APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2008, p. 1687–1695 Vol. 74, No. 6
0099-2240/08/$08.00+0 doi:10.1128/AEM.01208-07

Demonstration of Allelic Exchange in the Slow-Growing Bacterium *Mycobacterium avium* subsp. *paratuberculosis*, and Generation of Mutants with Deletions at the *pknG*, *relA*, and *lsr2* Loci[▽]

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Received 18 May 2007/Accepted 30 December 2007

***Mycobacterium avium* subsp. *paratuberculosis* is the causative pathogen of Johne's disease, a chronic inflammatory wasting disease in ruminants. This disease has been difficult to control because of the lack of an effective vaccine. To address this need, we adapted a specialized transduction system originally developed for *M. tuberculosis* and modified it to improve the efficiency of allelic exchange in order to generate site-directed mutations in preselected *M. avium* subsp. *paratuberculosis* genes. With our novel optimized method, the allelic exchange frequency was 78 to 100% and the transduction frequency was 1.1×10^{-7} to 2.9×10^{-7}. Three genes were selected for mutagenesis: *pknG* and *relA*, which are genes that are known to be important virulence factors in *M. tuberculosis* and *M. bovis*, and *lsr2*, a gene regulating lipid biosynthesis and antibiotic resistance. Mutants were successfully generated with a virulent strain of *M. avium* subsp. *paratuberculosis* (*M. avium* subsp. *paratuberculosis* K10) and with a recombinant K10 strain expressing the green fluorescent protein gene, *gfp*. The improved efficiency of disruption of selected genes in *M. avium* subsp. *paratuberculosis* should accelerate development of additional mutants for vaccine testing and functional studies.**

Johne's disease (paratuberculosis) is a chronic wasting disease of the intestine of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis*. It causes significant economic loss to animal producers, especially in the dairy industry, due to an increase in forage consumption, decreased milk production, and early culling due to poor health of affected animals (6, 24, 31). This disease has been difficult to control because of the lack of sensitive specific diagnostic assays and the lack of an efficacious vaccine. Available diagnostic assays, such as *M. avium* subsp. *paratuberculosis* antigen enzyme-linked immunosorbent assays and the gamma interferon assay, vary in the capacity to detect infected animals in the early stages of the disease (29). This is a major problem since infected animals begin to shed *M. avium* subsp. *paratuberculosis* in their feces early in the course of the disease before clinical signs appear. Available vaccines have been shown to reduce the severity of pathology but not to stop the shedding of bacteria (20). Consequently, there is a continuing need to develop both a better diagnostic assay and a better vaccine that, at a minimum, stops shedding of bacteria during the productive life of dairy cattle.

An important prerequisite before strategies can be developed to control this disease is a better understanding of the molecular mechanisms of *M. avium* subsp. *paratuberculosis* pathogenesis. To increase our knowledge of the genetic basis of virulence and persistence in the host and to develop efficacious potential live vaccines, an efficient method for generating targeted gene knockouts is urgently needed. In contrast to the successful gene disruption in fast-growing mycobacteria, such as *M. smegmatis* (8, 10, 26, 36), gene disruption in slow-growing mycobacteria has traditionally been inefficient, in part due to the high frequency of illegitimate recombination and the characteristic clumping of cells in culture (1, 25, 28).

Recent major advances in the methods used for genetic manipulation have overcome some of the difficulties encountered in attempts to disrupt genes in slow-growing mycobacteria. The ability to selectively disrupt genes of interest has improved our understanding of pathogenic mycobacterial virulence based on specific gene function. For example, allelic exchange using either linear DNA fragments or suicide vectors, insertion mutagenesis using transposons, and specialized transduction have been successful in *M. tuberculosis* and *M. bovis* (2–4, 7, 11). Although random transposon mutagenesis has been reported for *M. avium* subsp. *paratuberculosis* (12, 21, 35), directed allelic exchange mutagenesis has remained intractable. The inability to inactivate specific genes has impeded progress in the use of the recently completed genome sequence of *M. avium* subsp. *paratuberculosis* K10 (27). A new methodology or adaptation of an existing methodology to generate *M. avium* subsp. *paratuberculosis* allelic exchange mutants would provide an opportunity to gain insight into specific gene functions related to virulence and, importantly, would increase the potential for developing an effective live attenuated *M. avium*

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▽ Published ahead of print on 11 January 2008.

TABLE 1. Plasmid, phage, and bacterial strains used in this study

Bacterial strain, phage, or plasmid	Description	Source or reference(s)
Bacterial strains		
E. coli Top10	Commercial strain used as a cloning host	Invitrogen
E. coli HB101	*E. coli* strain without F factor	9
M. smegmatis mc^2 155	High-frequency transformation derivative of *M. smegmatis* mc^2 6	36
M. avium subsp. *paratuberculosis* K10	Virulent clinical isolate and sequencing project strain	13, 18
M. avium subsp. *paratuberculosis* K10-GFP	*M. avium* subsp. *paratuberculosis* K10 containing pWES4 for GFP expression	22
Phage or plasmid		
phAE87	Conditionally replicating shuttle phasmid derivative of TM4	4
pYUB 854	Derivative of pYUB572; *bla* gene was replaced with *hyg* cassette	5

subsp. *paratuberculosis* vaccine by disrupting genes needed for in vivo survival.

Here we report the first case of targeted gene disruption and improvement in the efficiency of allelic exchange mutagenesis in a virulent clinical isolate of *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *paratuberculosis* K10, the prototype isolate used to sequence the *M. avium* subsp. *paratuberculosis* genome (13, 18, 27). The improvement in the efficiency of allelic exchange mutagenesis was demonstrated by selective disruption of orthologues of two genes known to contribute to virulence in *M. tuberculosis* and *M. bovis* (*relA* and *pknG*) (17, 38) and one gene (*lsr2*) known to affect colony morphology, biofilm formation, and antibiotic resistance in *M. smegmatis* (14, 15). In addition, we describe the use of the improved technology to generate deletion mutants expressing green fluorescent protein (GFP), using GFP-tagged *M. avium* subsp. *paratuberculosis* K10 (*M. avium* subsp. *paratuberculosis* K10-GFP) as the parental strain (22). This study established an efficient allelic exchange system for use with *M. avium* subsp. *paratuberculosis* that can be used to elucidate specific gene functions and develop novel live attenuated vaccines.

MATERIALS AND METHODS

Bacterial strains, vectors, and culture conditions. All strains of bacteria, plasmids, and phages used in this study are listed in Table 1. The *Escherichia coli* Top10 strain was cultured in LB broth or on LB agar (Difco, Maryland) and was used for cloning homologous regions and for construction of allelic exchange substrates (AESs) in pYUB854. The *E. coli* HB101 strain was used in an in vitro λ-packaging reaction (Gigapack III; Stratagene, California). *M. smegmatis* mc^2 155 was grown in basal Middlebrook 7H9 (Difco, Maryland) broth medium containing 0.05% Tween 80 and prepared for generation of phage lysates as previously described (9). *M. avium* subsp. *paratuberculosis* strains were grown in Middlebrook 7H9 medium containing 6.7% para-JEM GS (Trek Diagnostic Systems, Ohio) for oleic acid-albumin-dextrose-catalase supplementation, 2 μg/ml of mycobactin J (Allied Monitor, Missouri), and 0.05% Tween 80 (7H9 broth medium) or on Middlebrook 7H9 medium supplemented with 6.7% para-JEM GS, 6.7% para-JEM EYS (Trek Diagnostic Systems, Ohio) for egg yolk supplementation, 2 μg/ml of mycobactin J, and 1.5% agar base (Difco, Maryland) (7H9 agar medium). *M. avium* subsp. *paratuberculosis* liquid cultures were grown at 37°C in a shaking incubator (100 rpm), unless stated otherwise. Hygromycin (Hyg) was used at a concentration of 50 or 75 μg/ml for selection and subsequent culture of mutant colonies. Kanamycin (Kan) was used at a concentration of 25 μg/ml for subculture of GFP-tagged mutants.

Generation of specialized transducing mycobacteriophage containing AES. All primers used to generate upstream and downstream homologous regions and target genes are shown in Table 2. For the *relA* gene, two primer sets were designed in order to compare the efficiencies of allelic exchange for small (873-bp) and large (1737-bp) sequence deletions at the same genetic locus. The primer sets used for *pknG* deletion and two types of *relA* mutants were designed to obtain in-frame deletions (replacing 1,737, 1,737, or 873 bp in the genes with a 1,915-bp insertion sequence), while the primer set used for the *lsr2* mutant was designed to obtain an out-of-frame deletion (replacing 314 bp in the *lsr2* locus with the 1,915-bp insertion sequence) due to the efficiency of primers for the PCR (Table 2). The *lsr2* mutation introduced a stop codon at the 11th amino acid position from the start codon. Construction of each AES and subsequent delivery to the specialized transducing phage were done as previously reported (4, 9). Briefly, up- and downstream flanking fragments were amplified by PCR with primers designed to contain restriction sites corresponding to the restriction sites in the multiple cloning sites in cosmid pYUB854. Up- and downstream fragments were digested with appropriate enzymes (Table 2) and directionally cloned into pYUB854 on either side of the Hyg resistance (Hyg^r) gene to generate the AESs. The pYUB854 plasmids containing AESs were packaged into phasmid phAE87 using an in vitro λ-packaging solution (Gigapack III; Stratagene). The packaging solution was incubated with *E. coli* HB101, which was plated on LB agar containing 100 μg/ml of Hyg. The phAE87 phasmid DNA containing the AESs was prepared from the pooled Hyg^r colonies and electroporated into *M. smegmatis* mc^2 155 to generate transducing mycobacteriophage. After incubation at the permissive temperature (30°C) for 3 to 4 days, each plaque was tested for the temperature-sensitive phenotype. After the correct construct for each AES was confirmed by PCR with locus-specific primers and restriction analysis, high-titer transducing mycobacteriophage ($>10^{10}$ PFU/ml) were prepared in MP buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 10 mM $MgCl_2$, 2 mM $CaCl_2$) as previously described (9).

Generation of targeted gene disruption in *M. avium* subsp. *paratuberculosis*. The first transducing experiment with *M. avium* subsp. *paratuberculosis* K10 or *M. avium* subsp. *paratuberculosis* K10-GFP was performed using transducing phage containing AESs for *pknG*, *relA*S (S indicates that the small 873-bp sequence deletion at the *relA* locus was present [Table 2]), and *relA*L (L indicates that the large 1,737-bp sequence deletion at the *relA* locus was present [Table 2]), as previously described for *M. tuberculosis* and *M. bovis* BCG (4), with slight modifications (referred to as method A in this study). Briefly, *M. avium* subsp. *paratuberculosis* was cultured in 10 ml of 7H9 broth medium with 1 ml of frozen stock in a 50-ml tube at 37°C until the optical density at 600 nm (OD_{600}) was 0.6 (approximately 6×10^8 CFU/ml). The culture was centrifuged at 12,000 × *g* for 10 min, resuspended, and incubated in 10 ml of 7H9 broth medium without Tween 80 at 37°C for 24 h to remove any residual Tween 80 that could inhibit phage infection (9). Pelleted *M. avium* subsp. *paratuberculosis* cells were resuspended in 2 ml of 7H9 broth medium without Tween 80, and the suspension was divided into two halves. Each half of the suspension was incubated with 1 ml of MP buffer containing 10^{10} PFU of each phage in a 2-ml screw-cap tube at the nonpermissive temperature (37°C) for 4 h. The mixtures were added to 30 ml of 7H9 broth medium and cultured at 37°C for an additional 24 h for recovery. The cultures were centrifuged as described above and resuspended in 400 μl of 7H9 broth medium. Each half of the resuspended cultures was then plated on 7H9 agar medium containing 50 μg/ml Hyg. After 8 weeks of incubation 100 to 300 colonies were selected for analysis in each experiment. Because of the appearance of numerous spontaneous Hyg^r colonies in the initial plates containing transduced bacteria, the method used for preparation of the transduced bacteria and culture was modified in a second trial. In this method (referred to as method B in this study), mycobacteriophage containing AESs for *relA*S or *relA*L were transduced into *M. avium* subsp. *paratuberculosis* K10 and *M. avium* subsp. *paratuberculosis* K10-GFP. Because we hypothesized that the high level of spontaneous Hyg^r colonies was due in part to excessive clumping of *M. avium* subsp. *paratuberculosis* during culture, strong aggregation in the centrifugation steps, and insufficient selective pressure in the selective medium, in method B the

TABLE 2. Targeted genes and primers used for construction of allelic exchange substrates

Targeted gene[a]	Primer[c]	Oligonucleotide sequence (5′ to 3′)[d]	Tagged restriction enzyme site	Expected deletion size (bp)[e]
pknG	pknGU-F	GTCAGATCTTCGTGGTGTCGGTGGTCAACT	BglII	1,737
	pknGU-R	GCTAAGCTTGCCCTTGCTCTTCTTGGTGGA	HindIII	
	pknGD-F	GTCTCTAGACACATCCTGGGCTTCCCGTTCA	XbaI	
	pknGD-R	TGTCTTAAGTACCTGCGGCTGCTGCTCATCG	AflII	
lsr2	lsr2U-F	CTGAGATCTTAGAAATGTACCCGTCGCTGTC	BglII	311
	lsr2U-R	GTCAAGCTTTTTGCCATTGGCTTACCCTC	HindIII	
	lsr2D-F	GTCTCTAGACCTTCCACGCCGCAACCT	XbaI	
	lsr2D-R	TGTCTTAAGGGCTCAGCTCCAGCACCTTC	AflII	
*relA*S[b]	relASU-F	GTCAGATCTCGACCGAATCGCTCAAGACG	BglII	873
	relASU-R	CTGAAGCTTGCGAACGACAGGTCCTCCAAC	HindIII	
	relASD-F	TCATCTAGAGCAGTGGTTCGCCAAGGAG	XbaI	
	relASD-R	TGACTTAAG GGGTCGCCCATCTCAAAGG	AflII	
*relA*L[b]	relALU-F	GTCAGATCTAAGAAGATGTACGCGGTGAGC	BglII	1,737
	relALU-R	GCTAAGCTTCTTGAGCGATTCGGTCGG	HindIII	
	relALD-F	GTCTCTAGAATCGACCAGACCGAGGAGGAC	XbaI	
	relALD-R	TGACTTAAGCCACAGACCAACGGCAAGG	AflII	

[a] GenBank accession no. AE16958.
[b] S and L after *relA* indicate a relatively small sequence deletion and a large sequence deletion at the *relA* gene locus, respectively.
[c] The primer designations include the designation of the gene, followed by a letter indicating the presence of an upstream (U) or downstream (D) homologous region and (after the hyphen) the direction (F, forward; R, reverse).
[d] Restriction sites are underlined.
[e] The sizes of inserted sequences are the same in all cases (1,915 bp).

number of bacterial clumps present in cultures was reduced by including several cycles of gravity sedimentation and by using a lower *g* force during centrifugation. The concentration of Hyg was also increased. Briefly, bacteria were cultured in 10 ml of 7H9 broth medium in each 50-ml tube until the OD_{600} was 0.6. The cultures from four tubes were mixed together and vigorously shaken. Then the culture was allowed to stand for 10 min to allow large clumps of bacteria to sediment by gravity. Twenty milliliters of the top layer of the culture was then transferred into a new 50-ml tube and vigorously vortexed. The tube was then allowed to stand for an additional 20 min without disturbance to allow further sedimentation of residual clumps. The top 10 ml of the culture was then carefully collected for use. The OD_{600} of the culture after sedimentation was about 0.5 (approximately 5×10^8 CFU/ml). The rest of the procedures were the same as the procedures described above for method A, with three exceptions. First, the preparations were centrifuged at $3,700 \times g$ for 30 min. Second, the amount of Hyg used in the selective agar was increased from 50 to 75 μg/ml. Third, in the experiments in which the Δ*relA*S construct in *M. avium* subsp. *paratuberculosis* K10 was transduced, bacteria were washed two times with 10 ml of MP buffer to remove residual Tween 80 (9) instead of incubation in 7H9 broth medium without Tween 80, which was used in all other experiments. As a control, *M. avium* subsp. *paratuberculosis* that received no phage was plated on the same selective agar. Subsequently, the third gene, *lsr2*, was mutagenized using method B.

In addition, to evaluate whether the recovery time used in the experiments described above had a critical effect on the efficiency of allelic exchange, *M. avium* subsp. *paratuberculosis* receiving AES for *relA*L or *lsr2* was directly plated onto the selective agar without the 24-h recovery time. The results were compared to those obtained in the experiment in which a recovery time was used.

Isolation and confirmation of allelic exchange mutants. After 8 weeks of incubation on selective agar containing Hyg, each Hyg^r colony was recultured on new selective agar containing Hyg alone or Hyg plus Kan to expand bacterial cultures for subsequent analyses. After a colony was recultured, the correct structure of the disrupted gene in the colony was confirmed by PCR. For Δ*relA*S and Δ*lsr2*, each PCR was performed with a specific primer set binding the flanking regions of the homologous section because the sizes of the amplified fragments of the wild type and mutant are clearly distinguished by PCR (>1-kb difference) (Fig. 1 and Table 2). The following primer sets were used: for Δ*relA*S, relL-3F (5′-TTCGGAGGTGAGCATCGTGG-3′) and relR-3R (5′-CCGACAACGGGTCCTGCTAC-3′); and for Δ*lsr2*, lsrL-1F (5′-CCCCAATGTTGCAGACGC-3′) and lsrR-1R (5′TCACCCGCTCGATTTCCTT-3′). For Δ*pknG* and Δ*relA*L, correct construction of each side was confirmed separately with site-specific primer sets because the sizes of PCR fragments obtained with a primer set binding the flanking regions of the homologous section were not well distinguished for the mutant and the wild type (178-bp difference) (Fig. 1 and Table 2). Each primer set was designed so that one primer bound within the *hyg* gene and the other primer bound up- or downstream of the homologous region (Fig. 1). The following primer sets were used: for the left side of Δ*pknG*, pknL-1F (5′-ACCAGAACTGCGACCTGACGG-3′) and hyg-R (5′-GCCCTACCTGGTGATGAGCC-3′); for the right side of Δ*pknG*, hyg-F (5′-CACGAAGATGTTGGTCCCGT-3′) and pknR-1R (5′-TCCACCACAACACTCGTGCC-3′); for the left side of Δ*relA*L, relL-1F (5′-CAGGTGGACAACGCGATCG-3′) and hyg-R; and for the right side of Δ*relA*L, hygF and relR2R (5′-TGCGTCGTTGATGAGGGTT-3′). For further confirmation, a sequencing analysis was performed with one or two isolates from the *M. avium* subsp. *paratuberculosis* K10 and *M. avium* subsp. *paratuberculosis* K10-GFP mutant groups. Transduction frequencies were calculated as follows: $(X - Y)/Z$, where X is the number of Hyg^r colonies obtained, Y is the number of spontaneous Hyg^r colonies from control cells which received no phage, and Z is the number of input cells for each experiment. The allelic exchange frequency was calculated by determining the percentage of allelic exchange in the population of Hyg^r colonies (4).

Expression analysis of disrupted *M. avium* subsp. *paratuberculosis* genes. RNA expression of the disrupted gene was also checked by reverse transcription (RT)-PCR. Total RNA from 2×10^9 cells of each strain was isolated using a FastRNA Pro Blue kit (Q-Biogene, Ohio) and treated two times with DNase I (Invitrogen, California). Five hundred nanograms of RNA was used for RT-PCR with a specific primer set for each gene using SuperScript One-Step RT-PCR systems (Invitrogen, California) according to the manufacturer's instructions. Negative (no RT) and positive (*gapDH* gene) (19) controls were also included in each RT-PCR.

Southern blot analyses. Southern blot analyses were performed to show that the allelic exchange occurred at the correct position, as well as to show that illegitimate recombination at another site did not occur. DNA was extracted from each strain as previously described (23). Two micrograms of DNA from each strain was digested with an appropriate restriction enzyme (BamHI for the Δ*relA*L, Δ*relA*S, Δ*lsr2*, and K10 strains; ClaI for the Δ*pknG* and K10 strains). One microgram of digested DNA from a mutant or the wild type was electrophoresed on a 1% agarose gel and transferred to a Hybond-N+ membrane (Amersham, New Jersey) by the capillary method. Generation of digoxigenin-labeled probes for specific binding sites (Fig. 1), hybridization, and chemiluminescence detection were performed with a DIG-High Prime DNA labeling and detection starter kit II (Roche Applied Science, Germany) used according to the manufacturer's recommendations.

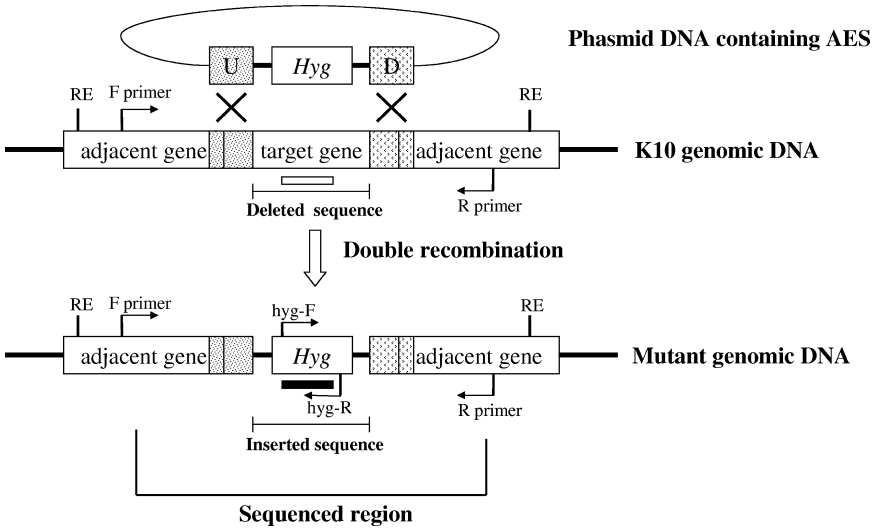

FIG. 1. Schematic diagram of allelic exchange mutagenesis in *M. avium* subsp. *paratuberculosis*. The inserted sequence containing the Hyg gene is the same size in all mutants (1,915 bp), but the size of the deleted sequence varies in the mutants developed in this study (Δ*pknG* and Δ*relA*L, 1,737 bp; Δ*relA*S, 873 bp; Δ*lsr2*, 311 bp [Table 2]). Arrows indicate the schematic binding sites and the directions of primers used for PCR identification. The F and R primers are the primers designed to bind outside up- and downstream homologous regions in each mutant. PCRs for Δ*relA*S and Δ*lsr2* were performed with a primer set consisting of F and R primers, because the PCR fragments for the mutant and wild-type strains were clearly distinguished (>1-kb difference). PCRs for Δ*pknG* and Δ*relA*L were performed with specific primer sets consisting of both F and hyg-R primers and hyg-F and R primers because the size differences between the mutant and wild-type strains with the F and R primers were not well distinguished in these cases (178-bp difference). The schematic restriction (RE) and probing (open and filled bars) sites for Southern blot analysis are also shown. *Hyg*, Hyg^r gene; U and D, up- and downstream homologous regions; RE, restriction enzyme site; open and filled bars, probes for the deleted gene and the *hyg* gene, respectively.

RESULTS

Disruption of *pknG* in *M. avium* subsp. *paratuberculosis*. Using method A, which was based on a protocol developed for *M. tuberculosis* and *M. bovis* BCG (4), *M. avium* subsp. *paratuberculosis* K10 and *M. avium* subsp. *paratuberculosis* K10-GFP were infected with a specialized transducing phage carrying AES for *pknG* or *relA*S. More than 1,000 colonies were visible after 8 weeks of incubation in each of the cultures of *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *paratuberculosis* K10-GFP transduced with the *pknG* or *relA*S AES. Screening of 300 colonies from each experiment by PCR revealed there was a high level of spontaneous Hyg^r. This initial trial yielded only seven Δ*pknG* mutants each for *M. avium* subsp. *paratuberculosis* K10 and *M. avium* subsp. *paratuberculosis* K10-GFP. No mutants were detected when cultures of *M. avium* subsp. *paratuberculosis* K10 and *M. avium* subsp. *paratuberculosis* K10-GFP transduced with the *relA*S AES were screened (Table 3). Furthermore, no mutants were detected in

TABLE 3. Efficiency of allelic exchange in *M. avium* subsp. *paratuberculosis*

Host strain	Genotype[a]	Method[b]	No. of allelic exchanges/ no. of tested Hyg^r mutants (%)[d]	Total no. of Hyg^r mutants	Transduction frequency
M. avium subsp. *paratuberculosis* K10	Δ*pknG*	A	7/300 (2.3)	NA[e]	NA
	Δ*relA*S	A	0/300 (0.0)	NA	NA
	Δ*relA*L	A	0/150 (0.0)	NA	NA
	Δ*relA*S	B[c]	2/35 (5.7)	35	1.1×10^{-8}
	Δ*relA*L	B	48/50 (96.0)	291	1.1×10^{-7}
	Δ*lsr2*	B	50/50 (100.0)	738	2.9×10^{-7}
M. avium subsp. *paratuberculosis* K10-GFP	Δ*pknG*	A	7/300 (2.3)	NA	NA
	Δ*relA*S	A	0/300 (0.0)	NA	NA
	Δ*relA*L	A	0/150 (0.0)	NA	NA
	Δ*relA*S	B	33/35 (94.3)	499	2.0×10^{-7}
	Δ*relA*L	B	39/50 (78.0)	448	1.8×10^{-7}
	Δ*lsr2*	B	50/50 (100.0)	438	1.7×10^{-7}

[a] S and L after *relA* indicate a small sequence deletion and a large sequence deletion at the *relA* gene locus, respectively.
[b] Method A was used in the first trial, and method B was used in the second trial. For detailed information, see Materials and Methods.
[c] The difference from other method B experiments was that *M. avium* subsp. *paratuberculosis* was washed with MP buffer to remove residual Tween 80 before absorption of phage. For detailed information, see Materials and Methods.
[d] The values in the parentheses are the allelic exchange frequencies.
[e] NA, not available.

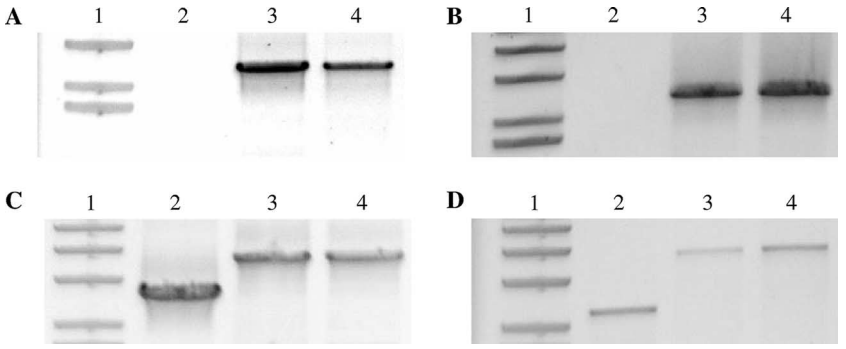

FIG. 2. PCR identification for specific gene construction in mutants. (A) PCR for Δ*pknG*; (B) PCR for Δ*relA*L; (C) PCR for Δ*relA*S; (D) PCR for Δ*lsr2*. Lane 1, DNA size marker; lane 2, wild type (*M. avium* subsp. *paratuberculosis* K10); lane 3, mutant in *M. avium* subsp. *paratuberculosis* K10; lane 4, mutant in *M. avium* subsp. *paratuberculosis* K10-GFP. The primer sites for Δ*pknG* (A) and Δ*relA*L (B) PCRs were located in the Hyg gene (inserted gene) for the forward primer and outside the downstream homologous region of each disrupted gene for the reverse primer. Note that the wild-type gene was not amplified in panels A and B because of the primer design. The primer sites for Δ*relA*S (C) and Δ*lsr2* (D) PCRs were located outside up- and downstream homologous regions of each disrupted gene, which allowed identification of mutants based on the sizes of the amplified fragments.

two additional experiments with the *relA*S AES (data not shown). Because the size of the inserted sequence was similar to the size of the deleted sequence in Δ*pknG* (1,915 bp versus 1,737 bp) but greater than the size of the deleted sequence in Δ*relA*S (1,915 bp versus 873 bp), we performed an experiment to determine whether the sizes of inserted and deleted sequences at the recombination locus might interfere with the efficiency of allelic exchange. Another transducing phage carrying an AES for the *relA* deletion (*relA*L) was designed to delete 1,737 bp in the *relA* locus and tested using the same method. However, no mutants were detected when 150 colonies each of *M. avium* subsp. *paratuberculosis* K10 and *M. avium* subsp. *paratuberculosis* K10 GFP transduced with the *relA*L AES were screened (Table 3).

Although some mutants were generated in the Δ*pknG* experiments, the frequency of allelic exchange compared to the frequencies for *M. tuberculosis* and *M. bovis* (4) was very low (0 to 2.3% versus 90 to 100%). These findings underscored the difficulties encountered when working with *M. avium* subsp. *paratuberculosis* and suggested that the methodology would have to be modified to use this system of transduction as a routine laboratory procedure.

Efficiency of allelic exchange mutagenesis in *M. avium* subsp. *paratuberculosis* by specialized transduction. After a high rate of spontaneous Hyg^r was observed in the first trial with method A, the procedure was modified to determine if the efficiency of allelic exchange could be increased by modifying the method. With method B, in which the procedure was modified to decrease spontaneous Hyg^r as described above, 35 to 500 Hyg^r colonies were obtained in the *relA* deletion experiment after 8 weeks of incubation. Colonies having each type of targeted gene deletion were transferred onto new agar plates containing Hyg or Hyg plus Kan. The mutant colonies were first identified by PCR using locus-specific primers (Fig. 2). Then the correct position of allelic exchange was confirmed by performing a sequencing analysis of one or two mutant isolates from each mutant group (*M. avium* subsp. *paratuberculosis* K10 and *M. avium* subsp. *paratuberculosis* K10-GFP) (data not shown). In addition, the lack of RNA expression for deleted genes was also confirmed by RT-PCR using two isolates from each mutant group (*M. avium* subsp. *paratuberculosis* K10 and *M. avium* subsp. *paratuberculosis* K10-GFP) (Fig. 3). Both target genes were expressed in the control strain (*M. avium* subsp. *para-*

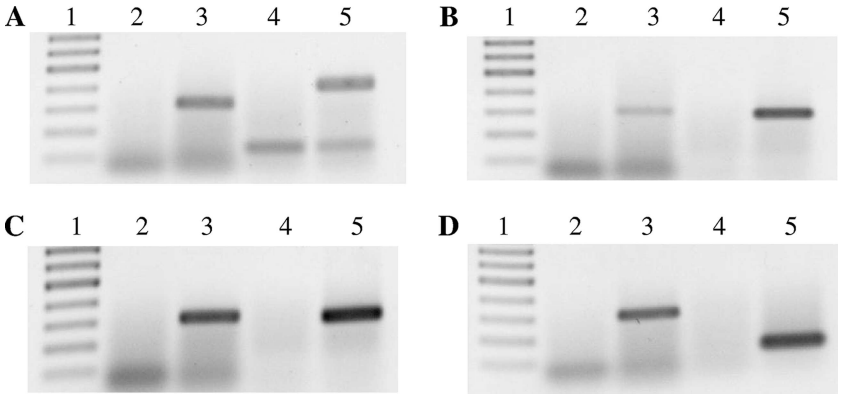

FIG. 3. RT-PCR analysis of gene expression in *M. avium* subsp. *paratuberculosis* strains. (A) RT-PCR for *pknG* (380 bp); (B and C) RT-PCR for *relA* from Δ*relA*L (303 bp) (B) and Δ*relA*S (303 bp) (C) mutants; (D) RT-PCR for *lsr2* (145 bp). Lane 1, DNA marker; lanes 2 and 3, negative (without RT) and positive controls (*gapDH*) for RT-PCR, respectively; lanes 4 and 5, target gene expression in mutant and wild-type (*M. avium* subsp. *paratuberculosis* K10) strains, respectively. Negative and positive controls for RT-PCR of *M. avium* subsp. *paratuberculosis* K10 RNA were also analyzed (data not shown).

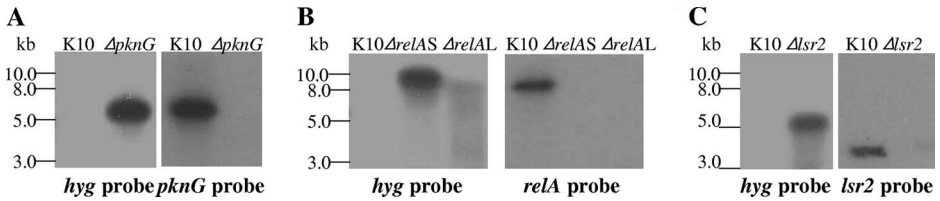

FIG. 4. Southern blot analysis of genomic DNA from mutant and wild-type strains. (A) Southern blot characterization for Δ*pknG*; (B) Southern blot characterization for Δ*relA*S and Δ*relA*L; (C) Southern blot characterization for Δ*lsr2*. The restriction and probing sites are shown in Fig. 1. The membrane was hybridized with the first probe (*hyg* probe). After chemiluminescence detection, the first probe was stripped out, and the membrane was reprobed with the second probe (probe for the deleted gene) and examined. All bands are consistent with the expected sizes.

tuberculosis K10), but they were absent in the corresponding gene deletion mutants. Furthermore, these results were confirmed by Southern blot analysis, as shown in Fig. 4. Southern blotting with a *hyg* gene-specific probe for a mutant, as well as a target gene-specific probe for the wild type, revealed single hybridization bands at the expected sizes, which indicated that the target gene was deleted and replaced with the inserted fragment containing the *hyg* gene. Compared with the allelic exchange frequencies obtained in first trial using method A, the allelic exchange frequencies in the second trial using method B were greatly increased (from 0 to 2.3% to 78 to 96%) (Table 3). Compared to incubation in 7H9 broth medium without Tween 80, washing with MP buffer to remove the residual Tween 80 resulted in decreases in the allelic exchange frequency and the transduction frequency (Table 3). Contrary to our assumption about the sizes of the deletions and inserts with allelic exchange, the size of the deletions in *relA* did not have a significant effect on the frequency of mutants generated with method B (873-bp deletion versus 1,915-bp insertion and 1,737-bp deletion versus 1,915-bp insertion) (Tables 2 and 3) in this study.

To test whether the optimized method (method B) works well with additional gene deletions, a third gene, *lsr2*, was selected for disruption. Lsr2 is a cytosolic protein implicated in cell wall lipid synthesis, which has an important role in colony morphology and biofilm formation in *M. smegmatis* (14). The confirmation method used for *lsr2* deletion was exactly the same as the method described above (Fig. 2 and 3). As shown in Table 3, generation of Δ*lsr2* with method B showed that there was 100% correlation of Hyg^r with successful allelic exchange. These data indicate that method B works equally well with additional genes.

We also compared the effects of recovery times between 0 and 24 h in three knockout experiments. For the Δ*relA*L mutation in *M. avium* subsp. *paratuberculosis* K10-GFP and the Δ*lsr2* mutation in *M. avium* subsp. *paratuberculosis* K10, the total numbers of Hyg^r colonies were about twofold greater after 24 h of incubation in 7H9 broth medium before plating, which is consistent with one replication cycle of *M. avium* subsp. *paratuberculosis* (24 to 48 h). However, for the Δ*relA*L mutation in *M. avium* subsp. *paratuberculosis* K10, the number of Hyg^r colonies decreased after 24 h of incubation. In contrast to previous findings with *M. bovis* BCG (4), which showed the highest allelic exchange frequency with a recovery time of 24 h, the allelic exchange frequencies in each experiment were virtually the same with and without the recovery time in the present study (Table 4).

Generation of GFP-tagged mutants of *M. avium* subsp. *paratuberculosis*. Expression of GFP in *M. avium* subsp. *paratuberculosis* is variable, and only a few transformants express high GFP levels (32). Thus, to construct GFP-tagged mutants with equivalent high fluorescence levels, it may be useful to carry out allelic exchange directly with an *M. avium* subsp. *paratuberculosis* host with optimal GFP expression, such as our strain *M. avium* subsp. *paratuberculosis* K10-GFP. The data show that allelic exchange mutagenesis occurred in *M. avium* subsp. *paratuberculosis* K10-GFP at the same rate as it occurred in *M. avium* subsp. *paratuberculosis* K10 (Tables 3 and 4). Every 10 isolates of each mutant made from *M. avium* subsp. *paratuberculosis* K10-GFP, except Δ*pknG* (7 isolates), were examined by fluorescence microscopy for the presence of GFP. Even after extensive incubation without antibiotic pressure for the GFP plasmid (Kan), some mutant strains still expressed GFP. The percentages of GFP-expressing mutants in the colonies examined ranged from 20% (Δ*lsr2*) to 100% (Δ*relA*S). We also examined the potential of the GFP-tagged mutants to be a useful tool for tracing the mutants within bovine macrophages after infection. The presence of GFP-expressing mutants in macrophages was clearly detected by fluorescence microscopy (data not shown).

TABLE 4. Effect of recovery time on the efficiency of allelic exchange mutagenesis

Recovery time (h)	Δ*relA*L K10		Δ*relA*L K10-GFP		Δ*lsr2* K10	
	No. of Δ*relA*L mutants/ no. of Hyg^r mutants (%)[a]	Total no. of Hyg^r mutants	No. of Δ*relA*L mutants/ no. of Hyg^r mutants (%)[a]	Total no. of Hyg^r mutants	No. of Δ*lsr2* mutants/ no. of Hyg^r mutants (%)[a]	Total no. of Hyg^r mutants
0	50/50 (100)	656	39/50 (78)	266	50/50 (100)	402
24	48/50 (96)	291	42/50 (84)	448	50/50 (100)	738

[a] Values in parentheses are the allelic exchange frequencies.

DISCUSSION

Allelic exchange mutagenesis using specialized transduction has been used successfully with some slow-growing mycobacteria, including *M. tuberculosis*, *M. bovis*, and *M. avium* (4, 30). However, successful use of this technology has not been reported previously for *M. avium* subsp. *paratuberculosis*. We explored the use of this approach to develop targeted gene disruptions in *M. avium* subsp. *paratuberculosis*, one of the slowest-growing mycobacterial species, whose generation time is 24 h or longer (34). The ability to obtain directed gene knockouts in *M. avium* subsp. *paratuberculosis* is a major breakthrough in Johne's disease research. Results from sequencing the *M. avium* subsp. *paratuberculosis* genome have shown that 41.6% of the annotated genes in the genome are unknown or hypothetical open reading frames (27). Only through specific gene disruptions can potential phenotypes be assigned to these unknown genes. Rational design and construction of attenuated mutants as possible vaccines are now within reach. Persistence within host macrophages is a key feature of mycobacterial pathogenesis that needs to be understood further. By selectively disrupting *pknG* and *relA* by allelic exchange, we took an important step in this direction as both *pknG* and *relA* have been shown to be key virulence determinants in *M. tuberculosis* and *M. bovis* (17, 38). The ability to selectively disrupt genes in *M. tuberculosis* has already facilitated increases in our knowledge of specific gene functions in *M. tuberculosis* (2, 3, 7, 11, 16, 33).

In the first trial (method A), which was similar to a previous study of *M. tuberculosis* and *M. bovis* BCG (4), we obtained a high rate of spontaneous Hyg^r or illegitimate recombination. A previous study with *M. avium* produced similar results (30). To overcome the very low efficiency of allelic exchange in that study, the authors used an *M. avium leuD* deletion mutant as a genetic host with the *Streptomyces clelicolor ledD* gene as a selective marker. In contrast, we obtained a high efficiency of allelic exchange in *M. avium* subsp. *paratuberculosis* (allelic exchange frequency, up to 100%; transduction frequency, 2.9×10^{-7}) (Table 3) after modifying the original methodology (method B). Importantly, the successful optimization of the method should now allow this tool to be used routinely to generate directed gene deletions in an isogenic virulent strain of *M. avium* subsp. *paratuberculosis*. To obtain the very efficient allelic exchange in the second trial, we reduced the chance that clumped bacteria would be plated on the selective agar by consecutive gravity sedimentations and by use of a low *g* force in the centrifugation steps. To disrupt cell clumps, other mechanical methods might be used, such as passage through a syringe or sonication. However, these physical disruptions might cause damage to the cells, which might decrease the viability of transduced bacteria on Hyg-containing medium. In addition, we also increased the concentration of Hyg from 50 to 75 μg/ml in the second trial and did not use the drug concentration typically used for other mycobacteria (23, 30). When the concentration of Hyg was increased to 75 μg/ml, the rate of spontaneous Hyg^r was greatly diminished. When 50 μg/ml Hyg was used, many spontaneous Hyg^r mutants were generated in the experiments with *M. avium* (30) and *M. avium* subsp. *paratuberculosis* (Table 3). In contrast, 75 μg/ml Hyg provided excellent selective pressure for isolating mutant colonies of *M. tuberculosis*, *M. bovis* (4), and *M. avium* subsp. *paratuberculosis* in this study (Table 3). The effects of gravity sedimentation, a lower *g* force in centrifugation ($3{,}700 \times g$ versus $12{,}000 \times g$), and a higher concentration of Hyg (75 μg/ml versus 50 μg/ml), which was assumed to contribute to the high efficiency of allelic exchange in method B, were evaluated separately. Each procedure had a significant effect, reducing the number of spontaneous Hyg^r colonies by 50 to 90% (data not shown), thus supporting our hypothesis.

All transduction frequencies obtained with method B, except the transduction frequency for Δ*relA*L in *M. avium* subsp. *paratuberculosis* K10, were calculated to be between 1.1×10^{-7} and 2.9×10^{-7} per recipient cell, and these values were similar to the values obtained in previous studies of transposon mutagenesis in *M. avium* subsp. *paratuberculosis* by specialized transduction (21). However, we estimated that the concentration of cells at an OD_{600} between 0.5 and 0.6 was 5×10^8 to 6×10^8 CFU/ml based on the results of CFU counting in our lab, while in other studies of transposon mutagenesis of *M. avium* subsp. *paratuberculosis* the workers interpreted similar optical density values as indicating that the concentration was 1.5×10^8 to 2.0×10^8 CFU/ml (12, 21, 35). If we had used a concentration of 2.0×10^8 CFU/ml for recipient cells, the calculated transduction frequencies in this study would have been three times greater. In contrast to the previous findings for *M. bovis* BCG (4), the recovery time for transduced *M. avium* subsp. *paratuberculosis* with specialized transducing mycobacteriophage did not have much effect on the allelic exchange frequency in the current study (Table 4). This suggests that the recovery time is not a critical factor for achieving a high efficiency of allelic exchange.

One novel benefit of present study was the ability to create defined mutants in a GFP-expressing strain of *M. avium* subsp. *paratuberculosis* K10. This feature should enable easy tracking of mutants in a variety of downstream assays, including infection of macrophages, as shown in this study. We demonstrated that the efficiencies of allelic exchange in *M. avium* subsp. *paratuberculosis* K10-GFP mutants were similar to those in wild-type *M. avium* subsp. *paratuberculosis* strain K10 (Tables 3 and 4), and some of these mutants still expressed GFP after lengthy incubation without selective pressure for the GFP plasmid. Importantly, the pWES4 plasmid (the plasmid encoding GFP in *M. avium* subsp. *paratuberculosis* K10-GFP) introduced into *M. avium* did not alter bacterial virulence (32). Therefore, it is evident that *M. avium* subsp. *paratuberculosis* mutants containing GFP would have an advantage in investigations of the functions of deleted genes in host cells. In addition, GFP can be a potential antigenic marker for differentiation between wild-type and potential vaccine strains used as a live attenuated vaccine. By making GFP-expressing mutants from the parent strain *M. avium* subsp. *paratuberculosis* K10-GFP, we can save at least several months which would be required to introduce the GFP plasmid into mutant strains for this purpose. Moreover, we maximized the chance that mutants would express optimal GFP fluorescence like that in the original *M. avium* subsp. *paratuberculosis* K10-GFP host.

Lsr2 has been shown to be a cytosolic protein related to lipid biosynthesis in the cell wall and antibiotic resistance in *M. smegmatis* (14, 15). Deletion of the *lsr2* gene in *M. smegmatis* resulted in an alteration in colony morphology (generation of

smooth colonies) and an alteration in biofilm and pellicle formation. The *lsr2* mutant of *M. avium* subsp. *paratuberculosis* generated in this study also had a distinctive smooth-colony morphology and was defective in pellicle formation (unpublished data), which indicates that the role of *lsr2* in *M. avium* subsp. *paratuberculosis* is also related to lipid biosynthesis of cell wall components. The lipid-rich cell wall is a distinctive feature of mycobacteria. The cell wall components of *M. tuberculosis* have been shown to contribute to the virulence of this organism and modulate the host immune response (16, 37). Therefore, *lsr2* in pathogenic mycobacteria may be related to virulence. Indeed, deletion of *lsr2*, as well as deletion of *pknG* and *relA*, resulted in a reduction in survival in bovine macrophages (unpublished data). Recently, Colangeli et al. proposed that *lsr2* may be an essential gene in *M. tuberculosis* since they were unable to delete the gene in their study (15). Whether this is true or not, *lsr2* can be deleted in *M. avium* subsp. *paratuberculosis*, one of the slow-growing mycobacterial species.

In conclusion, we established an efficient allelic exchange mutagenesis system for *M. avium* subsp. *paratuberculosis* by generating three different targeted gene disruptions, one of which involved two deletions that were different sizes (*relA*), in *M. avium* subsp. *paratuberculosis* K10 and *M. avium* subsp. *paratuberculosis* K10-GFP. The three genes are assumed to have important roles in the virulence of *M. avium* subsp. *paratuberculosis*, as they do in other mycobacterial species. Along with the recently completed genome sequence (27) and a random transposon mutagenesis system for *M. avium* subsp. *paratuberculosis* (12, 21, 35), this tool should allow us to gain more insight into pathogenesis and further efforts to develop an effective vaccine for *M. avium* subsp. *paratuberculosis*.

ACKNOWLEDGMENTS

This study was supported in part by the Washington State Monoclonal Antibody Center, the Johne's Disease Integrated Program funded by the Animal Biosecurity program of USDA-CSREES National Research Initiative 2004-356051-14243, USDA-APHIS grants 03-9100-0788-GR and 03-9100-07-GR, and USDA Animal Health intramural grant WNV-00150.

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