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Characterization of Mycobacterial Isolates Phylogenetically Related to, but Different from, *Mycobacterium simiae*

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The use of high-performance liquid chromatography (HPLC) revealed four previously unreported profiles within a group of mycobacteria consisting of 14 clinical isolates. These mycobacteria, whose identification by conventional tests appeared problematic, mostly resembled *Mycobacterium avium* complex or *Mycobacterium simiae*. Genetic analysis revealed, within this group, six different nucleic acid sequences in a hypervariable 16S rRNA segment, but all the isolates appeared to be phylogenetically related to *M. simiae*. Six isolates representing the largest of groups defined by means of genetic sequencing turned out to belong to the newly described species *Mycobacterium lentiflavum*. Furthermore, three such clusters precisely coincided with three of those defined by HPLC, while the three remaining clusters shared almost identical HPLC profiles. All but one strain (which, although clearly not belonging to the *M. avium* complex, hybridized with specific commercial DNA probes) showed high-grade resistance to the majority of antimycobacterial drugs. Three of the isolates were clinically significant according to stringent criteria. Sophisticated techniques, like genetic sequencing or HPLC, by now seem indispensable for differentiating unusual and new mycobacteria from well-established ones.

The number of mycobacteria of clinical interest had remained practically unchanged for many years and few new species had been added to the group of nontuberculous mycobacteria (mycobacteria other than *M. tuberculosis*) classified by Runyon (10) in 1959. Recently, however, the recognition and identification of novel mycobacterial species has become rather frequent (1, 2, 4, 7, 8, 12, 13) due to the advancement of genetic techniques, which has led to the description of hypervariable nucleic acid sequences characterized by species specificity (5, 6). Although 16S rRNA gene sequencing is not the only genetic technique suitable for identification purposes (17), it is the one which has been most exploited to achieve a better knowledge of mycobacterial taxonomy.

In the meantime, the cultural and biochemical characteristics, the traditional bases for the identification and classification of mycobacteria, have become less and less important, mainly because of their inadequacy in recognizing new species. By the traditional approach, novel mycobacteria, about whose phenotypic features little is known, are in fact often misidentified as members of some of the traditionally established species (15).

In recent years, another technique, based on the analysis of cell wall mycolic acids by high-performance liquid chromatography (HPLC), has been introduced. This technique has turned out to be useful for the identification of mycobacterial species. The finding of unusual HPLC profiles allowed us to differentiate several mycobacteria from other previously known species which, on the basis of partial 16S rRNA gene sequencing, seem to belong to a group of organisms phylogenetically related to, but different from, *Mycobacterium simiae.*

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MATERIALS AND METHODS

The isolates (isolates FI-3692, FI-17193, FI-18893, FI-2994, FI-7294, FI-7794, FI-9994, FI-5995, FI-9395, FI-16795, FI-22895, FI-22995, FI-23395, and FI-25996) were grown from clinical samples in different laboratories by standard cultural procedures (9); their clinical relevance has been assessed by a retrospective review of the clinical records on the basis of the criteria proposed by the American Thoracic Society (21).

The investigation of biochemical and cultural features was carried out by conventional techniques (9).

Because of the phenotypic similarity of several isolates with the members of *M. avium* complex (MAC), they were all tested with commercially available DNA probes (AccuProbe *M. avium* complex; Gen-Probe, San Diego, Calif.) and, when positive, with the species-specific AccuProbe for *M. avium* and *M. intracellulare*. The recommendations of the manufacturer for hybridization and reading with a PAL luminometer (Gen-Probe) were followed.

The analysis of cell wall mycolic acids by HPLC was performed as reported previously (3) with a reverse-phase C_{18} Ultrasphere-XL cartridge column on an HPLC instrument (System Gold model; Beckman Instruments Inc., Berkeley, Calif.).

Nucleic acid sequence analysis of PCR-amplified 16S rRNA gene fragments was performed as described previously (5). Sequences alignments in the regions corresponding to positions from positions 129 to 266 (hypervariable region A) and from positions 430 to 468 (hypervariable region B) of *Escherichia coli* 16S rRNA were used for identification and to determine the phylogenetic relatedness of the strains.

The susceptibilities of the strains were assessed in liquid radiometric medium by following the procedure developed for MAC (11), whose requirements were fulfilled because our isolates had growth kinetics very close to those for MAC.

RESULTS

The main conventional features shared by our isolates included slow growth at 25 and 37°C, but not at 45°C, and

TABLE 1. Comparison of results of conventional biochemical, cultural and inhibition tests for 14 isolates arranged on the basis of their 16S rRNA sequences[a]

Test	A	B	C	D	E	F
Niacin	−	−	−	−	−	−
Nitrate reduction	−	−	−	−	−	−
Thermostable catalase	+	+/−	+	+	+	−
β-Glucosidase	−	−	−	−	−	−
Tween 80 hydrolysis (10 days)	−	−	−	−	−	+
Tellurite reduction	v	+	+	−	+	−
Arylsulfatase (3 days)	−	−	−	−	−	−
Urease	+/−	+	+	+	−	+
Catalase (over 45 mm of foam)	−/+	+/−	+	−	−	−
Photochromogenicity	v	−	v	−	−	−
Scotochromogenicity	v	−	−	−	+	+
Growth at 25°C	+	+	+	+	+	+
Growth at 37°C	+	+	+	+	+	+
Growth at 45°C	−	−	−	−	−	−
Growth on MacConkey agar	v	−	−	−	−	−
Growth rate	Slow	Slow	Slow	Slow	Slow	Slow
Colonial morphology	v	Smooth	v	Smooth	Smooth	Smooth
Tolerance to:						
p-Nitrobenzoate (500 μg/ml)	+	+	+	+	+	+
NaCl (5%)	−	−	−	−	−	−
Thiophene-2-carboxylic hydrazide (5 μg/ml)	+	+	+	+	+	+
Thiacetazone (10 μg/ml)	+	+	+	+	+	+
Hydroxylamine (500 μg/ml)	v	+	+	+	+	+
Isoniazid (1 μg/ml)	+	+	+	+	+	+
Oleate (250 μg/ml)	+	+	+	−	+	−

[a] v, variable; +/−, prevalently positive; −/+, prevalently negative (in the different isolates present in each grouping). A, isolates FI-7294, FI-3692, FI-7794, FI-5995, FI-9395, and FI-25996; B, isolates FI-17193, FI-18893, and FI-2994; C, isolates FI-9994 and FI-22995; D, isolate FI-23395; E, isolate FI-22895; F, isolate FI-16795.

negative results in biochemical tests for niacin and nitrate reduction and the 3-day arylsulfatase test. Major differences within the group concerned pigmentation (scotochromogenic, photochromogenic, and nonchromogenic strains were present), urease, and tellurite reduction (Table 1).

On the basis of such test results, the best-likelihood identifications, with proper software (19), were MAC or *M. simiae* in the majority of cases, but several patterns were also compatible with the newly described species *M. interjectum.*

All but one of the isolates (isolate FI-16795) gave a negative result with the AccuProbe MAC test; however, this one did not hybridize with species-specific probes for *M. avium* and *M. intracellulare*, thus behaving as a MAIX strain (20).

Partial 16S rRNA sequencing revealed that all of the isolates shared the same sequence within the hypervariable region B, which was characterized by a short helix 18. This sequence is identical to that of *M. simiae* and of the recently described species *M. intermedium* and *M. interjectum* (8, 12). The sequence alignments in region A showed six different and novel sequences within the investigated isolates (Fig. 1); three of the sequences were shared by six isolates (isolates FI-7294, FI-3692, FI-7794, FI-5995, FI-9395, and FI-25996), three isolates (isolates FI-17193, FI-18893, and FI-2994), and two isolates (isolates FI-9994 and FI-22995), respectively; the remaining three sequences were presented by only one strain each.

The most frequent of such sequences turned out to be identical to that from the very recently described new species *M. lentiflavum* (14), and the six strains were assigned to this species.

Four different lipid profiles were detected by HPLC (Fig. 2), and they were presented by nine, three, and two single isolates, respectively. The more common HPLC pattern closely resembled those of *M. simiae* and *M. genavense* (Fig. 2, phenotype i). Of the other patterns, two were clearly different from any other pattern reported previously (Fig. 2, phenotypes ii and iv), while the last pattern presented some resemblance to that of MAC (Fig. 2, phenotype iii).

As a group, all but one of the isolates were resistant to the majority of antimicrobial agents tested, with rifabutin appearing to be the most active; quinolone MICs for three of the isolates were very high (Table 2).

The review of records of patients from which isolates were obtained revealed a large variety of sources and clinical situations; human immunodeficiency virus infection was the underlying condition in two patients (Table 3). A rigorous reexamination of clinical and microbiological data according to the stringent criteria set forth by the American Thoracic Society (21) permitted us to ascertain the clinical significance of the mycobacteria isolated from three patients.

Patient 1. A 6-year-old girl developed over several days a local swelling at the right mandibular edge. The swelling showed no improvement after 4 weeks of antibiotic and mild steroid therapy. The child was living in an urban area and had not received *M. bovis* BCG vaccination; neither she nor her family had remarkable medical histories. On hospital admission three lymph nodes were palpable, with the largest one measuring 2 by 3 cm; an ultrasonic scan revealed abscess formation in all of them. Routine laboratory investigations and chest film were normal; skin tests with standard tuberculin antigens and antigens of mycobacteria other than *M. tuberculosis* gave weakly positive results, with a slightly more evident reaction against *M. avium*. Chemotherapy with rifampin and clarithromycin was started, and a surgical excision was performed 3 weeks later; at that time a pronounced abscess had formed in the lymph node. Acid-fast bacilli were seen in the pus, and the culture of tissue fragments yielded a slowly growing nonpigmented mycobacterium (isolate 7 in Table 3).

Patient 2. A 58-year-old man with a history of rheumatoid arthritis was hospitalized because of complaints of painful tenderness in small joints, low-grade fever, and weight loss. A

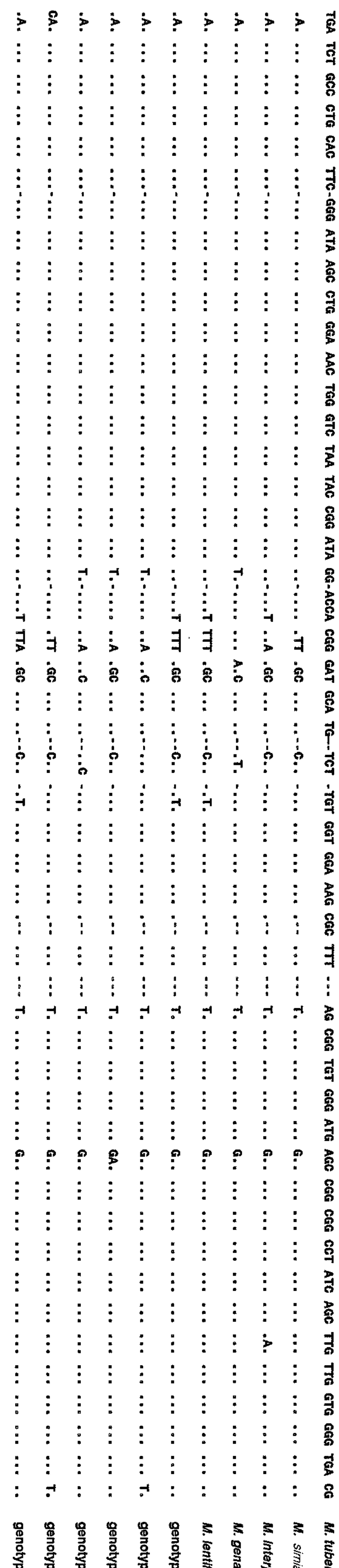

FIG. 1. Alignment of selected mycobacterial 16S rRNA sequences within hypervariable region A. The sequence of *M. tuberculosis* was used as the reference sequence. Nucleotides different from those of *M. tuberculosis* are indicated; dashes indicate deletions, and dots indicate identity. The first nucleotide corresponds to *E. coli* 16S rRNA position 129 (genotype A, isolates FI-7294, FI-3692, FI-7794, FI-5995, FI-9395, and FI-25996; genotype B, isolates FI-17193, FI-18893, and FI-2994; genotype C, isolates FI-9994, and FI-22995; genotype D, isolate FI-23395; genotype E, isolate FI-22895; and genotype F, isolate FI-16795).

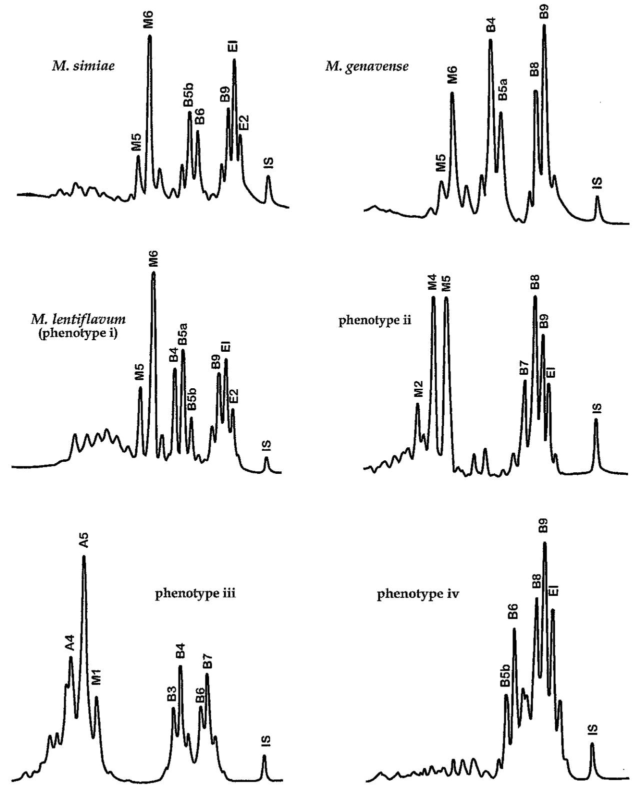

FIG. 2. Comparison of representative HPLC phenotypes of 14 isolates investigated with the phenotypes of *M. simiae* and *M. genavense*. IS, internal standard (phenotype i, isolates FI-7294, FI-3692, FI-7794, FI-5995, FI-9395, FI-25997, FI-9994, FI-22995, and FI-23395; phenotype ii, isolates FI-17193, FI-18893, and FI-2994; phenotype iii, isolate FI-22895; and phenotype iv, isolate FI-16795).

chest computed tomography scan revealed two small infiltrates in the upper lobe of the left lung and a pleural effusion. A standard tuberculin test was strongly positive. No bacterial pathogen was grown from sputum, bronchial secretions, or pleural fluid. Histopathological examination of a pleural biopsy specimen showed noncaseating granulomas with central necrosis and Langerhans giant cells; no acid-fast bacilli were seen. Routine cultures for mycobacteria gave negative results; however, on biphasic medium (Septi-Chek AFB; Becton Dickinson, Cockeysville, Md.) the pleural biopsy specimen yielded slowly growing nonpigmented mycobacteria (isolate 11 in Table 3) by the third week. On the basis of histological and microbiological results, empirical therapy with isoniazid, rifabutin, ethambutol, and pyrazinamide was started. After 4 months of therapy, the patient was stationary, and no significant reduction of lung infiltrates and pleural effusion was shown by computed tomography scan.

Patient 3. A 4-year-old girl was first hospitalized with a clinical and radiological picture of bronchopneumonia, which cleared up after 2 weeks of antimicrobial treatment, at which time she was released from the hospital. Fifteen days later a local swelling appeared at the right submandibular region. Serological (including tests for toxoplasmosis and mononucleosis), biochemical, and hematological data, except for erythrocyte sedimentation rate (50 mm/h), were normal; the tuberculin test was positive. An unspecified antimicrobial therapy given at home was ineffective, and the child was hospitalized again. Physical examination revealed a lightly aching volumi-

TABLE 2. MICs for the isolates tested

Drug	MIC (μg/ml) for the following isolates, 16S rRNA genotype (HPLC phenotype):													
	FI-7294, A (i)	FI-3692, A (i)	FI-7794, A (i)	FI-5995, A (i)	FI-9395, A (i)	FI-25996, A (i)	FI-17193, B (ii)	FI-18893, B (ii)	FI-2994, B (ii)	FI-9994, C (i)	FI-22995, C (i)	FI-23395, D (i)	FI-22895, E (iii)	FI-16795, F (iv)
Amikacin	4	8	2	8	16	8	2	0.5	4	4	8	32	2	4
Azithromycin	32	32	64	32	64	32	16	8	32	16	32	32	32	1
Capreomycin	8	16	8	16	16	8	8	4	8	8	16	8	8	32
Ciprofloxacin	0.5	1	1	1	1	0.5	4	128	128	16	32	16	256	0.12
Clarithromycin	1	4	2	4	4	4	2	2	2	4	8	4	1	0.25
Clofazimine	0.25	0.12	0.5	0.25	0.5	0.12	0.12	0.12	0.5	0.5	0.5	1	0.25	0.008
Ethambutol	8	4	4	8	4	8	8	4	8	4	4	32	4	1
Isoniazid	4	16	8	1	16	0.5	4	4	8	4	8	8	32	8
Kanamycin	1	1	2	1	1	2	4	2	4	1	1	4	2	0.5
Ofloxacin	4	32	1	2	4	2	8	128	128	16	16	32	64	2
p-Aminosalicylic acid	8	16	8	16	16	16	4	8	16	16	32	8	16	4
Rifabutin	0.25	0.12	0.5	0.5	0.5	0.5	0.25	0.06	0.25	0.5	1	1	0.03	1
Rifampin	4	16	2	16	16	64	16	8	4	32	32	32	0.5	0.25
Sparfloxacin	0.5	4	4	8	4	1	1	16	16	2	1	4	16	0.06
Streptomycin	4	1	8	4	8	8	2	2	4	4	4	16	2	1

TABLE 3. Clinical and microbiological data

Patient no.	Gender[a]-age (yr)	Isolate no.	Source of specimen for culture	Microscopy result	Isolation	Clinical presentation	Underlying disease	Clinical significance	Isolation site[b]	Yr of isolation	Genotype	HPLC phenotype	Conventional identification
1	M-74	FI-7294	Bronchial aspirate	Negative	Single	Pulmonary fibrosis	Adenocarcinoma	−	Milan-1	1994	A	i	*M. interjectum*
2	F-73	FI-3692	Sputum	Negative	Single	Pneumonia	None	−	Milan-1	1992	A	i	*M. simiae*
3	M-76	FI-7794	Sputum	Negative	Single	Pneumonia	Lymphoma	−	Milan-2	1994	A	i	*M. simiae*
4	M-36	FI-5995	Gastric aspirate	Negative	Single	Hemophilia	AIDS	−	Vicenza	1994	A	i	*M. simiae*
5	F-77	FI-9395	Sputum	Positive	Single	Pneumonia	None	−	Milan-2	1995	A	i	*M. interjectum*
6	M-25	FI-25996	Sputum	Positive	Single	Pneumonia	Miliary tuberculosis	−	Sondalo	1996	A	i	*M. interjectum*
7	F-6	FI-17193	Lymph node	Positive	Single	Lymphadenopathy	None	+	Ancona	1993	B	ii	MAC
8	F-82	FI-18893	Sputum	Negative	Twice	Emphysema	Cancer	−	Udine	1993	B	ii	*M. simiae*
9	M-33	FI-2994	Stools	Negative	Single	Cirrhosis	AIDS	−	Ancona	1993	B	ii	MAC
10	M-53	FI-9994	Sputum	Negative	Single	Cough	None	−	Milan-3	1988	C	i	*M. simiae*
11	M-58	FI-22995	Pleural biopsy specimen	Negative	Single	Pleural effusion	Rheumatoid arthritis	+	Ancona	1995	C	i	*M. simiae*
12	M-31	FI-23395	Sputum	Negative	Single	Interstitial pneumonia	AIDS	−	Bergamo	1995	D	i	MAC
13	M-81	FI-22895	Sputum	Positive	Single	Lung cavitations	None	−	Florence	1995	E	iii	MAC
14	F-4	FI-16795	Lymph node	Positive	Single	Lymphadenopathy	None	+	Vicenza	1995	F	iv	*M. gordonae*

[a] M, male; F, female.
[b] The numbers 1, 2, and 3 indicate three different clinical settings in Milan.

nous lymph node with reddened overlying skin. Ultrasonic investigation showed a single sonographically hypodense mass (major axis, 2.5 cm) without evidence of colliquation; a week later colliquation areas had developed and a second swelling lymph node was present. Abdominal echographic scan and chest roentgenogram were normal. Under the hypothesis of a tubercular lymphadenitis, a therapeutic regimen with isoniazid and rifampin was started. Microbiological investigations performed on cervical lymph node biopsy specimens revealed scanty acid-fast bacilli which grew in culture and were identified with genetic probes as belonging to MAC (isolate 14 in Table 3). A fistula secreting necrotic yellowish material developed a few days later; the whole lymph node group was surgically removed and the girl was dismissed completely healed. No relapse has occurred so far.

DISCUSSION

Recent genetic studies revealed within the genus *Mycobacterium* a much greater complexity than was previously believed to be the case and has led to the description of a number of new species. Although the rarity of reports concerning the isolation of new mycobacteria seems to suggest a limited dissemination of such organisms, we believe that their extent is grossly underestimated. The identification of mycobacteria for which commercial genetic probes are not yet available largely relies, in fact, on conventional tests which tend to assign them to one of the well-established species (15).

Genetic sequencing of mycobacterial 16S rRNA revealed differences in the number of nucleotides forming helix 18, which was at first considered a marker of phylogenetic distance between the rapidly and the slowly growing organisms (16). A short helix 18, initially considered a distinctive feature of the rapidly growing mycobacteria, subsequently appeared to be shared by *M. simiae* and related species such as *M. intermedium* (8), *M. interjectum* (12), and *M. genavense* (1). Most remarkably, *M. simiae* and related organisms show an identical nucleic acid sequence in helix 18, which therefore serves as a molecular marker for this intermediate group (5). This intermediate branch of the phylogenetic tree is now enlarged by the group of mycobacteria investigated here. Although the taxonomic status of these isolates (except *M. lentiflavum* [14]) has not been firmly defined, they are closely related to the species *M. simiae*, from which they can be differentiated on the basis of specific genetic sequences. Interestingly, HPLC patterns seem to support the genetic results, because the related species *M. genavense*, *M. simiae* (18), and the prevailing type in this study (Fig. 2, phenotype i) are characterized by similar HPLC profiles.

Depending on the approach used, the mycobacteria investigated here clustered differently. Identifications based on conventional tests appear to be little more that arbitrary labels, because they are reached with a very low level of confidence. The clusters determined by genetic and lipidic analyses, albeit not completely overlapping, appear, on the contrary, to be more solidly based; in fact, three genotypes (genotypes B, E, and F) of the six different genotypes (A, B, C, D, E, and F) identified by genetic sequencing coincide with three phenotypic clusters (phenotypic clusters ii, iii, and iv) emerging from HPLC, while the remaining three genotypes (genotypes A, C, and D) show a common mycolic acid pattern (phenotypic cluster i) (Table 3).

Apart from the six isolates fitting the newly described species *M. lentiflavum*, the taxonomic status of the other strains remains undetermined. It appears certain, however, that they are distinct from the commonly encountered species to which they could be assigned on the basis of conventional test results. Isolate FI-16795 appears to be of interest because it could be confused, on the basis of the AccuProbe test, with a MAIX isolate, but it differs from this group in both its mycolic acid and susceptibility patterns. In our experience, all MAIX isolates investigated so far present a HPLC profile typical of that of MAC (18) and are quite resistant to antimycobacterial drugs.

Although the clinical significance of the present set of novel mycobacteria as potential pathogens was proved for only three isolates, the potential clinical significance cannot be excluded for the majority of the other isolates, all the more so because in immunocompromised patients, from which three of our isolates were obtained, the transition from colonization to disease almost regularly accompanies a decrease in the CD4 level.

In conclusion, at present, only two techniques seem to have the potential to recognize new species of mycobacteria: genetic analysis and HPLC. So far, in fact, all reports concerning such microorganisms evolved from laboratories that used at least one of these approaches. Genetic sequencing, particularly of 16S rRNA, has been confirmed to be the method of choice for the ultimate discrimination of mycobacterial taxa, while within phenotypic investigations, HPLC analysis appears to be the method which best correlates with genotypic results.

ADDENDUM IN PROOF

An HPLC profile identical to the one presented by our phenotype ii is reported in the very recent proposal of *Mycobacterium triplex* sp. nov. (M. M. Floyd, L. S. Guthertz, V. A. Silcox, P. S. Duffey, Y. Jang, E. P. Desmond, J. T. Crawford, and W. R. Butler, J. Clin. Microbiol. 34:2963–2967, 1996); the respective 16S rRNA sequences differ, however, for the nucleotide at position 201.

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