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Transcriptional Regulation of the *virR* Operon of the Intracellular Pathogen *Rhodococcus equi*▽

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The *virR* operon, located on the virulence plasmid of the intracellular pathogen *Rhodococcus equi*, contains five genes, two of which (*virR* and *orf8*) encode transcriptional regulators. The first gene of the operon (*virR*), encoding a LysR-type transcriptional regulator, is transcribed at a constitutive low level, whereas the four downstream genes are induced by low pH and high growth temperature. Differential regulation of the *virR* operon genes could not be explained by differential mRNA stability, as there were no major differences in mRNA half-lives of the transcripts representing each of the five genes within the *virR* operon. Transcription of *virR* is driven by the P_{virR} promoter, with a transcription start site 53 bp upstream of the *virR* initiation codon. The four genes downstream of *virR* are transcribed from P_{virR} and from a second promoter, P_{orf5}, located 585 bp downstream of the *virR* initiation codon. VirR binds to a site overlapping the initiation codon of *virR*, resulting in negative autoregulation of the *virR* gene, explaining its low constitutive transcription level. The P_{orf5} promoter is induced by high temperature and low pH, thus explaining the observed differential gene expression of the *virR* operon. VirR has a positive effect on P_{orf5} activity, whereas the response regulator encoded by *orf8* is not involved in regulating transcription of the *virR* operon. The P_{virR} promoter is strikingly similar to those recognized by the principal sigma factors of *Streptomyces* and *Mycobacterium*, whereas the P_{orf5} promoter does not share sequence similarity with P_{virR}. This suggests that P_{orf5} is recognized by an alternative sigma factor.

The mycolic acid-containing actinomycete *Rhodococcus equi* is an intracellular pathogen of macrophages and the causative agent of foal pneumonia. Although foals are the primary host of this pathogen, other animals, including pigs, goats, and cattle, are sporadically infected. In addition to these animal hosts, *R. equi* infections are increasingly encountered in immunocompromised humans, in particular in those diagnosed with AIDS (20, 24). Virulence relies on the ability of *R. equi* to replicate inside macrophages (13), a process which is dependent on the pathogen interfering with endosomal maturation following phagocytosis and preventing acidification of the vacuole in which it resides (9, 37, 38). Eventually, intracellular proliferation of the pathogen leads to necrosis of the macrophage, accompanied by massive damage to lung tissue characterized by cavitation and granuloma formation (18, 20, 24).

All *R. equi* strains isolated from foals harbor an 81-kb plasmid that has been shown to be essential for intracellular replication and virulence (10, 35, 36). This virulence plasmid contains a region of 27.5 kb that is characterized by a lower G+C content than the remainder of the virulence plasmid and the *R. equi* genome, indicating that it probably was acquired via lateral gene transfer (33). It was proposed that this region represents a pathogenicity island, a hypothesis that was lent credence by the fact that the transcription of genes located within it is upregulated following phagocytosis of *R. equi* (25, 33). Among these is a group of genes encoding a family of small proteins, one of which, virulence-associated protein A (VapA), has been shown to be a virulence factor (14).

Transcriptional regulation of pathogenicity island genes is subject to at least six different environmental parameters, including temperature and pH (3, 25, 32, 34). In contrast to this apparent regulatory complexity, only two genes encoding transcriptional regulators, located in a five-cistron operon (the *virR* operon, containing *virR*, *orf5*, *vapH*, *orf7*, and *orf8*), have been identified in the pathogenicity island (28, 33). It therefore is likely that regulators encoded by the *R. equi* genome play an important role in controlling the expression of pathogenicity island genes. This may be accomplished, at least in part, through controlling the expression levels of the two transcriptional regulators within the pathogenicity island. The *virR* gene encodes a LysR-type transcriptional regulator (LTTR) that is required for transcription of the *vapA* gene (28). The *orf8* gene encodes a response regulator; interestingly, a cognate sensor kinase protein is not encoded on the virulence plasmid (33). Disruption of either gene attenuates *R. equi*, demonstrating their importance in controlling virulence (26). A recent report describing a low level of constitutive transcription of the *virR* gene while the transcript levels of the four downstream genes responded to changes in growth conditions (25) appeared to contradict our previous finding that the five genes constitute an operon (28). Considering the importance of the *virR* operon in controlling the virulence of *R. equi*, this apparent paradox was examined further. This paper reports that the observed difference in regulation of the *virR* operon genes is not due to differential mRNA stability of the *virR* operon transcript but to the presence of a regulated promoter within the *virR* gene driving transcription of *orf5*, *vapH*, *orf7*, and *orf8*.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain, plasmid, or oligonucleotide	Genotype or characteristics	Source or reference
Strains		
E. coli DH5α	*supE44* Δ*lacU169* (ϕ80*lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Bethesda Research Laboratories
E. coli BL21(DE3)pLysE	F^- *ompT hsdS*$_B$(r_B^- m_B^-) *dcm gal* λ(DE3) (pLysE) Cm^r	31
R. equi ATCC 33701	Virulent strain with 81-kb virulence plasmid p33701	American Type Culture Collection
R. equi ATCC 33701 (P^-)	Avirulent strain; virulence plasmid cured	35
R.equi orf8 mutant	*orf8* deletion	26
Plasmids		
pBluescript II KS(+)	Ap^r α*lacZ′*	Stratagene
pREV5	Apr^r oriV(pMF1) oriV(ColE1)	19
pBlueRegP1	pBluescript KS with 6,680-bp fragment containing *orf3* to *orf8* from the *R. equi* ATCC 33701 virulence plasmid	28
pET3bvirRhis	pET3b with 931-bp fragment containing *virR* with a six-His tag at the 3′ end	28
pJOE814.2	Amp^r ColE1 *ori xylE* reporter gene	23
pREV6	pREV5 with t tag located within two terminators derived from pJOE814.2	This study
pORF3PEX	pREV6 with a 2,705-bp DNA fragment containing promoters P_{virR} and P_{orf5}, *virR*, and *orf5*	This study
pVirRT	pREV6 with a 2,183-bp DNA fragment containing P_{orf5}, *virR*, and *orf5*	This study
p004	pREV6 with a 1,840-bp DNA fragment containing *virR* and *orf5*	This study
pVirRT-LysR	pVirRT with *virR* located outside the transcriptional terminators	This study
pInterVir	pBluescript KS with a 459-bp DNA fragment containing the *virR-orf3* intergenic region	This study
pCodingVir	pBluescript KS with an 846-bp DNA fragment containing 690 bp of the *virR* coding region	This study
pREV531	pREV5 with a 1.5-kb DNA fragment containing *orf3* and *virR′* derived from pBlueRegP1	This study
pREV5341	pREV5 with a 3.1-kb DNA fragment containing *orf3*, *virR*, and *orf5′* derived from pBlueRegP1	This study

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1.

Media and growth conditions. *Escherichia coli* DH5α (Bethesda Research Laboratories) and *E. coli* BL21(DE3)pLysE (Novagen) were used for general cloning procedures and for expression of *virR-his*, respectively. Bacterial strains were grown in Luria-Bertani (LB) broth (29). Growth of *R. equi* at 37°C and pH 6.5 (inducing conditions) was used to induce virulence plasmid gene expression, whereas these genes were transcribed at low levels following growth at 30°C and pH 8.0 (noninducing conditions). Where appropriate, the following supplements were added: kanamycin, 50 μg ml^{-1} (*E. coli*) or 200 μg ml^{-1} (*R. equi*); ampicillin, 50 μg ml^{-1}; apramycin, 30 μg ml^{-1} (*E. coli*) or 80 μg ml^{-1} (*R. equi*); chloramphenicol, 30 μg ml^{-1}; 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 20 μg ml^{-1}; isopropyl-β-D-thiogalactopyranoside (IPTG), 0.1 mM. For solid medium, agar was added at 1.5% (wt/vol).

DNA manipulations. Chromosomal DNA was isolated as described previously (28). Plasmid DNA was isolated via the alkaline lysis method of Birnboim and Doly (5) or by using a Wizard Plus SV miniprep kit as described by the manufacturer (Promega). DNA fragments were isolated from agarose gels by using a Genelute DNA purification kit as described by the manufacturer (Sigma-Aldrich). PCR was carried out using *Taq* DNA polymerase (Promega) or Deep Vent DNA polymerase (New England Biolabs) as described by the manufacturer. Other DNA manipulations were done in accordance with standard protocols (29).

Plasmid construction. A region located on plasmid pJOE814.2 containing two transcriptional terminators and a *xylE* gene was amplified with Deep Vent DNA polymerase, using primers XylF and XylR. This 1,424-bp fragment was cloned into DraI-digested pREV5 to make pREV81. The *xylE* gene was subsequently excised by digesting pREV81 with ClaI, followed by religation of the 4,948-bp fragment, resulting in pREV6.

A series of DNA fragments serially shortened from the 5′ end was constructed by PCR with the oligonucleotide BCMRev in conjunction with either ORF3PEX2, VirRT1, or 004F, using pBlueRegP1 as a template. These fragments were ligated into the unique EcoRV restriction site of pREV6 to yield pORF3PEX, pVirRT, and p004, respectively. An intact *virR* gene was amplified from pBlueRegP1 by using oligonucleotides 005R and Orf3BlnI. The resulting 1,717-bp product was digested with BlnI, and the 1,296-bp fragment was cloned into SpeI-digested pVirRT.

pInterVir was constructed by amplifying a 459-bp fragment containing the *orf3-virR* intergenic region from pBlueRegP1, using oligonucleotides GM-Lys-F and GM-Lys-R; this fragment was subsequently ligated into EcoRV-digested pBluescript II KS. pCodingVir was constructed by amplifying an 846-bp fragment containing the *virR* coding region, using oligonucleotides VirRT1 and −70−50REV; this fragment was subsequently ligated into EcoRV-digested pBluescript II KS. The sequences of the oligonucleotides used in the construction of these plasmids are listed in Table 2.

Electroporation of *R. equi*. Plasmids were introduced into *R. equi* strains by electroporation as described previously (19).

RNA isolation and real-time RT-PCR. RNAs were isolated from *R. equi* as described previously (28). Reverse transcriptase (RT) reactions using random primers (Promega) were performed with 1 U Improm II RT following the manufacturer's recommendations, with 100 ng of total RNA as the template in a final volume of 20 μl. The product was subsequently amplified using a QuantiTect SYBR green real-time kit following the manufacturer's instructions (QIAGEN). Reaction mixtures were subjected to 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s in a LightCycler instrument (Roche) with a temperature transition rate of 20°C/s. Melting curve analysis was performed at 50 to 95°C (temperature transition rate, 0.2°C/s), with stepwise fluorescence detection following amplification. Cycle threshold (C_T) values were obtained and used to calculate the number of RNA copies per μg of total RNA, using a standard curve of known amounts of DNA target with r^2 coefficients of >0.997 in the range of 5×10^3 to 5×10^8 molecules per reaction. *gyrB* mRNA was used as a housekeeping gene to compare the amounts of RNA in each reaction. The data reported in this paper represent the results of three independent experiments in which each sample was analyzed in duplicate. The sequences of oligonucleotides used for real-time PCR are listed in Table 2.

Fluorescent primer extension and DNA sequencing. The D4-labeled oligonucleotides D4-VIR5022 and D4-2IntPex5564 (Table 2), complementary to sequences 62 to 80 bp and 624 to 643 bp, respectively, downstream of the initiation codon of *virR*, were used in primer extension reactions to determine the tran-

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′ to 3′)	Purpose	Reference
XylF	TATATAACTAGTGCCTCTTCGCTATT	Plasmid construction	This study
XylR	TATAGGCCGGCCCGATTCATTAATGCA	Plasmid construction	This study
BCMRev	GTTATCTAGGGGCAGGCGACAG	Plasmid construction	This study
VIRRT1	CGCATTGAACGACAGGTTG	Plasmid construction	This study
ORF3PEX2	TGACGAGCACCAATGTTTTC	Plasmid construction	This study
GM-Lys-R	CGGCTGCCGCAATATGAC	Plasmid construction	This study
GM-Lys-F	CACCCCAACCTGTCGTTC	Plasmid construction	This study
−70−50 REV	GACCACCTGCGATGCGTGAT	Plasmid construction	This study
GyrCOOH1	GTCGAGCAGGGTCAAGTGTA	Quantification of *gyrB*	This study
GyrCOOH25′	AGCTCCTTGGCGTTCATCT	Quantification of *gyrB*	This study
16SrRNAF200	ACGAAGCGAGAGTGACGGTA	Quantification of 16S rRNA	21
16SrRNAR200	ACTCAAGTCTGCCCGTATCG	Quantification of 16S rRNA	21
003F	GTTTCGTCTTCCACCGTCTT	Quantification of *orf3*	This study
003R	AGCCTTATCGTCGCAACTGT	Quantification of *orf3*	This study
004F	CGGACGAGTTCGACTGGTAT	Quantification of *virR*	This study
004R	CAAAGACGATTTGGGGTACG	Quantification of *virR*	This study
005F	CTCTTCCTGATCGGAGTTGC	Quantification of *orf5*	This study
005R	GAGTCGCAGACGAGGTAAGC	Quantification of *orf5*	This study
006F	AGGGTTATGCAGGTGGATTG	Quantification of *vapH*	This study
006R	TACCGATTACGGAGCTCACC	Quantification of *vapH*	This study
007NF	ATGCACTCCCTGAAAACTATC	Quantification of *orf7*	This study
007NR	GGTGGGCTGGATTGACGCGCA	Quantification of *orf7*	This study
008F	GAACAACTGGGAATGGTGGT	Quantification of *orf8*	This study
008R	GTTCGCCGTTTCTAGACGAA	Quantification of *orf8*	This study
D4-VIR5022	GATGTGCAGGGCGTCAGC	Primer extension	This study
VIR5022	GATGTGCAGGGCGTCAGC	Sequencing	This study
D4-2IntPex5564	GGTACGACGCCAGCAGCCGC	Primer extension	This study
2IntPex5564	GGTACGACGCCAGCAGCCGC	Sequencing	This study

scriptional start sites of the *virR* operon. Total RNA (2 μg) and 1 μM of D4-VIR5022 were incubated at 70°C for 5 min, followed by reverse transcription at 42°C for 60 min, using 5 U of Superscript III RT in a volume of 20 μl as recommended by the manufacturer (Invitrogen). After treatment of the sample with 20 μg of RNase A at 37°C for 30 min, cDNA was precipitated and dissolved in 12 μl of nuclease-free water. The primer extension product (0.5 ng) was combined with 0.5 μl of DNA size standard kit 600 (Beckman Coulter) and 40 μl of CEQ sample loading solution (Beckman Coulter) and analyzed with a CEQ 8000 fragment analysis system on a CEQ 8000 DNA sequencer (Beckman Coulter). In addition, dideoxy sequencing reactions using either VIR5022 or 2IntPex5564 (Table 2) and 60 ng of NheI-digested pORF3PEX (Table 1) were performed using a CEQ DCTS kit as described by the manufacturer (Beckman Coulter). The D4-labeled primer extension products (50 pg) were added to the sample prior to analyzing the sequence on a CEQ 8000 DNA sequencer to identify transcriptional start sites.

mRNA half-life determination. RNAs were isolated from *R. equi* in the mid-logarithmic phase of growth (optical density at 600 nm = 0.5) at selected intervals following inhibition of transcription by the addition of 200 μg ml^{-1} rifampin (Sigma). The number of mRNA copies was determined using real-time RT-PCR, followed by linear regression to determine the half-life. 16S rRNA was used for internal normalization in this study.

EMSA. Expression and purification of *virR-his* were done as previously described (28). A DNA fragment containing the *virR* promoter region, pInterVir, was digested with EcoRI and HindIII and labeled with [α-^{32}P]dATP, using Klenow DNA polymerase as described previously (28). The labeled fragment was subsequently purified using a QIAquick PCR purification kit according to the manufacturer's instructions (QIAGEN). Radiolabeled DNA fragments (2 ng) were incubated with purified VirR-His at 30°C for 30 min in electrophoretic mobility shift assay (EMSA) binding buffer, 20 μg of bovine serum albumin, and 1 μg of poly(dI-dC) DNA (Amersham Biosciences) in a volume of 20 μl. The samples were separated by electrophoresis in a prerun 5% nondenaturing polyacrylamide gel containing TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA) and run at 4°C and 10 V cm^{-1}. Following drying, the gel was analyzed by autoradiography.

DNA restriction protection assay. A 459-bp fragment containing the *virR* promoter region was amplified using the oligonucleotides GM-Lys-F and GM-Lys-R, with pInterVir as the template. To obtain a negative control DNA region, an 846-bp fragment was amplified using the oligonucleotides VirRT1 and −70−50REV. DNA fragments (500 ng) were incubated with purified VirR-His at 30°C for 30 min in EMSA binding buffer. HincII (10 units) was added to each sample, which was further incubated at 30°C for 30 min. The samples were separated by electrophoresis and visualized using SYBR green (Sigma) per the manufacturer's instructions.

RESULTS

Transcription of the *virR* operon is controlled by temperature and pH. Using a microarray to study the transcription levels of pathogenicity island genes, Ren and Prescott (25) showed that the transcription of four genes (*orf5*, *vapH*, *orf7*, and *orf8*) downstream of *virR*, but not that of *virR* itself, is upregulated when cells are grown at pH 6.5 and 37°C compared to that at pH 8.0 and 30°C. Since this observation appeared to contradict our previous findings that these genes are organized in an operon (28), mRNAs from *R. equi* grown under these two conditions were isolated and quantified using real-time RT-PCR (Fig. 1). The transcription levels of the four genes downstream of *virR* were significantly increased (threefold) when cells were grown at pH 6.5 and 37°C compared to those for cells grown at pH 8.0 and 30°C. In contrast, there was no significant difference in transcription levels of *virR*, the first gene of the operon, thus confirming the earlier microarray data (25).

mRNA stability of the *virR* operon transcript. Although *virR*, *orf5*, *vapH*, *orf7*, and *orf8* are organized in an operon (28), their transcription is not coordinately regulated (25). A possible explanation for this apparent paradox may be that mRNAs derived from *orf5*, *vapH*, *orf7*, and *orf8* are more stable than that of *virR*, allowing the former four transcripts to accumulate. To examine this possibility, the mRNA half-lives of these genes

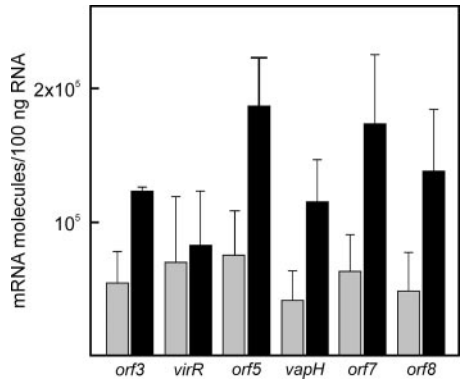

FIG. 1. Regulation of *virR* operon gene transcription by temperature and pH. mRNAs were isolated from *Rhodococcus equi* cells grown under noninducing (30°C, pH 8.0) or inducing (37°C, pH 6.5) growth conditions, followed by absolute quantification of the mRNA molecules using real-time RT-PCR. Transcription levels are indicated for each gene of the *virR* operon (*virR*, *orf5*, *vapH*, *orf6*, *orf7*, and *orf8*) and the divergently transcribed *orf3* gene following growth under noninducing (gray bars) and inducing (black bars) conditions.

were determined following inhibition of transcription by the addition of rifampin (Table 3). The half-lives of the transcripts representing each of the five genes within the *virR* operon varied from 1.3 to 2.3 min. Since the *virR* transcript had a half-life of 1.8 min, differential mRNA stability of the *virR* operon transcript does not account for the observed differences in transcript levels of *virR* and its four downstream genes.

The *virR* operon contains an internal promoter regulated by pH and temperature. An alternative explanation for the observed differential regulation of the *virR* operon genes is the presence of a promoter located downstream of the *virR* operon promoter (P_{virR}) driving transcription of the *orf5*, *vapH*, *orf7*, and *orf8* genes. Transcription levels were analyzed using the promoter probe vector pREV6, which contains a small DNA fragment (t tag) located between two transcriptional terminators which is not present in *R. equi* (Fig. 2A). Since there is no promoter, transcription of the t tag does not occur (Fig. 2B). Introduction of a DNA fragment containing the *orf3-virR* intergenic region as well as *orf5* into pREV6 resulted in transcription of the t tag, demonstrating the presence of an active promoter. Deletion of the *orf3-virR* intergenic region, including the 5′ end of *virR* (1 to 111 bp), reduced but did not abolish transcription of the t tag. Transcription of the t tag was completely abolished when a larger 5′ fragment (1 to 404 bp) of *virR* was deleted, demonstrating that a promoter (P_{orf5}) or sequences required for its activity are located between bp 111 and 404 of the *virR* gene (Fig. 2B).

To examine whether the internal promoter P_{orf5} is regulated by temperature and pH, the transcription levels of the t tag were examined following growth of *R. equi*(pVirRT) under either inducing (pH 6.5, 37°C) or noninducing (pH 8.0, 30°C) growth conditions. Transcription of the t tag increased 67-fold under inducing compared to noninducing conditions, whereas transcription of the t tag did not occur from pREV6 (Fig. 2C).

TABLE 3. Transcript half-lives of genes within the *virR* operon

Gene	Half-life (min)
virR	1.8 ± 0.2
orf5	1.3 ± 0.3
vapH	2.3 ± 0.3
orf7	2.2 ± 0.1
orf8	1.3 ± 0.1

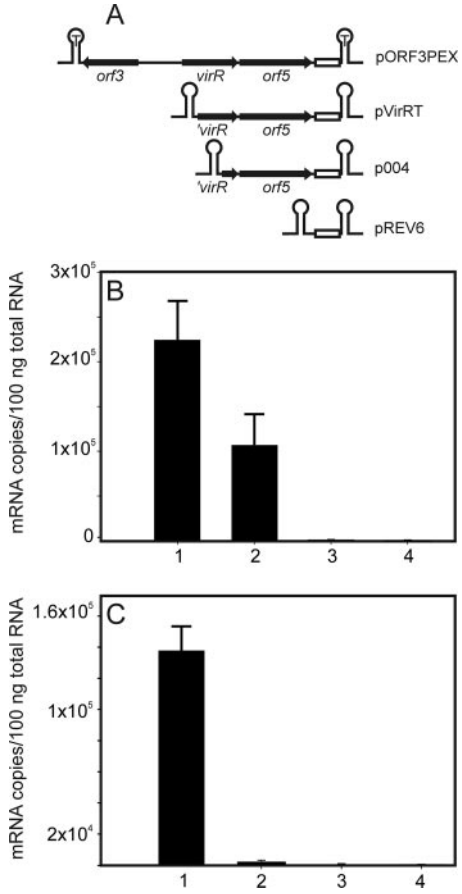

FIG. 2. Determination of P_{virR} and P_{orf5} promoter activities by real-time RT-PCR. (A) Schematic representation of inserts in the promoter probe vector pREV6. The hairpin structure marked with a "T" represents the transcriptional terminators. Genes and their direction of transcription are indicated by black arrows. The open white box represents the t tag. The transcription level of the t tag was measured by real-time RT-PCR. (B) mRNA transcript levels of the t tag in *R. equi* cells harboring pORF3PEX, containing both P_{virR} and P_{orf5} (1); pVirRT, containing P_{orf5} (2); p004 (3); or pREV6 (4). (C) mRNA transcript levels of the t tag in *R. equi* cells harboring pVirRT containing only P_{orf5} grown at 37°C and pH 6.5 (1) or at 30°C and pH 8.0 (2) and in *R. equi* cells harboring pREV6 grown at 37°C and pH 6.5 (3) or 30°C and pH 8.0 (4).

Mapping of the transcriptional start sites of the *virR* operon. To determine the transcriptional start site of the *virR* gene, a primer extension reaction using the fluorescently labeled oligonucleotide D4-VIR5022, complementary to the *virR* gene, was carried out. Using mRNA isolated from *R. equi* grown under inducing conditions as the template, a 133-bp DNA fragment was observed (Fig. 3A). The transcriptional start site of P_{virR} was subsequently determined to be an adenine located 53 bp upstream of the *virR* initiation codon (Fig. 3A and 4D). Interestingly, the −10 sequence (nucleotides −12

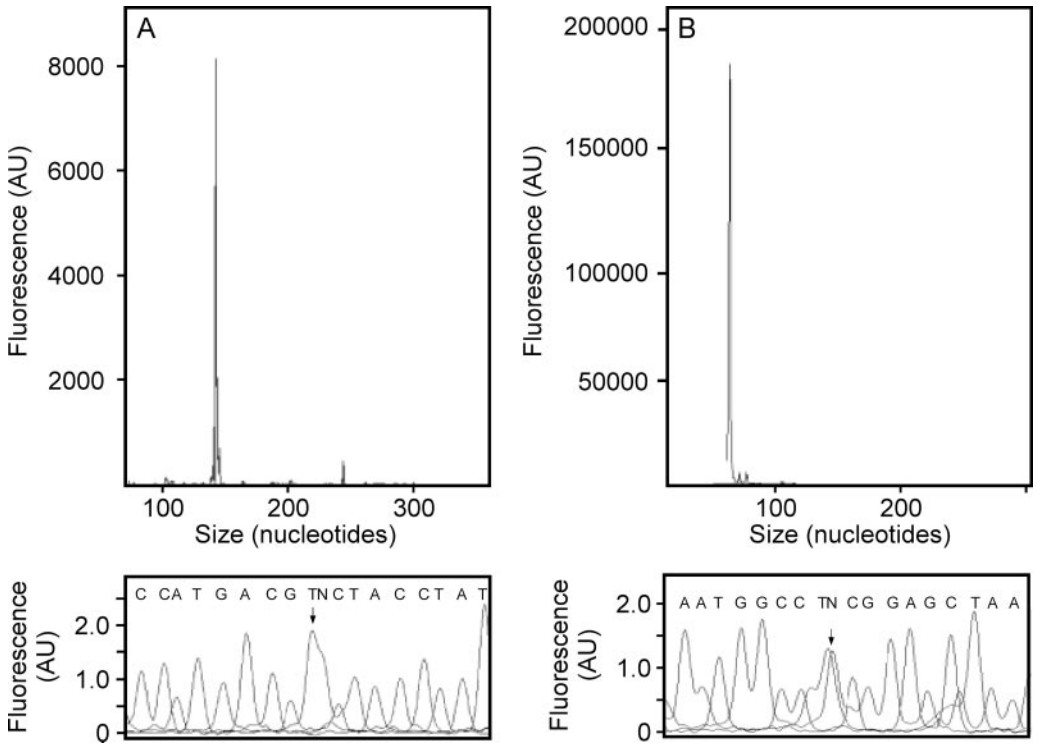

FIG. 3. Determination of transcriptional start sites of the *virR* operon of *R. equi*. Fluorescent primer extension was carried out with a Cy5-labeled primer and 5 μg of total cellular RNA extracted from *R. equi* cells grown under inducing conditions (37°C and pH 6.5). The upper panels show the D4-labeled primer extension products combined with DNA size standards and analyzed with a CEQ 8000 fragment analysis system. The lower panels show dideoxy sequencing reactions spiked with the D4-labeled primer extension products. The arrows indicate the transcriptional start sites where the D4-labeled cDNA and sequencing products overlapped. (A) Determination of the P_{virR} transcriptional start site, using D4-VIR5022. (B) Determination of the P_{orf5} transcriptional start site, using D4-2IntPex5564. AU, arbitrary units.

to −7 [TAGCAT]) of the P_{virR} promoter is strikingly similar to that of the consensus σ^{hrdB} promoter (TAGART), which is recognized by the principal sigma factor of *Streptomyces coelicolor* (7, 15). However, there is no clear similarity in the −35 region to the consensus σ^{hrdB} promoter (TTGACA). Using the same approach, the internal transcriptional start site of P_{orf5} was mapped to a guanidine 585 bp downstream of the *virR* initiation codon (Fig. 3B and 4D). The P_{virR} and P_{orf5} promoters do not share any obvious sequence similarity in either the −10 or −35 area.

VirR binds to the *orf3-virR* intergenic region. To determine whether VirR interacts with the P_{virR} and P_{orf5} promoters, band shift experiments were carried out. Incubation of purified VirR-His with a 459-bp radiolabeled DNA fragment containing the *orf3-virR* intergenic region resulted in retardation of the DNA fragment. At low VirR-His concentrations, a single retarded band was visible, whereas a second retarded band became visible at higher concentrations (Fig. 4A, lane 5). In contrast, VirR-His failed to bind to a DNA fragment containing only the internal P_{orf5} promoter (Fig. 4B).

To further corroborate that VirR-His binds to a DNA fragment containing the P_{virR} promoter, a restriction enzyme protection assay was carried out. The 459-bp DNA fragment containing the *orf3-virR* intergenic region was incubated with VirR-His and subsequently subjected to digestion with HincII, whose recognition site is located 59 to 64 bp downstream of the P_{virR} transcription initiation site. While VirR-His protected this 459-bp DNA fragment from HincII digestion, it failed to prevent digestion by HincII of the adjacent 846-bp DNA fragment. These data show that the VirR binding site overlaps the HincII site downstream of P_{virR} within the *virR* coding region (Fig. 4C).

VirR is a negative regulator of the *virR* gene. The results of the restriction enzyme protection assay show that VirR protects a HincII site located 59 to 64 bp downstream from the P_{virR} transcriptional start site. This strongly suggests that VirR acts as a repressor of P_{virR} driving the transcription of *virR*. To examine this possibility, plasmids with DNA fragments containing either *orf3*, an intact *virR* gene and the 5′ end of *orf5* (pREV5341), or *orf3* and the 5′ end of *virR* (pREV531) were electroporated into *R. equi* P^-. Transcription of *virR* was subsequently analyzed by RT-PCR, using primers that amplified a *virR* fragment located upstream of P_{orf5}, thus analyzing only the activity of P_{virR}. While transcription of *virR* was below the detection limit in strains harboring pREV5341 (Fig. 5, lane 2), it was clearly detectable in the absence of an intact *virR* gene (pREV531) (Fig. 5, lane 3).

VirR is a positive regulator of the *orf5-orf8* cluster. Since the *orf5* and *orf8* genes are transcribed from the P_{virR} promoter as well as the P_{orf5} promoter, it is likely that VirR is involved in transcription from P_{orf5} as well as that from P_{virR}. To investigate this possibility, pVirRT, containing P_{orf5} and the 3′ end of *virR*, was introduced into *R. equi* P^-. The transcription levels of the t tag in this strain were 14-fold lower than those in the

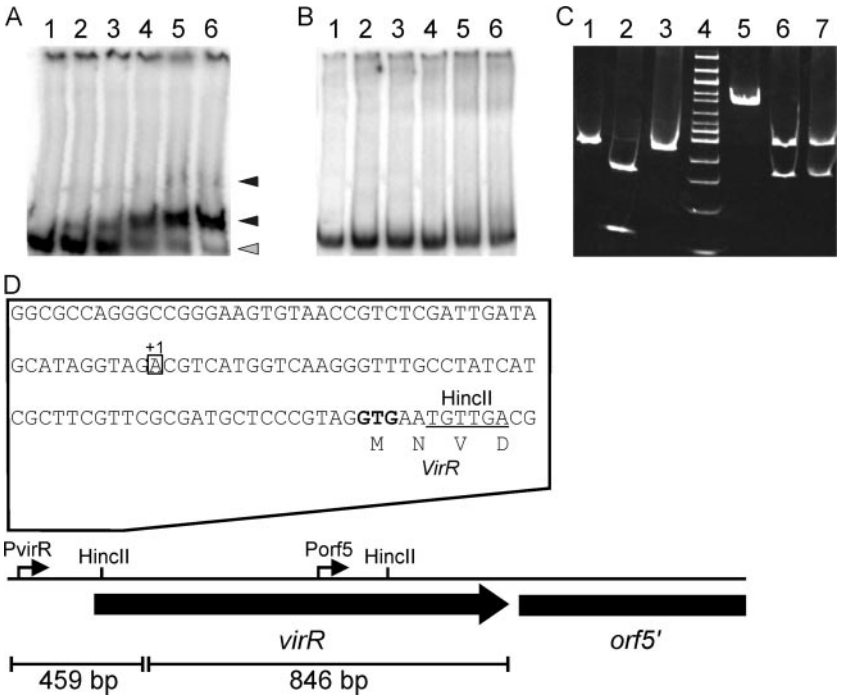

FIG. 4. Analysis of VirR DNA binding. Various concentrations of VirR were incubated with 2 ng of radiolabeled DNA containing (A) the P_{virR} promoter region in a 459-bp DNA fragment or (B) the P_{orf5} promoter region in an 846-bp DNA fragment. The amount of protein added to each lane was as follows: lanes 1, radiolabeled DNA fragment only; lanes 2, 50 ng VirR-His; lanes 3, 100 ng VirR-His; lanes 4, 200 ng VirR-His; lanes 5, 300 ng VirR-His; and lanes 6, 400 ng VirR-His. The reaction volume was 20 μl. Protein-DNA complexes are indicated with black arrowheads. Nonbound DNA is indicated with a gray arrowhead. (C) HincII restriction protection assay. DNAs were incubated with VirR and HincII, followed by analysis of the restriction digest by gel electrophoresis. Lanes 1 to 3 contain a 459-bp DNA fragment with P_{virR}, and lanes 5 and 6 contain an 846-bp DNA fragment with P_{orf5}. Lanes 1 and 5, no VirR or HincII added; lanes 2 and 6, HincII added; lanes 3 and 7, HincII and VirR added. (D) Schematic representation of the 5′ end of the *virR* operon. Genes and the direction of transcription are indicated by black arrows. The HincII sites used in the restriction protection assay and the positions of P_{virR} and P_{orf5} are indicated above the arrows. The nucleotide sequence of the sequence upstream of *virR* containing the P_{virR} promoter is shown. The P_{virR} transcriptional start site is indicated (+1).

wild-type strain, indicating that the presence of virulence plasmid genes is required for wild-type transcription levels. The transcription levels were restored only partially (twofold increase) following introduction of an intact *virR* gene into pVirRT (but outside the transcriptional terminators of pREV6), demonstrating that VirR is required but not sufficient for full activity of the P_{orf5} promoter.

***orf8* does not regulate transcription of the *virR* operon.** The *orf8* gene encodes a response regulator that probably interacts with an as yet unidentified chromosomally encoded sensor kinase protein to regulate virulence plasmid gene expression. To determine whether *orf8* is involved in transcriptional regulation of the *virR* operon, the transcription levels of *orf5* were compared in the wild-type strain and an *orf8* disruption mutant. The *orf5* transcription level in the wild-type strain was 5.6×10^5 molecules mRNA/100 ng RNA $\pm$ 7.6×10^4 molecules mRNA/100 ng RNA, whereas this level was 4.7×10^5 molecules mRNA/100 ng RNA $\pm$ 5.6×10^4 molecules mRNA/100 ng RNA for the *R. equi orf8* mutant, indicating that *orf8* does not play a role in regulating transcription of the *virR* operon.

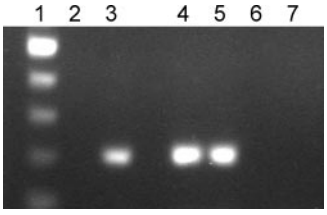

FIG. 5. Autoregulation of *virR* transcription. mRNAs were isolated from *R. equi* P^- harboring either pREV531 (*orf3-virR′*) or pREV5341 (*orf3-virR-orf5′*). *virR* and *gyrB* transcripts were subsequently detected by RT-PCR as 200-bp amplification products. The oligonucleotides used for this experiment (Table 2) amplified a region upstream of P_{orf5}. Lane 1, 100-bp marker; lane 2, *R. equi*(pREV5341) (*virR*); lane 3, *R. equi*(pREV531) (*virR*); lane 4, *R. equi*(pREV5341) (*gyrB*); lane 5, *R. equi*(pREV531) (*gyrB*); lanes 6 and 7, RT-PCR of *gyrB* genes of *R. equi*(pREV5341) and *R. equi*(pREV531), respectively, without RT.

DISCUSSION

The *virR* operon contains two genes encoding transcriptional regulators which are required for the virulence of *R. equi*; one of these, the LTTR protein VirR, is required for expression of the virulence factor VapA (26, 28). The transcriptional organization of *virR* is unusual in that it is the first gene in a five-cistron operon (28); in most instances, LTTR-encoding genes are transcribed as monocistronic transcripts. In addition, an analysis of virulence plasmid transcript levels following growth under a variety of conditions showed that *virR* is transcribed at a low and constant level, whereas the transcription of

the downstream genes is regulated (25). The aim of this paper was to analyze the transcriptional regulation of the *virR* operon in order to explain the observed differential regulation of *virR* compared to the four downstream genes.

Many LTTRs autoregulate their expression by acting as a repressor of the LTTR-encoding gene (30). The data presented here show that in this respect, *virR* behaves as a typical LTTR. Inactivation of *virR* in a virulence plasmid-free background resulted in a dramatic increase of *virR* transcription, which is consistent with the notion that VirR acts as a repressor of *virR* transcription. This is supported by the finding that VirR binds to a site that overlaps a HincII restriction site located 59 to 64 bp downstream of P_{virR} within the 5′ end of the *virR* gene, which is a typical location for a repressor binding site (8). The VirR autoregulatory circuit thus results in a constant low level of transcription of the *virR* operon from the P_{virR} promoter, independent of growth temperature and pH.

Differential mRNA stability within a polycistronic transcript has been shown to lead to vastly different mRNA levels for individual genes within an operon, resulting in differential gene expression (11, 17). This could account for the observed increase in *orf5*, *vapH*, *orf7*, and *orf8* transcripts under inducing conditions. However, the half-life of *virR* mRNA (1.8 min) was the same as the average half-life of the transcripts of all five genes within the *virR* operon and therefore does not account for the observed differential regulation of the four genes downstream of *virR* in the *virR* operon. The observed short half-lives are typical of the majority of bacterial transcripts, as recently shown for *Escherichia coli* and *Bacillus subtilis*, where over 80% of transcripts are unstable, with half-lives of <8 min (4, 12).

Deletion analysis of the *virR* operon showed that in addition to the P_{virR} promoter, transcription of the four genes downstream of *virR* is also driven by a second promoter located within the *virR* gene. In contrast to P_{virR}, the activity of the P_{orf5} promoter is regulated by temperature and pH, resulting in a 67-fold increase in transcript levels following growth under inducing conditions compared to those obtained under noninducing growth conditions. Interestingly, the activity of the P_{orf5} promoter in a virulence plasmid-free *R. equi* strain was significantly lower than that in the presence of the virulence plasmid, suggesting that one or more components encoded by the virulence plasmid are required for full activity of P_{orf5}. One of these components is the *virR* gene, as inclusion of this gene on the plasmid containing P_{orf5} resulted in a twofold higher activity, indicating that *virR* is required but not sufficient for full activity of P_{orf5}.

The p004 plasmid contains the P_{orf5} promoter and its upstream region (−140 nucleotides) but did not display any promoter activity, indicating that a far upstream region required for P_{orf5} activity had been deleted. The requirement for far upstream sequences for promoter activity has been shown for many bacterial systems, for example, *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* (22, 27). Often these involve two-component regulatory systems interacting with either σ^{54}- or σ^{70}-type RNA polymerases (6, 8). However, disruption of *orf8*, encoding a response regulator, had no significant effect on *orf5* transcription levels, showing that it is not involved in controlling transcription of the *virR* operon under the experimental conditions used. This strongly suggests that genome-encoded transcriptional regulators are involved in regulating the activity of the P_{orf5} promoter, and therefore the expression levels of the response regulator Orf8.

The −10 regions of promoters recognized by the principal sigma factors of *Streptomyces coelicolor*, *Mycobacterium smegmatis*, and *M. tuberculosis* (HrdB and MysA) are highly conserved (2, 7, 15). Interestingly, the −10 regions of P_{virR} and the previously characterized *vapA* promoter (28) are highly similar to those recognized by HrdB and MysA, indicating that these promoters are recognized by the main principal sigma factor of *R. equi*. In contrast, the −10 and −35 sequences of P_{virR} and P_{orf5} do not share any obvious sequence similarities, strongly suggesting that these two promoters are recognized by different sigma factors. The involvement of alternative sigma factors in the regulation of virulence factor expression, as proposed here, is increasingly being observed (1, 16). The *R. equi* genomic sequence was recently completed (http://www.sanger.ac.uk/Projects/R_equi/), and a preliminary genome analysis revealed the presence of at least 20 potential sigma factor-encoding genes (R. J. Fahey and W. G. Meijer, unpublished results). We are currently analyzing the functions of these sigma factors.

The model that emerges from these studies is that the *virR* operon is transcribed at a low constitutive level under noninducing conditions from the P_{virR} promoter, which is most likely recognized by the principal sigma factor. The noninducing growth conditions resembled those encountered during saprophytic growth of *R. equi*, where expression of virulence factors is not required. The constitutive low-level transcription of the *virR* operon is maintained through binding of VirR to a site overlapping the *virR* initiation codon, resulting in an autoregulatory circuit. Following a change to inducing growth conditions, which are detected by an as yet unidentified genome-encoded signal transduction pathway, P_{orf5} becomes active, resulting in a significant increase of *orf5*, *vapH*, *orf7*, and *orf8*, but not *virR*, expression. Transcription from P_{orf5} is dependent on an alternative sigma factor and involves far upstream sequences. The *orf8* gene encodes a response regulator which is required for virulence (26) but not for P_{orf5} activity, whereas the roles of *orf5*, *orf7*, and *vapH* in virulence are not clear. Upregulation of P_{orf5} activity by inducing growth conditions may act as a master switch, resulting in increased expression of the response regulator Orf8, leading to subsequent full induction of the virulence genes. The validity of this model for the regulation of virulence gene expression is currently under investigation.

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