The Hydroxamate Siderophore Rhequichelin Is Required for Virulence of the Pathogenic Actinomycete Rhodococcus equi

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We previously showed that the facultative intracellular pathogen Rhodococcus equi produces a nondiffusible and catecholate-containing siderophore (rhequabactin) involved in iron acquisition during saprophytic growth. Here, we provide evidence that the rhbABCDE cluster directs the biosynthesis of a hydroxamate siderophore, rhequichelin, that plays a key role in virulence. The rhbC gene encodes a nonribosomal peptide synthetase that is predicted to produce a tetrapeptide consisting of N5-formyl-N5-hydroxyornithine, serine, N5-hydroxyornithine, and N5-acyl-N5-hydroxyornithine. The other rhb genes encode putative tailoring enzymes mediating modification of ornithine residues incorporated into the hydroxamate product of RhbC. Transcription of rhbC was upregulated during growth in iron-depleted medium, suggesting that it plays a role in iron acquisition. This was confirmed by deletion of rhbCD, rendering the resulting strain R. equi SID2 unable to grow in the presence of the iron chelator 2,2-dipyridyl. Supernatant of the wild-type strain rescued the phenotype of R. equi SID2. The importance of rhequichelin in virulence was highlighted by the rapid increase in transcription levels of rhbC following infection and the inability of R. equi SID2 to grow within macrophages. Unlike the wild-type strain, R. equi SID2 was unable to replicate in vivo and was rapidly cleared from the lungs of infected mice. Rhequichelin is thus a key virulence-associated factor, although nonpathogenic Rhodococcus species also appear to produce rhequichelin or a structurally closely related compound. Rhequichelin biosynthesis may therefore be considered an example of cooption of a core actinobacterial trait in the evolution of R. equi virulence.

The genus Rhodococcus, which contains over 40 species, is widely distributed in the environment, including in soil (3). The enormous metabolic diversity of these species is exploited in a large number of biotechnological applications, ranging from bioremediation of soils to production of fine chemicals (3, 45). Rhodococcus equi, the only animal pathogen in this genus, proliferates rapidly as a saprophyte in soil, especially when these soils are enriched with manure of grazing herbivores (2, 20). R. equi is a multisite pathogen infecting a wide range of animals as well as humans. However, as the name implies, R. equi is predominantly an equine pathogen, in particular of young foals, which become infected in the first 6 months of life (31, 48). Equine R. equi disease most frequently presents as pyogranulomatous catarrhal pneumonia, while ulcerative enteritis and osteomyelitis are also common manifestations (31, 48). The success of R. equi as a pathogen depends on its ability to prevent phagosomal maturation and the production of microbialicidal compounds following uptake by phagocytic cells (19, 43). R. equi subsequently proliferates within these compartments, eventually killing the macrophage in a necrotic manner (16, 26). Proliferation in macrophages and development of disease in foals are dependent on VapA, a member of the virulence-associated protein (Vap) family encoded within a pathogenicity island of the virulence plasmid (18, 21).

Iron plays a critical role as an electron carrier and biocatalyst and is therefore an essential micronutrient in most bacteria. While iron is abundant in nature, it is usually present as Fe3+, which is insoluble at neutral pH in aerobic environments (1). In animals iron is sequestered by proteins such as lactoferrin and transferrin or is bound to heme. In addition, mammals respond to infection by further lowering the iron concentration as part of the acute-phase response (35). The concentration of free iron both in soil and in the host is therefore too low to sustain bacterial growth.

To gain access to iron, many saprophytic and pathogenic bacteria produce siderophores, a structurally diverse group of low-molecular-weight compounds that are characterized by an extremely high affinity for iron (51). The majority of siderophores have hydroxamate, catecholate, or carboxylate iron coordinating groups; however, mixed siderophores that have more than one type of functional group have also been identified (12, 51). Siderophores may be produced by nonribosomal peptide synthetases (NRPS) or by NRPS-independent pathways (9, 12). Following chelation of Fe3+ in the medium, siderophores are taken up by their cognate ABC transport systems, and Fe3+ release subsequently occurs either by reduction of Fe3+ to Fe2+ or by hydrolysis of the siderophore (23, 25, 28).

We previously demonstrated that R. equi produces a diffusible catecholate-containing siderophore (rhequabactin) during growth in iron-depleted medium (30). The synthesis of
TABLE 1 Bacterial strains and plasmids used in this study

<table>
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<th>Strain or plasmid</th>
<th>Genotype or characteristics</th>
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<td>E. coli DH5α</td>
<td>supE44 ΔlacU169 (ΔlacZΔM15) hisD17 recA1 endA1 gyrA96 thi-1 relA1</td>
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<td>pSId2K</td>
<td>pSelAct derivative containing the upstream region of rhibC (coordinates 788716–790175) and the downstream region of rhibD (coordinates 808254–809630) producing an in-frame deletion of rhibCD</td>
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rhequibactin depends on a six-cistron operon containing two NRPS genes, iupS and iupT, and four genes encoding siderophore biosynthesis and transport proteins. Upstream and transcribed divergently of this operon is a four-cistron operon encoding en-zyms required for the production of 2,3-dihydroxybenzoic acid, which is also present in the mixed-type catecholate-hydroxamate siderophores heterobactin A, rhodobactin, and rhodochelin produced by R. erythropolis, R. rhodochrous, and R. jostii, respectively (4, 7, 14). In addition to rhequibactin, R. equi appears to synthe-size a nonsoluble siderophore that is produced by an NRPS protein encoded by iupU (30). While disruption of either iupS or iupU prevented growth of R. equi in medium supplemented with the iron chelator 2,2-dipyridyl, it did not attenuate R. equi (30).

This work analyzed the rhibABCE gene cluster, which, based on a bioinformatic analysis, is predicted to direct the synthesis of a tetrapeptide hydroxamate siderophore, rhequichelin. We show here that this putative siderophore is essential for virulence of R. equi as judged by its requirement for bacterial proliferation in macrophages and mice.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown at 37°C in brain heart infusion broth (BHI), in Luria-Bertani (LB) broth (40), or in polypropylene flasks in minimal medium (22) supplemented with 20 mM l-lactate. Water used for minimal medium was treated with Chelex-100 ion-exchange resin to remove iron as directed by the manufacturer (Bio-Rad). Vishniac-Santer trace elements (46) with FeSO4 were added (4, 7, 14). In addition to rhequibactin, R. equi appears to synthesis a nonsoluble siderophore that is produced by an NRPS protein encoded by iupU (30). While disruption of either iupS or iupU prevented growth of R. equi in medium supplemented with the iron chelator 2,2-dipyridyl, it did not attenuate R. equi (30).

This work analyzed the rhibABCE gene cluster, which, based on a bioinformatic analysis, is predicted to direct the synthesis of a tetrapeptide hydroxamate siderophore, rhequichelin. We show here that this putative siderophore is essential for virulence of R. equi as judged by its requirement for bacterial proliferation in macrophages and mice.

RNA isolation. RNA was isolated from R. equi grown in vitro as described previously (39). R. equi RNA was isolated from macrophages following phagocytosis of the pathogen using a guanidine thiocyanate-based lysis buffer (4 M guanidine thiocyanate, 0.5% [wt/vol] sodium N-lauryl sarcosine, 25 mM sodium citrate, and 0.1 M β-mercaptoethanol) as previously reported (44), and the loss of the plasmid was confirmed by apramycin sensitivity. Excision of the plasmid by homologous recombination produced either the deletion mutant, R. equi SIDD2, or the revertant wild-type strain, R. equi SIDD2rev. The genotypes of the mutant and revertant strains were confirmed by PCR analysis using primer pairs that are complementary to sequences within (SIDD2_210F and SIDD2_210R) and outside (SIDD2_205EF and SIDD2_205ER) the deleted region. The presence of the virulence plasmid in wild-type, mutant, and revertant strains was confirmed by amplification of vapA using primer pair VapA_182F and VapA_182R (Table 2).

**RNA isolation.** RNA was isolated from R. equi grown in vitro as described previously (39). R. equi RNA was isolated from macrophages following phagocytosis of the pathogen using a guanidine thiocyanate-based lysis buffer (4 M guanidine thiocyanate, 0.5% [wt/vol] sodium N-lauryl sarcosine, 25 mM sodium citrate, and 0.1 M β-mercaptoethanol) as previously described (6, 37). Samples were vortexed and passed 10 times through a 21-gauge needle to shear macrophage DNA and to reduce viscosity. Intracellular bacteria were recovered by 30 min of centrifugation (3,220 × g). Pelleted bacteria were lysed using TRIZol (Sigma) and physically disrupted with zirconia beads in a MagNALyser instrument (Roche). Total RNA was isolated by chloroform extraction followed by DNA digestion with Turbo DNase (Ambion) and application to a Qiagen RNeasy column, with a second, in-column, DNA digestion with the RNase-free DNase as previously described (29).

**Reverse transcription and reverse transcription-PCR (RT-PCR).** cDNA was produced by extension of hexamer random primers with ImProm-II reverse transcriptase and 1 U/μl Ribonuclease inhibitor using...
TABLE 2 Oligonucleotides used in this study

<table>
<thead>
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<td>16SrRNA200R</td>
<td>ACTCAAGTCTGCGCCGATCG</td>
<td>qPCR of the 16S rRNA gene</td>
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80 ng of total RNA following the manufacturer’s directions (Promega). DNA contamination of RNA samples was ruled out by including controls with no addition of reverse transcriptase. Endpoint PCR was performed using oligonucleotides listed in Table 2 and KAPA2G Fast DNA polymerase following the manufacturer’s instructions (Kapa Biosystems).

qPCR and analysis of data. Quantitative PCR (qPCR) using appropriate oligonucleotides (Table 2) was performed using the hot-start LightCycler 480 SYBR green I Master as recommended by the manufacturer. Samples were subjected to 45 cycles of 94°C for 15 s, 60°C for 15 s, and 72°C for 15 s with temperature transition rates of 4.2, 2.2, and 4.2°C/s, respectively. A melting curve analysis from 50 to 99°C (temperature transition, 0.2°C/s) was performed after amplification. At least two independent experiments in duplicate were performed for each sample. The efficiency of amplification (E) was determined for each pair of primers with the equation $E = 10^{-1/\text{slope}}$, where s is the slope of the standard curve. Fold changes were calculated using the $2^{-\Delta\Delta C_{T}}$ method. 16S rRNA was used as a reference for normalization. Absolute quantification of transcripts made use of standard curves of known amounts of template DNA in the range of $10^{2}$ to $10^{6}$ molecules.

Eukaryotic cell cultures. Macrophage-like J774A.1 cells (ATCC TIB-67) were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum and 2 mM l-glutamine and grown at 37°C with 5% CO$_{2}$. Primary bone marrow-derived macrophages were obtained from femurs and tibias of BALB/c mice (Charles River, Wilmington, MA) aged 6 to 8 weeks by flushing with 5 ml each of cold phosphate-buffered saline (PBS) (without CaCl$_{2}$ and MgCl$_{2}$) supplemented with penicillin-streptomycin (100 units · ml$^{-1}$ penicillin and 100 μg · ml$^{-1}$ streptomycin) and collected in 50-ml conical tubes. The cells were spun for 10 min at 1,100 rpm. The supernatant was discarded, and the cell pellet was resuspended in 24 ml per mouse of DMEM, 10% (vol/vol) fetal calf serum (FCS), 10% (vol/vol) CSF-1 conditioned supernatant, and 2 mM glutamine. The cells were plated in 6-well non-tissue-culture-treated plates (4 ml/well) and incubated at 37°C with 5% CO$_{2}$. On day 3, a further 4 ml/well medium was added, and cells were incubated for an additional 3 days. On day 6, nonadherent cells were removed by aspiration of the medium, and adherent cells were washed once with 4 ml PBS. Adherent cells were then resuspended by addition of 8 ml/well cold PBS and incubation of plates at 4°C for 15 min. After incubation, any remaining adherent cells were gently resuspended using a sterile cell scraper. Cells were subsequently transferred to sterile 50-ml tubes and spun at 1,100 rpm for 10 min. The resulting pellet was suspended in medium composed of DMEM, 10% (vol/vol) FCS, 10% (vol/vol) CSF-1 conditioned supernatant, and 2 mM glutamine. The total cell number was determined by using a hemocytometer, and the cell concentration was adjusted to obtain $2 \times 10^{6}$ cells · ml$^{-1}$. These cells were then used directly for macrophage assays or frozen in 90% (vol/vol) FCS containing 10% (vol/vol) dimethyl sulfoxide (DMSO) in liquid nitrogen till further use.

Macrophage infections. Both macrophage-like J774A.1 cells and primary bone marrow macrophages were used to perform infection assays to compare the intracellular proliferation of R. equi and its derivative mutant R. equi SID2. Macrophages were seeded at $2 \times 10^{5}$ cells per well into 24-well tissue culture plates. Overnight broths cultures of bacteria at an optical density at 600 nm of 1.0 ($2 \times 10^{6}$ CFU ml$^{-1}$) were pelleted, washed once with PBS, and resuspended in PBS. Macrophage monolayers were washed once with warm DMEM, and the medium was replaced with fresh DMEM supplemented with 10% (vol/vol) FCS, 10% (vol/vol) CSF-1 conditioned supernatant, and 2 mM glutamine. Bacteria were added at a multiplicity of infection (MOI) of 10 bacteria per macrophage. Bacterial incubation with macrophages proceeded for 60 min at 37°C, followed by repeated washing of the macrophage monolayer with prewarmed DMEM to remove unbound bacteria. The medium was subsequently replaced with complete DMEM supplemented with amikacin sulfate (20 μg · ml$^{-1}$), and the infected cells were incubated at 37°C in the presence of 5% CO$_{2}$. At various times postinfection, macrophage monolayers were washed repeatedly, and 500 μl sterile water was then added to lyse the macrophages upon further incubation at 37°C for 20 min. Bacterial growth was determined by dilution plating of macrophage lysates. CFU were enumerated after 1, 24, 48 h, and 72 h postinfection.
For the intramacrophage gene expression analysis, J774A.1 cells were seeded at 6 × 10⁵ cells ml⁻¹ in a 6-cm tissue culture plate (Sarstedt) and cultured overnight at 37°C with 5% CO₂. Bacteria grown in BHI broth were harvested by centrifugation (10 min, 3,220 g) in the exponential phase of growth and were washed twice with cation-free PBS. J774A.1 cells were infected with *R. equi* at an MOI of 20 bacteria per macrophage. Infections were initiated by centrifugation (160 g, 3 min) of bacteria onto confluent macrophage monolayers to synchronize internalization. The first samples were harvested 1 h after addition of medium supplemented with vancomycin (5 μg·ml⁻¹), which was considered t = 0 h. Infected monolayers were also harvested at different time points until 48 h postinfection. Fold changes in transcript level were normalized to that of the 16S rRNA gene in qPCRs as described above.

**Infection of mice.** Female severe combined immunodeficient (SCID) mice were obtained from Charles River (Wilmington, MA). Mice were received at 6 weeks of age and were used when they were approximately 8 weeks old. For the infection of mice, frozen aliquots of the bacterial strains for which titers had been determined were thawed and grown for 1 h at 37°C in BHI broth. Bacteria were pelleted and resuspended in PBS at the desired concentration. Groups of mice were infected intravenously through the tail vein with approximately 5 × 10⁵ bacteria. The concentration of the injection stock was determined retrospectively by dilution plating. To confirm that the mice received the expected amounts of bacteria, the first group of mice was sacrificed 2 h after infection. This time was defined as t = 0 h. At that time and at 2 and 14 days postinfection, five mice from each group were euthanized, and their livers, spleens, and lungs were removed. Each organ was placed in sterile PBS and homogenized with a tissue homogenizer (Seward, Bohemia, NY). Serial 10-fold dilutions of the homogenate were plated onto BHI agar, and CFU counts were determined after 48 h of incubation at 37°C.

**Statistical analysis.** Statistical analyses were performed using the SigmaPlot statistical package (SigmaPlot version 11.2.0.3; Systat Software, San Jose, CA). Comparison of the means of intracellular bacterial numbers between bacterial strains was assessed using a one-way analysis of variance (ANOVA). When appropriate, multiple pairwise comparisons were done using Tukey’s honestly significant difference (HSD) test. Significance was set at a *P* value of <0.05.

**RESULTS**

**Identification of a hydroxamate siderophore biosynthetic gene cluster.** We previously showed that *R. equi* produces a diffusible catecholate-containing siderophore (*rhequibactin*) while growing under iron-limiting conditions, requiring the activity of *iupS* (REQ08140) (30). Disruption of the *iupS* gene abolished growth in the presence of the iron chelator 2,2-dipyridyl yet did not affect intracellular growth of the *iupS* mutant in macrophages. This suggested that *R. equi* deploys an additional siderophore that allows the pathogen to obtain iron during infection (30).

In order to identify additional genes that may direct the synthesis of a siderophore, a bioinformatic analysis of the *R. equi* genome was carried out, which identified a gene cluster ranging from REQ07620 to REQ07650 (24) (Fig. 1). Considering the small intergenic space between these genes, it is highly likely that this gene cluster is transcribed as a four-cistron operon. mRNA isolated from *R. equi* grown under iron-depleted conditions was reverse transcribed, and the resulting cDNA was amplified by PCR using oligonucleotide primers complementary to sequences in adjacent genes (Fig. 2). This demonstrated that the genes REQ07620 to REQ07650 form an operon. An amplicon was not observed when oligonucleotides that were complementary to REQ07610 and REQ07620, which are divergently transcribed, were used (Fig. 2).

REQ07630 encodes a 596-kDa protein that is homologous to...
The exochelin-producing NRPS enzymes FxbB and FxbC of *Myco bacterium smegmatis* (52, 53). It contains four adenylation (A) domains that are required for incorporation of amino acids into the peptide assembly line. The amino acids lining the active site of the A domain of an NRPS module define the amino acid that is activated by the A domain (10). The eight-amino-acid fingerprint of the A domain of the first (D648, I649, N652, specificity of the NRPS protein (11). Challis et al. identified eight site of the A domain of an NPRS module define the amino acid into the peptide assembly line. The amino acids lining the active site that are required for incorporation of amino acids (A) domains that are required for incorporation of amino acids in module 1 of FxbB and FxbC (lanes 4 to 6), 7630F/7640R (predicted size, 282 bp) complementary to rhbC and rhbD (lanes 7 to 9), and 7640F/7650R (predicted size, 221 bp) complementary to rhbD and rhbE (lanes 10 to 12). Lanes 1, 4, 7, and 10, control reactions without reverse transcriptase; lanes 2, 5, 8, and 11, 1 μl of the reverse transcriptase reaction mixture (cDNA); lanes 3, 6, 9, and 12, reactions using genomic DNA as a template. Lane M, molecular size standard in base pairs. Genes are not drawn to scale.

FIG 2 Transcriptional organization of the rhbABCDE cluster. Shown are the results of the reverse transcriptase analysis of the rhbABCDE cluster using oligonucleotide pairs (Table 2) 7610F/7620R (predicted size, 241 bp) complementary to rhbA and rhbB (lanes 1 to 3), 7620F/7630R (predicted size, 280 bp) complementary to rhbB and rhbC (lanes 4 to 6), 7630F/7640R (predicted size, 282 bp) complementary to rhbC and rhbD (lanes 7 to 9), and 7640F/7650R (predicted size, 221 bp) complementary to rhbD and rhbE (lanes 10 to 12). The eight-amino-acid fingerprint of the A domain of an NRPS module define the amino acid into the peptide assembly line. The amino acids lining the active site that are required for incorporation of amino acids in module 1 of FxbB and FxbC. The amino acids lining the active site that are required for incorporation of amino acids in module 1 of FxbB and FxbC, respectively, to A domains that bind, respectively, N5-formyl-N3-hydroxoyrhodamine in module 1 of FxbB and N3-hydroxyrhodamine in A domains 1 and 3 of FxbC, as identified by Challis et al. (10). The amino acids that are activated by A domains 2 and 4 were predicted to be serine and N3-acyl-N3-hydroxoyrhodamine by NRPS predictor 2 (38). The REQ07630 protein therefore appears to synthesize a hydroxamate that is predicted to consist of N3-formyl-N3-hydroxyrhodamine, serine, N3-hydroxyrhodamine, and N3-acyl-N3-hydroxyrhodamine. The genes flanking REQ07630 encode putative enzymes that are likely required for modification and activation of the amino acids that are incorporated in the hydroxamate compound produced by REQ07630 and the release of this compound from the NRPS (Fig. 1). REQ07640 encodes a putative l-ornithine-5-monooxygenase that most likely is responsible for hydroxylation of l-ornithine to N3-hydroxyrhodamine. REQ07650 encodes an MbtH-like protein, a protein family that was recently shown to form an integral part of NRPS synthetases and play a role in amino acid activation (15). REQ07660 encodes an α/β-hydrolase fold protein that may play a role as a thioesterase to release hydroxamate from the NRPS synthetase at the end of the assembly line. The gene upstream and transcribed divergently of the putative hydroxamate biosynthetic operon (REQ07610) is annotated as encoding a methionyl-tRNA formyltransferase, which may play a role in the synthesis of N3-formyl-N3-hydroxyrhodamine, the likely substrate of NRPS module 1. The predicted activities of the proteins encoded by the gene cluster encompassing REQ07610 to REQ07650 are consistent with the biosynthesis of a hydroxamate siderophore (rhequichelin). We therefore propose to use the nomenclature rhb (rhequichelin biosynthesis) for REQ07610 (rhbA), REQ07620 (rhbB), REQ07630 (rhbC), REQ07640 (rhbD), and REQ07650 (rhbE).

**FIG 3** Transcriptional regulation of genes encoding siderophore-producing NRPS (rhbC, iupS, iupT, and iupU) (30) and a siderophore ABC transporter (iupA) (29) in response to the iron concentration in the medium. *R. equi* was grown in iron-replete LMM medium (white bars) or in iron-depleted LMM–Fe medium (black bars). Shown are the averages from two independent experiments in which each sample was analyzed in duplicate. Error bars denote the standard error of the mean.

Transcription of rhbC is regulated by iron. Transcription of the rhequibactin biosynthetic genes iupS and iupT, as well as that of the iupABC operon, which encodes a siderophore uptake system, is upregulated when *R. equi* is grown in medium containing low iron concentrations (29, 30), highlighting their role in iron acquisition. *R. equi* was grown in LMM (iron replete) and LMM–Fe (iron depleted) to determine whether rhbC transcription is also controlled by the concentration of iron. Transcription of the rhbC gene was clearly regulated by the iron concentration in the medium, since rhbC transcript levels increased more than 1,000-fold during growth in iron-depleted medium compared to iron-replete medium (Fig. 3). As was observed previously, the transcription levels of iupS, iupT, and iupA increased by two to three orders of magnitude following growth of *R. equi* in LMM–Fe compared to growth in LMM, whereas transcription levels of iupU and 16S rRNA were not affected (29, 30) (Fig. 3).

**rhbCD** is required for growth at low iron concentrations. In silico analysis of the rhb gene cluster strongly suggested that it is required for the production of a hydroxamate siderophore, which is supported by the observation that transcription of rhbC is dependent on the concentration of iron in the medium. To further analyze the function of the rhb gene cluster, rhbCD was deleted from the genome. Initially plasmid pSid2K was inserted into the genome via a single recombination event, rendering the resulting *R. equi* strain resistant to apramycin. The correct integration of pSid2K into rhbCD in an apramycin-resistant first recombinant colony was confirmed by PCR (data not shown). A subsequent second recombination between the integrated plasmid and chromosome leads to excision of the plasmid. In the resulting apramycin-sensitive strains, either rhbCD is deleted or the wild-type genotype is restored. Phenotypic analysis showed that all of the double recombinants in which rhbCD was deleted were unable to grow in the presence of an 80 μM concentration of the iron chelator 2,2-dipyridyl (data not shown). In contrast, growth of all of the recombinants that reverted to the wild-type genotype was indistinguishable from that of the parent wild-type strain. Thus,
Rhequichelin of *R. equi* is Required for Virulence

**FIG 4** *R. equi* produces a diffusible compound that stimulates growth of *R. equi* SID2 under iron-limiting growth conditions. *R. equi* SID2 was grown in iron-depleted LMM–Fe medium to which filtered culture supernatant of the *R. equi* wild-type (WT) strain grown in LMM–Fe was added. The growth rates shown are the averages from three independent experiments. Error bars denote the standard error of the mean.

**FIG 5** Transcriptional profile of genes encoding siderophore-producing NRPS proteins following infection of the murine macrophage-like cell line J774A.1. Monolayers were infected with *R. equi*; following a 1-h incubation to allow phagocytosis, monolayers were washed and treated with vancomycin to kill remaining extracellular bacteria (*t* = 0 h). ○, *iupA*; ▼, *rhbC*; △, *iupS*; □, *iupT*; ●, *iupU*; ◆, *iupS*. Transcript fold changes were normalized against the levels of expression of 16S rRNA and calibrated to time 0 h. Dashed lines indicate 2-fold changes in gene expression. The experiment was carried out in triplicate. Error bars denote the standard error of the mean.

The phenotype of the Δ*rhbCD* deletion mutants is unlikely to be due to secondary mutations that could have occurred elsewhere on the genome during the gene disruption process. Growth of the *rbhCD* deletion strain *R. equi* SID2 in the presence of 2,2-dipyridyl was restored when FeSO₄ was added to the medium, demonstrating that lack of growth was due not to 2,2-dipyridyl toxicity but to iron limitation. The data are therefore entirely consistent with the notion that *rbhCD* is required for the production of a siderophore that supports *R. equi* growth under iron-limiting growth conditions.

*rbhCD* is required for the production of a diffusible siderophore. The data suggested that the *rbhABCDE* cluster directs the synthesis of a diffusible hydroxamate siderophore. To test this hypothesis, filtered culture supernatant of the *R. equi* wild-type strain grown in LMM–Fe (WT-Spent medium) was added to iron-depleted culture medium (LMM–Fe) of *R. equi* SID2. The addition of WT-Spent medium increased the maximum growth rate (µmax) of *R. equi* SID2 in a dose-dependent manner, increasing from 0.26 ± 0.1 h⁻¹ to 0.36 ± 0.1 h⁻¹, which is comparable to that of the wild-type strain in iron-depleted medium (µmax ≈ 0.39 ± 0.1 h⁻¹) (Fig. 4). Addition of culture supernatant of the wild type to that of *R. equi* SID2 is thus able to rescue the iron-deficient phenotype of the latter. This demonstrates that *R. equi* secretes a diffusible compound that is required for growth under iron-limiting growth conditions, which supports the hypothesis that *rbhCD* is required for the production of a siderophore.

**Transcription of *rbhC* is upregulated in macrophages.** Uptake of *R. equi* by phagocytic cells reduces access of the pathogen to essential nutrients, including iron. In order to acquire sufficient iron for growth, it is highly likely that *R. equi* adapts to this environment by upregulating the transcription of genes required for iron acquisition, including *rbhC*. To determine whether this is the case, the macrophage-like cell line J774A.1 was infected with *R. equi*, and at various time points postinfection the transcription levels of the iron acquisition genes *iupA*, *iupS*, *iupT*, *iupU*, and *rhbC* were analyzed by RT-qPCR. The transcription levels of *rhbC* increased 100-fold within 10 h postinfection, indicating that *R. equi* experiences iron limitation following uptake by macrophages. Except for *iupU*, the transcription levels of the other iron acquisition genes also increased in this period (Fig. 5).

**The *rbhCD* genes are required for intracellular growth of *R. equi*.** Upregulation of *rbhC* transcription in macrophages may suggest that the *rhbABCDE* cluster is required for intracellular growth and virulence of *R. equi*. In order to test this hypothesis, bone marrow-derived macrophages were infected with wild-type *R. equi* *R. equi* SID2, and *R. equi* SID2rev. The intracellular levels of the wild-type strain and the revertant strain *R. equi* SID2rev increased 9-fold at 72 h postinfection. In contrast, *R. equi* SID2 was significantly (*P* = 0.015) attenuated in its ability to proliferate in macrophages (Fig. 6). Similar results were obtained when the macrophage-like cell line J774 was infected with wild-type *R. equi*, *R. equi* SID2, and *R. equi* SID2rev (data not shown). These data thus show that *rbhCD* is essential for intracellular proliferation of *R. equi*.

**rbhCD** is required for bacterial proliferation in vivo. Having demonstrated that the *rbhCD* gene cluster was required for opti-
We previously showed that *R. equi* produces a soluble catecholate-containing siderophore, rhequibactin, and a nondiffusible siderophore (29, 30). Disruption of *iupS* and *iupU*, required for biosynthesis of the former and latter compounds, respectively, prevented growth under iron-limiting conditions but did not attenuate virulence in macrophages or mice, strongly suggesting that *R. equi* produces a third siderophore that provides the pathogen with iron following infection (29, 30). Our analysis of the *R. equi* genome identified a gene cluster, *rhbABCDE*, that may direct the biosynthesis of this siderophore. The *rhbBCDE* genes form a four-cistron operon, whereas *rhbA* is located upstream and transcribed divergently from this operon (Fig. 2). The transcription of *rhbC* was controlled by the concentration of iron in the medium, increasing 1,000-fold in iron-depleted compared to iron-replete medium. A similar observation was made for the *iupS* and *iupT* genes, encoding NRPS proteins required for the biosynthesis of the catecholate containing siderophore rhequibactin (30).

RhbC is an NRPS protein that is predicted to synthesize a tetrapeptide composed of a serine and three modified ornithine residues (N⁵-formyl-N⁵-hydroxyornithine, serine, N⁵-hydroxyornithine, and N⁵-acyl-N⁵-hydroxyornithine). The rhodococcal siderophores characterized to date (heterobactin A, rhodobactin, and rhodochelin) all contain ornithine or modified ornithine residues (4, 7, 14). The remaining genes in this gene cluster are predicted to encode proteins required for the modification of ornithine by formylation and hydroxylation (RhbA and RhbD), amino acid activation (RhbE), and release of the tetrapeptide from the NRPS (RhhB).

A survey of rhodococcal genomes shows that *R. jostii*, *R. eurythropolis*, *R. imtechensis*, and *R. opacus* contain a region syntenic to the *rhbABCDE* gene cluster (Fig. 1). The NRPS proteins encoded by these syntenic regions share a high degree of identity with RhbC (62 to 67%) and have the same domain structure. An analysis of the four adenylation domains of these proteins (see Table S1 in the supplemental material) showed that the active-site residues conferring substrate specificity are identical in A domains 1, 3, and 4. These domains activate modified ornithine residues for incorporation in the resulting hydroxamate. The active-site residues in A domain 2 of the *R. jostii*, *R. opacus*, and *R. imtechensis* NRPS proteins are identical to each other. They differ in active-site residue 5 compared to RER0980 of *R. eurythropolis* and in residues 3 and 5 in RhhC (see Table S1 in the supplemental material). Despite these differences, NRPSpredictor2 (36, 38) predicts that A domain 2 in all five proteins activates a serine for incorporation in the resulting hydroxamate. Interestingly, the genetic organization of the NRPS loci of *R. jostii*, *R. opacus*, and *R. imtechensis* NRPS proteins are identical to each other. They differ in active-site residue 5 compared to RER0980 of *R. eurythropolis* and in residues 3 and 5 in RhhC (see Table S1 in the supplemental material). The organization of the NRPS loci of *R. equi* and *R. eurythropolis*, which differ slightly in their A domain 2 active sites from the former three species, is also different from these three (Fig. 1). The conserved genetic context of the NRPS-encoding genes, in which the same tailoring enzymes are encoded, and the identical active-site residues in A domains 1, 3, and 4 and highly similar residues in A domain 2 of the NRPS proteins suggest that it is likely that all five rhodococcal species produce structurally highly similar or identical hydroxamate compounds.

The bioinformatic and transcriptional analysis of the *rhbABCDE* cluster strongly suggested that it directs the synthesis of a siderophore. This was further supported by a mutational analysis of this cluster. Disruption of the *rhbC* genes prevented growth of *R. equi* SID2 in the presence of low concentrations of the iron chelator 2,2-dipyridyl and, in addition, reduced the growth rate of this mutant in iron-depleted medium compared to that of the wild type. Addition of culture supernatant of the wild type grown in iron-depleted medium to that of *R. equi* SID2 restored
the growth rate of the latter to close to that of the wild type in a dose-dependent manner. These data, together with the bioinformatic analysis of the rhbABCDE cluster, lead us to conclude that these genes are required for the production of a diffusible hydroxamate siderophore, rhequichelin.

Transcription of the rhequichelin and related rhequibactin biosynthetic genes was strongly upregulated in the first 10 h following infection of nonactivated macrophages, suggesting that R. equi encounters iron restriction following macrophage infection. However, since R. equi proliferates in macrophages, it clearly is able to obtain sufficient iron for growth. We previously demonstrated that rhequichelin is not essential for virulence (30). However, disruption of the rhequichelin biosynthesis genes attenuated bacterial growth in macrophages, showing that iron acquisition in the phagosomal compartment in which R. equi resides is dependent on this siderophore. These observations correspond to those made for Mycobacterium tuberculosis and Mycobacterium avium. Infection of macrophages with these pathogens resulted in an increase in the phagosomal iron concentration, which was derived from transferrin. In sharp contrast, the iron concentration in phagosomes harboring the nonpathogenic M. smegmatis decreased following infection (49, 50). The ability of M. tuberculosis to proliferate in macrophages is dependent on the biosynthesis of the siderophore mycobactin (13); furthermore, the increase in phagosomal iron concentration seen following infection of macrophages with M. tuberculosis did not occur following infection with an M. tuberculosis strain unable to produce mycobactin (49). These data strongly suggest that mycobactin competes with transferrin for iron, preventing a decrease in the phagosomal iron concentration. The data presented here suggest that rhequichelin fulfills a role similar to that of mycobactin.

In addition to preventing proliferation in macrophages, disruption of rhequichelin biosynthesis also attenuated growth and survival of R. equi in SCID mice, resulting in clearance of the pathogen in lungs and a dramatic reduction in bacterial numbers in liver and spleen, further supporting the importance of this siderophore in virulence. In stark contrast, previous disruption of iupS or iupU, which are required for biosynthesis of rhequibactin and a nondiffusible siderophore, respectively, had no effect on the establishment of a chronic infection in mice (30). These data suggest that rhequichelin may have characteristics that make it better at obtaining iron from iron sources present in the phagosome than rhequibactin. The observation that a specific siderophore out of several is preferred in virulence has been described for a number of other pathogenic bacteria (8, 42, 47). Uropathogenic E. coli strains produce up to four siderophores: the catecholates enterobactin and salmochelin and the hydroxamates aerobactin and yersiniabactin. However, only the latter two contributed significantly to urinary tract infection, despite the fact that aerobactin has a lower affinity for iron than enterobactin (5, 17, 33, 34). In comparison to enterobactin, aerobactin preferentially derives iron from cell components, which may explain its role in virulence (5).

Comparative analysis of rhodococcal genomes showed that the pathogenic R. equi acquired only a few virulence-specific genes through horizontal gene transfer, most notably the pathogenicity island of the virulence plasmid. The majority of potential virulence-associated factors identified in the genome are also present in nonpathogenic Rhodococcus species, suggesting that R. equi virulence evolved by cooption of existing core actinobacterial traits which were not initially involved in virulence or which did not evolve primarily to play a specific role in pathogenesis (24). The rhequichelin biosynthetic cluster is an excellent example of this: while a bioinformatic analysis provides strong evidence that nonpathogenic rhodococci produce rhequichelin or a structurally highly related compound, it clearly is indispensable for virulence of R. equi and hence is a virulence-associated factor.

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