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Can whole genome analysis refine the taxonomy of the genus *Rhodococcus*? ☆

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Abstract

The current systematics of the genus *Rhodococcus* is unclear, partly because many members were originally included before the application of a polyphasic taxonomic approach, central to which is the acquisition of 16S rRNA sequence data. This has resulted in the reclassification and description of many new species. Hence, the literature is replete with new species names that have not been brought together in an organized and easily interpreted form. This taxonomic confusion has been compounded by assigning many xenobiotic degrading isolates with phylogenetic positions but without formal taxonomic descriptions. In order to provide a framework for a taxonomic approach based on multiple genetic loci, a survey was undertaken of the known genome characteristics of members of the genus *Rhodococcus* including: (i) genetics of cell envelope biosynthesis; (ii) virulence genes; (iii) gene clusters involved in metabolic degradation and industrially relevant pathways; (iv) genetic analysis tools; (v) rapid identification of bacteria including rhodococci with specific gene RFLPs; (vi) genomic organization of *rrn* operons. Genes encoding virulence factors have been characterized for *Rhodococcus equi* and *Rhodococcus fascians*. Based on peptide signature comparisons deduced from gene sequences for cytochrome P-450, mono- and dioxygenases, alkane degradation, nitrile metabolism, proteasomes and desulfurization, phylogenetic relationships can be deduced for *Rhodococcus erythropolis*, *Rhodococcus globerulus*, *Rhodococcus ruber* and a number of undesignated *Rhodococcus* spp. that may distinguish the genus *Rhodococcus* into two further genera. The linear genome topologies that exist in some *Rhodococcus* species may alter a previously proposed model for the analysis of genomic fingerprinting techniques used in bacterial systematics.

Keywords: *Corynebacterineae*; *Gordonia*; *Nocardia*; *Rhodococcus*; Molecular systematics; Genome topology

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Abbreviations: RFLP, restriction fragment length polymorphism; PFGE, pulsed-field gel electrophoresis; RAPD, randomly amplified polymorphic DNA; AFLP, amplified fragment length polymorphisms.

☆ Bacterial names consistent with current recommendations [1].

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1. Introduction

Bacteria of the order *Actinomycetales* were originally classified as fungi because of their hyphal and mycelial morphology, and include the mycolic acid containing *Corynebacterineae* which presently contain the genera *Gordonia*, *Rhodococcus* and *Nocardia* [2–6]. This review discusses the significance and inter-relationships of some of the recently discovered or re-classified members of the *Nocardia*, *Rhodococcus* and *Gordonia*.

The medical, veterinary, environmental and industrial significance of members of the genera *Nocardia* [7–9] and *Rhodococcus* [5,10–12] have been extensively reviewed. In the last 5 years the number of new species assigned to these two genera has almost doubled. Furthermore, (i) several new genera (e.g., *Gordonia*, *Tsukamurella*) and species have been recognized to account for observed variations among newly characterized isolates; (ii) a number of actinomycete species have been reclassified; (iii) many environmental rhodococcal isolates have been biochemically characterized but not formally described and validly named; (iv) the unusual genome topology found in some of these species has either not been assessed for its taxonomic value, not determined or both and (v) species variation among members of the genus *Rhodococcus* may require formal recognition of additional genera within this family.

This review summarizes what is known about the recently discovered and reclassified species within the genera *Nocardia*, *Rhodococcus* and *Gordonia* paying particular attention to the criteria outlined above. The discussion of genome characteristics will use the phylogenetic relationship of *Rhodococcus* to *Nocardia* and *Gordonia*, as a framework to determine if *Rhodococcus* needs to be divided into further genera. This revised phylogeny will then be used as a framework to discuss what is known about members of the genus *Rhodococcus* at the whole genome level, the genetics of their cell wall composition/biosynthesis and the genetics of xenobiotic degradation, to propose genetic studies aimed at providing further information for the clarification of relationships within the genus *Rhodococcus*.

Some of the innovative technologies that have driven the advance of bacterial genomics during the last decade have been reviewed [13], as have the genomic approaches to bacterial classification, typing and evolution [14,15]. The circular genome paradigm has been used as the basis of a whole genome model devised as a framework for the analysis of DNA fingerprinting gel patterns [14]. This model is extended in this review to include the linear genome topologies that exist in some actinomycete genera. The phylogeny of the genus *Rhodococcus*, together with other closely related actinomycete genera [16] is used here as the basis for analysis at the whole genome level of the poorly characterised members of the genus *Rhodococcus*, by listing what is known about genome organization in the closely related streptomyces [17], mycobacteria [18], corynebacteria [19] and amycolata [20]. This approach may have potential value as a model for analysis at the whole genome level of other bacterial taxa.

2. Phylogeny of the genera *Gordonia*, *Nocardia*, *Rhodococcus* and related genera

With the advent of 16S rRNA sequencing, a new hierarchic classification system, *Actinomycetales* classis nov., was proposed [2,20]. Included in this class are the bacteria that form persistent filamentous hyphae and complex mycelial structures (e.g., *Streptomyces* and *Nocardia*), generating spore bodies, motile or non-motile spores and which are capable of fragmenting to produce coccal (e.g., *Rhodococcus* and *Dietzia*) or rod (e.g., *Mycobacterium* and *Corynebacterium*)-shaped forms during their life cycles. The 16S rRNA-based phylogenetics [20] and the 100 nt 23S rRNA insertion

shared by all *Actinomycetales* [21] have enabled the systematics of this group, that were previously based mainly on morphological variation, to be clarified [20].

2.1. Genera recognized currently within the mycolic acid containing actinomycetes

Within the *Actinomycetales*, the mycolic acid containing genera (including some of the *Nocardiaceae*)[1] form a clade that lies between the *Frankiaceae* (e.g., *Geodermatophilus obscurus* and *Blastococcus aggregatus*) and the *Actinoplanaceae* (e.g., *Actinoplanaceae philippinensis*) [20]. The type species of the genera originally included in the *Nocardiaceae*, i.e., *Nocardia* and *Rhodococcus* are *Nocardia asteroides* [22] and *Rhodococcus rhodochrous* [23]. Since then a number of other mycolic acid containing genera have been described (or reassigned) that are phylogenetically related to *Nocardia* and *Rhodococcus* spp. (Fig. 1 and number of species shown in Tables 1 and 2 in parentheses). These currently include *Tsukamurella* (1) [24–26], *Dietzia* (2) [3], *Corynebacterium* (3) [19], *Williamsia* (1) [27], *Turicella* (1) [28], *Mycobacterium* (3) [18,29], *Gordonia* (13) [30,31] *Skermania* (1) [32], in addition to *Rhodococcus* (22 and 12 unnamed) and *Nocardia* (30) [2,4–6]. The proposed placement of the genera *Mycobacterium* and *Corynebacterium* into this family has not been entirely resolved in the literature (see [12,17,20,29,33]. It has been suggested that the genus *Nocardia* radiates from a rhodococcal ancestor [5], although the inter-relationships and placement of some of the species within these two genera is very unclear and largely unexplored.

2.2. Reclassified actinomycete species

An important part of this review will focus on the genomic studies available that may help to clear up the blurred distinction between some members of the genus *Rhodococcus*. In order to identify those isolates that have not been comfortably placed as *Nocardia* or *Rhodococcus* spp., the high numbers of reassignments of isolates from the genus *Nocardia* to the genus *Rhodococcus*, *Amycolatopsis*, *Gordonia* or *Skermania* are shown in Table 3. The species names shown have all been used in publications describing studies on these organisms, but the new names are those that are now generally accepted. Most of the species have been moved from the genus *Nocardia* into the genus *Rhodococcus*. Species of industrial importance are classified particularly unclearly (e.g., Table 3: *Nocardia corallina* is now known as either *Rhodococcus corallinus* or *Gordonia rubripertinctus*; *R. globerulus* was previously named *Acinetobacter* sp6, *Nocarida corynebacterium* or *Corynebacterium* sp. MB1; *Nocardia lactamdurans* is now *Amycolatopsis lactamdurans* and *Nocardia italica* is *Streptomyces griseus* subsp. *griseus* (e.g., {actinomycete expression systems} [34]). When reading the current review it should be kept in mind that: (i) the species with the current name of *Rhodococcus ruber* was previously named *Nocardia ruber or Gordonia terrae*; (ii) the aim of the summary of reassignments presented in this review is to provide a guide; (iii) the identification of most (if not all) reassignments of species within the genus *Rhodococcus* highlights the need for extreme caution when reading publications in this area and (iv) even though the main focus of this review is on the genus *Rhodococcus*, the phylogeny of the genera *Gordonia* and *Nocardia* have been included because of the large number of taxonomic reassignments.

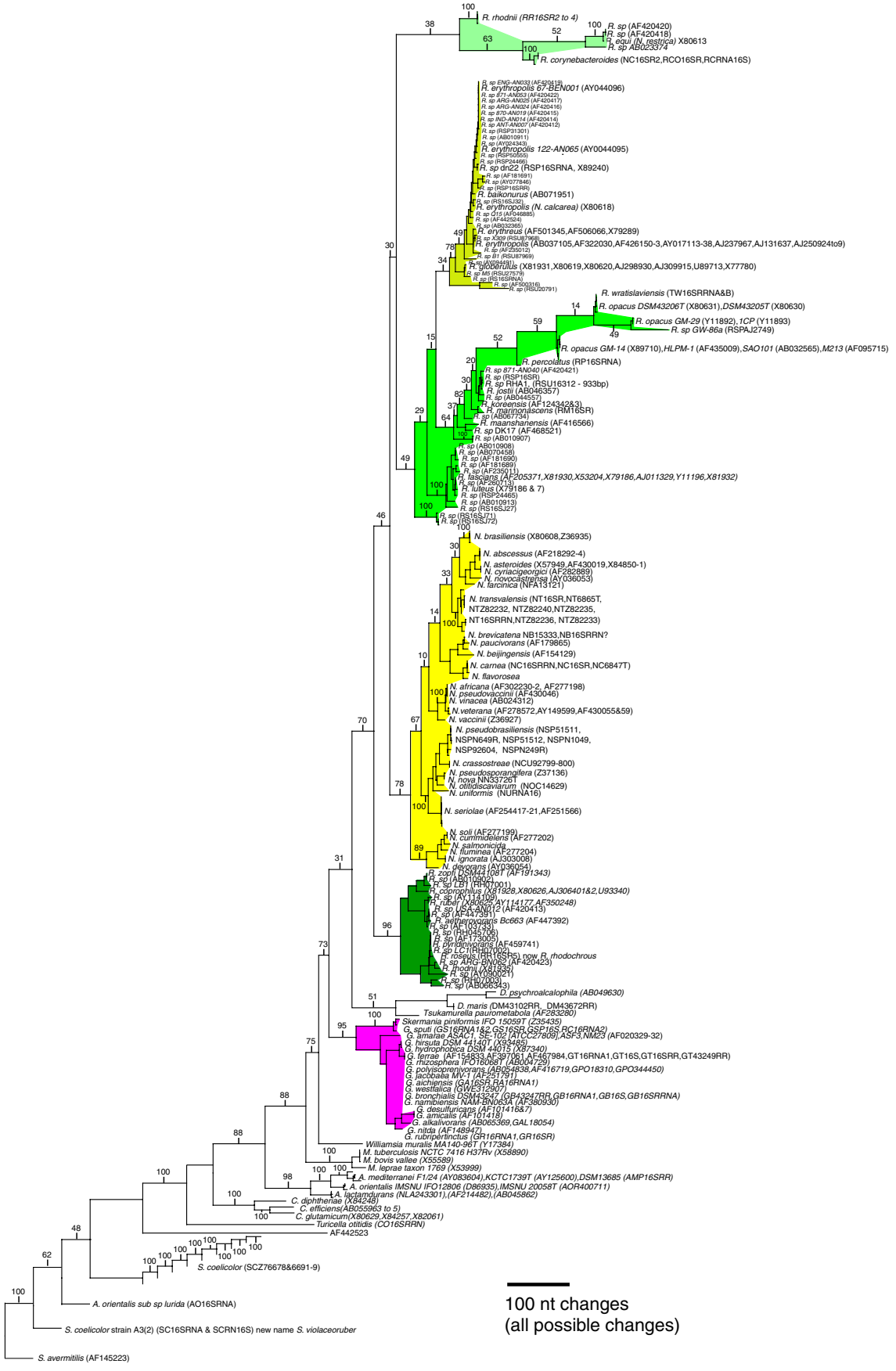
2.3. The genus Nocardia

Bovine farcy was the first reported clinical disease caused by an aerobic actinomycete isolate described in 1888 by the French veterinarian Edmond Nocard which he characterized and named *Nocardia farcinica* in 1889. It was formally named as *N. asteroides* in 1896 ([7] and references therein). From the end of the 19th century until the mid 1990s, classification and identification of members of this genus was generally restricted to the *N. asteroides* complex which includes *N. asteroides*, *N. farcinica* and *Nocardia nova* [7]. Often an isolate was identified only as far as whether it belonged to this complex, and attempts at further speciation were hindered by a lack of suitable distinguishing characters. Some isolates were identified occasionally as *N. brasiliensis*, *N. transvalensis* and *N. otitidiscaviarum* [7,35–37]. The clinical importance of *N. nova* and *N. brasiliensis* has only been demonstrated recently [35, 36] and this *N. asteroides* complex (Fig. 1 and Table 1) has now been expanded to contain 30 species [38]. However, it must be emphasised that the medical, veterinary and environmental significance of most of the more recently designated *Nocardia* species are still largely unknown.

2.4. The genus Rhodococcus

The genus *Rhodococcus* was first proposed by Zopf [39] in 1891 to describe two species of red-pigmented bacteria and was later viewed as a coherent taxon encompassing species of veterinary, clinical and industrial importance [40]. Since then many environmental isolates have been placed in this genus. Table 2 lists the current valid *Rhodococcus* species and also isolates that have not been formally classified but probably deserve species status. Many of these have been shown to possess novel metabolic functions in their abilities to degrade chemically diverse xenobiotics. One of the aims of this review

[1] *Nocardiaceae* includes *Nocardia* and *Rhodococcus*.


R. rhodnii (RR16SR2 to 4)
R. sp (AF420420)
R. sp (AF420418)
R. equi (N. restricta) X80613
R. sp AB023374
R. corynebacteroides (NC16SR2,RCO16SR,RCRNA16S)
R. erythropolis 67-BEN001 (AY044096)
R. erythropolis 122-AN065 (AY0044095)
R. sp dn22 (RSP16SRNA, X89240)
R. baikonurus (AB071951)
R. erythropolis (N. calcarea) (X80618)
R. erythreus (AF501345,AF506066,X79289)
R. erythropolis (AB037105,AF322030,AF426150-3,AY017113-38,AJ237967,AJ131637,AJ250924to9)
R. globerulus (X81931,X80619,X80620,AJ298930,AJ309915,U89713,X77780)
R. wratislaviensis (TW16SRRNA&B)
R. opacus DSM43206T (X80631),DSM43205T (X80630)
R. opacus GM-29 (Y11892),1CP (Y11893)
R. sp GW-86a (RSPAJ2749)
R. opacus GM-14 (X89710),HLPM-1 (AF435009),SAO101 (AB032565),M213 (AF095715)
R. percolatus (RP16SRNA)
R. sp 871-AN040 (AF420421)
R. sp RHA1, (RSU16312 - 933bp)
R. jostii (AB046357)
R. koreensis (AF124342&3)
R. marinonascens (RM16SR)
R. maanshanensis (AF416566)
R. sp DK17 (AF468521)
R. taiyangi (AF205371,X81930,X53204,X79186,AJ011329,Y11196,X81932)
R. luteus (X79186 & 7)
N. brasiliensis (X80608,Z36935)
N. abscessus (AF218292-4)
N. asteroides (X57949,AF430019,X84850-1)
N. cyriacigeorgici (AF282889)
N. farcinica (NFA13121)
N. transvalensis (NT16SR,NT6865T, NTZ82232, NTZ82240,NTZ82235, NT16SRRN,NTZ82236, NTZ82233)
N. brevicatena NB15333,NB16SRRN?
N. paucivorans (AF179865)
N. beijingensis (AF154129)
N. carnea (NC16SRRN,NC16SR,NC6847T)
N. flavorosea
N. africana (AF302230-2, AF277198)
N. pseudovaccinii (AF430046)
N. vinacea (AB024312)
N.veterana (AF278572,AY149599,AF430055&59)
N. vaccinii (Z36927)
N. pseudobrasiliensis (NSP51511, NSPN649R, NSP51512, NSPN1049, NSP92604, NSPN249R)
N. crassostreae (NCU92799-800)
N. pseudosporangifera (Z37136)
N. nova NN33726T
N. otitidiscaviarum (NOC14629)
N. uniformis (NURNA16)
N. seriolae (AF254417-21,AF251566)
N. soli (AF277199)
N. cummidelens (AF277202)
N. salmonicida
N. fluminea (AF277204)
N. ignorata (AJ303008)
N. devorans (AY036054)
R. zopfii (DSM441081 (AF191343)
R. sp LB1 (RH07001)
R. coprophilus (X81928,X80626,AJ306401&2,U93340)
R. sp (AY114109)
R. rubber (X80628,AY114177,AF350248)
R. sp USA-AN012 (AF420413)
R. sp (AF447391)
R. aetherovorans Bc663 (AF447392)
R. sp (RH07002)
R. sp (AF173005)
R. pyridinivorans (AF459741)
R. sp LC1(RH07004)
R. roseus,(RR16SR5) now R. rhodochrous
R. sp ARG-BN062 (AF420423)
R. rhodnii (X81932)
R. sp (AY0900021)
R. sp (RH07003)
R. sp (AB066343)
D. psychroalcalophila (AB049630)
D. maris (DM43102RR, DM43672RR)
Tsukamurella paurometabola (AF263280)
Skermania piniformis IFO 15059T (Z35435)
G. sputi (GS16RNA1&2,GS16SR,GSP16S,RC16RNA2)
G. amarae ASAC1, SE-102 (AY027809),ASF3,NM23 (AF020329-32)
G. hirsuta DSM 44149T (X93465)
G. hydrophobica DSM 44015 (X87340)
G. ferrae (AF154833,AF397061,AF467984,GT16RNA1,GT16S,GT16SRR,GT43249RR)
G. rhizosphera IFO16066T (AB004729)
G. polyisoprenivorans (AB054838,AF416719,GPO18310,GPO344450)
G. jacobaea MK-1 (AF251791)
G. aichiensis (GA16SR,RA16RNA1)
G. westfalica (GWE3129071)
G. bronchialis DSM43247 (GB43247RR,GB16RNA1,GB16S,GB16SRRNA)
G. namibiensis NAM-BN0634A (AF380930)
G. desulfuricans (AF101418&7)
G. amicalis (AF101418)
G. alkalivorans (AB065369,GAL18054)
G. nitda (AF148947)
G. rubripertinctus (GR16RNA1,GR16SR)
Williamsia muralis MA140-96T (Y17384)
M. tuberculosis NCTC 7416 H37Rv (X58890)
M. bovis vallee (X55589)
M. leprae taxon 1769 (X53999)
A. mediterranei F124 (AY083604),KCTC1739T (AY125600),DSM13685 (AMP16SRR)
A. orientalis IMSNU IFO12806 (D86935),IMSNU 20058T (AOR400711)
A. lactamdurans (NLA243301),(AF214482),(AB045862)
C. diphtheriae (X84248)
C. efficiens(AB055963 to 5)
C. glutamicum(X80629,X84257,X82061)
Turicella otitidis (CO16SRRN)
AF442523
S. coelicolor (SCZ76678&6691-9)
A. orientalis sub sp lurida (AO16SRNA)
S. coelicolor strain A3(2) (SC16SRNA & SCRN16S) new name S. violaceoruber
S. avermitilis (AF145223)
100 nt changes
(all possible changes)

is to summarize what is known about these isolates and to suggest how these might be formally classified.

The existence of six distinct groups (clades) within the genus *Rhodococcus* was suggested by Rainey et al. [5] and Goodfellow et al. [12]. The phylogenetic data presented here suggests that there may be as many as nine distinct groups (Fig. 1). Furthermore members of the *Gordonia*, *Skermania*, *Tsukamurella*, *Dietzia*, *Williamsia* and *Turicella* may in fact separate some of these groups. The four groups that are of particular taxonomic interest are those containing *R. equi*, *Rhodococcus rhodnii*, *R. corynebacteroides* and *R. zopfii*. It is still not clear whether *R. equi* should be placed in the genus *Nocardia*, to which it may be more closely related than to other members of the genus *Rhodococcus*, or whether it merits recognition as a genus in its own right. The latter proposal is supported by evidence suggesting that *R. equi* isolates from veterinary, human and environmental

Table 1
Characteristics of members of the genus *Nocardia*

Species	Diagnosis	Site	Authors
N. abscessus	Endoprosthesis; immunosuppressed; leg infection; neotype of *N. asteroides*	Abscess joint/fibula; leg drainage; Tsukamura	[184]
N. africana (4)	Pulmonary nocardiosis	Sputum 400 sputa tested	[185]
N. asteroides (4)	Nocardiosis	Various	[4–6]
N. beijingensis	Environmental source	Soil	[186]
N. brasiliensis (2)	Mycetoma	Various	[4,5,53]
N. brevicatena (2)	Tuberculosis	Sputum	[4,6]
N. carnea	Environmental	Various	[4–6]
N. caishijiensis	Environmental source	Soil	[187]
N. cerradoensis	Environmental source	Soil	[188]
N. crassostreae (3)	Nocardiosis of fish	Oysters	[189]
N. cyriacigeorgici	Chronic bronchitis	Bronchial secretions	[190]
N. cummidelens	Environmental source	Rubber-water	[191]
N. devorans	Unavailable	Clinical isolate	na
N. farcinica	Nocardiosis	Various	[4–6]
N. flavorosea	Environmental source	Soil	[192]
N. fluminea	Environmental source	Soil	[191]
N. ignorata	New species	QAP for mycobacteria	[193]
N. nova	Nocardiosis	Blood culture	[6,194]
N. novocastrensa	Unavailable	Clinical isolate	AY036054
N. otitidiscaviarum	Pulmonary nocardiosis	Sputum	[4–6]
N. paucivorans	Lung disease-chronic	Sputa and bronchial secretions	[195]
N. pseudobrasiliensis	Human mycetoma; Nocardiosis	Abscess-leg/buttocks/ brain; sputum; blood	[6]
N. pseudosporangifera	Environmental source	Soil	[151]
N. pseudovaccinii	Environmental source	Soil	[196]
N. salmonicida	Nocardiosis of fish	Salmon	[197]
N. seriolae	Nocardiosis of fish	Fish	[4,6]
N. soli	Environmental source	Water	[191]
N. transvalensis	Mycetoma	Various	[4,6,53]
N. uniformis	Environmental source	Soil	[198]
N. vaccinii	Plant	Stem galls on blueberry	[4]
N. veterana	Pulmonary nocardiosis; human mycetoma	Bronchial lavage	[38,194,199–201]
N. vinacea	Environmental source	Soil	[202]

Fig. 1. The 16S rRNA phylogenetic tree for 217 actinomycete isolates from the genera *Dietzia*, *Gordonia*, *Rhodococcus*, *Nocardia*, *Skermania*, *Tsukamurella*, *Turicella* and *Williamsia*, including for comparison 11 isolates from the closely related actinomycete genera of *Amycolatopsis*, *Corynebacterium*, *Mycobacterium* and *Streptomyces*. The comparative overall phylogeny is shown – more detailed phylogenies can be found in the references listed in Tables 1 and 2 for the genera *Nocardia*, *Rhodococcus* and *Gordonia* (complete species complements for these three genera are shown). The 16S rRNA sequences available in GenBank (1400–1550 nt unless stated otherwise) were used to construct 100 bootstrapped sequence alignments with ClustalW [168], 100 distance matrices with DNAdist [169,170], 100 phylogenetic trees with Neighbor [169,170] (using Neighbor-Joining or UPGMA options) to obtain 1 phylogenetic tree with Consensus [169,170] at bootstrap percentages shown on branches with either Neighbor-Joining or UPGMA options. The 16S rRNA sequence for *R. corallinus* was not available in the databases (see *Gordonia rubripertinctus* to which it has been reclassified). The relationship of the species is shown by the horizontal lines proportional to the number of nt changes in the 16S rRNA (the scale is shown by the bar). The colored shading corresponds to the shading in Tables 5–7 for *Rhodococcus* (four shades of green for each clade), *Nocardia* (yellow), *Gordonia* (pink) and other genera (grey). The GenBank accession numbers are shown next to the species names (strain numbers and original phylogenetic studies are listed in Tables 1 and 2).

Table 2
Characteristics of members of the *Corynebacterineae*

Genus	Species	Significance	Site	Authors or GenBank
Amycolatopsis	*A. lactamdurans*	Cephamycin production	Industry	[157]
	A. mediterranei (4)	Rifamycin SV production	Industry	[203]
	A. orientalis (4)	Vancomycin production	Industry	[204–206]
Corynebacterium	*C. diphtheriae*	Diphtheria	Soil?	[207–209]
	C. efficiens	Glutamic-acid-producer	Soil and vegetables	[209,210]
	C. glutamicum[a]	Glutamic-acid-producer	Industry	[5,209]
Dietzia	*D. maris*[a]	Bacteremia, hip prosthesis	Human	[3]
	D. psychralcaliphila	Psychrophilic alkaliphile	Water	[211]
Gordonia	*G. aichiensis*	Pneumonia	Human sputum	[31]
	G. alkalivorans	Alkane degradation	Soil	[212]
	G. amarae[a]	Pneumonia	Human sputum	[31]
	G. amicalis	Dibenzothiophene-desulphurization	Soil	[213]
	G. bronchialis[a]	Infection in various sites	Human	[5,19,30,31,214]
	G. desulfuricans	Benzothiophene-desulphurization	Soil	[215]
	G. hirsuta	Environmental	Biofilter	[216]
	G. hydrophobica	Environmental	Biofilter	Unpublished
	G. jacobaea	Production of canthaxanthin	Food industry	[217,218]
	G. namibiensis	Nitrile metabolism	African sand	[219,220]
	G. nitida	3-Ethylpyridine/3-methylpyridine degradation	Water	[221]
	G. polyisoprenivorans	Isoprene rubber degradation	Rubber	[222,223]
	G. rhizosphera	Mangrove rhizosphere	Mangrove	[224]
	G. rubripertinctus (2)	Alkane oxidization	Soil	[5,19,31,105,225]
	G. sputi	Bacteremia, endocarditis	Human	[31,226–228]
	G. terrae[a]	Human immunocompromised infection	Human/ environment	[19,30,226,227]
	G. westfalica (2)	Rubber degradation	Rubber	[229]
Mycobacterium	*M. bovis*	Bovine tuberculosis	Bovine	[230]
	M. leprae	Leprosy	Human	[231]
	M. tuberculosis (2)	Tuberculosis	Human	[230,232,233]
Rhodococcus	*R. aetherovorans strain Bc663*	Methyl-*t*-butyl ether-degrading	Environmental	Unpublished
	R. coprophilus[a]	Pollution of rivers by faeces	Animal faeces	[5,19,234,235]
	R. corynebacteroides[a]	Human arthritis/vermilion formation	Water	[3,104,176,236]
	R. equi[a] *(N. restrica)*	Equine pneumonia	Horses	[5,19,86,87]
	R. erythreus[a]	Mercury biodegradation	Soil	[3,5,19]
	R. erythropolis[a] *(N. calcarea)*	2,4-Dinitrophenol-degradation; dibenzothiophene desulfurization	Soilticks	[5,19,30,117,237–245]
	R. fascians[a]	Phytopathogen	Plants	[5,19,30,82,83,158, 159,240,246]
	R. globerulus[a] (2)	Polychlorobiphenyl-degradation	Soil	[5,19,247–250]
	R. jostii	Medieval grave	Soil	[251]
	R. koreensis	2,4-Dinitrophenol-degradation	Soil	[252]
	R. luteus[a]	Endophthalmitis	Environment	[5,19,44,246,253, 254]
	R. maanshanensis	Unknown	Soil	[255]
	R. marinonascens	Environmental	Marine sediment	[5]
	R. opacus SAO101	Degradation of aromatics	Soil	[256]
	R. opacus M213	Degradation of aromatics	Soil	[257]
	R. opacus HL PM-1	Degradation of aromatics	Soil	Unpublished
	R. opacus GM-14	Degradation of aromatics	Soil	[258]
	R. opacus 1CP	Degradation of aromatics	Soil	[259]
	R. opacus GM-29	Degradation of aromatics	Soil	[259]
	R. opacus[a] *DSM43205T*	Degradation of aromatics	Soil	[5,260]
	R. opacus[a] *DSM43206T*	Degradation of aromatics	Soil	[5,260]
	R. percolatus	2,4,6-Trichlorophenol degradation	Percolator	[261]
	R. pyridinivorans	Biphenyl degradation	Industrial waste water	[262]; AF459741
	R. pyridinivorans strain PA	Benzothiozole degradation	Environment	[263]
	R. rhodnii[a] (*RR16SR2–4*)	Pulmonary diseases/insect symbiont	Insects	[5,264,265]
	R. rhodnii[a] (*X81935*)	Pulmonary diseases/insect symbiont	Insects	[19,264,265]

Table 2 (*continued*)

Genus	Species	Significance	Site	Authors or GenBank
	R. rhodochrous[a] (formerly *R. roseus*) (3)	Immunocompromised infection; hexahydro-1,3,5-Trinitro-1,3,5-triazine; 2-ethoxyphenol/4-methoxybenzoate degradation; Dimethylsulfide (DMS) production	Human/environmental	[5,11,19,45,173, 266–270]
	R. ruber[a]	Degradation of aromatics	Soil	[5,19,271]
	Rhodococcus sp. *LB1*	Toluene degradation	Compost biofilter	[272]
	Rhodococcus sp. *122-AN065*	Nitrile metabolizing	Deep-sea sediment-5425m	[219,273]
	Rhodococcus sp. *67-BEN001*	Nitrile metabolizing	Deep-sea sediment-6475m	[219,273]
	Rhodococcus sp. *870-AN019*	Nitrile metabolizing	Deep-sea sediment-1151m	[219]
	Rhodococcus sp. *871-AN040*	Nitrile metabolizing	Deep-sea sediment-1521m	[219]
	Rhodococcus sp. *871-AN053*	Nitrile metabolizing	Deep-sea sediment-1521m	[219]
	Rhodococcus sp. *ANT-AN007*	Nitrile metabolizing	Antarctic lake sediment	[219]
	Rhodococcus sp. *ARG-AN024*	Nitrile metabolizing	Subtropical soil	[219]
	Rhodococcus sp. *ARG-AN025*	Nitrile metabolizing	Subtropical soil	[219]
	Rhodococcus sp. *ARG-BN062*	Nitrile metabolizing	Sub-tropical soil	[219]
	Rhodococcus sp. *B1*	Sulfur oxidation	Soil	[116]
	Rhodococcus sp. *DK17*	Monocyclic aromatic hydrocarbon degradation	Soil	[274]
	Rhodococcus sp. *dn22*	Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) biodegradation	Environmental	[275]
	Rhodococcus sp. *ENG-AN033*	Nitrile metabolizing	Peat	[219]
	Rhodococcus sp. *GW-86a*	Biphenyl degradation	Soil	[276]
	Rhodococcus sp. *IND-AN014*	Nitrile metabolizing	Mangrove mud	[219]
	Rhodococcus sp. *LC1*	Toluene degradation	Compost biofilter	[272]
	Rhodococcus sp. *M5*	Biphenyl/chlorobiphenyl degradation	Contaminant	[277]
	Rhodococcus sp. *Q15*	Alkane degradation	Ontario lake sediment	[103]
	Rhodococcus sp. *RHA1*	Polychlorinated biphenyl degradation	Contaminated soil	[278–280]
	Rhodococcus sp. *USA-AN012*	Nitrile metabolizing	Cultivated soil	[219]
	Rhodococcus sp. *X309*	Sulfur oxidation (ATCC 55309)	Soil	[116]
	R. wratislaviensis (2)	Alkanesulfonate degradation	Soil	[281,282]
	R. zopfii DSM44108(T)	Toluene-degradation	Bioreactor	[262]
	R. zopfii[a] *ATCC51349T*	Toluene-degradation	Bioreactor	[19,283,284]
Skermania	*S. piniformis* (3)	Foaming	Activated sludge	[32,285,286]
Streptomyces	*S. avermitilis* (1)	Avermectin production	Soil	[287]
	S. coelicolor (11)	Antibiotic production	Soil	[162,288–291]
Tsukamurella	*T. inchonensis (X85955)*	Human infection	Clinical material	[292]
	T. paurometabola[a]	Human infection	Human	[5,25,26,292–295]
	T. pulmonis (X92981)	Human lung infection	Sputum	[295]
	T. tyrosinosolvens (AF475083)	Human infection/chronic lung infection	Blood culture	[294]
Turicella	*T. otitidis*[a]	Otitis media	Human middle ear	[28]
Williamsia	*W. maris*	Environmental	Deep-sea	[254]
	W. muralis	Environmental	Day care centre	[27]

Included for comparison are some members of the non-mycolic acid containing genera *Amycolatopsis* and *Streptomyces*.
[a] Cited in the corresponding GenBank entry by one or more of the following references [3,5,19,30,31].

sources are phylogenetically very diverse [41–44]. It also appears that *R. rhodnii* is more closely related to the genus *Tsukamurella* than it is to other rhodococci.

Among the *Rhodococcus* species that have been analysed (Fig. 1) there are three species that have 16S rDNA sequences acquired from several isolates sharing the same species name but which cluster with different species. These are: (a) *R. zopfii*, one isolate of which clusters with *G. bronchialis* and the other with *R. coprophilus*; (b) *R. opacus*, where one isolate clusters with *R. jostii* but another seven with *R. koreensis* and two others with *R. wratislaviensis*; (c) *R. rhodnii*, one isolate of which clusters closest to *T. paurometabola* and the other clusters with *R. rhodochrous*. In each case, one or more of these isolates cluster with a member of the same previously described species, but the others cluster

Table 3
Recently reclassified *Corynebacterineae*

Previous name	Current name	Reference
Nocardia opaca	*Rhodococcus opacus*	[260,296,297]
Nocardia lactamdurans	*Amycolatopsis lactamdurans*	[34,157]
Nocardia orientalis	*Amycolatopsis orientalis*	[140]
Nocardia mediterranei	*Amycolatopsis mediterranei*	[122,298,299]
Nocardia italica	*Streptomyces griseus* subsp. *griseus*	[34,300]
Nocardia piniformis	*Skermania piniformis*	[32]
Nocardia corallina or *Rhodococcus corallinus* or *Gordonia rubropertincta*	*Gordonia rubripertinctus*[a]	[105,106,225,271,301,302]
Nocardia ruber or *Gordonia terrae*	*Rhodococcus ruber*	[5,47]
Nocardia amarae or *Rhodococcus australis*	*Gordonia amarae*	[5,303]
Streptomyces salmonicida	*Nocardia salmonicida*	[197]
Nocardia calcarea	*Rhodococcus erythropolis*	[5]
Nocardia restrica	*Rhodococcus equi*	[5]
Nocardia corynebacterium or *Acinetobacter* sp. P6 or *Corynbacterium* sp. *MB1*	*Rhodococcus globerulus*	[5,248]
Tsukamurella wratislaviensis	*Rhodococcus wratislaviensis*	[281,282]
Rhodococcus roseus	*Rhodococcus rhodochrous*	[5]

[a] According to seven GenBank entries for this organism.

distally from these. This suggests that the taxonomic status of these strains requires reassessment.

2.5. *The genus* Gordonia

The original isolates included in this genus (then named *Gordona*) were described as opportunistic pathogens [45,46]. However, most of the 15 species that have been described so far are environmental isolates, many of which are capable of degrading xenobiotics (see Table 2). The genus is relatively homogeneous and well defined with all known species taxonomically well characterized. The members of this genus are closely related to the genera *Rhodococcus* and *Nocardia* containing a number of species that have been reassigned (Table 3): (a) from *Gordonia terrae* to *Rhodococcus ruber* [47]; (b) from *N. corallina* to *R. corallinus* or *G. rubripertinctus* and (c) from *Nocardia amarae* to *Gordonia amarae* or *Rhodococcus australis* (ambiguity still exists for b and c). The reassignment of these species demonstrates the close phylogenetic association of the genus *Gordonia* with the genera *Nocardia* and *Rhodococcus* and highlights the importance of including all three genera in the genomic analysis of the genus *Rhodococcus* that is to follow.

2.6. *Environmental* Rhodococcus *isolates not formally recognized as valid species*

A number of *Rhodococcus* spp. exist for which 16S rDNA sequences are available but their formal descriptions have not yet been published. Those that have been published are shown in Table 2 and Fig. 1 as a species designation with the GenBank accession number in parenthesis, whereas those not published at all are listed with only the GenBank accession number. The isolates with a species designation have been phylogenetically and biochemically characterized as part of the published studies but await formal species descriptions. The isolates in which linear plasmids have been found are listed in Table 4.

3. Genome analysis of *Rhodococcus* species; comparisons with other actinomycetes

The genomes of the *Nocardia* species have not yet been well characterized. Apart from rRNA, *hsp60*, *IS* and plasmid gene sequence data, the only other gene sequences available for any *Nocardia* species are that for the superoxide dismutase-encoding gene (*sod*) of *N. asteroides* strain GUH-2 [48] and the β-lactamase from *N. asteroides*, AST-1 [49]. The β-lactamase is important because infection by many *Nocardia* species requires treatment with specific antibiotics [50–53]. An improvement of the paucity of information on the structure and organization of the *Nocardia* genome may be facilitated by a summary of what is known about the genome organization of the closely related genus *Rhodococcus*, which will be the subject of the remainder of this review.

The most recent reviews on the genetics of the genus *Rhodococcus* cover genetic tools [54], applied aspects of *Rhodococcus* species genetics [10] and the cloning of genes that have environmental and clinical importance [11]. Since these reviews, the past five years have seen substantial progress on *Rhodococcus* species genetics that would benefit from a systematic appraisal. Furthermore, since no completed rhodococcal whole ge-

nome sequence was publically available at the time of writing, the best comparative genome analysis available was from gene databases (e.g., GenBank). A summary of the deposited sequence entries that have also been published in peer reviewed journals is provided here.

This section will be divided into sub-sections that discuss the basic cellular functions unique to groups within the genus *Rhodococcus* (compared to other actinomycetes) including the genetics of their cell envelope biosynthesis and virulence genes; enzymatic functions; genome structure and practical applications; and universal molecular typing methods.

3.1. Genetics of cell envelope biosynthesis in the Corynebacterineae

3.1.1. Environmental and industrial significance of cell envelope components

The cell envelope in the genera *Nocardia*, *Rhodococcus*, *Gordonia*, *Tsukamurella*, *Dietzia*, *Corynebacterium* and *Mycobacterium* contain mycolic acids, with the exception of *Corynebacterium amycolatum* [55,56], *C. atypicum* [57] and *C. kroppenstedtii* [58] which are unable to synthesize mycolic acids. The chemical structures of mycolic acids (Fig. 2) show considerable variation in the lengths of the four carbon chains, extent and positions of hydroxyl substitutions and the types of associated sugar moiety [59–62]. Unlike the nocardias most of the rhodococci (except *R. equi*, a human and veterinary pathogen) are of potential commercial significance (Table 2) based on their rich source of mycolic acid surfactants (Fig. 2) and the presence of metabolic and cometabolic biodegradative enzymes [63]. The mycolic acid containing cell envelope creates an impermeable barrier that prevents large and small molecules, including antibiotics, from readily entering the cell. The model for the organization of the cell envelope (Fig. 3) was determined mainly from studies carried out with *Mycobacterium* species [64–68], although the same model probably applies to the other mycolic acid containing genera, *Nocardia* [61,69], *Rhodococcus* [70,71] and *Corynebacterium* [72] because their cell envelopes all have similar chemical compositions (Fig. 3).

The model recognizes a number of cell surface components including (from inside to outside) the cell membrane, peptidoglycan, arabinogalactan, mycolic acid layers and capsule-like material (Figs. 2 and 3). Apart from mycolic acids with carbon chain lengths of 40–60 (which overlap the carbon chain lengths of the other genera), the presence of a hexahydrogenated menaquinone isoprene side chain represents a valuable phylogenetic marker that distinguishes members of the *Nocardia* from *Rhodococcus* and all known bacteria [73].

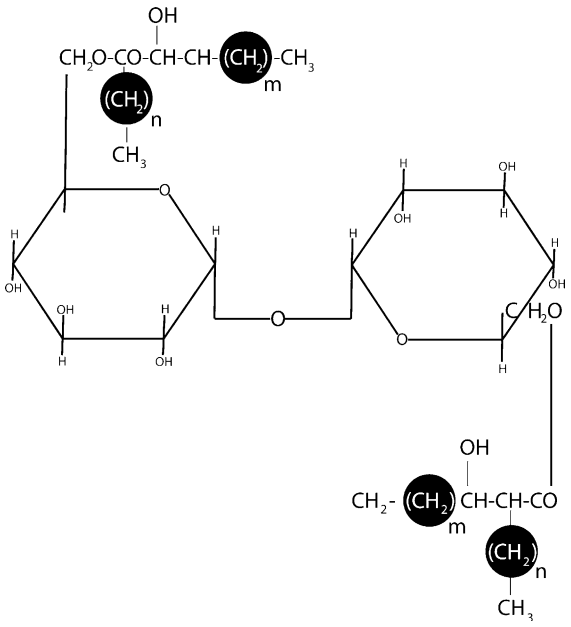


Fig. 2. The chemical structure and possible biosynthetic pathways of mycolic acids in the mycolic acid containing *Corynebacterineae*. The chemical structure of the core mycolic acid structure showing the central sugar moiety (either glucose, sucrose or mannose) from which two main carbon chains extend with the variable length CH_2 group shown as white inside a black filled circle and where $(m + n) = 22$–90 (see Fig. 3 and adapted from [71]). Both the carbon chain length and the central sugar moiety are key biosynthetic points that vary between genera.

With the availability of whole genome data it should now be possible to elucidate the genetics of the complex biochemical pathways that are thought to be involved in cell envelope biosynthesis in these organisms (see [68] for a review of cell envelope biosynthesis in *Mycobacterium* species). Even though members of these different genera share a large number of common features, it is probable that marked differences between cell envelope biosynthesis among members of the mycolic acid containing *Actinomycetales* exist. Thus, to understand what these differences might be, in addition to those shown in Fig. 6, whole genomic characterization of more representatives of the *Corynebacterineae* is urgently required.

3.1.2. Porins

Porins are a family of proteins that form channels through the outer membrane of gram-negative bacteria allowing solutes to enter the cell. They have also been detected in some of the mycolic acid producing bacteria although their current location is not well understood. The published data on the protein biochemistry, electrophysiological properties and gene sequencing of the porin familes of several *Nocardia*, *Rhodococcus*, *Corynebacterium* and *Mycobacterium*

Table 4
Chromosome/plasmid size, topology and characteristics for members of the *Corynebacterineae*

Genus and species	Strain	Plasmid	Significance	Chromosome size (Mb)	Chromosome topology	Chromosome	Chromosome	Plasmid size (Mb)	Plasmid topology	Plasmid protein	Plasmid invertron	Reference
A. mediterranei			Lethal/zygosis fertility		L?			0.0237	C			[203]
A. orientalis	G24			8.1	C							[140]
C. glutamicum	ATCC 13032		Chemical industry	3	C	N	N		C			[135,304, 305]
G. rubripertinctus	B-276	pNC40			L?			0.235	L			[271]
G. rubripertinctus	B-276	pNC10			L?			0.07	L			[271]
G. rubripertinctus	B-276	pNC20			L?			0.085	L			[271]
G. rubripertinctus	B-276	pNC30	Alkene monooxy-genase TCE degradation		L?			0.185	L			[271]
M. avium				4?	C?			0.32, 0.025, 0.14, 0.18	L			[306,307]
M. branderi				4?	C?			0.32, 0.025, 0.14, 0.18	L			[306,307]
M. celatum				4?	C?			0.32, 0.025, 0.14, 0.18	L			[306,307]
M. tuberculosis				4.4	C							[133]
M. xenopi				4?	C?			0.32, 0.025, 0.14, 0.18	L			[306,307]
N. asteroides	N3			7.3	L			0.22	L			[140]
R. equi	ATCC33701 and 103				C?			0.09	C	N	N	[86,87]
R. erythropolis		pBD2	Degredation of isopropylbenze and co-oxidation of trichloroethene		C?			0.208	L			[172,308]
R. erythropolis		pTA421	Biphenyl degradation		C?			0.5	L			[309]
R. erythropolis	MP50		Enantioselective amidase		C?			0.04	C			[310]
R. fascians	DSM20669		Avirulent phytopathogen	5.6	C	nd	nd	N	N	N	N	[159]
R. fascians	D188		Virulent phytopathogen	5.8	C	nd	nd	0.2	L	nd	nd	[159]

R. fascians	D188.2		Virulent phytopathogen	5.8	C	nd	nd	0.2	L	nd	nd	[159]
R. fascians	D188.5		Virulent phytopathogen	5.8	C	nd	nd	N	N	N	N	[159]
R. fascians	CECT3001		Avirulent phytopathogen	8	C	nd	nd	0.64	L	nd	nd	[159]
R. fascians	CECT3001		Avirulent phytopathogen	8	C	nd	nd	0.12	C	nd	nd	[159]
R. fascians	D188		Virulent phytopathogen	5.8	C	nd	nd	0.138	C	nd	nd	[159]
R. opacus	DSM3346	pHG205	H2 autotrophy		C?			0.28	L	Y	PH	[296,311]
R. opacus	DSM3346	pHG206	Cryptic		C?			0.51	L	N	N	[296,311]
R. opacus	DSM43205	pHG201	H2 autotrophy		C?			0.27	L	Y	PH	[296,311]
R. opacus	DSM43205	pHG202	Cryptic		C?			0.4	L	N	N	[296,311]
R. opacus	DSM43205	pHG203	Cryptic		C?			0.42	L	N	N	[296,311]
R. opacus	DSM 43250		Cryptic	6.4	C	nd	nd	1.6	L	nd	nd	[140]
R. opacus	DSM 43250		Cryptic	6.4	C	nd	nd	0.3	L	nd	nd	[140]
R. opacus	DSM3346	pHG204	Cryptic		C?			0.18	L	Y	Y	[296,311]
R. opacus	MR2253	pHG207	Cryptic		C?			0.225	L	Y	PH	[296,311, 312]
Rhodococcus sp.	DK17	pDK1			L?			0.388	L?			[274]
Rhodococcus sp.	DK17	pDK2	Alkylbenzene metabolism		L?			0.3395	L?			[274]
Rhodococcus sp.	DN22		RDX biodegradation					Large	C?			[313]
Rhodococcus sp.	RHA1	pRHL1	Polychlorinated biphenyl degradative genes		C?			1.1	L	Y	Y?	[278]
Rhodococcus sp.	RHA1	pRHL2	Polychlorinated biphenyl degradative genes		C?			0.45	L	Y	Y	[278]
Rhodococcus sp.	RHA1	pRHL1	Polychlorinated biphenyl degradative genes		C?			0.33	L	Y	Y?	[278]
Sp.[a] *erythraea*				8	L	Y	Y					[139,140]
St.[b] *avermitilis*			Avermectin production									[155]
St.[b] *coelicolor*	A3(2)		Antibiotic production									[154]
St.[b] *rimosus*				8	L	Y	Y	0.387	L	Y	Y	[138]

Included for comparison are some members of the non-mycolic acid containing genera *Amycolatopsis* and *Streptomyces*.

[a] *Saccharopolyspora.*

[b] *Streptomyces.*

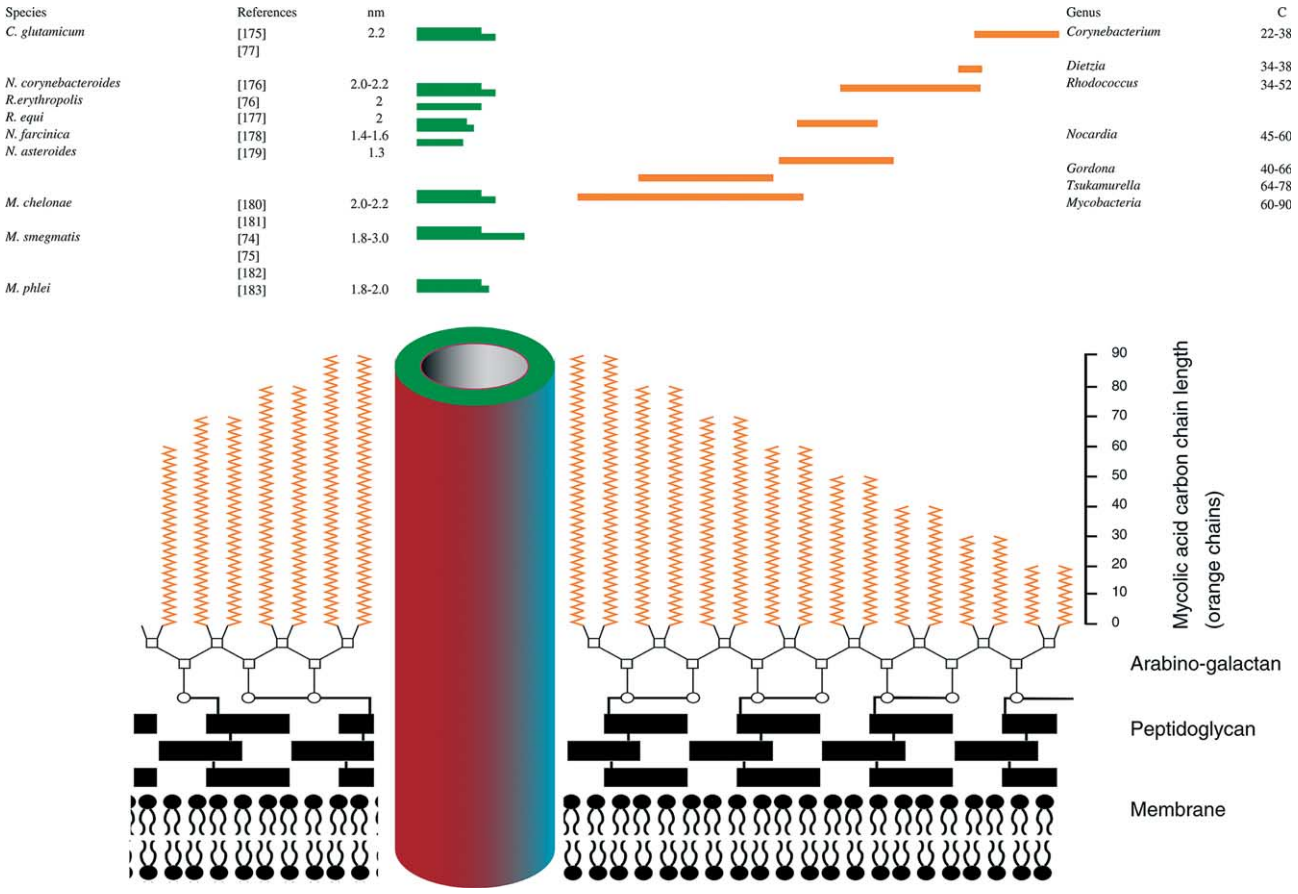


Fig. 3. Model for cell envelope composition in the mycolic acid containing *Corynebacterineae*. The model shown is based on those developed for other *Corynebacterineae* [64,67,68,71,175]. The length of the orange horizontal lines are drawn above the jagged vertical lines that represent the carbon chain lengths found in each genus of each mycolic acid linked by a sugar moiety (Fig. 2) to arabino-galactan, peptidoglycan and the cell membrane. The length of the mycolic acid carbon chain length is on the right (C†) and the orange horizontal bar indicates the carbon chain length. The red, blue and green cylinder represents the porin with the pore size indicated by a green bar immediately above the porin for each species. The respective studies are also shown. †Carbon chain length (orange bars); nm, pore diameter in nanometers (green bars) [177–183].

species are available in the sources listed in Fig. 3. The porin channel diameters of *N. asteroides* and *N. farcinica* are smaller than those of the other *Actinomycetales*, but the significance of this finding is unknown. The most completely characterized porin gene family (*MspABCD*) is that from *M. smegmatis* (Fig. 3) which shows significant homology within the family but not to other members of the *Actinomycetales* [74,75]. These data together with the available partial protein sequences of porins from *R. erythropolis* [76] and *C. glutamicum* [77] would suggest that homology only exists within porin families of each individual species and not between genera. A complete genetic characterization of porins may show that porin gene families are either species- or genus-specific for each mycolic acid containing genus, and if so this information could greatly assist in the systematics of the *Nocardiaceae* as well as increasing our understanding of their well-known antibiotic resistance.

3.2. Virulence genes

3.2.1. Chromosomal syntenic gene clusters

In the context of gene structure and evolution, chromosomal syntenic[2] gene families (Fig. 4) are those where significant homology exists between species at the primary structural, protein structural and organizational levels [78]. This section will present an example of a syntenic gene cluster in *R. fascians*, and in Section 3.3 more examples of rhodococcal syntenic gene clusters will be presented.

The *vicA* locus of *R. fascians* encodes a malate synthase (functional in the glyoxylate shunt of the TCA cycle) that is required for efficient *R. fascians* intracellular growth in plants with leafy gall formation provoked: a mutation in *vicA* prevents virulence [79]. The

[2] Of or relating to two or more genes that are present on the same chromosome regardless of whether linkage has been demonstrated between the loci.

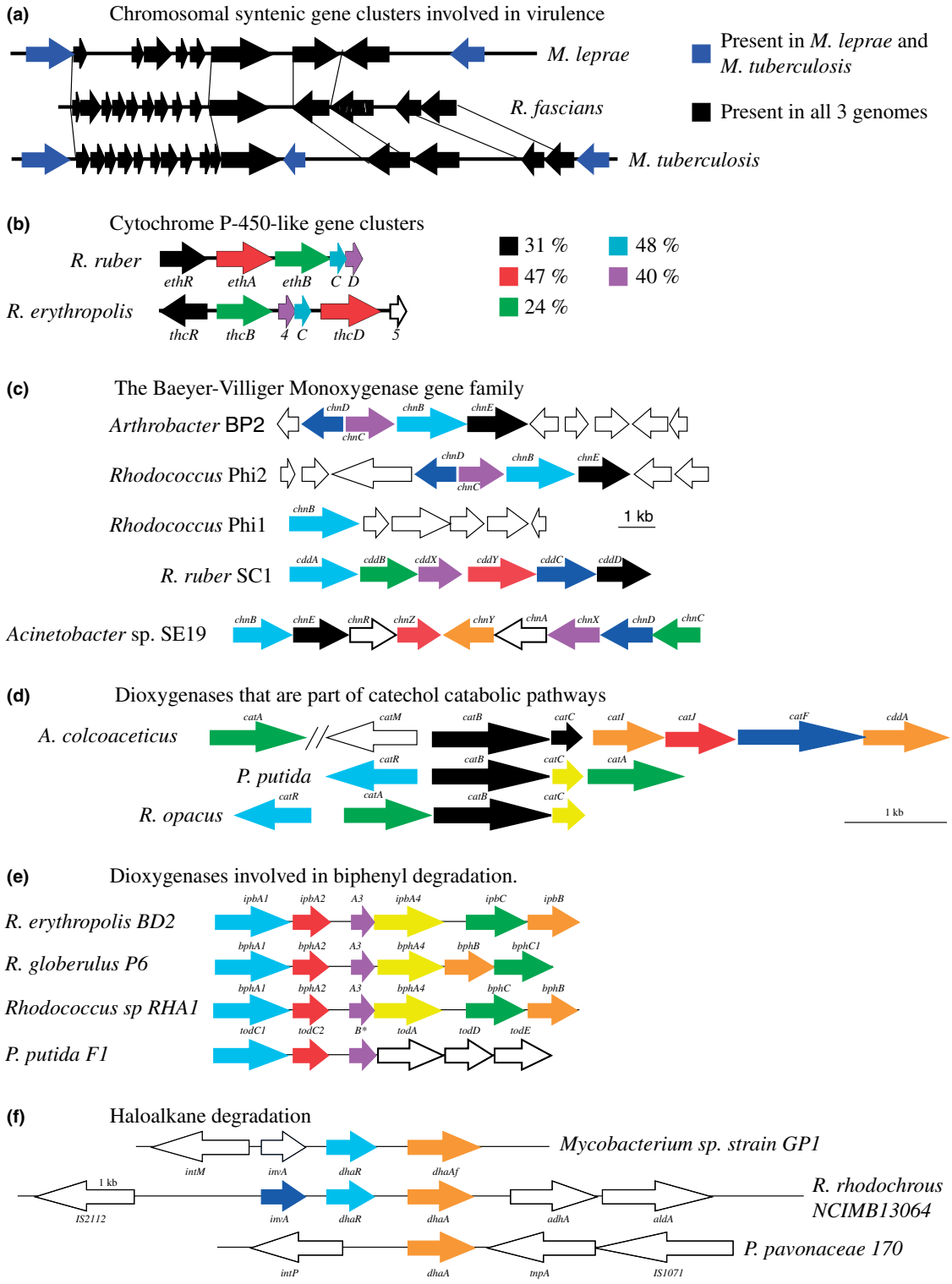


Fig. 4. Gene clusters from members of the genus *Rhodococcus* with sequence and organizational homologues in other species. Each gene cluster for the respective bacterial species is shown in boxes A–F. The enzymes encoded by the respective genes are listed in Table 5. The maps in each respective box have been modified from the respective references A [78]; B [47]; C [97,98]; D [171]; E [172] and F [173]. For each boxed set of gene clusters the encoded protein sequences that are similar between species are represented by the same colour and where available the level of similarity is indicated.

loci surrounding the *vicA* locus have syntenic regions on the chromosomes of *M. leprae* and *M. tuberculosis* [78]. The syntenic regions have been termed "persistence" loci because it has been suggested that they encode shared metabolic or nutritional factors between plant and bacteria that are required for virulence [79]. This

hypothesis proposes an adaptive role of chromosomal synteny between bacterial species.

3.2.2. Genes located on plasmids and bacteriophages

To date there has been only one report of an association of bacteriophage-like particles and virulence [80], but no bacteriophage gene sequences were found in the databases nor were any reports of gene characterization found in the literature [81].

Another gene involved in leafy gall formation is the AraC-type transcription regulatory gene *fasR* located on a 200-kb linear plasmid (Table 4) [82–84]. The *fasR* gene belongs to the AraC family with significant protein homology to other AraC-type transcriptional regulators, including those from *R. fascians*, *R. rhodochrous*, *E. coli* and *Streptomyces hygroscopicus* [84].

Virulence in *R. equi* is usually dependent on the presence of a recently sequenced 81 kb circular plasmid containing a pathogenicity island with seven virulence associated protein (*vap*) genes and the genes encoding the 15 and 17 kDa antigens [85–90]. However, chromosomal genes are also involved in the disease process because the virulence plasmid is present in only a subset of disease causing isolates [91]. In order to identify chromosomal genes, a transposon-mutagenesis system was developed and able to identify phenotypic mutants [92] involved in attenuation of *R. equi* that may be useful for vaccine development. Taking into account techniques that have been developed, including random insertion mutagenesis of transposomes [93] and a plasmid shuttle vector [94], the elucidation of virulence mechanisms in *R. equi* is reliant on the development of new genetic analysis tools.

3.3. Gene clusters involved in metabolic, degradation and industrially relevant pathways

The number of genes that code for proteins with homologues in closely and distantly related species is growing rapidly [95]. Often the homologues perform the same function. Many of these genes encode products involved in biodegradation of xenobiotic compounds. Some of the genes characterized in the genus *Rhodococcus* that have homologues in other species may be useful in taxonomic studies. Many of these proteins have been arranged into families on the basis of functional homology and they have been phylogenetically arranged in the Protein families database of alignments (Pfam; pfam.wustl.edu). Tables 5–7 list the studies characterizing gene clusters involved in catabolic pathways and Fig. 4 illustrates examples of homologous gene clusters between species. The mechanisms of action of these enzymes are described in the original papers. The following sections describe three levels of protein sequence conservation that have been observed when collating the evolutionary studies carried out on the gene clusters from rhodococci.

3.3.1. Homologues with conserved protein sequence and enzyme function

This group of proteins includes those enzymes where function and sequence is conserved across phyla (including rhodococci, other bacteria, fungi and eukaryotes) for Baeyer–Villiger monooxygenases (BVMOs), *n*-alkane catabolism, proteasomes and some housekeeping genes (e.g., RNA polymerase-*rpoB*).

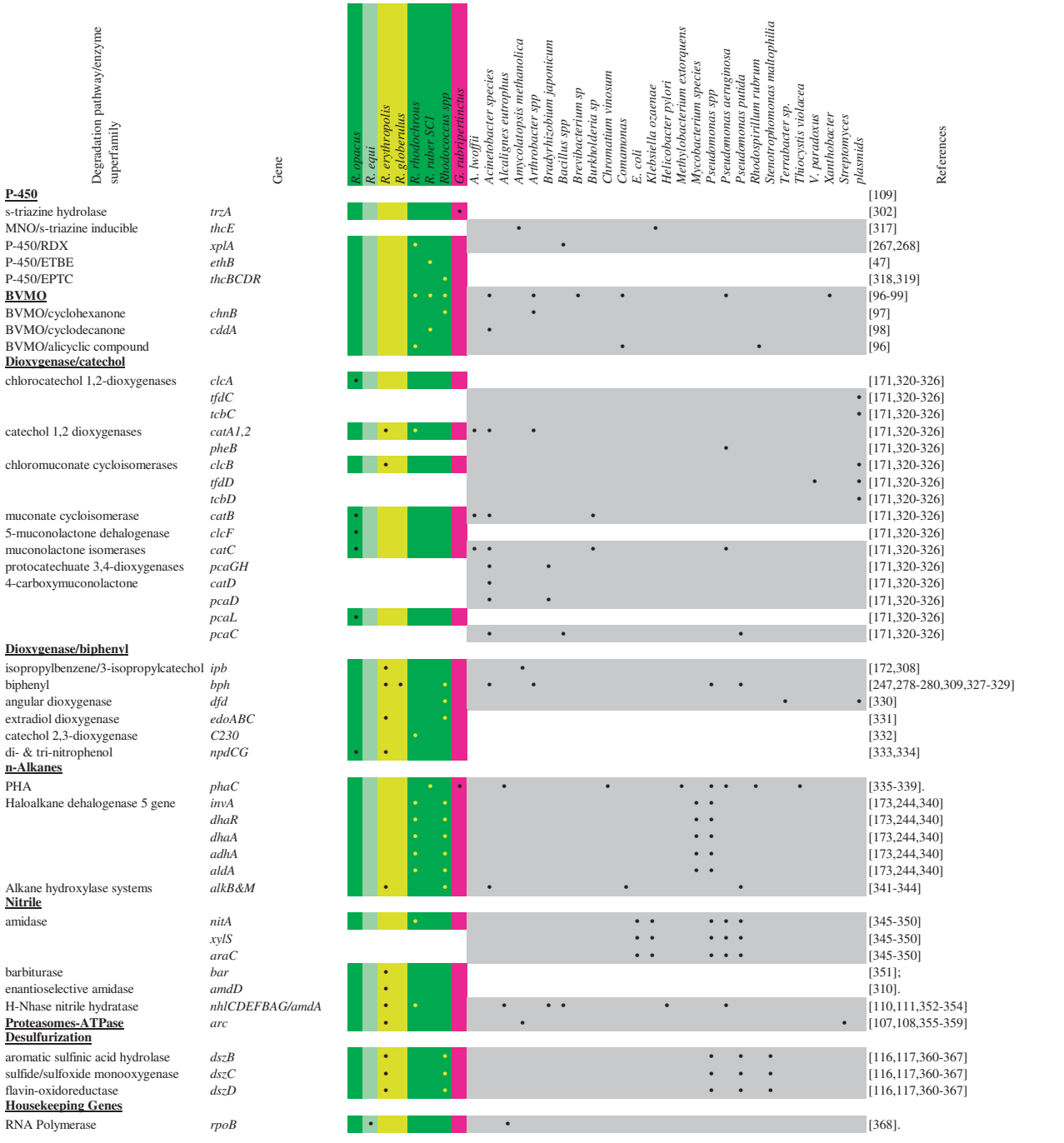
The comparison of primary sequences from the industrially valuable biocatalyst, BVMO, has so far only been done in a few diverse bacterial and fungal species, but homologues have been found [96–99].

The surface-active lipids in rhodococci [71] may in part contribute to their ability to utilize alkane as sole carbon source [100–105]. The polyhydroxyalkanoate (PHA) synthase gene (*phaCNc*) from *N. corallina* (now *R. corallinus*) has been characterized, but the level of homology with other rhodococci is unclear from the literature [106]. The haloalkane dehalogenase five gene cluster was highly conserved in six *Rhodococcus* isolates geographically separated and partially conserved between *P. pavonaceae*, *R. rhodochrous* and *Mycobacterium* sp. strain GP1 (Fig. 4 and Table 5). Alkane degradation is encoded primarily by the alkane hydroxylase gene (*alk*) that is found in various conserved forms between bacteria. The high conservation of *alkB* and the large copy number of *alkB* types between various bacterial species has made it possible to study the phylogeny of both gram-positive and gram-negative bacteria, relying on the prerequisite that isolates contain the alkane hydroxylase homologue. The method used was to design highly degenerate primers based on the various *alk* homologues found and then to use PCR to amplify the *alk* homologues.

The proteasome is an intracellular assembly that represents the major non-lysosomal proteolytic system in eukaryotes [107]. The first bacterial 20S proteasome core found was in *R. erythropolis* and contains a cylinder-shaped particle with four different subunits [108], compared to cylinder-shaped particles with two and 14 different subunits for archaea and eukaryotes, respectively [107]. Recent genome sequence comparisons of the genes encoding these energy-dependent proteases have revealed that various combinations occur in a wide range of bacteria.

Most of the above enzymes have been found in *R. rhodochrous*, *R. erythropolis*, *G. rubripertinctus* (*R. corallinus*), *R. globerulus*, *R. ruber* and undesignated *Rhodococcus* spp. (see Table 5 for exceptions) as well as bacterial, fungal and other eukaryotic species. In general they are enzymes that may be useful as alternative phylogenetic markers. The potential advantage of some

Table 5
Gene clusters from members of the genus *Rhodococcus* encoding products from enzyme families/superfamilies found in a range of bacterial species [317–368]



Abbreviations

MNO	N,N'-dimethyl-4-nitrosoaniline-dependent alcohol oxidoreductases
P-450	cytochrome P-450 monoxygenase homologues
BVMO	Baeyer-Villiger monooxygenase
RDX	(hexahydro-1,3,5-trinitro-1,3,5-triazine) military explosive
(ETBE)	gasoline pollutant ethyl tert-butyl ether
(EPTC)	herbicide S-ethyl dipropylthiocarbamate
(PHA)	Polyhydroxyalkanoic acid synthesis
3-CC	3-chlorocatechol
4-CC	4-chlorocatechol

The green pink and yellow shading represents the presence of genes in species within the *Rhodococcus*, *Gordonia* and *Nocardia* clades that have been shaded the same colors as those in Fig. 1.

The grey shading represents the presence of genes in a species other than members of *Rhodococcus* and the white corresponds to the absence of the respective genes in the corresponding species.

Table 6
Gene clusters from members of the genus *Rhodococcus* encoding proteins with sequence homology to single products in unrelated species [369–378]

Rhodococcal		Homologue		*R. opacus*	*R. fascians*	*R. globerulus*	*R. erythropolis*	*Alcaligenes eutrophus*	*Brucella abortus*	*E. coli*	*M. tuberculosis H37Rv*	*P. denitrificans*	*Pseudomonas*	*S. typhimurium*	*Human and rat*	References
Enzyme	Gene	Enzyme	*Gene/Protein*													
oxidase/reductase	*3′ of thcA*	oxidase/reductase	*3′ of thcA*				•				•					[369]
nonheme haloperoxidase	*thcF*	chloroperoxidase	chloroperoxidase				•						•			[113]
cobalamin biosynthetic	*cobKLM*	precorrin-6x reductase	CobK				•					•				[370]
cobalamin biosynthetic	*cobKLM*	corrin methylation/decarboxylation	CbiE/CbiT				•							•		[370]
ethanolamine ammonia-lyase	*eutB*	ethanolamine ammonia-lyase	*eutB*				•							•		[371]
ethanolamine ammonia-lyase	*eutC*	ethanolamine ammonia-lyase	*eutC*				•							•		[371]
unknown function	*ORF*	17-kDa protein antigen	AsnC family				•		•							[372]
unknown function	*ORF*	propionyl-CoA carboxylase	beta subunits				•								•	[373]
monoterpene epsilon-lactone hydrolase	MLH	monoterpene epsilon-lactone hydrolase	GDXG family				•									[374]
maleylacetate reductase	*macA*	aromatic compound degradation	MacA like	•												[375]
NAD-reducing hydrogenase	*hoxFUYHW*	NAD-reducing enzyme	hox-like	•					•							[376]
Hydroxylase	*hppCBKR*	3-(3-hydroxyphenyl)propionate	mhp operon			•				•						[377]
unknown function	*ORF*	enoyl-CoA hydratase	*echA1*		•						•					[378]

The green pink and yellow shading represents the presence of genes in species within the *Rhodococcus*, *Gordonia* and *Nocardia* clades that have been shaded the same colors as those in Fig. 1.

The grey shading represents the presence of genes in a species other than members of *Rhodococcus* and the white corresponds to the absence of the respective genes in the corresponding species.

Table 7
Genes from members of the genus *Rhodococcus* encoding products with industrial applications [379–392]

Application/degradation pathway & enzyme	Gene	*R. fascians*	*R. erythropolis*	*R. rhodochrous*	*R. equi*	*N. asteroides*	*A. simplex*	*C. testosteroni*	References
Steroid Production/precursor synthesis									
monooxygenase gene cluster	*kshAB*		•						[379]
3-ketosteroid Delta(1)-dehydrogenase	*ktsD/ksdD*		•	•			•	•	[380-382]
AIDS drug Crixivan: indene to indandiol			•						[383-386]
Food Production: limonene degradation									
limonene-1, 2-epoxide hydrolase	*limA*		•						[241,387-389]
limonene-1,2-diol dehydrogenase	*limC*		•						[241,387-389]
Antibiotic Resistance									
ß-lactamase	*AST-1*					•			[49]
chloramphenicol-resistance protein	*cmr*	•							[390]
rifampin resistance	*iri*				•				[391].
Pesticides				•					[236,392]

The green pink and yellow shading represents the presence of genes in species within the *Rhodococcus*, *Gordonia* and *Nocardia* clades that have been shaded the same colors as those in Fig. 1.

The grey shading represents the presence of genes in a species other than members of *Rhodococcus* and the white corresponds to the absence of the respective genes in the corresponding species.

of these enzymes (e.g., alkane degradation and BVMO) is that they perform functions or contain sequence motifs (e.g., proteasomes) that may be more specific to members of the genus *Rhododococcus* than other phylogenetic markers used currently such as 16S rRNA gene sequences.

3.3.2. Homologues with conserved protein sequences but different enzyme functions

This group includes enzymes that share protein sequence homology with each other, but have different enzyme functions. Included are those proteins that are part of large families and superfamilies (Table

5), and those where homology has only been demonstrated with one other protein from an unrelated species (Table 6).

A novel class of self-sufficient cytochrome P-450 monoxygenases have been shown to be present in many prokaryotes (including actinomycetes) and fungi [109]. Genetic organization and primary structure similarities (Fig. 4 and Table 5) for cytochrome P-450-like gene clusters were found in *R. ruber*, *R. rhodochrous*, *R. erythropolis*, *Rhodococcus sp* strains Phi1 and 2 and other diverse species, but associated with the biodegradation of different substrates. This suggests that further work is required to determine if other *Rhodococcus* species contain homologues to these gene clusters, their biodegradation capabilities and if the homologues have a role to play in the systematics of members of the genus *Rhodococcus*.

A number of genes encoding different dioxygenase enzymes (oxygenation catalysts) are involved in the biodegradation of environmental pollutants belonging to the biphenyl and catechol groups of aromatic compounds. From the literature it is unclear how many of the proteins encoded by these genes are homologues. Catechol degradation is performed in a wide range of bacterial species with sequence conservation observed in four enzyme families encoded by conserved structural gene clusters that all include at least 1 dioxygenase enzyme (Fig. 4 and Table 5). Furthermore, other phenol compounds are degraded by various gene clusters containing dioxygenase enzymes. Table 5 shows that the catechol degradation pathways may be more common in *R. opacus*-related isolates, while the biphenol degradation pathways are more common in the *R. erythropolis* related isolates.

Many *Rhodococcus* sp. can utilize nitriles as carbon and/or nitrogen sources (see Table 5), a process catalyzed by two main enzymes: (i) nitralase cleavage of the nitrile group to the corresponding acids and ammonia (amidohydralases); (ii) nitrile hydratase hydration of nitriles to amides (e.g., in *R. rhodochrous* J1 high and low molecular mass nitrile hydratase gene clusters [110,111] with significant homology between them contain cobalt or iron). Furthermore, evolutionary relationships have been identified in both prokaryotic and eukaryotic cells for the active sites of the amide bond- and peptide bond-cleaving enzymes (amidases and proteinases, respectively).

The *ideR* gene encoding the iron-dependent regulatory protein was found in both *R. erythropolis* and *R. equi* and is a homologue of the DtxR protein of *C. diphtheriae* [112]. Homologues of the LuxR family of MalT-related ATP-dependent regulators [95] have been found in *Rhodococcus* spp.: (i) herbicide biodegradation by chloroperoxidase *thcF* from *R. erythropolis* [113]; (ii) *bpdTS* encodes a response regulator and a transmembrane protein in *Rhodococcus* sp. strain M5 [114] and (iii) genes involved in the degradation of 2,4-dinitrophenol in *R. erythropolis* strain HL PM-1 [115].

The P-450-like and Nhase families include protein sequences homologous to those found in many members of *Rhodococcus* including *G. rubripertinctus* (*R. corallinus*), *R. erythropolis*, *R. rhodochrous* and *R. ruber*, but the functions of these enzymes varies (see Table 5 for the various enzyme functions reported). The dioxygenase family is mainly present in *R. opacus* and *R. globerulus* (Table 5), and Table 6 lists a number of species-specific enzyme functions. The variable functions of these protein sequence homologues may also be useful in differentiation of the four *Rhodococcus* clusters shown in Fig. 1.

3.3.3. Enzyme functions found only in Rhodococcus

The last group of enzymes are those that perform specific functions (e.g., food and drug production or sulfur decontamination) that are largely species-specific. In some cases sequence homologues have been found in single unrelated species. These functions may be useful in identifying some of the individual *Rhodococcus* species listed in Tables 6 and 7.

The desulfurization (*dsz*) genes (Table 5) are conserved among the genus *Rhodococcus* and located on a large 100–150 kb plasmid [116]. Analysis of bacterial community structure of sulfurous-oil-containing soils showed that many isolates were *R. erythropolis*, with at least one of the *dsz* genes present in each isolate [117].

There are also a number of enzymes that have been found in *Rhodococcus* species for which protein sequence homologues (up to 73%) occur in single unrelated bacterial species (Table 6). Isocitrate lyase is the first enzyme of the glyoxylate shunt which is required for the assimilation of fatty acids and acetate. It has not been determined whether the isocitrate lyase protein from *R. equi* [118] and *R. fascians* [119] are homologous, even though both genes (*aceA* and *icl*, respectively) have been sequenced [118,119]. Finally protein purification and partial protein sequencing, but no phylogenetic studies, have been performed for two proteins from *R. opacus*: (i) involved in the oxidation of L-amino acids to keto acids [120] and (ii) encoded by the 1:2-dehydrogenase gene [121].

The genes relevant to industrial applications that have been isolated and characterized from *Rhodococcus* species are listed in Table 7 and include: steroid production; production of the AIDS drug Crixivan by indene bioconversion to 1,2-indandiol; limonene degradation, the major ingredient of citrus peel essential oil; antibiotic resistance genes and pesticide degradation pathways. In the genera *Nocardia*, *Rhodococcus* and *Gordonia*, only three antibiotic resistance genes have so far been characterized (Table 7).

3.4. Genetic analysis tools

This section will first list some general methods that have been developed and then proceed to a discussion under the following headings: (i) mobile genetic elements and (ii) linear and circular chromosomes.

A transformation system has been developed in *Amycolatopsis (Nocardia) mediterranei* LBG A3136 [122]. In order to aid in the discovery of environmental bacterial species, environmental libraries were constructed using vectors capable of stably maintaining large segments of actinomycete DNA in *E. coli* [123].

3.4.1. Mobile genetic elements

The only report of insertion sequences (IS) in any *Nocardia* species (Table 8) is the 1452 bp IS*204* element of *N. asteroides* [124], which has terminal inverted repeat sequences showing high homology with those of the IS*1096* from *M. smegmatis*. The situation in the mycobacteria is complex, with the identification of 30 different insertion sequences and 56 loci with homology to insertion sequences [125,126].

One of the first characterized insertion elements in a *Rhodococcus* sp. was IS*2112* of the haloalkane degrading *R. rhodochrous*, which does not possess terminal repeats, in most cases does not duplicate upon integration into the target sequence, and is a member of the IS*110* family isolated from *M. avium* and *M. paratuberculosis* [127]. In addition to those genetic analysis tools already mentioned in Section 3.2. for *R. equi*, other developments include: (i) a 6.0-kb cryptic plasmid from *R. erythropolis* (N186/21) was used to construct a shuttle vector that could be shuttled from *E. coli* to *R. erythropolis*, *R. fascians*, *R. rhodochrous* and *R. ruber* [128]; (ii) construction of unmarked deletion mutants of the *kstD* gene using the *sacB* counter-selection system and conjugative mobilization of an *E. coli* plasmid to *R. erythropolis* [129]; (iii) transposition of IS*1415* [130] and characterization of IS*1676* from *R. erythropolis* [131] demonstrated insertion sequence homology and shuttle vector transferability, respectively, and (iv) pKA22 cryptic plasmid from the naphthalene degrading *R. rhodochrous* [132].

Therefore, the preliminary evidence reviewed above would suggest that in the mycolic acid containing group, these IS are members of related insertion element families, an observation consistent with the relationships revealed from the 16S rRNA phylogenetic comparisons of these organisms.

3.4.2. Linear and circular, genome and plasmid topologies; recombination and band pattern analysis

In order to understand the possible chromosome topologies among members of the rhodococci, *Nocardia and Gordonia*, the current state of knowledge is summarized in Table 4. The three main plasmid-chromosome topology combinations from *Corynebacterium diphtheriae*, *C. glutamicum*, *Mycobacterium bovis*, *M. leprae*, *M. tuberculosis* and *S. coelicolor* A3(2) are schematically represented in Figs. 5 and 6.

Whole genome sequence data for *M. tuberculosis*, *M. leprae* and *M. bovis* [126,133,134], *Corynebacterium glutamicum* [135] and *C. diphtheriae* have revealed circular chromosome topologies. Large linear plasmids have also been reported in many mycobacterial species (Table 4). Furthermore, whole genome comparison of one clinical and one laboratory isolate of *M. tuberculosis* has shown many more single nucleotide polymorphisms and genome rearrangements in their circular chromosome than were previously thought to exist [136].

The chromosomes of most, if not all, *Streptomyces* species are linear [137], about 8 MB in length, with long inverted repeats (up to 200 kb) and with covalently bound proteins on ends that are subject to large rearrangements including the chromosomal integration of the 387-kb linear plasmid pPZG101 of *Streptomyces rimosus* R6 [138] (Table 4). *Saccharopolyspora erythraea* is a mycelial forming actinobacterium that has a linear chromosome with 4 *rrn* operons [139]. Many of the rhodococci have large linear plasmids with inverted repeats and covalently bound terminal proteins, similar to the chromosomes in members of the genus *Streptomyces* (Table 4). However, only preliminary evidence would suggest that some rhodococci have circular chromosomes (Table 4 and [140]). Very little is known about genome topology in the genus *Nocardia*, although linear chromosomes have been detected in *N. asteroides* [140]. The same authors have suggested that the presence of such linear chromosomes may correlate with the production of vegetative mycelia (e.g., as in *Nocardia* spp.

Table 8
Insertion sequences and tansponsable elements found in members of the *Corynebacterineae*

Insertion sequence	Origin	Host	Size (bp)	Reference (see [54])
IS31831, Tn31831	*Corynebacterium glutamicum*	*Brevibacterium flavum*		[314,315]
IS1676	*Rhodococcus erythropolis SQ1*	*Rhodococcus erythropolis SQ1*	1693	[131]
Transposome	Tn5	*Rhodococcus equi*		[93]
IS204	*Nocardia asteroides (YP21)*	*Nocardia asteroides (YP21)*	1452	[124]
IS2112	*Rhodococcus rhodochrous*	*Rhodococcus rhodochrous*	1415	[127]
IS1166, IS1295	*Rhodococcus* sp. *1GTSB*	*Rhodococcus* sp. *1GTSB*		[316]

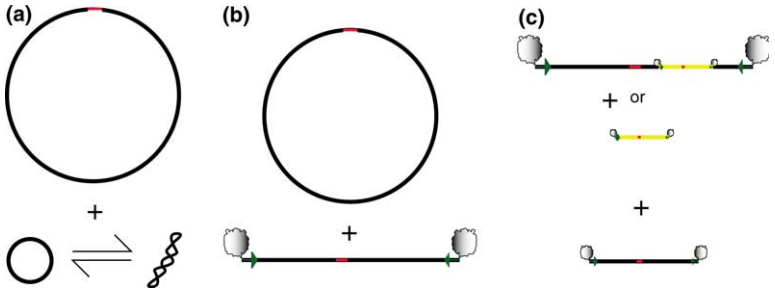


Fig. 5. Linear and circular genome topologies in bacteria. Three different chromosome-plasmid topology possibilities are shown. (a) classic paradigm of circular chromosome and plasmid (open circular and covalently closed circular forms); (b) circular chromosome and linear plasmid and (c) linear chromosome and linear plasmid. The shaded circles represent covalently bound terminal proteins, the green inward pointing arrows represent terminal inverted repeat sequences (invertrons), the red region is the origin of replication and the yellow region is a linear plasmid that has significant homology with chromosomal DNA.

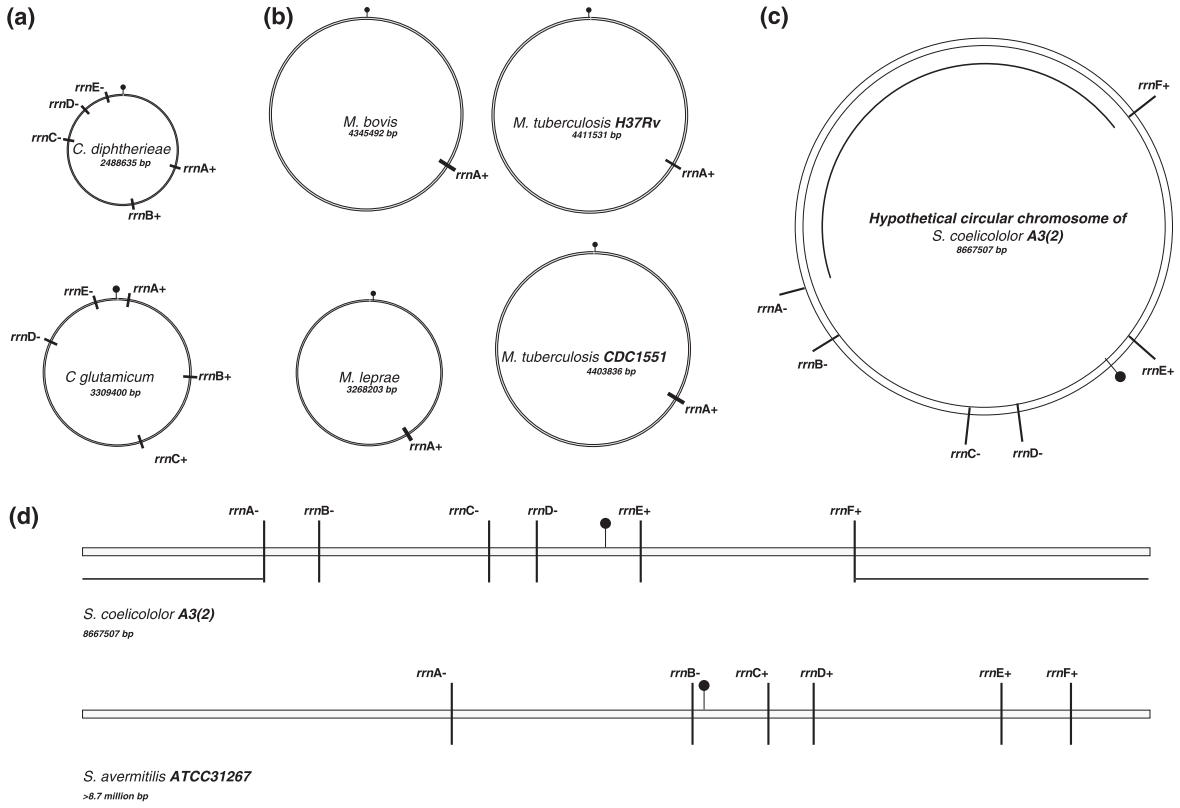


Fig. 6. Chromosomal maps (drawn to scale with sizes in base pairs) derived from the complete genome sequences for species of (a) *Corynebacterium* (wit.integratedgenomics.com/GOLD), (b) *Mycobacterium* [126,133,134,136] and (d) *Streptomyces* [154,155]. A hypothetical circular genome for *S. coelicolor* A3(2) is drawn in (c). The vertical line with a filled circle on top represents the origin of replication, the *rrn* operon positions are marked and the solid lines under the *S. coelicolor* A3(2) genomes between *rrn* A and *rrn* F represent I-*Ceu* I restriction fragments. *S. coelicolor* A3(2) has been re-classified to *S. violaceruber* [174].

and *Streptomyces* spp.). Among *Rhodococcus* species, mainly circular chromosome topologies have been detected, and this may correspond to a growth cycle where mycelia are only occasionally detected, if at all [140]. However, the presence so far of linear chromosome topology in the genus *Nocardia* has not been confirmed. Furthermore, it is not clear whether a direct relationship exists between the type of genome/plasmid topologies and growth cycles in *Nocardia* and *Rhodococcus* species.

The generally accepted paradigm for genome topology (Fig. 5(a)) predicts circular chromosomes and plasmids with covalently closed, supercoiled DNA and open circular forms of plasmids and perhaps chromosomes [141]. The presence of linear plasmids alone (Fig. 5(b)) and together (Fig. 5(c)) with linear chromosomes has challenged the classical circular genome paradigm [137,142]. Genomic approaches to typing, taxonomy and evolutionary relationships of bacterial

isolates have been reviewed [14], and a model based on the following three categories of genomic fingerprinting band pattern studies proposed: (i) random whole-genome analysis (ii) specific gene variation and (iii) mobile genetic elements. The predictive influence of linear genomes on this model is discussed next.

The effect of linear chromosomes on each of the three band pattern study categories can be summarized as: (i) Relative to the analysis of specific genes, the band patterns produced by pulsed-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD) and amplified fragment length polymorphisms (AFLP) are all generated by random sequences, the positions of which can be determined from whole genome sequence data. Fig. 6(c) shows the single I-*Ceu* I fragment between *rrn* A and *rrn* F that would be resolved by PFGE if the *S. coelicolor* A3(2) genome was circular, whereas digestion of a linear *S. coelicolor* A3(2) chromosome would produce two fragments of very different size, demonstrating the effect of genome topology on band pattern analysis (Fig. 6(d)). (ii) The *rrn* operon is one example of a number of specific genes that can be used for band pattern analysis. The restriction enzyme I-*Ceu* I is 23S rRNA-specific, thereby acting as a phylogenetic marker by giving an indication of the number and skeletal arrangement of *rrn* operons on all bacterial chromosomes (reviewed in [14]). Furthermore, the *rrn* operon appears to be prone to macro-genomic recombination [14,143,144] causing macro-genomic/micro-genomic rearrangements of the whole *rrn* operon/ISR that produce differences in band pattern analysis. Fig. 6(c) and (d) shows how circular and linear *S. coelicolor* A3(2) chromosomes, respectively, can affect the position of *rrn* operons, and further investigations are needed to determine the possible effects of genome topology on macro-genomic recombination of *rrn* operons. (iii) In a similar way the mechanisms that determine the mobility of DNA may also be affected by genome topology.

3.5. Rapid identification of bacteria including rhodococci with specific gene RFLPs

The need for accurate, rapid laboratory diagnosis of *Rhodococcus* and *Nocardia* species has resulted in the development of identification procedures based on the RFLP analysis of the *hsp60* gene [35,37,145–147] and the 16S rRNA gene [148–150]. Using these procedures, simple, rapid and accurate identification of isolates can be performed allowing the initial identification of new species (for, e.g., see [145]). However, typing of isolates and rapid identification from clinical specimens are not always possible using 16S rRNA or *hsp60* sequencing and RFLP, and thus a RAPD method has been developed that accurately and rapidly identifies *Nocardia* species [151,152]. However, RAPD is not as reproducible as sequencing or RFLP [14].

3.6. Genomic organization of rrn operons

An insertion within the central part of the 23S rRNA gene is common to all *Actinomycetales* analysed so far [20,21]. As little information on *rrn* operon structure and organization at the level of the whole genome is available for *Rhodococcus* species, the literature on *rrn* operon structure and organization in the small number of actinomycete species analysed so far will also be reviewed. The streptomycetes all have six *rrn* operons, detected using mapping or whole genome data (see Fig. 6). These operons are known to exist in *S. lividans* [153], *S. coelicolor* A3(2) [154], *S. avermitilis* [155] and *S. ambofaciens* [144]. However, *S. venezuelae* has seven *rrn* operons [156]. The cephamycin-producer *N. lactamdurans* (reclassified as *A. lactamdurans*) has four *rrn* operons and the primary and secondary structure of the whole *rrn* D operon was analyzed [157]. Similarly, the plant pathogen *R. fascians* contains four *rrn* operon copies [158] and preliminary evidence would also suggest that the chromosome is circular [159].

In addition to sequence variation in the genome location-dependent flanking regions of the *rrn* operon, considerable intra-genomic sequence variability in the 16S-23S rRNA spacer (ISR) is common and often involves the presence or absence of different tRNA genes (e.g., $tRNA^{ile}$, $tRNA^{ala}$, $tRNA^{glu}$, $tRNA^{val}$ and $tRNA^{met}$) in many bacterial species [160]. However, none of the streptomycete ISRs studied to date appear to contain any tRNA genes (see Table 3 for references]. In those *Nocardioides* species where the ISR has been characterized, considerable intra- and interspecies sequence and length variation have been found, but no tRNA sequences were detected [161]. The number and size of ISR fragments varied from 2 to 5 and 347 to 363 bp, respectively, in *Streptomyces albidoflavus* [162] and a more complete analysis of the ISRs of *Streptomyces ambofaciens* demonstrated mosaic variable regions interspersed with constant sequence regions [144]. The three slow growing *Mycobacterium* species for which whole genome data are available all have a single rRNA operon with no tRNA genes [160]. However, rapidly growing *Mycobacterium* species have up to two copies of the rRNA operon with intra-genomic variation within the ISR observed in *M. smegmatis* [163], *M. fortuitum* [164] and *M. celatum* [165]. Taken altogether these results have formed the basis of a new scheme for grouping the mycobacteria proposed by Menendez et al. [164] that is phylogenetic in concept and based on the properties of the *rrn* operons of a cell.

4. Conclusions and proposals: further subdivision of the genus *Rhodococcus*

The classification of the *Corynebacterineae* has changed markedly over the last 10 years. Descriptions have now been made for 30 *Nocardia* species (15 new), 17 *Gordonia* species, 25 validly named and 16 designated *Rhodococcus* species, 1 or 2 species each of *Williamsia*, *Skermania*, *Tsukamurella*, *Dietzia* and *Turicella*. The large number of members in the closely related *Corynebacterium* and *Mycobacterium* were not discussed here. Furthermore, seven species have been reclassified as *Rhodococcus* species, and it has been proposed that a new genus may need to be created to accommodate the isolates that cluster closely with *R. equi* [5,12]. A genetic characterization of the genus *Nocardia* is now urgently required to increase our knowledge about this important group of organisms, many of which may be medically significant.

Unlike the genus *Nocardia*, a large number of studies have characterized a variety of novel genes from members of the genus *Rhodococcus*. This review has attempted to summarize all those gene sequences that have simultaneously appeared in GenBank and been published in peer reviewed journals. The gene families that have been studied can be classified into five categories that each resolve the taxonomy of the genus *Rhodococcus* in different ways:

A. Virulence factors have only been identified in *R. equi* and *R. fascians.* Many of the catabolic functions that have been described for other *Rhodococcus* species have not been described for *R. equi* and *R. fascians.*
B. The use of protein signatures for evolutionary studies has been recently discussed based on Hsp60, succinyl-CoA synthetase and signature sequences from other proteins [166], including tRNA-synthetases [166,167] not studied in members of *Rhodococcus*. Instead, the proteins that have been studied in members of the genus *Rhodococcus* are catabolic enzymes relevant to the unique metabolic activities of this genus, including enzymes with (i) protein sequence and function phylogenetically conserved; (ii) protein sequence conserved but enzyme function not conserved and (iii) protein sequence, function or both that are *Rhodococcus* species-specific. These three categories phylogenetically identify different clusters of the genus *Rhodococcus* (see Section 3.3).
C. *Genome structure and analysis*. A number of genetic analysis tools have been developed to overcome and capitalize on some of the unique genomic properties of actinomycetes such as DNA topology, recombination, mobile genetic elements and transformation.
D. *Band pattern analysis*. The DNA topology will affect the way RFLPs are analysed for the analysis of isolates.
E. *rRNA operons*. The operon number, genome position of operons and the intra-genomic variability of the 16S-23S rDNA spacer sequence may be useful for taxonomic studies.

The genome categories (A–E) may be useful in determining whether the genus *Rhodococcus* needs to be divided into more genera because the Fig. 1 tree topology suggests there may be at least four *Rhodococcus* groups that are as phylogenetically distinct as *Nocardia* and *Gordonia*. Specific and multilocus genetic tests may demonstrate more differences such as the virulence factors (see A above) within the five genome categories. Many genetic tests that are applicable to rhodococci have been described and some are summarized in a number of comprehensive reviews [14,15,20,107,123,141,166,167]. With expansion of knowledge for the five genome categories (A–E) listed above for each *Rhodococcus* member, a clarification of the systematics of members of the genus *Rhodococcus* is highly likely.

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