Journal of Industrial Microbiology (1996) 17, 205–213

Phylogeny of mycolic acid-containing actinomycetes

J Chun[1], S-O Kang[1], YC Hah[1] and M Goodfellow[2]

[1]*Department of Microbiology and Research Center for Molecular Microbiology, Seoul National University, Seoul 151–742, Republic of Korea;* [2]*Department of Microbiology, The Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK*

Almost-complete 16S rRNA sequences of 32 representatives of the genera *Corynebacterium, Gordona, Mycobacterium, Nocardia, Rhodococcus, Tsukamurella* and *Turicella* were examined using the neighbor-joining, Fitch-Margoliash, maximum parsimony and maximum likelihood methods in order to clarify the suprageneric relationships of these taxa. The topology of the resultant phylogenetic trees was only marginally affected by the use of the different algorithms. Several outgroup strains were used to try and establish the position of the root in the mycolata evolutionary tree. Most of the outgroup strains gave estimates of root positions that proved to be inconsistent when the different tree-making algorithms were used. However it was possible to assign the test strains to two suprageneric lineages the members of which can be distinguished using chemical markers. The family *Corynebacteriaceae* encompasses the genera *Corynebacterium, Dietzia* and *Turicella* and the family *Mycobacteriaceae*, the genera *Gordona, Mycobacterium, Nocardia, Rhodococcus* and *Tsukamurella. Nocardia pinensis* formed a distinct clade that was most closely related to the *Gordona* lineage. Emended descriptions are given of the two suprageneric taxa.

Keywords: mycolata; suprageneric classification; rRNA sequencing; chemotaxonomy

Introduction

Small subunit ribosomal (r) RNA sequences are widely used in bacteriology to construct phylogenetic trees which in turn provide an essential framework for the classification of bacteria [40,64]. However, evolutionary relationships between bacteria need to be interpreted with care as phylogenetic reconstruction is based on relatively simple assumptions which can be violated by the data to a greater or lesser extent [30,40,59]. Potential problems in nucleotide sequence data include non-independence of sites, inequalities in base substitutions between sequences, and lineage-dependent inequalities in rates of change [43]. None of the currently available methods of phylogenetic inference can be relied upon to give the 'correct' tree topology when compositional bias or rate effects are large. There is, therefore, a need to evaluate phylogenetic relationships in light of data derived from other taxonomic approaches. There is good evidence in bacterial systematics of congruence between the distribution of specific chemical markers and the relative position of taxa in phylogenetic trees [23].

A reliable and comprehensive strategy for the delineation of bacterial taxa is emerging based on the integrated use of genotypic and phenotypic data [27]. This approach, known as polyphasic taxonomy, was introduced by Colwell [10] to signify successive or simultaneous taxonomic studies on groups of organisms using methods designed to provide complementary genotypic and phenotypic information. Such all-embracing studies by their very nature can be expected to yield well-defined groups, a stable nomenclature and improved description of taxa, as is the case with actinomycetes, notably mycolic acid-containing actinomycetes [12,21].

The classification of actinomycetes which contain mycolic acids (high-molecular-weight, long-chain, 3-hydroxy fatty acids with an alkyl branch at position 2) has undergone frequent revision, the direct result of improvements in chemotaxonomic, numerical phenetic and molecular systematic procedures [21]. Mycolic acid-containing actinomycetes, the mycolata, have many properties in common [20–22] and form a distinct phyletic line within the evolutionary radiation encompassed by actinomycetes [6,12,46,48,49,51–53,58]. Mycolata strains are either strict or facultative aerobes, have walls which contain *meso*-diaminopimelic acid, arabinose and galactose (wall chemotype IV *sensu* Lechevalier and Lechevalier [37] and an A1γ peptidoglycan [55]. The seven taxa forming the group, namely the genera *Corynebacterium, Dietzia, Gordona, Mycobacterium, Nocardia, Rhodococcus* and *Tsukamurella*, can be distinguished using a combination of chemical and morphological properties [21,50].

Little is known about the detailed taxonomic structure of the mycolata above the genus level possibly because of difficulties inherent in distinguishing between monophyletic and paraphyletic groups [11,45]. However, it is important to establish taxonomic relationships at this level in order to secure a coherent framework for the classification of novel mycolata strains. It is clear from numerical taxonomic and molecular systematic studies that many mycolic acid-containing actinomycetes have still to be fully characterised and named [26,56]. There is also a need to establish the relative branching order of the major suprageneric groups of actinomycetes, including the mycolata [12].

The mycolata are currently classified in three suprageneric taxa, namely the families *Corynebacteriaceae* [39],

Correspondence: Professor M Goodfellow, Department of Microbiology, The Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK
Received 15 March 1996; accepted 29 August 1996

Mycobacteriaceae [4] and *Nocardiaceae* [3]. The family *Corynebacteriaceae* encompasses the genus *Mycobacterium* [38]; and the family *Nocardiaceae* the genera *Gordona* [57,61], *Nocardia* [60], *Rhodococcus* [62,65] and *Tsukamurella* [8]. The suprageneric position of *Dietzia* is not clear; this monotypic taxon encompasses organisms previously classified as *Rhodococcus maris* [50]. *Turicella otitidis*, which lacks mycolic acids, is morphologically and phylogenetically related to *Corynebacterium* [17,46,53]. This organism shares a close phylogenetic relationship with *Corynebacterium amycolatum* [9] which also lacks mycolic acids.

In the present investigation, almost-full 16S rRNA sequences of representatives of the genera *Corynebacterium, Dietzia, Gordona, Mycobacterium, Nocardia, Rhodococcus, Turicella* and *Tsukamurella* were the subject of detailed phylogenetic analyses designed to determine the suprageneric structure of the mycolata within the context of the discontinuous distribution of specific chemical markers.

Materials and methods

Alignment of small subunit rRNA sequences

16S rRNA sequences of 32 representative mycolata and related strains obtained from the EMBL (European Molecular Biology Laboratory), GENBANK and RDP databases (Ribosomal Database Project [36]), were aligned manually using the AL16S program [5], as described previously [6]. The names and accession numbers of the examined type strains were as follows: *Corynebacterium amycolatum* (X84244), *C. cystitidis* (X84252), *C. diphtheriae* (X84248), *C. glutamicum* (Z46753), *C. propinquum* (X84438), *C. xerosis* (X84446), *Dietzia maris* (X79290), *Gordona amarae* (X80635), *G. bronchialis* (X79287), *G. hydrophobica* (X87340), *G. sputi* (X80634), *G. terrae* (X79286), *Mycobacterium chlorophenolicum* (X79094), *M. fortuitum* (X52933), *M. intermedium* (X67847), *M. simiae* (X52931), *M. tuberculosis* (X52917), *Nocardia asteroides* (Z36934), *N. brasiliensis* (Z36935), *N. farcinica* (Z36936), *N. otitidiscaviarum* (M59056), *N. pinensis* (Z35435), *N. vaccinii* (Z36927), *Rhodococcus equi* (X80614), *R. erythropolis* (X79289), *R. opacus* (X80630), *R. rhodnii* (X80621), *R. rhodochrous* (X79288), *R. ruber* (X80625), *Tsukamurella inchonensis* (X85955), *T. paurometabola* (Z46751) and *Turicella otitidis* (X73976). The final database contained information on 1379 nucleotide positions.

Construction of phylogenetic trees

Unrooted evolutionary trees were inferred by using four algorithms, namely the Fitch–Margoliash [FM; 16], maximum parsimony [MP; 35], neighbor-joining [NJ; 54], and maximum likelihood methods [ML; 13]. Evolutionary distance matrices for the FM and NJ methods were generated according to Jukes and Cantor [32] using the DNADIST program in the PHYLIP package [15]. The PHYLIP package was used for the NJ, FM and MP analyses; the fastDNAml program [44] was applied for the ML method. The resultant unrooted tree topologies were evaluated in bootstrap analyses [14] of the NJ method based on 1000 resamplings.

Evolutionary trees

The root positions of the unrooted phylogenetic trees were estimated by using outgroup strains individually or in combination; the position of the root is the joining point of the outgroup strain(s) to the ingroup strains in evolutionary trees [59]. The outgroup strains, namely *Actinokineospora riparia* (accession number X76953), *Actinoplanes philippinensis* (X72864), *Amycolatopsis orientalis* (X76958), *Arthrobacter globiformis* (M23411), *Bacillus subtilis* (K00637), *Bifidobacterium bifidum* (M38018), *Propionibacterium freudenreichii* (X53217), *Saccharomonospora cyanea* (Z38018), *Saccharothrix longispora* (X76964), *Streptomyces coelicolor* (Y00411) and *Thermocrispum agreste* (X79183), were chosen to represent a relatively wide spectrum of evolutionary distances relative to the ingroup strains.

Results and discussion

Phylogenetic relationships among mycolic acid-containing genera

It is now established that mycolic acid-containing actinomycetes and some mycolateless strains form a monophyletic clade [6,12,49,42,53]. In the present investigation, almost-complete 16S rRNA sequences of representatives of this lineage were examined using four algorithms; the resultant unrooted phylogenetic trees are summarized in Figure 1. It is encouraging that the relationships between the test strains were only marginally affected by the use of the different algorithms for Kim [33] has shown that confidence can be placed in branching patterns when several inference methods based on different assumptions indicate a single topology. The recovery of the genera *Gordona, Mycobacterium* and *Tsukamurella* as distinct lineages within the evolutionary radiation encompassed by the mycolata is in good agreement with the results of previous studies [6,49,52].

Members of the genus *Nocardia*, apart from *Nocardia pinensis*, formed a monophyletic clade with a bootstrap value of 87% (Figure 1). Rainey *et al* [49] also found that nocardiae formed a monophyletic line albeit with a bootstrap value of 71%; this relatively low value can be attributed to the fact that these workers only examined polymorphic sites in the resampling process. In contrast, the report that *Rhodococcus equi* lies within the evolutionary radiation encompassed by *Nocardia* strains is more apparent than real [52]. The separation of these taxa is evident both in the present and previous studies [2,49] and is strongly supported by the results of chemotaxonomic [6,21] and AT L30 protein analyses [42].

The taxonomic position of *Nocardia pinensis* has been equivocal since its inception [1,2]. This organism, which causes extensive foams or scums on the surfaces of aeration plants in activated-sludge sewage-treatment plants, was assigned to the genus *Nocardia* using a combination of chemotaxonomic, morphological and physiological properties. Atypical features included the relatively slow-growth rate of the organism, mycolic acids unsaturated in the two-position and a distinctive antimicrobial sensitivity pattern. Representative strains were considered to be most closely related to *Nocardia amarae*, another causal agent of acti-

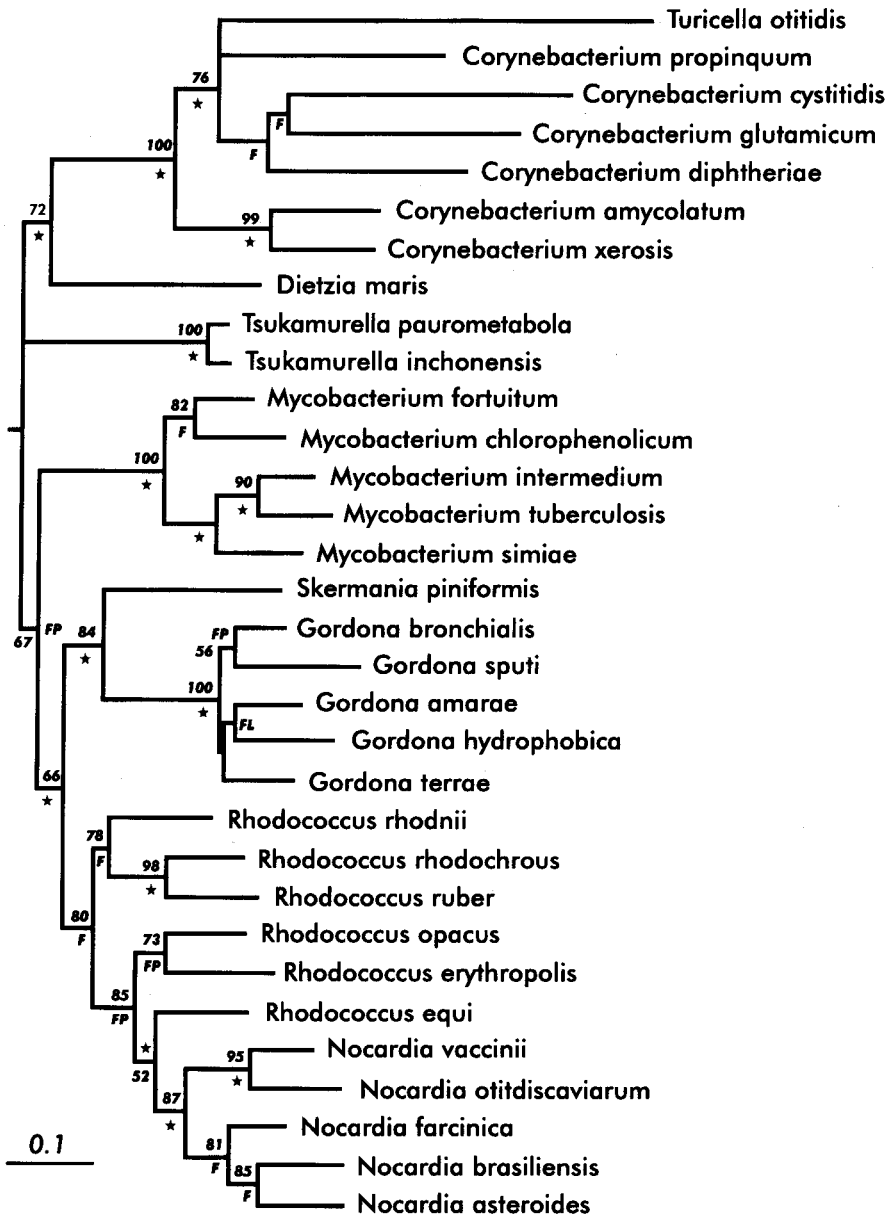


Figure 1 A neighbor-joining tree [54] based on 16S rDNA sequences of representative mycolata strains. The letters F, P and L indicate branches that were also found using the Fitch–Margoliash [16], maximum parsimony [35] and maximum likelihood methods [13], respectively; asterisks indicate branches that were recovered using all four methods. The numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analysis of 1000 resampled datasets; only values more than 50% are given. The scale bar indicates 0.01 substitutions per nucleotide position. The tree is based on 1379 nucleotide positions.

vated sludge foam, which has been transferred to the genus *Gordona* as *Gordona amarae* [25,34,52]. *Nocardia pinensis* forms a phyletic line that is most closely related to the *Gordona* lineage (Figure 1). The nucleotide sequence data taken together with the discontinuous distribution of key chemical markers (Table 1) indicate that *Nocardia pinensis* merits generic status.

The present data underpin those of Rainey *et al* [49] who were the first to clearly show that the validly described species of *Rhodococcus* form several phyletic lines. Their finding that nocardiae have evolved from a rhodococcal ancestor is also substantiated in the present study. The close relationship between nocardiae and rhodococci is also supported by amino acid sequence data generated from the analysis of ribosomal protein AT-L30 [42]. This relationship also helps to explain the difficulties previous investigators had in recognising that nocardiae and rhodococci belong to different genera [18,24].

Extensive 16S rRNA sequencing studies carried out on representative corynebacteria have clarified the internal taxonomic structure of the genus *Corynebacterium* [46,53]. The present data also show that members of the genus *Corynebacterium* and *Turicella otitidis* form a monophyletic group (Figure 1). It is possible that organisms like

Table 1 Differential chemotaxonomic markers of mycolic acid-containing actinomycetes and related taxa[a]

Taxon	Number of carbons in mycolic acids	Tuberculostearic acid	Phophatidyl-ethanolamine	Phophatidylinositol and phophatidylinositol mannosides	Predominant menaquinone	Muramic acid type in peptidoglycan	Guanine plus cytosine content of DNA (mol%)
Corynebacterium	22–36[b]	Absent[c]	Absent[d]	Present	MK-8(H_2), MK-9(H_2)	Acyl	51–67
Dietzia	34–38	Present	Present	Absent	MK-8(H_2)	Acyl	73
Gordona	48–66	Present	Present	Present	MK-9(H_2)	Glycolyl	63–69
Mycobacterium	60–90	Present	Present	Present	MK-9(H_2)	Glycolyl	61–71
Nocardia	44–64	Present	Present	Present	MK-8(H_4, ω-cycl)[e]	Glycolyl	64–72
Nocardia pinensis	58–64	Present	Present	Present	MK-8(H_4, ω-cycl)	Glycolyl	67.5
Rhodococcus	34–52	Present	Present	Present	MK-8(H_2)	Glycolyl	63–73
Tsukamurella	64–78	Present	Present	Present	MK-9	Glycolyl	67–68
Turicella	Absent	Present	ND[f]	ND	MK-10, MK-11	ND	65–72

[a]Data taken from Goodfellow [21], Chun and Goodfellow ([6]; unpublished), Rainey *et al* [50] and Funke *et al* [17].
[b]*Corynebacterium amycolatum* lacks mycolic acids [9].
[c]Some corynebacteria have tuberculostearic acid [29,47].
[d]Some corynebacteria have phophatidylethanolamine [29,47].
[e]Hexahydrogenated menaquinone with eight isoprene units in which the end two units are cyclized [31].
[f]ND, not determined.

Corynebacterium amycolatum and *Turicella otitidis*, which lack mycolic acids, have lost the ability to synthesise mycolic acids. Such a situation would be analagous to that of certain rhizobia which have lost their nitrogenase genes during the course of evolution [28]. However, the taxonomic position of mycolate-free organisms classified within the mycolata needs to be examined by sequencing alternative molecules such as 23S rRNA, elongation factors and ATPase subunits.

Suprageneric relationships among mycolic acid-containing actinomycetes

Several outgroup strains were used individually and in combination to try and establish the position of the root in the mycolata evolutionary tree. The outgroup strains ranged from relatively closely related organisms belonging to, or associated with, the family *Pseudonocardiaceae*, namely *Actinokineosporia riparia, Amycolatopsis orientalis, Saccharomonospora cyanea, Saccharothrix longispora* and *Thermocrispum agreste*, to distantly related organisms such as *Bacillus subtilis*. The overall nucleotide sequence similarity values found between the ingroup and outgroup strains are shown in Table 2. The phylogenetic tree depicting relationships between these strains is presented in Figure 2. The relatively close relationship that can be observed between the mycolata clade and the phyletic line encompassing the family *Pseudonocardiaceae* and related taxa is in line with the results of earlier studies based on ribosomal protein [42] and 16S rRNA [46,53] sequence data.

The best outgroups are usually considered to be the ones which show the closest taxonomic relationships to the ingroup strains [15,59] though Ludwig and Schleifer [40] advocated the use of outgroups that were only moderately related to the ingroup strains. A broad range of outgroup strains were employed in the present investigation to determine their effect on the topology of the resultant evolutionary trees. The root positions identified using the various outgroups are summarised in Table 3. It is evident that the positions of the estimated roots are influenced by the outgroups.

Table 2 Overall 16S rDNA sequence similarity values between individual outgroup strains and the ingroup mycolata strains. The values are given in order of similarity

Outgroup taxa	Similarity (%)
Actinokineosporia riparia	92.6
Saccharothrix longispora	92.5
Saccharomonospora cyanea	91.8
Amycolatopsis orientalis	91.5
Thermocrispum agreste	91.0
Arthrobacter globiformis	90.3
Actinoplanes philippinensis	90.2
Streptomyces coelicolor	89.7
Propionibacterium freudenreichii	89.4
Bifidobacterium bifidum	84.9
Bacillus subtilis	80.3

The variations in estimated root positions are most apparent when individual outgroups were used (Table 1). Most of the outgroups when used individually gave estimates of root positions that proved to be inconsistent when the different tree-making algorithms were employed. The only exceptions were *Arthrobacter globiformis* and *Bifidobacterium bifidum* which are relatively distantly related to the ingroup strains (Figure 2). These organisms gave a consistent root position, that is, position 1. It is also interesting that, with a single exception, the outgroups representing the family *Pseudonocardiaceae* and allied taxa gave different estimated root positions when the same phylogenetic algorithms were used. It is evident that the position of the root in the mycolate evolutionary tree cannot be resolved solely on the basis of 16S rRNA sequence data.

Good congruence has been found between the emerging actinomycete phylogeny and the discontinuous distribution of key chemical markers, notably wall amino acids, sugars and lipids [12,20]. Mycolic acid-containing actinomycetes

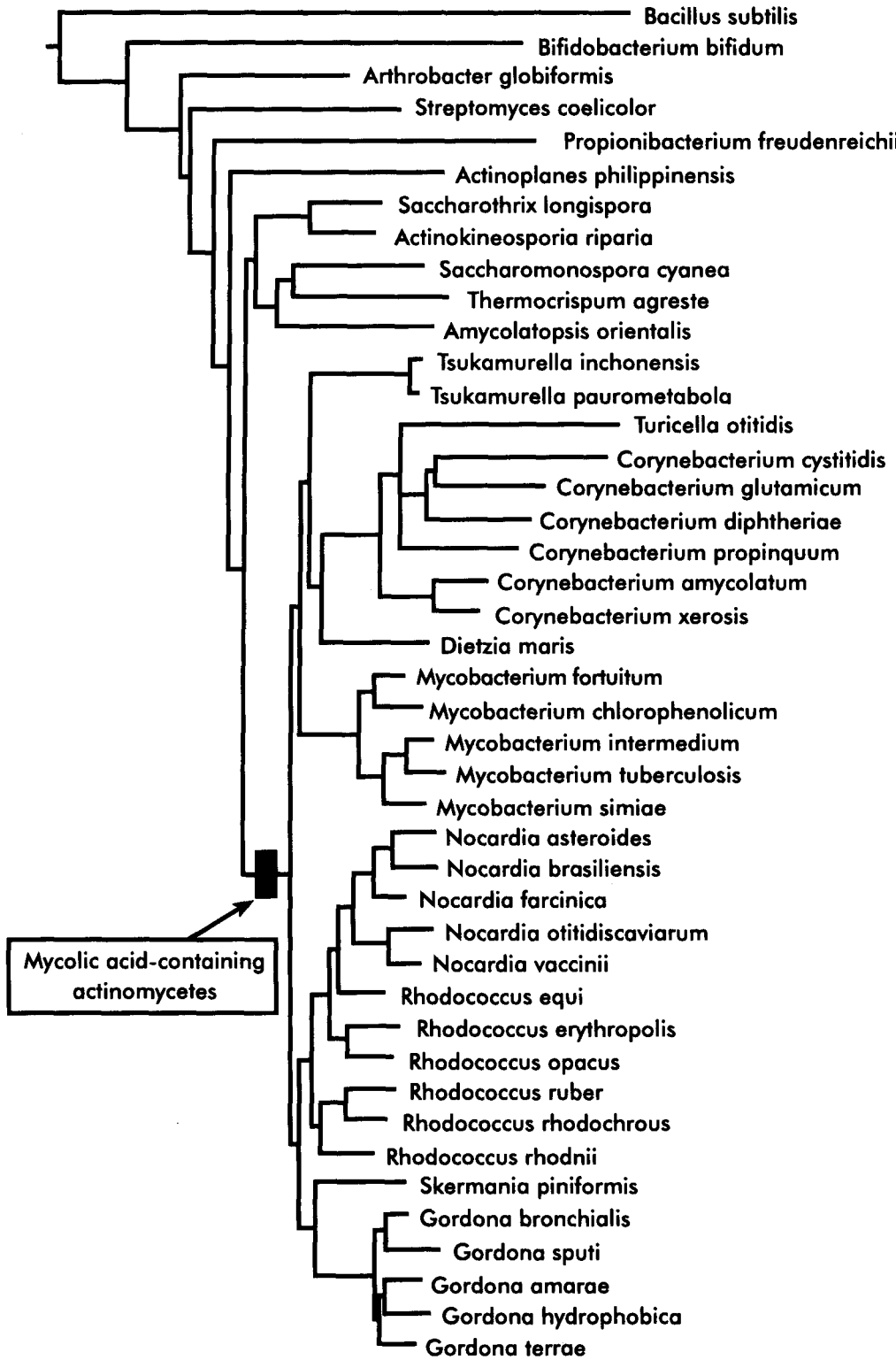
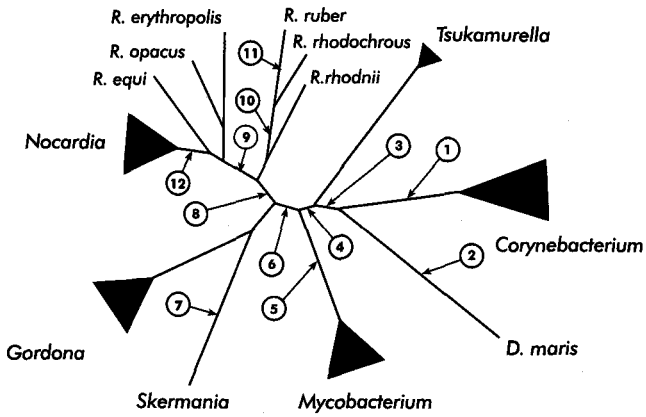


Figure 2 A neighbor-joining tree [54] depicting relationships between outgroup strains and ingroup organisms, that is, the mycolata strains.

can be assigned to two suprageneric groups according to their mycolic acid chain length and whether they contain N-acetylated or N-glycolated muramic acid (Table 1). Members of the genera *Gordona, Mycobacterium, Nocardia, Rhodococcus* and *Tsukamurella* contain N-glycolated muramic acid and relatively long chain mycolic acids, attributes probably shared by the common ancestor of these organisms. In contrast, *Corynebacterium* and *Dietzia* strains contain N-acetylated muramic acid and in nearly all cases relatively short chain mycolic acids.

Table 3 Summary of outgroup analyses. The positions of the estimated roots are indicated by the circled numbers



	Inference method		
	Neighbor-joining	Fitch–Margoliash	Maximum parsimony
• Multi-membered outgroup			
All outgroup strains	6	3	6
Pseudonocardiaceae and related organisms[a]	6	3	7
• Single-membered outgroup			
Actinokineosporia riparia	6	8	5
Actinoplanes philippinensis	9	9	12
Amycolatopsis orientalis	8	8	7
Arthrobacter globiformis	1	1	1
Bacillus subtilis	2	3	2
Bifidobacterium bifidum	1	1	1
Propionibacterium freudenreichii	1	1	5
Saccharomonospora cyanea	4	3	1
Saccharothrix longispora	9	9	12
Streptomyces coelicolor	3	3	11
Thermocrispum agreste	10	6	11

[a]*Actinokineosporia riparia, Amycolatopsis orientalis, Saccharomonospora cyanea, Saccharothrix longispora* and *Thermocrispum agreste.*

The estimated evolutionary root of the mycolata can be narrowed down to positions 1, 2 and 3 in light of the discontinuous distribution of the key chemical markers (Table 3). It is not possible to choose between the branching patterns of the three topologies defined by these estimated root positions due to the chemical heterogeneity of the *Corynebacterium/Turicella* clade and a dearth of suitable chemical markers. However, it is possible to derive a presumptive evolutionary tree for the mycolata and related organisms based on the combined use of 16S rDNA sequence and chemical data (Figure 3).

The suprageneric classification of the mycolata needs to be revised in light of current knowledge to provide a framework for the classification of existing and potentially novel mycolic acid-containing actinomycetes of clinical and industrial importance [26,41,56]. To this end, it is proposed that the mycolata be assigned to two families, a revised family *Corynebacteriaceae* for the genera *Corynebacterium, Dietzia* and *Turicella*, and a revamped family *Mycobacteriaceae* for the genera *Gordona, Mycobacterium, Nocardia, Rhodococcus* and *Tsukamurella* and *Nocardia pinensis.*

Emended description of the family *Corynebacteriaceae* Lehmann and Neumann 1907, 500[AL]: Co.ry.ne.bac.te.ri.a.ce.ae. *Corynebacterium*, the type genus of the family; *aceae* ending to denote family; M.L. fem.pl.n. *Corynebacteriaceae*, the *Corynebacterium* family.

The following description is based upon data taken from several sources [7,17,50]: aerobic to facultatively anaerobic, non-motile, non-spore-forming, catalase-positive actinomycetes which are pleomorphic. Straight to slightly curved rods with tapered ends and club-shaped elements may be observed. Snapping division produces an angular and pallisade arrangement of cells. Gram-positive though some strains stain unevenly; some stains are partially acid-alcohol-fast. Aerial hyphae are not formed.

The wall peptidoglycan contains N-acetylated muramic acid, major amounts of *meso*-diaminopimelic acid, arabinose and galactose, and is of the A1γ murein type. Short chain mycolic acids (22–38 carbons) are usually present, exceptions include *Corynebacterium amycolatum* and *Turicella otitidis*. The fatty acids released on pyrolysis gas chromatography of mycolic acid esters contain 8–18 carbon atoms. Non-hydroxylated long chain fatty acids are primarily of the straight chain saturated and monounsaturated types; some strains may also produce substantial amounts of 10-methyl branched chain acids, notably 10-methyloctadecanoic acid. *Anteiso-* and *iso*-methyl branched fatty acids are either absent or present in only trace amounts. Menaquinones are the sole respiratory isoprenoid quinones; strains typically contain dihydrogenated menaquinones with either eight or nine isoprene units as the predominant component. Most strains contain phosphatidylinositol and phosphatidylinositol dimannosides but lack phosphatidylethanolamine though dietziae contain the latter component but not the former two compounds. The guanine-plus-cytosine ratio of the DNA ranges from 51 to 73 mol %.

Type genus: *Corynebacterium* Lehmann and Neumann 1896, 350[AL].

Emended description of the family *Mycobacteriaceae* Chester 1897, 63[AL]. My.co.bac.te.ri.a.ce.ae. M.L. neut.n. *Mycobacterium* type genus family; *aceae* ending to denote a family; M.L. p. fem. n. *Mycobacteriaceae*, the *Mycobacterium* family (syn. *Nocardia* Castellani and Chalmers 1919[AL])

The following description is based on data taken from several sources [8,19,22,63]: aerobic to microaerophilic, non-motile, non-spore-forming, catalase-positive actinomycetes which are morphologically heterogeneous. Some strains form slightly curved or straight rods, others are pleomorphic and some produce an extensively branched mycelium which fragments into rod- and coccoid-like elements. Cells are acid-alcohol-fast at some stage of growth, and are usually Gram-positive though some strains are not readily stained by Gram's method. Aerial hyphae are produced by some strains.

The wall peptidoglycan contains N-glycolated muramic acid, major amounts of *meso*-diaminopimelic acid, arabinose and galactose and is of the A1γ murein type. Strains contain major proportions of straight-chain saturated and unsaturated fatty acids with 10-methyloctadecanoic acid,

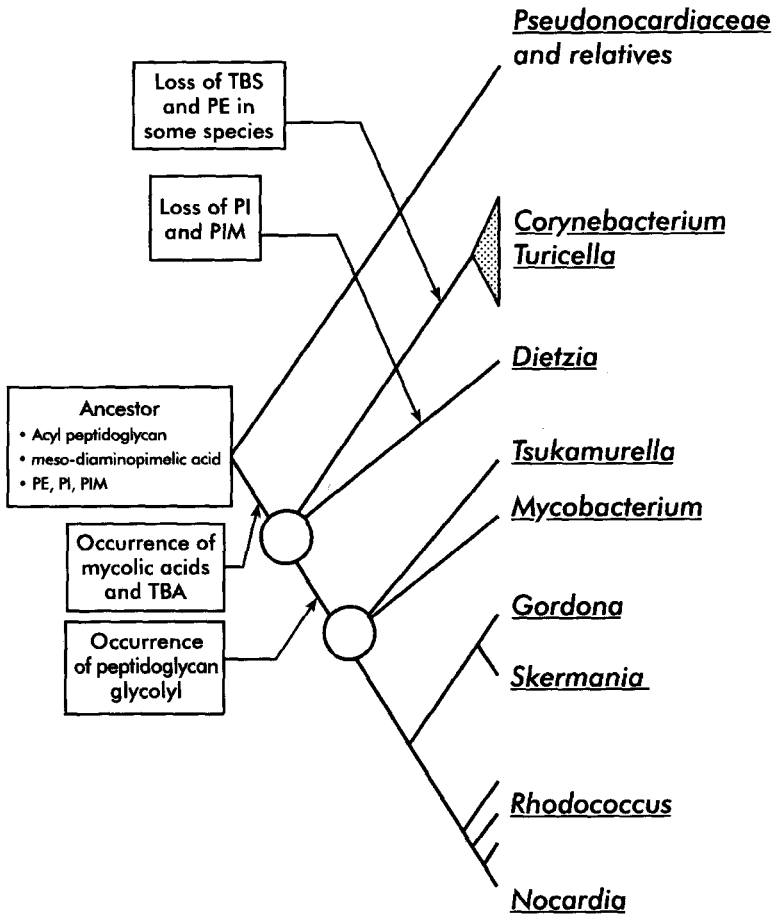


Figure 3 A hypothetical evolutionary tree for mycolata taxa based on 16S rDNA sequence and chemotaxonomic data. Uncertain branching points are indicated by circles. Abbreviations: PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIM, phosphatidylinositol mannoside; and TBS, tuberculostearic acid.

diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and phophatidylinositol mannosides as major polar lipids, and mycolic acids with 44–90 carbon atoms and up to 6 double bonds. The fatty acids released on pyrolysis gas chromatography of mycolic acid esters have between 12 and 26 carbon atoms. Menaquinones, the sole respiratory isoprenoid quinones, are varied. The guanine-plus-cytosine content of the DNA ranges from 61 to 73 mol %.

Type genus: *Mycobacterium* Lehmann and Neumann 1896, 363[AL].

Acknowledgements

The authors are grateful to the International Collaborative Research Programme of the British Council (Seoul, Republic of Korea) and the Korea Science and Engineering Council for support.

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