

The Genus *Mycobacterium*—Nonmedical

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Introduction

Because new *Mycobacterium* species are being described in rapid order and because awareness of the importance of these organisms in the clinical and nonclinical environment is increasing, the editors of *The Prokaryotes* think that a thorough coverage of the rapidly growing species should await extensive comparative studies and a deeper analysis of their biotechnological and ecological role. Several genomes of slowly growing mycobacteria have been or are in the process of being sequenced, e.g., several strains of *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium leprae*, and the two subspecies of *M. avium*. Only one genome of rapidly growing mycobacteria, i.e., *Mycobacterium smegmatis* MC2155 (see The Institute for Genomic research website), has been sequenced; this approach will certainly be applied to more members of these organisms. For the time being, the reader is referred to the recent descriptions of novel species (Table 2) to obtain information on specific properties and to the original chapter by Hartmans and De Bont (1991) in *The Prokaryotes*. The phylogenetic placement of nonmedical strains next to their medically relevant neighbors is shown in Figs. 1–4.

Mycobacteria are aerobic, acid-fast actinomycetes that usually form slightly curved or straight nonmotile rods ($0.2\text{--}0.6 \times 1.0\text{--}10 \mu\text{m}$). Branching and mycelium-like growth may take place with fragmentation into rods and coccoid elements. Many species form whitish or cream-colored colonies, but especially among the rapid growers, there are also many bright yellow or orange species containing carotenoid pigments (David, 1984). In some cases, the pigments are only formed in response to light (photochromogenic species), but most pigmented species also form these pigments in the dark (scotochromogenic species).

Mycobacterium is the only genus listed in the Family Mycobacteriaceae in *Bergey's Manual of Systematic Bacteriology* (Wayne and Kubica, 1986), but the genus is considered to be closely related to the other mycolic acid-containing gen-

era of cell wall chemotype IV: *Caseobacter*, *Corynebacterium*, *Nocardia* and *Rhodococcus* (Goodfellow and Cross, 1984).

Chemical differentiation of mycobacteria from the other mycolic acid-containing genera is possible by analysis of the fatty acid esters formed upon pyrolysis of the mycolic acid esters, in combination with the identification of the major menaquinone present in the plasma membrane (Table 1). The Ziehl-Neelsen stain for acid fastness, however, remains the most obvious method to quickly identify mycobacteria (Barksdale and Kim, 1977).

Mycobacteria are the causal agents of two important diseases, tuberculosis and leprosy, and there has thus been a significant clinical interest in the two responsible species. This started with the work of Koch (1882), who detected the tubercle bacillus in stained infected tissues. The generic name *Mycobacterium* was introduced by Lehmann and Neumann (1896) to include the tubercle and leprosy bacilli. For many years after this work, isolates other than *M. tuberculosis*, but resembling it in staining characteristics, were described as “atypical mycobacteria.” After the discovery in the early 1950s that several of these “atypical mycobacteria” could also produce disease in humans (see Kubica, 1978), it was recognized that identification of these strains was required. The classification of Runyon, separating the mycobacteria into four groups (photochromogens, scotochromogens, nonphotochromogens, and rapid growers) was introduced in the late 1950s as a systematic base for the description of the “atypical mycobacteria” (Wayne, 1984). This division, based on pigmentation and rate of growth, is still of use to the clinical mycobacteriologist (see The Genus *Mycobacterium*—Medical in this Volume).

The separation of the genus into two major groups on the basis of the growth rate of the individual species forms the basis of mycobacterial taxonomy. Although not exactly following this division, many slow growers are either associated with or the causal agents of human or other animal diseases. Most rapid growers are not known to be associated with human diseases

Table 1. Differential characteristics of the mycolic acid-containing genera of wall chemotype IV.

Genus	Mycolic acids			N-glycolyl in glycan moiety of cell wall
	Overall size (number of carbons)	Ester pyrolysis products (number of carbons in chain)	Predominant menaquinone	
<i>Caseobacter</i>	30–36	14–18	MK-8(H ₂), MK-9(H ₂) ^a	–
<i>Corynebacterium</i>	22–36	8–18	MK-8(H ₂), MK-9(H ₂)	–
<i>Mycobacterium</i>	60–90	22–26	MK-9(H ₂)	+
<i>Nocardia</i>	44–60	12–18	MK-8(H ₄)	+
<i>Rhodococcus</i>	34–64	12–18	MK-8(H ₂), MK-9(H ₂)	+
<i>Tuskamurella</i>	64–78	20–22	MK-9	+

and consequently are often considered as non-pathogens, although in a strict sense, “nonpathogens” may not really exist (Tsukamura, 1984). Rapidly growing mycobacteria are common saprophytes in natural habitats but have received much less attention than the clinically more relevant slow-growing species.

Phylogeny and Taxonomy

Taxonomic studies traditionally have relied heavily on morphological characteristics, but subsequently numerical taxonomy and chemotaxonomic characteristics have played an important role in determining taxonomic relationships. Cell wall and mycolic acid analyses have proved to be of great value in separating the actinomycete genera, while numerical taxonomy has had the most impact on the subgeneric level (Goodfellow and Wayne, 1982). DNA hybridization of genomic DNA and, more recently, 16S rRNA similarity data are of course important tools in determining phylogenetic relations on both the generic and subgeneric levels.

Phylogeny

The number of *Mycobacterium* species has increased from about 40 in 1980 (Skerman et al., 1980) to about 110 in 2004. The description of novel species is paralleled by the development of molecular methods and by the increased recognition that slow growing mycobacteria are clinically important and fast-growing mycobacteria are ecologically important. By the end of 1983, there were 52 described species. Only 6 new species were added between 1984 and 1991 and about 4 new species per year between 1992 and 2003. In 2004, 12 new species were described or are in the process of being validated (Table 2).

The often very close relationship among mycobacterial strains and the finding that the slow growing strains possess a single copy of the *rrn* operon only, thus lacking sequence microheterogeneity, made the 16S rRNA gene an

ideal target to be used in the differentiation of strains (De Smet et al., 1996; Holberg-Petersen et al., 1999). Many mycobacterial species can be differentiated on the basis of the sequence of the variable stretch between positions 175 and 238 (*Escherichia coli* nomenclature; Böddinghaus et al., 1990a; Stahl and Urbance, 1990; Pitulle et al., 1992). More than in other bacterial genera, small differences are used as a basis for describing species (Patel et al., 2000), determining intraspecific subclusters (Böddinghaus et al., 1990b; Frothingham and Wilson, 1994). A specific database of high sequence quality (the Ribosomal Differentiation of Medical Microorganisms database; RIDOM) was specifically established for routine diagnosis of these organisms (Harmsen et al., 2003). For those mycobacterial taxa that cannot be discriminated on the basis of their 16S rRNA gene sequence, other genes, such as *gyrB* (Kasai et al., 2000; Niemann et al., 2000), *hsp65* (Devallois et al., 1997; Ringuet et al., 1999), and intraspecific spacers (De Smet et al., 1995; Roth et al., 2000b), were targeted. In addition, the presence of differences in the sequence of other genes (Aranaz et al., 2003) was used in the differentiation of taxa highly related to *Mycobacterium tuberculosis* and *Mycobacterium bovis*.

In the early 1990s, several studies were published which covered the phylogenetic structure of the fast- and slow-growing mycobacteria (Smida et al., 1988; Rogall et al., 1990a; Stahl and Urbance, 1990; Pitulle et al., 1992). The data by and large supported the clustering of mycobacteria according to their growth behavior and indicated that the mostly clinical, slow growing strains evolved from their fast growing relatives (Pitulle et al., 1992). The genomic separateness of the two groups was supported by a sequence idiosyncrasy: the majority of slow growing strains contained an insert (i.e., a long helix between nucleotide positions 451 and 482 [*E. coli* nomenclature]), while the fast growers contained a short helix only. However, some slow growers, such as *Mycobacterium simiae* and *Mycobacterium triviale*, branching deeply within

Table 2. *Mycobacterium* species, the year of their description, and growth velocity.

Taxon	Growth Velocity	Author and validation date ^a
<i>Mycobacterium abscessus</i>	Rapid	Kusunoki and Ezaki, 1992
<i>Mycobacterium africanum</i>	Slow	Skerman et al., 1980 (AL)
<i>Mycobacterium agri</i>	Rapid	Tsukamura, 1981
<i>Mycobacterium aichiense</i>	Rapid	Tsukamura, 1981
<i>Mycobacterium alvei</i>	Rapid	Ausina et al., 1992
<i>Mycobacterium asiaticum</i>	Slow	Skerman et al., 1980 (AL)
<i>Mycobacterium aurum</i>	Rapid	Skerman et al., 1980 (AL)
<i>Mycobacterium austroafricanum</i>	Rapid	Tsukamura et al., 1983c
<i>Mycobacterium avium</i> subsp. <i>avium</i>	Slow	Skerman et al., 1980 (AL) emend. Thorel et al., 1990
<i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i>	Slow	Thorel et al., 1990
<i>Mycobacterium avium</i> subsp. <i>Silvaticum</i>	Slow	Thorel et al., 1990
<i>Mycobacterium bohemicum</i>	Slow	Reischl et al., 1998
<i>Mycobacterium bonnickei</i>	Rapid	Schinsky et al., 2004
<i>Mycobacterium botniense</i>	Slow	Torkko et al., 2000
<i>Mycobacterium bovis</i> subsp. <i>bovis</i>	Slow	Skerman et al., 1980 (AL)
<i>Mycobacterium branderi</i>	Slow	Koukila-Kähkölä et al., 1995
<i>Mycobacterium brisbanense</i>	Rapid	Schinsky et al., 2004
<i>Mycobacterium brumae</i>	Rapid	Luquin et al., 1993
<i>Mycobacterium canariense</i>	Rapid	Jiménez et al., 2004
<i>Mycobacterium caprae</i>	Slow	Aranaz et al., 2003
<i>Mycobacterium celatum</i>	Slow	Butler et al., 1993
<i>Mycobacterium chelonae</i> subsp. <i>chelonae</i>	Rapid	Skerman et al., 1980 (AL)
<i>Mycobacterium chimaerae</i>	Slow	Tortoli et al., 2004
<i>Mycobacterium chitae</i>	Rapid	Skerman et al., 1980 (AL)
<i>Mycobacterium chlorophenicum</i>	Rapid	Häggbloom et al., 1994
<i>Mycobacterium chubuense</i>	Rapid	Tsukamura, 1981
<i>Mycobacterium confluentis</i>	Rapid	Kirschner et al., 1992
<i>Mycobacterium conspicuum</i>	Slow	Springer et al., 1995a
<i>Mycobacterium cookii</i>	Slow	Kazda et al., 1990
<i>Mycobacterium cosmeticum</i>	Rapid	R. C. Cooksey et al., unpublished
<i>Mycobacterium diernhoferi</i>	Rapid	Tsukamura et al. 1983c
<i>Mycobacterium doricum</i>	Cluster rapid, designated slow	Tortoli et al., 2001
<i>Mycobacterium duvalii</i>	Rapid	Skerman et al., 1980 (AL)
<i>Mycobacterium elephantis</i>	Rapid	Shojaei et al., 2000
<i>Mycobacterium fallax</i>	Rapid	Levy-Frebault et al., 1983
<i>Mycobacterium farcinogenes</i>	Rapid	Skerman et al., 1980 (AL)
<i>Mycobacterium flavescens</i>	Rapid	Skerman et al., 1980 (AL)
<i>Mycobacterium fortuitum</i> subsp. <i>acetamidolyticum</i>	Rapid	Tsukamura et al., 1986
<i>Mycobacterium fortuitum</i> subsp. <i>fortuitum</i>	Rapid	Skerman et al., 1980 (AL)
<i>Mycobacterium frederiksbergense</i>	Rapid	Willumsen et al., 2001
<i>Mycobacterium gadium</i>	Rapid	Skerman et al., 1980 (AL)
<i>Mycobacterium gastri</i>	Slow	Wayne, 1966
<i>Mycobacterium genavense</i>	Slow	Böttger et al., 1993
<i>Mycobacterium gilvum</i>	Rapid	Skerman et al., 1980 (AL)
<i>Mycobacterium goodii</i>	Rapid	Brown et al., 1999
<i>Mycobacterium gordonae</i>	Slow	Skerman et al., 1980 (AL)
<i>Mycobacterium haemophilum</i>	Slow	Skerman et al., 1980 (AL)
<i>Mycobacterium hassiacum</i>	Cluster slow, designated rapid	Schröder et al., 1997
<i>Mycobacterium heckeshornense</i>	Slow	Roth et al., 2000a
<i>Mycobacterium heidelbergense</i>	Slow	Haas et al., 1997
<i>Mycobacterium hiberniae</i>	Slow	Kazda et al., 1993
<i>Mycobacterium hodleri</i>	Rapid	Kleespies et al., 1996
<i>Mycobacterium holsaticum</i>	Rapid	Richter et al., 2002
<i>Mycobacterium houstonense</i>	Rapid	Schinsky et al., 2004
<i>Mycobacterium immunogenum</i>	Rapid	Wilson et al., 2001
<i>Mycobacterium interjectum</i>	Slow	Springer et al., 1993
<i>Mycobacterium intermedium</i>	Slow	Meier et al., 1993
<i>Mycobacterium intracellulare</i>	Slow	Skerman et al., 1980 (AL)
<i>Mycobacterium kansasii</i>	Slow	Skerman et al., 1980 (AL)
<i>Mycobacterium komossense</i>	Rapid	Skerman et al., 1980 (AL)
<i>Mycobacterium kubicae</i>	Slow	Floyd et al., 2000
<i>Mycobacterium lacus</i>	Slow	Turenne et al., 2002

(Continued)

Table 2. *Continued*

Taxon	Growth Velocity	Author and validation date ^a
<i>Mycobacterium lentiflavum</i>	Slow	Springer et al., 1996
<i>Mycobacterium</i>	Slow	Skerman et al., 1980 (AL)
<i>Mycobacterium lepraemurium</i>	Slow	Skerman et al., 1980 (AL)
<i>Mycobacterium madagascariense</i>	Rapid	Kazda et al., 1992
<i>Mycobacterium mageritense</i>	Rapid	Domenech et al., 1997
<i>Mycobacterium malmoense</i>	Slow	Skerman et al., 1980 (AL)
<i>Mycobacterium marinum</i>	Slow	Skerman et al., 1980 (AL)
<i>Mycobacterium microti</i>	Slow	Skerman et al., 1980 (AL)
<i>Mycobacterium montefiorensis</i>	Slow	Levi et al., 2003
<i>Mycobacterium moriokaense</i>	Rapid	Tsukamura et al., 1986
<i>Mycobacterium mucogenicum</i>	Rapid	Springer et al., 1995
<i>Mycobacterium murale</i>	Rapid	Vuorio et al., 1999
<i>Mycobacterium nebraskense</i>	Slow	Mohamed et al., 2004
<i>Mycobacterium neoaurum</i>	Rapid	Skerman et al., 1980 (AL)
<i>Mycobacterium neworleansense</i>	Rapid	Schinsky et al., 2004
<i>Mycobacterium nonchromogenicum</i>	Slow	Skerman et al., 1980 (AL)
<i>Mycobacterium novocastrense</i>	Rapid	Shojaei et al., 1997
<i>Mycobacterium obuense</i>	Rapid	Tsukamura and Mizuno, 1981
<i>Mycobacterium palustre</i>	Slow	Torkko et al., 2002
<i>Mycobacterium parafortuitum</i>	Rapid	Skerman et al., 1980 (AL)
<i>Mycobacterium parascrofulaceum</i>	Slow	Turenne et al., 2004
<i>Mycobacterium parmense</i>	Slow	Fanti et al., 2004
<i>Mycobacterium peregrinum</i>	Rapid	Kusunoki and Ezaki, 1992
<i>Mycobacterium phlei</i>	Rapid	Skerman et al., 1980 (AL)
<i>Mycobacterium pinnipedii</i>	Slow	Cousins et al., 2003
<i>Mycobacterium porcinum</i>	Rapid	Tsukamura et al., 1983b
<i>Mycobacterium poriferae</i>	Rapid	Padgett and Moshier, 1987
<i>Mycobacterium psychrotolerans</i>	Rapid	Trujillo et al., 2004
<i>Mycobacterium pulveris</i>	Rapid	Tsukamura et al., 1983a
<i>Mycobacterium rhodesiae</i>	Rapid	Tsukamura, 1981
<i>Mycobacterium saskatchewanense</i>	Slow	Turenne et al., 2004b
<i>Mycobacterium scrofulaceum</i>	Slow	Skerman et al., 1980 (AL)
<i>Mycobacterium senegalense</i>	Rapid	Skerman et al., 1980 (AL)
<i>Mycobacterium septicum</i>	Rapid	Schinsky et al., 2000
<i>Mycobacterium shimoidi</i>	Slow	Tsukamura, 1982
<i>Mycobacterium shottsii</i>	Slow	Rhodes et al., 2003
<i>Mycobacterium simiae</i>	Slow	Skerman et al., 1980 (AL)
<i>Mycobacterium smegmatis</i>	Rapid	Skerman et al., 1980 (AL)
<i>Mycobacterium sphagni</i>	Rapid	Kazda, 1980
<i>Mycobacterium szulgai</i>	Slow	Skerman et al., 1980 (AL)
<i>Mycobacterium terrae</i>	Slow	Skerman et al., 1980 (AL)
<i>Mycobacterium thermoresistibile</i>	Rapid	Skerman et al., 1980 (AL)
<i>Mycobacterium tokaiense</i>	Rapid	Tsukamura, 1981
<i>Mycobacterium triplex</i>	Slow	Floyd et al., 1996
<i>Mycobacterium triviale</i>	Slow	Skerman et al., 1980 (AL)
<i>Mycobacterium tuberculosis</i>	Slow	Skerman et al., 1980 (AL)
<i>Mycobacterium tusciae</i>	Cluster rapid, designated slow	Tortoli et al., 1999
<i>Mycobacterium ulcerans</i>	Slow	Skerman et al., 1980 (AL)
<i>Mycobacterium vaccae</i>	Rapid	Skerman et al., 1980 (AL)
<i>Mycobacterium vanbaalenii</i>	Rapid	Khan et al., 2002
<i>Mycobacterium wolinskyi</i>	Rapid	Brown et al., 1999
<i>Mycobacterium xenopi</i>	Slow	Skerman et al., 1980 (AL)

^aSkerman et al. (1980 [AL]) refers to the Approved Lists in which the respective descriptions were validated.

their cluster, lacked the long helix, thus showing the signature of their fast-growing ancestors (Pitulle et al., 1992). Since 1990, the vast majority of the species descriptions contained an indication (from 16S rRNA gene sequences) of the phylogenetic position of the novel species. How-

ever, the topologies of most phylogenetic dendrograms differed in detail from each other because of the influence of the treeing algorithm used, the selection of reference sequences, and the addition of novel sequences into the database.

The phylogenetic dendrograms (Figs. 2–4) are from a comprehensive neighbor-joining tree based on 16S rRNA gene sequences of all valid and yet-to-be validated species (Fig. 1). The dendrogram includes new sequences, for which the public records indicated incomplete datasets only (i.e., sequences for type strains of *Mycobacterium agri*, AJ429045; *M. confluentis*, AJ634379; *M. moriokaense*, AJ429044; *M. pulveris*, AJ429046; *M. rhodesiae*, AJ429047; and *M. margaritense*, AJ699399). As compared to the dendrograms based on a very limited dataset in 1992, the complete dataset of *Mycobacterium* type strains has not changed significantly: the rapidly growing strains are more deeply branched (Fig. 1, cluster B) and are ancestors of the slow growing strains (Fig. 1, cluster A). However, it should

be noted that the statistical significance is low for the majority of branching points, indicating that the order at which the species branch from each other is not settled and may change within the group to which they belong when new sequence data are added to the database.

SLOW GROWING MYCOBACTERIA The vast majority of slow growing mycobacteria form three clusters with high intracluster relationships (Fig. 1, cluster A, groups A1–A3). Group A1 contains the well known pathogens such as *Mycobacterium tuberculosis*, *Mycobacterium ulcerans*, *Mycobacterium intracellulare*, *Mycobacterium leprae* and their relatives (Fig. 2). These organisms are characterized by a long helix between position 451 and 482 of the 16S rRNA sequence.

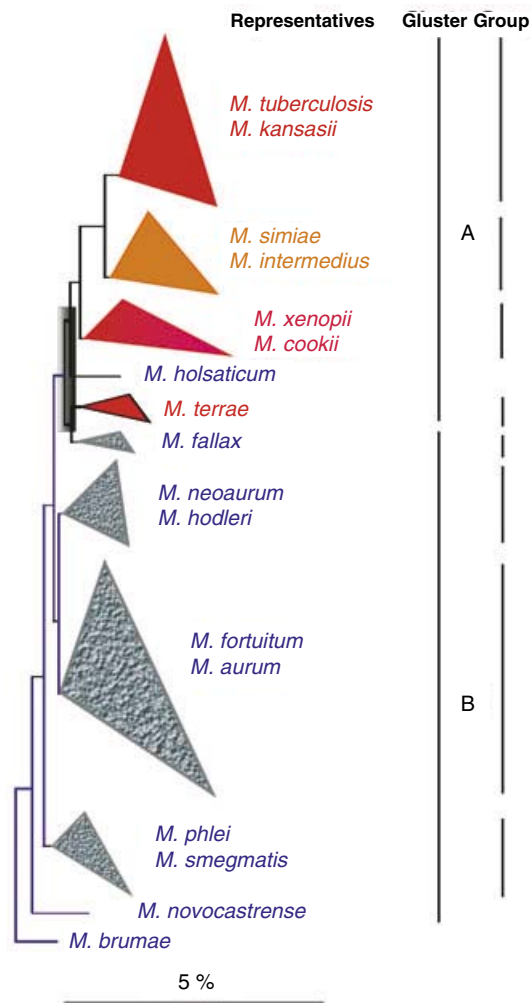


Fig. 1. Schematic graph of a neighbor-joining 16S rRNA gene sequence analysis of type strains of slow growing mycobacterial species. Red triangle: slow growing species containing a long helix between positions 451 and 482; yellow triangle: slow growing species containing a short helix between position 451 and nucleotide position; and gray triangle: fast growing species. Bar: 5% sequence difference.

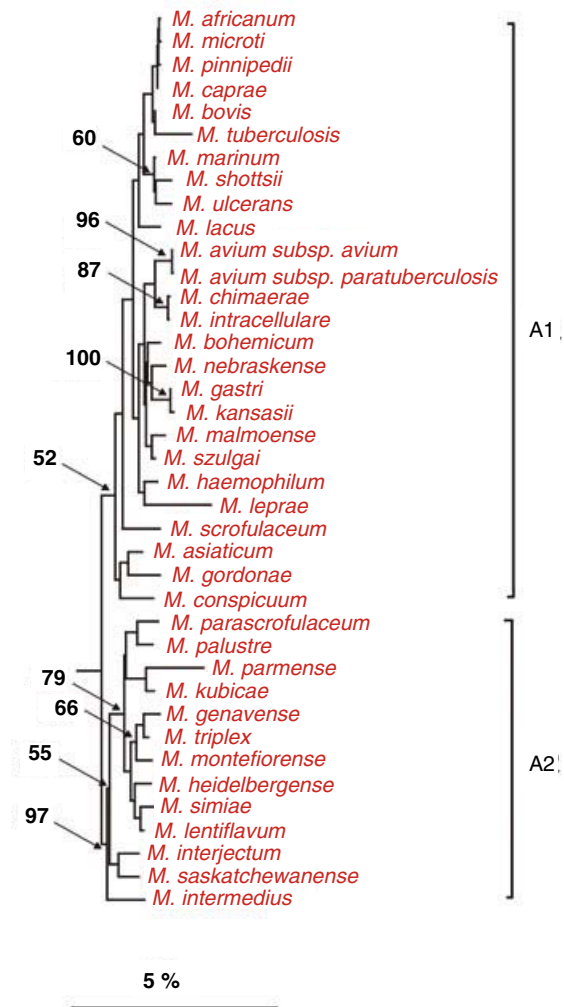


Fig. 2. Neighbor-joining dendrogram of 16S rRNA gene sequences showing the position of type strains of slow growing mycobacterial species. Numbers at branching points refer to bootstrap values. For references to strain number and 16S rRNA gene sequence accession number, see Table 1. Bar: 5% sequence difference.

Group A2 members, embracing *Mycobacterium simiae*, *Mycobacterium gevanense* and relatives (Fig. 2), however, lack this insert.

Group A3 (Figs. 1 and 3) is interesting from an evolutionary point of view in that this assemblage unites slow growing organisms without the characteristic helix insert (*Mycobacterium shimoidei* and *Mycobacterium triviale*) with other members of this group lacking this insert.

The fourth group of slow growers (Figs. 1 and 3), containing the insert (group A4), occupies a distinctly separate position. The three species *Mycobacterium hiberniae*, *Mycobacterium nonchromogenicum* and *Mycobacterium terrae* cluster next to some fast growing members of *Mycobacterium* (cluster B1). Two species, *Mycobacterium doricum* and *Mycobacterium tusciae*, are defined as slow growing mycobacteria but cluster within the phylogenetic radiation of rapidly growing mycobacteria (Fig. 4).

RAPIDLY GROWING MYCOBACTERIA These organisms form four phylogenetic clusters, three of which (together with two individual lineages defined by *Mycobacterium novocastrense* and *Mycobacterium brumae*) are located at the root of the mycobacterial tree (Figs. 1 and 4). The internal structure allows the definition of four groups (B1–B4), of which only group B4 is well separated from the others. Within group B3, the three species *Mycobacterium abscessus*, *Mycobacterium chelonae* and *Mycobacterium immunogenum* are located at the tip of a longer branch which may indicate that these organisms are subjected to a different evolutionary rate. Interestingly, *Mycobacterium chitae* and *Mycobacterium fallax* (group B1) as well as *Mycobacterium holsaticum* show a closer relatedness to slow growing mycobacteria and thus serve as a bridge between fast and slow growers. Most of the fast growing mycobacterium species have been isolated from environmental samples and some are involved in the degradation of polycyclic aromatic hydrocarbons (e.g., *Mycobacterium vanbaalenii*, *M. hodleri* and *M. frederickbergense*) and pentachlorophenol (*M. chlorophenicum*). Others were isolated from wound infections (*M. goodii* and *M. wolinskyi*), but their role as pathogens has not been evaluated. The pathogenicity of other fast growing mycobacterial species is well studied (members of cluster B3, e.g., *Mycobacterium fortuitum*, *Mycobacterium septicum* and their relatives). The ecological role of most fast growing mycobacteria has yet to be determined.

Taxonomy

On the basis of chemotaxonomic studies, the genus *Mycobacterium* was placed within the CNM (*Corynebacterium*–*Nocardia*–*Mycobacte-*

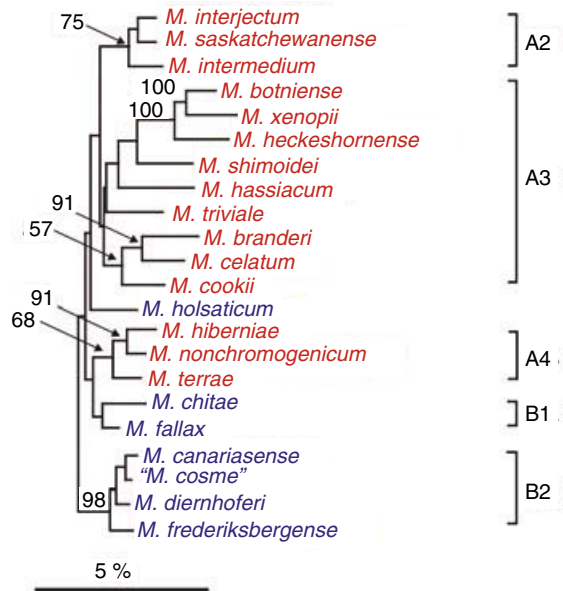
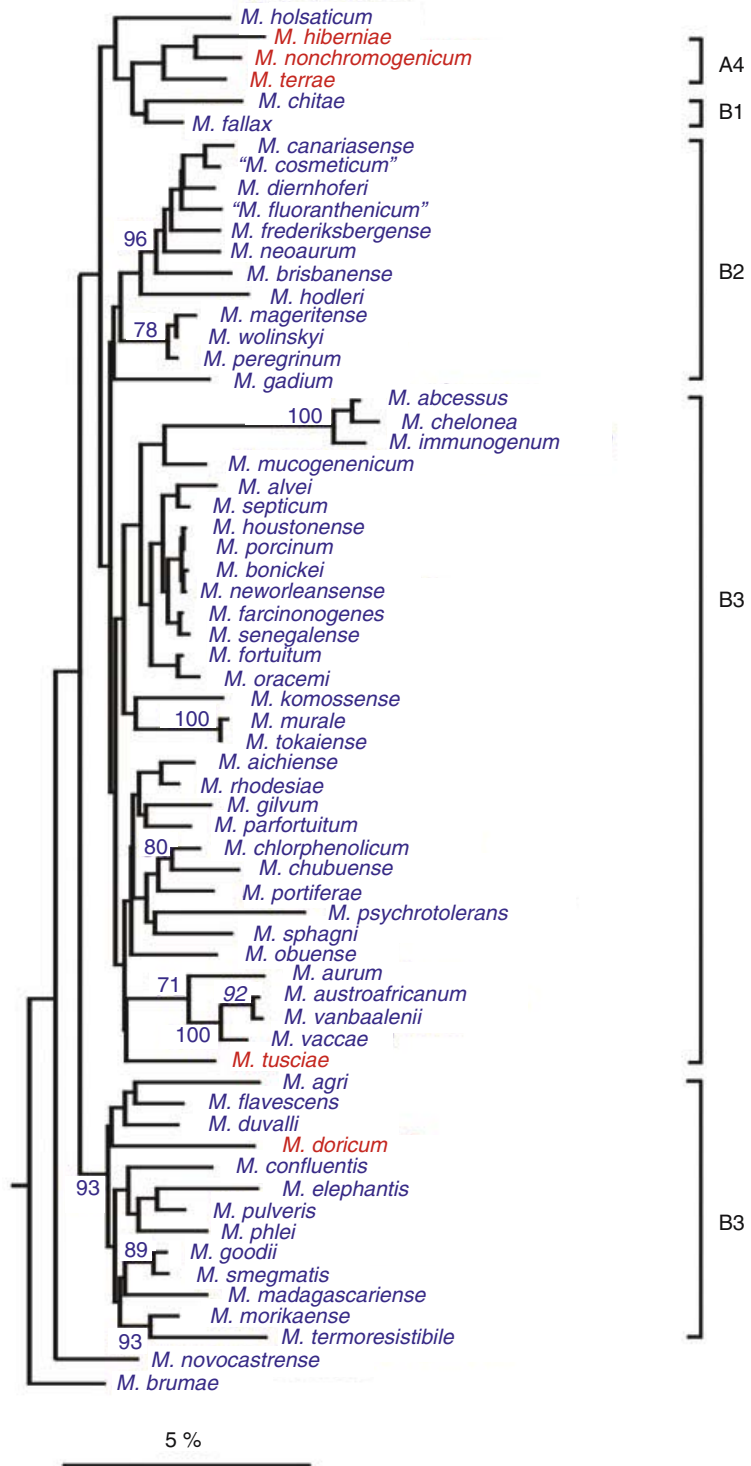


Fig. 3. Neighbor-joining dendrogram of 16S rRNA gene sequences showing the position of type strains of mycobacterial species and the evolution of slow growers (in red) from fast growing ancestors (in blue). Numbers at branching points refer to bootstrap values. For references to strain number and 16S rRNA gene sequence accession number, see Table 1. Bar: 5% sequence difference.

rium) complex. Together with the genera *Caseobacter*, *Rhodococcus* and *Tsakamurella*, these genera all have cell wall chemotype IV (Lechevalier and Lechevalier, 1970) and contain mycolic acids (Collins et al., 1988).

DNA hybridization studies of the genomic DNA have been performed with a number of mycobacteria (Bradley, 1973; Baess, 1982; Lévy-Frébault et al., 1984; Lévy-Frébault et al., 1986b; Garcia and Taberés, 1986; Imaeda et al., 1988; Hurley et al., 1988; Estrada-Garcia et al., 1989) and have generally confirmed the species status already derived from numerical taxonomy studies (Wayne, 1984). More discriminative techniques involving specific DNA probes hybridizing with total DNA (Zainuddin and Dale, 1989; McFadden et al., 1990) or with DNA amplified with the polymerase chain reaction (PCR; Hartskeerl et al., 1989) have been used, but they have focused mainly on the slowly growing species. Sequences of 16S rRNA have also been obtained by direct sequencing of DNA amplified by the PCR method. They were demonstrated to be useful in the differentiation of mycobacteria at the species level (Rogall et al., 1990b). Clearly, the PCR technique will have a significant impact on the identification of mycobacteria in the near future (also see The Genus *Mycobacterium*—Medical in this Volume). DNA-DNA homology studies can be used to dis-

Fig. 4. Neighbor-joining dendrogram of 16S rRNA gene sequences showing the position of rapidly growing type strains of the genus *Mycobacterium* (in blue). Members in red are slow growing species but they cluster with the fast growers phylogenetically. Numbers at branching points refer to bootstrap values. For references to strain number and 16S rRNA gene sequence accession number, see Table 1. Bar: 5% sequence difference.



criminate between more closely related taxa, e.g., the different species of a genus. Note however that DNA-DNA hybridization data from different laboratories can only be compared when reference strains are included. DNA homology studies with selected strains of *M. tuberculosis*, *M. bovis*, *M. bovis* BCG (Bacillus

Calmette-Guérin), *M. microti* and *M. africanum* revealed that all strains exhibited more than 90% DNA relatedness, whereas DNA relatedness between *M. tuberculosis* and other slowly growing mycobacteria ranged from 9–53% (Imaeda, 1985). Although *M. microti*, *M. tuberculosis*, *M. bovis* and *M. africanum* can be distin-

guished phenotypically, numerical taxonomy places them all in a cluster distinct from other slowly growing mycobacteria (Wayne and Kubica, 1986). The 16S rRNA sequences of 14 *M. tuberculosis*, two *M. bovis* and two *M. africanum* isolates were also identical (Rogall et al., 1990b). Considering the above, it is anticipated that a proposal will be made to reduce these four species to one species of *M. tuberculosis*, which possibly will be subdivided into subspecies (Wayne and Kubica, 1986).

DNA-DNA hybridization studies have also led to a better understanding of the relationship between *M. paratuberculosis* and the MAIS (*M. avium-intracellulare-scrofulaceum*) complex. The MAIS complex strains have been grouped together on the basis of phenotypic characteristics (Wayne, 1984), but DNA hybridization studies indicate that *M. scrofulaceum* shows little DNA similarity with *M. avium* and *M. intracellulare* (Hurley et al., 1988). The suggestion that *M. avium*, *M. intracellulare* and *M. paratuberculosis* should be grouped together as biovars of a single species is based on these studies (Hurley et al., 1988). The 16S rRNA sequences can however be used to differentiate between representatives of each of these species (Rogall et al., 1990a).

DNA homology studies with rapidly growing mycobacteria have also been performed (Baess, 1982; Lévy-Frébault et al., 1984; Lévy-Frébault et al., 1986b; Garcia and Taberés, 1986). In the study of Baess (1982), *M. chelonae* subsp. *chelonae* and *M. chelonae* subsp. *abscessus* could not be distinguished using the spectrophotometric method, but with the more sensitive S1 nuclease method, the two subspecies were clearly distinct (Lévy-Frébault et al., 1986b). Within the subspecies, homology values were higher than 73% and changes in melting temperature (ΔT_m) less than 2°C, whereas homology values between the *M. chelonae* groups were 26–52% with ΔT_m values of more than 8°C. Both studies indicate that the unrecognized species “*M. perigrinum*,” often grouped within the *M. fortuitum* complex, has been revived as an independent species (Table 2).

Habitats

Mycobacteria have been isolated from a very diverse array of biotopes including material of both mammalian and nonmammalian origin, as for instance fresh and salt water, soil, and dust. Some species of the nonpathogenic saprophytes may also occur as opportunistic pathogens. The pathogenic species and their habitats are covered in The Genus Mycobacterium—Medical in this Volume and will only be discussed in this chapter in relation to nonmammalian environments.

Although many *Mycobacterium* species have been isolated from environmental samples, this does not necessarily imply that these strains can all grow under these conditions. Essential in assigning a species to the natural flora of a specific environment is that it should be capable of multiplying actively in these environments (Kazda, 1983). If it lacks this property then it should be regarded as a contaminant. Discriminating between these two possibilities is very difficult because the chance of isolating “contaminating” mycobacteria from environmental samples is quite large as a result of the ability of mycobacteria to survive for very long periods under nongrowth conditions. *Mycobacterium paratuberculosis*, for example, was reported to survive for 252 days in a soil-water slurry (Kazda, 1983).

The most recent reviews on the ecology of mycobacteria are those of Kazda (1983), Collins et al. (1984), and Tsukamura (1984). Kazda (1983) classified the mycobacteria into four groups on the basis of ecologically relevant properties. He distinguished obligate pathogenic, facultative pathogenic, potentially pathogenic, and saprophytic species.

Included in the group of obligate pathogens, which presumably are unable to multiply outside living beings, are the species *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. asiaticum*, *M. malmoense*, *M. microti*, *M. simiae*, *M. szulgai* and *M. haemophilum*. Of these species, *M. tuberculosis* and *M. bovis* have been isolated from wastewater (Kazda, 1983). *Mycobacterium farcinogenes* and *M. shimodei* can also be included in the group of obligate pathogens as they have been isolated from mammalian sources only (Tsukamura, 1982; Wayne and Kubica, 1986).

Kazda assigned *M. leprae*, *M. paratuberculosis* and *M. ulcerans* to the provisional group of the facultative pathogens as their incidence in natural biotopes has not been investigated in detail. Future research may result in the assignment of these species to either the potential or obligate pathogens (Kazda, 1983).

Examples of potential pathogens are *M. avium*, *M. chelonae*, *M. fortuitum*, *M. intracellulare*, *M. kansasii*, *M. marinum*, *M. senegalense*, *M. scrofulaceum* and *M. xenopi* (Kazda, 1983). These species can grow in natural biotopes without losing their pathogenic properties, and they have been isolated from a variety of biotopes (Kazda, 1983; Tsukamura, 1984).

The saprophytic mycobacteria include among others the slowly growing species *M. gordonae*, *M. nonchromogenicum*, *M. triviale*, *M. terrae* and *M. gastri* and most of the rapidly growing species (Table 2), with the exception of *M. chelonae*, *M. fortuitum* and *M. senegalense* and their relatives, which are classified as potential pathogens

(Kazda, 1983). The saprophytic and potentially pathogenic groups (Kazda, 1983) are found in many environments (Tsukamura, 1984) and only occasionally occur as an accompanying flora in pathogenic processes. The ability of organisms from these groups to utilize many different growth substrates and their capacity to survive and multiply under a wide range of environmental conditions apparently enable them to compete successfully with other organisms in many biotopes.

The abundance of the fast-growing species in soils was already demonstrated by Jones and Jenkins (1965). In screening soil samples, 101 acid-fast strains were isolated. Of the 93 isolates studied, five were scotochromogens and all but three of the nonphotochromogens were rapidly growing mycobacteria, forming colonies within 7 days at 25°C.

The most commonly occurring *Mycobacterium* in the environment is probably *M. fortuitum*, as it is often the major *Mycobacterium* species (40–80%) isolated from soil samples (Tsukamura, 1984). Strains of the slowly growing *M. nonchromogenicum* complex (*M. nonchromogenicum*, *M. terrae* and *M. triviale*) and the rapidly growing species *M. aurum*, *M. smegmatis* and *M. agri* have also been isolated frequently from soil samples (Tsukamura, 1984). Isolates from hospital dust were also predominantly *M. fortuitum* (43%), with significant numbers of strains of the *M. nonchromogenicum* complex (25%) and *M. gordonae* (18%), whereas from house dust the majority of the strains isolated belonged to the MAIS (*M. avium*–*intracellulare*–*scrofulaceum*) complex (55%), and significant numbers belonged to the *M. nonchromogenicum* complex (23%) and were also *M. gordonae* (11%; Tsukamura, 1984).

The “tap water scotochromogen,” *M. gordonae*, is the strain most often isolated from water samples of various origins (Collins et al., 1984). Besides *M. gordonae*, strains of *M. terrae*, *M. phlei* and *M. fortuitum* have also been isolated at a high frequency from various nonmarine aquatic environments (Viallier and Viallier, 1973). Analysis of municipal water supplies and water supplies in hemodialysis centers found strains of *M. fortuitum*, *M. chelonae*, *M. scrofulaceum*, *M. gordonae* and of the *M. avium* and *M. terrae* complexes (Carson et al., 1988). The average number of mycobacteria detected in the municipal water supplies was 74 ± 42 per 100 ml. Marine water samples, usually from coastal waters, predominantly yielded strains of the MAIS complex, although strains of *M. gordonae* and *M. terrae* were also isolated (Collins et al., 1984).

However, when considering studies on the isolation of various *Mycobacterium* species from

the environment, note that environmental samples are routinely decontaminated and that the distribution of the species isolated need not necessarily reflect the distribution of these species in the original sample. It may well be that certain species, or cells in a particular physiological state, exhibit a higher- or lower-than-average survival rate during the decontamination procedure. Application of the decontamination procedure therefore may result in an over- or underestimate of the prevalence of a particular species.

Another consequence of routinely applied decontamination procedures is that no reliable data can be obtained on the numbers of viable mycobacteria present in natural environments. The actual numbers present in soil samples may also be considerably higher than anticipated from viable counts of nondecontaminated samples because of the hydrophobic characteristics of the mycobacterial cell wall, which may result in significant bacterial adhesion to surfaces.

Isolation

Mycobacteria are not always readily isolated from natural samples. They grow relatively slowly and are therefore easily overgrown by faster-growing organisms. However, taking advantage of the resistance of mycobacteria to adverse conditions, decontamination procedures and selective media have been developed to increase the efficiency of isolation procedures (Kubica and Good, 1981). Most of the decontamination methods were developed for the isolation of mycobacteria from specimens originating from diseased humans or animals and exploit the resistance of the acid-fast mycobacteria to alkaline and acidic conditions. The most commonly applied decontamination procedures involve treatment of the samples with NaOH (2–4%) for 15–30 min at room temperature or at 37°C (Jenkins et al., 1982). Methods for the selective isolation of mycobacteria from environmental samples have been reviewed by Songer (1981), who, besides reviewing decontamination methods, also discussed numerous selective growth media.

Realize however that no information is available on the number and type(s) of mycobacteria that are lost as a result of the decontamination procedure. Furthermore, the selectivity of the growth conditions (medium composition and incubation temperature) also affects the number and species of mycobacteria that eventually are isolated.

Portaels et al. (1988) have recently compared different decontamination procedures and selective media for the isolation of mycobacteria from soil samples. The best results (low contamination

in combination with high positivity rates) were obtained using the following procedure:

Isolation of Mycobacteria from Soil (Portaels et al., 1988)

Add 0.5 g of soil (wet weight) to 5 ml of sterile trypticase soy broth, shake vigorously, and incubate at 37°C for 5 h. After sedimentation of the soil particles, add 5 ml of malachite green (0.2%), 1 ml of cycloheximide (500 µg per ml), and 5 ml of NaOH (1 M) to the supernatant. After 30 min at room temperature, neutralize the mixture with HCl (1 M) and centrifuge at 2,000 g for 20 min. Inoculate the pellet on Ogawa egg medium containing cycloheximide (500 µg per ml). Incubate at 30°C and inspect every 2 weeks until growth is observed.

Ogawa Egg Medium (Tsukamura et al., 1986b)

Add to 100 ml of water containing sodium glutamate (1%) and KH₂PO₄ (1%), 200 ml of whole eggs, 6 ml of glycerol, and 6 ml of a malachite green solution (2%). The medium is made up as slopes by heating for 60 min at 90°C.

Using the above method, mycobacteria were isolated from 91% of the soil samples with only 4% of the tubes being contaminated, i.e., exhibiting growth of non-acid-fast microorganisms within 6 months (Portaels et al., 1988). Omission of cycloheximide, malachite green, or both compounds from the egg medium resulted in contamination rates of 14%, 49%, and 63%, respectively.

The decontamination method and incubation conditions may even select for a specific mycobacterial species. *Mycobacterium moriokaense*, for instance, is the predominant species isolated from soil samples using the following procedure:

Isolation of *M. moriokaense* (Tsukamura et al., 1986b)

Shake soil sample (20 g) with 100 ml of 0.9% NaCl for 30 min and allow to settle for 15 min. Filter the supernatant and centrifuge the filtrate for 20 min at 700 g. Mix the precipitate in the centrifuge tube with 3 ml of KOH (1%). After 5 min, inoculate 0.02 ml portions of this suspension on slopes of Ogawa egg medium. Incubate the inoculated slopes at 42°C for 7–10 days and subculture the colonies.

The nonpigmented acid-fast colonies isolated using the above procedure were characterized as *M. moriokaense* (80%) and as *M. fortuitum* (20%). The strong selectivity of this procedure is probably due to the combination of the relatively high incubation temperature and the short incubation time. Unfortunately, no mention was made of the numbers of any pigmented acid-fast colonies which might also have grown on the slopes (Tsukamura et al., 1986b).

Another approach for selectively isolating mycobacteria is to use specific carbon sources in simple mineral media. Many years ago, Söhngen (1913) demonstrated that paraffin could be used to enrich mycobacteria from soil and water samples. Likewise, enrichment cultures with ethene

as the sole carbon source also exclusively led to the isolation of mycobacteria (Hartmans et al., 1989). Other genera, for instance *Xanthobacter* and *Nocardia* species, may also grow on ethene (Van Ginkel et al., 1987b). However, the very low growth rates of these species with ethene may explain why enrichment cultures with ethene result in the isolation of the faster-growing mycobacteria.

Morpholine (1-oxa-4-azacyclohexane), which for many years was considered nonbiodegradable because of its persistence in waste-water-treatment plants, has recently been demonstrated to be degraded by *Mycobacterium* isolates (Cech et al., 1988; Knapp and Brown, 1988). All strains isolated on morpholine from municipal activated sludge systems and river water were identified as mycobacteria (Knapp and Brown, 1988).

Another approach combines the selectivity of a single carbon source with the selectivity of an antibiotic in enrichment cultures. Gram-positive methanol-utilizing bacteria were isolated from different soil samples with methanol as carbon source in the presence of the antibiotic polymyxin B (Urakami and Yano, 1989). The 14 isolates were all very similar and were identified as mycobacteria closely resembling *M. fortuitum*. The growth rates with methanol, though, varied quite significantly. Enrichment procedures on methanol in the absence of polymyxin B usually result in the isolation of Gram-negative genera.

Although mycobacteria are isolated quite easily from many different environments, they are difficult to quantify, especially in heterogeneous matrices such as soil. As mentioned by Songer (1981), the attachment of mycobacteria to surfaces has not yet been investigated. Considering the hydrophobic character of mycobacteria (Van Loosdrecht et al., 1987), conceivably a large percentage of the mycobacteria present in soil adhere to the surfaces of solids. Biofouling of cellulose diacetate membranes used in reverse-osmosis water purification plants is an example of this characteristic. It has been proposed that the initial step in this process is the attachment of mycobacteria (Ridgway et al., 1984). Therefore, many of the mycobacteria present in environmental samples may be overlooked, especially if interfaces are present.

Cultivation

Mycobacteria can utilize a wide range of carbon compounds. Very often glycerol is designated the preferred carbon since it is utilized as sole source of carbon and energy by all cultivable mycobacteria (Ratledge, 1982b). For clinical isolates, an absolute requirement for carbon diox-

ide has been reported (Ratledge, 1982b). It has been observed repeatedly that the lag phase of rapidly growing mycobacteria is shortened considerably when liquid cultures are incubated stationary rather than shaken. This effect may be due to a stimulatory effect of carbon dioxide accumulating in the medium of the stationary culture.

Mycobacteria can use a variety of nitrogen sources, including amino acids and ammonium. In many of the frequently used media for the cultivation of mycobacteria, asparagine is the nitrogen source (Jenkins et al., 1982). Many species can also reduce nitrate (Table 3) and use it as a source of nitrogen. Mycobacteria cannot fix nitrogen. The “*M. flavum*” 301 capable of fixing nitrogen (Ratledge, 1982b) is in fact a *Xanthobacter flavus* strain (see The Genus *Xanthobacter* in Volume 5). Table 3 lists a number of charac-

teristics of rapidly growing species that are useful in identification.

Most mycobacteria do not require any specific growth factors or vitamins in the growth medium. Exceptions are *M. haemophilum*, which requires hemin, and *M. paratuberculosis*, which requires mycobactin, and of course *M. leprae*, which has until now not been cultivated in vitro (Goodfellow and Wayne, 1982).

Problematic in the cultivation of many strains of mycobacteria in liquid culture is the tendency of these organisms to form aggregates and to adhere to the surfaces of growth vessels, probably as a result of the hydrophobic nature of the cell wall. Detergents such as Tween 80 are therefore often added to the growth medium to reduce clumping and to stimulate growth (Ratledge, 1982b). Owing to this tendency of most mycobacteria to form clumps

Table 3. Identifying characteristics of some rapidly growing species of mycobacteria.

Species	Characteristic															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
<i>M. acetamidolyticum</i> ”	N	+	-	-	+	-	+	+	+	+	-	-	-	-	-	-
<i>M. agri</i>	N	+	+	+	-	+	+	+	v	+	+	-	+	-	-	-
<i>M. aichiense</i>	S		-	+	+	-	v	-	-	-	-	-	-	v	+	-
<i>M. aurum</i>	S	-	-	+	v	-	+	v	v	v	v	-	+	v	+	-
<i>M. austroafricanum</i>	S	-	-	+	+	v	+	+	-	+	-	-	+	-	+	-
<i>M. chelonae</i>																
Subsp. <i>abscessus</i>	N	+	-	+	+	v	+	-	-	v	-	-	-	v	-	-
Subsp. <i>chelonae</i>	N	-	-	-	+	v	+	-	-	v	-	-	-	v	-	-
<i>M. chitae</i>	N	-	-	+	-	+	+	+	+	+	-	-	-	-	-	-
<i>M. chubuense</i>	S	-	-	+	-	-	+	+	-	+	-	-	+	v	v	v
<i>M. diernhoferi</i>	N	-	-	+	-	-	+	+	+	+	v	-	+	-	+	-
<i>M. duvalii</i>	S		-	-	-	-	+	+	-	+	-	-	-	-	+	-
<i>M. fallax</i>	N	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-
<i>M. flavescens</i>	S	v	-	-	v	+	+	+	-	v	-	-	-	-	v	v
<i>M. fortuitum</i>	N	v	-	+	+	v	v	+	+	v	+	-	-	v	v	-
<i>M. gadium</i>	S		-	-	-			+			-	-				
<i>M. gilvum</i>	S		-		v	-	v	+	-	+	v	-	-	-	+	-
<i>M. komossense</i>	S		-	+	-	+	+	-	-	-	-	+	-	+	+	+
<i>M. moriokaense</i>	N	+	-	+	+	+	+	+	-	-	v	-	-	v	+	+
<i>M. neoaurum</i>	S	v	-	+	+	-	+	v	+	+	+	-	v	+	+	-
<i>M. obuense</i>	S		-	+	+	v	+	-	-	+	+	-	-	v	+	v
<i>M. parafortuitum</i>	P	v	-	+	v	-	v	v	v	+	-	-	v	-	+	-
<i>M. phlei</i>	S	+	+	+	-	+	+	+	v	+	v	-	v	v	+	v
<i>M. porcinum</i>	N	+	-	+	+	v	+	-	+	+	+	+	-	+	+	-
<i>M. poriferae</i>	S	-	-	+	-	-	-	-	-	v	-	-	+	+	+	+
<i>M. pulveris</i>	N	+	-	v	-	+	+	+	-	+	-	-	-	-	-	-
<i>M. rhodesiae</i>	S		+	+	+	-	-	-	-	-	-	-	+	-	+	-
<i>M. senegalense</i>	N							+	+	+	+	+			+	
<i>M. smegmatis</i>	N	+	+	+	+	-	-	+	+	+	-	+	+	+	+	+
<i>M. sphagni</i>	S		-	-	-	-	-	+	-	-	-	-	-	v	+	-
<i>M. thermoresistibile</i>	S	+	+	-	-	+	+	+	-	+	-	-	-	-	-	-
<i>M. tokaiense</i>	S		-	+	+	v	+	-	+	+	+	+	+	v	+	+
<i>M. vaccae</i>	P	+	-	+	v	-	v	v	+	+	+	+	v	v	+	-

Symbols: N, nonchromogenic; S, scotochromogenic; P, photochromogenic; +, >85% positive; v, variable; -, <15% positive. ^aNumbers correspond to the following characteristics: 1, pigmentation; 2, growth at 42°C; 3, growth at 45°C; 4, tolerates 0.2% picric acid; 5, arylsulfatase (3 days); 6, α-esterase; 7, β-esterase; 8, nitrate reduction (24 h); 9, acetamidase; 10, nicotinamidase; 11, allantoinase; 12, succinamidase; 13, xylose utilized; 14, trehalose utilized; 15, mannitol utilized; 16, sorbitol utilized. Data adapted from Kubica et al. (1972); Saito et al. (1977); Tsukamura (1981); Tsukamura et al. (1981, 1983c, 1986); Tsukamura and Ichiyama (1986); Wayne (1984); Wayne and Kubica (1986); and Padgett and Moshier (1987).

and to adhere to the surfaces of laboratory fermentors, there have been very few studies of mycobacteria using continuous cultures. One exception was reported by Lowrie et al. (1979). Using a special fermentor in which all stainless steel had been replaced by glass, Teflon, or titanium, *M. bovis* BCG and *M. microti* were grown with 0.08% Tween 80 in the growth medium and by aerating with air containing 5% carbon dioxide. The air supply was introduced in the gas phase of the fermentor, and the stirring rate was kept low to prevent the formation of bubbles.

Some species, however, may be grown in chemostat cultures without having to take any specific precautions. *Mycobacterium phlei* can be grown dispersed in a fermentor (Girbal et al., 1989) without the addition of a detergent, and consequently it has been used quite often for metabolic studies (Ratledge, 1982b). Two other examples of mycobacteria cultivated in continuous culture without detergents are *M. aurum* strain L1 growing on vinyl chloride (Hartmans and De Bont, 1992) and the ethene-utilizing strain E3 growing on ethene (Van Ginkel et al., 1987a).

The mineral salts medium (Wiegant and De Bont, 1980) used in the chemostat studies of *M. aurum* L1 contained 0.2 ml of the Vishniac and Santer (1957) trace element solution per liter. Subsequent chemostat studies using this medium (S. Hartmans and J. A. M. de Bont, unpublished observations) with vinyl chloride as the carbon source revealed that the culture was iron-limited rather than carbon-limited. An improved mineral salts medium with an increased iron content was therefore formulated.

Basal Mineral Salts Medium for the Cultivation of Mycobacteria

K ₂ HPO ₄	1.55 g
NaH ₂ PO ₄ · 2H ₂ O	0.85 g
(NH ₄) ₂ SO ₄	2.0 g
MgCl ₂ · 6H ₂ O	0.1 g
EDTA	10 mg
ZnSO ₄ · 7H ₂ O	2 mg
CaCl ₂ · 2H ₂ O	1 mg
FeSO ₄ · 7H ₂ O	5 mg
Na ₂ MoO ₄ · 2H ₂ O	0.2 mg
CuSO ₄ · 5H ₂ O	0.2 mg
CoCl ₂ · 6H ₂ O	0.4 mg
MnCl ₂ · 2H ₂ O	1 mg
Deionized water	1 liter

Identification

The simplest test for discriminating mycobacteria from other prokaryotes is the Ziehl-Neelsen acid-fast stain. Mycobacteria stained with carbol fuchsin resist decolorization with hydrochloric

acid-alcohol. Barksdale and Kim (1977) have termed this property "mycobacterial acid-fastness" or acid-alcohol-fastness, in contrast to the acid-fastness of several other mycolic acid-containing genera, which resist decolorization with dilute mineral acids but which can be decolorized with hydrochloric acid-alcohol. A detailed discussion of the acid-fast stain is presented in the excellent review of Barksdale and Kim (1977).

An alternative method to reliably distinguish mycobacteria from other genera containing mycolic acids (Table 1) is to analyze the mycolic acid methyl esters by thin-layer chromatography (TLC; Minnikin et al., 1980b; Daffé et al., 1983) and to characterize the major menaquinone (Collins et al., 1977). Alternatively, the fatty acid methyl esters formed during pyrolysis gas liquid chromatography of mycolic acid methyl esters can also be analyzed (Kusaka and Mori, 1986).

Identification schemes for mycobacteria are based on a clear distinction between slowly growing and rapidly growing species. Species forming colonies from dilute inocula within 7 days under optimal conditions are classified as rapid growers. Those requiring more than 7 days are designated slow growers. The difference in growth rates is actually quite distinct, with the slow growers usually requiring 10 days or more to form colonies, whereas all rapidly growing species, including the relatively slowly growing *M. flavescens* and *M. thermoresistibile*, form colonies within 4–5 days (Jenkins et al., 1982).

It has been argued that the importance attributed to this characteristic may lead to erroneous results in the identification of mycobacteria, as isolates are usually only compared with known species of the same growth rate category. However, evidence that the majority of the rapidly growing and slowly growing mycobacteria are also clearly separated by 16S RNA studies (Stahl and Urbance, 1990; see Figs 1 and 4) supports the significance usually attributed to this characteristic in identifying mycobacteria.

The identification of slowly growing strains is described in *The Genus Mycobacterium—Medical in this Volume* as well as in other reviews (Jenkins et al., 1982; Wayne, 1984; Wayne and Kubica, 1986).

Identification of Rapidly Growing Mycobacteria

Identification of rapidly growing mycobacteria can be based on a number of characteristics. Chromogenicity is often used as an initial criterion, primarily of course because of its obvious nature. Numerical studies based on a large number of biochemical and physiological properties

have been used to define the presently accepted species, and consequently many of these characteristics can be used in the identification of new isolates. Chemical analyses of cell constituents such as mycolic acids and mycobactins are also very powerful tools in the identification of mycobacteria. DNA analyses including hybridization studies with specific probes and the identification of specific sequences are the most recently developed techniques in mycobacterial identification.

Chromogenicity and Morphology

Chromogenicity is often used as a convenient criterion for identifying or classifying mycobacterial species, but some caution should be taken, as the significance of pigment production in the physiology of mycobacterial species and its taxonomic value are not very well studied (Kaneda et al., 1988). Thus, novel isolates should be compared with the pigmented as well as with the nonpigmented species (e.g., Tsukamura et al., 1986b). When determining chromogenic properties, the effect of light on pigmentation or even on growth itself should also be considered (De Bont et al., 1980).

A description of the morphological characteristics of the different species has been given by Wayne and Kubica (1986), although, according to Tsukamura et al. (1981b), colonial morphology is not very useful in differentiating mycobacterial species.

Biochemical and Physiological Characteristics

A probability matrix for the identification of the slowly growing mycobacteria has been developed on the basis of a large pool of collected data (Wayne et al., 1980). Although many species of rapidly growing mycobacteria also have been subjected to numerical analysis of a large number of biochemical characteristics (Saito et al., 1977; Tsukamura, 1981a; Tsukamura et al., 1981b; Tsukamura et al., 1983c), such a probability matrix has not yet been developed for the rapidly growing species (Wayne, 1984). Identification of the many rapidly growing mycobacterial species that have been proposed during the last 10 years was, however, usually based on studies involving numerical analysis (Kazda, 1980; Tsukamura, 1981a; Tsukamura et al., 1981b; Tsukamura, 1982; Tsukamura et al., 1983a; Tsukamura et al., 1983b; Tsukamura et al., 1983c; Tsukamura et al., 1986b; Lévy-Frébault et al., 1983; Tsukamura and Ichiyama, 1986a; Padgett and Moshier, 1987). Besides the numerical taxonomic data, additional criteria such as DNA hybridization experiments are generally required to warrant the proposal of a new species (Wayne, 1984).

On the basis of a number of these numerical analysis studies of a large number of strains (Kubica et al., 1972; Saito et al., 1977; Tsukamura, 1981a; Tsukamura et al., 1981b; Tsukamura et al., 1983c; Tsukamura and Ichiyama, 1986a), we have listed several physiological and biochemical characteristics which should be of help in the identification of newly isolated rapidly growing species (Table 3). A detailed description of the methods is given by Vestal (1975). The diagnostic table for rapidly growing species published by Wayne and Kubica (1986) and the references cited therein are also very useful.

Generally, additional tests, such as mycolic acid analysis and DNA-hybridization experiments, are required before an exact identification can be made. Identification of strains of the "*M. parafortuitum* complex," which includes *M. parafortuitum*, *M. diernhoferi*, *M. aurum* and *M. neoaurum*, for example, is sometimes rather difficult. Several strains that clustered with the type strain of *M. neoaurum* in the numerical study of Saito et al. (1977) clustered with the *M. aurum* type strain in a subsequent study by Tsukamura et al. (1983c). In the second study, the above species and the novel species *M. austroafricanum* could be separated satisfactorily. Nevertheless, in view of the similarities observed, the preservation of the *M. parafortuitum* complex containing the five species mentioned above was considered to be justified (Tsukamura et al., 1983c).

The identification of *M. fortuitum* is another example showing that a small number of tests is not sufficient for identifying strains of rapidly growing mycobacteria. This clinically most important rapidly growing species is often identified solely on the basis of its positive 3-day arylsulfatase test. To distinguish *M. fortuitum* from *M. chelonae*, NaCl tolerance, nitrate reduction, and Fe uptake are also often determined (Kubica and Good, 1981). However, as can be seen in Table 3, several other nonphotochromogenic species are also positive in the 3-day arylsulfatase test.

Chemical Analysis of the Mycobacterial Cell

Cell wall and fatty acid analysis has had much impact on the classification of those Gram-positive prokaryotes that have wall chemotype IV (Goodfellow and Cross, 1984), i.e., prokaryotes with a cell wall containing *meso*-diaminopimelic acid (*meso*-A₂pm), arabinose, and galactose (Lechevalier and Lechevalier, 1970). In the mycobacterial peptidoglycan, the peptidoglycan residues are *N*-glycolated rather than *N*-acetylated as in most other *meso*-A₂pm containing prokaryotes (Brennan, 1988; Table 1). Also very characteristic for mycobacteria is the

lipid-rich thick cell envelope containing very long-chain mycolic acids (Draper, 1982; Minnikin and O'Donnell, 1984a; Rastogi et al., 1986; Mamadou et al., 1989; Table 1). The characteristic mycolic acids have received much attention in relation to the identification of mycobacteria, especially at the species level.

Mycolic acids are high-molecular-weight, 3-hydroxy fatty acids substituted with an aliphatic side chain at the C₂ position (Fig. 5). The molecular mass of mycobacterial mycolic acids varies from C₆₀ to C₉₀ (Table 1). Pyrolysis of the methyl esters of mycobacterial mycolic acids yields long-chain meroaldehydes and fatty acid methyl esters (Fig. 6) with chain lengths of 22–26 carbon atoms (Table 1). This is a very useful characteristic in separating mycobacteria from the other mycolic acid-containing genera, although discrimination between the genera *Tsukamurella* and *Mycobacterium* also requires identification of the major menaquinone (Table 1).

Methanolysates of mycobacterial mycolic acids can be resolved into several classes based on the presence or absence of different functional groups in the longer carbon chain, i.e., R₁ in Fig. 5, of the mycolic acid molecule. Both one-dimensional (Daffé et al., 1983) and two-dimensional (Minnikin et al., 1980b) TLC methods have been used. The least polar compounds are termed "α-mycolic acids" and "α'-mycolic acids." They do not contain any oxygen functions apart from the 3-hydroxy and carboxy functions. In the more polar mycolic acids, the longer chain is substituted with methoxy, keto, epoxy, or carboxy functions (Minnikin, 1982). The various epoxy mycolic acids produce differing characteristic TLC patterns, depending on the method by which the methanolysate (acidic or basic methanolysis) was prepared (Minnikin et al., 1984b).

The predominant α-mycolic acids generally contain 74–82 carbon atoms, whereas the α'-mycolic acids are of a lower molecular weight,

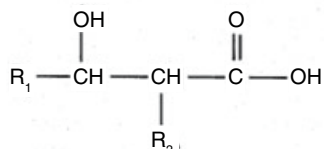


Fig. 5. General formula for mycolic acids. R₁ and R₂ represent variable side chains.

with the predominant type containing 60–68 carbon atoms (Kaneda et al., 1988). Five different types of long-chain α-mycolic acids have been separated and identified using argentation TLC, gas chromatography, and mass spectrometry (Minnikin et al., 1984c; Kaneda et al., 1988). These types are I, dicyclopropanoyl; II, monocyclopropanoyl monoenoic; III, methylated monocyclopropanoyl monoenoic; IV, dienoic; and V, methylated dienoic.

The α'-mycolic acids generally contain only one or two double bonds without any cyclopropanoyl functions (Kaneda et al., 1988). Very often (e.g., Collins et al., 1988) the α-mycolic acids of mycobacteria are described as always containing one or two double bonds. This is probably due to the fact that mycolic acids containing a cyclopropanoyl ring and mycolic acids containing a double bond are not separated by TLC. In fact, many species contain only dicyclopropanoyl α-mycolic acids without any double bonds as shown in Table 4, which lists the mycolic acid content by species (Kaneda et al., 1988).

For a number of species, e.g., *M. fortuitum*, *M. smegmatis* (Minnikin et al., 1984b), *M. phlei*, *M. thermoresistibile* (Lévy-Frébault et al., 1986a) and *M. chitae* (Minnikin et al., 1985), the mycolic acids of large numbers of strains have been analyzed, all resulting in a characteristic pattern for each species. Analysis of seven *M. aurum* strains, however, revealed two different mycolic acid patterns (Table 4), with two strains also containing α'-mycolic acids. This discrepancy was also observed by Lanéelle et al. (1988).

The composition of the α- and α'-mycolic acids of a number of strains has been further analyzed by Kaneda et al. (1988) using gas chromatography and mass spectrometry. These authors separated the rapid growers into three groups on the basis of the types of α-mycolic acids present. Group A only contained dienoic α-mycolic acids, group B contained α-mycolic acids containing double bonds and cyclopropanoyl rings, and group C only contained dicyclopropanoyl α-mycolic acids. Group A could be further divided into two groups on the basis of the number of double bonds in the α'-mycolic acids (Table 4). No α'-mycolic acids were detected in *M. gilvum* by Kaneda et al. (1988), whereas several other authors reported that TLC detected α'-mycolic acids in this species. Unfortunately, *M. fallax* was

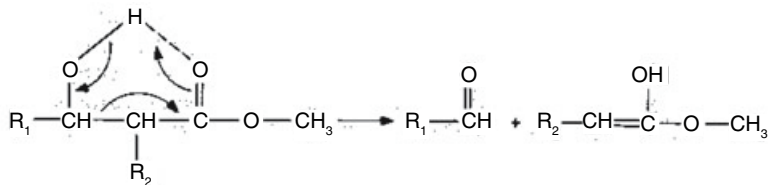


Fig. 6. Rearrangement of the mycolic acid methyl ester during pyrolysis to the meroaldehyde (containing R₁) and the fatty acid methyl ester (containing R₂).

Table 4. Classification of the mycolic acids and pyrolysis products of some of the rapidly growing species of mycobacteria.

Species	Mycolic acids ^a	α -Mycolic acids ^b	α' -Mycolic acids ^b	Ester pyrolysis products ^c		
				1	2	3
<i>M. acetamidolyticum</i> "		III, III'	III	24		
<i>M. agri</i>	$\alpha\alpha$ m	I	IV	24	24	
<i>M. aichiense</i>	α kc				24	
<i>M. aurum</i>	$\alpha\alpha$ 'kc/ α kc	I/III ^a	IV	22, 24	22/24	22B
<i>M. austroafricanum</i>						
<i>M. chelonae</i>	$\alpha\alpha'$	III, III'	IV		24	22B
<i>M. chitae</i>	$\alpha\alpha'e$	III, III'	IV	24		24B
<i>M. chubuense</i>	$\alpha\alpha'$ kc				22, 24	
<i>M. diernhoferi</i>	α kc	I, II, III		22	24	22A
<i>M. duvalii</i>	$\alpha\alpha'$ kc	I	IV	22, 24	22, 24	
<i>M. fallax</i>	α					
<i>M. flavescens</i>	α kc				24	24B
<i>M. fortuitum</i>						
Subsp. <i>fortuitum</i>	$\alpha\alpha'e$	III, III'	III	24	24	24B
Subsp. <i>peregrinum</i>	$\alpha\alpha'e$	III, III'	III	24	24	24B
<i>M. gadium</i>	α kc					
<i>M. gilvum</i>	$\alpha\alpha'$ kc	I		22, 24	22	
<i>M. komossense</i>	α kmc/ α kc					
<i>M. moriokaense</i>	α kc					
<i>M. neoaurum</i>	α kc				22	22A
<i>M. obuense</i>	$\alpha\alpha'$ kc				22	
<i>M. parafortuitum</i>	$\alpha\alpha'$ kc	I, II, II', III, III'	IV	22	22	22A
<i>M. phlei</i>	α kc	I, II, II'		22, 24	24	24A
<i>M. porcinum</i>	$\alpha\alpha'$ / α e	III, III'	III	24		
<i>M. poriferae</i>	α kc					
<i>M. pulveris</i>		I	IV	22, 24		
<i>M. rhodesiae</i>	α kc	I, II, II', III, III'		22, 24	24	24A
<i>M. senegalense</i>	$\alpha\alpha'e$			24 ^c		
<i>M. smegmatis</i>	$\alpha\alpha'e$	III, III'	IV	24	24	24B
<i>M. sphagni</i>	α kc				24	
<i>M. thermoresistibile</i>	$\alpha\alpha'$ km	I	IV	24		24B
<i>M. tokaiense</i>	α kc				24	
<i>M. vaccae</i>	$\alpha\alpha'$ kc	I	IV	22, 24	22	24B

^aAbbreviations: m, methoxy-; k, keto-; c, dicarboxy mycolic acids. Data adapted from Daffé et al. (1983); Minnikin et al. (1984b, 1985); Valero-Guillén and Martín-Luengo (1986); Lévy-Frédault et al. (1986b); Tsukamura et al. (1986); Luquin et al. (1987); Padgett and Moshier (1987).

^bAbbreviations: I, dicyclopropanoyl; II, monocyclopropanoyl monoenoic; II', methylated monocyclopropanoyl monoenoic; III, dienoic; III', methylated dienoic; IV, monounsaturated α' -mycolic acids. Data adapted from Kaneda et al. (1988).

^cData adapted from: 1, Kaneda et al. (1988); 2, Valero-Guillén and Martín-Luengo (1986); 3, Kusaka and Mori (1986). Chain lengths of the fatty acid methyl esters formed upon pyrolysis: 22A, C₂₂ > 80%; 22B, C₂₂ > 50% and C₂₄ > 20%; 24A, C₂₄ > 50% and C₂₂ > 20%; 24B, C₂₄ > 80%.

^dType strain data adapted from Lanéelle et al. (1988).

^eData adapted from Daffé et al. (1983).

not included in the study of Kaneda et al. (1988), as *M. fallax* was previously reported to contain unique α -mycolic acids containing three double bonds (Lévy-Frédault et al., 1983). Comparison of the data from these authors, however, also reveals a discrepancy in the reported composition of α -mycolic acids of *M. triviale*. Lévy-Frédault et al. (1983) could not detect unsaturated α -mycolic acids in the type strain of *M. triviale*, whereas Kaneda et al. (1988) detected only dienoic α -mycolic acids in the *M. triviale* strain they analyzed.

The chain lengths of the fatty acid esters released upon pyrolysis of mycolic acid methyl esters have also been analyzed for a number of

strains (Table 4). Usually only the major chain length is reported. With the exception of *M. aurum* and *M. diernhoferi* (Table 4), no contradictory results were reported. *M. aurum* (ATCC 25793) contained almost equal amounts of C₂₂ and C₂₄ (Kusaka and Mori, 1986), whereas *M. aurum* (ATCC 25797) mainly contained C₂₄ (Valero-Guillén and Martín-Luengo, 1986), while the type strain *M. aurum* (ATCC 23366) only contained C₂₂ (Valero-Guillén and Martín-Luengo, 1986; Lanéelle et al., 1988) although it had previously been reported to contain mainly C₂₄ (Daffé et al., 1983).

As the analysis of mycobacterial mycolic acid methyl esters by TLC provides a sensitive and

relatively easy method to determine mycolic acid patterns, the technique clearly can be of great use in the identification of mycobacteria and in mycobacterial systematics. For a number of species, however, more strains need to be analyzed to confirm the consistency of this characteristic within the species. Especially the situation observed within the *M. aurum* species requires further research, possibly resulting in the reassignment of several strains presently recognized as belonging to this species or to other species or subspecies. These studies should be combined with the data from biochemical numerical studies. The chain length of the fatty acid methyl ester formed during pyrolysis may be of additional use in characterizing new isolates. The detailed characterization of the α -mycolic acids, as was reported by Kaneda et al. (1988), would seem to be less practical in view of the required analytical equipment.

The use of mycobactins (lipid-soluble intracellular siderophores of mycobacteria) as chemotaxonomic markers was proposed in the past. Their use was, however, often hampered by the difficulty of acquiring sufficient amounts of material for analysis. Mycobactin yields of 1–8% (w/w) were reported for *M. smegmatis* using a simple glycerol/asparagine/phosphate medium solidified with agar (Hall and Ratledge, 1982). Subsequently, the same method was used to analyze the mycobactins of a number of different rapidly growing mycobacteria using TLC and high-performance liquid chromatography (HPLC; Hall and Ratledge, 1984). With most strains tested, mycobactin yields of 4–6% (w/w) were obtained. *Mycobacterium aurum*, *M. parafortuitum* and *M. thermoresistibile* did not form detectable amounts of mycobactin under the standard conditions, though more than two-thirds of these strains did yield mycobactins (3–5%) when grown on glucose/yeast extract/agar. The *M. vaccae*, *M. chelonae* subsp. *chelonae*, and *M. komossense* strains tested did not produce detectable amounts of mycobactin on either growth medium. TLC analysis of 32 strains of 15 species of rapidly growing mycobacteria using two different solvent systems revealed that all strains within a single species (except for *M. flavescens*) produced mycobactin with the same R_f value.

TLC analysis of mycobactins is a relatively simple technique, and on the basis of the data presented by Hall and Ratledge (1984), it appears to be a very useful chemotaxonomic character with a high discriminatory power. However, just as in the case of mycolic acid patterns, much larger numbers of strains have to be analyzed to confirm the chemotaxonomic utility of the mycobactin R_f value in identifying mycobacterial species.

Physiology

The physiology of mycobacteria will not be discussed in an exhaustive manner in this chapter as this subject has been dealt with in excellent reviews by Ratledge (Ratledge, 1976; Ratledge, 1982b) and also in the reviews of Ramakrishnan et al. (1972) and Masood et al. (1985). Furthermore, contrary to what was sometimes suspected in the past, mycobacterial physiology differs from that of other aerobic saprophytes only in minor aspects. This section will focus on the areas of mycobacterial physiology that are typical for the genus. The metabolism of unsaturated gaseous hydrocarbons is one area where the role of the mycobacteria is especially important. Another area to be discussed is fatty acid biosynthesis, because mycobacteria clearly differ in this respect from most other prokaryotes. Reserve materials, fatty acid composition, and iron uptake are also discussed.

Catabolic Activities

Mycobacteria are metabolically versatile organisms. They grow not only on common substrates such as sugars, alcohols, and organic acids, but also on a large variety of hydrocarbons including branched-chain, unsaturated, aromatic, and cyclic hydrocarbons (Söhngen, 1913; Lukins and Foster, 1963). Mycobacteria also degrade polycyclic aromatic hydrocarbons, such as pyrene (Heitkamp et al., 1988a; Heitkamp et al., 1988b) and phenanthrene (Guerin and Jones, 1988). Some mycobacteria grow on the simple one-carbon compounds methanol and methylamines (Kato et al., 1988; Urakami and Yano, 1989). In one strain of *M. gastri*, 3-hexulose-6-phosphate synthase was present, indicating formaldehyde incorporation via the ribulose-monophosphate pathway in this strain (Kato et al., 1988). Autotrophic growth on carbon dioxide and hydrogen gas of several strains of *M. smegmatis*, *M. marinum* and *M. fortuitum* was already reported by Lukins and Foster in 1963 (20% of the strains tested). The propene-utilizing strain called "*Mycobacterium* Py1" (De Bont et al., 1980) also grew autotrophically. Under these conditions, ribulose-1,5-bisphosphate carboxylase and a membrane-bound hydrogenase were induced (C. G. van Ginkel and J. A. M. de Bont, unpublished observations). Reports in the older literature on mycobacteria growing on methane should be treated skeptically since such isolates were either lost before a rigorous identification was performed or no longer grew on methane when investigated by others later.

Metabolism of Gaseous Hydrocarbons

In the past, one of the incentives for studying microorganisms degrading gaseous hydrocarbons was the suspected relation between the numbers of these organisms present in soil and the presence of fossil fuel reserves. Such a relationship has, however, not been substantiated (Brisbane and Ladd, 1972).

Growth of mycobacteria with the C₂ hydrocarbons ethane and ethene (ethylene) has been described several times. Using ethane as carbon source, Davis et al. (1956) isolated several types of ethane-utilizing bacteria, with mycobacteria predominating. The isolated mycobacteria could be divided into two groups on the basis of their capacity to grow on complex media. About half of the isolates did not grow on nutrient agar or with glycerol as sole carbon source and were placed in a separate novel species, "*M. paraffinicum*." The assignment of "*M. paraffinicum*" to the genus *Mycobacterium* is, however, questionable, as it was shown to contain trehalose mycolates of a relatively low molecular weight (Minnikin and Goodfellow, 1980a). Using ethene as carbon source, several mycobacteria were isolated (De Bont, 1976). *Mycobacterium* E20 also grew with ethane. Ethane metabolism was via acetate and ethene metabolism via epoxyethane (De Bont and Harder, 1978). Epoxyethane was further degraded to acetyl-coenzyme (Co)A in a CoA- and nicotinamide adenine dinucleotide (NAD)-dependent reaction (De Bont and Harder, 1978). In contrast to the alkene monooxygenase induced by growth on ethene, the ethane hydroxylase activity of *Mycobacterium* E20 could not be detected in cell-free extracts of ethane-grown cells (De Bont et al., 1979).

Of the short-chain hydrocarbons, the metabolism of propane and its derivatives has received the most attention in the past. The initial step in propane degradation has been the subject of speculation and study for quite some time. Oxidation of both the primary and secondary carbon atoms of propane has been shown to occur (Perry, 1980). *Mycobacterium vaccae* JOB5 has been used in several cases to study three-carbon metabolism (see Perry, 1980). In this strain, acetone and acetol as well as acetate have been detected as intermediates in propane metabolism. These observations contrast with acetone metabolism in several unidentified Gram-positive bacteria that degrade acetone via pyruvate (Taylor et al., 1980).

Mycobacterium Py1, which was isolated with propene as carbon source (De Bont et al., 1980), did not grow on propane or acetone. However, it utilized acetol, which was oxidatively transformed into acetate and formaldehyde by acetol monooxygenase through a Bayer-Villiger type of

reaction (Hartmans and de Bont, 1986). Propene metabolism in *Mycobacterium* Py1 proceeds via an initial oxidation to epoxypropane, which is subsequently carboxylated, presumably to acetoacetate.

Fatty Acid Biosynthesis

Mycobacterial lipids have been the subject of numerous studies, often with an emphasis on taxonomic aspects (Ratledge, 1982a; Minnikin, 1982; Brennan, 1988). Besides the characteristic lipid-rich cell wall, mycobacteria also differ from most other prokaryotes in aspects of fatty acid biosynthesis. Fatty acid synthetases are generally divided into two types: the type I "eukaryotic" system and the type II "prokaryotic" system. The type II system, which readily dissociates into separate proteins with discrete catalytic activities, has been detected in all bacteria studied, with the exception of some mycobacteria and corynebacteria. The type I system present in animals and eukaryotic microorganisms has also been found in *M. smegmatis* and *Corynebacterium diphtheriae*.

Fatty acid biosynthesis in *M. smegmatis* has been studied in detail by Bloch and coworkers (Bloch, 1977). They described two fatty acid synthetase (FAS) activities in this species. One is the extensively studied multienzyme complex called "FAS-I," which is probably very much like the FAS of eukaryotes. FAS-I is, however, unique in that it produces both very long (C₂₄ and C₂₆) as well as the more common (C₁₆ and C₁₈) saturated CoA esters of fatty acids, and its activity is stimulated by certain polysaccharides. The 3-*O*-methylmannose polysaccharide (MMP) and 6-*O*-methylglucose polysaccharide (MGLP) affect the K_m for both acetyl-CoA and malonyl-CoA and can bind palmitoyl-CoA, thus restricting further chain elongation and consequently influencing the bimodal product distribution. The biosynthesis of these polysaccharides has recently been studied by Ballou and coworkers (Weisman and Ballou, 1984a; Weisman and Ballou, 1984b; Kamisango et al., 1987).

The second FAS in *M. smegmatis* studied by Bloch and coworkers is similar to the type II FAS found in other prokaryotes in its requirement for an acyl-carrier protein (ACP). De novo fatty acid synthesis is, however, not observed with the type II FAS of *M. smegmatis*, so that it should actually be regarded as a fatty-acid-elongating system, elongating acyl-CoA esters of C₁₆ to C₂₈ (Odriozola et al., 1977). The polysaccharides which affect the type I FAS do not affect the type II elongating system of *M. smegmatis*, and FAS II activity is not inhibited by palmitoyl-CoA.

Another fatty-acid-elongation system (FES I), isolated from *M. smegmatis*, requires acetyl-CoA and is apparently ACP-independent (Shimakata

et al., 1977). It exhibits optimal activity with the C₈ and C₁₀ acetyl-CoA esters. The enzymatic activities of this fatty acid-elongation system, however, under physiological conditions may be involved in the β -oxidation of fatty acids and utilize the stereospecificity of the 3-oxoacyl-CoA reductase, which forms L-hydroxyacyl-CoA esters (Shimakata et al., 1979). L-Hydroxyacyl-CoA esters normally are intermediates in the degradation of fatty acids.

Subsequently, a third elongation system has been described which was apparently also ACP-independent, but which required malonyl-CoA instead of acetyl-CoA. This FES exhibits activity with C₁₀ to C₂₄ acetyl-CoA esters, with an optimum for stearyl-CoA (Kikuchi and Kusaka, 1982). Also, a very long-chain fatty acid-elongation system was isolated from *M. avium* (Kikuchi et al., 1989), which possibly is involved in the synthesis of mycolic acids. It differs from the ACP-independent, malonyl-CoA-incorporating-elongation system of *M. smegmatis* (Kikuchi and Kusaka, 1982) in its cofactor requirements and its sensitivity towards isoniazid. The authors suggest that isoniazid possibly affects the 3-oxoacyl-CoA and enoyl-CoA reductase activities. This would explain the previously observed effect of isoniazid specifically inhibiting the synthesis of mycolic acids (Winder, 1982).

In contrast to the common bacterial pathway of unsaturated fatty acid formation by elongation of decenoyl-CoA, resulting in the formation of palmitoleic and *cis*-vaccenic (C_{18:1 Δ^{11}) acid, biosynthesis of unsaturated fatty acids in mycobacteria is accomplished by desaturation of stearyl-CoA to oleoyl-CoA, and to a lesser extent palmitoyl-CoA to palmitoleoyl-CoA, by the particulate Δ^9 desaturase (Ratledge, 1982a). A very long-chain, soluble, fatty acid Δ^{15} desaturase was isolated from *M. smegmatis*, which exhibited optimal activity with lignoceroyl-CoA (C_{24:0}; Kikuchi and Kusaka, 1986).}

The most common mycobacterial branched-chain fatty acid is tuberculostearic acid (D-10-methylstearic acid). It is formed by methylation of oleic acid residues already esterified in phospholipids (Ratledge, 1982a). The methyl group is derived from *S*-adenosylmethionine.

The various different fatty acid synthases and elongating systems that have been found in mycobacteria are of course a reflection of the many different fatty acids present in these organisms. The different constituents will not be discussed in detail here, as excellent reviews are available (Minnikin, 1982; Brennan, 1988).

Reserve Materials

Besides the mycolic acid composition (see Identification), other lipid components of mycobacte-

ria have also been studied. Mycobacteria can contain considerable amounts of triacylglycerols, especially in glycerol-grown cells, resulting in the formation of fat bodies (lipid vacuoles; Brennan, 1988). These lipids may be utilized as a reserve material, though glycogen and trehalose have also been suggested as reserve materials (Ratledge, 1982b). In nitrogen-limited batch cultures of *M. phlei*, both lipid and glycogen accumulation were observed (Antoine and Tepper, 1969). Transfer of these cells to a medium with a high nitrogen content without carbon resulted in restoration of growth and a decrease of the lipid and glycogen content. Similar experiments with *M. smegmatis* (Elbein and Mitchell, 1973) focused on glycogen and trehalose levels. *Mycobacterium smegmatis* grown under nitrogen-limiting conditions had an increased glycogen content, which was rapidly utilized when these cells were transferred to a medium containing sufficient carbon and nitrogen. The trehalose levels were more or less the same under all growth conditions, indicating that under these conditions trehalose is not a reserve material, although the turnover rates were very high (Elbein and Mitchell, 1973).

Starvation experiments performed with *Mycobacterium* sp. strain E3, a "*M. parafortuitum* complex" species, grown under nitrogen or carbon limitation in chemostat cultures have been performed (Habets-Crützen, 1985a). Cells grown under nitrogen limitation contained 10 times more glycogen and only 50% more trehalose and lipid than cells grown under carbon limitation. The reserve materials of these cells were monitored during a 2-day incubation in buffer in the absence of carbon or nitrogen sources. Trehalose levels fell from 2–3% to less than 0.5% within 8 h. The lipid/protein ratio remained constant during the 2-day starvation experiment. Glycogen was only consumed in the cells grown under nitrogen limitation. The 2% glycogen present in cells grown under carbon limitation was not consumed during the 2-day-starvation experiment. Experiments monitoring the NADH-dependent oxidation of propene to 1,2-epoxypropane by starving cells showed that nitrogen-limited-grown cells, containing the higher glycogen levels, produced more of the epoxide (De Haan et al., 1991).

Lipid Composition

The phospholipids of *M. smegmatis* and *M. phlei* have been studied in some detail (Dhariwal et al., 1976). Major phospholipids present in mycobacteria are phosphatidylinositol, phosphatidylethanolamine, diphosphatidylglycerol (cardiolipin), and the phosphatidylinositol mannosides. The phosphatidylinositol mannosides

are highly characteristic for actinomycetes and coryneform bacteria (Brennan, 1988).

The phospholipid and fatty acid composition are to a large extent dependent on the culture conditions. The growth temperature and the carbon source as well as the ratio of carbon and nitrogen sources affect the lipid composition (King and Perry, 1975; Dhariwal et al., 1977). Dhariwal et al. (1976) also reported the effect of culture age on the fatty acid composition. The major change they observed was an increase in tuberculostearic acid accompanied by a decrease in oleic acid (18:1) content. This composition is probably a reflection of the biosynthesis of tuberculostearic acid, which is formed by methylation of esterified oleic acid residues. This perhaps also explains why relatively high tuberculostearic acid contents have often been reported, as, very often, cultures were analyzed which were already in the stationary growth phase.

An important aspect which should be emphasized is that these studies were all performed with batch-grown cultures. This implies that the growth conditions and growth rates were rarely constant, thus making it difficult to ascribe the observed changes in fatty acid composition to a specific factor.

More reliable experimental data require the application of chemostat cultures. As discussed in the section on cultivation, the continuous culture of mycobacteria in chemostats has been demonstrated several times. Therefore the regulation of lipid composition, or indeed many other aspects of mycobacterial physiology, should be studied using chemostat cultures.

Iron Uptake

Mycobacterial iron metabolism, especially iron uptake, has been extensively studied by the group of Ratledge (Ratledge, 1982b; Ratledge, 1984). Mycobacteria appear to be unique in producing two different siderophores (exochelins and mycobactins), probably necessitated by the thick lipoidal nature of the cell envelope. Exochelins are extracellular siderophores, which until now have only been poorly characterized. Two types of exochelins, differing in their solubility in organic solvents, have been described (Ratledge, 1982b). The very hydrophobic mycobactins, which are located within the cell envelope, have been studied to a greater extent. The potential for using the mycobactin composition in the identification of rapidly growing mycobacteria (Hall and Ratledge, 1984) is discussed in the section on identification. Recent work has focused on the regulation of the biosynthesis of the different siderophores and other iron-regulated proteins (Sritharan and Ratledge, 1989). For *M.*

neoaurum, these components were coordinately expressed in the presence of low iron concentrations (<0.2 mg/liter). Increasing the iron concentration to 0.5 mg/liter (or more) resulted in repression of the synthesis of all three components (Sritharan and Ratledge, 1989).

Genetics

Our knowledge of the genetics and the molecular biology of mycobacteria lags behind that of thoroughly characterized species such as *Escherichia coli* or *Bacillus subtilis*. This is partly due to the low growth rate of mycobacteria but also to the ineffectiveness of many of the standard molecular biology techniques when applied to the mycobacteria. Recently, however, significant progress has been made, part of which has already been reviewed (Grange, 1982; Hopwood et al., 1988; Konicek et al., 1988).

Organization of Genetic Information

The estimated genome sizes of mycobacteria vary from 3 to 5.5×10^9 daltons (Baess and Mansa, 1978). As a comparison, the genome size of *E. coli* is 2.5×10^9 daltons. Mycobacterial DNA has a high G+C content (66–71 mol% for the strains examined by Baess and Mansa, 1978).

The presence of extrachromosomal DNA (plasmids) has been demonstrated conclusively in several species. Most of the strains studied were, however, slow-growing pathogens (Hopwood et al., 1988). Plasmids have also been described in fast-growing species although no selective markers could be attributed to them (Labidi et al., 1984). A 173-kb plasmid isolated from a *M. scrofulaceum* species (isolated from the environment) encoded mercury and copper resistance (Meissner and Falkinham, 1984; Erardi et al., 1987). This strain contained a total of four plasmids varying in size from 15 to 300 kb. Two smaller mycobacterial plasmids have been studied in more detail: Plasmid pLR7 (15.3 kb) from *M. intracellulare* has been mapped (Crawford and Bates, 1984), and the complete nucleotide sequence of pAL5000, a 4,837-bp plasmid from *M. fortuitum*, has been determined (Rauzier et al., 1988).

The organization and sequences of mycobacterial genes coding for ribosomal RNA (rRNA) have been studied quite intensively. The rRNA can be easily isolated and sequenced, and these sequences have potential as taxonomic markers (Cox and Katoch, 1986) and are used in determining phylogenetic relationships (Woese, 1987; also see Identification and Phylogeny and Taxonomy).

Using rRNA probes derived from *E. coli*, hybridization with DNA from *M. phlei* and *M.*

smegmatis revealed that these fast-growing strains contain two rRNA operons, whereas the slow-growing *M. tuberculosis* and *M. intracellulare* appeared to possess only one rRNA operon (Bercovier et al., 1986). The slow-growing *M. bovis* BCG also contains only one set of rRNA genes (Suzuki et al., 1987). It is tempting to speculate that the number of rRNA operons present in the genome forms the genetic basis for the difference in growth rate between the rapidly and slowly growing mycobacteria. *Escherichia coli* grows much faster than the fast-growing mycobacteria and possesses seven rRNA operons (Bercovier et al., 1986).

The rRNA genes of *M. smegmatis* have been studied by restriction analysis (Bercovier et al., 1989), and, like the *M. bovis* BCG rRNA genes (Suzuki et al., 1987), the genes coding for the different rRNAs are organized in operons in the order 16S—23S—5S, as in other eubacteria (Woese, 1987; Clark-Curtiss, 1990).

Genetic Recombination

Papers concerning the transfer of mycobacterial DNA through transduction and conjugation published in the 1970s have been reviewed (Grange, 1982; Konicek et al., 1988) and will not be discussed here. Genetic recombination of mycobacteria by spheroplast fusion to produce genetically modified strains for sterol transformation has also been reported (Jekkel et al., 1989).

However, other methods of genetic recombination are essential for a better genetic characterization of mycobacteria. Methods to efficiently transfer DNA between mycobacteria and *E. coli* and vice versa are consequently an important tool. The approach of Jacobs et al. (1987) to attack this problem was to construct a vector which replicates as a plasmid in *E. coli* and as a phage in mycobacteria. This was achieved by introducing an *E. coli* plasmid replicon into a nonessential region of mycobacteriophage TM4, a temperate phage of *M. avium*. The resulting "phasmid" phAE1 grows as a lytic phage in *M. smegmatis* and replicates as a plasmid in *E. coli*. DNA transfer into *M. smegmatis* was achieved by transfection of protoplasts, and introduction into *E. coli* was done by in vitro packaging with lambda proteins.

Subsequently, efforts were undertaken to use the same approach to construct lysogenic phasmids that would allow the introduction and maintenance of DNA in growing mycobacteria. "Shuttle phasmids" were constructed in a similar manner as described above using the temperate phage L1 which stably lysogenizes *M. smegmatis* by integrating in the chromosome (Snapper et al., 1988). With one of these phasmids (phAE19),

it was possible to lysogenize *M. smegmatis* protoplasts and generate kanamycin- and chloramphenicol-resistant colonies, thus illustrating the possibility of introducing and expressing foreign genes in mycobacteria (Snapper et al., 1988).

A much larger stimulation of research of mycobacterial genetics is expected from the construction of plasmids capable of replicating in both *E. coli* and mycobacteria. Snapper et al. (1988) have constructed such hybrid shuttle plasmids by randomly inserting the *E. coli* plasmid pIJ666, containing an origin of replication and the genes for kanamycin and chloramphenicol resistance in pAL5000 from *M. fortuitum*. Transformation of the pIJ666::pAL5000 library into *M. smegmatis* protoplasts was not successful. Subsequently the high voltage electroporation technique was applied. This method had previously been demonstrated to be useful in the transformation of other Gram-positive bacteria (Chassy and Flickinger, 1987). Electroporation conditions were optimized for the uptake of lytic D29 phage DNA by intact *M. smegmatis* cells, resulting in $>5 \times 10^3$ plaque-forming units per μg DNA. Electroporation of *M. smegmatis* using the optimized procedure with the pIJ666::pAL5000 recombinant library yielded 1–10 kanamycin-resistant transformants per μg DNA. Plasmids isolated from the transformants were used in retransformation experiments yielding kanamycin-resistant *E. coli* and *M. smegmatis* colonies.

Gicquel-Sanzey et al. (1989) used a similar approach to construct the 9.2-kb vector pAL8 by combining pAL5000 with an *E. coli* plasmid and a gene coding for kanamycin resistance. However, transformation of spheroplasts with the pAL8 vector was not successful using conditions under which transformation with the lytic mycobacteriophage D29 resulted in transformation efficiencies of 10^4 to 10^5 per μg DNA. Using the electroporation technique, Gicquel-Sanzey et al. (1989) reported transformation frequencies for *M. smegmatis* of 10 per μg pAL8 DNA. Electroporation of *M. smegmatis* with phage D29 resulted in transformation frequencies of 10^3 per μg DNA, similar to the rate reported by Snapper et al. (1988). Both groups report much higher transformation efficiencies (10^3 per μg DNA) for *M. bovis* BCG. Recently though, high-efficiency-transforming mutants of *M. smegmatis* have been isolated, yielding more than 10^5 transformants per μg DNA (Snapper et al., 1990b). Interestingly, these mutants do not show enhanced transformation frequencies with an integrating vector that recombines into the *M. smegmatis* chromosome. It is suggested that the mutation possibly affects plasmid replication and maintenance in *M. smegmatis*. Using these mutants, an essential replication region of pAL5000 was mapped and the gene coding for the 65-kDa

stress-protein antigen of *M. leprae* was expressed (Snapper et al., 1990a).

Deletion experiments with pAL8 have resulted in the construction of a smaller shuttle plasmid of 6.6 kb (pRR3) incorporating only 2.58 kb of pAL5000 (Ranes et al., 1990). The transformation rates of *M. bovis* BCG and a high-transforming mutant of *M. smegmatis* (Snapper et al., 1990b) using the electroporation technique with this plasmid were 10^4 per μg pRR3 DNA.

Transformation by electroporation of *M. aurum* and *M. smegmatis* with the broad-host-range, Gram-negative vector pJRD215 has also been demonstrated (Hermans et al., 1991). In contrast to the constructs described above, this cosmid vector does not contain mycobacterial DNA, i.e., a mycobacterial origin of replication. An advantage of pJRD215 is that it contains the phage lambda *cos* site, allowing the cloning of relatively large DNA fragments in *E. coli* and thereby facilitating the construction of genomic libraries. Expression of pJRD215 in mycobacteria should allow the screening of such libraries by the complementation of mutants. The kanamycin- and streptomycin-resistance genes carried by pJRD215 were both expressed in *M. aurum*. The transformation efficiency, as determined by screening for kanamycin resistance, was rather low at 2×10^2 transformants per μg DNA. Enhancement of the transformation frequency by pretreatment of the cells with isoniazid, as was demonstrated for the transformation of *M. aurum* with pAL8 (Hermans et al., 1990), might also be applied successfully for the transformation of mycobacteria with pJRD215.

Another approach to stably introduce DNA in mycobacteria, using a vector without a mycobacterial origin of replication, requires homologous recombination to take place. This was demonstrated with a shuttle vector that can replicate autonomously in *E. coli* but must integrate into homologous DNA for survival in *M. smegmatis* (Husson et al., 1990). The vector, pY6002, contained an *E. coli* origin of replication and the *pyrF* gene of *M. smegmatis* with the kanamycin-resistance gene *aph* as an insert. The *pyrF*-negative mutants are both uracil auxotrophic and fluorouracil resistant. The transformation frequency of *M. smegmatis* by electroporation with this vector was 10–500 transformants per μg DNA. Integration of pY6002 in the chromosomal DNA of the prototrophic “wild-type” *M. smegmatis* gave two types of kanamycin-resistant recombinants resulting from either a single or double recombination event. Class I recombinants resulting from a single recombination event contained the entire plasmid as well as a functional *pyrF* gene, whereas class II recombinants were uracil auxotrophic and fluorouracil

resistant. In class II recombinants, the plasmid had apparently integrated in the chromosome at the *pyrF* locus and replaced it. Class II transformants could be retransformed with a plasmid containing the intact *pyrF* gene without an *aph* insert (pY6001). Selection for uracil prototrophs gave both classes of transformants. Class I, resulting from a single recombination event, contained both the disrupted and the wild-type *pyrF* gene, while class II kanamycin-sensitive transformants only contained the wild-type *pyrF* gene. Using this technique, the 65-kDa stress-protein antigen of *M. leprae* was expressed in *M. smegmatis* at detectable levels. Two vectors were used with the *M. leprae* gene on a 3.6-kb fragment inserted at different sites of pY6002. The transformation frequencies were comparable to those of pY6002. The two transformants expressing the 65-kDa antigen were kanamycin resistant and fluorouracil sensitive, indicating them to be class I transformants (Husson et al., 1990).

The methods and tools allowing the transformation and molecular genetic manipulation of mycobacteria described above (Jacobs et al., 1987; Snapper et al., 1988; Snapper et al., 1990a; Husson et al., 1990; Hermans et al., 1991) should result in major advances in mycobacterial research in the near future.

Applications

The most obvious applications of mycobacteria are of course associated with the disease tuberculosis. Examples are the BCG (*Bacillus Calmette-Guérin*) vaccine, which is derived from the *Mycobacterium bovis* BCG strain, and the production of tuberculin, which is extracted from *Mycobacterium* cultures. Tuberculin is used diagnostically in a delayed hypersensitivity type of skin test for the detection of a current or previous infection with *M. tuberculosis*.

In this section we will focus on nonmedical applications. Mycobacteria are used mainly in the area of biocatalysis to perform specific transformation reactions. The justification for using microorganisms or enzymes for a particular transformation is usually the selectivity and specificity (regio- or stereospecificity) exhibited by the biocatalyst (Meijer et al., 1985).

One area in biotechnology that has a large number of commercial applications utilizing mycobacteria is the biotransformation of steroids. These processes involve either the modification of the steroid nucleus of natural or synthetic sterols or the selective degradation of the side chain of naturally occurring sterols such as cholesterol and β -sitosterol (Martin, 1984). The products formed can subsequently be chemically transformed to pharmacologically active

sterols (Martin, 1984). Cholesterol degradation by mycobacteria has been known for a long time (Söhngen, 1913) and usually involves simultaneous degradation of both the side chain and the steroid nucleus. Selective modification of only the nucleus or the side chain can be achieved by employing inhibitors in the biotransformation process or by using mutants (Martin, 1984). Wovcha et al. (1978) for example obtained *M. fortuitum* mutants blocked in various steps of sitosterol degradation. With these mutants, intermediates of the steroid-degradation pathway can be produced. Some of these intermediates can be used as substrates for the production of medically useful steroids. Most commercial processes for the selective side-chain cleavage of sterols employ mutant strains of *M. fortuitum* or *M. parafortuitum*, although other *Mycobacterium* species and bacteria of related genera are also used (Martin, 1984).

Many biocatalytic processes comprise only one enzymatic step, using enzyme preparations or whole (permeabilized) cells. An example is the utilization of *M. neoaurum* (ATCC 25795) containing an L-specific aminopeptidase with a very high stereospecificity and broad substrate specificity. Using permeabilized *M. neoaurum* cells, a wide range of L- or D- α -methyl-substituted amino acids can be produced by stereoselective hydrolysis of racemic mixtures of the corresponding amides (Kamphuis et al., 1987).

Another potential application of mycobacteria in the field of biocatalysis is the production of optically active epoxides from alkenes (Habets-Crützen et al., 1985b; Hartmans et al., 1989). Optically pure epoxides form versatile starting materials for the chemical synthesis of optically active pharmaceutical compounds. Screening a number of bacteria from different genera revealed that alkene-grown mycobacteria produce the epoxides examined (1,2-epoxypropane, 1,2-epoxybutane, and 2,3-epoxy-1-chloropropane) in the highest enantiomeric excess (Weijers et al., 1988). However, substantial research efforts will be required to increase the specific activity and the operational stability of the biocatalysts before commercial production of epoxides by the alkene-utilizing mycobacteria can be realized. In this respect, product toxicity and cofactor regeneration have been studied for the production of 1,2-epoxypropane with the ethene-utilizing *Mycobacterium* strain E3 (Habets-Crützen and De Bont, 1985a; Habets-Crützen and De Bont, 1987).

One aspect that has received some attention is the use of organic solvents and immobilization of mycobacteria for application in continuously operated processes (Steinert et al., 1986; Flygare and Larsson, 1987). The advantages of immobilization (i.e., increased reactor productivity or a

better operational stability) were however not very evident. One advantage that immobilization of cells can offer is that organic solvents, which can function as a reservoir for substrates and products with a low water solubility, can be used without the problems of cell aggregation and biocatalyst/solvent separation associated with the use of organic solvents with free cells (Linko and Linko, 1985). However, immobilization does not protect the cells against the adverse effects many solvents have on mycobacteria (Brink and Tramper, 1985; Steinert et al., 1986). In the study of Brink and Tramper (1985), complete retention of activity was observed in the presence of dioctyl and didecyl phthalate, but most solvents resulted in decreased biocatalytic activity.

The effect of water-immiscible organic solvents on growth has also been tested with the rapidly growing strain *Mycobacterium* E3 and several other bacteria from different genera (Rezessy-Szabó et al., 1986). From these experiments, *Mycobacterium* E3 was not particularly resistant to organic solvents compared to other bacteria, as for instance *Pseudomonas* species.

Another area of research concerning the potential application of mycobacteria is in environmental biotechnology. Removal of traces of the plant hormone ethene from storage facilities for fruit using immobilized ethene-utilizing mycobacteria is one example (Van Ginkel et al., 1986). Unfortunately, the activity of the ethene-utilizing mycobacteria was very low at the ethene levels (often less than 1 part per million [ppm]) prevailing in the fruit storage facilities. Another example is the application of the vinyl chloride-utilizing *Mycobacterium* strain L1 to remove the carcinogenic vinyl chloride from industrial waste gases (Hartmans and De Bont, 1992). The compound is metabolized by an initial oxidation step to the corresponding epoxide by alkene monooxygenase. Accumulation even of very low concentrations of this reactive intermediate, which can occur because of fluctuations in the vinyl chloride supply, irreversibly inhibited the alkene monooxygenase. As such fluctuations will probably frequently occur, practical application of this strain does not seem realistic.

The use of mycobacteria in the bioremediation of contaminated sediments has also been suggested. Addition of a pyrene-degrading *Mycobacterium* strain to sediments resulted in an enhancement of the mineralization rates of several polycyclic aromatic hydrocarbons. Further investigations were suggested to assess the potential of this strain in the bioremediation of contaminated sediments (Heitkamp and Cerniglia, 1989).

A disadvantage of mycobacteria in general is their relatively low growth rate and hence their low catalytic activities. However, mycobacteria

are likely to play an increasingly important role as a source of interesting biocatalytic capacities now that the development of the molecular genetic tools required to increase the expression of desired activities, either in mycobacteria or other hosts, is well under way.

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