SUPPORTING INFORMATION

Materials and Methods

Mutant construction The *R. equi* MDM strain lacking all *vap* genes bar *vapA* was constructed by deletion of the *vapI-vapF* gene locus (containing *vapI, -C, -D,-E, -F*) in *R. equi* Δ*vapG*Δ*icgA-vapH-orf7*. A 715 bp region of DNA upstream of *vapI* was amplified using primers vapI-up-F and vapI-up-R and the amplicon was digested with *Bam*HI and *Eag*I and ligated into pSelAct (1) that had been previously digested with the same enzymes, yielding pGBC17. Subsequently, a 709 bp fragment of DNA downstream of *vapF* was amplified using primer pair vapF-up-F and vapF-up-R, and digested with *Bam*HI and *Eco*RI. This fragment was then ligated into pGBC17 which had been digested with *Bam*HI and *Eco*RI creating pGBC18, which was electroporated into *R. equi* Δ*vapG*Δ*icgA-vapH-orf7*. Apramycin resistant transformants were grown for 20 h in LB medium at 37 ^{o}C. Aliquots (100 µl) of these cultures were plated in 10^{-4} to 10^{-6} dilutions onto acetate containing minimal medium agar plates supplemented with 100 µg/ml 5-fluorocytosine and incubated for 3 days at 37 ^{o}C.

Expression of *virR, virS* and *vapA*. Plasmid pVirR (for expression of *virR*) was generated by amplification of *virR* with its promoter region (1872 bp) from *R. equi* 103S using primers PAI38d12_2080F and PAI4_1872R (**Table S2**), which carried *Bcl*I and *Pci*I restriction sites, and cloning of this fragment into pSET152 previously digested with *Bam*HI and *Pci*I. Plasmid pVirRS (for expression of *virR* and *virS*) was generated using total genomic DNA from *R. equi* Δ*vapG*Δ*icgA-vapH-orf7,* which carries an in-frame deletion of *icgA-orf7,* as template for a PCR reaction, amplifying a DNA fragment of 2890 bp starting at the terminal part of *orf3* and continuing until the stop codon of *virS*. Primers used (PAI38d12_2890F and Orf8 InterSeq_918R) introduced *Bcl*I and *Pci*I restriction sites, which facilitated cloning of the resulting PCR product into the respective *Bam*HI/*Pci*I sites of pSET152 giving rise to plasmid pVirRS. Plasmid pVirS (for expression of *virS*) was

generated by using the Quick Change Kit (Invitrogen) according the manufacturer's recommendations and primers 4aa8_9468F and 4aa8_9468R to introduce two adenine residues at position 484 of *virR* in pVirRS producing a frameshift mutation. The *vapA* gene with its native promoter was amplified (using primer pair vapA_PciI F/vapA_PciI R) and inserted into the *Pci*I site of pVirRS yielding plasmid pVirRSvapA. *R. equi* strains $103S^{P-}$/pVirR and $103S^{P-}$/pVirS were co-transformed with the episomal plasmid pMV261.vapA (2) in which *vapA* is constitutively expressed from the mycobacterial *hsp60* promoter, thus yielding $103S^{P-}$/pVirR::hsp60vapA and $103S^{P-}$/pVirS::hsp60vapA

Microarray expression analysis. Briefly, purified cDNA (400 ng) was random-primer labeled with Cy3 nonamers using the Nimblegen One-Color DNA Labeling Kit (Roche). The array was then hybridized for 16 hours at 42°C, washed, dried, and scanned at 2 µm resolution using a NimbleGen MS 200 Microarray Scanner (Roche). The NimbleScan v2.6 software (Roche) was used to extract fluorescence intensity signals from the scanned images and perform Robust Multi-Array (RMA) analysis to generate gene expression values. Analysis of normalized expression values, calculation of fold changes and P values were performed with ArrayStar software (DNASTAR, Madison, WI).

Supporting Information Legends

Figure S1. Verification of the genotype of *R. equi* MDM. Verification of the *R. equi* MDM genotype. Total *R. equi* DNA was used as template in a PCR reaction using primers specific for either *vapA, -C, -D, -E, -F, -G, -H* or *-I* to determine the presence of these genes in *R. equi* MDM. Shown are the amplicons for each primer pair amplifying a specific *vap* gene. Lane M: DNA size standards; lane +: *R. equi* 103S; lane -: *R. equi* $103S^{P-}$; Lane MDM: *R. equi* MDM.

Figure S2: Complementation of *R. equi* MDM with *icgA* restores wild type intracellular growth. Intracellular growth of *R. equi* strains was assessed in murine bone marrow derived macrophages infected with *R. equi* 103S, MDM and MDM/pAI35 at a MOI of 10. Following incubation for one hour to allow phagocytosis, monolayers were washed and treated with amikacin to kill remaining extracellular bacteria (t=0 h). Monolayers were lysed in triplicate 24 h and 48 h post-infection (hpi). Error bars represent the standard deviation of the mean. Horizontal lines represent the statistical significance of fold changes in CFU per monolayer for *R. equi* strains represented by bars at the end of each line.

Figure S3. Reverse transcription PCR confirming transcription of *virR, virS* and *vapA* in *R. equi* $103S^{P-}$/pVirRSvapA. Total RNA was extracted from *R. equi* strains $103S^{P-}$/pVirRSvapA (lanes 1, 3, 5 and 7) and 103S (lanes 2, 4, 6 and 8). cDNA was synthesized using equivalent concentrations of total RNA as template. The presence of *virR* (lanes 1 and 2), *icgA* (lanes 3 and 4), *virS* (lanes 5 and 6) and *vapA* (lanes 7 and 8) was assessed by PCR using primer pairs (Table S2) that specifically amplify internal regions of each of these genes. Standard DNA markers are indicated on the left of the panel (M).

Figure S4. Western blot analysis confirming VapA expression in recombinant R. equi strains. Whole-cell extracts were prepared from *R. equi* 103S (lane 1), virulence plasmid-

cured 103S^{P-} (lane 2), 103S^{P-}/pVirR::hsp60vapA (lane 3), 103S^{P-}/pVirS::hsp60vapA (lane 4) and 103S^{P-}/pVirRSvapA (lane 5), cultured at 37°C as described in the Materials and Methods. The presence of VapA protein was detected with rabbit polyclonal antiserum to VapA.

Figure S5. Validation of microarray data with reverse transcription qPCR. (A) The transcript levels of 18 genes were determined in three biological replicates, each measured in duplicate by reverse transcription qPCR using specific primers listed in Table S2. Log_{10} fold changes in gene expression of *R. equi* 103S (black bars) or *R. equi* 103S^{P-}/pVirRSvapA (white bars) relative to *R. equi* 103S^{P-}. Error bars represent the standard error of fold changes. Dotted lines above and below the zero line represent a 2-fold increase or decrease in transcription levels. (B) Comparison of Log_2 fold changes in gene expression as determined by RT-qPCR and microarray analysis. Correlation coefficients of 103S (black symbols) and 103S^{P-}/pVirRSvapA (white symbols) are r^2=0.965 and r^2=0.969, respectively. (C) List of genes used for validation purposes.

Table S1: Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics	Source/Reference
Escherichia coli		
DH5α	F- Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA*1 *endA*1 *hsdR*17 (r_K^-, m_K^+) *phoA supE*44 λ- *thi*-1 *gyrA*96 *relA*1	Zymo Research
Rhodococcus equi		
103S	Virulent strain containing an 81kb virulence plasmid	(3)
$103S^{P-}$	Plasmid-cured variant of strain 103S	(4)
Δ*vapG*	Δ*vapG* Hyg^R	(5)
Δ*vapG*Δ *icgA-vapH-orf7*	Δ*vapG* Δ(*icgA-vapH-orf7*) Hyg^R	(6)
MDM	Δ*vapG* Δ(*icgA,vapH,orf7*) Δ(*vapICD*, pVAPA_0680, *orf16, orf17, orf18*, pVAPA_0740, *vapE, vapF*) Hyg^R	This study
MDM/pAI35	Δ*vapG* Δ(*icgA,vapH,orf7*) Δ(*vapICD*, pVAPA_0680, *orf16, orf17, orf18*, pVAPA_0740, *vapE, vapF*) Hyg^R,pAI35	This study
$103S^{P-}$/pVirR	$103S^{P-}$, pVirR	This study
$103S^{P-}$/pVirRSvapA	$103S^{P-}$, pVirRSvapA	This study
$103S^{P-}$/pVirS	$103S^{P-}$, pVirS	This study
$103S^{P-}$/pVirR ::hsp60vapA	$103S^{P-}$, pVirR, *hsp60::vapA*	This study
$103S^{P-}$ /pVirS::hsp60vapA	$103S^{P-}$, pVirS *hsp60::vapA*	This study
Plasmids		
pSelAct	Apr^R, *lacZ*, *codA*:*upp*	(1)
pSET152	ϕC31 integrase *att*P Apr^R	(2)
pGBC10	pSelAct containing 734 bp upstream of *icgA*	This study
pGBC11	pGBC10 containing 726 bp downstream of *orf7*	This study
pGBC17	pSelAct containing 751 bp upstream of *vapI*	This study
pGBC18	pGBC17 containing 709 bp downstream of *vapF*	This study
pVirR	pSET152 containing a 1872 bp fragment including the 5' end of *orf3* and *virR*	This study
pVirRS	pSET152 containing a 2890 bp fragment including *virR* and *virS*.	This study
pVirS	Derivative of pVirRS incorporating a two nucleotide insertion after position 484 of *virR*	This study
pVirRSvapA	Derivative of pVirRS carrying a 1268 bp fragment containing *vapA* downstream of *virS*	This study
pMV261vapA	Shuttle plasmid pMV261 containing *vapA* gene under expression of the *Mycobacteria* spp. *hsp60* promoter	(2)
pAI35	pSET152 derivative containing *virR* and *icgA*	(6)

Table S2. Oligonucleotides used in this study.

Name	Sequence (5' – 3')[a]	Purpose	Reference
VapA-hsp60-F	CGC*TGGCCA*CTCTTCACAAGACG	Genotyping of *R. equi* MDM	(5)
VapA-hsp60-R	CTA*TGGCCA*CTAGGCGTTGTGCCA	Genotyping of *R. equi* MDM	(5)
vapC-int-F	GGGTCGTCCATCCAAATC	Genotyping of *R. equi* MDM	This study
vapC-int-R	CATTCCCACCGCCTATAC	Genotyping of *R. equi* MDM	This study
VapD1	ATATAT*TCTAGA*ATGGGTCGGAAGGTAAAC	Genotyping of *R. equi* MDM	(4)
VapD1c	ATATAT*GTTAAC*ACTTGTTCCTCACGCAGC	Genotyping of *R. equi* MDM	(4)
VapE1	ATATAT*TCTAGA*GTCGCGCTTGAAGTGCGG	Genotyping of *R. equi* MDM	(4)
VapE1c	ATATAT*GTTAAC*CAGCTATCGCCAGGCG	Genotyping of *R. equi* MDM	(4)
VapF1	ATATAT*TCTAGA*CTGACGATAGCTGGGCCT	Genotyping of *R. equi* MDM	(4)
VapF1c	ATATAT*GTTAAC*CAATCATTGCGCTAACAC	Genotyping of *R. equi* MDM	(4)
vapG-int-F	CCGCCAGAATCACCAGTAAAC	Genotyping of *R. equi* MDM	(5)
vapG-int-R	GCGAACGCGGAAACTTCAATG	Genotyping of *R. equi* MDM	(5)
vapI-int-F	GCCAGTGCGCAAGAAATG	Genotyping of *R. equi* MDM	This study
vapI-int-R	CCGTGTACCGATACTGATTCC	Genotyping of *R. equi* MDM	This study
ORF6F-RTPCR	AGCTGTGCCTGCAACATTCG	Genotyping of *R. equi* MDM	(5)
ORF6R-RTPCR	CTACGCTACATCGCCTATCC	Genotyping of *R. equi* MDM	(5)
vapI-upstream-F	AATT*CGGCCG*TAATGCGACCGTTCTTG	Plasmid construction	This study
vapI-upstream-R	AATT*GGATCC*TGCAGTCAGCGCGATTG	Plasmid construction	This study
vapF-downstream-F	AAGT*GGATCC*TGGCATTCTTGGGAGTG	Plasmid construction	This study
vapF-downstream-R	AAGT*GAATTC*CGCGTTGCATCAGGTGG	Plasmid construction	This study
004F	CGGACGAGTTCGACTGGTAT	Genotyping of *R. equi* $103S^{P-}$ /pVirRSvapA	(7)
004F	CAAAGACGATTTGGGGTACG	Genotyping of *R. equi* $103S^{P-}$ /pVirRSvapA	(7)
005F	CTCTTCCTGATCGGAGTTGC	Genotyping of *R. equi* $103S^{P-}$ /pVirRSvapA	(7)
005R	GAGTCGCAGACGAGGTAAGC	Genotyping of *R. equi* $103S^{P-}$ /pVirRSvapA	(7)
008F	GAACAACTGGGAATGGTGGT	Genotyping of *R. equi* $103S^{P-}$ /pVirRSvapA	(7)

008R	GTTCGCCGTTTCTAGACGAA	Genotyping of *R. equi* 103S^{P-}/pVirRSvapA	(7)
VapA_182F	AATGCGACCGTTCTTGATTC	Genotyping of *R. equi* 103S^{P-}/pVirRSvapA	(8)
VapA_182R	TCTCCGTGAACGTCGTACTG	Genotyping of *R. equi* 103S^{P-}/pVirRSvapA	(8)
Orf8 InterSeq_918F	AAT*GCGGCCGC*TGAGTTCAGCGACAGGAGTG	Plasmid construction	This study
Orf8 InterSeq_918R	GCCG*ACATGT*GCAGGTCCGGAATACATGA	Plasmid construction	This study
vapA_PciI F	GCA*ACATGT*AGACCAACATCGTTCGCG	Plasmid construction	This study
vapA_PciI R	GATC*ACATGT*CTAGGCGTTGTGCCAGCTAC	Plasmid construction	This study
PAI38d12_2890 F	GTC*TGATCA*CCTCAAGGGCGTCGACCAAG	Plasmid construction	This study
PAI4_1872R	TGCG*ACATGT*AGTGTTCGACGCTAGGAGGG	Plasmid construction	This study
4aa8_9468F	CACCCCTTAGCGGGGCGTGCAAGACTATCCGG	Plasmid construction	This study
4aa8_9468R	CCGGATAGTCTTGCACGCCCCGCTAAGGGGTG	Plasmid construction	This study
rpoA_201F	TCGACGTGGAGACCAAGAAC	qPCR/microarray validation	This study
rpoA_201R	TGTAGGAACGAACCGTCAGG	qPCR/microarray validation	This study
GyrCOOH1	GTCGAGCAGGGTCACGTGTA	qPCR/microarray validation	(7)
GyrCOOH25′	AGCTCCTTTGCGTTCATCT	qPCR/microarray validation	(7)
REQ140_191F	GGTCACCATCCTCAAGTCGT	qPCR/microarray validation	This study
REQ140_191R	GCGTCGACCACAGGATCT	qPCR/microarray validation	This study
REQ4990_186F	GACTCGACGTCCTGGTCAAC	qPCR/microarray validation	This study
REQ4990_186R	CGGACGTGGAGCTGAAGT	qPCR/microarray validation	This study
REQ7620_191F	CCCCTGAACTACCAGGTCAA	qPCR/microarray validation	This study
REQ7620_191R	ATCGCTTCGATGGTGATCC	qPCR/microarray validation	This study
REQ14460_198F	CAACCTCTTCCTGGACATGG	qPCR/microarray validation	This study
REQ14460_198R	TCGATGTCACACAGGACCAC	qPCR/microarray validation	This study
REQ15980_171F	AGGTGATCTCCCAGCTGATG	qPCR/microarray validation	This study
REQ15980_171R	AGATGTTGCCGATGTGTTTG	qPCR/microarray validation	This study
REQ31040_150F	TCGAAAGTCTCAACGACGTG	qPCR/microarray validation	This study
REQ31040_150R	ACACGTTTCCGTGAGAGAGC	qPCR/microarray validation	This study

REQ33310_154F	TCAAGCGTGACGTCCACTAC	qPCR/microarray validation	This study
REQ33310_154R	TTGATCGCGTTGTTCAGGTA	qPCR/microarray validation	This study
REQ37050_195F	AAGATGGTGGGCCAGAAGTT	qPCR/microarray validation	This study
REQ37050_195R	GTTGAACGCGTACTTGAGCA	qPCR/microarray validation	This study
REQ41960_158F	CTGTACATGGGGCAGATCG	qPCR/microarray validation	This study
REQ41960_158R	GAGTGGTCCTCGATGGTGTC	qPCR/microarray validation	This study
20280_168F	GGCTCTCGTACAAGGAGTGG	qPCR/microarray validation	This study
20280_168R	TCAGCAGAGCTGCAGGAAC	qPCR/microarray validation	This study
42130_185F	ACCCGCAGTACCTCGTGAT	qPCR/microarray validation	This study
42130_185R	GGTGAGTTCGACGACCAGTT	qPCR/microarray validation	This study
2910_204F	GGAGATCAAACGCATGTGG	qPCR/microarray validation	This study
2910_204R	GCGTAGGCATTGTCGTTGTA	qPCR/microarray validation	This study
41890_160F	CGCTGTTCGAGGTGAAGAAC	qPCR/microarray validation	This study
41890_160R	TAGTGCACGAGAACGGAGTG	qPCR/microarray validation	This study
19090_170F	GATCCCGAGAACGAGGAACT	qPCR/microarray validation	This study
19090_170R	GTGCGGATCTGGGTGTAGAT	qPCR/microarray validation	This study
22420_158F	AGGTCAGCGAAGACCATCTC	qPCR/microarray validation	This study
22420_158R	CTTGTCCTTGAAATGGTGGAA	qPCR/microarray validation	This study
37390_201F	GAACCTTGGAGGATCGATGA	qPCR/microarray validation	This study
37390_201R	GACGAGAGGAGTGCCATCAC	qPCR/microarray validation	This study
24440_178F	GTCAGCGGCTGTACTCCTTC	qPCR/microarray validation	This study
24440_178R	GTGCACTCGTAGACGGTGGT	qPCR/microarray validation	This study
23320_155F	ACCAAAGTCGAACGGTTGAT	qPCR/microarray validation	This study
23320_155R	AGTTCGCTCTTGTCCCGTTC	qPCR/microarray validation	This study

[a] Nucleotides underlined and italicized indicated restriction enzyme sites used for sub-cloning.

Figure S1.

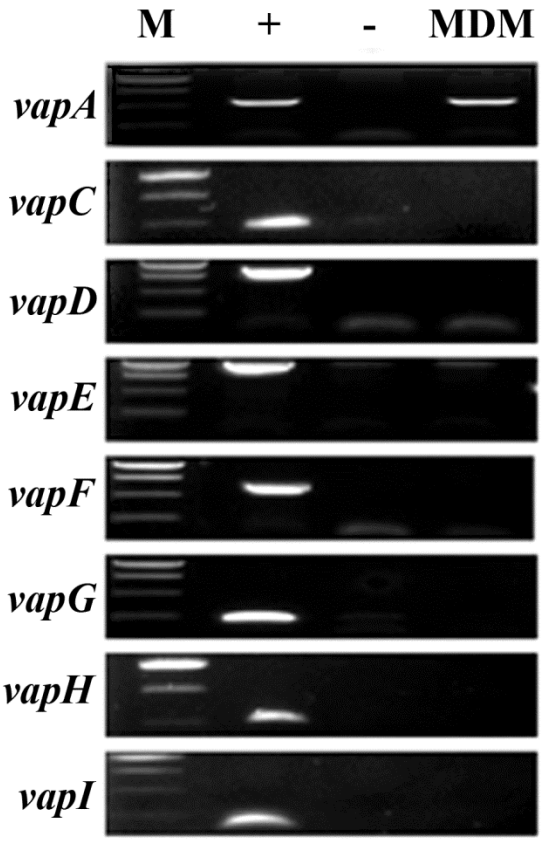


Figure S2.

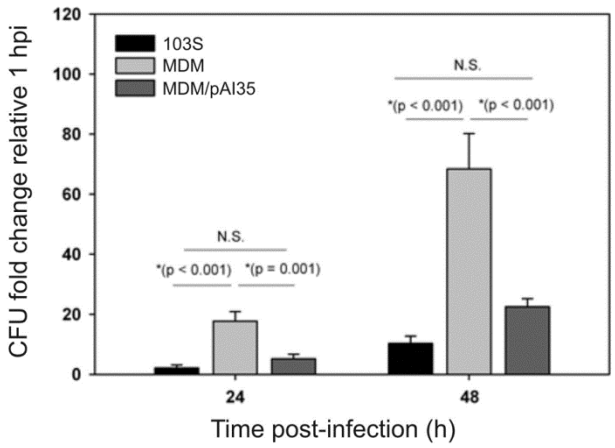

120
100
80
60
40
20
0
CFU fold change relative 1 hpi
103S
MDM
MDM/pAI35
N.S.
*(p < 0.001)
*(p = 0.001)
N.S.
*(p < 0.001)
*(p < 0.001)
24
48
Time post-infection (h)

Figure S3.

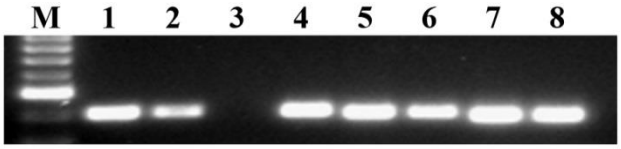


Figure S4.

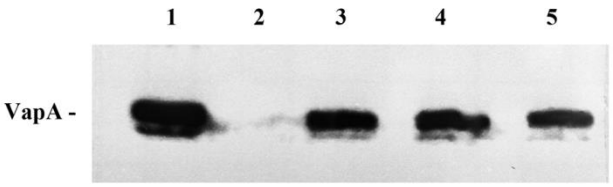
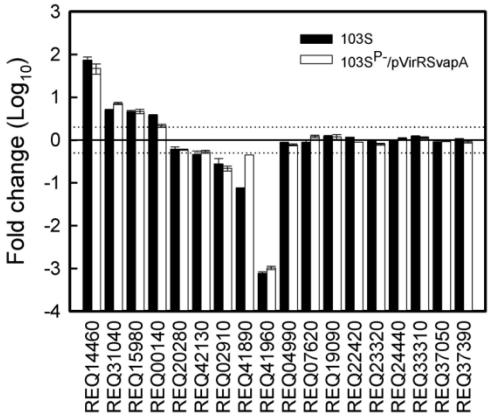
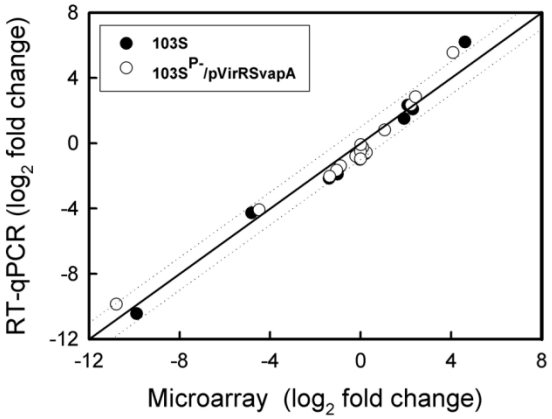


Figure S5.

A



B



C

Gene	Description	Change in transcript level
REQ14460	Sigma-70 factor	Upregulated
REQ31040	Two component system sensor kinase KpdD	Upregulated
REQ15980	LuxR family transcriptional regulator	Upregulated
REQ00140	TetR family transcriptional regulator	Upregulated
REQ20280	Rok family transcriptional regulator	Downregulated
REQ42130	MarR family transcriptional regulator	Downregulated
REQ02910	MarR family transcriptional regulator	Downregulated
REQ41890	GntR family transcriptional regulator	Downregulated
REQ41960	IclR family transcriptional regulator	Downregulated
REQ04990	Short chain dehydrogenase	Unchanged
REQ07620	alpha/beta hydrolase	Unchanged
REQ19090	TetR family transcriptional regulator	Unchanged
REQ22420	TetR family transcriptional regulator	Unchanged
REQ23320	Transcriptional regulator	Unchanged
REQ24440	MerR family transcriptional regulator	Unchanged
REQ33310	Preprotein translocase SecA	Unchanged
REQ37050	Naphthoate synthase	Unchanged
REQ37390	MerR family transcriptional regulator	Unchanged

SUPPLEMENTARY REFERENCES

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117