vol/vol), and cells were incubated for an additional 24 h. All the following steps were performed at room temperature. Cells were harvested by centrifugation, washed several times with 10% glycerol, and finally resuspended in a volume of 5 ml. For electroporation, 400 μl of competent cells was mixed with 1 μg of supercoiled plasmid DNA and electroporated (Gene pulser II; Bio-Rad) with the following settings: 2.5 kV, 1,000 ohms, 25 μF. After electroporation cells were resuspended in 4 ml of 7H9-OADC–TWEEN and incubated for 20 h with vigorous shaking at 37°C. Following incubation, appropriate dilutions were plated on selective agar. Single colonies were picked, restreaked, and grown in liquid broth.

Transformants which had undergone a homologous single crossover were grown in liquid broth until an optical density of approximately 0.5 was achieved. Afterwards, appropriate dilutions were spread on plates containing either kanamycin or kanamycin plus streptomycin. After 4 weeks of incubation, the efficiency of counterselection was determined by dividing the number of colonies obtained on plates containing kanamycin plus streptomycin by the number of colonies obtained on kanamycin.

**EMS and MMS assay.** 7H9-OADC medium containing ethylmethane sulfonic ethyl ester (EMS) or ethylmethane sulfonic methyl ester (MMS) was inoculated with a 1/50 volume of a freshly grown culture (OD, 0.5). After 6 days of incubation, the OD was determined. 7H9-OADC medium without alkylating chemicals served as a control.

**UV irradiation assay.** For the UV irradiation assay, 100-μl aliquots of a freshly grown culture (OD at 600 nm [OD<sub>600</sub>] = 0.1) were placed in an inverted lid of a 24-well culture plate and put under a standard germicidal UV lamp (distance, 20 cm). Cells were irradiated for different time periods, and samples were removed and plated. Mean values and standard deviations from three independent experiments are shown. Appropriate dilutions of each culture were plated out in duplicate.

**In vitro induced dormancy.** Experiments were performed as described previously using an in vitro dormancy model (29). Briefly, screw-cap test tubes (20 by 125 mm) with a total fluid capacity of 25.5 ml were used. An early-log-phase culture was diluted to an OD<sub>600</sub> of 0.005 in a total volume of 17 ml of Dubos broth (Difco). Solid caps with latex liners were tightly screwed down (limited gas exchange). Cultivation of mycobacteria was divided to an OD<sub>600</sub> of 0.8; 0.2 ml (approximately 10<sup>8</sup> CFU) was inoculated into the tail vein. Mice were sacrificed according to ethical guidelines at various times (three mice per BCG strain for each time point), and the spleens and lungs were removed, weighed, and homogenized. The suspensions were serially diluted in saline and then plated on 7H10 agar supplemented with OADC. The plates were incubated for 3 weeks. The results were calculated and expressed as CFU per organ.

**RESULTS**

**Generation of *M. bovis* BCG *recA*.** Strain *M. bovis* BCG SMRI (for a list of strains and plasmids, see Table 1) is a streptomycin-resistant derivative of *M. bovis* BCG; this strain has a mutation in *rpsL* codon 88 (Lys→Arg), a mutation known to confer a streptomycin-resistant phenotype.

For generation of *recA* knockout mutants, *M. bovis* BCG SMRI was transformed with suicide vector precA::aph-rpsL. This vector carries an *M. tuberculosis recA* fragment; part of the
coding region and part of the intein coding region of this fragment have been replaced by a kanamycin resistance cassette. The wild-type rpsL flanking the inactivated target gene facilitates isolation of allelic replacement mutants (44). Transformants were selected on medium containing kanamycin (efficiency in the range of 10 to 50 transformants per μg of plasmid DNA compared to 2 × 10^4 transformants per μg of plasmid DNA when using plasmid pMV361 as a control). Transformants obtained with plasmid precA::aph-rpsL were chosen at random for further investigations.

**Genetic analysis of transformants and counterselection.** Nine of 11 kanamycin-resistant (KanR) clones investigated contained the aph cassette, indicating that these colonies arose from transformation with the suicide vector rather than representing spontaneous KanR mutants (data not shown). Genomic DNA was isolated and investigated by Southern blot analyses using a recA fragment as the probe (Fig. 1). Two of the nine transformants revealed a pattern indicative of a 5' single crossover at the recA locus (a 2.6-kbp fragment and a 4.9-kbp fragment; the wild type shows a 2-kbp fragment) and were subjected to counterselection on medium containing kanamycin plus streptomycin. Transformants resistant to kanamycin plus streptomycin (frequency, 10^{-2} to 10^{-5}) were screened by PCR for the absence of the deleted recA intein coding sequence (7 of 40 transformants were investigated; data not shown). recA deletion was confirmed by Southern blot analyses. Following digestion with Smal the kanamycin- and streptomycin-resistant transformants revealed a single 2.6-kbp fragment (Fig. 1), indicating a second crossover event, resulting in loss of the functional recA copy. For further investigations, one of the recA mutants was chosen.

**Western blot analyses.** Western blot analyses using an antiserum raised against M. tuberculosis RecA was performed with the parental strain, a single-crossover transformant, and the corresponding knockout mutant (Fig. 2). A single band with a molecular mass of approximately 40 kDa corresponding to the mature, spliced form of M. tuberculosis RecA was observed in extracts of both the parental strain and the single-crossover transformant but not in the recA mutant, indicating the ab-

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**TABLE 1. Strains and vectors used**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
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<tr>
<td>E. coli XL1-Blue MRF</td>
<td>Stratagene</td>
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<tr>
<td>M. bovis BCG strain Pasteur</td>
<td>ATCC 35734</td>
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<td>M. bovis BCG SMR1</td>
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<td>M. bovis BCG recA'/recA::aph</td>
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<td><strong>Plasmids</strong></td>
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<td>pUC4K</td>
<td>10</td>
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<tr>
<td>pBluescript KSII(−)</td>
<td>Pharmacia</td>
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<tr>
<td>ptrpA-1-rpsL</td>
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<td>pBluescript-recA::aph</td>
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**FIG. 1.** (A) Southern blot analysis of the recA locus. Lane 1, parental M. bovis BCG; lane 2, single-crossover transformant obtained by transformation with plasmid precA::aph-rpsL; lanes 3 to 5, recA (recA::aph) knockout mutants obtained after counterselection of a single-crossover transformant on medium containing kanamycin plus streptomycin. Approximately 200 ng of genomic DNA was digested with Smal and hybridized to a recA probe. (B) Schematic drawing of the BCG recA locus: the wild-type locus is shown along with the vector used for inactivation, a 5' single-crossover transformant, and a knockout mutant. S, Smal recognition site.
The ability of tests with different DNA-damaging agents were performed to investigate the physiological effects of their increased sensitivity to DNA-damaging agents (26). One of the most noticeable phenotypes of recA mutant strains is their sensitivity to DNA-damaging agents. Approximately 30 μg of protein was separated on a polyacrylamide gel, transferred to a polyvinylidene fluoride membrane, and probed with an antibody raised against M. tuberculosis RecA.

The presence of 0.05% EMS only slightly inhibited growth of the recA+ strain. In contrast, growth of the recA mutant strain was reduced 10-fold compared to that of the control. Similar results were obtained with MMS. At a concentration of 0.006% MMS growth of the recA+ strain was not significantly affected, whereas growth of the recA mutant strain was inhibited by approximately 90%. In the absence of DNA-alkylating agents the growth rates of the recA+ and the recA mutant strains were indistinguishable. The single-crossover transformant essentially behaved like the recA+ strain in the presence of DNA-damaging agents.

(ii) M. bovis BCG recA mutants are sensitive to UV irradiation. Irradiation with UV light is frequently used to compare the levels of effectiveness of DNA repair mechanisms. Inactivation of recA rendered BCG sensitive to UV irradiation: the recA strain exhibited a 10,000-fold-decreased viability after 30 s of irradiation compared with a 25-fold decrease for the recA+ strain (Fig. 3). The survival rate decreased dramatically at higher irradiation dosages; very few mutant survivors were detected after 60 s of irradiation (<0.0001%), compared to about a 0.01% survival rate for the parental strain. These results support previous observations that, despite the presence of an intein, M. bovis BCG RecA is functionally expressed and promotes DNA repair mechanisms in mycobacteria (18, 40).

(iii) In vitro induced dormancy and metronidazole resistance. The obligate aerobe M. tuberculosis complex can enter an anaerobic dormant state in which it survives for extended periods of time (54). To determine whether recA is involved in the dormancy response, we investigated recA− and recA+ mutant strains with respect to their abilities to survive in an in vitro dormancy model (57). Mycobacteria were grown in sealed and stirred tubes to achieve self-generated oxygen depletion, a signal which triggers entry into the dormant state. Growth of the cultures was monitored by measuring the OD; the number of cells surviving oxygen depletion was determined by plating. Self-generated oxygen depletion was judged by fading or decolorization of the indicator methylene blue.

With respect to OD, recA− and recA+ mutant strains behaved identically (Fig. 4). A plateau was achieved after approximately 8 days. After day 9 and after day 15, respectively, fading and decolorization of the oxygen indicator methylene blue were observed in each of the cultures. After incubation for an additional 5 days, cultures were harvested and the numbers of...
CFU were determined (Fig. 5). The \( \text{recA} \) genotype did not affect the number of viable cells after oxygen depletion, indicating that RecA does not affect in vitro induced-dormancy survival.

Metronidazole is the first lead compound active against dormant \( M. \text{tuberculosis} \). Metronidazole does not affect the growth of aerobically grown cultures but acts exclusively on the anaerobically grown \( M. \text{tuberculosis} \) complex (56). We next investigated the effect of metronidazole on the growth of \( \text{recA}^+ \) and \( \text{recA} \) mutant \( M. \text{bovis} \) BCG in the in vitro dormancy model described above. Compared to \( \text{recA}^+ \) \( M. \text{bovis} \) BCG, the \( \text{recA} \) mutant showed increased susceptibility to metronidazole, i.e., the number of viable cells was reduced about 20-fold by metronidazole for the \( \text{recA}^+ \) strain, but a 100-fold reduction in the number of viable cells was observed in the \( \text{recA} \) mutant (Fig. 5).

**Survival of the \( M. \text{bovis} \) BCG \( \text{recA} \) mutant in mice.** DNA repair mechanisms in general and RecA in particular are important virulence factors for the survival of intracellular pathogens, such as \( \text{Salmonella} \) (5). Although wild-type \( M. \text{bovis} \) BCG does not cause a progressive infection in mice, it does persist in tissue for a significant period of time. We thus investigated the effect of \( \text{recA} \) inactivation on the survival of \( M. \text{bovis} \) BCG in a high-dose animal infection model. BALB/c mice were infected by intravenous injection with \( M. \text{bovis} \) BCG \( \text{recA}^+ \), the single-crossover transformant, and the \( \text{recA} \) mutant strain. After days 1, 28, and 84, organs (spleen and lung) were removed and homogenized and appropriate dilutions were plated on 7H10 agar. The plates were incubated for 3 weeks, and the numbers of CFU per organ were calculated. As shown in Fig. 6, the knockout mutant showed no difference in the course of infection compared to the wild-type strain, either in the spleens or in the lungs of infected BALB/c mice. These data indicate that RecA is not essential for survival in the high-dose mouse infection model.

Athymic mice were inoculated to assess the virulence of the \( \text{recA} \) mutant in a more progressive infection model. As shown in Fig. 7, a comparison of the three strains in athymic mice did not show significant difference in growth in spleens or lungs.

**DISCUSSION**

Infections with \( M. \text{tuberculosis} \) are a major cause of human morbidity and mortality. Vaccines are the most cost-effective intervention to prevent disease, and \( M. \text{bovis} \) BCG is a widely...
M. bovis used vaccine; it can be given as a single dose at birth and confers long-lasting immunity. M. bovis BCG not only is used as vaccine against tuberculosis but also offers great potential for innovative approaches for development of polyvalent vaccines (1, 33, 48, 58). Genetic stability and in vivo persistence are of special importance for the use of live vaccines; a mutant BCG which is rapidly eliminated is unlikely to be an effective vaccine (2).

RecA is a multifunctional and ubiquitous protein involved both in general recombination and in DNA repair. Thus, RecA-mediated DNA repair mechanisms have been shown to be essential for intracellular survival and persistence (5), while simultaneously RecA-dependent recombination mediates genetic rearrangements resulting in increased genetic instability (25). As an inducer of the SOS response RecA regulates at least 20 genes, most of which are usually suppressed by LexA (36); mycobacteria possess the key elements of a functional SOS system (13, 37).

Investigations of M. tuberculosis RecA so far have been performed in vitro, with Escherichia coli and M. smegmatis (10, 11, 12, 18, 27, 40, 51). These investigations demonstrated that the mature RecA is able to promote DNA repair mechanisms and homologous recombination (18, 40). However, as no iso-
genic M. tuberculosis complex mutants were available, the possibility that a homologous gene, e.g., radA (Rv3585) (8), could compensate for RecA function could not be excluded. The results presented here show that BCG recA mutants are sensitive to DNA-damaging agents (DNA alkylation and UV irradiation) and thus have an in vitro phenotype similar to those of recA mutants of other species (36). These investigations suggest that M. bovis BCG has a nonredundant recA gene, which is essential to promote DNA repair mechanisms.

M. bovis BCG and M. tuberculosis are able to enter a dormant state (29). This response is triggered by slow self-generated depletion of oxygen (54). Entry into the dormant state is an adaptive process, as sudden oxygen depletion results in cell death (55). Experiments in an in vitro dormancy model were performed to investigate whether RecA is involved in dormancy survival. As the numbers of viable bacterial cells after oxygen depletion were essentially identical for the recA1 and recA mutant strains, RecA does not appear to play an essential role in entry, survival, or exit from the dormant state.

Originally developed as an antiparasitic agent, metronida-
zo12zole has been recognized as an effective drug for treatment of infections with anaerobic bacteria. Metronidazole is also active against M. tuberculosis and M. bovis BCG when grown under anaerobic conditions (29, 56). Our investigations in the in vitro dormancy model demonstrated that M. bovis BCG recA knock-out mutants show increased susceptibility to metronidazole. These results support previous findings that metronidazole acts by damaging DNA after reduction to form a toxic metabolite (15).

Numerous reports have demonstrated that recA represents an important virulence factor: RecA is involved in stress survival (14), mediates aerotolerance in microaerophilic bacteria (9), induces production of colicins, pyocins (36), and extracellular degradative enzymes (30), and mediates amplification of toxin genes (19). Most notably, Salmonella recA mutant strains are highly attenuated, both in cultured macrophage cells (4) and in a mouse infection model (5). This effect has been attributed to the DNA-damaging effect of the oxidative burst and the reduced ability of the mutants to perform DNA repair (47). However, a recA mutation does not necessarily affect bacterial virulence, as demonstrated, e.g., for Campylobacter jejuni (20), Corynebacterium pseudotuberculosis (43), Brucella abortus (50), and some Vibrio cholerae strains (49). It was thus of interest to investigate the contribution of RecA to the survival of M. bovis BCG in mice. The results indicate that RecA does not contribute to the establishment and maintenance of infection. This is an important finding since persistence of BCG following vaccination is thought to be a significant contributory factor to its immunogenicity; a mutant BCG which is rapidly eliminated is unlikely to be an effective vaccine.

In analogy to M. tuberculosis (3, 16) differences between strains of M. bovis BCG can be attributed to RecA-dependent genetic rearrangements. The BCG recA mutant constructed in this study is deficient in the major recombination pathway but is not affected in its in vivo survival. Due to the increased genetic stability of recA mutants, this strain is of interest as a tuberculosis vaccine and for further development of M. bovis BCG as an antigen delivery system for expression of foreign antigens.

The BCG mutant was generated by adapting an rpsL-based strategy which has previously been used to generate allelic exchange mutants in M. smegmatis (44). As transformation efficiencies are a critical issue, the original strategy has been modified to a two-step allelic exchange procedure incorporating successive steps of positive and negative selection. The procedure described is a valuable alternative to strategies which use sacB and thermosensitive vectors for the generation of allelic exchange mutants in mycobacteria (42).

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