Horizontal spread of Rhodococcus equi macrolide resistance plasmid pRErm46 across environmental Actinobacteria

Horizontal spread of *Rhodococcus equi* macrolide resistance plasmid pRErm46 across environmental *Actinobacteria*

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Keywords: *R. equi*, conjugation, pRErm46, *erm*(46), soil, fitness

ABSTRACT

Conjugation is one of the main mechanisms involved in the spread and maintenance of antibiotic resistance in bacterial populations. We recently showed that the emerging macrolide resistance in the soil-borne equine and zoonotic pathogen *Rhodococcus equi* is conferred by the *erm*(46) gene carried on the 87-kb conjugative plasmid pRErm46. Here we investigated the conjugal transferability of pRErm46 to 14 representative bacteria likely encountered by *R. equi* in the environmental habitat. *In vitro* mating experiments demonstrated conjugation to different members of the genus *Rhodococcus* as well as to *Nocardia* and *Arthrobacter* spp. at frequencies ranging from ~10^{-2} to 10^{-6}. pRErm46 transfer was also observed in mating experiments in soil and horse manure, albeit at a low frequency and after prolonged incubation at 22-30ºC (environmental temperatures), not 37ºC. All transcojugants were able to transfer pRErm46 back to *R. equi*. Conjugation could not be detected with *Mycobacterium* and *Corynebacterium* spp. or several members of the more distant phylum *Firmicutes* such as *Enterococcus*, *Streptococcus* or *Staphylococcus*. Thus, pRErm46 host range appears to span several actinobacterial orders with certain host restriction within the *Corynebacteriales*. All bacterial species that acquired pRErm46 expressed increased macrolide resistance with no significant deleterious impact on fitness, except in the case of *Rhodococcus rhodnii*. Our results indicate that actinobacterial members of the environmental microbiota can both acquire and transmit the *R. equi* pRErm46 plasmid and thus potentially contribute to the maintenance and spread of *erm*(46)-mediated macrolide resistance in equine farms.

IMPORTANCE

This study demonstrates the efficient horizontal transfer of the *R. equi* conjugative plasmid pRErm46, recently identified as the cause of the emerging macrolide resistance among equine isolates of this pathogen, to and from different environmental *Actinobacteria* including a variety of rhodocci as well as *Nocardia* and *Arhtrobacter* spp. The reported data support the notion that environmental microbiota may act as reservoirs for the endemic maintenance of antimicrobial resistance in an antibiotic pressurized farm habitat.

INTRODUCTION

Rhodococcus spp. are present in diverse environments owing to their unique capacity for niche adaptation [1-3]. Much of their environmental plasticity relies on extrachromosomal genetic elements of circular or linear topology that carry key niche-adaptive traits[4, 5]. While environmental *Rhodococcus* spp. typically harbor plasmids encoding catabolic pathways[3, 6], *R. equi*, the only animal pathogen of this genus, carries the pVAP virulence plasmids essential for pathogenesis and survival in host macrophages[5, 7]. Three pVAP plasmid types have been described so far, each adapted to a specific animal host: the equine-associated pVAPA, porcine-associated pVAPB and ruminant-associated pVAPN[8-10]. Although *R. equi* can infect a variety of animal species including humans, young foals are particularly susceptible and develop a severe respiratory disease characterized by focal purulent brochopneumonia[11, 12].

R. equi is a ubiquitous soil organism that becomes endemic in horse breeding farms where it causes high morbidity and mortality in foals [12, 13]. Due to the lack of an effective vaccine and the insidious nature of the initial stages of the infection, many farms rely on thoracic ultrasonographic screening followed by antibiotic treatment of foals presenting subclinical lung lesions[11]. Field studies indicate, however, that this practice may constitute an example of unjustified antibiotic misuse because many subclinically infected foals would spontaneously recover independently of antibiotic prophylaxis [14, 15]. Not surprisingly, the inception of the mass antibioprophylaxis in 2001 has resulted a few years later in the emergence of resistance to the antimicrobials used in such treatments, a combination of a macrolide and rifampin [13, 16-18]. The emergence of this dual resistance is problematic because only a few antimicrobials are clinically effective to combat rhodococcal foal pneumonia and the macrolide-rifampin combination remains the mainstay of antimicrobial therapy against equine *R. equi*

infection [19].The macrolide-rifampin resistance emerged upon horizontal transfer of a novel rRNA methylase gene, *erm*(46), which confers resistance to macrolides, lincosamides and streptogrnim B (MLS_B) [20], to a specific *R. equi* strain carrying a novel chromosomal *rpoB* mutation (S531F), which then gave rise to a clonal population[21].

We recently reported that *erm*(46) is acquired and mobilized as part of a conjugative plasmid, pRErm46 [20, 21]. This macrolide resistance plasmid has so far only been identified in equine (pVAPA positive) *R. equi* isolates, likely as a result of the selective pressure exerted by the mass antimicrobial treatments systematically applied to control foal pneumonia in endemic farms. Whether pRErm46 can also be maintained and spread by *R. equi* bacteria carrying the porcine-associated (pVAPB) or ruminant-associated (pVAPN) virulence plasmid types is not known. Homology analysis of the coding sequences of pRErm46's replicon indicated an actinobacterial origin, particularly rhodococcal, but whether pRErm46 can actually be mobilized and replicate in other *Actinobacteria* remains also to be determined. The purpose of this study was to explore the host range of pRErm46 and elucidate whether other members of the environmental microbiota can act as its reservoir in the absence of *R. equi*, thus potentially contributing to the maintenance and perpetuation of the macrolide resistance determinant in the farm habitat.

RESULTS

1. pRErm46 can be self-transferred to and maintained by different actinobacterial species

In a first series of experiments, mating assays were carried out using macrolide-resistant strain $103^{+}Apra^{R}$ (pRErm46) as a donor and porcine clinical isolate REPB1 Rmp^{R} (pVAPB positive) and bovine isolate REPN1 Rmp^{R} (pVAPN-positive) as recipient strains (see Table 6).

pRErm46 was mobilized to the *R. equi* porcine and bovine isolates at conjugation frequencies of ~10^{-5} and ~10^{-4} respectively (Table 1). The presence of the *R. equi* virulence plasmids (pVAPB and pVAPN) and macrolide resistance plasmid pRErm46 was verified by PCR in the dual resistant (to erythromycin [Erm^R] and rifampin [Rmp^R]) trasnconjugant colonies. The porcine isolate transconjugants were confirmed to still carry pVAPB in addition to the pRErm46 macrolide resistance plasmid. However, none of the bovine isolates that received pRErm46 kept the pVAPN virulence plasmid. Plasmid incompatibilities are based on similarities in the origin of replication of the amplicons, whereby competition for replication factors favors plasmids which, due e.g. to smaller size (as would be the case of the 87-kb pRErm46 vs the 120-kb pVAPN), have a faster replication [22, 23]. We searched the pVAPN putative origin of replication sequence 5′-AAAACCCCCAGGTGGGGGTGGGTTTT [9] in the pRErm46 DNA sequence using Blast and we identified a 33-nt segment (5- AAAACCCCCAGCCATGCGGGGCT GAGGGTTTCT) upstream the open reading frame (ORF) PRERM_0270 (23985-24018 bp) that shared 25 of the 27 nt of pVAPN's sequence (Figure S1). No such sequence was identified in the replicon of the pVAPA/B plasmids.

We next explored the host range of pRErm46 by performing bacterial conjugation assays using the same donor and 14 representative bacterial species including six non-*equi Rhodococcus* spp. (*Rhodococcus defluvii* DSM45893, *Rhodococcus fascians* DSM20669, *Rhodococcus rhodochrous* JCM2156, *Rhodococcus erythropolis* JCM2892, *Rhodococcus ruber* JCM3205, *Rhodococcus rhodnii* JCM3203); five different *Actinobacteria* (*Corynebacterium pseudotuberculosis*, *Mycobacterium smegmatis* MKD8, *Mycobacterium fortuitum* and *Nocardia globerula* ATCC21505 from the Order *Corynebacteriales, Arthrobacter paraffineus* ATCC19958 from the Order *Micrococcales*); and three species from the more distant Phylum

Firmicutes (*Enterococcus faecalis* ATCC29212, an equine field *Streptococcus zooepidermicus* subsp. *equi* isolate and *Staphylococcus aureus* ATCC29213). All *Rhodococcus* species successfully acquired pRErm46 at varying transfer frequencies (Table 1). Similarly, pRErm46 was conjugally transferred to *N. goberula* and *A. paraffineus* at comparable ratios to *Rhodococcus* spp. (Table 1). In contrast, transfer of pRErm46 could not be detected to the tested mycobacteria, *C. pseudotuberculosis* and the non-actinobacterial species. pRErm46 transconjugants were subsequently used as donors in conjugation assays with *R. equi* recipients. Notably, all primary recipients of pRErm46 were able to mobilize the plasmid back to macrolide susceptible *R. equi* at similar transfer frequencies (Table 1).

The macrolide resistance phenotype of five randomly selected transconjugant colonies per recipient bacteria from the *in vitro* mating assays with the *R. equi* $103^{+}Apra^{R}$ (pRErm46) donor (see above) was evaluated by susceptibility testing using eTest strips. Minimum inhibitory concentrations (MIC) to macrolides were tested before and after acquisition of pRErm46. The MIC of recipients prior and after gaining pRErm46 were 0.5-8 μg/ml and ≥ 256 μg/ml, respectively, for all isolates. The only exception was *R. fascians* in which the MIC increased from 0.19-0.38 μg/ml to 8 -12 μg/ml (Table 2).

2. Transfer of pRErm46 in soil and manure

To assess whether pRErm46 transfer can take place in conditions approximating the equine farm habitat, bacterial mating experiments were performed in soil and horse manure. Macrolide and rifampin resistant equine clinical isolate PAM2287[21] (prototype strain of the pRErm46-harboring *R. equi* clone) was used as pRErm46 donor and susceptible (pRERrm46-negative) avirulent *R. equi* 103^{-} with an apramycin resistance *aac(3)IV* cassette [$103^{-}Apra^{R}$][24]

as the recipient in the mating assay, enabling transconjugant selection via dual resistance to erythromycin (Erm^R) and apramycin ($Apra^R$). Three different conjugation ratios (1:1, 1:10 and 10:1), and four different temperatures (4°C, 22°C, 30°C and 37°C) to mimic seasonal temperature changes, were tested. Soil and manure samples were screened for conjugation at 7, 30, 90 and 180 days. pRErm46 transfer in both soil and horse manure was sporadically observed after 30 days incubation at 22˚C and 30˚C, independently of the donor:recipient ratio (Table 3). Transconjugants were detected in all cases, although mostly in only one of the triplicate soil samples per time point and tested temperature. Calculated transfer ratios ranged from 10^{-6} to 10^{-2} transconjugants/recipient bacteria. For confirmation, the presence of the $Apra^R$ cassette, *erm*(46) gene, pRErm46, in addition to the virulence plasmid pVAPA, was tested by PCR in up to 10 (depending on transconjugant numbers) Ery^R and $Apra^R$ colonies. All transconjugants screened by PCR carried the the *aac(3)IV* ($Apra^R$) cassette and the *erm*(46) (Erm^R) gene, providing molecular confirmation of the transconjugant phenotype. pRErm46 was detected by PCR in all transconjugants tested except for the those isolated after 90 days at 30˚C with a 10:1 donor:recipient ratio, despite these testing positive for the *erm*(46) gene. Interestingly, these transconjugants are those in which pVAPA acquisition had been detected in our experiments. Based on these data, optimal environmental mating conditions appear to be 22˚C-30˚C for a length of between 8 to 30 days.

Under the above optimal conditions for pRErm46 environmental conjugation, we used the same assay to test the mobilization of pRErm46 from *R. equi* to *R. erythropolis* (as a representative of the *Rhodococcus* genus), *N. globerula* and *A. paraffineus* in soil (Table 4). The three species successfully acquired pRErm46 after 15 days. All soil samples tested contained transconjugant bacteria, with *N. globerula* showing the highest transfer ratio ranging from 10^{-1} to

143 10^{-4} transconjugants/recipient bacteria. The mobilization of pRErm46 to *R. erythropolis* occurred
144 at similar frequencies to those observed for *R. equi* in soil and ranged between 10^{-5} and 10^{-7}
145 transconjugants/recipient bacteria, while slightly lower transfer ratios were observed using *A.*
146 *parraffineus* as recipient (10^{-6}-10^{-9} transconjugants/recipient).

147

148 **3. pRErm46 fitness cost varies in different recipients.**

149 To gain further insight into the determinants of pRErm46 maintenance, we measured the
150 impact of pRErm46-mediated macrolide resistance on bacterial fitness. *R. erythroplis A.*
151 *paraffineus* and *N. globerula* showed no significant differences in exponential growth rate and
152 maximum growth upon acquisition of pRErm46 neither in complex medium (BHI) nor *R. equi*
153 chemically defined medium (mREMM) (Table 5, Fig. 1). Surprisingly, some of the tested strains
154 (*R. fascians* and *R. rhodnii*) even showed significantly improved fitness in mREMM medium
155 when carrying pRERm46 (Table 5). This was particularly evident with *R. fascians* which failed
156 to grow in mREMM in the absence, but not presence, of pRErm46, suggesting the intriguing
157 possibility that some plasmid-encoded determinants may contribute to regulate bacterial growth
158 in nutrient-limiting conditions. The only detrimental effect of pRErm46 on bacterial fitness was
159 observed with *R. rhodnii*, which when harboring pRErm46 manifested slightly impaired growth
160 (Table 5, Fig 5).

161

162 **DISCUSION**

163 Horse breeding farms that use macrolides and rifampin as a mass treatment for *R. equi*
164 subclinical pneumonia likely represent highly antibiotic-pressurized environments [17, 25],
165 where bacterial survival is contingent upon acquisition of a suitable resistant phenotype. This

166 study explores how the horizontal transfer dynamics of the *R. equi* MLS_B resistance plasmid
167 pRErm46 to indigenous members of the environmental microbiota may contribute to the
168 maintenance of a resistant microbial pool in equine farms. pRErm46 was successfully mobilized
169 *in vitro* to six different *Rhodococcus* spp. as well as to *N. globerula.* This was unsurprising as
170 most of the genes of the pRErm46 genetic backbone have an obvious rhodococcal origin[21] and
171 because phylogenetic evidence supports that *Nocardia* is the closest *Corynebacteriales* genus to
172 *Rhodococcus* [26, 27]. The fact that no conjugal transfer could be detected with *Firmicutes*
173 species nor representative species of other *Corynebacteriales* such as *Corybebacterium* or
174 *Mycobacterium* while it was observed with the *Actinomycetales* species *A. paraffineus* suggests
175 that pRErm46's host rage is essentially actinobacterial while at the same time exhibits genus-
176 specific restrictions. Further work is required with additional representative bacteria commonly
177 found in the equine farm habitat or equine microbiome to more specifically delineate the
178 conjugal transfer range of the *R. equi* macrolide resistance determinant and the potential
179 mechanisms underpinning its persistence in equine farms.

180 *erm*(46)-driven macrolide resistance has only been reported in *R. equi* equine isolates
181 carrying the pVAPA virulence plasmid [21]. This study shows that pRErm46 can be also
182 acquired by *R. equi* carrying the pVAPB porcine plasmid, thus indicating that porcine
183 environments/isolates may also theoretically contribute to the spread and maintenance of
184 *erm*(46)-mediated *R. equi* macrolide resistance. This might not to be the case for ruminant-
185 associated *R. equi* isolates based on the observed potential incompatibility of the pRERm46 and
186 pVAPN replicons. Our findings with the pVAPN plasmid imply that differences in compatibility
187 among plasmids, typically abundant among soil-dwelling *Actinobacteria*, may be a critical factor
188 in shaping pRErm46's host range.

Despite the ease with which pRErm46 is conjugally transferred between *R. equi* isolates [21, 28], and to other rhodococci or other susceptible *Actinobacteria* as shown here, the MLS_B resistance determinant *erm*(46) has until now only been found in a specific *R. equi* clonal population[21]. This may be explained by the requirement of a strong antibiotic selective pressure for the maintenance of pRErm46[21], apart from the fact that no systematic searches have been undertaken to detect pRErm46 in the environmental microbiome. In addition, our *in vitro* data may not be an accurate reflection of what occurs in the farm environment. To approximate such conditions, we carried out mating assays in soil and horse manure, at four different incubation temperatures to mimic seasonal changes. Despite using a large conjugation mix of ~10^7 CFU/g of soil (100 times higher than the concentrations at which *R. equi* is typically found in soil in endemic farms [16]), conjugation remained sporadic but consistently detectable after 8 to 30 days at temperatures of 22-30˚C, independently of the donor:recipient ratio used. These results demonstrate that conjugal transfer of the *R. equi* macrolide resistance plasmid can occur in the equine farm environment, potentially contributing to its spread and endemicity.

Interestingly, in the soil experiments we noted that all transconjugant colonies that resulted from the 10:1 (donor:recipient) matings after a 90 day incubation at 30˚C had acquired both the *erm*(46) gene and the pVAPA virulence amplicon while pRErm46's transfer was not detected. *erm*(46) is actually carried within a highly mobile transposon (Tn*RErm46*) harbored by pRErm46, from which we previously found it readily transposes to the *R. equi* genome including the virulence plasmid[21]. We therefore assume that the observed pRErm46-independent transfer of *erm*(46)-mediated macrolide resistance reflects the co-option of the transfer functions of an indigenous mobile element (most likely pVAPA, but potentially other extrachromosomal elements present in the microbiota present in the soil sample tested as well). This finding

highlights the extraordinary horizontal spread potential of the *erm*(46) determinant via the transposition functions of the highly mobile Tn*RErm46* element[21].

Finally, we explored potential bacterial fitness costs associated with the acquisition of the pRErm46 plasmid by components of the environmental microbiota. Similar to what we had previously observed in *R. equi* [21], pRErm46 showed a neutral effect on fitness *in vitro*, or even promoted higher growth rates than the corresponding isogenic strains lacking the plasmid, in all bacteria species tested excepting *R. rhodnii*. Although *in vitro* fitness assays may not accurately reflect the growth dynamics of bacteria in soil and manure, our results indicate that under suitable conditions environmental bacteria could serve as a potential reservoir for pRErm46 and, hence, MLS_B resistance, in the absence of the primary host organism organism *R. equi* (and even antibiotic selective pressure).

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 6. *R. equi* isolates were routinely cultured in Brain-Heart Infusion medium (BHI, Difco Laboratories-BD) at 30°C, 200 rpm, unless otherwise stated. Agar media were prepared by adding 1.6 % of bacteriological agar (Oxoid). Media were supplemented with antibiotics (erythromycin 10 μg/ml, rifampin 100 μg/ml, apramycin 50 μg/ml; Sigma) whenever required.

Rifampin resistant strain derivation. Prior to the mating experiments, rifampin resistant (Rmp^R) derivatives of recipient species were obtained as previouisly described [9]for selection of transconjugants by double antimicrobial resistance (Erm^R and rifampin Rmp^R). Briefly, several well-isolated colonies were collected, resuspended in PBS and streaked onto a BHI plate supplemented with 25 μg/ml of rifampin. After incubation for 48 h at 30°C, the Rmp^R phenotype was selected and stabilized by restreaking a few colonies in a fresh BHI plate supplemented with 100 μg/ml rifampin.

***In vitro* bacterial conjugation assay**. Conjugation assays were carried out as described in Anastasi et al. 2015[28]. *R equi* donor and recipient strains were grown overnight in BHI (in the presence of antibiotic when required), washed twice with PBS and adjusted to OD_{600} = 1. Then, 100 μl of donor suspension were mixed with 100 μl of the recipient 1:1 in a microtube. The mixture was centrifuged (6000 rpm, 10 min), resuspended in 5 μl of sterile BHI and spotted in a thick drop onto a BHI plate. After 72 h incubation at 30°C, the bacterial mixture was scraped and resuspended in PBS, and serial dilutions plated onto BHI agar supplemented with rifampin (recipient selection) or rifampin plus erythromycin (trasnconjugant selection). Transconjugants

were confirmed by PCR. Conjugation ratios were calculated using the following formula: Conjugation ratio = n. of transconjugant cell/ n. recipient cells

Conjugation assay in soil and horse manure. Bacterial mating assays were performed in soil and horse manure (collected from University of Georgia teaching farm) in parallel. For each time point and condition, 3g of soil/manure were placed in three 5ml test tubes (1gr/tube) and inoculated with 10^7 CFU/g containing 10:1, 1:1 or 1:10 donor:recipient ratios. Then, soil/manure was stirred during 30 min for an even bacterial distribution and incubated at four different temperatures 4˚, 22˚, 30˚ and 37℃ for up to 180 days. The presence of macrolide mobilization was checked at 5 time points: Day 0 [control], 7, 30, 90 and 180. For each time point and condition, 3 g of soil/manure (coming from 3 independent test tubes) were quantitatively cultured by serial 10-fold dilutions on *R. equi* selective NANAT[29] supplemented with corresponding antibiotics for transconjugant and recipient bacteria selection. < 10 transconjugant colonies were confirmed by PCR (S2). Conjugation ratios were calculated as stated above.

Polymerase chain reaction. PCRs were carried out using C100 thermal cycler (Bio-Rad) and GoTaq® Flexi DNA Polymerase (Promega) following general parameters.

DNA sequencing and analysis: Sanger sequencing was performed by Eurofins (Louisville, Kentucky). Sequences were analyzed using Ape plasmid editor (Wayne Davis) and the Basic Local Alignment Search Tool (BLAST www.ncbi.nlm.nih.gov/BLAST/) was used for the alignment of DNA sequences against the reference sequence.

Antimicrobial susceptibility testing. Bacteria were prepared from overnight cultures in tryptic soy agar (TSA) by the direct colony suspension method according to the guidelines established by the CLSI, resulting in the recommended inoculum of ~ 1 to 5 × 10^5 CFU as verified by colony counting. The MICs of erythromycin were determined by use of ETEST® strips (bioMerieux).

Bacterial growth assays. Potential bacterial fitness costs associated with the acquisition of the pRErm46 were investigated by monitoring the growth rate of macrolide -susceptible and -resistant isogenic strains (that received pRErm46 by conjugation) in complex media BHI and in chemically defined medium mREMM[21],24. Bacteria were grown overnight in BHI, washed twice with PBS and adjusted to OD_{600} = 1. Then. 400 µl/well of bacteria in the selected media were added in triplicate to 48-well plates (Corning). The assays were conducted using an automated plate reader (Synergy HT, BioTeK) at 37° C, at 200rpm. Measurements were taken every 30 min. Data were analyzed using BioTeK Gen5 Data Analysis Software (BioTeK). Data were processed by biological growth curve fitting package "growthrates" [30] in statistical software R (version 3.6.1). Growth parameters (i) exponential growth and (ii) maximal bacterial yield were analyzed for significant differences using paired t-test also in R (version 3.6.1).

ACKNOWLEDGEMENTS

We would like to acknowledge Dr. Mary Hondalus for providing *N. globerula*, *A. paraffineus, M.fortuitum, R. equi* 103^+Apra^R and *R. equi* 103^-Apra^R. This work was supported by the Morris Animal Foundation (Grant # D18EQ-401) and the Hodgson Chair of Equine Research, University of Georgia. JV-B thanks the financial support of the Horserace Betting Levy Board (grants vet/prj 764/70 and 796) and Grayson-Jockey Club Research Foundation.

AUTHOR CONTRIBUTIONS

Álvarez–Narváez, S.: Manuscript writing, experimental design, data analysis and research implementation.

Giguere, S.: Experimental design and data analysis.

Berghaus, L.J.: Antimicrobial susceptibility testing, help in manuscript preparation.

Dailey C.: Data analysis.

Vázquez-Boland J.A.: Experimental design, conceptualization, data analysis and manuscript writing.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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TABLE 1. *R. equi* pRErm46 heterologous conjugation experiments.

Donor	Recipient	Transfer frequency[a]
R. equi 103S (pRErm46)	*R. equi* porcine isolate (pVAPB)	$9.14 \pm 6.98 \times 10^{-5}$
	R. equi bovine isolate (pVAPN)	$4.41 \pm 1.42 \times 10^{-4}$
	R. defluvii	$1.62 \pm .85 \times 10^{-5}$
	R. erythropolis	$2.49 \pm 1.12 \times 10^{-5}$
	R. rhodochrous	$4.31 \pm 2.91 \times 10^{-2}$
	R. rhodnii	$7.51 \pm 6.94 \times 10^{-4}$
	R. ruber	$3.39 \pm 1.55 \times 10^{-5}$
	R. fascians	$1.6 \pm .28 \times 10^{-7}$
	N. globerula	$7.9 \pm 5.8 \times 10^{-3}$
	A. paraffineus	$5.1 \pm 3.8 \times 10^{-5}$
	M. smegmatis	$< 10^{-10}$
	M. fortuitum	$< 10^{-10}$
	C. pseudotuberculosis	$< 10^{-10}$
	S. aureus	$< 10^{-10}$
	S. zooepidemicus	$< 10^{-10}$
	E. faecalis	$<10^{-10}$
R. equi porcine isolate (pVAPB, pRErm46)	Susceptible *R. equi* 103$^-$	$4.09\pm 2.37 \times 10^{-6}$
R. equi bovine isolate (pRErm46)		$1.46 \pm .62 \times 10^{-5}$
R. defluvii (pRErm46)		$8.47 \pm 4.60 \times 10^{-6}$
R. erythropolis (pRErm46)		$1.95 \pm 1.12 \times 10^{-5}$
R. rhodochrous (pRErm46)		$5.97 \pm 2.85 \times 10^{-5}$
R. rhodnii (pRErm46)		$2.71 \pm 2.21 \times 10^{-5}$
R. ruber (pRErm46)		$2.68 \pm 2.67 \times 10^{-6}$
R. fascians (pRErm46)		$1.11 \pm .39 \times 10^{-7}$
N. globerula (pRErm46)		$1.18 \pm .66 \times 10^{-5}$
A. paraffineus (RErm46)		$1.23 \pm 1.22 \times 10^{-5}$

[a] Number of transconjugant/recipient CFU. Data represent means ± SD (n = 3 experiments).

TABLE 2. Erythromycin MICs of different bacteria upon *erm*(46) conjugal acquisition. Data refers to MIC in mg/L determined using Etest (n = 3).

Species	***erm*(46) negative**	***erm*(46) positive**
R. equi porcine isolate	0.25 - 0.5	>256
R. equi bovine isolate	0.5	>256
R. defluvii	6 - 8	>256
R. erythropolis	0.25 - 0.38	>256
R. fascians	0.19 - 0.38	8 - 12
R. rhodnii	0.38 - 0.75	>256
R. rhodochrous	3 - 4	>256
R. ruber	0.64	>256
A. paraffineus	0.50 - 0.75	>256
N. globerula	0.38 - 1	>256

TABLE 3. Conjugal transfer of pRErm46 in soil and horse manure.

Sample	Ratio[a]	Temperature	day 30			day 90			day 180		
			sample1	sample2	sample3	sample1	sample2	sample3	sample1	sample2	sample3
Soil	1:1	4°C	-	-	-	-	-	-	-	-	-
		22°C	-	-	-	-	2.56×10^{-4}	-	-	-	-
		30°C	-	-	-	-	-	-	-	-	-
		37°C	-	-	-	-	-	-	-	-	-
	1:10	4°C	-	-	-	-	-	-	-	-	-
		22°C	9.43×10^{-6}	1.58×10^{-6}	2.38×10^{-5}	-	-	-		-	1.14×10^{-4}
		30°C	-	-	-	-	-	-	-	-	-
		37°C	-	-	-	-	-	-	-	-	-
	10:1	4°C	-	-	-	-	-	-	-	-	-
		22°C	-	-	-	-	-	-	6.25×10^{-4}	-	-
		30°C	-	-	-	-	-	$*5.10 \times 10^{-2}$	1.14×10^{-2}	-	-
		37°C	-	-	-	-	-	-	-	-	-
Manure	1:1	4°C	-	-	-	-	-	-	-	-	-
		22°C	-	2.11×10^{-2}	-	-	-	-	-	-	-
		30°C	-	2.21×10^{-5}	-	-	-	-	-	-	-
		37°C	-	-	-	-	-	-	-	-	-
	1:10	4°C	-	-	-	-	-	-	-	-	-
		22°C	-	-	-	-	-	-	-	-	-
		30°C	-	-	-	-	1.25×10^{-5}	-	-	2.21×10^{-5}	-
		37°C	-	-	-	-	-	-	-	-	-
	10:1	4°C	-	-	-	-	-	-	-	-	-
		22°C	-	-	-	-	-	-	-	-	-
		30°C	-	-	-	-	-	-	-	-	-
		37°C	-	-	-	-	-	-	-	-	-

Data represents conjugation transfer frequencies per sample of soil/horse manure. 3 samples were collected each time point and incubation temperature.

[a] Ratio refers to the ratio donor:recipient bacteria used in each conjugation assay

TABLE 4. pRErm46 transfer from *R. equi* to other species in soil.

Species	Ratio[a]	Temperature	day 15	day 30
R. erythropolis	1:1	22°C	$2.43 \pm 2.02 \times 10^{-5}$	$3.32 \pm 1.99 \times 10^{-5}$
		30°C	$9.66 \pm 5.69 \times 10^{-6}$	$3.05 \pm 1.46 \times 10^{-6}$
	1:10	22°C	$1.24 \pm .40 \times 10^{-6}$	$1.29 \pm .97 \times 10^{-7}$
		30°C	$1.91 \pm 1.06 \times 10^{-5}$	$6.29 \pm 2.20 \times 10^{-6}$
A. paraffineus	1:1	22°C	$9.98 \pm 9.98 \times 10^{-9}$	$1.66 \pm .98 \times 10^{-7}$
		30°C	$2.78 \pm 1.15 \times 10^{-6}$	$2.18 \pm 1.55 \times 10^{-6}$
	1:10	22°C	$5.61 \pm 6.47 \times 10^{-8}$	$3.63 \pm 1.98 \times 10^{-7}$
		30°C	$4.94 \pm 2.39 \times 10^{-7}$	$9.07 \pm 6.67 \times 10^{-7}$
N. globerula	1:1	22°C	$1.39 \pm .81 \times 10^{-1}$	$2.32 \pm 2.07 \times 10^{-2}$
		30°C	$5.62 \pm 2.22 \times 10^{-1}$	$0.97 \pm 1.19 \times 10^{-3}$
	1:10	22°C	$9.38 \pm 8.25 \times 10^{-3}$	$4.82 \pm 2.37 \times 10^{-4}$
		30°C	$6.06 \pm 3.47 \times 10^{-2}$	$8.33 \pm 8.33 \times 10^{-3}$

Data represent means ± SDs of 3 samples collected per time point and incubation temperature.

[a] Ratio refers to the ratio donor:recipient bacteria used in each conjugation assay.

TABLE 5. Growth data of isogenic bacteria in the presence and absence of the macrolide resistance plasmid pRErm46 in BHI and chemically defined medium. The maximum growth rate during exponential growth and maximal bacterial growth during the growth curve were estimated from fits of the OD600 values using Growrates package in R. Asterisk represents significant (*P<0.05*) increase based on paired t-test statistical analysis (n=3).

(A) BHI

	Maximum Exponential Growth Rate (h^{-1})		Maximal Bacterial Growth (OD600)	
	pRErm46	No pRErm46 -	pRErm46	No pRErm46
R. erythropolis	0.24 (± .007)	0.24 (± .009)	1.60 (± .05)	1.59 (± .05)
R. fascians	0.17 (± .02)	0.21 (± .02)*	0.71 (± .20)	0.63 (± .14)
R. rhodnii	0.13 (± .002)*	0.11 (± .004)	0.69 (± .03)	1.07 (± .03)*
A. paraffineus	0.26(± .06)	0.26 (± .02)	1.35 (± .03)	1.42 (± .03)
N. globerula	0.12 (± .01)	0.14 (± .02)	2.07 (± .29)	2.04 (± .28)

(B) mREMM

	Maximum Exponential Growth Rate (h^{-1})		Maximal Bacterial Growth (OD600)	
	pRErm46	No pRErm46	pRErm46	No pRErm46
R. erythropolis	0.22 (± .02)	0.22 (±.02)	0.36(± .006)	0.36 (±.01)
R. fascians	0.39 (± .12)*	0.005 (± .004)	0.54 (± .06)*	0.02 (± .02)
R. rhodnii	0.96 (± .01)*	0.86 (±.003)	0.46(± .008)*	0.42 (±.003)
A. paraffineus	0.46(± .21)	0.41 (± .07)	0.36 (± .04)	0.38 (± .01)
N. globerula	0.23 (± .06)	0.26 (±.06)	0.43 (± .05)	0.44 (±.05)

TABLE 6. Bacterial strains used in this study.

Species	Description	Source
Rhodococcus equi		
103^{-} ApraR	Plasmidless 103 strain containing the *aac(3)IV* apramycin resistance gene integrated on the chromosome	Tripathi *et al.* 2012
PAM2287	Macrolide and Rifampin clinical isolate	Alvarez *et al.* 2019
103^{+}ApraR, pRERM46	Derivative strain from 103- ApraR. pRERM46 and pVAPA plasmids introduced by conjugal transfer	Giguère collection
REPB1	Rifampin resistant derivative strain from porcine clinical isolate	This study
REPN1	Rifampin resistant derivative strain from bovine clinical isoalte	This study
Rhodococcus spp		
R. defluvii RmpR	Rifampin resistant derivative strain from *R. defluvii DSM45893*	This study
R. erythropolis RmpR	Rifampin resistant derivative strain from *R. erythropolis* JCM 2892	This study
R. fascians RmpR	Rifampin resistant derivative strain from *R. fascians*DSM20669	This study
R. rhodnii RmpR	Rifampin resistant derivative strain from *R. rhodnii* JCM 3203	This study
R. rhodochrous RmpR	Rifampin resistant derivative strain from *R. rhodochrous* JCM 2156	This study
R. ruber RmpR	Rifampin resistant derivative strain from *R. ruber* JCM 3205	This study
Other bacteria		
Nocardia globerula RmpR	Rifampin resistant derivative strain from *N. globerula* ATCC 21505	This study
Arthrobacter paraffineus RmpR	Rifampin resistant derivative strain from *A. paraffineus* ATCC19958	This study
Mycobacterium smegmatis RmpR	Rifampin resistant derivative strain from *M. smegmatis* MKD8	This study
Mycobacterium fortuitum RmpR	Rifampin resistant derivative strain from *M. fortuitum* from Hondalus strain collection	This study
Staphilococcus aureus RmpR	Rifampin resistant derivative strain from *S.aureus* ATCC 29213	This study
Enterococcus faecalis RmpR	Rifampin resistant derivative strain from *E. faecalis* ATCC 29212	This study
Streptococcus equi subspecies zooepidemicus RmpR	Rifampin resistant derivative strain from *S. zooepidermicus* wild-type clinical isolate (from Giguère strain collection)	This study

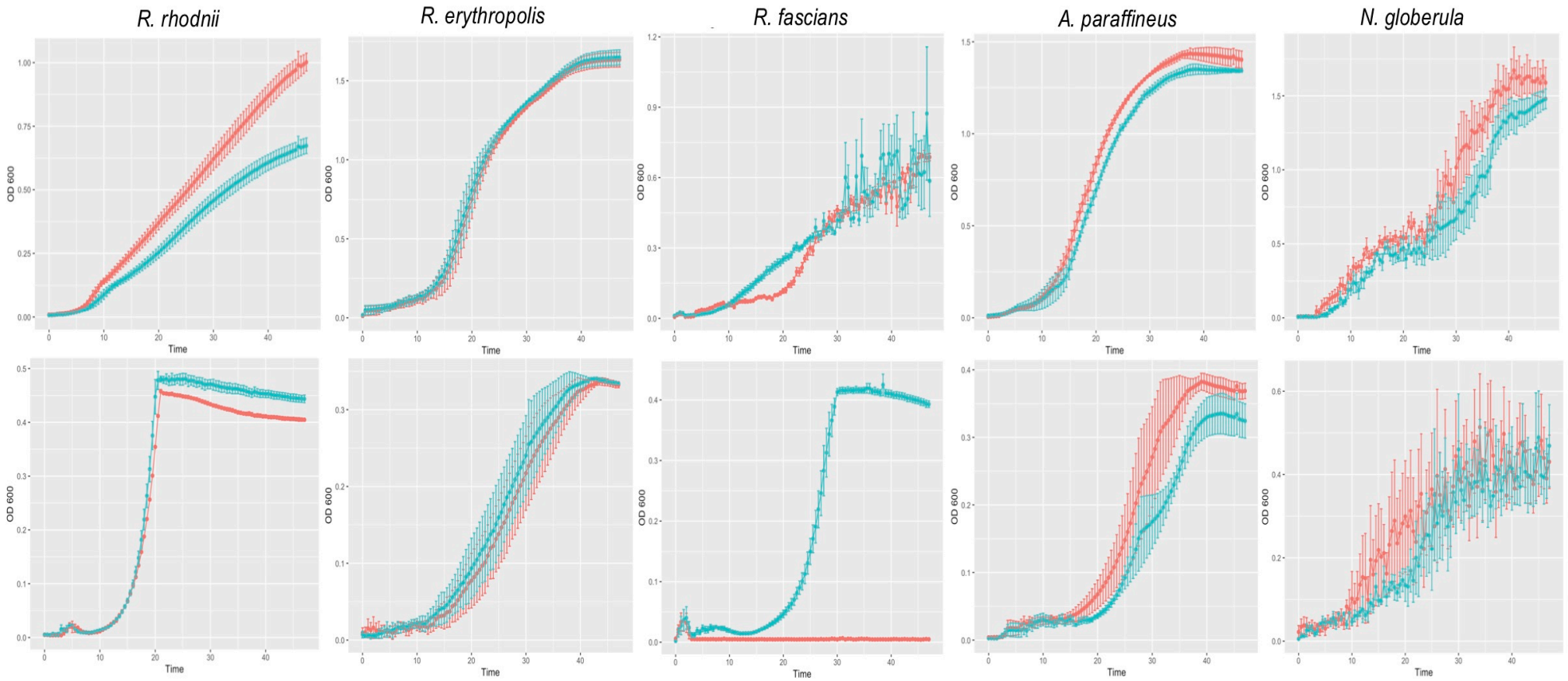

Figure 1. Acquisition of pRErm46 has a different fitness cost depending on the recipient species. pRErm46 was conjugally transferred from *R. equi* to (i) *R. rhodnii*, (ii) *R. erythropolis*, (iii) *R. fascians* (iv) *A. paraffineus* and (v) *N. globerula*. Growth curves with each isogenic set composed by a pRErm46+ (red) and a pRErm46- (turquoise) isolate in rich complex medium (BHI) and chemically defined medium (mREMM, see Materials and Methods).