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NOTES

16S rRNA Sequence Diversity in *Mycobacterium celatum* Strains Caused by Presence of Two Different Copies of 16S rRNA Gene

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Direct sequencing of the 16S rRNA gene (16S rDNA) of *Mycobacterium celatum* isolates showed ambiguities, suggesting heterogeneity. Cloned 16S rDNA yielded two copies of the gene, which differed by insertion of a thymine at position 214 and by additional mismatches. Restriction fragment length polymorphism analysis confirmed the presence of two copies of 16S rDNA within the bacterial chromosome.

Mycobacterium celatum has been isolated from samples of immunocompetent patients with pulmonary disease (5) and from patients infected with human immunodeficiency virus in whom it had caused disseminated disease (20). While its resistance to antituberculosis treatment requires rapid and accurate diagnosis, identification of *M. celatum* by conventional techniques often takes several weeks because of the slow growth of this organism (11). Furthermore, rapid identification techniques, such as commercially available DNA probes, may yield false-positive results for *Mycobacterium tuberculosis* when they are applied to *M. celatum* (4, 11). Direct sequencing of the bacterial 16S rRNA gene (16S rDNA) has proven to be a stable and specific marker for mycobacterial identification (15, 16, 24); while the number of copies of 16S rRNA genes may vary among bacterial species, their sequences are assumed to be identical, with only minor differences (14, 21). Fast-growing members of the genus *Mycobacterium* generally have two identical copies of the 16S rRNA gene (9), while slow growers are thought to have only one (13) and, therefore, yield unambiguous sequence patterns. However, two different 16S rRNA genes were recently detected in a slowly growing mycobacterium belonging to the *Mycobacterium terrae* complex (19). Here, we report the observation of 16S rDNA heterogeneity in three isolates of the clinically important *M. celatum.*

Mycobacterial isolates. Three clinical laboratories each provided one clinical isolate of *M. celatum* (1732, T322, and MI1581). The isolates were grown on Loewenstein-Jensen medium and examined for growth rate, microscopic colony morphology, and pigmentation. Biochemical tests of all three clinical isolates yielded identical results, which were consistent with those published by Butler et al. (5).

Amplification and sequencing of genomic DNA. A single colony of each isolate was harvested and chromosomal DNA was extracted as previously described (22). One microgram of bacterial DNA was subjected to PCR with each of two primer pairs, i.e., biotinylated M285 in combination with M264 (15) and primer R247 (GTAGTCCACGCCGTAAACGG) in combination with M261 (15), thus yielding the complete 16S rDNA in two fragments. Nonradioactive sequencing of all three isolates was performed with forward primer M285 and reverse primer M259 (15) by using a commercially available cycle sequencing kit (PRISM Ready Reaction Dye Deoxy Terminator cycle sequencing kit; Applied Biosystems GmbH, Weiterstadt, Germany). Electrophoresis of sequenced DNA and data collection were performed with an automated sequencer (ABI 373A; Applied Biosystems). The results revealed identical patterns, showing heterogeneity consecutive to position 214 within helix 10 (first line of Fig. 1); in position 214, all isolates showed a partial insertion of an additional T (TTTTT instead of TTTT), thus leading to a duplication of the peaks seen on the electropherograms, consecutive to the point of insertion (Fig. 1). Clear peak patterns were obtained for the stretch between the sequencing primer and the insertion. These results were confirmed by manual sequencing (data not shown).

RFLP. Single colonies of each isolate were grown for as many as 3 weeks in 5 ml of Middlebrook 7H9 broth supplemented with 0.05% Tween 80. The cultures were centrifuged, resuspended in 0.85% NaCl, and inactivated by heat (30 min at 80°C). Thereafter, bacterial DNA was extracted with cetytrimethylammonium bromide as described elsewhere (26). After digestion with 10 U of *Pvu*II (Boehringer Mannheim GmbH, Mannheim, Germany), fragments were electrophoresed (1% agarose gel), transferred by Southern blotting onto a nylon membrane, and hybridized to a PCR-generated, genus-specific, digoxigenin-labelled probe (positions 9 to 341) (23). In the absence of a known *Pvu*II restriction site within the 16S rDNA of *M. celatum*, restriction fragment length polymorphism (RFLP) patterns of all three isolates yielded two bands of equal sizes (Fig. 2).

Cloning of 16S rDNA amplification products. The complete 16S rDNA from *M. celatum* was amplified from single colonies of isolate MI1581 with primers M285 and M261. Amplicons were directly cloned into a pCR 2.1 vector (Invitrogen Corp.,

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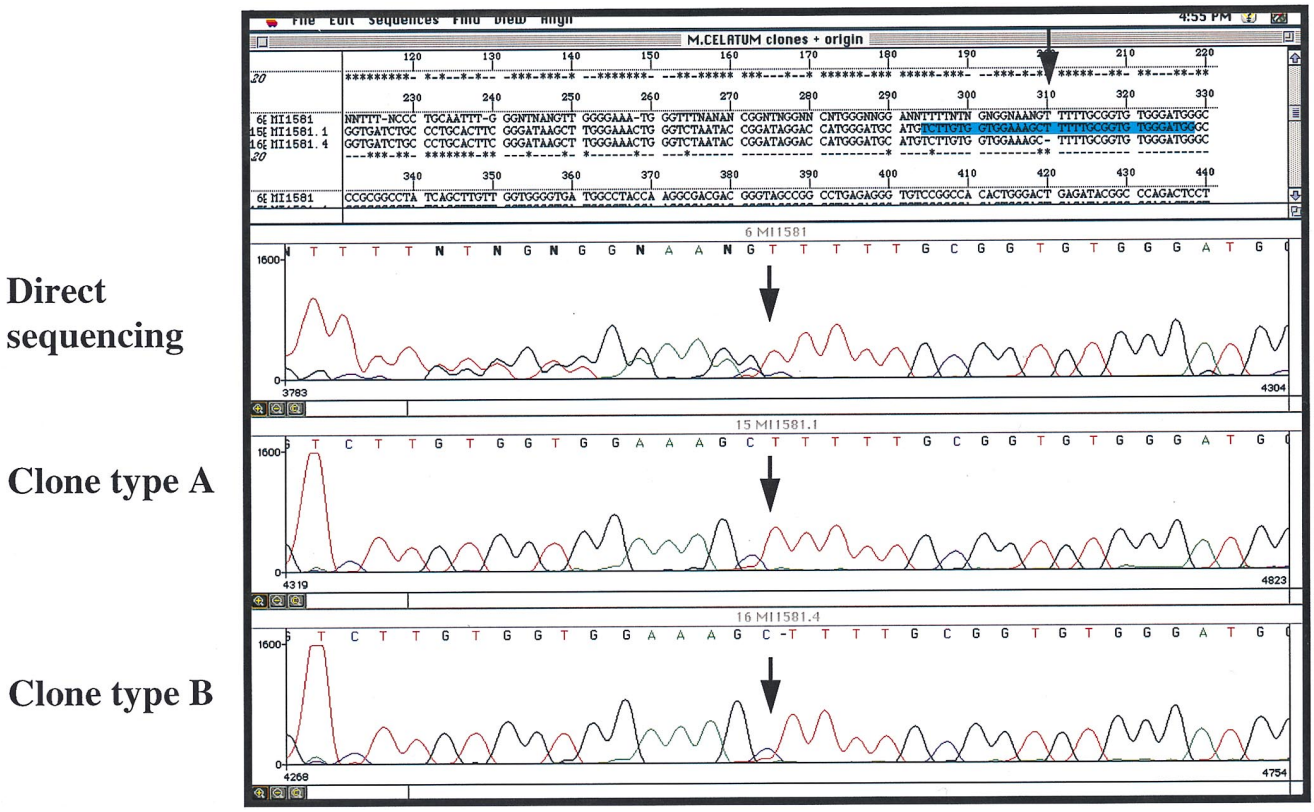

FIG. 1. Graphic plot of the direct sequencing (lane 1) of PCR-amplified 16S rDNA and cloned 16S rDNA (clones 1 and 4) of the clinical isolate MI1581, obtained by automated sequencing. The stretch of consecutive T's indicates the start of ambiguities in the pattern obtained by direct sequencing with reverse primer M259.

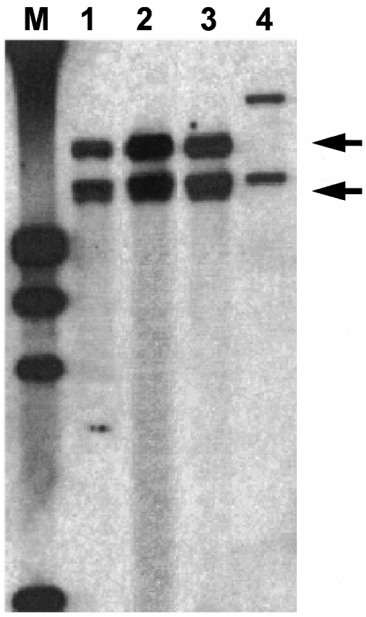

FIG. 2. RFLP analysis. Southern blot of genomic DNAs of all three clinical isolates of *M. celatum*, i.e., 1732 (lane 1), T322 (lane 2), and MI1581 (lane 3), which were digested with *Pvu*II and hybridized with a 16S rDNA-specific probe. The rapidly growing *Mycobacterium hassiacum* (lane 4) is shown as a reference. Digoxigenin-labelled DNA molecular weight marker III (Boehringer Mannheim GmbH) was applied (lane MW).

San Diego, Calif.) and transformed into competent cells (TOP10F′; Invitrogen Corp.). Plasmid DNA extracted from 20 transformed colonies yielded seven interpretable sequences of both hypervariable regions (>500 bp). In contrast to direct sequencing, two unambiguous but slightly different types of sequences were obtained; types A and B showed four and five consecutive thymine residues, respectively, at positions 210 to 214 (Fig. 1 and 3). Both types could be further differentiated within positions 78 to 85. When the sequences of both types of clones were compared with published sequences for *M. celatum*, type A was identical to *M. celatum* type 3 (MC3RNA16S [GenBank accession no. Z46664]) and type B resembled *M. celatum* type 1 (MCRGDSA [GenBank accession no. L08169]), differing only at positions 77 to 79 (additional CCT) and 110 (A/G) (Fig. 3).

Advances in technology have made direct sequencing of 16S rDNA a powerful tool for the identification of bacteria in medical microbiology. 16S rDNA sequence analysis has become a standard in bacterial identification and in systematic bacterial taxonomy (1, 16). In mycobacteriology, this approach has been particularly valuable in clinical diagnosis, since slow growth hampers diagnosis by conventional tests (2, 10). Furthermore, 16S rDNA sequencing has contributed to the description of many new species of this family, among them *M. celatum* (3, 5, 15).

16S rDNA analysis for mycobacterial identification depends on the assumption that the sequences obtained from reference strains represent functional rRNA molecules typical of their taxa (12, 27). Although the level of intraspecific sequence variability is assumed to be low, some species are represented in international databases by an unexpectedly high number of

FIG. 3. Alignment of a portion of 16S rDNA sequences (*M. celatum* types 1 (L08169) and 3 (Z46664) with clones derived from MI1581).

deviating sequences (e.g., 11 different 16S rDNA sequences for *Mycobacterium paratuberculosis*). Some authors have traced these deviations back to errors in laboratory procedures, e.g., to sequencing techniques (7) or to subspecies variations (14, 21). However, recent findings indicate the presence of heterogeneity between different copies of 16S rDNA in *Escherichia coli* (6) and in *Thermospora bispora* (25), as well as in a slowly growing mycobacterium resembling *M. terrae* (19).

In *M. celatum*, we suspected heterogeneity when identical sequence patterns were obtained in different laboratories from clinical strains from different patients; all patterns were characterized by the same ambiguities starting at position 214, regardless of the strand sequenced. However, the phenotypical features of all strains were consistent with *M. celatum* type 1. Cloning of 16S rDNA finally resolved the ambiguities, yielding two types of unambiguous sequences. Interestingly, the two types were almost identical to the reference sequences for *M. celatum* type 1 (5) and *M. celatum* type 3 (3), respectively. We take it for granted that analysis of single colonies from pure isolates ruled out contamination. Thus, our results indicate the presence of two different 16S rRNA genes within the genome of *M. celatum.*

Little is known about heterogeneity in bacterial 16S rDNA. When present, distinct types of 16S rDNA seemed to be equally expressed (19, 25), suggesting their functionality. With regard to the origin of divergent 16S rDNAs, lateral gene transfer has been discussed (18) but has not yet revealed unsuspected relationships to other species (19, 25). However, in the present study both sequences obtained by cloning were identical with either one of the GenBank reference sequences for subtypes 1 and 3 of *M. celatum*, which may not be distinguished by conventional techniques (3, 5).

With regard to unambiguous identification of mycobacteria, sequence diversity in 16S rRNA genes may raise a problem. In agreement with Clayton et al. (7), we would recommend that to define a new bacterial or mycobacterial taxon, one should investigate multiple isolates. Sequence analysis of cloned genes may complement the characterization of ambiguous positions, and possible heterogeneity should be indicated in the published reference sequence by using ambiguity codes such as IUPAC (Nomenclature Committee of Biochemistry) (8). Thus, the quality of sequence data in public databases such as GenBank, EMBL, or the Ribosomal Database Project (17) will improve and lead to higher accuracy in the identification of clinically important bacteria, including mycobacteria, via 16S rDNA sequence analysis.

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