Classification of the Genus *Mobiluncus* Based on Comparative Partial 16S rRNA Gene Analysis

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On the basis of partial 16S rRNA gene sequences and the results of Southern blot analyses, we confirmed the division of the genus *Mobiluncus* into the species *Mobiluncus curtisii* and *Mobiluncus mulieris*. Division of *M. curtisii* into *M. curtisii* subsp. *curtisii* and *M. curtisii* subsp. *holmesii* was not supported by our data.

Spiegel and Roberts (15) proposed that the genus *Mobiluncus* comprises two distinct species, *Mobiluncus curtisii* and *Mobiluncus mulieris*, and these investigators also suggested that *M. curtisii* should be separated into *M. curtisii* subsp. *curtisii* and *M. curtisii* subsp. *holmesii*, a distinction that is still a matter of debate. The phylogenetic position of the genus *Mobiluncus* based on partial 16S rRNA sequences was determined later (8), and it has been suggested that the genus *Mobiluncus* belongs to the order *Actinomycetales*.

However, characterization of *M. curtisii* and *M. mulieris* that are not type strains is a troublesome task since the phenotypes of both species vary. Identification of *Mobiluncus* strains has been based on morphological and biochemical characteristics (14), but proper identification sometimes is not possible because of phenotypic complexity. To improve species characterization, serological and nucleic acid methods have been used to supplement the classical methods (14). Neither polyclonal and monoclonal antibodies, nor nucleic acid hybridization in which genomic DNA probes are used, nor dot blot assays based on *Mobiluncus*-specific 16S rRNA sequences have substantially improved *Mobiluncus* classification.

The presence of evolutionarily conserved regions and variable sequence motifs within bacterial 16S rRNA genes has turned out to be valuable for microorganism identification. Gray et al. (7) identified eight universal regions and nine variable regions within the 16S rRNA gene, and workers have shown that unculturable bacteria can be detected by using bacterial broad-range PCR primers based on conserved 16S ribosomal DNA (rDNA) sequence motifs (13).

To further clarify the relationship between *M. curtisii* and *M. mulieris* and to determine whether the differentiation of *M. curtisii* subspecies is manifested evolutionarily within the 16S rRNA gene, we performed a comparative sequence analysis of partial 16S rDNA sequences. In addition, we constructed *M. curtisii*-specific DNA hybridization probes by using variable motifs within the 16S rRNA gene in order to establish a method which can be used to discriminate between *M. curtisii* and *M. mulieris*.

Practical approach. The *Mobiluncus* strains (Table 1) used in this study were cultured as described previously (6). DNA was extracted by the modified procedure of Woo et al. (19). Briefly, this modified method includes treatment with Triton X-100, proteinase K, lysozyme, and RNase and a final phenol-chloroform extraction. Bacterial DNA was amplified by using Promega PCR reagents (Promega Corp., Madison, Wis.) and 200 pmol of primers pJB-1 and P13B, respectively, or 200 pmol of primers Mob-s and Mob-as, respectively (Table 2). The

following PCR conditions were used: 94°C for 2 min, 60°C for 1 min, and 72°C for 1 min (1 cycle); 94°C for 20 s, 60°C for 20 s, and 72°C for 20 s (29 cycles); and a final extension step consisting of 72°C for 10 min. Subsequently, the PCR products were purified and the DNA sequences were determined by using the primers described in Table 2 and established standard procedures (commercial kits).

Southern blot analysis. The PCR products were separated on a 1.5% agarose gel, blotted onto a Hybond-N membrane (Amersham Corp.), and finally hybridized with 5'-end-labelled broad-range primer pJB-1 or *M. curtisii*-specific primers Mob-V3 and Mob-V4 by using [γ -³²P]ATP (3,000 Ci/mmol; Amersham Corp.) and a commercially available 5'-end-labelling kit (Boehringer Mannheim GmbH, Mannheim, Germany). The posthybridization treatment of the filter is described in the legend to Fig. 1. The membrane was exposed to type MP X-ray film (Amersham Corp.) at -70°C for 5 to 24 h. After each hybridization experiment the hybridized primer was stripped off the membrane by boiling it for 2 min.

Using *Mobiluncus*-specific PCR primers (8) which covered variable regions V6, V7, and V8 according to the nomenclature of Gray et al. (7), we were able to amplify genomic rDNAs from *Mobiluncus* strains, including atypical strains A294, A345, A543, and 30185, as indicated in Table 1. However, the genomic rDNA of atypical strain 0960 was not amplified. A DNA sequence analysis of the ~400-bp PCR products of type strains ATCC 35241, ATCC 35242, and ATCC 35243 revealed a 1-base transition (G to A) at position 755 when we compared *M. curtisii* and *M. mulieris* (all positions are the positions in the *Escherichia coli* 16S rRNA sequence [1]). No detectable DNA sequence differences were found in *M. curtisii* strains at the subspecies level.

Broad-range PCR amplification of *Mobiluncus* strains listed in Table 1 with PCR primers pJB-1 and p13B, which covered variable regions V3, V4, and V9, yielded a ~450-bp product with all of the *Mobiluncus* strains tested. Direct DNA sequencing was performed by using the same primers. An alignment of the 16S rDNA sequences revealed that there was distinct variability within motifs V3 and V4 of *M. curtisii* and *M. mulieris*, but no base deviations were found elsewhere in the ~370-bp sequence obtained. The sequences of variable regions V3 and V4 of *M. curtisii* ATCC 35241^T (T = type strain), ATCC 35242^T, CCUG 21018^T, CCUG 17762^T, 1, 3, 8, 9, 2507, 79363, A63, Vib 7, Vib 16, M101f, M136-23, M119-3, L796, and L904c were determined by using sense primer pJB-1. The DNA sequences of type strains were also determined by using anti-sense primer p13B. We observed variability in the V3 region between positions 1006 and 1008, where the *M. curtisii* DNA sequence had a base insertion. Another hypervariable motif in variable region V3 was also localized; *M. curtisii* ATCC 35241^T

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TABLE 1. *Mobiluncus* strains used in this study and results obtained in Southern blot analyses when *M. curtisii*-specific hybridization probes were used

Expt	Strain	Taxon as determined by Garlind et al. ^a	Southern blot analysis results obtained with:		
			Probe Mob-V3 with 2× SSC wash ^b	Probe Mob-V3 with 0.1× SSC wash ^c	Probe Mob-V4 ^b
1	ATCC 35241 ^T	<i>M. curtisii</i> subsp. <i>curtisii</i>	+	+	+
2	ATCC 35242 ^T	<i>M. curtisii</i> subsp. <i>holmesii</i>	+	+	+
3	ATCC 35243 ^T	<i>M. mulieris</i>	+	—	—
4	CCUG 20071 ^T	<i>M. mulieris</i>	+	—	—
5	CCUG 21018 ^T	<i>M. curtisii</i> subsp. <i>curtisii</i>	+	+	+
6	CCUG 17762 ^T	<i>M. curtisii</i> subsp. <i>holmesii</i>	+	+	+
7	A63	<i>M. curtisii</i>	+	+	+
8	PG491	<i>M. mulieris</i>	+	—	—
9	PG632	<i>M. mulieris</i>	+	—	—
10	A294 ^c	Atypical <i>Mobiluncus</i> sp.	+	+	+
11	A345 ^c	Atypical <i>Mobiluncus</i> sp.	+	+	+
12	A543 ^c	Atypical <i>Mobiluncus</i> sp.	+	+	+
13	L796	<i>M. curtisii</i>	+	+	+
14	M101f	<i>M. curtisii</i>	+	+	+
15	M119-3	<i>M. curtisii</i>	+	+	+
16	M101a ^c	<i>M. mulieris</i>	+	+	+
17	BV108-27	<i>M. mulieris</i>	+	—	—
18	79363	<i>M. curtisii</i>	+	+	+
19	Vib7	<i>M. curtisii</i>	+	+	+
20	0960 ^f	Atypical <i>Mobiluncus</i> sp.	—	—	—
21	90061 ^c	Atypical <i>Mobiluncus</i> sp.	+	+	+
22	30185 ^f	Atypical <i>Mobiluncus</i> sp.	—	—	—
23	01466.1 ^c	Atypical <i>Mobiluncus</i> sp.	+	+	+
24	15.18 ^g	Atypical <i>Mobiluncus</i> sp.	+	—	—
25	36.6 ^g	Atypical <i>Mobiluncus</i> sp.	+	—	—
26	1 ^h	<i>M. curtisii</i>	+	—	—
27	L2006	<i>M. curtisii</i>	+	+	+
28	3	<i>M. curtisii</i>	+	+	+
29	8 ^h	<i>M. curtisii</i>	—	—	—
30	9 ^f	<i>M. curtisii</i>	—	—	—
31	2507	<i>M. curtisii</i>	+	+	+
32	L904c	<i>M. curtisii</i>	+	+	+
33	Vib16	<i>M. curtisii</i>	+	+	+
34	M136-23	<i>M. curtisii</i>	+	+	+
35	12158	<i>M. mulieris</i>	+	—	—
36	L33 ^c	<i>M. mulieris</i>	+	+	+
37	M142-3	<i>M. mulieris</i>	+	—	—
38	L2313 ^c	<i>M. mulieris</i>	+	+	+
39	1005	<i>M. mulieris</i>	+	—	—

^a See reference 6.^b The preparation was washed three times (20 min each) with 2× SSC–0.1% sodium dodecyl sulfate at 50°C (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).^c The preparation was washed three times (20 min each) with 0.1× SSC–0.1% sodium dodecyl sulfate at 50°C.^d +, positive; —, negative.^e Strain shown to be an *M. curtisii* strain in this study.^f Strain shown to be an atypical *Mobiluncus* strain in this study.^g Strain shown to be an *M. mulieris* strain in this study.^h Strain not identified in this study.

and ATCC 35242^T had the DNA sequence motif 5'-GCCA-G CCTTCG-3' between positions 1021 and 1032 (Fig. 2, box II). In variable region V4, *M. curtisii* had the DNA sequence motif 5'-G-GTTCGGCC-3' at positions 1133 to 1141 (Fig. 2, box III). Most strains that have been characterized by other workers as *M. curtisii* strains had these DNA sequence motifs in regions V3 and V4; the exceptions were strains 1, 8, and 9. Strain 1 of *M. curtisii* had an *M. mulieris*-like DNA sequence pattern, and strain 8 appeared to be unrelated to *M. curtisii*. Strain 8 was isolated from an external ear exudate (6). Strain 9 exhibited sequence variability identical to that exhibited by atypical *Mobiluncus* sp. strain 0960 (see below).

The DNA sequences of the PCR products of *M. mulieris* ATCC 35243^T, CCUG 20076^T, 1005, 12158, PG491, PG632, L33, M101a, M142-3, BV108-27, and L2313 were determined

as described above for *M. curtisii* strains. The DNA sequence of strain ATCC 35243^T spanning part of variable region V3 (positions 1021 to 1032) was 5'-TGTG-GCCTTCG-3' (Fig. 2, box II), and the DNA sequence 5'-ACGTTATGGT-3' (Fig. 2, box III) was found in variable region V4 (positions 1133 to 1141) (bases that differ from the bases in the *M. curtisii* DNA sequence are underlined). Most *M. mulieris* strains had the same DNA sequence as strain ATCC 35243^T; the exceptions were strains L33, M101a, and L2313, all of which had a DNA sequence identical to the *M. curtisii* sequence.

Mobiluncus sp. strains 0960, 30185, 90061, 15.18, 36.6, A294, A345, A543, and 01466.1, which were previously classified as atypical *Mobiluncus* strains, were also subjected to broad-range PCR amplification and subsequent DNA sequencing. Interestingly, strains 90061, A294, A345, A543, and 01466.1 had the

FIG. 1. Southern blot analyses of broad-range amplified PCR products from *Mobiluncus* strains. (A) Membranes were hybridized with 5'-end-labelled probe pJB-1 and then washed three times (20 min each) with 2× SSC–0.1% sodium dodecyl sulfate at 50°C (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). (B and C) After the hybridized probe was stripped off, the membranes were rehybridized with 5'-end-labelled *M. curtisii*-specific probe Mob-V3 and then washed three times (20 min each) with 2× SSC–0.1% sodium dodecyl sulfate at 50°C (B) and three times (20 min each) with 0.1× SSC–0.1% sodium dodecyl sulfate at 50°C (C). (D) After the probe was stripped off, the membranes were finally rehybridized with 5'-end-labelled *M. curtisii*-specific probe Mob-V4 and washed three times (20 min each) with 2× SSC–0.1% sodium dodecyl sulfate at 50°C. The film was exposed at –70°C overnight. The numbers at the top are the experiment numbers (see Table 1).

same DNA sequence as *M. curtisii*, but the DNA sequences of atypical strains 15.18 and 36.6 were *M. mulieris*-like. Only the sequences of atypical strains 0960 and 30185 differed from the *M. curtisii* and *M. mulieris* sequences in the broad-range amplified fragment. A similarity analysis (9) of aligned 16S rDNA sequences between positions 990 and 1360 revealed that the levels of sequence homology between the atypical strains and *M. curtisii* and *M. mulieris* strains were approximately 90%. As shown in Fig. 2, strains 0960 and 30185 had homologous DNA sequences in variable region V4 (5'-AAGTTGTGTT-3'; positions 1133 to 1141) and in one of the hypervariable motifs in region V3 (5'-GTTTTCCTTTT-3'; positions 1021 to 1032) (bases that differ from the bases in the *M. curtisii* DNA sequence are underlined). However, strains 0960 and 30185 exhibited sequence variability in the first hypervariable motif in region V3 (Fig. 2, box I), where they differed at two positions. Strain 9 of *M. curtisii* had the same DNA sequence as atypical strain 0960.

No base changes in the ~370-bp 16S rDNA sequence were detected at the subspecies level in *M. curtisii*.

On the basis of the different DNA sequences in variable regions V3 and V4 of *M. curtisii*, *M. mulieris*, and atypical *Mobiluncus* strains, we constructed two *M. curtisii*-specific 16S rRNA gene hybridization probes, Mob-V3 and Mob-V4. These probes were used to hybridize broad-range amplified 16S rDNA products from *Mobiluncus* strains (Table 1). The PCR products were separated electrophoretically on an agarose gel, transferred to a Hybond-N membrane, and hybridized with

5'-end-labelled broad-range primer pJB-1 (Fig. 1A). Then pJB-1 was stripped off by boiling the membrane in sterile water, and the filters were rehybridized with 5'-end-labelled probes Mob-V3 and Mob-V4; this was followed by a posthybridization treatment under moderately stringent conditions. Hybridization with Mob-V3 revealed that the probe was specific for the genus *Mobiluncus*, since it hybridized to all *Mobiluncus* strains listed in Table 1 except atypical strains 0960 and 30185 and *M. curtisii* 8 and 9 (Table 1 and Fig. 1B). More stringent posthybridization conditions resulted in higher levels of *M. curtisii* specificity of probe Mob-V3 since *M. mulieris* probes were removed (Table 1 and Fig. 1C). This species specificity of probe Mob-V3 was expected since the probe sequence derived from *M. curtisii* differs at four positions from the *M. mulieris* sequence. Hybridization with 5'-end-labelled probe Mob-V4 that differed at six positions from the *M. mulieris* sequence under moderately stringent conditions resulted in an *M. curtisii*-specific hybridization pattern (Table 1 and Fig. 1D). The differences in band intensity may have been due to multiple uses of the filters in hybridization experiments. However, in all cases classification of the strains analyzed was confirmed by DNA sequence data.

The level of DNA sequence homology between *M. curtisii* and *M. mulieris* in the *Mobiluncus*-specific amplified region was not consistent with previously published *Mobiluncus* 16S rDNA sequence data, which indicated that the interspecies variation was greater (8). It is debatable whether a single base change in the *Mobiluncus*-specific region is enough to draw

TABLE 2. Oligodeoxyribonucleotide primers used for PCR amplification, to determine DNA sequences, and to hybridize 16S rRNA genes of members of the genus *Mobiluncus*

Primer	Sequence (5' to 3')	Specificity (orientation)	Position ^a	<i>T_m</i> (°C) ^b
pJB-1	ATTCGATGCAACGCGAAGAACCCTTACC	Broad range (sense)	959	54.6
p13B	GTGTACTAGGCCCGGGAACGTATTC	Broad range (antisense)	1397	55.9
Mob-s	GTGAACCTCTTTTCTCGTGAA	<i>Mobiluncus</i> specific (sense)	452	45.3
Mob-as	CGCAGAAACACAGGATTGCATCC	<i>Mobiluncus</i> specific (antisense)	855	51.9
Mob-V3	GCCAGCCTTCGGGGTGGTGT	<i>M. curtisii</i> specific (sense)	1021	54.9
Mob-V4	TCACGAGTCCCCGGCCGAACC	<i>M. curtisii</i> specific (antisense)	1153	57.0

^a 5' position in the *Escherichia coli* 16S rRNA sequence (1).

^b The melting temperature (*T_m*) was calculated by using the following formula: $T_m = 81.5 + 16.6(\log[K^+]) + 0.41(\text{mol\% G+C}) - (675/n)$, where $[K^+]$ is 0.050 M and *n* is the chain length (17).

FIG. 2. Alignment of 16S rRNA genes within variable regions V3 and V4 (underlined). Gaps indicate base deletions, and dashes indicate positions at which the sequence is homologous to the reference sequence (*E. coli* [1]). The locations of the Mob-V3 and Mob-V4 oligonucleotide primers are indicated. Abbreviations: E.c., *Escherichia coli*; M.m., *Mobiluncus mulieris*; M.c.c., *Mobiluncus curtisii* subsp. *curtisii*; M.c.h., *Mobiluncus curtisii* subsp. *holmesii*. Strains 0960, 30185, and 9 are clinical isolates (Table 1). Boxes indicate DNA sequences which are different in different *Mobiluncus* strains (see text).

taxonomic conclusions. Instead, we focused on another part of the 16S rDNA sequences which were amplified with broad-range bacterial PCR primers pJB-1 and p13B and subsequently directly sequenced. The 16S rDNA sequences of members of the genus *Mobiluncus* revealed that there were species differences in variable regions V3 and V4. Furthermore, *M. curtisii*-specific DNA hybridization probes were constructed from these variable regions to ensure proper identification of the genus *Mobiluncus*.

The genetic drift observed in variable regions V3 and V4 of the 16S rDNA sequences supports the taxonomic division of members of the genus *Mobiluncus* into two distinct species, *M. curtisii* and *M. mulieris*. *M. curtisii* and *M. mulieris* exhibited base differences at 14 positions in these variable regions. The potential of our *M. curtisii*-specific hybridization probes, Mob-V3 and Mob-V4, was clearly demonstrated, and the data obtained with these probes supported this classification (Fig. 1). Hybridization with probe Mob-V3 resulted in specificity for the genus *Mobiluncus* under moderately stringent posthybridization conditions. More stringent posthybridization conditions resulted in specificity for *M. curtisii*. *M. curtisii*-specific probe Mob-V4 was discriminatory even under moderately stringent posthybridization conditions (Fig. 1). These probes did not react with other, non-*Mobiluncus*-related bacterial strains (data not shown). To show that clinical strains exhibited sequence homologies that were consistent with hybridization results, the DNA sequences of the *M. curtisii* strains collected from investigators around the world (6) and listed in Table 1 were determined in the sense direction; thus, variable regions V3 and V4 were included. The DNA sequence analysis results were consistent with the hybridization results.

The complexity of the genus *Mobiluncus* has forced some investigators to classify several *Mobiluncus* strains as atypical

strains (Table 1). The strains described by Garlind et al. (6) were subjected to 16S rDNA analyses and species-specific hybridization. Interestingly, strains 90061, A294, A345, A543 and 01466.1 had partial 16S rDNA sequences identical to those of *M. curtisii* strains. All of these strains except strain 01466.1 exhibited biochemical differences when they were compared with the *M. curtisii* type strains (6). Atypical strains 15.18 and 36.6 exhibited sequence homology to *M. mulieris* and also produced identical phenotypic patterns (6). Interestingly, atypical strain 30185, but not strain 0960, was amplified when these strains were subjected to *Mobiluncus*-specific PCR amplification. Atypical strains 0960 and 30185 exhibited a high level of homology, and significant sequence differences compared with *Mobiluncus* strains were detected, especially in variable regions V3 and V4 but also elsewhere in the partial 16S rDNA sequences.

Spiegel and Roberts (15) proposed that the members of *M. curtisii* should be separated into subspecies *M. curtisii* subsp. *curtisii* and *M. curtisii* subsp. *holmesii* on the basis of different migration patterns in soft agar and the ability of *M. curtisii* subsp. *holmesii* to reduce nitrate. Using a variety of techniques, other investigators had difficulty finding evidence which supported this proposal (2, 3, 6, 11, 18). Unpublished data from workers in our group confirmed the nitrate-reducing properties of the American Type Culture Collection type strains. Vetere et al. (18) proposed that members of the genus *Mobiluncus* should be separated into more than two species, but their data did not support division of *M. curtisii* because all of the strains that were not type strains tested were nitrate reducers. Our 16S rDNA sequence analyses revealed that the sequences of *M. curtisii* subsp. *curtisii* and *M. curtisii* subsp. *holmesii* are completely homologous, which is in conflict with the proposed division of *M. curtisii* into subspecies (15). How-

ever, the conflicting genotypic and phenotypic results add a further argument to the discussion whether 16S rRNA gene sequences alone provide enough information to distinguish *M. curtisii* subspecies (5, 10, 16). Fohn et al. (4) raised a series of monoclonal antibodies to *M. curtisii* subsp. *curtisii* and *M. mulieris* and found that at a 1/50 dilution of the culture fluids these antibodies could distinguish between *M. curtisii* subsp. *curtisii* and *M. curtisii* subsp. *holmesii*. These findings are not consistent with those of Pålsson et al., who were not able to distinguish between *M. curtisii* subsp. *curtisii* and *M. curtisii* subsp. *holmesii* with a set of monoclonal antibodies (11). No taxonomic conclusion can be drawn from the findings of Fohn et al. (4) since dilution experiment data are not reported for the various monoclonal antibodies. On the basis of DNA hybridization data and G+C contents, Christiansen et al. suggested that the more homologous long curved rods (LCR) (*M. mulieris*) is a distinct species, but the more heterologous short curved rods (SCR) (*M. curtisii*) should be separated into several species (3). However, these authors felt that these species designations should be supported by biochemical and serological evidence.

Several investigators have described the phenotypic complexity of the genus *Mobiluncus* and have characterized several atypical *Mobiluncus* strains (3, 6, 12, 18). Spiegel stated that the differences are due mainly to differences in the media and methods used, as well as to normal strain variation (14). However, when partial 16S rDNA sequences were examined, no differences between *M. curtisii* subsp. *curtisii* and *M. curtisii* subsp. *holmesii* sequences were found. These results are not consistent with the previously described division of *M. curtisii* strains into subspecies, which was based on biochemical and serological results.

Nucleotide sequence accession numbers. Partial DNA sequences derived from broad-range amplified 16S rRNA genes have been deposited in the European Molecular Biology Laboratory Data Library under the following accession numbers: *M. mulieris* ATCC 35243^T, X82602; *M. curtisii* subsp. *curtisii* ATCC 35241^T, X82603; *M. curtisii* subsp. *holmesii* ATCC 35242^T, X82604; *Mobiluncus* sp. strain A294, X82605; *Mobiluncus* sp. strain A345, X82606; *Mobiluncus* sp. strain A543, X82607; and atypical *Mobiluncus* sp. strain 0960, X82608. Partial DNA sequences derived from *Mobiluncus*-specific amplified 16S rRNA genes have been deposited under the following accession numbers: *M. mulieris* ATCC 35243^T, X86004; *M. curtisii* subsp. *curtisii* ATCC 35241^T, X86005; and *M. curtisii* subsp. *holmesii* ATCC 35242^T, X86006.

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