Phylogenetic analysis of *Mycobacterium aurum* and *Mycobacterium neoaurum* with redescription of *M. aurum* culture collection strains

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We examined American Type Culture Collection (ATCC) strains of *Mycobacterium aurum* and *Mycobacterium neoaurum* by using multilocus DNA target sequencing. Apart from the type strain, all 10 ATCC *M. aurum* strains examined were classified incorrectly, with most being reclassified as belonging to the *M. neoaurum*–‘*Mycobacterium lacticola*’ relatedness group. All four *M. neoaurum* strains were tightly clustered, but heterogeneity was observed within the cluster. As a result of the incorrect annotation of the *M. aurum* strains, two commonly used methods of identification are compromised and two case reports implicating *M. aurum* as a human pathogen are probably incorrect, with the isolates probably belonging to the *M. neoaurum*–‘*M. lacticola*’ relatedness group. These findings together with a review of isolates identified at two large reference laboratories suggest that *M. aurum* is not a clinically significant isolate.

An accurate taxonomic classification of culture collection strains for *Mycobacterium* species is important for providing the framework for reference databases used in microbiology laboratories. A widely accepted approach for determining the species of *Mycobacterium* is DNA target sequence analysis with the 16S rRNA gene. However, sequencing multiple DNA targets for identification is an increasingly recognized tool for assigning more accurate taxonomic classifications (Lan & Reeves, 2001; Maiden *et al.*, 1998) and for describing novel species (Adékambi *et al.*, 2004, 2006a, b). In particular, *rpoB* and *hsp65* genes have served as excellent targets to discriminate closely related *Mycobacterium* species (Adékambi *et al.*, 2003, 2006a; Williams *et al.*, 2007).

Abbreviations: ARUP, Associated Regional and University Pathologists; ATCC, American Type Culture Collection; PRA, PCR-restriction fragment length polymorphism analysis; UTHSCT, University of Texas Health Science Center.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are given in Supplementary Table S1 in IJSEM Online.

Neighbour-joining trees based on *rpoB* and *hsp65* gene sequences and a table of the ATCC strains used and GenBank accession numbers for the genes investigated in this study are available as supplementary material with the online version of this paper.

Since 2001, Associated Regional and University Pathologists (ARUP) Laboratories, a large national reference laboratory that provides a service to all 50 United States, have identified *Mycobacterium* species by sequencing the first 500 bp of the 16S rRNA gene. Upon comparison of sequence-based identifications with a PCR-restriction fragment length polymorphism analysis (PRA) of *hsp65* (Devallois *et al.*, 1997; Telenti *et al.*, 1993), we observed discrepancies in the species identifications of *Mycobacterium aurum* and *Mycobacterium neoaurum*, which prompted an investigation. In general, a laboratory's confidence in a test result that is based on either gene sequencing or PRA methods increases when isolates share high identity scores or have patterns that match with culture collection strains. The American Type Culture Collection (ATCC) has 11 strains of *M. aurum* and four strains of *M. neoaurum*. Both *M. aurum* and *M. neoaurum* were described prior to 1975 (Tsukamura & Mizuno, 1972; Tsukamura & Tsukamura, 1966) and, to our knowledge, these ATCC strains have not been interrogated by using modern genetic techniques to determine the accuracy of their identification. In this study, we aimed to clarify the phylogenetic relationships of culture collection strains of *M. aurum* and *M. neoaurum*.

All available ATCC strains of *M. aurum* and *M. neoaurum* were obtained directly from the ATCC, and 19 clinical

isolates identified by *hsp65* PRA as *M. aurum* or *M. neoaurum* by the University of Texas Health Science Center (UTHSCT) were also obtained from stocks that had been stored at -70°C since isolation. In addition, microbiological records were reviewed for clinical information on all clinical isolates that had been identified as *M. aurum* and *M. neoaurum* between July 2003 and March 2008 at ARUP Laboratories and between January 1995 and March 2008 at UTHSCT. Strains were grown according to the instructions provided by the ATCC. DNA extraction, PCR and sequencing reactions were performed as described previously (Simmon *et al.*, 2007). Amplification and sequencing reactions were performed using primers targeting ~1500 bp of the 16S rRNA gene: 5F (5'-TTGGAGAGTTTGATC-CTGGCTC-3') and 1492R (5'-ACGGITACCTTGTTA-CGACTT-3'); and ~750 bp of *rpoB* (Adékambi *et al.*, 2003) and ~440 bp of *hsp65* genes (Telenti *et al.*, 1993). PCR products were purified and sequenced as described previously (Simmon *et al.*, 2006). Sequence alignments and phylogenetic trees were constructed using the neighbour-joining method with Kimura's two-parameter distance correction model and 1000 bootstrap replications in the MEGA version 4 software package (Tamura *et al.*, 2007). All clinical isolates were subjected to *hsp65* PRA analysis of a 441 bp fragment, as described previously by Telenti *et al.* (1993). This study was conducted under IRB approved protocols at both ARUP Laboratories and UTHSCT.

Evaluation of *M. aurum* and *M. neoaurum* culture collection strains

Comparisons of the full 16S rRNA gene sequences from the culture collection strains showed that the type strain of *M.*

aurum (ATCC 23366^T) shared very little identity with the other 10 culture collection strains identified as *M. aurum* (Fig. 1). All four *M. neoaurum* strains shared high identity with one another, with identical 16S rRNA sequences for the first 500 bp of the gene, with one strain showing 4 bp differences when the entire gene was analysed (Fig. 1). Analysis of the *hsp65* and *rpoB* genes showed heterogeneity among all four *M. neoaurum* strains (Supplementary Fig. S1, available in IJSEM Online). Sequences from the ATCC strains were compared with GenBank references to ascertain the most closely related species for each strain (Table 1). The ATCC 23366^T type strain for *M. aurum* differed by 6 bp from the reference sequence from GenBank (X55595), which was derived from the same type strain. The ATCC 23366^T type strain did share 100% identity with the equivalent reference in RIDOM (Ribosomal Differentiation of Medical Microorganisms; <http://www.ridom-rdna.de/>). This observation was confirmed with repeat testing. Seven strains (ATCC 27277, 25791, 25803, 25800, 25799, 25798, 23071) were most closely related to *M. neoaurum*. The remaining three *M. aurum* ATCC strains shared greater identities with emerging or novel pathogens; namely, *Mycobacterium fluoranthenvivorans* (*M. aurum* ATCC 23070), '*Mycobacterium petroleophilum*' (*M. aurum* ATCC 21498), and *Mycobacterium chlorophenolicum* (*M. aurum* ATCC 25793). Further characterization using *hsp65* and *rpoB* gene sequencing corroborated these phylogenetic relationships. Based on multi-DNA target sequence data and currently accepted practices for mycobacterial identification, we propose that the identification of all *M. aurum* ATCC strains, excluding the type strain of *M. aurum*, should be

Fig. 1. Neighbour-joining tree based on full 16S rRNA gene sequences with percentage bootstrap values (based on 1000 replications) recorded at each node. Bar, 0.002 substitutions per nucleotide position.

Table 1. Taxonomic reclassification of ATCC culture collection strains of *M. aurum* and *M. neoaurum* by using full 16S rRNA gene sequencing

ATCC number	ATCC identification	Top species match (GenBank number)	Per cent identity	Next species match (GenBank number)	Per cent identity	Corrected identification
25803	<i>M. aurum</i>	' <i>M. lacticola</i> ' ATCC 9626 (AF480582)	100.0	<i>M. neoaurum</i> ATCC 25795 ^T (AF480593)	99.4	<i>M. neoaurum</i> –' <i>M. lacticola</i> '
25800	<i>M. aurum</i>	' <i>M. lacticola</i> ' ATCC 9626 (AF480582)	100.0	<i>M. neoaurum</i> ATCC 25795 ^T (AF480593)	99.4	<i>M. neoaurum</i> –' <i>M. lacticola</i> '
25799	<i>M. aurum</i>	' <i>M. lacticola</i> ' ATCC 9626 (AF480582)	100.0	<i>M. neoaurum</i> ATCC 25795 ^T (AF480593)	99.4	<i>M. neoaurum</i> –' <i>M. lacticola</i> '
25798	<i>M. aurum</i>	' <i>M. lacticola</i> ' ATCC 9626 (AF480582)	100.0	<i>M. neoaurum</i> ATCC 25795 ^T (AF480593)	99.4	<i>M. neoaurum</i> –' <i>M. lacticola</i> '
27277	<i>M. aurum</i>	<i>M. neoaurum</i> ATCC 25795 ^T (AF480593)	100.0	' <i>M. lacticola</i> ' ATCC 9626 (AF480582)	99.4	<i>M. neoaurum</i> –' <i>M. lacticola</i> '
25791	<i>M. aurum</i>	' <i>M. lacticola</i> ' ATCC 9626 (AF480582)	99.7	<i>M. neoaurum</i> ATCC 25795 ^T (AF480593)	99.7	<i>M. neoaurum</i> –' <i>M. lacticola</i> '
23071	<i>M. aurum</i>	<i>M. neoaurum</i> ATCC 25795 ^T (AF480593)	99.7	' <i>M. lacticola</i> ' ATCC 9626 (AF480582)	99.4	<i>M. neoaurum</i> –' <i>M. lacticola</i> '
25793	<i>M. aurum</i>	<i>M. chlorophenolicum</i> (X79094)	99.7	<i>M. chubuense</i> ATCC 27278 (AF480597)	99.7	<i>M. chlorophenolicum</i>
23070	<i>M. aurum</i>	<i>M. fluoranthenorans</i> (AJ617741)	99.3	' <i>M. sacrum</i> ' (AY235429)	99.2	Most closely related to <i>M. fluoranthenorans</i>
21498	<i>M. aurum</i>	' <i>M. petroleophilum</i> ' ATCC 21497 (AF480587)	100.0	<i>M. gilvum</i> (CP000656)	99.2	' <i>M. petroleophilum</i> '
23366 ^T	<i>M. aurum</i>	<i>M. aurum</i> ATCC 23366 ^T (X55595)*	99.6	<i>M. aichiense</i> (AF498656)	98.4	No change
25801	<i>M. neoaurum</i>	<i>M. neoaurum</i> ATCC 25795 ^T (AF480593)	99.7	' <i>M. lacticola</i> ' ATCC 9626 (AF48052)	99.4	No change
25796	<i>M. neoaurum</i>	<i>M. neoaurum</i> ATCC 25795 ^T (AF480593)	100.0	' <i>M. lacticola</i> ' ATCC 9626 (AF480582)	99.4	No change
23072	<i>M. neoaurum</i>	<i>M. neoaurum</i> ATCC 25795 ^T (AF480593)	99.9	' <i>M. lacticola</i> ' ATCC 9626 (AF480582)	99.6	No change
25795 ^T	<i>M. neoaurum</i>	<i>M. neoaurum</i> ATCC 25795 ^T (AF480593)	100.0	' <i>M. lacticola</i> ' ATCC 9626	99.4	No change

*Confirmed with repeat testing.

changed to their respective taxonomic identifications as given in Table 1.

Identification of *M. aurum* and *M. neoaurum* by using partial 16S rRNA gene sequencing and *hsp65* PRA

To assess the impact of the incorrectly annotated strains on result reporting, laboratory results from two laboratories performing either partial 16S rRNA gene sequencing or *hsp65* PRA analysis were reviewed. Of 7413 mycobacterial isolates identified at ARUP Laboratories, no clinical isolates were identified as *M. aurum*, 20 were identified as belonging to the *M. neoaurum*–'*M. lacticola*' group and one was identified as *M. chlorophenolicum*. Only the *M. aurum* type strain is available in public and commercial reference databases for 16S rRNA gene sequence comparison. The

incorrectly annotated ATCC sequences of *M. aurum*, therefore, did not affect the accuracy of laboratory test results using partial 16S rRNA gene sequencing. This observation was supported by another large-scale study that identified *Mycobacterium* spp. by using partial 16S rRNA gene sequencing with the MicroSeq reference database. Of 773 clinical isolates, none were identified as *M. aurum* (Hall *et al.*, 2003).

Conversely, isolates at UTHSCT were identified by using both phenotypic and PRA methods, and 25 isolates were identified as *M. neoaurum* or *M. aurum* without a definitive result to the species level. The *hsp65* PRA database contained three different PRA patterns for *M. aurum* that were derived from strain ATCC 23366^T (*M. aurum*), *M. aurum* strain ATCC 23070 (Devallois *et al.*, 1997) and *M. aurum* strain CIP 14121.0005 (Telenti *et al.*, 1993). Strain CIP 14121.0005 is no longer listed in the

Institut Pasteur culture collection and we were unable to retrieve this isolate to verify its identification. None of the 25 UTHSCT clinical isolates matched the *hsp65* PRA pattern for the type strain of *M. aurum*, 24 matched the pattern that was more correctly annotated as *M. fluoranthenorans* (but also identical to the predicted PRA pattern of *M. neoaurum* and *Mycobacterium frederiksbergense*), and the final isolate shared a pattern with the isolate from the Institut Pasteur (Table 2).

Taxonomic concerns

Although we clarified the taxonomic classifications among *M. aurum* culture collection strains, our investigations highlighted the need for finer phylogenetic distinctions within the *M. neoaurum* relatedness group. From phylogenetic analyses of 16S rRNA, *rpoB* and *hsp65* gene targets, we classified a total of six *M. aurum* and three *M. neoaurum* culture collection strains in a *M. neoaurum* taxonomic cluster (Fig. 1). Of particular concern was the distinction between *M. neoaurum* and '*M. lacticola*', the name of which to date is not validly published, but has been reported in the literature (Kiska *et al.*, 2004). With full 16S rRNA gene sequencing, *M. aurum* strains ATCC 25803, 25800, 25799 and 25798 shared 100% identity with the type strain of '*M. lacticola*', which differed by 8 bp from the type strain of *M. neoaurum*. With the *hsp65* and *rpoB* targets, these four ATCC isolates remained closely clustered as '*M. lacticola*' group, but intraspecies variability differed (0.7% for *hsp65* and 1.5% for *rpoB*). Further phylogenetic distinctions for the remaining seven ATCC strains that comprise the *M. neoaurum* relatedness group could not be determined reliably in the absence of genealogical concordance among the three targets. We propose that all 11 culture collection strains as annotated in Fig. 1 be considered part of the *M. neoaurum* and '*M. lacticola*' relatedness groups, until studies are performed to evaluate their larger genomic differences and determine their biological relevance.

Clinical impact

Only two case reports have been reported in the literature that implicate *M. aurum* as a cause of infection, both of which were catheter-related bloodstream infections. (Esteban *et al.*, 1998; Koranyi & Ranalli, 2003). Esteban *et al.* (1998) did not specify their method of identification, and Koranyi & Ranalli (2003) reported the performance of high-performance liquid chromatography (HPLC). Of note, the published method for mycobacterial identification by HPLC includes the ATCC 25793 *M. aurum* strain, which is more closely related to *M. chlorophenolicum* (Butler & Guthertz, 2001). Without the benefit of a molecular-based test, we question the accuracy of identification methods from these two case reports. In addition, not finding *M. aurum* among any clinical isolates from our current and other longitudinal studies (Hall *et al.*, 2003) adds to our uncertainty that *M. aurum* is associated with invasive disease. It should also be noted that the type strain of *M. aurum* was isolated from soil and not associated with human disease (Tsukamura & Tsukamura, 1966). On the other hand, *M. neoaurum* has been well-recognized as a human pathogen associated with meningoencephalitis (Heckman *et al.*, 2004) and catheter-related bloodstream infections in immunocompromised hosts (Washer *et al.*, 2007).

We conclude that, except for the type strain, ATCC culture collection strains of *M. aurum* are incorrectly annotated. We also demonstrate that partial 16S rRNA gene sequencing is unaffected by these errors and can reliably distinguish between *M. aurum* and *M. neoaurum* as public and commercial databases currently have only sequences from the ATCC *M. aurum* type strain. Conversely, identification by *hsp65* PRA analysis can lead to inaccurate classification of isolates as *M. aurum* because reference patterns are derived from erroneously annotated ATCC strains. Laboratories using this method should modify their reference set based on our identifications shown in Table 2. Finally, we propose that the majority of *M. aurum* strains belong to the *M. neoaurum*–'*M. lacticola*' relatedness

Table 2. Comparison of clinical isolates identified by using *hsp65* PRA and 16S rRNA gene sequencing analyses

NA, Not applicable.

PRA pattern attributed to <i>M. aurum</i>	Source of PRA reference pattern for <i>M. aurum</i>	No. of clinical isolates	Identification by partial 16S rRNA (% identity)*	Identification by full 16S rRNA gene (% identity)
I	ATCC 23366 ^T	0	NA	NA
II	ATCC 23070	24	16 <i>M. neoaurum</i> ; 3 <i>M. frederiksbergense</i> (100%)	16 <i>M. neoaurum</i> ; 2 <i>M. fluoranthenorans</i> (99.3%); 1 <i>M. frederiksbergense</i> (99.2%)
III	CIP 14121.0005	1	<i>Mycobacterium</i> sp. (98.7%)	<i>Mycobacterium</i> sp. (98.5%)

*19 of the 24 isolates were analysed by using 16S rRNA gene sequencing, the remaining five were not available for further analysis.

group. Further phylogenetic distinctions within this group cannot be made with 16S rRNA, *hsp65* and *rpoB* gene targets because of a lack of genealogical concordance and a high degree of intraspecies variability. Further study is warranted to better define the need for greater phylogenetic distinctions within the *M. neoaurum*–*M. lacticola* group.

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