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molecular microbiology

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Journal:	*Molecular Microbiology*
Manuscript ID	MMI-2018-17472.R1
Manuscript Type:	MicroReview
Date Submitted by the Author:	n/a
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Key Words:	Rhodococcus equi, Rhodococcus genomics, Rhodococcus equi phylogenomics, Rhodococcus equi virulence, Rhodococcus equi plasmids

The pathogenic actinobacterium *Rhodococcus equi*: What's in a name

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This article is dedicated to the memory of our colleague and friend Dr. Steeve Giguère, equine veterinary practitioner and active *Rhodococcus equi* researcher.

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ABSTRACT

Rhodococcus equi is the only animal pathogenic species within an extended genus of metabolically versatile *Actinobacteria* of considerable biotechnological interest. Best known as a horse pathogen, *R. equi* is commonly isolated from other animal species, particularly pigs and ruminants, and causes severe opportunistic infections in people. As typical in the rhodococci, *R. equi* niche specialization is extrachromosomally determined, via a virulence plasmid that promotes intramacrophage survival. Progress in the molecular understanding of *R. equi* and its recent rise as a novel paradigm of multihost adaptation has been accompanied by an unusual nomenclatural instability, with a confusing succession of names: "*Prescottia equi*", "*Prescottella equi*", *Corynebacterium hoagii*, and *Rhodococcus hoagii*. This article reviews current advances in the genomics, biology and virulence of this pathogenic actinobacterium with a unique mechanism of plasmid-driven animal host tropism. It also discusses the taxonomic and nomenclatural issues around *R. equi* in the light of recent phylogenomic evidence that confirms its membership as a *bona fide Rhodococcus*.

Rhodococcus equi

Rhodococcus equi is a high-G+C Gram-positive, facultative intracellular coccobacillus that parasitizes macrophages, causing pulmonary and extrapulmonary pyogranulomatous infections in different animal species and people (Prescott, 1991; von Bargen and Haas, 2009; Vazquez-Boland et al., 2013). Since its discovery in 1923 by H. Magnusson in Sweden as the causative agent of purulent bronchopneumonic disease in foals (Magnusson, 1923) (Fig. 1A), it is well known in veterinary medicine as a major horse pathogen (Muscatello et al., 2007; Giguere et al., 2011). In humans, *R. equi* mostly affects immunocompromised individuals, notably HIV-infected patients, where the infection resembles pulmonary tuberculosis (Yamshchikov et al., 2010). *R. equi* is ubiquitous in soil, multiplies in herbivore manure and the large intestine, and spreads in the farm habitat presumably via fecal-oral cycling (Muscatello et al., 2007; Vazquez-Boland et al., 2013). Lung infections are likely contracted through inhalation of airborne dust particles carrying *R. equi* (Muscatello et al., 2006; Cohen et al., 2008; Petry et al., 2017).

Initially named *Corynebacterium equi* by H. Magnusson himself, *R. equi* was transferred in 1977 to the genus *Rhodococcus* (Goodfellow and Alderson, 1977), currently within the *Nocardiaceae* in the order *Corynebacteriales*. *R. equi* shares a protective mycolic acid-containing cell envelope with other members of this group of *Actinobacteria*. Like other rhodococci, it is strictly aerobic and non-motile, forms orange-salmon pigmented colonies (Fig. 1B), and shows coccus-to-rod or (occasionally) branched filament cell shape transition (Jones and Goodfellow, 2012). The genus *Rhodococcus* comprises at least 57 species and an ever-growing number of unclassified isolates. Many of these are of considerable significance for the environmental, pharmaceutical and energy sectors owing to their versatile catabolic and biocatalytic properties (van der Geize and Dijkhuizen, 2004). Two rhodococcal species are recognized as pathogenic, *Rhodococcus fascians*, which causes leafy gall in plants (Stes et al., 2013), and *R. equi*.

R. equi genome

The only complete and manually curated genome sequence available for *R. equi* is from strain 103S (= NCTC 13926 = DSM 104936), a prototypic equine clinical isolate (Letek et al., 2010). The reference 103S genome (NCBI RefSeq NC_014659.1, GenBank FN563149.1) consists of a circular chromosome of 5.04 Mbp with 4,598 predicted genes and a G+C content of 68.8% (Fig. 2A). A second key genome component is the virulence plasmid, which carries the *vap* pathogenicity island (PAI) (Takai et al., 2000). In 103S it is a circular plasmid of 80.6 kb designated pVAPA1037 (reference sequence GenBank AM947677) (Letek et al., 2008b). The *R. equi* chromosome appears to be genetically stable, as indicated by the rarity of DNA mobility genes or insertion sequences (Letek et al., 2010) and absence of significant recombination (Anastasi et al., 2016). A small number of pseudogenes (14 in 103S, most in horizontally acquired regions) suggests that it is under strong selection.

Comparative genomic analyses show that *R. equi* is genetically homogeneous and clonal, with a large core genome equivalent to ≈80% of the gene content plateauing at about 25 to 27 genomes in size estimation plots. It is a well-defined taxon with an Average Nucleotide Identity (ANI) of 99.13% and 100% 16S rDNA sequence identity. In a core-genome phylogenomic tree, *R. equi* isolates radiate at a short genetic distance from each other (0.001–0.002 substitutions per site) (Fig. 2B), consistent with a relatively recent evolutionary origin and a rapid clonal diversification from the common progenitor.

Like many other bacteria, *R. equi* has an open pangenome. Although non-core genes only represent ≈20% of each strain's gene content, a significant proportion of the accessory genome (60%) is only present in one or two isolates, accounting for the intraspecific variability. *R. equi* genome evolution is primarily driven by gene gain/loss processes, with a significant contribution of horizontal gene transfer (HGT) events (Letek et al., 2010; Anastasi et al., 2016) (Fig. 2A). Phages are abundant in *R. equi* (Summer et al., 2011;

Petrovski et al., 2013; Salifu et al., 2013) and probably play an important role in HGT-based genome plasticity.

Core *R. equi* traits

Comparative genomic studies confirmed that most traits predicted to be important for *R. equi* biology and niche adaptation belong to the core genome (Letek et al., 2010; Anastasi et al., 2016). This includes all putative pathogenicity determinants identified in the 103S chromosome, notably a number of mycobacterial virulence gene homologs. Genes involved in tolerance to desiccation and oxidative stress, presumably important for survival in dry soil and transmission via aerosolized dust, also belong to the core genome. There is also a conserved intrinsic resistome with several putative β-lactamases, aminoglycoside phosphotransferases and multidrug efflux pumps. These probably contribute to the variable susceptibility reported for *R. equi* to diverse antimicrobials, as observed for example with β-lactams and quinolones (Nordmann and Ronco, 1992; Mascellino et al., 1994; Soriano et al., 1998; Makrai et al., 2000; Jacks et al., 2003; Letek et al., 2010; Yamshchikov et al., 2010).

A distinctive characteristic of *R. equi* is the complete absence of phospho*enol*pyruvate:carbohydrate transport system (PTS) components, consistent with an eminently asaccharolytic metabolism. Among the rhodococci, only its close relative *Rhodococcus defluvii* (Kampfer et al., 2014) also lacks a PTS sugar transport system (Anastasi et al., 2016), indicative of specific gene loss in the common ancestor of the *R. equi*-*R. defluvii* sublineage (see below Fig. 5). The absence of PTS homologues is rather unique within the *Actinobacteria*; among the few examples are *Mycobacterium tuberculosis*, also a parasite of macrophages, and the obligate intracellular pathogen *Tropheryma whipplei* (Barabote and Saier, 2005; Letek et al., 2010), suggesting that loss of this sugar transport system might be associated with adaptation to intracellular parasitism in this bacterial group.

Recently, genes encoding putative non-PTS transporters for glucose (GlcP) and

ribose (RbsCB) have been identified in the *R. equi* core genome. Both permeases seem to be functional, although utilization of ribose and, particularly, glucose by *R. equi* 103S was inefficient (and variable for the latter) compared to preferred carbon sources such as lactate or acetate (Letek et al., 2010; Anastasi et al., 2016). Since *R. equi* assimilates carbon principally via short chain organic acids and lipid catabolism, these two sugar transporters might act as occasional "nutritional fitness" enhancers in specific habitats.

In addition to monocarboxylate and dicarboxylate transporters, the *R. equi* core genome encodes an extensive array of lipases (both secreted and intracellular) and β-oxidation enzymes. There are also three complete *mce* ("mammalian cell entry") systems, which form channel mechanisms specialized in lipid transport (Ekiert et al., 2017), for example cholesterol (Mohn et al., 2008; Pandey and Sassetti, 2008). Similar to *M. tuberculosis* (Munoz-Elias and McKinney, 2005), *R. equi* virulence requires the glyoxylate shunt enzyme isocitrate lyase (ICL) (Wall et al., 2005). ICL mediates the diversion of TCA cycle intermediates for gluconeogenesis and carbohydrate biosynthesis from acetylCoA generated through fatty acid β-oxidation or acetate oxidation, indicating that, like the tubercle bacillus, *R. equi* utilizes lipids as *in vivo* growth substrate. Interestingly, although not a fermentative organism, *R. equi* possesses a putative bifunctional D-xylulose 5-phosphate (X5P)/fructose 6-phosphate (F6P) phosphoketolase (Xfp) (Meile et al., 2001). This enzyme may provide flexibility in carbon and energy metabolism by converting pentose phosphate pathway (PPP) and glycolytic intermediates into acetyl phosphate (and acetate/acetyl-CoA) (Ingram-Smith et al., 2006).

R. equi appears to be particularly well adapted for growth on exogenous L-lactate, with a dedicated transporter (LldP) and determinants for its conversion into acetate, either directly (L-lactate monooxygenase) or via pyruvate (*lutABC* operon [Chai et al., 2009]) combined with pyruvate decarboxylation via pyruvate dehydrogenase [cytochrome]) (Letek et al., 2010). Moreover, *R. equi* has denitrification capacity, with a NarK nitrate/nitrite

transporter, NarGHIJ nitrate reductase and a NirBD nitrite reductase. It also has the ability to grow on urea as the sole nitrogen source through the action of a urease and an ATP-dependent urea carboxylase (Letek et al., 2010; Anastasi et al., 2016).

Another core characteristic is the disruption of the *thiCD* locus by an HGT island, rendering *R. equi* auxotrophic to thiamin (Letek et al., 2010; Anastasi et al., 2016). Apart from this, *R. equi* is otherwise not nutritionally demanding and can grow vigorously in the presence of just inorganic N (e.g. in the form of ammonium chloride) and an organic acid as a carbon source. Together with its alkalophily (optimal growth between pH 8.5 and 10) (Letek et al., 2010), the nutritional and metabolic profile of *R. equi* may confer a competitive advantage in manure and the intestine, its natural reservoirs, where there is easy access to microbiota-derived thiamine and lactate and short-chain fatty acids fermentation products (Letek et al., 2010; Anastasi et al., 2016). Via a NiFe-type hydrogenase, *R. equi* has the potential to utilize H_2, released through microbial metabolic activity, potentially contributing to survival in the intestinal habitat.

R. equi illuminates rhodococcal genome evolution

R. equi possesses a more compact genome compared to environmental rhodococci, exemplified by *Rhodococcus erythropolis* PR4 (6.52 Mb) and, particularly, *Rhodococcus jostii* RHA1 (7.80 Mb) or *Rhodococcus opacus* B4 (7.25 Mb), for which complete genomes are also available (McLeod et al., 2006; http://www.nite.go.jp/index-e.html). Analysis of gene duplication and HGT events, together with the slow rate of gene decay in the 103S chromosome, indicate that the genome size differences are due to genome expansion in the environmental species rather than genome contraction in *R. equi* (Letek et al., 2010). Rhodococcal genome expansion is due to amplification of paralogous families and acquisition of HGT DNA and extrachromosomal genes, often as part of plasmids as large as 1 Mb in size. These plasmids are particularly rich in HGT DNA (up to 50%), contain a much

higher density of mobility genes and pseudogenes, unique species-specific genes, and niche-adaptive determinants, specifically catabolic (McLeod et al., 2006; Letek et al., 2010). The metabolic complexity of the environmental *Rhodococcus* spp. is a likely reflection of the isolation criteria, seeking for specific abilities such as degradation of multiple aromatic pollutants, biotransformation, or production of secondary metabolites (van der Geize and Dijkhuizen, 2004; Larkin et al., 2005; Yamashita et al., 2007; Kitagawa and Tamura, 2008; Holder et al., 2011; Foster et al., 2014). For example, compared to *R. equi*, the polychlorinated biphenyl degrader *R. jostii* RHA1 contains a much larger complement of unique metabolic genes, aromatic gene clusters (29 vs only three), non-ribosomal peptide synthases (24 vs 11) and polyketide synthases (7 vs 1 in *R. equi*) (McLeod et al., 2006; Letek et al., 2010).

Circular and linear genomes: a matter of size

The determination of the complete 103S genome sequence made it apparent that *Rhodococcus* spp. differ in chromosome topology despite being monophyletic. While *R. equi* 103S and *R. erythropolis* PR4 both posses covalently closed circular chromosomes, *R. jostii* RHA1 and *R. opacus* B4 have linear ones. Remarkably, not only the four species belong to a same subdivision of the genus *Rhodococcus*, but *R. erythropolis* and *R. jostii*/*R. opacus* even belong to sister sublineages within the same terminal clade (no. 2, see below Fig. 5) (Anastasi et al., 2016). Since the four chromosomes share the same overall structure and synteny (Letek et al., 2010; Anastasi et al., 2016), the only obvious difference is a comparatively larger size for *R. jostii* and *R. opacus* (≥7.25 Mb), similar to *Streptomyces* spp (≥8 Mb), which also possess linear genomes. This suggests that actinobacterial chromosome linearization occurs as a function of increasing size rather than phylogenetic background. This mirrors the situation with the rhodococcal plasmids, which independently of the host species tend to be linear above 100 kb (Larkin et al., 2010; Valero-Rello et al., 2015).

Plasmid-determined virulence

A distinguishing feature of the genus *Rhodococcus* is the characteristic presence of large circular or linear conjugative plasmids carrying niche-adaptive DNA (Larkin et al., 2010). While these regions encode catabolic and detoxification pathways in rhodococcal species isolated from xenobiotic-contaminated ecosystems (McLeod et al., 2006; Sekine et al., 2006), in the pathogenic species *R. equi* and *R. fascians* they encode virulence (i.e. host-adaptive) determinants (Letek et al., 2008b; Francis et al., 2012; Valero-Rello et al., 2015). In the case of *R. equi*, the plasmid's HGT-acquired *vap* PAI (Fig. 3) supports intramacrophage survival and is essential for animal host colonization (Coulson et al., 2010).

The *vap* PAI encodes a set of homologous secreted virulence-associated proteins (Vap) (Letek et al., 2008b; Valero-Rello et al., 2015) (Fig. 4A) that fold in a cork-shaped eight-stranded antiparallel β-barrel structure (Geerds et al., 2014; Whittingham et al., 2014). One of them, designated VapA in the equine-type plasmid pVAPA, is essential for pathogenesis (Jain et al., 2003; Gonzalez-Iglesias et al., 2014; Valero-Rello et al., 2015). The exact mechanism of action of VapA and homologous proteins remains unknown but are thought to play a key role in the biogenesis of the modified, Rab7-positive endosome where the bacterium replicates within macrophages (aka the *R. equi*-containing vacuole'', RCV) (Fernandez-Mora et al., 2005; Sydor et al., 2013; Rofe et al., 2017). Consistent with this, VapA has recently been found to localize to the membrane of the RCV (Wright et al., 2018). Non-*vap* genes are also present in the PAI (Valero-Rello et al., 2015; MacArthur et al., 2017), notably the *vir* operon, which encodes two key regulators (VirR and VirS) required for *vap* PAI gene activation and virulence (Byrne et al., 2007) (Fig. 4A). Another *vir* operon product, IcgA, has been shown to modulate intracellular growth of *R. equi* (Wang et al., 2014).

A number of core chromosomal metabolic genes appear to have been coopted within the regulatory network of the *vap* PAI and exhibit expression patterns similar to those of the

plasmid virulence genes. Two of these genes, encoding chorismate mutase and anthranilate synthase enzymes involved in aromatic amino acid biosynthesis, were found to facilitate intracellular survival in macrophages (Letek et al., 2010).

Plasmid-mediated host tropism: a novel paradigm

Three *R. equi* virulence plasmid types have been identified to date, pVAPA and pVAPB associated with equine and porcine isolates, respectively, and pVAPN ("N" for no-A/B) associated with ruminants (bovines, ovines and caprines) (Ocampo-Sosa et al., 2007). pVAPA and pVAPB are variants of a same circular replicon which differ in *vap* PAI structure (Letek et al., 2008b) whereas pVAPN is an unrelated linear plasmid, with again a specific *vap* PAI (Valero-Rello et al., 2015; MacArthur et al., 2017) (Fig. 3). pVAPA/B/N type-host mismatch virtually never occurs among equine, porcine and ruminant isolates, suggesting stringent host-driven exclusion of non-adapted plasmids (Ocampo-Sosa et al., 2007; unpublished data from JV-B laboratory). Phylogenomic analyses did not find any association between host and chromosomal genotype but, instead, clear evidence of active exchange of the pVAPA/B/N plasmids across the *R. equi* population with corresponding host jumps (Anastasi et al., 2016; MacArthur et al., 2017). The *R. equi* virulence plasmid appears to be easily lost in the absence of host selection (Takai et al., 1994; Ocampo-Sosa et al., 2007) but can be readily reacquired via conjugation (Tripathi et al., 2012; Valero-Rello et al., 2012). The available evidence supports a model whereby *R. equi* host tropism is mediated by the virulence plasmids, with dynamic plasmid loss-regain allowing flexible adaptation to saprotrophic life in the environment and parasitization of different animal hosts.

Contrasting with their selectivity for certain animal species, the three host-adapted plasmids are commonly found in human isolates (Ocampo-Sosa et al., 2007; Anastasi et al. 2016). This suggests that animals are the source of infection for people, establishing *R. equi* as a novel zoonotic pathogen. It also implies that humans are essentially opportunistic hosts

for *R. equi* (Ocampo-Sosa et al., 2007; Vazquez-Boland, 2010, 2013). The situation appears to be analogous for other animal species which seem to be also accidental hosts for *R. equi*, as for example suggested by recent virulence plasmid characterization studies from dog isolates (Bryan et al., 2017).

Evolution of *R. equi* virulence

As mentioned above, the pVAPA/B/N plasmids each carry a type-specific *vap* PAI. The major differences lie in the *vap* multigene family (Fig. 4A). Phylogenetic reconstruction of *vap* multigene family evolution indicates that the nearest common ancestor of the *vap* PAI contained seven *vap* genes (Valero-Rello et al., 2015). These progenitor *vap* alleles originated via gene duplication from an ancestor *vap* determinant (Fig. 4A). This proto-*vap* gene was probably horizontally acquired because obvious homologs are absent from other *Actinobacteria* while they are found in bacteria from different phyla and even fungi, yet remaining relatively uncommon (Whittingham et al., 2014; Valero-Rello et al., 2015).

A likely hypothetical scenario is that the proto-*vap*, in combination with some non-*vap* determinant present in the common PAI ancestor, acquired at some stage the ability to promote intracellular survival. Perhaps initially a defence mechanism against predation by bacterivore environmental protozoa, eventually this also allowed the host bacterium to escape phagocytic killing by macrophages, paving the way to becoming an animal pathogen. Indeed, a critical intracellular survival determinant is obviously present in the extant *vap* PAIs, because the three host-adapted plasmids promote intracellular survival in cultured macrophages (Giguere et al., 1999; Coulson et al., 2010; Gonzalez-Iglesias et al., 2014; Valero-Rello et al., 2015; Willingham-Lane et al., 2016).

Cumulative epidemiological and experimental evidence indicates that the intracellular survival-promoting function is primordial and dissociable from host tropism, because the three plasmid types promote virulence in accidental (non-adapted) animal hosts

(e.g. humans and mice). This critical *vap* determinant is probably the common ancestor of *vapA* of the equine pVAPA type and its allelic variants *vapN* of the ruminant pVAPN type (Valero-Rello et al., 2015) and *vapK1/2* (and putative duplicate thereof, *vapB*) of the porcine pVAPB type (Valero-Rello et al., 2015; Willingham-Lane et al., 2018) (Fig. 4AB).

Subsequently, host-tropic properties evolved in the common ancestor of the *vap* PAI, presumably through adaptive evolution of the *vap* multigene family in equines, swines and ruminants. The process appears to have started in the pre-pVAPA/B plasmid, followed by horizontal transfer of the PAI from the pVAPA lineage to the pVAPN replicon (Valero-Rello et al., 2015) (Fig. 4C). The perfect conservation of the *vap* PAIs within each host-adapted virulence plasmid type indicates they are under strong selection, likely driven by species-specific host factors yet to be identified. The conservation of the DNA mobility gene remnants flanking the *vap* PAIs and the pseudogenes in each PAI type suggests that the PAI diversification process is relatively recent (MacArthur et al., 2017).

Common rhodococcal strategy for rapid niche adaptation

The *R. equi* virulence plasmids share similar backbones with other plasmids found in environmental rhodococci. Thus, the pVAPA/B replicon is homologous to that of pREC1 from the alkane degrader *R. erythropolis* PR4 (Sekine et al., 2006) or pKNR from the organic solvent-tolerant *R. opacus* B4 (Honda et al., 2012) (Fig. 3). All these circular plasmids posses a conjugation machinery based on a MOBf (TrwC) type relaxase (Garcillan-Barcia et al., 2009), designated TraA, together with a type IV secretion system (T4SS) which forms the transport channel. pVAPN, on the other hand, is closely related to the linear plasmid pNSL1 from the environmental *Rhodococcus* sp. NS1 (Zhu et al., 2010) (Fig. 3). Like the circular plasmids, pVAPN and pNSL1 share a conserved backbone but differ in a unique variable region (VR) adjacent to the replication/partitioning region (Fig. 3). Self-transmissibility relies in this case on a relaxase/T4SS-independent mechanism mediated by a

TraB translocase, a novel conjugation system first characterized in the *Streptomyces* linear plasmids. The TraB protein is evolutionarily related to FtsK/SpoIIIE involved in chromosome segregation (Guglielmini et al., 2013) and forms a hexameric channel through which dsDNA is conducted in an ATP-dependent manner (Vogelmann et al., 2011). While not obviously similar, pVAPN and pNSL1 replicons are phylogenetically related to other rhodococcal linear plasmids (Valero-Rello et al., 2015).

The VRs of all the rhodococcal plasmids, whether circular or linear, are typically flanked or contain DNA mobility gene remnants including a variety of recombinases and transposases (Letek et al., 2008b; Valero-Rello et al., 2015) (Fig. 4A). Integrative elements thus appear to play a key role in the formation and plasticity of the VRs. An intriguing feature is the conservation of the *rep-parA* module across the pVAPA/B and pREC1 circular plasmids and the pVAPN and pNSL1 linear plasmids (Fig. 3). The *rep-parA* module is detected as HGT DNA in pREC1 and pNSL1 and adjacent to it there is a putative phage excisionase gene that is also conserved in the circular and linear plasmids. This suggests that the *rep-parA* module adjoining the VRs itself forms part of a gene cassette that is horizontally exchangeable between different rhodococcal replicons despite their different ancestry (Letek et al., 2008b; Valero-Rello et al., 2015).

The *R. equi* virulence plasmids consolidate the notion that rapid niche adaptation through shared sets of self-transferable extrachromosomal replicons is a key common attribute of the actinobacterial genus *Rhodococcus*.

The lingering problem of *R. equi* taxonomy and nomenclature

Although sharing obvious physiological, compositional and genetic features with the other rhodococci, specifically the common plasmid-driven niche specialization strategy, the taxonomic status of *R. equi* within the genus *Rhodococcus* has been repeatedly questioned (McMinn et al., 2000; Jones and Goodfellow, 2012). These taxonomic difficulties are

mirrored in the nomenclature of the species. To several previous validly published names, i.e. *Corynebacterium equi* Magnusson 1923, *Nocardia restricta* (Turfitt 1944) McClung 1974, and *Rhodococcus equi* (Magnusson 1923) Goodfellow and Alderson 1977, additional names have been recently added in rapid succession, as discussed below.

At the root of the nomenclatural instability are a number of 16S rRNA phylogenetic studies, which placed *R. equi* at the periphery of the genus *Rhodococcus* (McMinn et al., 2000; Gurtler et al., 2004) or among the *Nocardia* (Rainey et al., 1995; Goodfellow et al., 1998). Their significance was, however, unclear because of the low bootstrap values (Rainey, 1995), or because *Nocardia* branched off from within the *Rhodococcus* radiation (Rainey, 1995; Goodfellow et al., 1998; McMinn et al., 2000) instead of forming a deep monophyletic lineage near the base of the *Corynebacteriales* (Ludwig et al. 2012).

A recent publication by Jones et al. (2013a) based on numerical phenetic and genotypic (PCR fingerprinting, 16S rDNA sequence) clustering further complicated the situation. While, as expected from their taxonomic status as a separate species, *R. equi* strains grouped in a distinct cluster, perfectly equivalent to that formed by other rhodococcal clades, this fact was used to justify the reclassification of *R. equi* into a new genus, as "*Prescottia equi*" gen. nov., comb. nov. (Jones et al., 2013a). The name "*Prescottia*" was proposed in honour of the *R. equi* research pioneer John F. Prescott from University of Guelph in Canada. However, the new genus name was found to be illegitimate according to the International Code of Nomenclature of Prokaryotes (aka the Bacteriological Code) (Lapage et al., 1992) because it already designated the *Orchidaceae* plant genus *Prescottia* Lindley 1824. The same authors corrected the mistake by proposing "*Prescotella*" gen. nov., and "*Prescotella equi*" comb. nov. as its sole species (Jones et al., 2013b).

The proposal to transfer *R. equi* to a new genus was presented to the international *R. equi* community at the 5th Havemeyer Workshop on *R. equi* held in Deauville (France) on 9-12 July 2012 and was largely met with disapproval for several reasons (Cauchard et al.,

2013). First, it clashed with the conclusions of the recently published 103S genome study, which found that *R. equi* was genomically well classified as a *Rhodococcus* sp. (Letek et al., 2010). Second, strong concerns were voiced that changing such a well-consolidated name in veterinary and medical science would cause considerable confusion. Another important caveat was whether the methodology used in the Jones et al. (2013a) study had adequate resolution to define taxa above the species level according to modern phylotaxonomic criteria. The opinion was expressed that reassigning *R. equi* to a novel genus would only be justified upon systematic re-examination of the taxonomy of the entire genus *Rhodococcus* using modern phylogenomic approaches (Cauchard et al., 2013). We transmitted these concerns on behalf of the international *R. equi* community to leading members of the International Committee for Systematics of Prokaryotes (ICSP) in correspondence exchanged in March 2013.

Probably due to the stir caused by the proposed name changes, the nomenclature of the species was re-examined and it became apparent that *R. equi* had an earlier heterotypic synonym in *Corynebacterium hoagii* (Morse 1912) Eberson 1918 (Tindall, 2014a). Evidence for the identity of *R. equi* and the nomenclatural type *C. hoagii* as the same species was provided by 16S rRNA gene phylogenies used in the description of the closely related species *R. defluvii* (Kämpfer et al., 2014). This meant that not only "*Prescotella equi*" was an illegitimate name but that the name *R. equi* also contravened the principle of priority of the Bacteriological Code (Tindall, 2014a). Since it was evident that *R. equi* is not a *Corynebacterium*, and Kämpfer et al. (2014) confirmed that the differentiation of *R. equi* from other members of the genus *Rhodococcus* was unsupported by chemotaxonomic and phylogenetic evidence, these authors proposed to retain *R. equi* within the genus *Rhodococcus* but with the priority epithet *hoagii*, as *Rhodococcus hoagii* comb. nov.

Truly, a "fine mess", to quote the authors who prompted this "nomenclatural storm" by proposing the new genus names "*Prescottia*/*Prescottella*" (Goodfellow et al., 2015).

R. equi is genomically a *bona fide Rhodococcus*

Anastasi et al. (2016) performed a detailed whole genome sequencing (WGS) phylogenetic analysis of a collection of representative *R. equi* isolates, including the type strain DSM 20307^T (=ATCC 6939^T), and available sequences from other rhodococcal and *Corynebacteriales* species (Fig. 5). An important first conclusion is that *R. equi* is a strictly monomorphic taxon (Fig. 2), thus settling the question of whether *R. equi* is phylogenetically heterogeneous. This had been often raised in the literature (McMinn et al., 2000; Gurtler et al., 2004; Jones, 2012), curiously based on just a single 16S rDNA study that reported similarity values of 97.9-98.3% between the *R. equi* type strain ATCC 6939^T / DSM 20307^T and some of the (only 10) isolates analyzed (McMinn et al., 2000). The reason for such rRNA sequence variability is unclear, but might be due to possible strain misidentifications (Vazquez-Boland et al., 2010) and/or 16S rDNA sequencing errors. In the genomes analyzed by Anastasi et al. (2016), representing a diversity of genetic backgrounds and isolation sources, all 16S rDNA sequences were 100% identical.

Secondly, the phylogenomic studies disambiguated the taxonomic relationship of *R. equi* with other *Rhodococcus* spp. All *R. equi* isolates group in a well-supported monophyletic cluster (no. 3 or "*equi*" subclade) deeply rooted in the rhodococcal phylogeny which also contains *R. defluvii* Ca11^{T}s as the closest relative, *Rhodococcus triatomae* BKS15-14 (a species not previously resolved into to any specific 16S rRNA gene clade) (Ludwig et al., 2012), and an unclassified isolate (Anastasi et al., 2016) (Fig. 5). Two other subclades are generally congruous with the 16S rDNA groupings "*rhodochrous*" (subclade 1) and "*erythropolis*" (subclade 2) (McMinn et al., 2000; Jones and Goodfellow, 2012; Ludwig et al., 2012). Subclade 1 splits into two distinct sublineages, one encompassing *R. ruber*, and the other, the type species of the genus, *R. rhodochrous*, and *Rhodococcus pyridinivorans*. Subclade 2 also splits into two sublineages, one containing *R. opacus*, *R. jostii*, *Rhodococcus imtechensis* and *Rhodococcus wratislaviensis*; the other, *R. erythropolis*

and *Rhodococcus qingshengii* (Fig. 5). The remaining major phyletic lines correspond to *R. rhodnii* LMG 5362 and *R. fascians* isolates, defining two novel subclades (nos. 4 and 5, respectively). Subclade 5 "*fascians*" branches off at an early bifurcation in the *Rhodococcus* phylogeny (Anastasi et al., 2016) (Fig. 5).

The general conclusion of these WGS studies is that *R. equi* is a prototypic *Rhodococcus*, and that if a new genus were to be created to accommodate the species, the same treatment would need to be applied for each of the major rhodococcal lineages (Anastasi et al., 2016). Such an atomization of the genus seems unjustified because *Rhodococcus* forms a distinct and biologically coherent and uniform monophyletic grouping comparable in rank and diversity to other well-established *Corynebacteriales* genera, such *Corynebacterium*, *Gordonia* or *Mycobacterium* (Anastasi et al., 2016) (Fig. 5). It would also defeat the very purpose of bacterial nomenclature in facilitating the coherent study of evolutionarily and biologically related organisms assembled under a common taxon name.

Corynebacteriales phylogenomics

The phylogenomic analyses by Anastasi et al. (2016) also illuminated the evolutionary relationships of the rhodococci with other *Corynebacteriales*, in particular *Nocardia*, inconsistently resolved by previous 16S rDNA phylogenies (Rainey et al., 1995; Goodfellow et al., 1998; McMinn et al., 2000; Jones and Goodfellow, 2012; Ludwig et al, 2012; Kampfer et al., 2014). *Rhodococcus* and *Nocardia* form two clearly distinct clades within a well-supported phyletic line that also comprises *Smaragdicoccus niigatensis* DSM44881^T, classified in the *Nocardiaceae* (as is *Rhodococcus*), as well as *Mycobacterium* spp. and *Amycolicicoccus subflavus* DQS3-9A1^T, a single-species genus of the *Mycobacteriaceae* (Fig. 5). Another major *Corynebacteriales* line of descent is formed by members of the genera *Gordonia* and *Williamsia*, classified in the *Nocardiaceae*, and *Tsukamurella* of the monogeneric *Tsukamurellaceae* (Fig. 5). Two additional major lines of descent are clearly

defined, one encompassing *Corynebacterium*, *Turicella otitidis* ATCC15513^T (*Corynebacteriaceae*) and *Dietzia* (family *Dietziaceae*), the other corresponding to *Segniliparus* spp. (family *Segniliparaceae*) (Anastasi et al., 2016) (Fig. 5).

The phylogenomic data therefore indicate that the current *Nocardiaceae* taxon is polyphyletic and call for a possible reclassification of the *Corynebacteriales* into four families: a) *Mycobacteriaceae*, including the genera *Mycobacterium*, *Amycolicicoccus*, *Smaragdicoccus*, *Rhodococcus* and *Nocardia*; b) *Gordoniaceae*, with the genera *Gordonia*, *Williamsia* and *Tsukamurella*; c) *Corynebacteriaceae*, with the genera *Corynebacterium*, *Turicella* and *Dietzia*; and d) *Segniliparaceae*.

Conservation of *Rhodococcus equi* (Magnusson 1923) Goodfellow and Alderson 1977 and rejection of *Rhodococcus hoagii* (Morse 1912) Kämpfer et al. 2014

While, as discussed above, there appears to be no reasonable grounds for transferring *R. equi* to a new genus, the problem remains with the epithet *hoagii*. Though valid and legitimate in strict nomenclatural terms, the name *R. hoagii* is met with rejection in the context where the organism is relevant. The name *R. equi* is very well established, widely accepted and in widespread use, not only among the veterinary community and equine industry but also the medical profession where the bacterium is recognised as a human opportunistic pathogen. The epithet *equi* suitably encapsulates the very essence of *R. equi* and its significance for the communities concerned and the public. On the other hand, the *hoagii* epithet has remained largely in disuse, restricted to an obscure type strain characterized by features such as production of oxoalkylxanthines and pregnadienes, with no obvious connection with the identity of *R. equi* as a well-known pathogen.

Apart from the confusion already generated due to the use of the new epithet *hoagii* in gene repositories and genomic databases, the application of *R. hoagii* is likely to cause

significant problems of traceability and interpretation of the literature. We believe that this falls under the concept of *nomen perplexum*, one of the exceptions allowing rejection of a bacterial name (Rule 56a.4 of the Bacterial Code, perplexing name: "a name whose application is known but which causes uncertainty in bacteriology") (Lapage, 1992). This situation would be definitely compounded if in addition the genus name were to be changed (see below).

Where there is no doubt that the epithet *hoagii* meets the provisions for rejection is under Rule 56a.5 *nomen periculosum*, "a name whose application is likely to lead to accidents endangering health or life or both or of serious economic consequences" (Lapage, 1992). We cannot think of a more accurate adherence to the notion of a perilous name. There are real chances of potential misdiagnoses or inaccurate risk appraisals due to confusion generated as a result of the introduction of the name *R. hoagii* to designate a pathogenic microbe with a previously well consolidated and recognized name.

The above points have been already evoked by G. Garrity (2014) in his Request for an Opinion (RfO) to the Judicial Commission of the ICSP for conservation of *Rhodococcus equi* as the valid and legitimate name for the taxon. We entirely align ourselves with the views expressed by our colleague. However, Garrity's RfO was formulated to reject the name *Corynebacterium hoagii* and since then the name *Rhodococcus hoagii* has been validated by inclusion in a Notification List of approved names (Oren and Garrity, 2014). A formal request –which we hereby are formulating– is therefore needed for the conservation of *Rhodococcus equi* (Magnusson 1923) Goodfellow and Alderson 1977 and rejection of *Rhodococcus hoagii* (Morse 1912) Kämpfer et al. 2014.

Concluding remarks

The same arguments as above apply for the reassignment of the species to a new genus "*Prescotella*", still advocated by its proposers (Goodfellow et al., 2015) despite its evident

biological inadequacy, the confusion that it will create, the rejection by the *R. equi* community (Cauchard et al., 2013), and the implications for the nomenclature of the whole genus *Rhodococcus* (Letek et al., 2010; Anastasi et al., 2016).

stemming from

Probably as a side effect of the nomenclatural debate originally triggered by the proposed reclassification of *R. equi* into a new genus "*Prescottia*" which turned out to be illegitimate, a significant potential new difficulty arose with the realization that *Rhodococcus* Zopf 1891 may be illegitimate itself, because a later homonym of the algal genus *Rhodococcus* Hansgirg 1884 (Tindall, 2014b). Can one reasonably conceive changing well-established bacterial names such as *Staphylococcus*, *Escherichia* or *Salmonella*? The situation with *R. equi* is essentially analogous. The bacterial genus *Rhodococcus* Zopf 1891 has a comparable standing in the scientific literature and a potential change may have disastrous consequences. The same applies to the name *R. equi* in veterinary and medical microbiology. This extends to the terminology of the infectious disease caused by the pathogen: "rhodococcal pneumonia", "rhodococcal infection" and "foal rhodococcosis" are all well established and commonly used terms in the veterinary, medical and professional literature.

The phylogenomic confirmation of *R. equi* as a *bona fide Rhodococcus* contributes to the picture of a highly adaptable genus of *Actinobacteria* with a diversity of lifestyles, from saprotrophic biodegraders to plant pathogens and animal intracellular parasites. Further illustrating the unique versatility of this group of bacteria, the major *Rhodococcus* phyletic line within which *R. equi* evolved contains species with both circular and linear chromosomes. The genus *Rhodococcus* as it currently stands, including *R. equi*, thus serves to consolidate the important notion that genome topology is primarily a consequence of genome size and has no intrinsic taxonomic value. Together with the shared plasmid-driven niche-adaptive strategy, it showcases the extraordinary flexibility of the bacterial genome to ensure rapid accommodation to different ecological scenarios.

ACKNOWLEDGEMENTS

We gratefully acknowledge the support and encouragement from many members of the international *R. equi* research community. We would also like to thank Josep Casadesús for suggesting the literary quote "What's in a name" in the title. *R. equi* work in JV-B's laboratory received support from Horserace Betting Levy Board (project grants nos 712, 753, 764 and 770), Grayson-Jockey Club Research Foundation (USA), Zoetis, and Roslin Institute ISP core funding from BBSRC.

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FIGURE LEGENDS

Fig. 1. *R. equi* lung infection.

A. Purulent bronchopneumonia in foal with multifocal abscesses. Courtesy of Dr. U. Fogarty, Irish Equine Centre.

B. Typical mucous appearance of *R. equi* colonies (LB agar incubated at 30ºC for 48 h).

Fig. 2. Genomic relatedness of *R. equi* isolates. Modified from Anastasi et al. (2016).

A. Circular diagram of *R. equi* 103S (=NCTC 13926, DSM 104936) chromosome (5.02-Mpb, outer ring with forward and reverse strands) compared to draft genomes of representative isolates from different sources and genetic lineages (inner rings). BLASTn alignments, red color indicates >98% sequence identity. HGT regions in 103S (arrows) coincide with gaps in the DNA alignments, indicating they are strain specific less conserved**.**

B. Core-genome maximum-likelihood phylogeny of *R. equi* isolates in A. Top, unrooted tree; reference genome isolate 103S and type strain of the species are indicated. Bottom, tree rooted with the closely related species, *R defluvii* Ca11^{T}. The star-like topology in the early branchings of the *R. equi* lineage suggests that the species' diversification occurred through rapid clonal radiation from the common progenitor. See also Fig. 5.

Fig. 3. The three host-specific *R. equi* virulence plasmids. Comparison of pVAPA (equine type) and pVAPB (porcine type) circular virulence plasmids and the recently characterized linear pVAPN plasmid (ruminant type) with closest homologs from environmental biodegrader *Rhodococcus* spp. Regions of significant similarity are connected with grey stripes. The *vap* PAIs are shaded in light blue. Gene color code: Hypothetical proteins (gray), conjugation orDNAreplication/recombination/metabolism (red),DNAmobility genes (magenta), transcriptional regulators (blue), secreted proteins (dark green), membrane proteins (pale green), metabolic functions (yellow), *vap* family genes (black), and pseudogenes (brown).Green and pale red bars below the genes indicate conjugation and replication/partitioning functional modules, respectively; dashed underline indicates HGT region. Modified from Valero-Rello et al. (2015).

Fig. 4. Structure and evolution of the host-specific *vap* PAIs. Modified from Valero-Rello et al. (2015).

A. Genetic structure of the *vap* PAIs from pVAPA (15.1 kb), pVAPB (21.5 kb) and pVAPN (15.9 kb). PAI genes in grey (non-*vap* genes, in darker shade the *vir* operon) or black (*vap*

genes). Genes outside the PAIs in white. PAI boundaries indicated by yellow arrowheads. The figure schematizes the evolutionary relationships of the *vap* genes as inferred from phylogenetic analysis, gene duplication/loss analysis (panel C) and genetic structure comparison. Straight lines connect allelic variants of same vap ancestor; those of *vapA* have red surround, curved lines indicate *vap* gene duplications. Crosses denote *vap* genes that were lost. Asterisks indicate pseudogenes.

B. Gene duplication and loss in *R. equi vap* multigene family. Constructed with NOTUNG v2.6 from a *vap* gene ML tree. The analysis indicates that the common ancestor of the three host-specific PAIs contained seven *vap* genes which evolved by gene duplicaton from a single ancestor *vap* gene.

C. Fate of the *vap* PAI during host-driven *R. equi* virulence plasmid evolution.

Fig. 5. Whole-genome *Corynebacteriales* ML tree. Nodes indicate bootstrap values. Tree constructed with five *R.equi* genomes, 47 non-*equi Rhodococus* genomes including representatives from the major 16S rRNA gene clades (Goodfellow et al., 1998; McMinn et al., 2000; Jones and Goodfellow, 2012; Ludwig et al., 2012), and 57 genomes from 11 *Corynebacteriales* genera. Rooted with *Streptomyces albus* NBRC 1304^{T} (outgroup). (T) indicates type strain. Major genera are highlighted in different colors. Black arrowheads indicate misclassifications revealed by the phylogenomic analysis. One of them is *R. rhodnii* NRRL B-16535^{T} (GenBank assembly acc. no. GCA_000720375.1); this probably represents a sequence mislabelling or strain mixup. Modified from Anastasi et al. (2016).

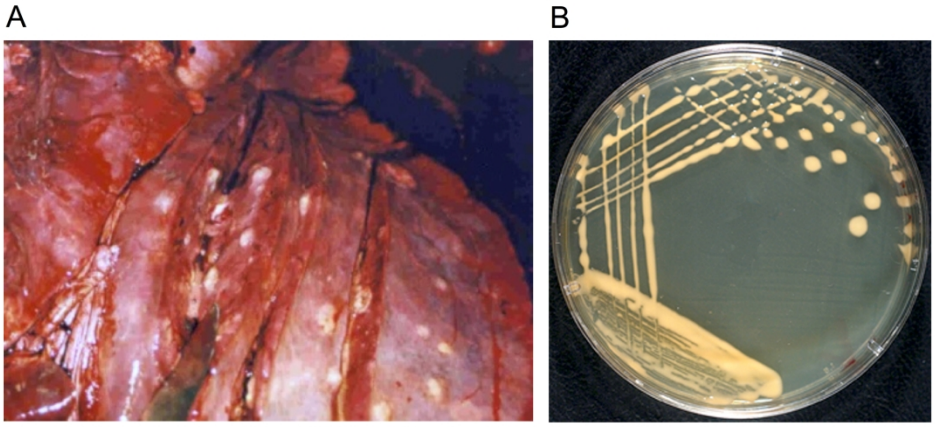

Fig. 1

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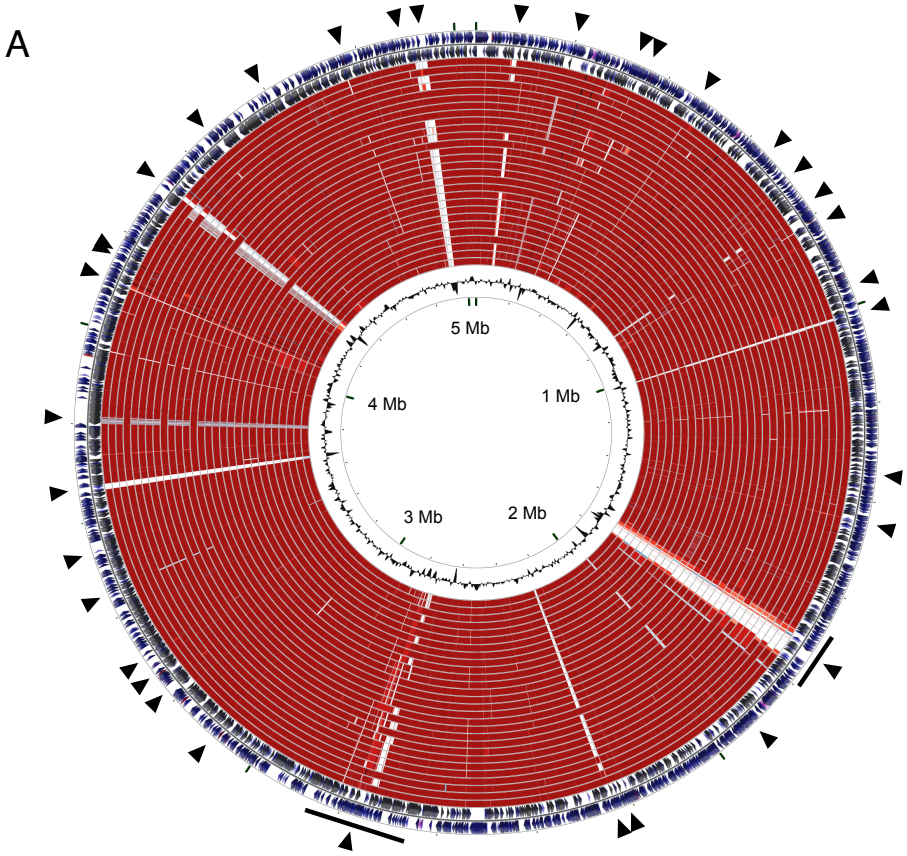
A
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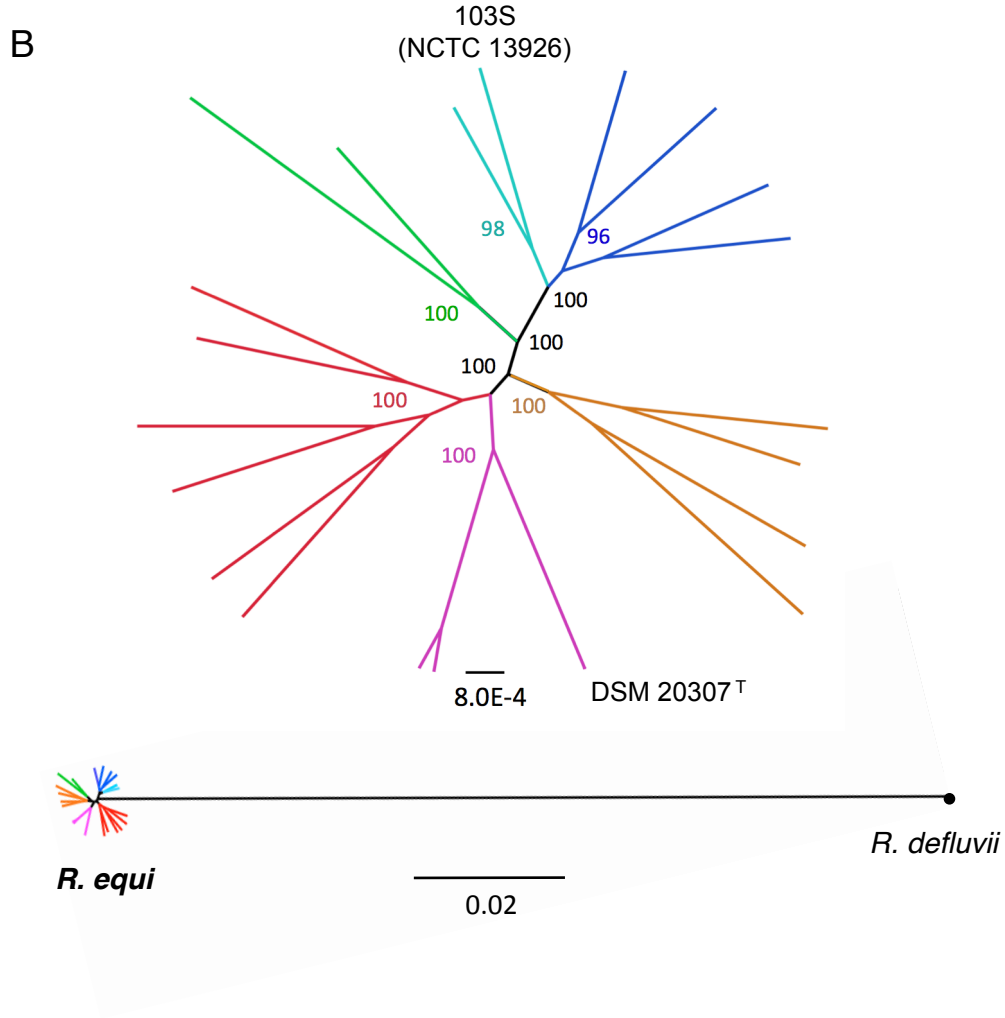
B
103S
(NCTC 13926)
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100
100
100
100
100
100
8.0E-4
DSM 20307 T
R. defluvii
R. equi
0.02

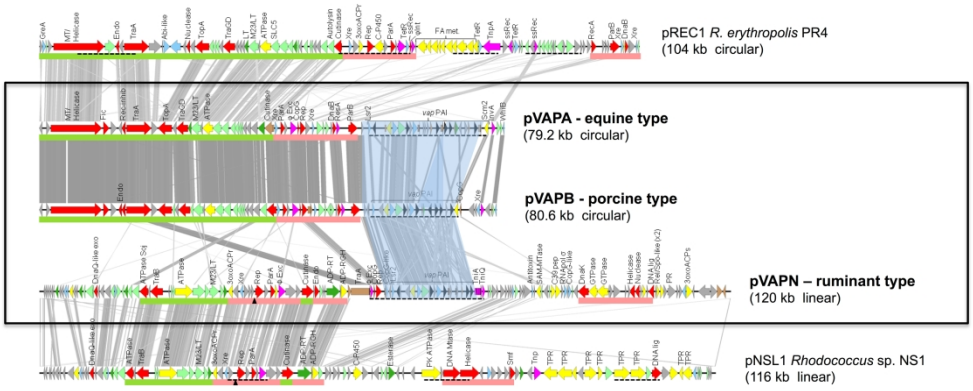

Fig. 3

139x58mm (600 x 600 DPI)

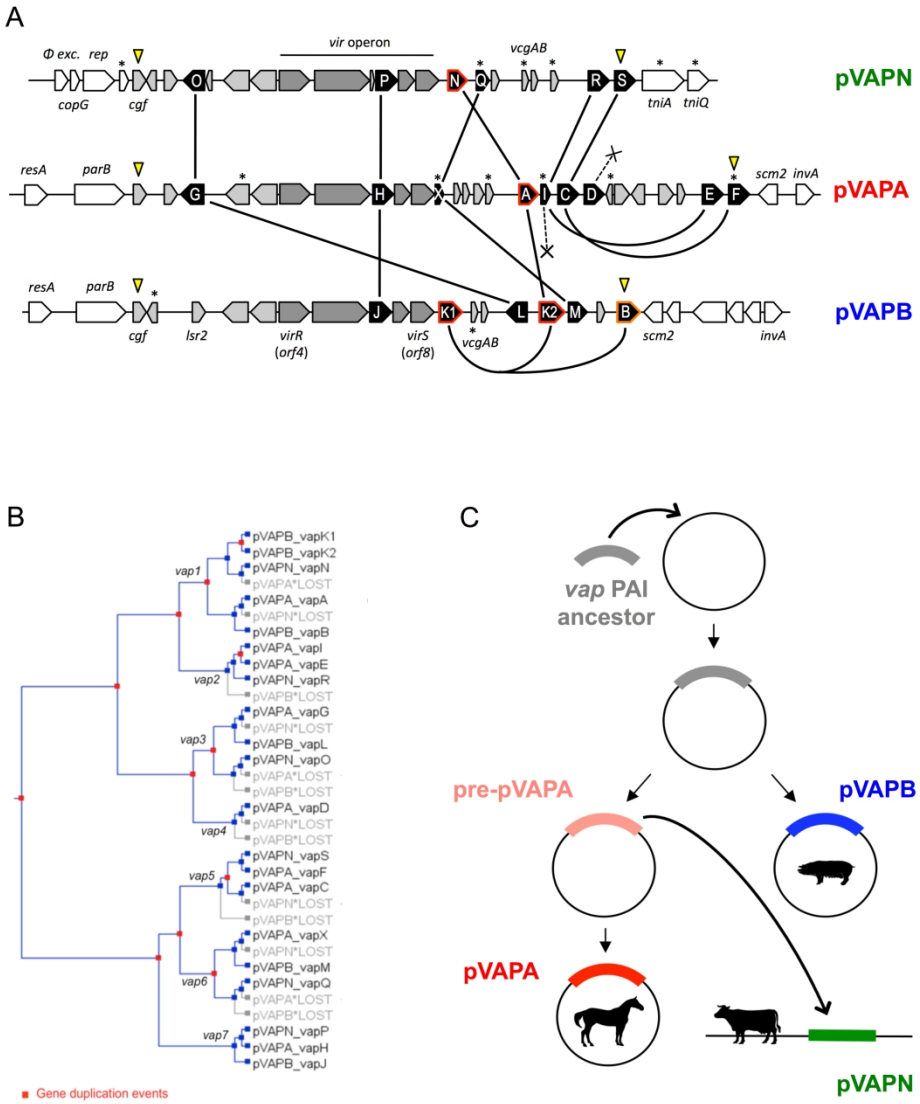

Fig. 4

139x159mm (600 x 600 DPI)


Tsukamurella paurometabola DSM20162 (T)
Tsukamurella sp 1534
Gordonia otitidis_NBRC100426
Gordonia soli NBRC108243
Gordonia bronchialis DSM43247 (T)
Gordonia alkanivorans CGMCC6845
Gordonia alkanivorans NBRC16433 (T)
Gordonia sp KTR9
Gordonia terrae NBRC100016 (T)
Gordonia araii NBRC100433 (T)
Gordonia neofelifaecis NRRLB-59395 (T)
Williamsia sp ARP1
Nocardia sp 348MFTsu51
Williamsia sp D3
R. rhodochrous BKS-46
Rhodococcus sp R04
R. pyridinivorans AK47
R. rhodochrous NBRC16069 (T)
Rhodococcus sp R1101
1
R. ruber chol4
Rhodococcus sp P14
R. ruber BKS20-38
Rhodococcus sp EsD8
Rhodococcus sp BCP1
R. rhodochrous ATCC21198
R. qingshengii TUHH-12
R. erythropolis JCM6824
R. erythropolis NRRLB-16532
R. erythropolis SK121
Rhodococcus sp P27
R. erythropolis DN1
R. erythropolis PR4
R. erythropolis R138
R. erythropolis XP
R. erythropolis CCM2595
R. opacus NRRLB-24011
R. rhodochrous ATCC17895
R. qingshengii BKS20-40
2
0.72
R. opacus B4
R. opacus PD630
R. wratislaviensis NBRC100605 (T)
Rhodococcus sp JVH1
Rhodococcus sp DK17
R. jostii RHA1
R. wratiswlaviensis IFP2016
R. opacus M213
R. imtechensis RKJ300 (T)
0.98
0.99
R. equi DSM20307 (T)
R. equi ATCC33707
R. equi PAM2282
R. equi 103S
R. equi PAM1216
0.97
3
R. defluvii Ca11 (T)
Rhodococcus sp UNC363MFT
R. triatomae BKS15-14
4
R. rhodnii LMG5362
Rhodococcus sp 114MFT
Rhodococcus sp 29MFTsu31
Rhodococcus sp AW25M09
R. fascians LMG3625
R. fascians D188
R. fascians LMG3623 (T)
R. fascians LMG3616
Rhodococcus sp JG3
5
Rhodococcus sp UNC23MF
Smaragdicoccus niigatensis DSM44881 (T)
Nocardia asteroides NBRB15531 (T)
Nocardia brasiliensis NBRC14402 (T)
Nocardia brasiliensis IFM10847
Nocardia sp CNY236
Nocardia araoensis NBRC100135 (T)
Nocardia asiatica NBRC100129 (T)
Nocardia abscessus NBRC100374 (T)
0.98
Nocardia cyriacigeorgica GUH2
Nocardia brevicatena NBRC12119 (T)
Nocardia farcinica IFM10152
Nocardia aobensis NBRC100429 (T)
Nocardia sp BMG111209
0.91
Amycolicicoccus subflavus DQS3-9A1 (T)
Mycobacterium abscessus 47J26
Mycobacterium fortuitum z58
Mycobacterium abscessus ATCC19977 (T)
Mycobacterium abscessus ssp bolletii BD
Mycobacterium fortuitum DSM46621 (T)
Mycobacterium smegmatis MC2155 (T)
Mycobacterium tuberculosis GM1503
Mycobacterium bovis BCGstrChina
Mycobacterium bovis AN5
Mycobacterium tuberculosis H37RvAE (T)
Mycobacterium avium ssp avium ATCC25291 (T)
Mycobacterium avium ssp silvaticum ATCC49884
Mycobacterium avium ssp paratuberculosis ATCC19698
Segniliparus rotundus DSM44985 (T)
Segniliparus rugosus ATCC BAA-974 (T)
Dietzia sp UCD-THP
Dietzia cinnamea P4
Dietzia alimentaria 72
Corynebacterium bovis DSM20582 (T)
Turicella otitidis ATCC51513 (T)
Corynebacterium ammoniagenes DSM20306 (T)
Corynebacterium accolens ATCC49726
Corynebacterium accolens ATCC49725 (T)
Corynebacterium glutamicum z188
Corynebacterium glutamicum ATCC13032 (T)
Corynebacterium pseudotuberculosis 258
Corynebacterium pseudotuberculosis 316
Corynebacterium diphtheriae NCTC13129
Corynebacterium diphtheriae C7 (T)
"Rhodococcus rhodnii" NRRLB16535 (T)
Streptomyces albus NBRC1304 (T)
0.3