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Identification and Mutagenesis by Allelic Exchange of *choE*, Encoding a Cholesterol Oxidase from the Intracellular Pathogen *Rhodococcus equi*

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The virulence mechanisms of the facultative intracellular parasite *Rhodococcus equi* remain largely unknown. Among the candidate virulence factors of this pathogenic actinomycete is a secreted cholesterol oxidase, a putative membrane-damaging toxin. We identified and characterized the gene encoding this enzyme, the *choE* monocistron. Its protein product, ChoE, is homologous to other secreted cholesterol oxidases identified in *Brevibacterium sterolicum* and *Streptomyces* spp. ChoE also exhibits significant similarities to putative cholesterol oxidases encoded by *Mycobacterium tuberculosis* and *Mycobacterium leprae*. Genetic tools for use with *R. equi* are poorly developed. Here we describe the first targeted mutagenesis system available for this bacterium. It is based on a suicide plasmid, a selectable marker (the *aacC4* apramycin resistance gene from *Salmonella*), and homologous recombination. The *choE* allele was disrupted by insertion of the *aacC4* gene, cloned in pUC19 and introduced by electroporation in *R. equi*. *choE* recombinants were isolated at frequencies between 10^{-2} and 10^{-3}. Twelve percent of the recombinants were double-crossover *choE* mutants. The *choE* mutation was associated with loss of cooperative (CAMP-like) hemolysis with sphingomyelinase-producing bacteria (*Listeria ivanovii*). Functional complementation was achieved by expression of *choE* from pVK173-T, a pAL5000 derivative conferring hygromycin resistance. Our data demonstrate that ChoE is an important cytolytic factor for *R. equi*. The highly efficient targeted mutagenesis procedure that we used to generate *choE* isogenic mutants will be a valuable tool for the molecular analysis of *R. equi* virulence.

The nocardioform actinomycete *Rhodococcus equi* is a primary pathogen of horses. Rhodococcal infection occurs in foals aged under 6 months old and results in severe pyogranulomatous bronchopneumonia and a high mortality rate. Respiratory disease is sometimes accompanied by mesenteric lymphadenitis and ulcerative enterocolitis. *R. equi* is widespread in its natural habitat, the soil, and rhodococcal infection is endemic in some horse farms. *R. equi* has recently emerged as an opportunistic pathogen in humans, especially in association with human immunodeficiency virus infection. Like in foals, human *R. equi* infection mainly affects the lungs, with clinical and pathological characteristics similar to pulmonary tuberculosis in immunocompromised patients. Although rare, granulomatous pneumonia, lymphadenitis, and abscesses caused by *R. equi* have been reported in a variety of mammals other than horses and humans (11, 17, 32, 40).

Despite its importance in veterinary medicine and as an emerging AIDS-associated pathogen, nothing is known about the virulence mechanisms that *R. equi* uses to colonize host tissues. The capacity of these bacteria to survive and to multiply inside the vacuolar compartment of macrophages is central to rhodococcal pathogenesis (16). Virulence in the natural host and in the mouse experimental model and the ability to replicate in macrophages has been related to the presence of an 80- to 90-kb plasmid (47). This plasmid is present in virtually all clinical isolates from foals, but it is absent from most environmental strains (10, 14, 45). The plasmid carries a cluster of seven *vap* genes encoding surface-associated proteins which react in the form of 15- to 18-kDa antigens with sera from pneumonic foals or from foals exposed to plasmid-containing, virulent *R. equi* isolates (46, 47). Because Vap antigens are upregulated at elevated temperatures (34 to 41°C) (46, 47) and have a role in the protective immune response against *R. equi* in foals (39), they are believed to play an important role in pathogenesis. To date, only one attempt has been made to assess the role of Vap proteins in virulence by using a genetic approach. The *vapA* gene was expressed in a plasmid-cured isogenic strain of *R. equi*, but this was not sufficient to restore the capacity to proliferate in macrophages and to colonize the lungs of experimentally infected foals (10), questioning a role for VapA in virulence. The virulence plasmid itself does not appear to be essential for pathogenesis in non-horse hosts, as it is not always detected in clinical isolates from humans and other animal species (4, 7, 33, 48, 49, 52). This suggests that chromosomally determined factors are involved in *R. equi* pathogenicity. Candidates for such chromosomal virulence factors include the following: the capsular polysaccharide, which might interfere with phagocytosis; mycolic acid-containing glycolipids, which are thought to be involved in granuloma formation; and, especially, cholesterol oxidase, a secreted enzyme that may act on eukaryotic membranes and be responsible for

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Description	Source or reference
R. equi		
ATCC 6939	Type strain	30
MAD	Cholesterol oxidase-hyperproducing strain	42
103^+	Clinical isolate with virulence plasmid	8
103^-	103 derivative lacking virulence plasmid	8
RHE3-15+	*choE* knockout mutant of 103^+	This work
RHE3-19	*choE* knockout mutant of 103^-	This work
L. ivanovii		
ATCC 19119	Type strain	Collection
E. coli		
DH5α	Cloning host strain	Our laboratory
HB101	Cloning host strain	Our laboratory
TG1	Cloning host strain	Our laboratory
Plasmids		
pUC19	Cloning vector	54
pGEM-Te	T-vector for cloning of PCR products	Promega
pPE207	*E. coli-Mycobacterium* shuttle vector containing apramycin resistance marker	37
pVK173-T	*E. coli-Mycobacterium* shuttle vector containing hygromycin resistance marker	37
pRE7	*E. coli-R. equi* shuttle vector	55
pRHE1	pUC19 inserted with *choE*	This work
pRHE2	pUC19 inserted with *accC4*	This work
pRHE3	pUC19 with *choE::accC4* mutant allele (suicide vector for *choE* mutagenesis by gene replacement)	This work
pRHE4	pGEM-Te inserted with *choE* and its natural promoter	This work
pRHE5	pVK173-T inserted with *choE* and its natural promoter	This work
pRHE6A	pRE7 inserted with *accC4*	This work

the observed cytotoxicity and macrophage destruction that accompany rhodococcal infection (17, 40). However, as for VapA, there is no direct proof that any of these putative virulence factors are involved in pathogenesis.

A major reason why the molecular mechanisms of *R. equi* pathogenesis remain unknown is the absence of genetic tools for creating isogenic mutants affected in individual loci in these bacteria. We have developed the first site-directed mutagenesis system that is functional in *R. equi*. This system is based on homologous recombination and on a suicide vector carrying a cassette that confers apramycin resistance. This system allowed us to generate cholesterol oxidase null mutants by insertional disruption of the structural gene of the enzyme, *choE*, which was also identified and characterized in this study.

MATERIALS AND METHODS

Bacteria, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1. *R. equi* and *Escherichia coli* were routinely grown at 37°C in Luria-Bertani medium, with rotary agitation in the case of fluid cultures. When required, antibiotics were added to culture media at the following concentrations: apramycin, 30 μg/ml; hygromycin, 150 μg/ml; ampicillin, 100 μg/ml.

DNA techniques. *R. equi* genomic DNA was prepared using a modification of a previously described protocol (2). Bacteria from 5-ml aliquots of a stationary-phase broth culture were collected by centrifugation at 10,000 × *g* for 10 min, washed in distilled water, resuspended in 0.25 ml of Tris-EDTA buffer containing 20 mg of lysozyme/ml and 50 mg of proteinase K/ml and incubated at 37°C for 2 h. Bacterial cells were then lysed by the addition of 0.25 ml of 0.1 M Tris containing 1% sodium dodecyl sulfate (SDS) and 400 μg of proteinase K/ml and incubated at 55°C for 1 h. The lysate was mixed with 0.1 ml of 5 M NaCl and 100 μl of cetyltrimethylammonium bromide-NaCl and incubated at 65°C for 10 min. DNA was then extracted with choloroform-isoamyl alcohol and phenol-choloroform, precipitated with isopropanol, and resuspended gently in distilled water. Plasmid DNA was extracted from *E. coli* using Qiagen plasmid purification kit. Single-stranded DNA (ssDNA) was prepared by mixing 1 μg of plasmid DNA with 20 μl of 0.2 M NaOH and 0.2 mM EDTA in distilled water. The mixture was incubated at 37°C for 30 min and DNA was precipitated with ethanol according to standard methodology. PCR products were purified from agarose gels with the Qiaquick purification system (Qiagen). Restriction enzymes and ligase were purchased from New England Biolabs and used according to the manufacturer's instructions. DNA was amplified using the Expand High-Fidelity PCR System (Roche). For Southern blotting, restriction endonuclease-treated total DNA was separated by agarose gel electrophoresis and immobilized on nylon membranes (Roche) by capillary blotting. Radiolabeling was performed with [α-^{32}P]dCTP (Amersham) by random priming using the Ready-to-Go kit from Pharmacia. Hybridization was performed at 65°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, 0.5% SDS, and 20 mg of salmon sperm DNA/ml. Blots were washed with 2× SSC, 0.5% SDS at room temperature for 5 min and then with 2× SSC, 0.1% SDS at 37°C for 30 min. DNA sequencing was performed on PCR products at the "Unidad de Secuenciación Automatizada de DNA" of the Universidad Complutense de Madrid using an Applied Biosystems 377 apparatus. Homology searches were performed with BLAST at the National Center for Biotechnology Information (Bethesda, Md.) website and with the Pfam (protein family database) search tool on the Sanger Centre (Cambridge, United Kingdom) internet server.

Electroporation of *R. equi*. Bacteria from 50 ml of a culture grown to an optical density at 600 nm (OD_{600}) of 0.6 were harvested by centrifugation, washed two times in 25 ml of washing buffer (cold 10% glycerol in distilled water), and resuspended in 0.2 ml of the same solution. Aliquots (40 μl) of the *R. equi* cell suspension were mixed with 200 ng of DNA in a prechilled 0.2-cm chamber and electroporated using the Gene Pulser apparatus (Bio-Rad) set at 2.5 kV/cm, 25 μF, and 1,000 Ω. After electroporation, the bacterial suspension was diluted with 1 ml of Luria-Bertani medium, incubated at 37°C for 2 h, and plated onto solid medium containing the appropriate antibiotics.

RNA techniques. Total RNA was extracted from 50-ml broth cultures grown to an OD_{600} of 0.6. Bacteria were harvested by centrifugation, resuspended in 2.5 ml of lysis buffer (0.02 M sodium acetate [pH 5.0], 1 mM EDTA, 20 mg of lysozyme/ml), and transferred to a 100-ml Erlenmeyer flask. The bacterial suspension was immediately frozen by placing the flask on dry ice and then thawed at 37°C. This treatment was repeated five times. After the last thawing, 0.25 ml of 10% SDS and 2.5 ml of 0.02 M sodium acetate-saturated phenol were added. The mixture was incubated at 70°C for 5 min with shaking and spun for 5 min in a microcentrifuge, and the aqueous phase was transferred to a fresh 100-ml flask. Phenol extractions were repeated two more times. Aliquots of 0.4 ml of the final aqueous phase were transferred to 2-ml microcentrifuge tubes, mixed with 40 μl of sodium acetate (pH 7), and precipitated with 3 volumes of ethanol. The RNA precipitate was collected by centrifugation at 12,000 × *g* for 15 min at 4°C, washed with 70% ethanol, and dried. The RNA pellet was resuspended in 100 μl of diethyl pyrocarbonate-treated distilled water and stored at −70°C. Northern blotting was carried out using 10 μg of RNA as previously described (12).

Cholesterol oxidase determinations. An assay based on the method described by Sojo et al. (43) was used. Cultures were grown to an OD_{600} of 0.6. After centrifugation, 0.1 ml of culture supernatant was mixed with 0.9 ml of reaction buffer (50 mM potasium phosphate [pH 7.0], 5 mM sodium cholate, 7 mM phenol, 0.4 mM 4-amino-antiprine, 1 mM cholesterol, 0.33% Triton X-100, and 6.7 U of horseradish peroxidase) and incubated at 37°C until a red color developed. Cholesterol oxidase activity units were calculated from the ratio between $A_{500} \times 10^4$ and $A_{600} \times d \times t$, where d is the dilution factor and t is the reaction time expressed in minutes. *choE*-derived cholesterol oxidase activity was calculated by subtracting the activity of the control ($ChoE^-$) strain from that of the isogenic test strain with functional *choE* allele.

Cooperative (CAMP-like) hemolysis assays. CAMP-like hemolysis tests were performed on sheep blood agar plates with Columbia base medium (Difco) as

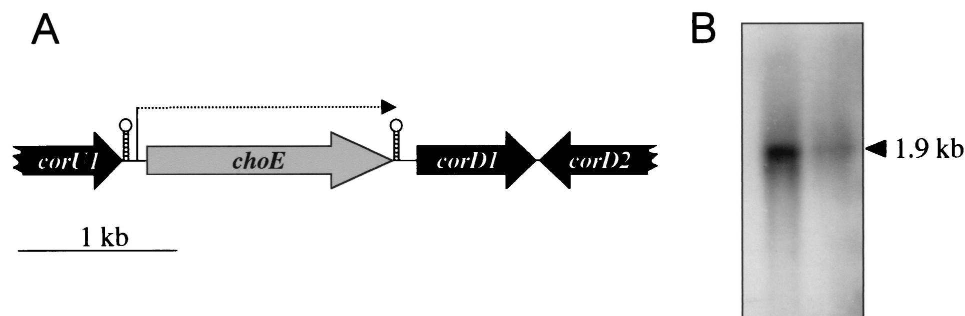

FIG. 1. Genetic organization of the *choE* region and transcriptional analysis of *choE*. (A) Physical map of the 3.9-kb chromosomal region encompassing the *choE* locus of *R. equi*. The location of the putative *choE* promoter and stem-loop transcription terminator is indicated. (B) Northern blot analysis of *choE* in strains *R. equi* MAD (left) and 103^- (right). In both strains, a single 1.9-kb transcript was detected.

described previously (42). The test and indicator bacteria were streaked perpendicularly to each other, leaving a distance between streaks of approximately 1 mm. Plates were incubated at 37°C overnight and the appearance of a shovel-shaped patch of hemolysis at the intersection of the streaks (see Fig. 5), due to the hemolytic cooperativity of the sphingomyelinase C produced by the indicator strain (*Listeria ivanovii*) and the *R. equi* cholesterol oxidase (9, 42), was recorded as a positive reaction.

Nucleotide sequence accession number. The sequence for the *R. equi choE* gene has been deposited in the EMBL database under accession number AJ242746.

RESULTS

Identification of the *R. equi* cholesterol oxidase gene *choE*. A number of gram-positive bacteria with a high G+C content, such as *Brevibacterium sterolicum, Streptomyces* spp., *Mycobacterium* spp., and *Rhodococcus* spp. (*R. equi, R. erythropolis*, and *R. rhodochrous*), have a cholesterol oxidase activity (29). This suggests that a common cholesterol oxidase genetic determinant is widespread among actinomycetes and related bacteria. Supporting this notion, the sequences of the cholesterol oxidase genes cloned to date, *choA* and *choM* from two *Streptomyces* spp. isolates (5, 19) and *choB* from *B. sterolicum* (35), show a high degree of similarity at both the nucleotide and amino acid levels. To determine whether *R. equi* contains *cho*-related sequences, Southern blots of genomic DNA digestions from three strains of this species (103^-, ATCC 6939, and MAD) (Table 1) and 20 additional clinical isolates of human and animal origin were probed with a *choB* DNA fragment. Positive signals were obtained in a 2.3-kb *Pst*I fragment for all of the *R. equi* strains tested (data not shown). To isolate the hybridizing *R. equi* DNA, a pair of degenerate oligonucleotide primers {CoXN [5′-AT(CT)TT(CT)TG(CT)GG(GC)ATGCT(AGCT)AA(CT)CC-3′] and CoXC [5′-C(GT)(GC)GC(AG)AA(CT)TT(AG)TACCA(CT)TC(AGCT)GT-3′]} were designed from the aligned amino acid sequences predicted from the known *cho* genes and used in PCRs with genomic DNA from strain 103^-. A 0.3-kb DNA fragment was amplified, cloned in pGEM-Te, and sequenced. The nucleotide sequence obtained was 99% similar to that of the corresponding segment of the *choB* gene. Several sets of primers were designed from *choB*, which allowed us to assemble a 3.9-kb region of the *R. equi* chromosome by direct and inverse PCR and subsequent primer-walking sequencing (Fig. 1). The *choB*-related sequence in this region belonged to a 1,656-bp open reading frame (ORF). The protein encoded by this ORF showed extensive similarity with the polypeptides encoded by the cholesterol oxidase genes *choB* from *B. sterolicum* and *choA* and *choM* from *Streptomyces* spp. (Fig. 2). It also exhibited significant similarity, albeit weaker, to polypeptides encoded by *Mycobacterium leprae, Mycobacterium tuberculosis*, and *Streptomyces coelicolor* described as probable cholesterol oxidases and named as ChoD (Fig. 2). The *R. equi* ORF was designated *choE* (for cholesterol oxidase of *R. equi*). *choE* was preceded at the correct distance by a putative ribosome-binding sequence, GAGG, and its stop codon was followed by a palindromic sequence of 44 nucleotides that might act as a transcription terminator. Putative −35 and −10 sites (GCGACG and CAGACC, respectively) were identified in the intergenic region upstream from *choE* on the basis of known *Rhodococcus* promoter sequences (1, 13, 23). The *choE* gene product, ChoE, is 552 amino acids long and has a predicted signal sequence of 45 residues (Fig. 2). A DNA fragment comprising the entire *choE* gene plus its putative ribosome-binding sequence and the transcription terminator was amplified by PCR with oligonucleotide primers CoEN (5′-TACCAAGCTTACCAAACCGCCGACAGAGGA-3′) and CoEC (5′-CAGTGAATTCCGCGTGAAGAAAACGTGGTC-3′) and inserted into pUC19, resulting in pRHE1 (Table 1). *E. coli* TG1 cells transformed with pRHE1 produced cholesterol oxidase activity (Table 2) and displayed cooperative, CAMP-like hemolysis with sphingomyelinase C-producing bacteria (a marker of cholesterol oxidase production; see below and Fig. 5). However, when TG1 was transformed with pRHE3, in which *choE* is disrupted by an antibiotic resistance cassette, neither cholesterol oxidase activity nor the CAMP-like hemolysis reaction was detected. These results confirmed that *choE* encodes *R. equi* cholesterol oxidase.

Genetic structure of the *choE* region and Northern blot analysis of *choE*. Three additional ORFs coding for polypeptides similar to known protein sequences were identified in the 3.9-kb DNA fragment that contains *choE* (Fig. 1). Upstream from *choE*, we found the 3′ region of an ORF, *cor* (for cholesterol oxidase region) *U1*, which encodes the last 159 residues of a protein with a high degree of similarity to a number of bacterial 3-oxoacyl-(acyl carrier protein) reductases. These enzymes belong to the short-chain dehydrogenase-reductase superfamily that is widespread in bacteria, archaea, and eu-

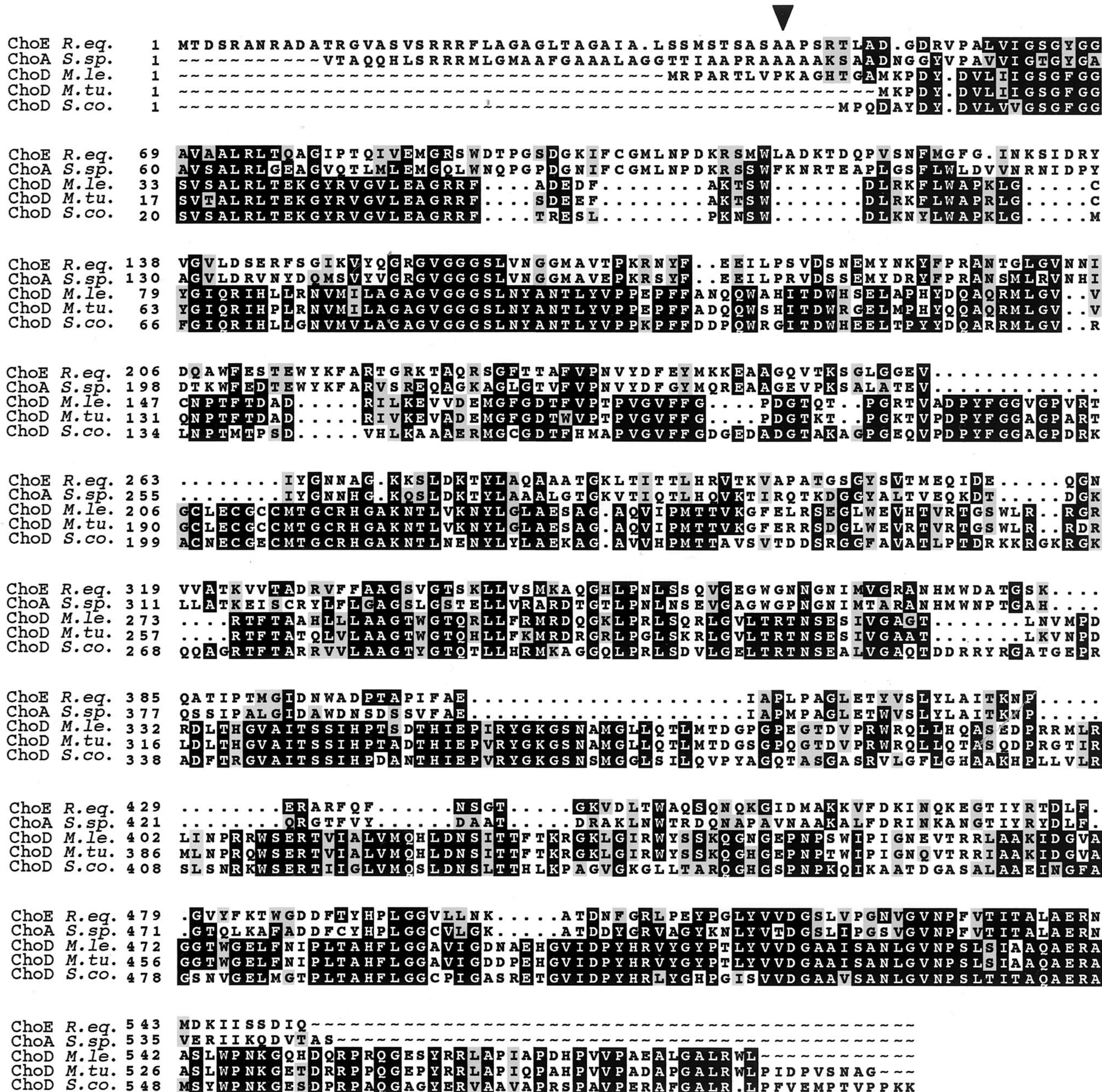

FIG. 2. Comparison of amino acid sequences of the cholesterol oxidases ChoE from *R. equi* (R. eq.; accession no. AJ242746) and ChoA from *Streptomyces* spp. strain SA-COO (S. sp.; accession no. A32260), and related ChoD polypeptides from *M. leprae* (M. le.; accession no. S72824), *M. tuberculosis* (M. tu.; accession no. F70736), and *S. coelicolor* (S. co.; accession no. AL161755). Identical amino acids are shaded in black and similar amino acids are in gray. The putative cleavage site of the signal peptide of ChoE is indicated by an arrowhead.

karyotes. Immediately downstream from *choE* and transcribed in the same orientation is *corD1*, which encodes a putative homolog of the *cfp30B* gene product (67% identity), a 27.3-kDa antigen from *M. tuberculosis*. The *corD1* product (CorD1) also shows a significant degree of similarity (32 to 39% identity) to polypeptides from *Streptomyces* and other actinomycetes, which are annotated in the databases as "probable hydrolases" or "hypothetical proteins." No obvious similarities were detected between CorD1 and any known protein sequence of archaeal or eukaryotic origin. Next to *corD1*, we found the 3′ portion of a divergently transcribed ORF, *corD2*. Its product shows significant similarity (26 to 35% identity) along most of its sequence with hypothetical proteins from *M. tuberculosis, Streptomyces* spp., and archaea, such as *Aeropyrum, Pyrococcus*, and *Sulfolobus*.

Thus, the *choE* gene is encompassed by two ORFs, which are transcribed in the same orientation (Fig. 1). In *Streptomyces* spp. strain SA-COO, *choA* and the upstream gene, *choP*,

TABLE 2. ChoE activity determinations

Strain	Enzyme activity[a]
E. coli	
TG1(pUC19)	0
TG1(pRHE1)	100.7
R. equi	
103^-	29.5
RHE3-19	0
RHE3-19(pRHE5)	37.3
103^+	32.3
RHE3-15^+	0
RHE3-15^+(pRHE5)	28.9

[a] *choE*-derived activity (see Materials and Methods).

which encodes a cytochrome P-450-like protein, are cotranscribed (18). To determine whether *choE* is expressed as part of an operon with *corU1* and/or *corD1*, total RNA from *R. equi* strains 103^- and MAD was subjected to Northern blot analysis using the entire *choE* gene as a probe (the CoEN-CoEC PCR product). In both strains, a single ≈1.9-kb transcript was detected (Fig. 1). This is consistent with the expected size of the *choE* message, indicating that *choE* is transcribed monocistronically.

Construction of *choE* mutants. An important factor in the development of a mutagenesis system is the availability of a convenient marker for positive selection of recombinational events. The following antibiotic resistance markers that had been used for selection in rhodococci and related bacteria were tested with *R. equi*: (i) the spectinomycin resistance gene, *aadA2*, from the *Corynebacterium glutamicum* plasmid pCG4 (22); (ii) the kanamycin resistance gene from Tn*903* (36) present in the *E. coli*-*R. equi* shuttle vector pRE7 (55); (iii) the apramycin resistance gene, *aacC4*, from the *Salmonella enterica* serovar Typhimurium plasmid Inc L/M (50), present in the *E. coli*-*Mycobacterium* shuttle vector pPE207 (37); and (iv) the chloramphenicol resistance gene from *R. erythropolis* plasmid pDA71 (41). Spectinomycin produced a high percentage of spontaneous resistant mutants in 103 and other *R. equi* strains and was discarded. In our hands, kanamycin, which was previously reported to be useful for plasmid selection in *R. equi* (55), also produced spontaneous mutants, although at a lower frequency than spectinomycin. Apramycin and chloramphenicol at concentrations of 30 and 100 μg/ml, respectively, gave no spontaneous resistant mutants, and the corresponding selection markers were further investigated. pRHE6A was constructed by cloning a *Not*I fragment containing the *aacC4* gene from pPE207 into the unique *Not*I site of pRE-7 (Table 1). When electroporated into *R. equi* 103^- and 103^+, apramycin-resistant transformants (Apr^r) appeared at a frequency of 1×10^6 and 1×10^5 per μg of DNA, respectively, which was suitable for the development of a mutagenesis tool based on a suicide vector. The chloramphenicol resistance gene from plasmid pDA71 was similarly tested but could not be expressed in *R. equi*.

The identified *choE* locus was used as a target for mutagenesis by allelic exchange in *R. equi*. A pair of suicide plasmids, pRHE2 and pRHE3, without and with *choE* target sequences, respectively, was constructed for this purpose (Table 1 and Fig. 3). For pRHE2, a 1.6-kb *Pst*I fragment from pPE207 containing the *aacC4* apramycin resistance gene was inserted into the corresponding restriction site of pUC19. For pRHE3, the *aacC4* gene was excised from pRHE2 and inserted into the unique *Bam*HI site of pRHE1, thus generating a recombinogenic cassette comprising the *aacC4* gene flanked by the 5′ and 3′ regions of *choE* (Fig. 3). The only origin of replication present in pRHE2/3 was that from pUC19, which is nonfunctional in *R. equi*. Thus, any Apr^r colonies arising after electroporation of these plasmids into *R. equi* should result from recombination of the plasmid with the genome of the host bacterium. Strains 103^- and 103^+ were electroporated with 200 ng of each of the plasmids and plated on solid medium containing apramycin. No recombinants were detected with pRHE2, but a large number of recombinants was obtained with pRHE3. These data were compatible with the Apr^r colonies resulting from site-specific chromosomal integration of the suicide plasmid via homologous recombination between *choE* sequences.

The use of ssDNA increased the number of Apr^r recombinants six-fold in both host strains. The number of recombinants per microgram of DNA was 10-fold higher in strain 103^- than in 103^+, consistent with the different transformation frequencies observed with the bifunctional plasmid pRHE6A (see above). Assuming that the suicide plasmids are taken up by *R. equi* at a frequency similar to that of pRHE6A, the recombination efficiencies would be between 1×10^{-2} and 1×10^{-3} for pRHE3 and between $<1 \times 10^{-5}$ and $<1 \times 10^{-6}$ for pRHE2 (Table 3).

To confirm that homologous recombination events were at the origin of the Apr^r phenotype in the pRHE3-transformed *R. equi*, the structure of the *choE* region was analyzed by PCR mapping and Southern blotting in 20 recombinants from each of the *R. equi* strains, 103^- and 103^+. For PCR mapping, primers CoN (5′-ACGCGTCCCGTCAGGCTCCGCT-3′), CoC (5′-ACGCGTGAAGAAAACGTGGTCG-3′), CoIN (5′-GTCAACAACATCGACCAGGCG-3′), and CoP1 (5′-GAC GCTGAATACCGCGTCCT-3′) were used. CoN and CoP1 target sequences were absent from the recombinogenic plasmid (Fig. 3). PCR amplifications with primers CoN and CoC, which normally produce a 2.3-kb product in wild-type *R. equi*, generated a 3.9-kb DNA fragment in three of the Apr recombinants analyzed from strain 103^- and two from the 103^+ recombinants (Fig. 3 and 4). Similarly, a 1.6-kb increase in size with respect to the parent strain was also observed when PCR was performed in these recombinants with the primers CoIN and CoP1. This is the expected size for a *choE* mutant allele resulting from the integration of the *aacC4* cassette by double-crossover recombination (DCR) in the *choE* chromosomal locus (Fig. 3 and 4). The occurrence of a double recombination event at the *choE* locus was verified by sequencing the junctions between *choE* and the *aacC4* cassette from two DCR mutants, RH3-19 (from 103^-) and RH3-15+ (from 103^+). The other 35 Apr^r transformants were single-crossover recombinants (SCRs), resulting from the integration of pRHE3 either via the upstream (SCR-1) or the downstream (SCR-2) *choE* target sequences. Of these SCRs, 33 were of the SCR-1 type, characterized by a larger CoN-CoC product of 3.9 kb (1.6-kb increase in size) and a wild-type CoIN-CoP1 product of 1.6 kb, with no PCR product being generated with primers CoN and CoP1 (Fig. 3 and 4). Only two recombinants (both from strain

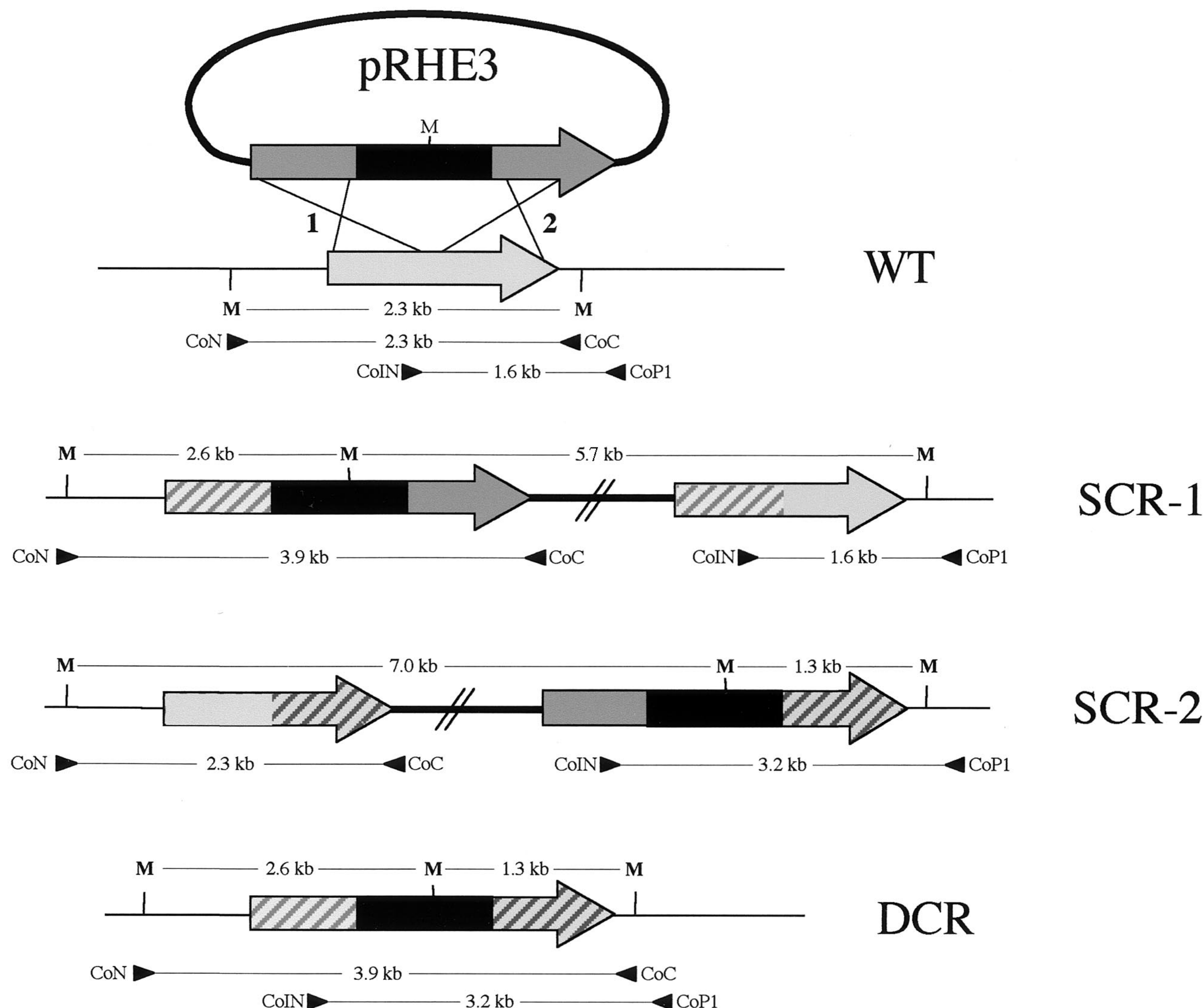

FIG. 3. Schematic diagram of the procedure for targeted mutagenesis of *choE* by homologous recombination and a physical map of the *choE* locus in the parent strain (WT) and the recombinants (SCR-1 and SCR-2, single crossover recombinants in sites 1 and 2, respectively, and DCR, double crossover recombinant). The recombinogenic cassette in the suicide plasmid, pRHE3, includes the *aacC4* apramycin resistance gene, shown in black, surrounded by the flanking *choE* target sequences, shown in dark gray. The wild-type *choE* allele is light gray. The crossover target sequences in each type of recombinant are dashed in light and dark gray. CoN-CoC and CoIN-CoP1 primer pairs were used for PCR mapping, and M indicates the position of the *Mlu*I restriction sites used in Southern blot analysis of the recombinants (Fig. 4).

103^+) were of the SCR-2 type, characterized by a CoN-CoC product of wild-type size (2.3 kb) and a larger CoIN-CoP1 product of 3.2 kb (1.6-kb increase), with no product with CoN and CoP1 (Fig. 3 and 4). Southern blot analyses of the insertion mutants were entirely consistent with the results of PCR mapping (Fig. 3 and 4).

Stability and phenotypic characterization of *choE* mutants. To assess the stability of the *choE* insertion mutants, RHE3-19 and RHE3-15^+ were grown for approximately 100 generations in the absence of selective pressure and plated out on antibiotic-free medium. One hundred colonies from each strain were picked onto agar plates containing apramycin. All replica colonies grew in the presence of the antibiotic, indicating that the *aacC4* cassette was stably inserted into the *R. equi* chromosome.

TABLE 3. Recombination frequencies of the suicide plasmid pRHE3 (containing *choE* target sequences) or pRHE2 (no *choE* target sequences) in *R. equi*

Suicide plasmid	Recombination frequency[a] in strain:	
	103^+	103^-
pRHE3	1.9×10^{-3}	7.5×10^{-3}
pRHE3ss	11.5×10^{-3}	47.5×10^{-3}
pRHE2	$<10^{-5}$	$<10^{-6}$
pRHE2ss	$<10^{-5}$	$<10^{-6}$

[a] Recombination frequencies were calculated as the ratio between transformation frequencies (expressed as Apr^r colonies per microgram of DNA) obtained with the suicide plasmids and with the pRHE6A vector, which replicates in *R. equi*.

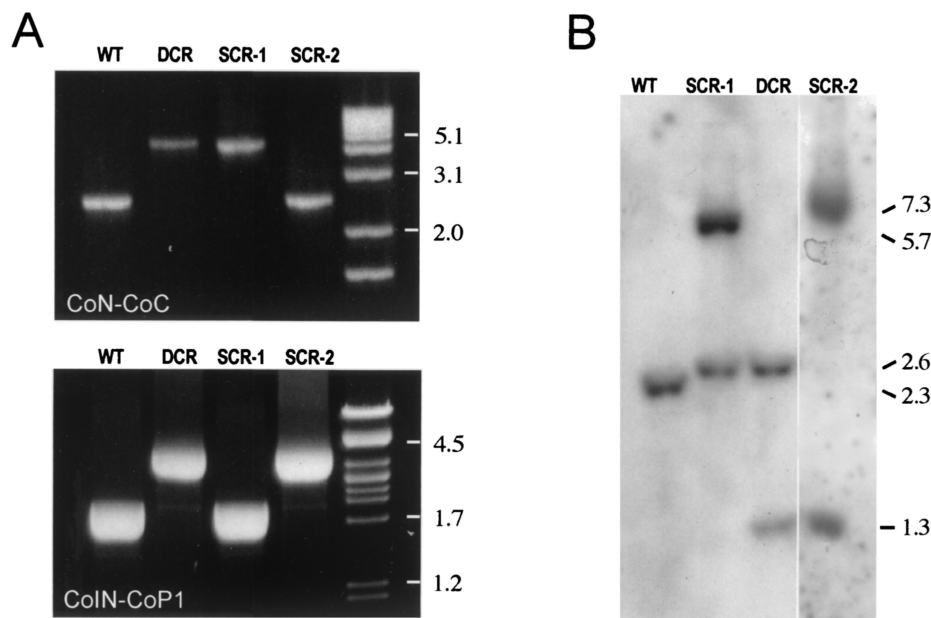

FIG. 4. PCR mapping and Southern blot analysis of *choE* recombinants. (A) PCR mapping with CoN-CoC (upper panel) and CoIN-CoP1 (lower panel) primer pairs. See text and Fig. 3 legend for details. (B) Southern blot analysis. Genomic DNA from representative mutants of each recombinational type were cut with *Mlu*I and hybridized against the CoEN-CoEC probe covering the entire *choE* gene. Hybridization patterns of *R. equi* (WT), DCRs, and the two types of SCRs are shown. In *R. equi, choE* is flanked by two *Mlu*I sites separated by 2.3 kb; thus, the WT displays a single 2.3-kb hybridization band. pRHE3 has a single *Mlu*I site located at the middle of the *aacC4* gene (Fig. 3); therefore, DCR shows two bands of 2.6 and 1.3 kb. In SCR-1, there were two bands of 2.6 and 5.7 kb. The 2.6-kb band is equivalent to the band of the same size observed in DCR and corresponds to the left side of the *aacC4*-disrupted *choE* allele; the 5.7-kb band includes the right side of the *aacC4*-disrupted *choE* allele, pUC19, and the intact copy of *choE* (Fig. 3). In SCR-2, two hybridization bands of 7.0 and 1.3 kb were observed (Fig. 3). When membranes were rehybridized with a pUC19 probe, hybridization signals were detected in SCR-1 and SCR-2, confirming the presence of the complete pRHE3 plasmid integrated at the *R. equi choE* locus as the result of an SCR event (data not shown).

Although *R. equi* is nonhemolytic on sheep blood agar, it develops a strong patch of synergistic hemolysis if streaked in the vicinity of sphingomyelinase C-producing bacteria, such as *L. ivanovii* or *Staphylococcus aureus* (42). Cholesterol oxidase is thought to be the *R. equi* factor responsible for this cooperative, CAMP-like lytic reaction (9, 26, 42). The five DCRs, in which only a disrupted *choE* allele is present on the chromosome, were negative in a CAMP-like hemolysis test with *L. ivanovii* (Fig. 5). This correlated with a loss of cholesterol oxidase activity in the culture supernatant of these mutants (Table 2). All of the SCR mutants, which bear an intact copy of *choE* in addition to the *choE*::*aacC4* allele (Fig. 3), tested positive in the CAMP-like reaction and produced cholesterol oxidase activity at wild-type levels (data not shown). These results were consistent with the involvement of cholesterol oxidase in the cooperative hemolysis shown by *R. equi* with *L. ivanovii*.

The *choE* mutation had no effects on colony morphology, bacterial growth rate, or biochemical profiles.

Complementation of *choE* mutants. To confirm directly that the disruption of *choE* was responsible for the observed loss of CAMP-like reactivity, we complemented the DCR mutants with *choE*. A DNA fragment containing *choE* plus its putative promoter region was inserted into pVK173-T, a pAL5000 derivative, giving rise to pRHE5 (Table 1). We previously verified that pVK173-T replicates and can be selected via its hygromicin resistance marker in *R. equi*. Introduction of pRHE5 restored cholesterol oxidase activity (Table 2) and CAMP-like reactivity with *L. ivanovii* (Fig. 5) in both RH3-19 and RH3-15^{+}. These results were entirely consistent with those obtained by complementation of *E. coli* with *choE* (see above) and confirmed that cholesterol oxidase is the *R. equi* membrane-damaging factor responsible for cooperative hemolysis with sphingomyelinase C-producing bacteria.

Finally, we assessed the stability of pRHE5 in *R. equi* by testing the number of bacteria that retained the hygromycin resistance (Hyg^r) phenotype after growth for 100 generations without selective pressure, as described above. The percentage of Hyg^r colonies decreased from 90% after 20 generations to 6% after 60 generations. These data show that the pAL5000-based mycobacterial replicon is largely unstable in the absence of selective pressure in *R. equi*.

DISCUSSION

We approached the molecular analysis of *R. equi* virulence by investigating cholesterol oxidase, a putative membrane-active virulence factor that is released into the culture supernatant by all isolates of this pathogenic actinomycete. We identified the gene encoding this enzyme by PCR using degenerate oligonucleotides designed from the known sequences of cholesterol oxidase genes previously identified in several high-G+C-content gram-positive bacteria. The *R. equi* cholesterol oxidase gene, *choE*, was almost identical (99.2% identity) to *choB* from *B. sterolicum*, and their corresponding protein products differed only in one residue. This high degree of sequence

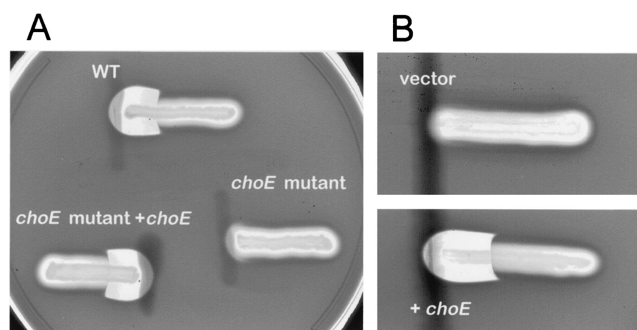

FIG. 5. Cooperative hemolysis assays with the sphingomyelinase-producing indicator species *L. ivanovii* (horizontal streaks). (A) Wild-type *R. equi* 103^- (WT) and its isogenic derivatives RHE3-19 (*choE*/pRHE5 mutant) and RHE3-19 (*choE* mutant *choE*). The *choE* mutant has lost the capacity to produce a shovel-shaped CAMP-like reaction and this property is recovered upon complementation with *choE*. (B) *E. coli* K-12 with the control vector pRHE2 (vector) and with the *choE*-containing plasmid pRHE1 (+*choE*); complementation with *choE* confers CAMP-like activity similar to that of wild-type *R. equi*.

conservation is unusual for two different bacteria, especially if we take into consideration that the primary structures of the cholesterol oxidases from two isolates belonging to the same taxon (strains SA-COO and A19249 of *Streptomyces* spp.) are 20% divergent. The sequence identity between *choE* and *choB* may be explained by a recent horizontal gene transfer event between *R. equi* and *B. sterolicum*. Another possibility is that ATCC 21387, the only known isolate of *B. sterolicum* (a species which, in addition, is not officially recognized as belonging to the genus *Brevibacterium* [21]), was missclassified and actually belongs to *R. equi*. Morphological, physiological, and genetic analyses on *B. sterolicum* ATCC 21387 suggest that this explanation may be correct (our unpublished observations).

The 552-amino-acid-long ChoE protein has a putative signal peptide of 45 residues and is similar (55 to 57% identity) to the above-mentioned cholesterol oxidases from the *Streptomyces* spp. strains SA-COO (19) and A19249 (5). These *Streptomyces* cholesterol oxidases were originally designated ChoA and ChoM (respectively) but now both are found in the protein databases with the name ChoD. ChoE is also related, although more distantly (24 to 27% identity), to a group of putative polypeptides found in mycobacteria and in *S. coelicolor* (Fig. 2). These polypeptides are also designated ChoD in the protein databases and are assumed to be cholesterol oxidases due to their structural similarities with known cholesterol oxidase enzymes. However, unlike the cholesterol oxidases from *R. equi* and *Streptomyces* spp. strains SA-COO and A19249, the mycobacterial and *S. coelicolor* ChoD proteins lack a signal sequence (Fig. 2), suggesting a cytoplasmic localization. This is also the case for the hypothetical proteins Y4NJ from *Rhizobium* sp. strain NGR234 (EMBL accession no. P55582) and Rv0492c from *M. tuberculosis* strain H37RV, classified as putative glucose-methanol-choline (GMC)-type oxidoreductases and which also exhibit significant similarity with ChoE (21 and 25% identity in a substantial sequence overlap, respectively). Production of cholesterol oxidase has been reported in *Mycobacterium* spp. (44), and membrane-bound or cytoplasmic forms of this enzymatic activity have been described in actinomycetes (20, 29). It remains to be determined whether the more distant ChoD polypeptides from *Mycobacterium* and *S. coelicolor* are indeed cytoplasmic cholesterol oxidases or enzymes without this activity but which belong to a group of oxidoreductases that share general structural features with cholesterol oxidases.

Targeted gene disruption via homologous recombination provided experimental evidence that *choE* encodes a cholesterol oxidase. Although there have been some previous attempts to use this genetic strategy in rhodococci, as for example in the plant pathogen *Rhodococcus fascians* (6) and in various unclassified strains of biodegradative *Rhodococcus* spp. (e.g., RHA1 and M5) (31, 53), this kind of mutagenesis procedure was not developed for *R. equi*. The previously described mutagenesis procedures for non-*R. equi* rhodococci used as selection markers the chloramphenicol resistance gene from *R. erythropolis* or the kanamycin resistance gene from Tn*903* (24). However, in our hands these markers were unsuitable for *R. equi* due either to the absence of expression (chloramphenicol) or the appearance of an excessively high rate of spontaneous resistance mutations (kanamycin).

We identified apramycin and the apramycin resistance gene, *aacC4*, from the *Salmonella* plasmid IncL/M (50) as a clean selection system for use in *R. equi*. This marker was previously found to be useful in mycobacteria (37). With this selection system, we constructed a suicide recombinogenic plasmid, pRHE3, which allowed us to produce chromosomal *choE* insertion mutants by allelic exchange following electroporation in *R. equi*. Specific recombination at the *choE* locus was confirmed by PCR mapping, Southern blotting, and DNA sequencing. These recombination events were 10-fold more frequently detected in *R. equi* 103^- than in 103^+, possibly due to the higher transformation efficiency of the former strain. The reason for this higher transformability of 103^- is presently unknown. As in other bacteria, such as *Streptomyces* (34) and *Mycobacterium* (15), the frequency of homologous recombination increased if ssDNA was used for transformation. This suggests that homologous recombination in *R. equi* proceeds through the general recombinational pathway described for *E. coli*, which involves a ssDNA intermediate (27). The use of ssDNA may be useful to enhance the mutational efficiency in *R. equi* strains more refractory to genetic manipulation than strain 103.

The *choE* gene was targeted in all of the Apr^r colonies analyzed, and no Apr^r transformants were obtained if the suicide plasmid did not contain *choE* target sequences. This indicates that homologous recombination works perfectly, and it suggests that the frequency of illegitimate rearrangement is negligible in *R. equi*. This is in contrast with the situation described for other rhodococci, such as the biodegradative *Rhodococcus globerulus*, in which most of the recombinants obtained resulted from nonhomologous recombination (24). The frequency of DCR we obtained (12% of the Apr^r population) was in the range of that reported in mycobacteria using a similar mutational strategy (38). SCR-1-type recombinants may have been obtained more frequently than SCR-2-type recombinants because the *choE* target sequence upstream from the *aacC4* cassette in pRHE3 (1 kb) was significantly longer than that situated downstream (0.6 kb).

Direct evidence that *choE* encodes a cholesterol oxidase was obtained by *trans*-complementation in *R. equi choE* mutants as

well as in *E. coli*. A shuttle vector, pRE-7, containing the origin of replication of the 80-kb virulence plasmid of *R. equi* and the *E. coli* CoE1 *ori* from pBluescript, was available (55). Selection with this plasmid was based on the *aph* kanamycin resistance gene of pACYC177, originally from Tn*903*, which was shown by us to be not suitable as it gives rise to spontaneous resistant mutants at a relatively high frequency. Therefore, we tried another shuttle vector, pVK173-T, containing an origin of replication derived from the mycobacterial plasmid pAL5000 and a hygromycin resistance gene (10, 37). The *choE*-inserted pVK173-T derivative (pRHE5) was able to replicate in *R. equi* and complemented the *choE* mutation. However, in contrast to pRE-7, which has been reported to be perfectly stable in *R. equi* (55), the pAL5000-derived mycobacterial replicon was unstable in *R. equi* in the absence of selective pressure.

In this study we have, finally, demonstrated that cholesterol oxidase is a major membrane-damaging factor of *R. equi*. Inactivation of *choE* abrogated the rhodococcal cohemolytic activity, which was restored upon complementation with *choE*. In addition, the expression of *choE* in *E. coli* K-12 conferred to this nonhemolytic bacterium the same membrane-damaging activity as that of wild-type *R. equi*. This provides support to the current belief that cholesterol oxidase is a major cytotoxic factor possibly involved in macrophage and leukocyte destruction and in the generation of the pyonecrotic lesions that characterize *R. equi* infection in humans and animals (26, 28, 40). The membrane-damaging activity of *R. equi* is observed in vitro on sheep blood agar as a cooperative, CAMP-like hemolytic reaction in the presence of bacterial sphingomyelinase C (12, 42). This requirement for a concomitant exposure of erythrocytes to a sphingomyelinase indicates that the cholesterol oxidase substrate is not directly accessible to the enzyme in intact membranes. Sphingomyelin is exposed on the outer lipid leaflet and its degradation by a sphingomyelinase leads to sublytic damage of the membrane, allowing cholesterol oxidase to reach its target substrate (9). The 3-hydroxyl of cholesterol, thought to mediate sterol-phospholipid interaction, is thus oxidized, leading to the formation of cholest-4-en-3-one and the total disorganization of the membrane (25). The relevance in pathogenesis of this dependency of cholesterol oxidase on another membrane-damaging enzyme for the exertion of a cytolytic effect remains to be determined. In pathogenic bacteria there are precedents for virulence factors that act cooperatively to cause membrane damage, as described for *Listeria* hemolysin and phospholipases (51). There is evidence that *R. equi* produces also a phospholipase activity (3), and it is possible that during infection this phospholipase forms part of a bipartite cytolytic system together with ChoE to efficiently alter the host cell membranes and cause cytotoxicity and tissue destruction. The *choE* isogenic mutants and the *choE*-complemented bacteria described here will be instrumental in determining the role of ChoE in *R. equi* virulence.

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