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Transcriptome Reprogramming by Plasmid-Encoded Transcriptional Regulators Is Required for Host Niche Adaption of a Macrophage Pathogen

Garry B. Coulson,[a]* Aleksandra A. Miranda-CasoLuengo,[b]* Raúl Miranda-CasoLuengo,[b] Xiaoguang Wang,[b] Jenna Oliver,[a] Jennifer M. Willingham-Lane,[a] Wim G. Meijer,[b] Mary K. Hondalus[a]

Department of Infectious Diseases, University of Georgia, Athens, Georgia, USA[a]; UCD School of Biomolecular and Biomedical Science and UCD Conway Institute, University College Dublin, Dublin, Ireland[b]

***Rhodococcus equi* is a facultative intracellular pathogen of macrophages, relying on the presence of a conjugative virulence plasmid harboring a 21-kb pathogenicity island (PAI) for growth in host macrophages. The PAI encodes a family of 6 virulence-associated proteins (Vaps) in addition to 20 other proteins. The contribution of these to virulence has remained unclear. We show that the presence of only 3 virulence plasmid genes (of 73 in total) is required and sufficient for intracellular growth. These include a single *vap* family member, *vapA*, and two PAI-located transcriptional regulators, *virR* and *virS*. Both transcriptional regulators are essential for wild-type-level expression of *vapA*, yet *vapA* expression alone is not sufficient to allow intracellular growth. A whole-genome microarray analysis revealed that VirR and VirS substantially integrate themselves into the chromosomal regulatory network, significantly altering the transcription of 18% of all chromosomal genes. This pathoadaptation involved significant enrichment of select gene ontologies, in particular, enrichment of genes involved in transport processes, energy production, and cellular metabolism, suggesting a major change in cell physiology allowing the bacterium to grow in the hostile environment of the host cell. The results suggest that following the acquisition of the virulence plasmid by an avirulent ancestor of *R. equi*, coevolution between the plasmid and the chromosome took place, allowing VirR and VirS to regulate the transcription of chromosomal genes in a process that ultimately promoted intracellular growth. Our findings suggest a mechanism for cooption of existing chromosomal traits during the evolution of a pathogenic bacterium from an avirulent saprophyte.**

The genus *Rhodococcus* comprises a large number of metabolically diverse species that have attracted considerable biotechnological interest because of their ability to metabolize a wide variety of substrates, which finds applications in bioremediation and in the synthesis of precursors of pharmaceutical compounds (1). The genus contains only two pathogenic species: the plant pathogen *Rhodococcus fascians* and the animal pathogen *Rhodococcus equi* (2). Although the latter species was initially isolated from young foals, it has subsequently been isolated from a wide range of animals and humans (3). Disease in foals and immunosuppressed humans usually presents as pyogranulomatous pneumonia, although other manifestations, including osteomyelitis, may also occur. In pigs and cattle, *R. equi* is usually associated with submandibular lymphadenitis (3). In addition to having a pathogenic lifestyle, *R. equi* grows readily as a saprophyte in soils, as well as in the equine intestinal tract (3).

R. equi is a parasite of macrophages, which prevents killing by the host cell through inhibition of phagosomal maturation, resulting in the formation of *R. equi*-containing vacuoles devoid of lysosomal markers, including cathepsin D and the proton-pumping vacuolar ATPase (vATPase) complex (4–6). The growth of *R. equi* is cytotoxic, resulting in the necrotic death of the phagocytic cell (7, 8). The inhibition of phagosomal maturation as a strategy for survival in macrophages is shared with the closely related organism *Mycobacterium tuberculosis* (9). However, despite the superficial similarities in virulence strategy, the underlying mechanisms are different. The subversion of the normal functioning of macrophages by *R. equi* is dependent on the presence of a conjugative plasmid harboring a pathogenicity island encoding a family of six virulence-associated proteins (Vap) that do not occur in *M. tuberculosis* (10–12). One of these, the cell envelope-associated protein VapA, is required, but not sufficient, for the inhibition of phagosomal maturation and for intracellular growth (13–16). It is not clear whether the remaining five *vap* genes (*vapC*, *vapD*, *vapE*, *vapG*, and *vapH*) play any role in macrophage parasitism.

The *vap* genes are organized into five transcriptional units (17–19). One of these, the *virR* operon, contains four genes in addition to *vapH*: *virR*, *icgA*, *orf7*, and *virS* (18). The *icgA* gene encodes a transport protein belonging to the major facilitator family. Ex-

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Address correspondence to Wim G. Meijer, wim.meijer@ucd.ie, or Mary K. Hondalus, hondalus@uga.edu.

* Present address: Garry B. Coulson, Department of Pathology, University of Georgia, Athens, Georgia, USA: Aleksandra A. Miranda-CasoLuengo, UCD School of Medicine and Medical Science, University College Dublin, Dublin, Ireland.

G.B.C., A.A.M.-C., and R.M.-C. contributed equally to this article.

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pression of this protein reduces the intracellular growth rate of *R. equi*, resulting in increased macrophage viability, allowing *R. equi* to reside longer in macrophages (20). VirR and VirS are transcriptional regulators belonging to the LysR-type transcriptional regulators and response regulators of two-component systems, respectively (12). Both VirR and VirS are required for wild-type-level expression of VapA, which has been offered as an explanation for the attenuation of mutants lacking either *virR* or *virS* (21–23).

In addition to the plasmid-encoded virulence factors, a number of virulence-associated factors, including cytoadhesive pili (24), the hydroxamate siderophore rhequichelin (25), and isocitrate lyase (26), are encoded by the chromosome. Comparative genome analysis demonstrated that many of these have orthologues in nonpathogenic actinobacteria. This suggests that in addition to the acquisition of a conjugative plasmid containing a pathogenicity island, virulence evolved by cooption of existing chromosomally encoded traits via an as yet unknown mechanism (24).

In this study, we demonstrate that the presence of just three pathogenicity island genes, *vapA*, *virR*, and *virS*, is required and sufficient for intracellular growth in macrophages. While this study confirms the central role of VapA in virulence, it also reveals that the two virulence plasmid-encoded transcriptional regulators, VirR and VirS, modify the chromosomal transcriptome, thus altering *R. equi* physiology to allow macrophage parasitism. This study thus provides evidence for the coevolution of the conjugative virulence plasmid and the ancestor of *R. equi*. In addition, it suggests a mechanism for the cooption of existing chromosomally encoded traits in the emergence of *R. equi* virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. Bacteria were grown on either brain heart infusion (BHI) or lysogeny broth (LB) medium. Antibiotics, when necessary, were used at the following concentrations: apramycin, 80 μg/ml (*R. equi*) or 30 μg/ml (*Escherichia coli*); hygromycin, 180 μg/ml.

Mutant construction. *R. equi* was made electrocompetent and was transformed by electroporation as described previously (20). Unmarked deletion mutants were constructed using the positive selection vector pSelAct (27). The construction of the *R. equi* multiple-deletion mutant (MDM) is described in the supporting Materials and Methods in the supplemental material.

Expression of *virR*, *virS*, and *vapA*. Plasmids pVirRSvapA, pVirRvapA, and pVirSvapA were constructed in the integrative vector pSET152 and contain the native promoters of the *virR* and *vapA* operons. The expression of *vapA* from its native promoter is dependent on both *virR* and *virS*. To drive the expression of *vapA* in the absence of either *virR* or *virS*, *vapA* was fused to the mycobacterial *hsp60* promoter. The construction of these expression plasmids is described in the supporting Materials and Methods in the supplemental material.

Intracellular growth of *R. equi* in macrophages. Murine J774.1 macrophages were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine and were incubated at 37°C under 5% CO_2. Murine bone marrow-derived macrophages were isolated and cultured as described previously (14). For intracellular growth assays, macrophages were seeded in 24-well tissue culture plates at 2×10^5 per well and were incubated at 37°C under 5% CO_2 overnight. Macrophages were infected at a multiplicity of infection (MOI) of 10 as described previously (20). At various times postinfection, macrophage monolayers were lysed with water (14), dilutions of the lysate were spread onto BHI agar, and plates were incubated (at 37°C for 48 h) to determine associated CFU.

Microarray expression analysis. Custom 12×135K gene expression microarrays (Roche NimbleGen) were designed with coverage of 4,525 (100%) chromosomal genes, 73 (100%) virulence plasmid-borne genes, and 20 pseudogenes. For each of these, seven individual probes (n = 32,319) of 60 nucleotides (nt) were designed and were spotted in quadruplicate. REQ01550 and REQ02340 are 100% identical and were covered by the same probes.

Total RNA was isolated from logarithmically grown *R. equi* bacteria using the guanidinium isothiocyanate method as described previously (20). Double-stranded cDNA was synthesized using the cDNA Synthesis System using random hexameric primers (Roche) and was cleaned using the High Pure PCR product purification kit (Roche). cDNA labeling, microarray hybridization, and signal detection were performed at Roche NimbleGen Systems (Iceland) (see the supplemental material for details). Network analysis was performed using BioLayout *Express*^{3D} using $\log_2$ robust multiarray average (RMA) expression values as described elsewhere (28). Four biological replicates were analyzed per strain. Gene Ontology (GO) enrichment analysis was performed with the customized microarray analysis tool of the Gene Ontology Enrichment Analysis Software Toolkit (GOEAST). P values were obtained using a Fisher exact test, adjusted for the false discovery rate (FDR) by the Benjamini-Yekutieli method, and corrected for overrepresentation of neighboring GO terms by the method of Alexa et al. (29, 30). *R. equi* Gene Ontology annotation was obtained from UniProt. Only 3,040 of 4,511 chromosomal genes have annotated GO terms.

RT-qPCR analysis. RNA isolation and reverse transcription-PCR (RT-PCR) using gene-specific primers (see Table S2 in the supplemental material) were carried out as described previously (19, 31). Quantitative PCR (qPCR) was carried out as described previously (20). The geNorm method was employed to determine the optimal number of reference targets from an initial set of 9 candidates. Normalization factor based on the REQ04990 and REQ23320 genes yielded a geNorm V value of <0.15 and a geNorm M value of ≤ 0.2, satisfying the requirements for use as reference targets for normalization under the experimental conditions used (32). qbasePlus software was used to evaluate quantitative variation in gene expression among strains (33). To rule out the presence of contaminating genomic DNA, controls without reverse transcriptase were routinely performed. In all experiments, each strain was represented by at least three independent biological replicates, and each sample was analyzed in duplicate.

Statistical analysis. Statistical analyses for bacterial growth assays were performed using the SigmaPlot (version 11.2.0.5) statistical package (Systat Software, San Jose, CA). The mean intracellular numbers of different bacterial strains were compared using one-way analysis of variance (ANOVA). When appropriate, multiple pairwise comparisons were carried out using Tukey's honestly significant difference (HSD) test. Significance was set at a P value of <0.05.

RESULTS

VapA is the sole virulence-associated protein family member essential for intramacrophage growth. The pathogenicity island encodes six Vaps, one of which, VapA, is essential for intracellular growth (14). To determine whether the remaining Vap are also required, a multiple-deletion mutant (MDM) was constructed in which 14 of the 26 genes of the pathogenicity island, including all *vap* genes except *vapA*, were removed (Fig. 1; see also Fig. S1 in the supplemental material). The intracellular growth of the MDM was compared to that of the wild-type strain *R. equi* 103S and the avirulent plasmid-cured strain *R. equi* 103S^{P-} in murine bone marrow-derived macrophages (Fig. 1). The *R. equi* MDM displayed a significantly ($P = 0.004$) better intracellular growth phenotype than wild-type *R. equi*. We showed previously that deletion of *icgA*, which is also absent in the *R. equi* MDM, resulted in enhanced intracellular growth (20). As expected, complementa-

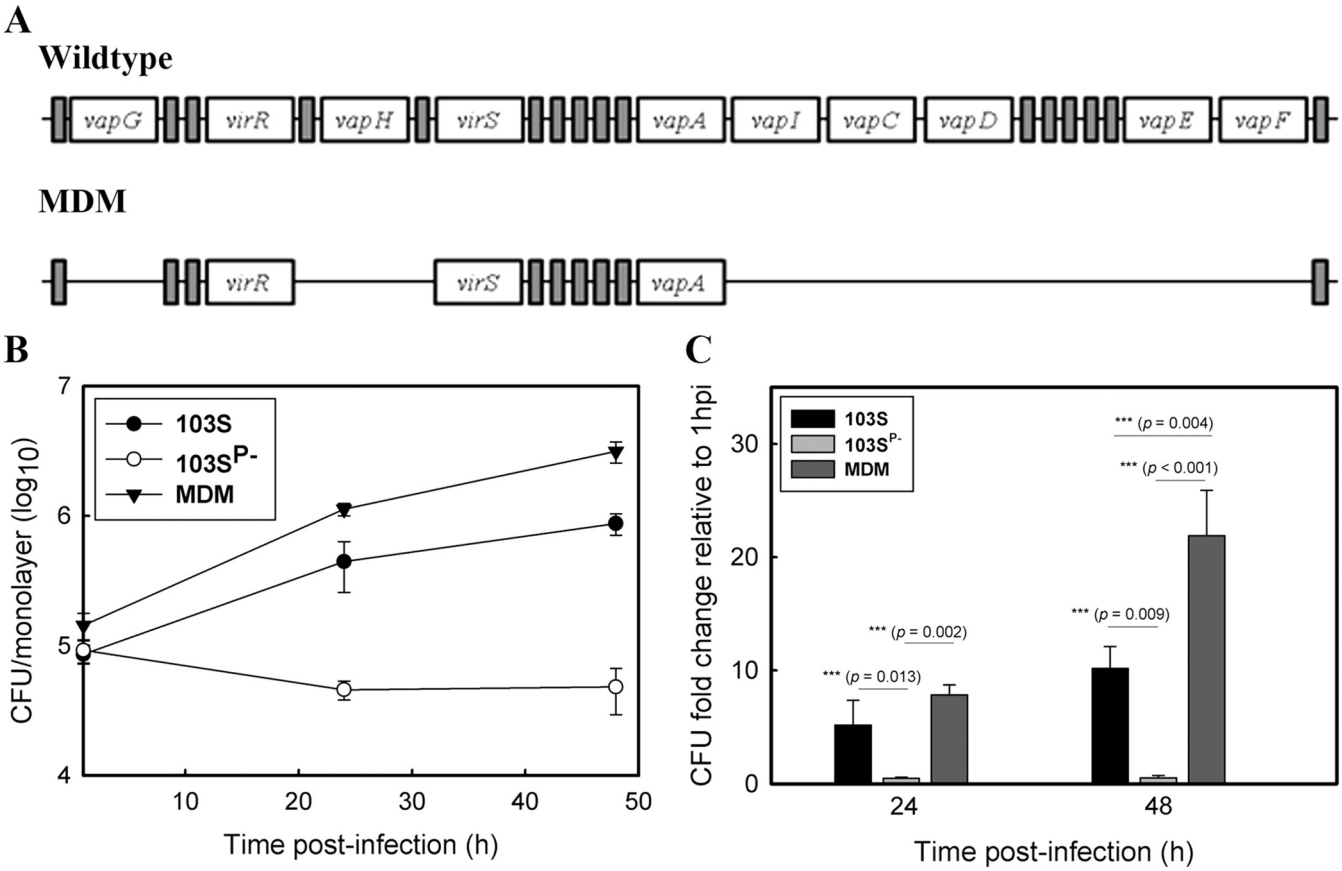

FIG 1 VapA is the only Vap family member required for growth in macrophages. (A) Schematic representation of the pathogenicity island in wild-type *R. equi* and the MDM, indicating the positions of genes deleted from the *R. equi* MDM. Open rectangles represent *vap* genes and the transcriptional regulators *virR* and *virS*. *vapI* and *vapF* are pseudogenes. Shaded rectangles represent all remaining genes of the pathogenicity island. (B and C) The intracellular growth of *R. equi* strains was assessed in murine bone marrow-derived macrophages infected with *R. equi* 103S, $103S^{P-}$, or the MDM at an MOI of 10. Following incubation for 1 h to allow phagocytosis, monolayers were washed and were treated with amikacin to kill remaining extracellular bacteria ($t = 0$ h). Monolayers were lysed in triplicate 24 h and 48 h postinfection. (B) Intracellular growth of *R. equi* strains following infection of macrophages. (C) Fold changes in the CFU count of intracellular bacteria at 24 h and 48 h postinfection relative to that at 1 h postinfection. Error bars represent the standard deviations of the means. The statistical significances of the differences in the fold change in CFU per monolayer between different *R. equi* strains are given. Data are representative of the results of three independent experiments.

tion of the *R. equi* MDM with *icgA* returned intracellular growth to wild-type levels (see Fig. S2 in the supplemental material). The data thus demonstrate that growth in macrophages does not require additional *vap* genes and that expression of *vapA* in the presence of the remaining pathogenicity island genes is sufficient for intracellular growth.

VapA and the transcriptional regulators VirR and VirS are the only virulence plasmid-encoded proteins required for intracellular growth. We subsequently postulated that *vapA* and the genes encoding the transcriptional activators VirR and VirS are the only genes of the pathogenicity island required for intracellular growth. To test this hypothesis, the avirulent, virulence plasmid-cured strain *R. equi* $103S^{P-}$ was transformed with pVirRSvapA, containing *vapA*, *virR*, and *virS* under the control of their native promoters. The transcription of *virR*, *virS*, and *vapA* was confirmed by RT-PCR (see Fig. S3 in the supplemental material). Macrophages were infected with *R. equi* $103S^{P-}$/pVirRSvapA, and its growth relative to that of the wild-type *R. equi* strain 103S and the virulence plasmid-free *R. equi* strain $103S^{P-}$ was assessed. As expected, the avirulent *R. equi* strain $103S^{P-}$ did not grow in macrophages (Fig. 2). Remarkably, however, expression of *virR*, *virS*, and *vapA* in the $103S^{P-}$ background restored intracellular growth, leading to levels of intracellular bacteria similar to those of the wild-type strain 103S at 48 h postinfection. These data demonstrate that the virulence plasmid-encoded proteins VapA, VirR, and VirS are sufficient to allow intramacrophage growth.

To determine if the presence of both transcriptional regulators was required for growth in macrophages, the plasmid-cured *R. equi* strain $103S^{P-}$ was engineered to express either *virR* or *virS* in the presence of constitutively expressed *vapA* by transformation of *R. equi* $103S^{P-}$/pVirR or $103S^{P-}$/pVirS with pMV261vapA, an episomal plasmid expressing *vapA* from the constitutive mycobacterial *hsp60* promoter (34). Expression of VapA in the resultant strains, $103S^{P-}$/pVirR::hsp60vapA and $103S^{P-}$/pVirS::hsp60vapA, respectively, was confirmed by Western blotting (see Fig. S4 in the supplemental material). In contrast to *R. equi* $103S^{P-}$/pVirRSvapA, strains expressing either VirR or VirS in conjunction with *vapA* did not grow in macrophages, suggesting that both regulators are required for intramacrophage replication

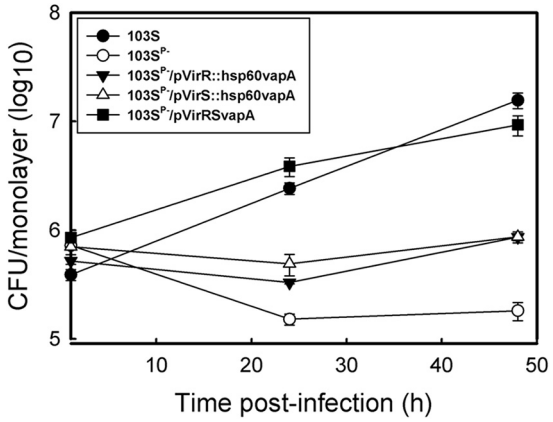

FIG 2 The transcriptional regulators VirR and VirS and the virulence-associated protein VapA are the only plasmid-encoded proteins required for growth in macrophages. Murine J774.1 macrophage monolayers were infected with *R. equi* 103S or 103S^{P-}, or with *R. equi* 103S^{P-} expressing *vapA* along with an individual regulator (103S^{P-}/pVirR::hsp60vapA or 103S^{P-}/pVirS::hsp60vapA) or with both regulators (103S^{P-}/pVirRSvapA). Intracellular bacterial growth was followed for 48 h postinfection. Values are means ± standard deviations for monolayers, each assessed in triplicate.

(Fig. 2). We therefore conclude that *vapA*, *virR*, and *virS* represent the minimum number of virulence plasmid genes that are required and sufficient for the intracellular growth of *R. equi*.

The virulence plasmid-encoded transcriptional regulators VirR and VirS remodel the *R. equi* transcriptome. Since *vapA* expression in the absence of VirR and VirS is not sufficient to allow intracellular growth, we hypothesized that in addition to regulating *vapA* expression, these two transcriptional regulators control the transcription of chromosomal genes. This was tested by comparing the transcriptional profiles of *R. equi* 103S and 103S^{P-}/pVirRSvapA to that of the virulence plasmid-cured strain *R. equi* 103S^{P-} grown under conditions that induce the transcription of *vapA* (pH 5.5, 37°C) (35). The presence of just these two virulence plasmid-encoded transcriptional regulators, VirR and VirS, had a significant effect (at least a 2-fold change [$P < 0.05$]) on the transcript levels of 18% of the transcriptome (Table 1). Introduction of the entire virulence plasmid had a similar effect, resulting in significantly altered transcript levels of 20% of the transcriptome (Table 1). The microarray data were validated by determining the changes in the transcript levels of 18 genes using reverse transcriptase qPCR (see Fig. S5 in the supplemental material).

Comparison of these transcriptomes with matrix scatter plots and a Euclidian distance-based dendrogram showed that expression of *virR* and *virS* in *R. equi* 103S^{P-}/pVirRSvapA resulted in a transcription profile closely resembling that of the virulence plasmid-containing *R. equi* strain 103S (Fig. 3). This was further underpinned by a 3-dimensional (3D) network analysis, which showed that introduction of the virulence plasmid resulted in the formation of two clusters of transcripts with highly correlated expression patterns (Fig. 4). The nodes at the core of each of these clusters represent genes with significantly increased (red) or decreased (blue) transcript levels. A network with very similar topology was observed when *virR* and *virS* were expressed in *R. equi* 103S^{P-} (Fig. 4). Inspection of individual genes showed that the presence of either the virulence plasmid or a plasmid driving the combined expression of *virR* and *virS* altered the expression levels (at least a 2-fold change; $P < 0.05$) of 614 genes in a similar manner (see Table S3 in the supplemental material). These data thus show that the modulation of the transcriptome by the virulence plasmid is due predominantly to the transcriptional regulators VirR and VirS.

VirR and VirS coopt chromosomally encoded regulatory networks. Among the chromosomal genes that were differentially expressed in the presence of VirR and VirS are 31 regulatory genes, encoding 28 transcriptional regulators, 2 sigma factors, and 1 antitermination regulator (Table 2). The transcriptional activity of these chromosomal regulators in *R. equi* 103S^{P-} transformed with *virR*, *virS*, and *vapA* closely mimics that of the plasmid-containing wild-type strain 103S (Fig. 5). While some of these regulators may control only a small regulon, others, in particular sigma factors, may control the transcription of a large number of genes. Of particular interest is the finding that VirR and VirS affected the transcript levels of 3 histidine sensor kinase- and 3 response regulator-encoding genes. These are part of two-component regulatory systems, which may respond to a wide range of environmental stimuli and often play a role in virulence, as highlighted by the response regulator VirS. The changes in the transcript levels of these regulatory genes resulting from the presence of VirR and VirS were tremendous, ranging from −805- to 24-fold. This undoubtedly has a profound impact on the regulatory network of the cell, offering an explanation for the major effect of VirR and VirS on the transcriptome of *R. equi*.

Enrichment in Gene Ontology terms of differentially expressed genes. GOEAST analysis of the 614 genes that were similarly differentially expressed in *R. equi* 103S^{P-}/pVirRSvapA and *R. equi* 103S compared to *R. equi* strain 103S^{P-} showed an enrichment in Gene Ontology terms (see Table S4 in the supplemental material). Within the "cellular component" domain, genes annotated with the terms GO:0005886 ("plasma membrane") and GO:0016021 ("integral to membrane") were enriched. Many of the genes within these ontologies encode transport proteins (44% and 63% of genes in GO:0005886 and GO:0016021, respectively), suggesting a major reconfiguration of transport activities when VirR and VirS are expressed. Interestingly, the majority of the genes encoding major facilitator superfamily (MFS) (88%) and ABC (64%) transporters were downregulated. Differentially expressed genes annotated with Gene Ontology terms that were enriched in the "biological process" and "molecular function" domains were predominantly related to energy and nitrogen metabolism. The enrichment of a limited number of Gene Ontology terms suggests that the effects of VirR and VirS on the transcriptome are not random but rather target a discrete number of processes that play a critical role in the adaptation of *R. equi* to an intracellular lifestyle.

TABLE 1 The virulence plasmid and the transcriptional regulators VirR and VirS have similar effects on the *R. equi* transcriptome

Strain	No. of genes with a >2-fold change in expression ($P < 0.05$) from that in *R. equi* 103S^{P-}		
	Upregulated	Downregulated	Total (%)[a]
R. equi 103S	347	572	919 (20)
R. equi 103S^{P-}/pVirRSvapA	386	406	792 (18)

[a] Percentage of the total number of chromosomal genes in the *R. equi* genome (n = 4,525).

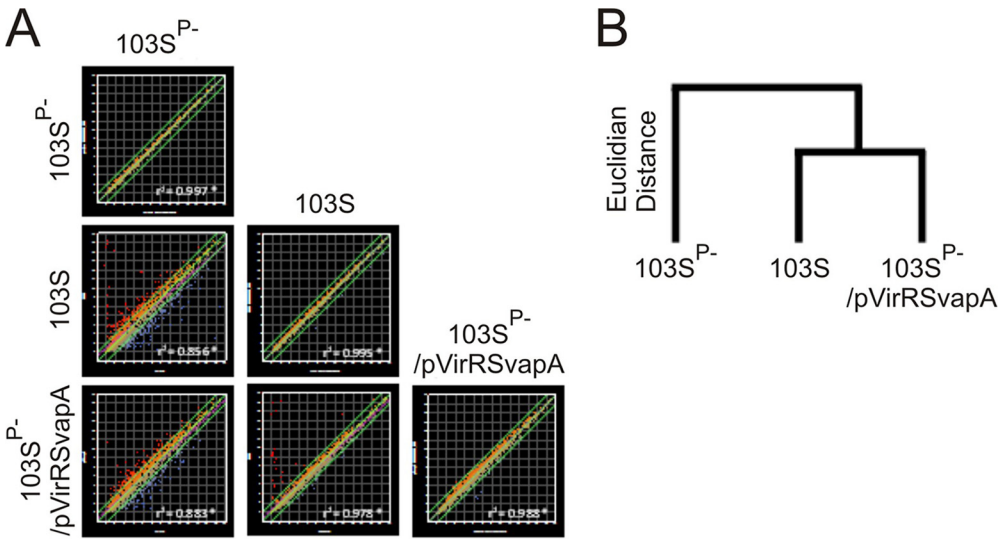

FIG 3 Transformation of *R. equi* 103S^{P-} with *virR*, *virS*, and *vapA* mimics the effect of the virulence plasmid on *R. equi* gene expression. Bacteria were grown to an optical density at 600 nm of 1 under *vapA*-inducing growth conditions (LB at 37°C and pH 5.5). RNA isolation, cDNA synthesis, and labeling and microarray hybridization were performed for four biological replicates as described in Materials and Methods. (A) Matrix of scatter plots comparing the $\log_2$ expression values between strains and between biological replicates. (B) Euclidian distance-based dendrogram obtained with the whole transcriptome, showing that the expression profile of *R. equi* 103S^{P-}/pVirRSvapA is more similar to that of *R. equi* 103S than to that of the parental strain lacking the virulence plasmid.

DISCUSSION

Although it is well established that the pathogenicity island of the virulence plasmid is essential for the host cell parasitism of *R. equi* (19), it remained unclear what role individual pathogenicity island genes play in this process, and which pathogenicity island genes are required and sufficient for intracellular growth. In addition to deepening our understanding of the virulence mechanisms of this pathogen, answering these questions also provides insight into the possible sequence of events in the evolution of this pathogenic bacterium from a saprophytic ancestor.

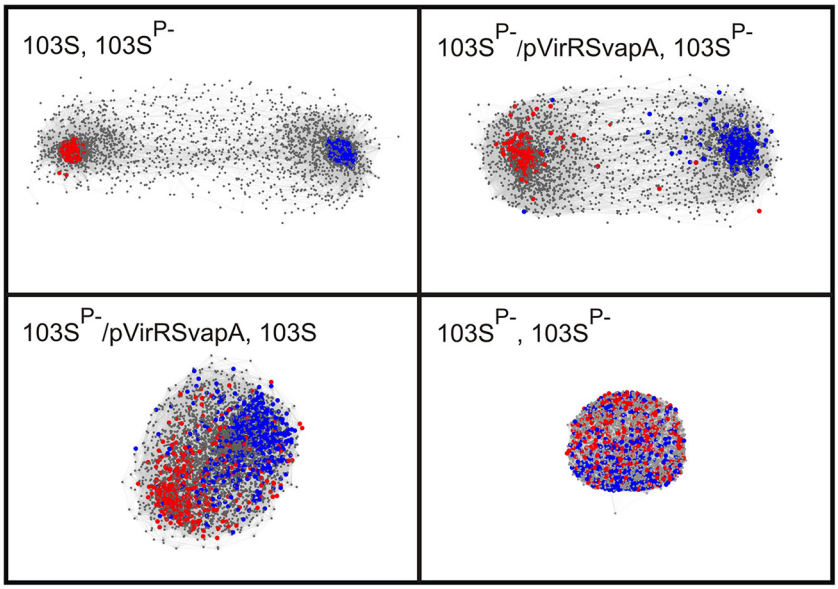

FIG 4 Network analysis to show the impact of the pathogenicity island regulators VirR and VirS on the expression of chromosomal genes. Shown are pairwise comparisons of the transcriptomes of *R. equi* 103S (plasmid containing, virulent), *R. equi* 103S^{P-} (plasmid cured, avirulent), and *R. equi* 103S^{P-} expressing the virulence plasmid pathogenicity island genes *virR*, *virS*, and *vapA* (*R. equi* 103S^{P-}/pVirRSvapA). 3D networks were made in BioLayout *Express*^{3D}, version 2.2, using $\log_2$ RMA expression values at a correlation threshold of 0.85, with an MCL inflation of 2.2, and an edge/node filter setting of 10. Each network shows a pairwise comparison of the transcriptomes between different strains or between biological replicates of strain 103S^{P-}. Genes are represented by nodes, which are connected by edges according to gene expression interrelationships above the threshold. Red and blue nodes represent genes that are up- and downregulated, respectively, in *R. equi* 103S relative to their expression in the plasmid-free avirulent strain *R. equi* 103S^{P-} (>2-fold change [$P < 0.05$]). The colored nodes in each of the four networks refer to the same set of genes. Genes with nonsignificant differences in transcript levels are shown in gray. Node size: gray, 1; colored, 3. Edge thickness, 0.1. The analysis was performed with $\log_2$ RMA expression values obtained by analysis of oligonucleotide microarrays for four biological replicates.

TABLE 2 Regulatory genes displaying at least a 2-fold ($P < 0.05$) change in transcript levels in both *R. equi* 103S and *R. equi* 103S^{P-}/pVirRSvapA from those in the virulence plasmid-cured strain *R. equi* 103S^{P-}

		Expression in:			
		103S		103S^{P-}/pVirRSvapA	
Gene name	Annotation	Fold change[a]	*P*	Fold change[a]	*P*
REQ00140	TetR family transcriptional regulator	3.66	0.0001	2.11	0.0012
REQ00980	TetR family transcriptional regulator	2.97	0.0019	4.93	0.0007
REQ02910	MarR family transcriptional regulator	−2.15	0.0044	−2.60	0.0004
REQ03830	IclR family transcriptional regulator	−2.47	<0.0001	−2.52	<0.0001
REQ08990	IclR family transcriptional regulator	−3.06	<0.0001	−2.87	0.0003
REQ10540	TCS[b] response regulator	2.58	0.0006	2.15	0.002
REQ13000	TetR family transcriptional regulator	2.68	0.0042	2.21	0.0072
REQ14460	Sigma 70 factor	24.20	<0.0001	15.61	0.0003
REQ15290	Transcriptional regulator	2.56	<0.0001	2.76	<0.0001
REQ15500	IclR family transcriptional regulator	−2.65	0.0006	−2.91	0.0002
REQ15980	LuxR family transcriptional regulator	4.96	0.0001	4.78	0.0002
REQ21040	TetR family transcriptional regulator	−2.42	0.0023	−2.60	0.0012
REQ23970	AraC family transcriptional regulator	2.96	0.0001	2.47	0.0008
REQ26080	RNA binding sensor regulator	2.05	0.0005	2.01	0.0001
REQ26260	MarR family transcriptional regulator	2.23	0.0016	3.57	0.0004
REQ31030	TCS response regulator KdpE	4.35	0.0002	4.29	0.0008
REQ31040	TCS sensor kinase KdpD	4.21	<0.0001	5.42	0.0002
REQ31260	AsnC family transcriptional regulator	2.26	0.0004	2.26	0.0009
REQ31580	Two-component system sensor kinase	−2.45	0.0007	−2.13	0.0013
REQ33120	Two-component system sensor kinase	2.85	<0.0001	2.95	0.0001
REQ34960	Sigma 70 factor	5.00	<0.0001	4.95	<0.0001
REQ38490	NmrA family transcriptional regulator	−7.68	<0.0001	−3.02	0.0019
REQ40160	PadR family transcriptional regulator	−2.92	0.001	−2.27	0.005
REQ41890	GntR family transcriptional regulator	−29.26	<0.0001	−22.74	<0.0001
REQ41960	IclR family transcriptional regulator	−805.27	<0.0001	−926.06	<0.0001
REQ41970	ArsR family transcriptional regulator	−176.15	<0.0001	−172.14	<0.0001
REQ42130	MarR family transcriptional regulator	−2.60	0.0027	−2.05	0.0031
REQ44180	TetR family transcriptional regulator	−3.16	<0.0001	−3.05	<0.0001
REQ45770	Purine catabolism regulatory protein	−4.25	<0.0001	−3.96	<0.0001
REQ46220	Transcriptional regulator	5.72	0.0001	3.84	0.0043
REQ46610	PadR family transcriptional regulator	−2.33	0.002	−2.42	0.0004

[a] From expression in the virulence plasmid-cured strain *R. equi* 103S^{P-}.
[b] TCS, two-component system.

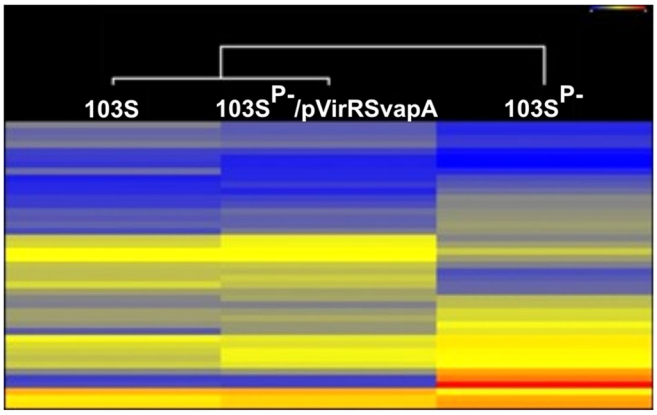

FIG 5 The regulatory transcriptome of *R. equi* 103S^{P-} transformed with *virR*, *virS*, and *vapA* closely resembles the wild-type *R. equi* regulatory transcriptome. Bacteria were grown to an optical density at 600 nm of 1 under *vapA*-inducing growth conditions (LB at 37°C and pH 5.5). RNA isolation, cDNA synthesis, and labeling and microarray hybridization were performed for four biological replicates as described in Materials and Methods. Shown is a heat map of differentially expressed genes annotated as regulators. Above the heat map is a Euclidian distance-based dendrogram of strains based on changed regulators. Microarray data were analyzed with ArrayStar (DNAStar).

The pathogenicity island encodes six homologous Vaps, which share a high degree of sequence similarity, in particular in their carboxy-terminal domains (12, 36). The recent structural determination of the C-terminal domains of VapD and VapB showed a novel 8-stranded β-barrel topology that is conserved among Vaps (37, 38). In addition to their structural relatedness, transcription of the *vap* genes is also regulated in a similar manner in response to changes in the pH and temperature of the growth medium (15, 35, 39). Deletion of *vapA* abolishes the capacity for intracellular growth and results in an attenuated phenotype in the mouse *R. equi* infection model (14). To date, it has remained unclear whether the VapA homologues encoded by the pathogenicity island also play a role in intracellular growth. Deletion of individual *vap* genes other than *vapA* (*vapG*, *vapH*) did not show a phenotype (19, 20). The similarities these proteins share in amino acid sequence, extracellular location, and expression patterns initially suggested that functional redundancy may exist. It was therefore surprising that deletion of all *vap* genes except *vapA* did not negatively affect the growth characteristics of *R. equi* in macrophages. Our data thus unequivocally demonstrate that VapA is the only Vap that is required and sufficient for intracellular growth in *in vitro*-cultured murine macrophages.

The transcriptional regulators VirR and VirS are required for wild-type-level *vapA* expression (21, 22). However, as was observed previously, VirR- and VirS-independent expression of *vapA* from the Hsp60 promoter in a virulence plasmid-cured strain did not result in intracellular growth (10). Surprisingly, introduction of a plasmid (pVirRSvapA) expressing all three genes did restore normal levels of intracellular growth. This observation has major implications for our understanding of the mechanisms by which *R. equi* blocks endosomal maturation. Analysis of *R. equi*-containing vacuoles demonstrated that early endosomal markers are acquired and lost normally; however, these phagosomal vacuoles do not acquire certain late endosomal markers, including cathepsin D and the proton-pumping vATPase complex (5). Deletion of *vapA* from the virulence plasmid allows phagosomes to develop into mature acidified phagolysosomes, resulting in the killing of *R. equi* (16). Although this clearly demonstrates that VapA is required for the prevention of phagosomal maturation, it does not exclude the possibility that other pathogenicity island-encoded proteins, including Vap homologues, may also be involved. Nonetheless, the data presented here demonstrate that VapA is the only structural pathogenicity island-encoded protein that is required and sufficient for normal intracellular growth. This suggests that VapA is critical for the inhibition of endosomal maturation without the need for other virulence plasmid-encoded structural proteins.

The results from this study show that VirR and VirS do not just regulate the expression of *vapA* and other pathogenicity island genes but have a major impact on global transcription. The VirR- and VirS-induced change in cellular physiology is a prerequisite for host cell parasitism, because expression of VapA in the absence of these two regulators does not support intracellular growth. Although a previous study demonstrated that the presence of the virulence plasmid affects the *R. equi* transcriptome (24), it remained unclear whether this was due to specific or pleiotropic effects, or whether these changes in gene expression are required for intracellular growth. We demonstrate here that the impact of the virulence plasmid on the transcriptome is specific and is due predominantly to the expression of just 2 (*virR* and *virS*) of the 63 genes of the virulence plasmid, affecting the transcript levels of 18% of chromosomal genes.

The global effect of VirR and VirS on transcription may be explained in part by the fact that their expression significantly altered the transcript levels of 31 transcriptional regulators, including those of 2 sigma factors. It therefore appears likely that in many cases, VirR and VirS have an indirect effect on gene expression by altering the transcription levels of other transcriptional regulators. Nevertheless, an analysis of gene ontology showed that the impact of VirR and VirS on global transcription was not random but resulted in enrichment of Gene Ontology term among differentially expressed genes. Most notably, these include genes playing roles in transport processes, e.g., ABC and MFS transporters, and in nitrogen and energy metabolism.

Acquisition of the pathogenicity island on a conjugative plasmid by a nonpathogenic ancestor of *R. equi* was undoubtedly the initial event in the evolution of this pathogen. However, the genome of this ancestral *R. equi* organism would not have had VirS and VirR DNA binding sites to allow these regulators to affect chromosomal gene expression in a nonrandom manner. In addition, VirS is an orphan response regulator, which may interact with a chromosomally encoded sensor kinase (12).

It is generally accepted that bacterial strategies to prevent or survive grazing by predatory protozoa were a major driving force in the evolution of pathogenesis (40–42). We therefore hypothesize that in the evolution of *R. equi*, acquisition of the pathogenicity island on a conjugative plasmid may have given an *R. equi* ancestor a selective advantage in surviving predation by protozoa, which are abundant both in the equine intestinal tract and in soil (43). Continued selective pressure exerted by these predatory protozoa may have subsequently selected for chromosomal mutations facilitating the binding of VirR and VirS to promoters that control the expression of genes influencing intracellular survival by altering bacterial physiology to enable adaptation to the host. In addition, predation may have selected for mutations in two-component systems allowing VirS to interact with one or more sensor kinase proteins, thus tapping into the sensor network of the ancestral *R. equi* organism. This scenario is supported by comparative analysis of the *R. equi* genome, which showed that the majority of genes implicated in virulence are conserved in nonpathogenic actinobacteria (24). Although the acquisition of the pathogenicity island may have been the initial event, the virulence of *R. equi* must therefore have evolved subsequently via adaptation of existing traits to the requirements of intracellular growth through nonrandom, VirR- and VirS-induced changes in the physiology of *R. equi*.

In conclusion, the pathogenicity island of the virulence plasmid fulfills at least three functions in relation to intracellular growth, by encoding (i) VapA, which alters the maturation of the early endosome, thus creating a niche conducive to the growth of *R. equi*, (ii) IcgA, an MFS transport protein, which tempers the intracellular growth rate of *R. equi*, resulting in increased macrophage viability, allowing *R. equi* to stay longer in this intracellular niche, and (iii) VirR and VirS, which selectively adjust chromosomal gene expression, thereby modifying the physiology of *R. equi* to enable survival and growth in an intracellular niche.

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