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Horizontal Spread of *Rhodococcus equi* Macrolide Resistance Plasmid pRErm46 across Environmental *Actinobacteria*

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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 soilborne 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 $\sim 10^{-2}$ 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 to 30°C (environmental temperatures), not 37°C. All transconjugants were able to transfer pRErm46 back to *R. equi*. Conjugation could not be detected with *Mycobacterium* or *Corynebacterium* spp. or several members of the more distant phylum *Firmicutes* such as *Enterococcus*, *Streptococcus*, or *Staphylococcus*. Thus, the 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 *Rhodococcus 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 rhodococci as well as *Nocardia* and *Arthrobacter* spp. The reported data support the notion that environmental microbiotas may act as reservoirs for the endemic maintenance of antimicrobial resistance in an antibiotic pressurized farm habitat.

KEYWORDS *R. equi*, antimicrobial resistance, conjugation, *erm*(46), macrolides, pRErm46, soil

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

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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 bronchopneumonia (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 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 streptogramin 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, pREerm46 (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 farms on which the pathogen is endemic. Whether pREerm46 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 pREerm46's replicon indicated an actinobacterial origin, particularly rhodococcal, but whether pREerm46 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 pREerm46 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

pREerm46 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 $103^{+}Apra^{r}$ (pREerm46) 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 below). pREerm46 was mobilized to the *R. equi* porcine and bovine isolates at conjugation frequencies of $\sim10^{-5}$ and $\sim10^{-4}$, respectively (Table 1). The presence of the *R. equi* virulence plasmids (pVAPB and pVAPN) and macrolide resistance plasmid pREerm46 in the dual-resistant (to erythromycin [Erm^{r}] and rifampin [Rmp^{r}]) transconjugant colonies was verified using PCR. The porcine isolate transconjugants were confirmed to still carry pVAPB in addition to the pREerm46 macrolide resistance plasmid. However, none of the bovine isolates that received pREerm46 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 to, e.g., smaller size (as would be the case of the 87-kb pREerm46 versus the 120-kb pVAPN), have a faster replication (22, 23). We searched the pVAPN putative origin of replication sequence 5′-AAAACCCCCAGGTGGGGGTGGGTTTT (9) in the pREerm46 DNA sequence using BLAST, and we identified a 33-nucleotide (nt) segment (5-AAAACCCCCAGCCATGCGGGGCTGAGGGTTTCT) upstream from the open reading frame (ORF) PRERM_0270 (base pairs 23985 to 24018) that shared 25 of the

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 103S (pRErm46)	*R. equi* bovine isolate (pVAPN)	$4.41 \pm 1.42 \times 10^{-4}$
R. equi 103S (pRErm46)	*R. defluvii*	$1.62 \pm 0.85 \times 10^{-5}$
R. equi 103S (pRErm46)	*R. erythropolis*	$2.49 \pm 1.12 \times 10^{-5}$
R. equi 103S (pRErm46)	*R. rhodochrous*	$4.31 \pm 2.91 \times 10^{-2}$
R. equi 103S (pRErm46)	*R. rhodnii*	$7.51 \pm 6.94 \times 10^{-4}$
R. equi 103S (pRErm46)	*R. ruber*	$3.39 \pm 1.55 \times 10^{-5}$
R. equi 103S (pRErm46)	*R. fascians*	$1.6 \pm 0.28 \times 10^{-7}$
R. equi 103S (pRErm46)	*N. globerula*	$7.9 \pm 5.8 \times 10^{-3}$
R. equi 103S (pRErm46)	*A. paraffineus*	$5.1 \pm 3.8 \times 10^{-5}$
R. equi 103S (pRErm46)	*M. smegmatis*	$<10^{-10}$
R. equi 103S (pRErm46)	*M. fortuitum*	$<10^{-10}$
R. equi 103S (pRErm46)	*C. pseudotuberculosis*	$<10^{-10}$
R. equi 103S (pRErm46)	*S. aureus*	$<10^{-10}$
R. equi 103S (pRErm46)	*Streptococcus zooepidemicus*	$<10^{-10}$
R. equi 103S (pRErm46)	*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)	Susceptible *R. equi* 103−	$1.46 \pm 0.62 \times 10^{-5}$
R. defluvii (pRErm46)	Susceptible *R. equi* 103−	$8.47 \pm 4.60 \times 10^{-6}$
R. erythropolis (pRErm46)	Susceptible *R. equi* 103−	$1.95 \pm 1.12 \times 10^{-5}$
R. rhodochrous (pRErm46)	Susceptible *R. equi* 103−	$5.97 \pm 2.85 \times 10^{-5}$
R. rhodnii (pRErm46)	Susceptible *R. equi* 103−	$2.71 \pm 2.21 \times 10^{-5}$
R. ruber (pRErm46)	Susceptible *R. equi* 103−	$2.68 \pm 2.67 \times 10^{-6}$
R. fascians (pRErm46)	Susceptible *R. equi* 103−	$1.11 \pm 0.39 \times 10^{-7}$
N. globerula (pRErm46)	Susceptible *R. equi* 103−	$1.18 \pm 0.66 \times 10^{-5}$
A. paraffineus (RErm46)	Susceptible *R. equi* 103−	$1.23 \pm 1.22 \times 10^{-5}$

[a]Number of transconjugants/recipient CFU. Data represent means ± SDs ($n = 3$ experiments).

27 nt of pVAPN's sequence (see Fig. S1 in the supplemental material). 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* DSM 45893, *Rhodococcus fascians* DSM 20669, *Rhodococcus rhodochrous* JCM2156, *Rhodococcus erythropolis* JCM2892, *Rhodococcus ruber* JCM3205, and *Rhodococcus rhodnii* JCM3203); four *Actinobacteria* species from the order *Corynebacteriales* (*Corynebacterium pseudotuberculosis*, *Mycobacterium smegmatis* MKD8, *Mycobacterium fortuitum*, and *Nocardia globerula* ATCC 21505), *Arthrobacter parraffineus* ATCC 19958 from the order *Micrococcales*, and three species from the more distant phylum *Firmicutes* (*Enterococcus faecalis* ATCC 29212, an equine field *Streptococcus zooepidemicus* subsp. *equi* isolate, and *Staphylococcus aureus* ATCC 29213). All of the *Rhodococcus* species successfully acquired pRErm46 at various transfer frequencies (Table 1). Similarly, pRErm46 was conjugally transferred to *Nocardia goberula* and *Arthrobacter paraffineus* at ratios comparable to those for *Rhodococcus* spp. (Table 1). In contrast, transfer of pRErm46 could not be detected to the tested mycobacteria, *C. pseudotuberculosis*, or the nonactinobacterial 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).

For each recipient bacterial species that successfully received pRErm46 in the *in vitro* mating assays (see above), five colonies were randomly selected and the macrolide resistance phenotype was determined using Etest strips. MICs to macrolides were tested before and after acquisition of pRErm46. The MICs of recipients prior to and after gaining pRErm46 were 0.5 to 8 μg/ml and $\geq$256 μg/ml, respectively, for all isolates. The only exception was *R. fascians*, in which the MIC increased from 0.19 to 0.38 μg/ml to 8 to 12 μg/ml (Table 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-

TABLE 2 Erythromycin MICs of different bacteria upon *erm*(46) conjugal acquisition[a]

Species	*erm*(46) negative (mg/liter)	*erm*(46) positive (mg/liter)
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

[a]Data determined using Etest ($n = 3$).

resistant equine clinical isolate PAM2287 (21) (prototype strain of the pRErm46-harboring *R. equi* clone) was used as the pRErm46 donor, and susceptible (pRERrm46-negative) avirulent *R. equi* 103^- with an apramycin resistance *aac(3)IV* cassette (103^-Apra^r) (24) was used as the recipient in the mating assay, enabling transconjugant selection via dual resistance to erythromycin (Erm^r) and apramycin (Apra^r). Three conjugation ratios (1:1, 1:10, and 10:1), and four 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 of incubation at 22°C and 30°C, independent 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, and pRErm46, in addition to the virulence plasmid pVAPA, was tested using PCR in up to 10 (depending on transconjugant numbers) Erm^r and Apra^r colonies. All transconjugants screened using PCR carried the *aac(3)IV* (Apra^r) cassette and the

TABLE 3 Conjugal transfer of pRErm46 in soil and horse manure[a]

			Day 30			Day 90			Day 180		
Sample	Ratio[b]	Temp (°C)	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
Soil	1:1	4									
Soil	1:1	22					2.56×10^{-4}				
Soil	1:1	30									
Soil	1:1	37									
Soil	1:10	4									
Soil	1:10	22	9.43×10^{-6}	1.58×10^{-6}	2.38×10^{-5}						1.14×10^{-4}
Soil	1:10	30									
Soil	1:10	37									
Soil	10:1	4									
Soil	10:1	22							6.25×10^{-4}		
Soil	10:1	30						5.10×10^{-2}	1.14×10^{-2}		
Soil	10:1	37									
Manure	1:1	4									
Manure	1:1	22		2.11×10^{-2}							
Manure	1:1	30		2.21×10^{-5}							
Manure	1:1	37									
Manure	1:10	4									
Manure	1:10	22									
Manure	1:10	30					1.25×10^{-5}			2.21×10^{-5}	
Manure	1:10	37									
Manure	10:1	4									
Manure	10:1	22									
Manure	10:1	30									
Manure	10:1	37									

[a]Data represent conjugation transfer frequencies per sample of soil/horse manure. Three samples were collected per each time point and incubation temperature. Blank entries indicate that no conjugation was observed under those specific conditions at that particular time.
[b]Ratio refers to the ratio of donor/recipient bacteria used in each conjugation assay.

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

Species	Ratio[b]	Temp (°C)	Day 15	Day 30
R. erythropolis	1:1	22	$2.43 \pm 2.02 \times 10^{-5}$	$3.32 \pm 1.99 \times 10^{-5}$
	1:1	30	$9.66 \pm 5.69 \times 10^{-6}$	$3.05 \pm 1.46 \times 10^{-6}$
R. erythropolis	1:10	22	$1.24 \pm 0.40 \times 10^{-6}$	$1.29 \pm 0.97 \times 10^{-7}$
	1:10	30	$1.91 \pm 1.06 \times 10^{-5}$	$6.29 \pm 2.20 \times 10^{-6}$
A. paraffineus	1:1	22	$9.98 \pm 9.98 \times 10^{-9}$	$1.66 \pm 0.98 \times 10^{-7}$
	1:1	30	$2.78 \pm 1.15 \times 10^{-6}$	$2.18 \pm 1.55 \times 10^{-6}$
A. paraffineus	1:10	22	$5.61 \pm 6.47 \times 10^{-8}$	$3.63 \pm 1.98 \times 10^{-7}$
	1:10	30	$4.94 \pm 2.39 \times 10^{-7}$	$9.07 \pm 6.67 \times 10^{-7}$
N. globerula	1:1	22	$1.39 \pm 0.81 \times 10^{-1}$	$2.32 \pm 2.07 \times 10^{-2}$
	1:1	30	$5.62 \pm 2.22 \times 10^{-1}$	$0.97 \pm 1.19 \times 10^{-3}$
N. globerula	1:10	22	$9.38 \pm 8.25 \times 10^{-3}$	$4.82 \pm 2.37 \times 10^{-4}$
	1:10	30	$6.06 \pm 3.47 \times 10^{-2}$	$8.33 \pm 8.33 \times 10^{-3}$

[a]Data represent means ± SDs of 3 samples collected per time point and incubation temperature.
[b]Ratio refers to the ratio of donor/recipient bacteria used in each conjugation assay.

erm(46) (Erm^r) gene, providing molecular confirmation of the transconjugant phenotype. pRErm46 was detected using 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, the optimal environmental mating conditions appear to be 22°C to 30°C for a length of 8 to 30 days.

Under the optimal conditions for pRErm46 environmental conjugation described above, 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 10^{-4} transconjugants/recipient bacteria. The mobilization of pRErm46 to *R. erythropolis* occurred at similar frequencies to those observed for *R. equi* in soil and ranged from 10^{-5} to 10^{-7} transconjugants/recipient bacteria, while slightly lower transfer ratios were observed using *A. paraffineus* as the recipient (10^{-6} to 10^{-9} transconjugants/recipient).

The pRErm46 fitness cost varies in different recipients. To gain further insight into the determinants of pRErm46 maintenance, we measured the impact of pRErm46-mediated macrolide resistance on bacterial fitness. *R. erythropolis*, *A. paraffineus*, and *N. globerula* showed no significant differences in exponential growth rate and maximum growth upon acquisition of pRErm46 in either complex medium (brain heart infusion [BHI]) or *R. equi* chemically defined medium (mREMM) (Table 5, Fig. 1). Surprisingly, some of the tested strains (*R. fascians* and *R. rhodnii*) even showed significantly improved fitness in mREMM medium when carrying pRERm46 (Table 5). This was particularly evident with *R. fascians*, which failed to grow in mREMM in the absence, but not presence, of pRErm46, suggesting the intriguing possibility that some plasmid-encoded determinants may contribute to the regulation of bacterial growth in nutrient-limiting conditions. The only detrimental effect of pRErm46 on bacterial fitness was observed with *R. rhodnii* which, when harboring pRErm46, manifested slightly impaired growth (Table 5, Fig. 1).

DISCUSSION

Horse breeding farms that use macrolides and rifampin as a mass treatment for *R. equi* subclinical pneumonia likely represent highly antibiotic-pressurized environments (17, 25), where bacterial survival is contingent upon acquisition of a suitable resistant phenotype. This study explores how the horizontal transfer dynamics of the *R. equi*

TABLE 5 Growth data of isogenic bacteria in the presence and absence of the macrolide resistance plasmid pRErm46 in BHI and chemically defined medium[a]

Species	Maximum exponential growth rate (h^{-1})		Maximal bacterial growth (OD_{600})	
	With pRErm46	Without pRErm46	With pRErm46	Without pRErm46
BHI				
R. erythropolis	0.24 (±0.007)	0.24 (±0.009)	1.60 (±0.05)	1.59 (±0.05)
R. fascians	0.17 (±0.02)	0.21 (±0.02)*	0.71 (±0.20)	0.63 (±0.14)
R. rhodnii	0.13 (±0.002)*	0.11 (±0.004)	0.69 (±0.03)	1.07 (±0.03)*
A. paraffineus	0.26 (±0.06)	0.26 (±0.02)	1.35 (±0.03)	1.42 (±0.03)
N. globerula	0.12 (±0.01)	0.14 (±0.02)	2.07 (±0.29)	2.04 (±0.28)
mREMM				
R. erythropolis	0.22 (±0.02)	0.22 (±0.02)	0.36 (±0.006)	0.36 (±0.01)
R. fascians	0.39 (±0.12)*	0.005 (±0.004)	0.54 (±0.06)*	0.02 (± 0.02)
R. rhodnii	0.96 (±0.01)*	0.86 (±0.003)	0.46 (±0.008)*	0.42 (±0.003)
A. paraffineus	0.46 (±0.21)	0.41 (±0.07)	0.36 (±0.04)	0.38 (±0.01)
N. globerula	0.23 (±0.06)	0.26 (±0.06)	0.43 (±0.05)	0.44 (±0.05)

[a]The maximum growth rate during exponential growth and maximal bacterial growth during the growth curve were estimated from fits of the OD_{600} values using the Growrates package in R. Asterisks represent a significant ($P < 0.05$) increase based on paired *t* test statistical analysis ($n = 3$).

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

erm(46)-driven macrolide resistance has only been reported in *R. equi* equine isolates

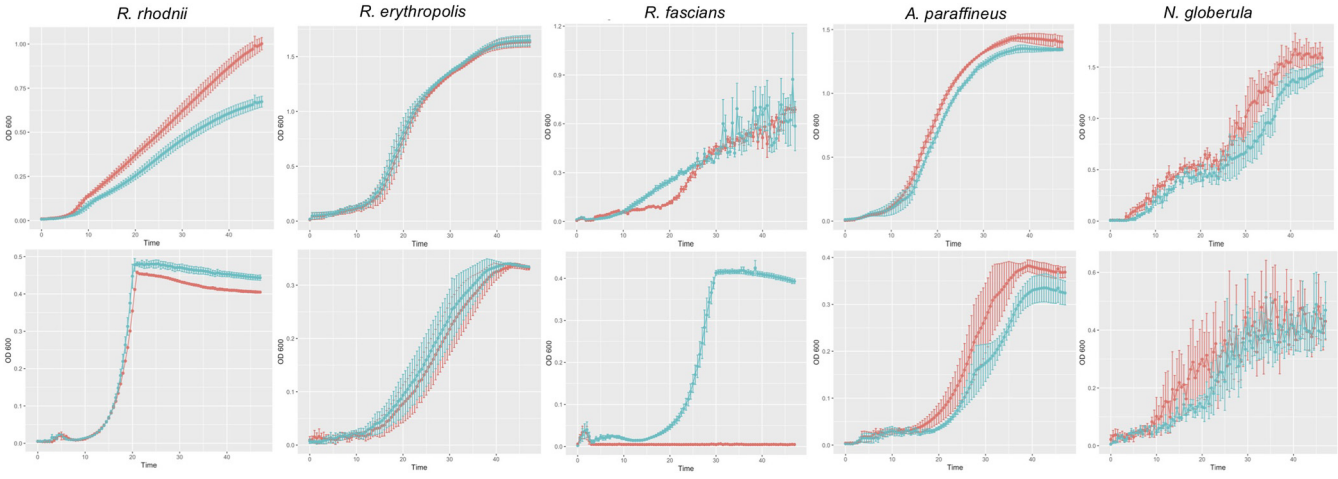

FIG 1 Acquisition of pRErm46 has a different fitness cost depending on the recipient species. pRErm46 was conjugally transferred from *R. equi* to *R. rhodnii*, *R. erythropolis*, *R. fascians*, *A. paraffineus*, and *N. globerula*. Shown are growth curves with each isogenic set composed by a pRErm46+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

carrying the pVAPA virulence plasmid (21). This study shows that pRErm46 can be also acquired by *R. equi* carrying the pVAPB porcine plasmid, thus indicating that porcine environments/isolates may also theoretically contribute to the spread and maintenance of *erm*(46)-mediated *R. equi* macrolide resistance. This might not be the case for ruminant-associated *R. equi* isolates based on the observed potential incompatibility of the pRERm46 and pVAPN replicons. Our findings with the pVAPN plasmid suggest that differences in compatibility among plasmids, typically abundant among soil-dwelling *Actinobacteria*, may be a critical factor 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 incubation temperatures to mimic seasonal changes. Despite using a large conjugation mix of $\sim 10^7$ CFU/g of soil (100 times higher than the concentrations at which *R. equi* is typically found in soil in farms where it is endemic [16]), conjugation remained sporadic but consistently detectable after 8 to 30 days at temperatures of 22 to 30°C, independent 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 that 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 except *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 *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 and 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 previously described (9) for selection of transconjugants by double antimicrobial resistance (Erm^r and 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 on a fresh BHI plate supplemented with 100 μg/ml rifampin.

TABLE 6 Bacterial strains used in this study

Species	Description	Reference or source
Rhodococcus equi		
103$^-$Apra^r	Plasmidless 103 strain containing the *aac(3)IV* apramycin resistance gene integrated on the chromosome	24
PAM2287	Macrolide and rifampin clinical isolate	21
103$^+$Apra^r, pRERM46	Derivative strain from 103$^-$Apra^r; 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 isolate	This study
Rhodococcus spp.		
R. defluvii Rmp^r	Rifampin-resistant derivative strain from *R. defluvii* DSM 45893	This study
R. erythropolis Rmp^r	Rifampin-resistant derivative strain from *R. erythropolis* JCM 2892	This study
R. fascians Rmp^r	Rifampin-resistant derivative strain from *R. fascians* DSM 20669	This study
R. rhodnii Rmp^r	Rifampin-resistant derivative strain from *R. rhodnii* JCM 3203	This study
R. rhodochrous Rmp^r	Rifampin-resistant derivative strain from *R. rhodochrous* JCM 2156	This study
R. ruber Rmp^r	Rifampin-resistant derivative strain from *R. ruber* JCM 3205	This study
Other bacteria		
Nocardia globerula Rmp^r	Rifampin-resistant derivative strain from *N. globerula* ATCC 21505	This study
Arthrobacter paraffineus Rmp^r	Rifampin-resistant derivative strain from *A. paraffineus* ATCC 19958	This study
Mycobacterium smegmatis Rmp^r	Rifampin-resistant derivative strain from *M. smegmatis* MKD8	This study
Mycobacterium fortuitum Rmp^r	Rifampin-resistant derivative strain from *M. fortuitum* from Hondalus strain collection	This study
Staphylococcus aureus Rmp^r	Rifampin-resistant derivative strain from *S. aureus* ATCC 29213	This study
Enterococcus faecalis Rmp^r	Rifampin-resistant derivative strain from *E. faecalis* ATCC 29212	This study
Streptococcus equi subsp. *zooepidemicus* Rmp^r	Rifampin-resistant derivative strain from S. *zooepidemicus* wild-type clinical isolate from Giguère strain collection)	This study

***In vitro* bacterial conjugation assay.** Conjugation assays were carried out as described by Anastasi et al. (28). The *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 an optical density at 600 nm (OD_{600}) of 1. Then, 100 μl of donor suspension was mixed with 100 μl of the recipient 1:1 in a microtube. The mixture was centrifuged (6,000 rpm, 10 min), resuspended in 5 μl of sterile BHI, and spotted in a thick drop onto a BHI plate. After 72 h of incubation at 30°C, the bacterial mixture was scraped and resuspended in PBS, and serial dilutions were plated onto BHI agar supplemented with rifampin (recipient selection) or rifampin plus erythromycin (transconjugant selection). Transconjugants were confirmed using PCR. Conjugation ratios were calculated using the following formula: conjugation ratio = no. of transconjugants cell/no. of recipient cells.

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

PCR. PCRs were carried out using a C100 thermal cycler (Bio-Rad) and GoTaq Flexi DNA polymerase (Promega) following general parameters, described as follows: 5 min at 95°C of initial denaturation, 30 cycles of amplification (involving 30 s at 95°C of denaturation, 30 s of oligonucleotide hybridization at the appropriate melting temperature, and 2 min of elongation at 72°C), plus another 10 min of final elongation at 72°C after the last cycle.

DNA sequencing and analysis. Sanger sequencing was performed by Eurofins (Louisville, Kentucky). Sequences were analyzed using the Ape plasmid editor (Wayne Davis), and the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) 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) using 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 with Etest strips (bioMérieux).

Bacterial growth assays. Potential bacterial fitness costs associated with the acquisition of pRErm46 were investigated by monitoring the growth rate of macrolide-susceptible and -resistant isogenic strains (which 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 an OD_{600} of 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 and 200 rpm. Measurements were taken every 30 min. Data were analyzed using BioTeK Gen5 data analysis software (BioTeK). Data were processed using the biological growth curve fitting package growthrates (https://cran.r-project.org/) in the statistical software R version 3.6.1. The 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.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 2.5 MB.

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We 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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