Mutation and virulence assessment of chromosomal genes of *Rhodococcus equi* 103

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Abstract

Rhodococcus equi can cause severe or fatal pneumonia in foals as well as in immunocompromised animals and humans. Its ability to persist in macrophages is fundamental to how it causes disease, but the basis of this is poorly understood. To examine further the general application of a recently developed system of targeted gene mutation and to assess the importance of different genes in resistance to innate immune defenses, we disrupted the genes encoding high-temperature requirement A (*htrA*), nitrate reductase (*narG*), peptidase D (*pepD*), phosphoribosylaminoimidazole-succinocarboxamide synthase (*purC*), and superoxide dismutase (*sodC*) in strain 103 of *R. equi* using a double-crossover homologous recombination approach. Virulence testing by clearance after intravenous injection in mice showed that the *htrA* and *narG* mutants were fully attenuated, the *purC* and *sodC* mutants were unchanged, and the *pepD* mutant was slightly attenuated. Complementation with the pREM shuttle plasmid restored the virulence of the *htrA* and *pepD* mutants but not that of the *narG* mutant. A single-crossover mutation approach was simpler and faster than the double-crossover homologous recombination technique and was used to obtain mutations in 6 other genes potentially involved in virulence (*clpB*, *fadD8*, *fbpB*, *glnA1*, *regX3*, and *sigF*). These mutants were not attenuated in the mouse clearance assay. We were not able to obtain mutants for genes *furA*, *galE*, and *sigE* using the single-crossover mutation approach. In summary, the targeted-mutation system had general applicability but was not always completely successful, perhaps because some genes are essential under the growth conditions used or because the success of mutation depends on the target genes.

Résumé

Rhodococcus equi *peut causer une pneumonie sévère ou fatale chez les poulains aussi bien que chez les animaux ou humains immunocompromis. Sa capacité à persister dans les macrophages est fondamentale à sa pathogénie, mais la base de ce phénomène est mal connue. Afin d'examiner plus en détails l'application générale d'un nouveau système de mutation génétique dirigée et d'évaluer l'importance de différents gènes dans la résistance à la réponse immunitaire innée, un dérèglement des gènes codant pour la protéase htrA (*htrA*), la nitrate réductase (*narG*), la peptidase D (*pepD*), la phosphoribosylaminoimidazole-succinocarboxamide synthétase (*purC*) et la superoxide dismutase (*sodC*) de la souche 103 de* R. equi *a été produit à l'aide d'une approche utilisant une recombinaison homologue à double croisement. Une évaluation de la virulence par évaluation de la clairance après injection intraveineuse chez la souris a permis de démontrer que les mutants* htrA *et* narG *étaient complètement atténués, les mutants* purC *et* sodC *étaient inchangés et le mutant* pepD *était légèrement atténué. Une complémentation avec le plasmide vecteur pREM a rétabli la virulence des mutants* htrA *et* pepD *mais pas celle du mutant* narG. *Une approche de mutation par croisement unique était plus simple et rapide que la technique de recombinaison homologue à double croisement et a été utilisée afin d'obtenir des mutations dans six autres gènes potentiellement impliqués dans la virulence (*clpB, fadD8, fbpB, glnA1, regX3 *et* sigF*). Ces mutants n'étaient pas atténués dans l'épreuve de clairance chez la souris. Il a été impossible d'obtenir des mutants pour les gènes* furA, galE *et* sigE *en utilisant l'approche de mutation par croisement unique. En résumé, le système à mutation dirigée a une application globale mais le résultat désiré n'est pas toujours obtenu, peut-être parce que certains gènes sont essentiels dans les conditions de culture utilisées ou parce que le succès de la mutation dépend des gènes ciblés.*

(Traduit par Docteur Serge Messier)

Introduction

Rhodococcus equi is a gram-positive bacterium that infects alveolar macrophages and can cause severe or fatal pneumonia in foals. The bacterium has also been increasingly identified as an opportunistic pathogen in immunocompromised humans, particularly AIDS patients (1). As a facultative intracellular organism, *R. equi* is also of comparative medical interest since understanding how it survives and replicates in macrophages has application to similar infections, particularly those caused by *Mycobacterium tuberculosis*. Although studies have shown that the virulence of *R. equi* depends on plasmids of 80 to 90 kb, the understanding of most aspects of the basis of this virulence is rudimentary (2).

Recently, chromosomal genes have been shown to play a role in the resistance of *R. equi* to oxidative stress (with H_2O_2) and low pH (3,4). A limited number of chromosomal genes have been disrupted by

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Table I. Oligonucleotide primers used in double-crossover homologous genetic recombination to construct mutants and for gene complementation

Gene	Primer for suicide plasmid construction by PCR[a]		Primer for gene complementation[a]
htrA	H1 up-F	cagttggccttccatgttcac	H9 gene 424F gtcgaccggtggcctctc
	H2 up-R	*cgtt**gaattc***gtccgacggactgatcatgacc	H10 gene 2873R cagatcggtgtcgggtag
	H3 down-F	*cctc**aagctt***gacgctcggcacgaccttg	
	H4 down-R	taccgccggaccgagcagat	
narG	N1 up-F	cttgccacgtgcgcgctggtag	N12 narG-R ctcgtagtagtcgcggatgttg
	N2 up-R	*cgtt**gaattc***gtggagtcacgacaaggtg	N13 narG-F gaaccaccggccgacgaaagac
	N3 down-F	*cctc**aagctt***gactttcgatcggtgcgggtg	
	N4 down-R	gacacgatgcccgtcgacatc	
pepD	P1 up-F	gacgtacgccaccagttgcttg	P10 pepD-R cgctcgggctcctgtgtc
	P2 up-R	*cgtt**gaattc***gttcaccgaggcgtacgtgttc	P11 pepD-F gagcgcacccatgtagtcg
	P3 down-F	*cctc**aagctt***ctccatcgcaccgaggttg	
	P4 down-R	gatccggtcgacgaagcgac	
purC	PU1 up-F	tcgaggttcttgaaccgcagc	ND
	PU2 up-R	*cgtt**gaattc***ggcctgtcgttcgaggactgg	
	PU3 down-F	*cctc**aagctt***gctcgtacgcctcgatgtag	
	PU4 down-R	gatacaagttcgaatcggtc	
sodC	S1 up-F	ccaccgttcttcaggtcctc	S7 sodC379F gtatccctcgaactgcgtcc
	S2 up-R	*cgtt**gaattc***tcgctcaaggatgccaag	S8 sodC2063R gaccggcaaatacagttacg
	S3 down-F	*cctc**aagctt***ggctcctcgttgttcgag	
	S4 down-R	gttacggggcagatccaag	

PCR — polymerase chain reaction; F — forward; R — reverse; ND — not done.

[a] The nucleotide sequences are shown 5′ to 3′. Non-*Rhodococcus equi*-linkers are italicized, and restriction-enzyme sites are bolded (*Hin*dIII and *Eco*RI).

allelic exchange or transposon mutagenesis (5–9). Such an approach, combined with gene complementation, identified *vapA* as a virulence gene (8). However, the precise survival strategies used by *R. equi* and the genes required for intracellular survival are largely unknown (2). A number of putative virulence and virulence-related chromosomal genes were identified in a partial (one-quarter) genome sequence of *R. equi* ATCC (American Type Culture Collection) 33701 (10), including homologs of genes for *M. tuberculosis* heat-shock proteins (*clpB* and *htrA*), 2-component regulatory systems (*senX3-regX3*), iron regulation (*furA*), sigma factors or sigma-factor regulation (*pepD, sigE,* and *sigF*), oxidative-stress detoxifying enzymes (*sodC*), and purine biosynthesis (*purC*), as well as genes involved in anaerobic growth (*narG*), nitrogen metabolism (*glnA1*), lipid metabolism (*fadD8*), and cell wall synthesis (*fbpB*). Many of these genes have been shown to be important in the survival of *M. tuberculosis* in macrophages.

A genome sequence of *R. equi* 103 is now complete (www.sanger.ac.uk/Projects/R_equi/), so that it may be possible to mutate any *R. equi* gene to better understand the basis of virulence. The purpose of the work described here was to examine further the application of a recently developed system for targeted gene mutation and complementation (9) using genes with a wide range of functions as described above. Mouse clearance studies were also used to assess whether these mutants were attenuated and whether complemented mutants regained virulence (9).

Materials and methods

Bacterial strains and growth conditions

Escherichia coli DH5α was the host for all plasmid constructions. We used *R. equi* 103^+, originally isolated from a pneumonic foal. All bacteria were grown on Luria Bertani (LB) agar or in LB broth. When required, antibiotics were used at the following concentrations: apramycin (Sigma Chemical Company, St. Louis, Missouri, USA), 50 μg/mL for both *E. coli* and *R. equi;* kanamycin (Sigma), 50 μg/mL for *E. coli* but 200 μg/mL for *R. equi;* and ampicillin (Sigma), 40 μg/mL. For blue–white selection, X-gal (Sigma), 50 μg/mL, was added to antibiotic-containing solid medium. All bacteria were grown at 37°C in broth with shaking at about 200 rpm. Electrocompetent *E. coli* and *R. equi* cells were prepared as previously described (10,11). All DNA electroporations were done with the use of a Pulser Electroporater (Bio-Rad Laboratories, Hercules, California, USA). To improve the efficiency of homologous recombination, we performed alkaline denaturation of the suicide plasmid DNA as previously described (6,9).

DNA manipulation

All restriction enzymes and T4 DNA ligase were from New England Biolabs (NEB, Beverly, Massachusetts, USA), and

Table II. Oligonucleotide primers used in single-crossover homologous genetic recombination and to amplify the *accC4* and *vapA* genes

Gene	Forward primers[a]		Reverse primers[a]	
clpB	clpB-F	*ccg**tctaga**ggccaccgaactcgacgacg*[a]	clpB-R	*ccg**ggtacc**ggccaccgaactcgacgacg*
fadD8	fadD-F	*cgtt**tctaga**ctcgacgacgtgatcgtccgtg*	fadD-R	*gcctc**ggtacc**cagcgaggcaccggagtactc*
fbpA	fbpA-F	*cgtt**tctaga**gtcgagcagcgtgggcagctc*	fbpA-R	*gcctc**ggtacc**gatccgagacgcgcggcgagac*
furA	furA-F	*ccgt**tctaga**cgcgttgcggttctgaacac*	furA-R	*gcct**ggtacc**gtggtggttgtccgcggtgc*
galE	galE-F	*ccgt**tctaga**ggatccggcaacagcacacc*	galE-R	*cggt**ggtacc**gaagtagcgcaggctggtgg*
glnA1	gln-F	*cgt**ttctaga**gatcgcgtcacgcaggtcgac*	gln-R	*gcct**ggtacc**ctgacgaacgcgggcttcgag*
regX3	regX-F	*ccgt**tctaga**gaagtcgtcggtgccggtc*	regX-R	*gcct**ggtacc**gcgcacggaagctcactcct*
sigF	sigF-F	*ccgt**tctaga**gatcgcctccatgaccgagt*	sigF-R	*ccgt**ggtacc**tcgctcgacgcttctccag*
accC4	APF	*cgtt**gaattc**cttcatgtgcagctccatcagc*	APR	*cct**caagctt**gcatatcatcagcgagctgaa*
vapA	F	taatgcgaccgttcttgattcc[b]	R	tgtagagacgctgaaggtcatttg[c]

[a] The restriction-enzyme sites bolded include *Hin*dIII, *Eco*RI, *Xba*I, and *Kpn*I.

[b] Positions 12632 to 12653 in the primer sequence of the virulence plasmid.

[c] Positions 12937 to 12960 in the primer sequence of the virulence plasmid.

the reactions used followed the manufacturer's instructions. The procedure for *R. equi* genomic DNA isolation is described below, under "Preparation of chromosomal DNA for Southern blotting". Plasmid DNA was extracted from *E. coli* by means of the Qiagen plasmid purification kit (Qiagen, Mississauga, Ontario). All polymerase chain reactions (PCRs) were conducted in a TGradient96 thermocycler (Biometra, Göttingen, Germany) with a touchdown program and annealing temperatures varying from 60°C to 45°C. The preparation of reaction mixtures followed the instruction manual of the *Taq* polymerase supplier (Applied Biosystems, Branchburg, New Jersey, USA), with the addition of acetamide (Sigma) at a final concentration (w/v) of 5%. The primers are described in Tables I and II. The PCR products were purified from agarose gels with the Qiaquick purification system (Qiagen).

Plasmids

The plasmids used were pBluescript II SK+ (Stratagene, La Jolla, California, USA), pUC18 (MBI Fermentas, Hamilton, Ontario), pAPvlacZ, used as the suicide plasmid backbone for *R. equi* gene mutation (9), and pREM, used for gene complementation in *R. equi* (9).

Plasmid construction and mutant selection

Targeted gene mutation with double-crossover homologous recombination — Construction of the suicide plasmid and mutant selection were performed as previously described (9), with the following modifications. Briefly, the PCR products were generated separately for the upstream and downstream homologous regions of the targeted gene and for the apramycin-resistance gene (*aacC4*). The oligonucleotide primers for each construction are listed in Table I. The primers incorporated *Hin*dIII or *Eco*RI sites. The digested PCR products for the upstream and downstream homologous regions, along with *aacC4*, were ligated into *Ssp*I-digested pBluescript II SK+. The ligation mixture was electroporated into *E. coli* DH5α, and apramycin-resistant transformants were selected. Apramycin-resistant colonies were tested by PCR with the use of forward and reverse primers related to the upstream and downstream homologous regions. The amplicon was ligated into *Sca*I/*Nru*I-digested pAPvlacZ, the suicide plasmid backbone used for subsequent gene mutation in *R. equi* (9). The resulting suicide plasmid construct was sequenced to confirm the fidelity of the cassette. First-stage and second-stage selection were as previously described (9).

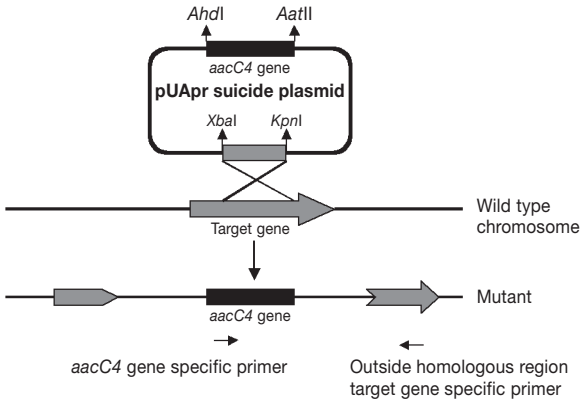

Figure 1. Schematic diagram of the 1-step strategy for single-crossover mutation. The homologous region is located in the middle of the target gene, so that the entire recombinant vector is integrated in the middle of the gene after a single crossover event. An *aacC4*-specific primer (right-pointing arrow at bottom) and a primer specific for part of the gene outside the homologous target region (left-pointing arrow at bottom) were used to confirm the single-crossover mutation.

Targeted gene mutation with single-crossover homologous recombination — For construction of the suicide vector pUApr, the *E. coli* plasmid pUC18 was chosen as the backbone of the suicide plasmid for a single-crossover strategy (Figure 1). The *aacC4* apramycin-resistance gene was PCR-amplified and cloned into *Aat*II (2617)- and *Ahd*I (1694)-digested sites of pUC18 to replace the ampicillin gene (*bla*), which resulted in the new suicide vector. This new suicide plasmid was used in targeted-mutation procedures, and single-crossover mutants were obtained. The genes *clpB*, *fadD8*, *fbpB*, *furA*, *galE*, *glnA1*, *regX3*, *sigE*, and *sigF* were amplified by PCR from purified chromosomal *R. equi* DNA with the oligonucleotide primers described in Table II. The PCR products were subcloned into the *Xba*I and *Kpn*I sites (positions 423 and 428, respectively) of the suicide vector. For electroporation of *R. equi* 103^+, nonreplicating vector (2 μg) was

alkali-denatured in order to stimulate homologous recombination. Single-crossover recombinants were selected with the use of apramycin. Plates were incubated at 37°C for 48 h.

Preparation of chromosomal DNA for Southern blotting

Briefly, 1.5 mL of cell culture was centrifuged at 12 000 × *g* for 1 min. The pellet was resuspended in 400 μL of TES buffer (50 mM Tris [pH 8.0], 1 mM ethylene diamine tetraacetic acid [pH 8.0], and 6.7% sucrose) to which was added 100 μL of lysozyme (10 mg/mL in TE), and the suspension was incubated for 30 min at 37°C. Next, 100 μL of 6% sodium dodecyl sulfate (SDS) in TE was added, and the suspension was vortexed for 10 s. An aliquot of 67 μL of 5 M NaCl was added and the tube placed on ice for 1 h. The mixture was then centrifuged at 12 000 × *g* for 10 min, after which the supernatant was mixed with an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1 v/v) and then centrifuged at 13 000 × *g* for 3 min. The aqueous layer was added to 0.1 volume of 3 M sodium acetate (pH 5.2). After the addition of 2 volumes of 95% ethanol to precipitate the DNA on ice for 1 h, the mixture was centrifuged at 13 000 × *g* for 10 min. The DNA pellet was air-dried for 10 min and then resuspended in 50 μL of TE buffer.

Southern blotting

Genomic DNA of the *R. equi* mutants was digested with *Sal*I, electrophoresed in a 0.8% agarose gel, and transferred to nylon membrane (Roche, Laval, Quebec) by capillary transfer, as previously described (12). The conditions for hybridization were as described by the manufacturer. The nylon membranes were prehybridized for at least 4 h at 42°C in hybridization solution without labeled probe, then hybridized separately at 42°C with the apramycin-specific DNA-didoxygenin (DIG) probes for 16 h. The membranes were washed at 68°C under high-stringency conditions.

Complementation studies

The entire *htrA, narG, pepD,* and *sodC* genes, including the potential regulatory and terminator regions flanking the structural genes, were PCR-amplified and cloned individually into the *Sma*I site of pREM (9) with the primers shown in Table I. The DNA inserted into the resulting plasmids was sequenced to verify the fidelity of the target-gene sequence, and the plasmids were then electroporated into the corresponding *R. equi* mutants, resulting in complemented strains of the mutants.

Assessment of *R. equi* virulence in mice

To assess the virulence of the mutants using clearance from mouse organs after intravenous injection, we diluted *R. equi* strains grown in log phase in saline to 5×10^6 colony-forming units (CFUs)/mL, then injected 100 μL of bacterial suspension intravenously into each of 7 adult female CD1 mice. The mice were euthanized after 4 d, and their livers were removed, homogenized individually in saline, and spread on trypticase soy agar (Difco, Detroit, Michigan, USA), with and without apramycin, in serial 10-fold dilutions. After 2 days' incubation at 37°C, the CFUs were counted, and the clearance of the mutant test strains was compared with that of virulent (103^+) and plasmid-cured nonvirulent (103^-) controls (13). Statistical evaluation of differences in the CFU counts between groups of mice was performed by Student's *t*-test.

To confirm the presence of the virulence plasmid, of the targeted gene mutations, and, when relevant, of the corresponding complementation plasmids, we conducted PCR on randomly picked colonies recovered from the mice, using primers to *vapA* (Table II).

Permission for the experiments in mice was obtained from the Animal Care Committee, University of Guelph, Guelph, Ontario, and care of the mice was in accord with the guidelines of the Canadian Council on Animal Care.

Comparison of growth of *R. equi* mutants with that of the parent 103^+ strain

Overnight cultures of strains were diluted to an optical density at 540 nm (OD_{540}) of 0.2. Then 2.5 mL of each strain was inoculated into 50 mL of trypticase soy broth (Difco). For mutants, 50 μg/mL of apramycin was added. Samples were withdrawn at 8-h intervals to determine the OD_{540} values.

Results

Nucleotide sequence analysis

The complete DNA sequence of *htrA, narG, pepD, purC,* and *sodC* was obtained using an inverse PCR approach based on the available partial sequences of these genes (10) (details not shown). The sequences reported in this work have been deposited in the GenBank database under accession numbers AY772449, AY922322, AY830683, AY854634, and AY762535, respectively.

Construction of suicide plasmids

Double-crossover recombinants — Table I summarizes the individual targeted-gene suicide-plasmid construction and organization. Double-crossover mutants were more frequent at the first-selection stage after electroporation of single-stranded DNA than at the second stage (6,9). For some genes, at the first stage white colonies were found to be single-crossover mutants. Therefore, we selected blue colonies at the first stage, confirmed these as single-crossover recombinants by PCR, and then carried out the second-stage selection to obtain double-crossover white colonies. These were confirmed by PCR as the desired mutants, and Southern blotting was used to confirm that only a single *aacC4* gene was present in the chromosome (data not shown).

Single-crossover recombinants — Using a 1-step strategy of single crossover was a rapid way to generate recombinants. Single-crossover recombinants of genes *clpB, fadD8, fbpB, glnA1, regX3,* and *sigF* were developed with the use of a design strategy to ensure that no gene duplication occurred, and recombinants were selected with the use of apramycin. By means of PCR amplification with vector-specific primers, in combination with gene-specific primers (Figure 1), we confirmed that the entire suicide-plasmid pUApr had been inserted through a single-crossover event of homologous recombination and that the recombination had occurred in the central region of each specific gene (data not shown).

Table III. Virulence assessment of *R. equi* mutants

Gene	Liver clearance in mice; mean $\log_{10}$ colony-forming units (and standard deviation)		
	Wild type, 103^{+}[a]	Mutant	Complemented mutant
clpB	4.98 (0.56)	4.73 (0.54)	ND
fadD8	5.25 (0.51)	5.32 (0.21)	ND
fbpB	5.25 (0.51)	5.29 (0.22)	ND
gln	5.25 (0.51)	5.38 (0.29)	ND
htrA	4.86 (0.14)	0	3.33 (1.49)
narG	5.74 (0.57)	0	0
pepD	5.25 (0.29)	4.06 (0.40), $P < 0.0002$[b]	4.85 (0.30), $P < 0.03$[c]
purC	5.83 (0.38)	5.53 (0.43)	ND
regX	4.98 (0.31)	5.0 (0.47)	ND
sigF	4.98 (0.31)	4.70 (0.64)	ND
sodC	5.31 (0.55)	5.53 (0.48), $P = 0.44$[b]	5.87 (0.39), $P < 0.05$[b]

[a] The clearance differs because it was assessed in different studies at different times. Plasmid-cured strains (*R. equi* 103^{-}) were totally cleared by day 4 in different experiments.
[b] Compared with the value for the wild type.
[c] Compared with the value for the mutant.

Virulence assessment in mice of *R. equi* mutants and their complementary strains

Double-crossover recombinants — Using liver clearance of intravenously injected bacteria as an indicator of virulence, we found the *htrA* and *narG* mutants to be fully attenuated, the *pepD* mutant to be slightly attenuated, and the *purC* and *sodC* mutants to be unaffected (Table III) in comparison with the parental *R. equi* virulent strain 103^{+}. Complementation restored the virulence of the *htrA* and *pepD* mutants but not of the *narG* mutant, and the *sodC* mutant became slightly more virulent than the parent strain. The PCR reactions showed that all colonies of *R. equi* mutants and their complementary strains recovered from mice possessed the correct genotype.

Single-crossover recombinants — None of the single-crossover mutants (*clpB*, *fadD8*, *fbpB*, *glnA1*, *regX3*, and *sigF*) was attenuated, as assessed by liver clearance in mice (Table III).

Comparison of growth of *R. equi* mutants with that of the parent 103^{+} strain

The in vitro growth phenotype of the 3 attenuated mutants (*htrA*, *narG*, and *pepD*) was similar to that of the wild type 103^{+} (Figure 2).

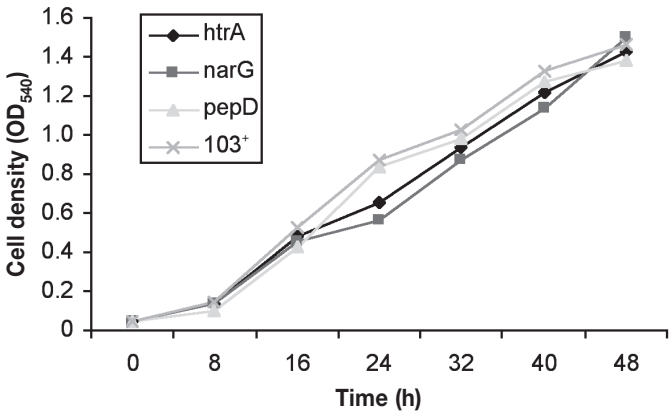

Figure 2. Growth curves for the *htrA*, *narG*, and *pepD* mutant strains and the parent strain (103^{+}) of *Rhodococcus equi* at 37°C. Results are presented as optical density at 540 nm (OD_{540}) at intervals after inoculation into growth medium.

Discussion

The overall objective of this study was to apply a recently developed system for targeted gene mutation and complementation (9) to genes with a wide range of functions identified in a partial genome sequence of *R. equi* ATCC 33701 (10). Southern blotting confirmed the lack of illegitimate recombination in strain 103^{+}, since there was shown to be only a single insertion in the chromosome, and the insertions were shown by PCR to be in the target gene (9). This contrasts with strain ATCC 33701, which has a high frequency of illegitimate recombination (9). The study also confirmed the value of the gene complementation approach using pREM. Mutants were generated by both the double-crossover homologous allelic exchange approach developed by Ren and Prescott (9) and a single-crossover approach. After making a number of double homologous recombination mutants, we adopted the single-crossover approach because it was a far faster way to obtain gene mutations, although, unlike the double-crossover approach, there is the potential for mutated genes to revert to wild type in the absence of antibiotic selection; for example, in chronic infections. No significant reversion should occur in the short term, such as in mouse-clearance or macrophage-survival studies, and none was observed in this study.

This study showed that most, but not all, attempts at targeted gene mutation were successful. The reason for the lack of success for individual genes may relate either to their essential nature or to variation between genes in their ability to be mutated. Differences between genes in ease of mutation have previously been noted (9). Clearance of the mutants in mice was compared with that of the virulent parent strain because mouse clearance data appear to correlate well with survival and growth in macrophages (8), and clearance studies are easier to perform.

The high-temperature requirement A protein encoded by *htrA* is a stress-induced serine protease involved in the folding and maturation of secreted proteins known to be involved in the virulence of many gram-negative bacteria, including *Salmonella* Typhimurium (14). This protease probably provides resistance to oxidative stress in vivo (15). The gene *narG* encodes nitrate reductase G, which plays a major role in respiration in the absence of oxygen. Anaerobic nitrate reduction has been shown to be essential for metabolism of *M. bovis* bacille Calmette–Guérin in immunocompetent but not immunodeficient mice (16). The complete attenuation of the *narG* mutant of *R. equi* in mice indicates that *narG* is important for full expression of virulence in *R. equi* and suggests that anaerobic or microaerophilic conditions may be important for *R. equi* growth during infection. In *M. tuberculosis*, genes encoding the subunits of nitrate reductase and

permitting anaerobic growth in the presence of nitrate are found in the *narGHJI* gene cluster (17). Complementation of the *narG* mutant in *R. equi* did not restore virulence, likely because *narG* and *narGHJI* are cotranscribed in *R. equi*. Our work shows that *htrA* and *narG* are apparently required for virulence in *R. equi* since these were fully attenuated in mice.

In *M. tuberculosis*, the products of the *sodA* and *sodC* genes are important for providing resistance against various oxidative stresses and for virulence (18). In a study in guinea pigs, mycobacterial *sodC* was found not to be essential for intracellular growth within macrophages and did not detectably contribute to the pathogenicity of *M. tuberculosis* (19). However, a copper or zinc-cofactored *sodC* mutant of *M. tuberculosis* had enhanced susceptibility to killing by gamma interferon (IFN-γ)-activated murine peritoneal macrophages producing an oxidative burst but was unaffected by macrophages not activated by IFN-γ and macrophages from respiratory-burst-deficient mice (20). Our mouse clearance studies showed no virulence attenuation in the *sodC* mutant of *R. equi*. The use of healthy adult CD1 mice to assess the virulence of mutant strains will, however, fail to identify any attenuation that may be observed only in activated macrophages and is a limitation of the model. The hypervirulence of the complemented sodC mutant compared with the wild type suggests that *sodC* activity contributes to survival in macrophages. Further studies using activated macrophages are required to determine whether this gene is involved in the survival of *R. equi* under these circumstances.

The product of the gene *purC* is a 1-phosphoribosylaminoimidazole-succinocarboxamide synthase involved in purine biosynthesis. Mutants of *M. bovis* and *M. tuberculosis* attenuated in mice conferred some level of protection in a challenge against aerosolized virulent *M. tuberculosis* in the guinea pig model (21). However, unlike the *M. tuberculosis* complex, the *purC* mutant of *R. equi* showed no attenuation in mice.

In *M. tuberculosis*, *pepD* (Rv0983), which encodes peptidase D, appears to be part of the *sigE* regulon; a *sigE* mutant showed defective growth in macrophages (22). The *pepD* gene is also controlled in *M. tuberculosis* by *mprA–mprB*, a 2-component regulatory system involved in establishing and maintaining persistent infection (23). Our study showed that *pepD* is involved in the survival of *R. equi* in mice, since the *pepD* mutant was slightly attenuated, and virulence was restored by complementation.

A heat-shock protein required for virulence in a murine model in *Listeria monocytogenes* (24), ClpB is regulated in *M. tuberculosis* by the alternative sigma factor SigH (25), which regulates the response to heat and oxidative stress. The lack of effect of mutation of *clpB* on mouse clearance suggests that this protein is not essential for short-term survival of *R. equi* in macrophages. In *M. tuberculosis*, the alternative sigma factor SigF is expressed under stationary-phase growth and stress conditions and provides protection against oxidative stress (26,27). Our results suggest that, as with *M. tuberculosis*, SigF is not essential for virulence in *R. equi* (18,28).

Glutamine synthase, encoded by the gene *glnA1*, is an enzyme of central importance in nitrogen metabolism that also catalyzes the extracellular synthesis of L-glutamine, an important component of the mycobacterial cell wall. This enzyme is important for the survival of *M. tuberculosis* in macrophages and essential for virulence in guinea pigs (29,30). Our study indicates that it is not essential for virulence in *R. equi*.

In *M. tuberculosis*, *fbpB* encodes the protein Ag85B, an immunodominant component of the mycobacterial antigen 85 complex (Ag85), which is a secreted protein with mycolyl transferase activity that is involved in the biogenesis of the mycobacterial cell wall (31). Of the 3 mycolyl transferase genes in *M. tuberculosis*, only *fbpA* when mutated will attenuate the growth of this organism in macrophages (32). Our study indicates that *fbpB* is not essential for virulence in *R. equi*. The *senX3–regX3* 2-component regulatory system may be a sensor of oxidative stress (33) and is required for virulence in *M. tuberculosis* (34). In *R. equi*, the *fbpB* mutant had unimpaired survival in mice.

The enzyme FadD8 is the homolog of an acyl-CoA synthase of *M. tuberculosis* involved in lipid degradation (10). We chose *fadD8* as a target acyl-CoA synthase gene because *M. tuberculosis* genes such as *fadD26* and *fadD28* that are involved in lipid metabolism are essential for virulence (18). The ability to metabolize fatty acids appears to be essential for the virulence of *R. equi* (35). In addition, a recent study of gene expression in *R. equi* showed that *fadD13*, a related-chain fatty acid CoA ligase gene, was upregulated by the organism inside macrophages (36).

We mutated *galE* since galactose is thought to be predominant in the outer lipid layer of the cell wall of *R. equi* as arabino-d-galactan linked to mycolic acids (37).

In summary, the described study results confirm the value of a system of targeted gene mutation and complementation for studies of *R. equi* infection. Because of speed, the single-crossover recombination approach is preferred, at least for screening for relevant attributes. There is still a need to improve targeted-mutation systems for double-crossover homologous recombination, particularly by use of a positive selection system equivalent to that of the *sacB* system used in targeted mutation in *M. tuberculosis*, although this approach is ineffective in *R. equi*.

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