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Molecular Study of *Mycobacterium avium-intracellulare* Complex Strains

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ABSTRACT

It is difficult to distinguish between clinically significant slowly-growing, non-pigmented mycobacteria, notably to separate *M. avium*and *M. intracellulare* from one another and from *M. scrofulaceum* strains. The purpose of this study was to evaluate the extent to which 16S rRNA sequencing could be used to highlight the taxonomic relationships of the mycobacterial strains, which are difficult to separate using conventional microbiologic methods. Almost the complete sequences of the 16S rRNA of several *M. avium-intracellulare* complex strains were determined following the isolation and direct sequencing of the amplified genes. The sequences were aligned with those of previously studied mycobacteria, and phylogenetic trees inferred by using the Fitch-Magoliash, neighbour-joining and maximum parsimony methods. It is evident from the result of the current study that the nucleotide signature regions of 16S rRNA provide valuable information for the differentiation of *M.avium-intracellulare* complex strains. *Iran. Biomed. J. 3 (3 & 4): 63-70, 1999*

Keywords: 16S rDNA sequencing; 16S rRNA; *Mycobacterium*

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INTRODUCTION

The members of the *Mycobacterium avium-intracellulare* Complexcause pulmonary disease in elderly patients, lymphadenitis in children and disseminated disease in HIV-infected patients. *Mycobacterium avium* subsp. *paratuberculosis* strains causes enteritis in ruminants and has been implicated in the pathogenesis of Crohn’s disease in humans [1].

Mycobacterial strains assigned to one or the other of these species on the basis of a few phenotypic characters do not always cluster with the appropriate species in more comprehensive taxonomic studies. It has been proposed that such strains be treated as *M. avium/ intracellulare/scrofulaceum* (MAIS) intermediates until their taxonomic standing is clarified [2-3]. Nevertheless, *M. avium* and *M. intracellulare* are clearly distinct species based on T-catalase serology [4-6] and DNA relatedness data [7].

The taxonomic structure of the MAI complex has been clarified though not resolved, in a series of *International Working Group on Mycobacterial Taxonomy* (IWGMT) co-operative studies [8-11]. It appears that the members of the complex are actively evolving, with the separation of the constituent taxa having been relatively recent, as shown by the shallow branching in the 16S rRNA tree. All members of the *M. avium* clade, including *M. intracellulare*, share a number of group specific antigens [12] and have a common mycolic acid pattern [13].

The purpose of this study was to evaluate the extent to which 16S rRNA sequencing could be used to highlight the taxonomic relationships of mycobacterial strains, which are difficult to separate using phenotypic properties.

MATERIALS AND METHODS

Organism and culture conditions. The sources and strain histories of the test organisms are given in Table1. The test strains were cultured on either Middlebrook 7H10 agar [14] or Löwenstein-Jensen slants [15] for up to 8 weeks at 37° C. Approximately 100 mg cells of each organism were harvested directly from cultures which showed heavy visible growth. The biomass preparations were washed twice with TE buffer and then used for DNA extraction.

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Table 1. Sources and histories of the test strains.

Laboratory No.	Other designation	Strains	Sources and strain histories
M539	539	*M. avium*-like strain	J.G. Magee, Regional Tuberculosis Reference Center, PHLS, General Hospital, Newcastle-upon-Tyne, 91/5131; chest swab
M501	501	*M. intracellulare*-like strain	R.C. Good, Center for Disease Control, Atlanta, Georgia, USA, serotype 11A, Vitoch
M660	660	*M. intracellulare*-like strain	NCTC 10424; M. Tsukamura, N 100616 (=ATCC 23068)
M666	666	*M. intracellulare*-like strain	R. C. Good, serotype 12B, P42
M495	495	*M. intracellulare*-likestrain	R. C. Good, Serotype 8A; ATCC 23435
M575	575	*M. scrofulaceum*-like strain	R. C. Good, serotype 26B; Mackenzie 2233
M782	782	*M. scrofulaceum*-like strain	V. Lévy-Frébault, 910486; gastric washing; AIDS patient
M784	784	*M. scrofulaceum*-like strain	R. C. Good, serotype 42B, 963
M786	786	*M. scrofulaceum*-like strain	R. C. Good, serotype 42B, Lunning

Abbreviations: ATCC, American Type Culture Collection, Rockville, Md., USA.; CIP, Collection de I' Institut Pasteur, Paris, France; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, UK.

16S rDNA sequencing. The extraction and purification of DNA and the amplification, cloning, and sequencing of the 16S rRNA gene were carried out as described previously [16]. The resultant 16S rDNA sequence was aligned manually with the available sequences of mycobacteria by using the AL16S program [17]. The additional sequence data were obtained from the GenBank and EMBL databases.

The similarity matrix and the phylogenetic trees were based on 1404 nucleotide positions. Evolutionary trees were inferred by using three algorithms: the neighbour-joining [18], least-squares [19] and maximum parsimony [20] methods. Evolutionary distance matrices for the neighbour-joining and least-squares methods were generated as described by Jukes and Cantor [21]. The PHYLIP package [22] was used for all analyses. The resultant unrooted tree topologies were evaluated by the performing bootstrap analyses [23] of the neighbour-joining method based on 1,000 resamplings. The least squares and maximum-likelihood analyses were not bootstrapped due to the unacceptably long computing time required for the necessary calculations.

RESULTS

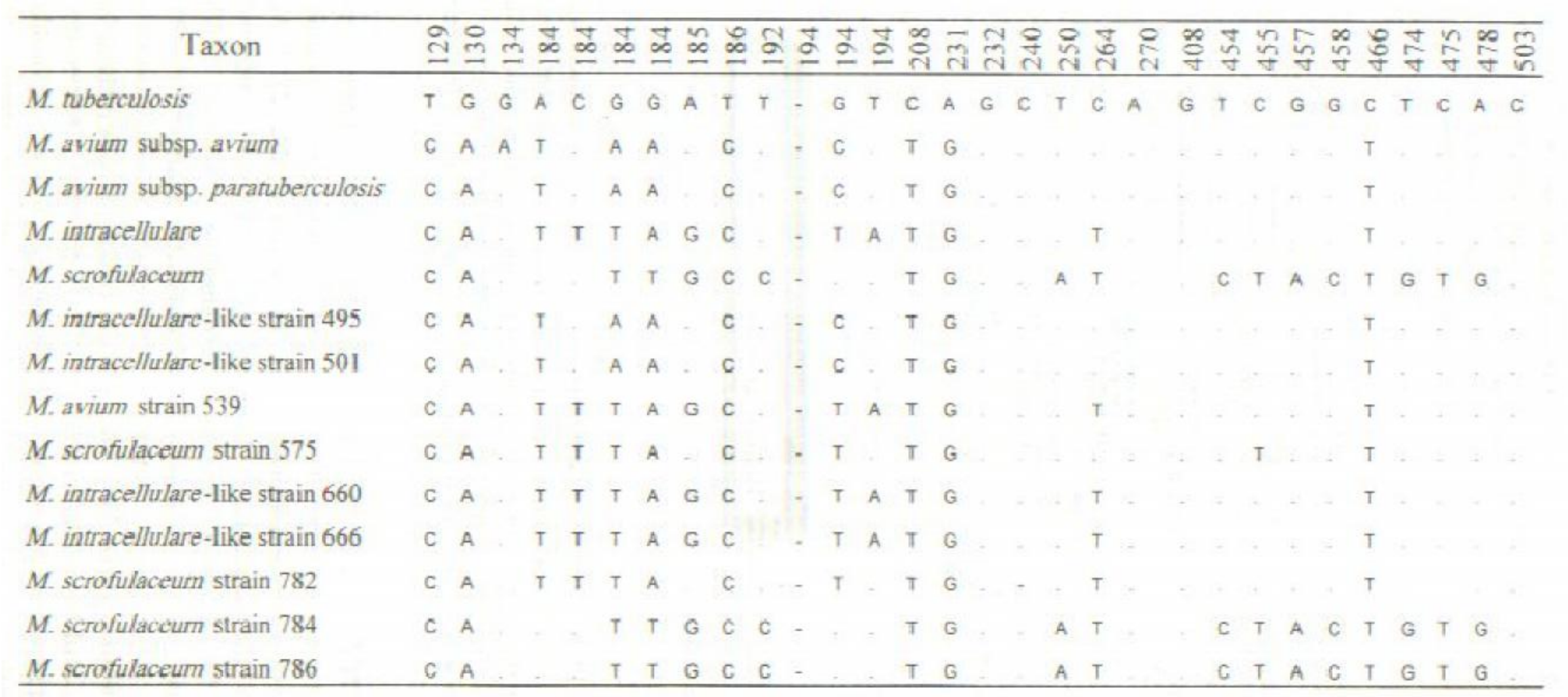
Almost the complete 16S rDNA sequences of the nine test strains (1517 nucleotides) were manually aligned with sixty-five corresponding mycobacterial 16S rDNA sequences.

All of the test strains showed mycobacterial nucleotide signature sequences that is, the sequences at positions 70-98 (A-T), 293-304 (G-T), 307 (T), 328 (T), 614-626 (A-T), 631 (G), 661-744 (G-C), 824-876 (T-A), 825-875 (A-T), 843 (C), and 1122-1151 (A-T) (*E. coli* numbering system; [24-25]). The 16S rRNA sequences of *M. avium*-like strain 539, *M. intracellulare*-like strains 495, 501, 660 and 666, and *M. scrofulaceum*-like strains 575, 782, 784, and 786, had the extended 16S rRNA helix at positions 451 to 482 which is the characteristic of slowly-growing mycobacteria.

The pairwise nucleotide similarity values determined for *M. intracellulare*-like strains 495 and 501 with the slowly-growing mycobacteria range from 95.1 to 99.8%. *Mycobacterium intracellulare*-like strains 495 and 501 show their highest levels of 16S rDNA similarity with *M. avium* subsp. *avium* DSM 43216 (99.6 to 99.8%, respectively) and *M. avium* subsp. *paratuberculosis* ATCC 19698^T (99.5 to 99.7%, respectively). The signature nucleotide sequences of *M. intracellulare*-like strains 495 and 501 at hypervariable region A (positions 125 to 266, *E. coli* numbering) were identical and similar to that of *M. avium* subsp. *paratuberculosis* ATCC 19698^T (Table 2).

The pairwise nucleotide similarity values determined for *M. scrofulaceum*-like strains 575 and 782 with the slowly-growing mycobacteria range

Table 2. Selected stretches of the first and second signature nucleotide regions in the 16S rDNA gene[a] of slowly-growing mycobacteri.[b,c,d]



Taxon	129	130	134	184	184	184	184	185	186	192	194	194	194	208	231	232	240	250	264	270	408	454	455	457	458	466	474	475	478	503
M. tuberculosis	T	G	G	A	C	G	G	A	T	T	-	G	T	C	A	G	C	T	C	A	G	T	C	G	G	C	T	C	A	C
M. avium subsp. *avium*	C	A	A	T	.	A	A	.	C	.	-	C	.	T	G	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
M. avium subsp. *paratuberculosis*	C	A	.	T	.	A	A	.	C	.	-	C	.	T	G	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
M. intracellulare	C	A	.	T	T	T	A	G	C	.	-	T	A	T	G	.	.	.	T	.	.	.	.	.	.	T	.	.	.	.
M. scrofulaceum	C	A	.	.	.	T	T	G	C	C	-	.	.	T	G	.	.	A	T	.	.	C	T	A	C	T	G	T	G	.
M. intracellulare-like strain 495	C	A	.	T	.	A	A	.	C	.	-	C	.	T	G	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
M. intracellulare-like strain 501	C	A	.	T	.	A	A	.	C	.	-	C	.	T	G	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
M. avium strain 539	C	A	.	T	T	T	A	G	C	.	-	T	A	T	G	.	.	.	T	.	.	.	.	.	.	T	.	.	.	.
M. scrofulaceum strain 575	C	A	.	T	T	T	A	.	C	.	-	T	.	T	G	.	.	.	.	.	.	.	T	.	.	T	.	.	.	.
M. intracellulare-like strain 660	C	A	.	T	T	T	A	G	C	.	-	T	A	T	G	.	.	.	T	.	.	.	.	.	.	T	.	.	.	.
M. intracellulare-like strain 666	C	A	.	T	T	T	A	G	C	.	-	T	A	T	G	.	.	.	T	.	.	.	.	.	.	T	.	.	.	.
M. scrofulaceum strain 782	C	A	.	T	T	T	A	.	C	.	-	T	.	T	G	.	-	.	T	.	.	.	.	.	.	T	.	.	.	.
M. scrofulaceum strain 784	C	A	.	.	.	T	T	G	C	C	-	.	.	T	G	.	.	A	T	.	.	C	T	A	C	T	G	T	G	.
M. scrofulaceum strain 786	C	A	.	.	.	T	T	G	C	C	-	.	.	T	G	.	.	A	T	.	.	C	T	A	C	T	G	T	G	.

[a]The numbers indicate the respective *E. coli* 16S rRNA positions. [b]*M.tuberculosis* was used as the reference sequence. [c]Dashes indicate deletions. [d]A"." Indicates that the the base pair was identical to the M. tuberculosis base pair.

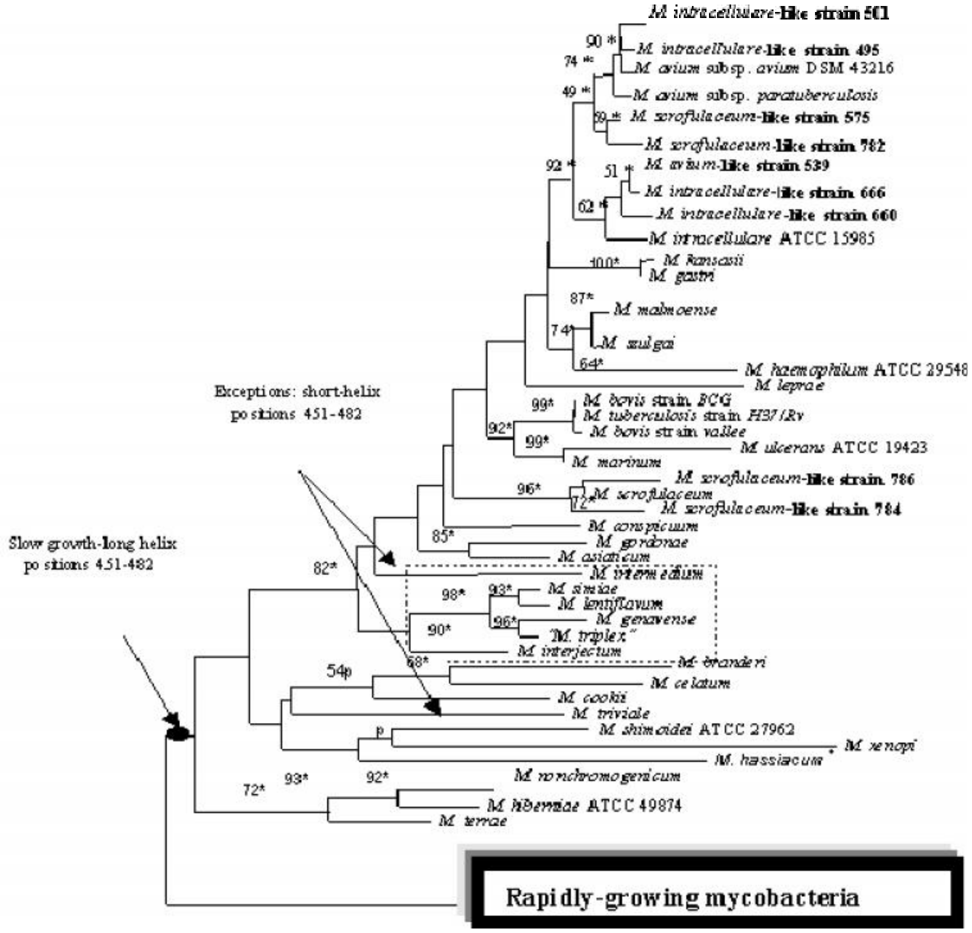


Fig. 1. Neighbor-joining tree [9) based on nearly complete 16S rDNA sequences of mycobacteria (1398 nucleotides) showing the phylogenetic position of test strains. The asterisks indicate branches that were recovered with all four methods (Neighbor-joining tree; the Fitch-Margoliash; maximum parsimony; and maximum-likelihood). The numbers at the nodes indicate the level of bootstrap support based on a neighbor-joining analysis of 1,000 re-sampled datasets; only values greater than 40% are given. The scale bar indicates 0.005 substitutions per nucleotide position.

from 95.7 to 99.4%. These strains show their highest levels of 16S rDNA similarity with one another (99.6%), *M. avium* subsp. *avium* DSM 43216 (99.3 to 98.9%, respectively) and *M. avium* subsp. *paratuberculosis* ATCC 19698^T (99.3 to 98.8%, respectively). These values correspond to 5 nucleotide differences from one another and 9 and 15 nucleotide differences from the sequence of *M. avium*subsp. *avium* DSM 43216 and 10 and 16 nucleotide differences from the corresponding nucleotide sequence of *M. avium* subsp. *paratuberculosis* ATCC 19698^T (Table 2). The signature nucleotide sequences of *M. scrofulaceum*-like strains 575 and 782 at hypervariable region A were different from one another by a base pair at position 264. These nucleotide signatures distinguished these strains from all other mycobacteria (Table 2). It was also interesting that *M. scrofulaceum*-like strains 575 and 782 formed a recognisable phyletic subline in all three phylogenetic analyses (Fig. 1). The integrity of this subline was also supported by the high bootstrap value based on the neighbour-joining method.

The pairwise nucleotide similarity values determined for *M. avium*-like strain 539 and *M. intracellulare*-like strains 660 and 666 with the slowly-growing mycobacteria range from 94.8 to 99.4% (Table 2). *Mycobacterium avium*-like strain 539 and *M. intracellulare*-like strains 660 and 666 show their highest 16S rDNA similarity levels with *M.*

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intracellulare ATCC 15985 (99.1%, 99.3% and 99.4%, respectively). These relationships are supported by the results obtained with all three treeing algorithms and by the high bootstrap value obtained with the neighbour-joining method (Fig. 1). All three strains have signature nucleotide sequences identical to that of *M. intracellulare* ATCC 15985 (Table 2).

The pairwise nucleotide similarity values determined for *M. scrofulaceum*-like strains 784 and 786 with the slowly-growing mycobacteria range from 95.8 to 99.6%. These strains show their highest level of 16S rDNA nucleotide similarity with the type strain of *M. scrofulaceum* (99.2 and 99.6%, respectively). The relationships between these strains and the type strain of *M. scrofulaceum* (ATCC 19981T) was highlighted with all three treeing algorithms and by the high bootstrap value obtained using the neighbour-joining method (Fig. 1). In addition, *M. scrofulaceum*-like strains 784 and 786 have signature nucleotide sequences identical to that of *M. scrofulaceum* ATCC 19981T (Table 2).

DISCUSSION

It is difficult to distinguish between clinically significant slowly-growing, non-pigmented mycobacteria, notably to separate *M. avium* and *M. intracellulare* from one another and from *M. scrofulaceum* strains [13]. Mycobacterial strains assigned to one or other of these species on the basis of a few phenotypic characters do not always cluster with the appropriate species in more comprehensive taxonomic studies. It has been proposed that such strains be treated as *M. avium/ intracellulare/ scrofulaceum* (MAIS) intermediates until their taxonomic standing is clarified [2-3]. However, it is evident from the present study that 16S rDNA sequencing and appropriate chemotaxonomic and microbiological data provide a means to characterise mycobacteria that are difficult to classify using conventional diagnostic procedures.

It is clear from the nucleotide similarity values (Table 2), the nucleotide signature sequences (Table 3) and from the phylogenetic trees (Fig. 1) that two out of the nine slowly-growing organisms, that is, *M. scrofulaceum*-like strains 575 and 782 can be distinguished from members of validly described mycobacterial species. The remaining test strains, namely, *M. avium*-like strain 539, *M. intracellulare*-like strains 495, 501, 660 and 666, *M. scrofulaceum*-like strains 784 and 786, have signature sequences identical to those of previously described mycobacteria.

The close relationship found between *M.intracellulare*-like strains 495 and 501 in the numerical phenetic survey of Magee [26] is supported by the 16S rRNA sequence data which show that these organisms have 1514 out of 1517 nucleotide sequences in common. The sequence data also show that these strains can be provisionally assigned to the taxon *M. avium* subsp. *paratuberculosis* as they share a 16S rRNA signature with the type strain of this sub spp. This result casts doubt on the assignment of these strains to the species *M. intracellulare* on the basis of the IWGMT serovar data [27] and their provisional classification as *M. intracellulare*-like strains using a battery of phenotypic properties [26]. However, further comparative taxonomic studies are required to clarify relationships between strains assigned to the various subspp. Of *M. avium*. Such studies should include a judicious selection of organisms, including the relevant type strains.

It is also clear from the 16S rRNA sequence data that *M. avium*-like strain 539 and *M. intracellulare*-like strains 660 and 666 are closely related to one another and with *M. intracellulare* ATCC 15985. In addition, these organisms form a distinct clade and share an identical nucleotide signature sequence. Strain 539 was classified as a *M. avium*-like organism in the numerical phenetic survey of Magee [26]. Strains 660 and 666 were classified as *M. intracellulare* in the IWGMT serovar study [27] and formed a minor cluster in the numerical phenetic survey of Magee [26]. It is clear that DNA:DNA relatedness experiments are needed to resolve the detailed taxonomic relationships between *M. avium*-like strain 539, *M. intracellulare*-like strains 660 and 666 and *M. intracellulare* ATCC 15985.

The 16S rRNA sequence data show that *M. scrofulaceum* like strains 575 and 782 are closely related not with *M. scrofulaceum* ATCC 19981T but with *M. avium* subsp. *avium* DSM 43216 and *M. avium* subsp. *paratuberculosis* ATCC 19698T. Strains 575 and 782 were consistently assigned to the same cluster in the numerical phenetic survey of Magee [26] but were considered to be *M. scrofulaceum* strains in the IWGMT serovar study [27]. It is also interesting that the two organisms have nucleotide signature sequences which differentiate them from one another, from *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis* and

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Table 3. phenotypic characteristics of members of closely related slowly-growing mycobacteria.

Characteristics	*M. avium*	*M. avium* subsp. *paratuberculosis*	*M. avium* subsp.*silvaticum*	*M. intracellulare*	*M. scrofulaceum*	*M. intracellulare*-like strain 495	*M. intracellulare*-like strain 501	*M. avium-like* strain 539	*M. scrofulaceum* strain 575	*M. intracellulare*-like strain 660	*M. intracellulare*-like strain 666	*M. scrofulaceum* strain 782	*M. scrofulaceum* strain 784	*M. scrofulaceum* strain 786
Enzymatic properties:														
Arylsulphatase														
14 days	–	v	–	v	–	–	–	–	–	+	–	+	–	–
21 days	v	ND	–	+	–	–	–	–	+	+	–	+	–	–
28 days	+	ND	+	+	+	+	+	–	+	+	+	+	+	+
Catalase (>45mm of foam)	–	–	–	–	v	–	–	–	–	–	–	+	+	+
Tellurite reduction	+	ND	+	+	+	+	+	–	–	–	–	+	+	+
Tween hydrolysis 10 days	–	v	–	–	–	–	–	+	–	–	–	–	–	–
Niacin accumulated	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Nitrate reduction	–	–	–	–	–	–	–	–	–	–	–	+	–	–
Beta-Galactosidase activity	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Morphological properties:														
Photochromogenic	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Scotochromogenic	–	–	–	v	+	+	+	–	–	–	–	–	+	+
Growth at:														
30°C	+	ND	+	+	+	+	+	+	+	+	+	+	+	+
36°C	+	ND	+	+	+	+	+	+	+	+	+	+	+	+
42°C	+	+	+	+	v	+	+	–	–	+	–	–	–	–
45°C	v	ND	v	–	–	–	–	–	–	–	–	–	–	–
50°C	–	ND	–	–	–	–	–	–	–	–	–	–	–	–
Colony colour:														
Buff	v	ND	–	–	–	–	–	–	+	–	–	+	–	–
None	–	ND	–	–	–	–	–	–	–	–	–	–	–	–
Yellow	–	ND	–	v	+	+	+	–	–	–	–	–	+	+
Growth in the presence of														
Ethambutol (1.6mg/ml)	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Isoniazid (10mg/l)	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NaCl (5%)	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Hydroxylamine (125mg/l)	+	ND	+	+	+	+	+	+	+	+	+	+	+	+
Hydroxylamine (500mg/l)	+	ND	–	+	+	+	+	+	+	+	+	+	+	+
p-Nitrobezoic acid	+	ND	+	+	+	+	+	+	+	+	+	+	+	+
Toluidine (300 mg/l)	+	ND	+	+	+	+	+	+	+	+	+	+	+	+
Mycobactin is required for growth	–	+	–	–	–	ND	ND	ND	ND	ND	ND	ND	ND	ND

Key: =, over 80% of strains positive; V, 20 to 80% of strains positive; -, over 80% of strains negative; ND, not determined; V, vaiable from all other species of *Mycobacterium*. Strains 575 and 782 may form the nucleus of two new sub spp. Of *M. avium* as they have a number of phenotypic properties, which distinguish them from the validly described sub spp. of this taxon. Moreover, *M. avium* strains 575 and 782 can readily be distinguished from one another and from *M. scrofulaceum* strains by several characters such as catalase activity and scotochromogenicity (Table 3). However, additional work is required, notably DNA: DNA relatedness studies, to clarify the finer taxonomic relationships of *M. avium* strains 575 and 782.

The 16S rRNA sequence data, together with the results of the IWGMT serovar study [27], clearly show that *M. scrofulaceum*-like strains 784 and 786 are bona fide members of the species *M. scrofulaceum*. This assignment is supported by high nucleotide similarity values, a species specific nucleotide sequence and by a number of key phenotypic properties.

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