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Characteristics of *Massilia timonae* and *Massilia timonae*-Like Isolates from Human Patients, with an Emended Description of the Species

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The description of *Massilia timonae*, a nonfermentative aerobic gram-negative rod, was based on a single strain. A subsequent report of a second isolate has been recently published. Phenotypic descriptions of these two strains were based primarily on commercial test kit results. We have identified three additional strains as *M. timonae* by 16S rRNA sequence analysis and have characterized them phenotypically in parallel with the type strain of *M. timonae*, CIP 105350, by conventional test methods. A fourth strain, designated *M. timonae*-like, was also characterized. All four strains were isolated from human patients: two were blood isolates, one was isolated from cerebrospinal fluid, and one was isolated from bone. The four strains and the type strain were quite similar phenotypically. However, in contrast to the original description, the strains were found to be oxidase positive and arginine dihydrolase negative and to have lateral flagella as well as a single polar flagellum. Additionally the strains produced acid oxidatively from some carbohydrates. Other phenotypic characteristics, including cellular fatty acids, agreed with the original description. Based on our emended description, *M. timonae* and *M. timonae*-like strains can be differentiated from other aerobic nonfermentative gram-negative rods by conventional biochemical tests combined with cellular fatty acid analysis.

Massilia timonae was described in 1998 based on a single isolate from the blood of an immunocompromised patient. It was classified as a novel bacterium based on its unique phenotypic and genotypic characteristics (11). Subsequently, the use of 16S rRNA sequence analysis led to the identification of a second isolate of *M. timonae* from a surgical wound infection in an immunocompetent 36-year-old male who had undergone orthopedic surgery (14). The phenotypic characteristics described in both these reports were based largely on commercial test kit results.

As the reference laboratory for the state of California, the Microbial Diseases Laboratory receives unusual bacteria for identification. Two isolates (99A9205 and 97A4424) that could not be identified by phenotypic testing by conventional algorithms (18) were subsequently identified by 16S rRNA analysis as *M. timonae*, a species we had not knowingly encountered before. In order to compare these strains with a known isolate of *M. timonae*, we characterized the type strain (CIP 105350) by the same test protocols. A search of our culture collection turned up two additional isolates (85A2206 and 96A14209) that appeared phenotypically similar to 99A9205 and 97A4424 as well as to the type strain of *M. timonae*. Herein we present our results, including 16S rRNA sequence analysis, conventional biochemical test results, morphological and flagellar characteristics, and cellular fatty acid (CFA) analysis, from examining our four strains and the type strain. Based on these results, we provide an emended description of this new species.

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MATERIALS AND METHODS

Bacterial strains. The strains studied are summarized in Table 1. Strain 85A2206 was isolated from the femur of a 29-year-old male who 10 years earlier had had a resection of a benign bone tumor. A biopsy performed at the time of culture showed osteomyelitis. Strain 96A14209 was isolated from the cerebrospinal fluid (CSF) of a 49-year-old female whose diagnosis was cerebral pseudotumor. The CSF showed a glucose level of 94 mg/dl and a protein level of 50 mg/dl. Microscopic examination showed one leukocyte and no erythrocytes. No bacteria were seen upon Gram staining. A more extensive history was associated with strain 97A4424. It was isolated from the blood of a 41-year-old male with end-stage renal disease secondary to diabetic nephropathy and hypertension. This patient had had insulin-dependent type I diabetes mellitus for 16 years and was on a waiting list for a combined kidney and pancreas transplant. He started intermittent hemodialysis in November 1996 and was doing well until April 1997, when he developed signs of sepsis (fever of 102.3°F and chills). Blood cultures were drawn, from which strain 97A4424 was recovered in pure culture. He was treated with vancomycin and tobramycin for 5 to 7 days, and follow-up blood cultures drawn 3 days posttreatment were negative. Shortly after his bout of sepsis, this patient was found to have two abscessed teeth that were then extracted. Strain 99A9205 was isolated from the blood of a 39-year-old female who had been hospitalized with a massive swelling of the left thigh. Upon admission a tentative diagnosis of sepsis was made. She was initially placed on cefazolin, and blood cultures were drawn, from which this strain was isolated in pure culture. Strain CIP 105350, the type strain of *M. timonae* as characterized and described by La Scola et al. (11), was obtained from the collection of the Pasteur Institute.

16S rRNA sequence analysis. Amplification and bidirectional sequencing of a 1,400-bp fragment of the 16S rRNA gene were performed for each isolate as previously described (9, 16). A standard nucleotide BLAST search of the National Center for Biotechnology Information sequence database (www.ncbi.nlm.nih.gov) was performed to identify related 16S rRNA sequences.

Phenotypic tests. Most tests were performed as previously described (18). For motility, wet mounts were made from growth in heart infusion (HI) broth incubated at 25°C. Reduction of nitrite was tested with 0.01% KNO_2 in HI broth. Both nitrite and nitrate reduction were tested for a total of 7 days of incubation. Indole production was tested with xylene extraction and Kovács' reagent. Difco oxidation-fermentation (OF) medium (Difco Laboratories, Detroit, Mich.) was used to test for oxidation of carbohydrates. Growth on MacConkey and salmonella-shigella (SS) agar was done with plated media. Growth temperatures were

TABLE 1. Summary of strains tested

Strain	Date received	Patient age (yr), sex	Source/diagnosis
85A2206	1985	25, male	Femur/osteomyelitis
96A14209[a]	1996	49, female	CSF/pseudotumor
97A4424	1997	41, male	Blood/end-stage renal disease
99A9205	1999	39, female	Blood/sepsis
CIP 105350[b]	2000	25, male	Blood/common variable immunodeficiency

[a] *M. timonae*-like strain.
[b] Type strain of *M. timonae*.

tested with HI broth. *N*,*N*-Dimethyl-*p*-phenylenediamine oxalate reagent was used for the oxidase test as performed on soaked filter paper and by flooding growth on HI agar. Catalase was tested by flooding growth on HI agar with 3% H_2O_2. Flagella were stained by the method of Ryu (18). Growth in HI broth was observed for pellicle formation, and pigment production was observed on HI agar. Starch hydrolysis was tested with 0.5% soluble starch in HI agar. After 3 days of incubation, the plate was flooded with Gram's iodine and observed for clearing around the growth. As a test for identification, susceptibility to polymyxin B (Becton Dickinson Microbiology Systems, Cockeysville, Md.) was determined by placing a 300-U disk on a blood plate inoculated to give confluent growth and incubating it overnight. Any zone of inhibition was interpreted as indicating a susceptible strain (13). Hydrolysis of arbutin was tested on an agar medium by the method of Frank and von Riesen (3). The extracellular enzymes staphylysin, RNase, DNase, tyrosinase, pectinase, chondroitinase, protease, mucinase, lecithinase, and chitinase were assayed as previously described (6, 10). Oxidation of potassium gluconate and urocanic acid utilization were tested according to the method of Janda et al. (8).

Except for strain 96A14209, test media were incubated for a total of 7 days at 35°C unless otherwise noted. Test media for strain 96A14209 were incubated at 25°C for 7 days with some indicated exceptions. Some test media were incubated at both 25 and 35°C (Table 2). Gelatin, litmus milk, and the OF media were incubated a total of 14 days unless they were positive by 7 days. In all cases the atmosphere was room air.

CFA analysis. CFA analysis was performed with a commercially available system (MIDI, Newark, Del.). Strains were grown overnight on HI-based 5% sheep blood agar plates incubated at 35°C (including strain 96A14209). The harvested cells were saponified, methylated, and analyzed by capillary gas-liquid chromatography. The system's software identified the fatty acid methyl esters based on their equivalent chain lengths (Sherlock Microbial Identification System MIS operating manual; MIDI). Unsaturated straight-chain fatty acids were confirmed by hydrogenation (18).

Nucleotide sequence accession number. The GenBank accession numbers for the 16S rRNA sequence of strains 85A2206, 97A4424, 99A9205, and 96A14209 are AY157759, AY157760, AY157761, and AY157762, respectively.

RESULTS

16S rRNA sequencing has proven to be a useful adjunct to conventional microbiological analysis in the identification of unknown bacterial pathogens. The inability of conventional microbiological analysis to identify four phenotypically similar gram-negative bacilli prompted our attempt to identify these strains by 16S rRNA sequencing. A BLAST search of the National Center for Biotechnology Information nucleotide database revealed that three of the isolates, 85A2206, 97A4424, and 99A9205, shared >99.4% 16S rRNA sequence identity with *M. timonae* (GenBank accession number U54470). The fourth isolate, 96A14209, shared only 97.8% sequence identity with *M. timonae*. According to the BLAST search algorithm, the highest-scoring sequence alignment for isolate 96A14209 was an uncultured beta-proteobacterium (GenBank accession number AF529109). The degree of sequence identity between this organism and 96A14209 was also 97.8%.

All five strains, including the type strain, were medium

TABLE 2. Conventional biochemical reactions of *M. timonae* and *M. timonae*-like bacteria

Test	Result for strain no.:				
	85A2206	97A4424	99A9205	CIP 105350[a]	96A14209[b]
Acid from:					
Glucose	Weak + (>7[c])	Weak + (>7)	Weak + (>7)	Weak + (>7)	Weak + (4–7)
Levulose	+ (2)	+	+	+ (3)	+ (2)
Xylose	+ (2)	+	+	+	+ (4–7)
Lactose	−	−	−	−	−
Maltose	+ (2)	+	+	+	+
Sucrose	−	−	−	−	−
Mannitol	−	−	−	−	−
Citrate	+ (3)	+	+ (2)	+ (2)	+
Nitrate reduction	−	−	+	−	−
Gelatin hydrolysis					
25°C	+ (3)	+ (3–5)	+ (5–7)	+ (3–5)	+ (2)
35°C	−	−	−	−	−
Litmus milk, peptonized					
25°C	+ (4)	+ (>7)	+ (>7)	+ (>7)	+ (>7)
35°C	+ (4)	+ (4–7)	+ (>7)	−	−
DNase					
25°C	+ (2)	+ (3–5)	+ (2)	+ (3–5)	+ (2)
35°C	Weak + (3–5)	−	Weak + (3)	Weak + (6–7)	NT[d]
Protease					
25°C	+	+ (2)	+ (2)	+	+ (2)
35°C	+ (2)	+ (3)	+ (2)	+ (5)	−
Urocanic acid utilization	+ (6)	+ (4)	+ (5)	+ (5)	−
Arbutin hydrolysis	−	−	+ (2)	−	+ (5)

[a] Type strain of *M. timonae.*
[b] *M. timonae*-like strain.
[c] Numbers in parentheses are the number of days to give a positive reaction, if greater than 1.
[d] NT, not tested.

TABLE 3. CFA composition of *M. timonae* and *M. timonae*-like bacteria

Fatty acid[a]	% of total CFA				
	85A2206	97A4424	99A9205	CIP 105350[b]	96A14209[c]
10:0	T[d]	T	T	1	T
3-OH-10:0	6	7	7	8	5
12:0	6	7	6	8	5
2-OH-12:0	3	3	3	3	2
14:0	T	T	T	T	T
15:0	T	T	1	T	1
16:1ω7c[e]	44	44	42	43	47
16:0	30	25	30	25	27
17:0	T	T	T	T	T
18:1ω7c	8	10	6	10	8
18:0	1	T	T	T	T

[a] The number before the colon indicates the number of carbons; the number after the colon is the number of double bonds; ω is the position of the double bond counting from the hydrocarbon end of the carbon chain; OH indicates a hydroxy group at the 2 or 3 position from the carboxyl end; c is *cis* isomer.
[b] The type strain of *M. timonae*.
[c] *M. timonae*-like strain.
[d] T, trace (0.4 to 0.8%).
[e] MIDI Sherlock version 4.0 software identifies this component as "Sum in Feature 3," either 16:1ω7c or i-2-OH-15:0. The identity of the peak as 16:1ω7c was confirmed by hydrogenation.

straight gram-negative rods. They showed visible growth in 1 day on routine plating media such as HI-based 5% sheep blood agar and HI agar. Acid was produced oxidatively from some carbohydrates (Table 2). When grown in Difco OF medium without added carbohydrate, these strains produced a distinctly alkaline reaction. Consequently, a neutral reaction in a tube with carbohydrate was interpreted as weakly positive for acid production. When tested in special OF medium (King's), reactions were weaker and more delayed (data not shown). All strains were motile. A single polar flagellum predominated, but cells with one to as many as three lateral flagella were also seen. The five strains were positive for catalase, oxidase, RNase, pectinase, chondroitinase, starch hydrolysis, esculin hydrolysis, sensitivity to polymyxin B, growth on MacConkey agar, and growth in nutrient broth with 0% NaCl. The following reactions were uniformly negative: indole production; urea hydrolysis; nitrite reduction; oxidation of gluconate; Voges-Proskauer; lysine and ornithine decarboxylase; arginine dihydrolase; production of soluble pigments on pseudomonas F and P agars; oxidation of amygdalin, salicin, and ribose; growth on SS agar; and growth in nutrient broth with 6% NaCl. None of the strains produced stapholysin, tyrosinase, mucinase, lecithinase, or chitinase. The triple sugar iron agar reaction was an alkaline slant with a neutral to alkaline butt, with no H_2S. None of the strains grew at 42°C. Growth at 35°C and that at 25°C were about equal except for strain 96A14209, which grew markedly better at the lower temperature. All strains produced a pellicle when grown in HI broth and a straw-colored growth pigment on HI agar. Results of additional reactions are shown in Table 2.

CFA results, shown in Table 3, agree with those originally reported (11). All five isolates had essentially the same fatty acid profile. Because the MIDI system software cannot differentiate 16:1ω7c and i-2-OH-15:0, it identifies this peak as a summed feature. We confirmed the identity of this peak as 16:1ω7c by hydrogenation. The CFA profile, predominantly saturated and monounsaturated straight-chain fatty acids and smaller but significant amounts of alpha- and beta-hydroxy acids, is typical of a gram-negative bacterium. The presence of both 3-OH-10:0 and 2-OH-12:0 is a salient feature of the CFA profile of this species. Among the encyclopedic collection of CFA profiles included in the work of Weyant et al. (18), there are few that have both of these fatty acids. CDC group OFBA-1 has both but also has 3-OH-12:0, appreciable amounts of 17:0 cyc and 19:0 cyc, and only 1% 16:1ω7c. *Chromobacterium violaceum*, *Pseudomonas aeruginosa*, "*Pseudomonas denitrificans*," *Pseudomonas fluorescens*, *Pseudomonas*-like group 2, and *Pseudomas putida* all have qualitatively similar profiles that include 3-OH-10:0 and 2-OH-12:0 but differ from *M. timonae* in having 3-OH-12:0 and, except for *C. violaceum*, 17:0 cyc. *Chryseomonas luteola*, *Flavimonas oryzihabitans*, and *Pseudomonas syringae* are perhaps the most like *M. timonae*. However, they differ in having 3-OH-12:0 and larger amounts of 18:1ω7c.

DISCUSSION

The identification of *M. timonae* and its placement in a new taxon were based largely on the unique 16S rRNA signature of this organism. As reported by La Scola et al. (11), the most closely related 16S rRNA sequence in GenBank displayed only 94.6% sequence identity with the 16S rRNA sequence of *M. timonae*. The most closely related 16S rRNA sequences in GenBank belonged to two nonpathogenic soil organisms, *Duganella zoogloeoides* and *Telluria mixta* (11). Similarly, our laboratory used 16S rRNA sequence analysis to first identify strains 97A4424 and 99A9205 and subsequently 85A2206 as *M. timonae*. The inclusion of strain 96A14209 as an *M. timonae*-like organism was established largely by phenotypic similarities. However, the significant 16S rRNA sequence divergence displayed between this strain and *M. timonae* precludes its classification as *M. timonae* in the strictest sense, hence the designation of strain 96A14209 as an *M. timonae*-like organism. Whether a marked preference for growth at 25 versus 35°C and lack of urocanic acid utilization will be phenotypic tests that can reliably differentiate *M. timonae*-like bacteria from true *M. timonae* strains will require the analysis of additional isolates.

The original description of *M. timonae* by La Scola et al. was based on strain CIP 105350 (11). They reported that this species does not form acid from carbohydrates, is oxidase negative and arginine dihydrolase positive, and has a single polar flagellum by electron microscopy (11). In our hands, *M. timonae*, including strain CIP 105350, produced acid oxidatively and was oxidase positive and arginine dihydrolase negative. In their report of a second human infection with *M. timonae*, Sintchenko et al. found their strain to be arginine negative (14). These discordant test results may reflect differences in test methodologies used between laboratories. While we found strains 85A2206, 96A14209, 97A4424, 99A9205, and CIP 105350 all to show cells with as many as three lateral flagella, a single polar flagellum was observed most frequently. This observation is similar to that seen with *Ochrobactrum anthropi* and *Ochrobactrum intermedium*. Both these species have been found to have both polar and lateral flagella (1, 5, 17). With the exception of the *M. timonae*-like strain, 96A14209, the strains did not dem-

onstrate a marked difference in growth between 25 and 35°C. La Scola et al. reported that the optimum growth temperature for *M. timonae* was 28°C (11). We found that the strains did demonstrate more proteolytic activity when incubated at the lower temperature than at the higher temperature (Table 2). Comparing our results with the descriptions of pathogenic gram-negative bacteria in the work of Weyant et al. (18), we find that this species closely resembles *Brevundimonas* (*Pseudomonas*) *vesicularis*. *B. vesicularis*, however, is citrate negative, while *M. timonae* is citrate positive. Further, the CFA profiles of the two species differ (18). Similarly, following the algorithms for the identification of nonfermenting gram-negative bacteria in the *Manual of Clinical Microbiology*, 7th ed., one is led to a number of possibilities, all of which can be differentiated from *M. timonae* by the phenotypic tests described in our report (4, 12, 13). Laboratories lacking the capacity to perform 16S rRNA sequence analysis can identify *M. timonae* and *M. timonae*-like bacteria, as we have described, by conventional biochemical tests supplemented with CFA analysis.

It has not been uncommon in recent years to base the description of a new bacterial species on a single strain. Recent publications have delineated some of the problems with this practice, and one has proposed a recommendation of a minimum number of strains on which to base the naming of a new taxon (2, 7). Basing the description of a new species on several strains provides an indication of the phenotypic variation of the species. If the strains are from human patients, it is possible to see if the range of clinical presentations and associations is narrow (e.g., usually associated with infected dog bites) or broad (e.g., isolated from a variety of body sites and from both immunocompetent and immunocompromised patients). The characterization of multiple strains may also provide evidence regarding the natural habitat of the species. The isolates that we studied, including the *M. timonae*-like strain, were phenotypically quite alike. We did find that nitrate reduction was a variable reaction for this species. Additional strains will provide a better picture of the range of phenotypic variation among *M. timonae* strains. Aside from the exceptions noted above, our phenotypic results agreed overall with those previously reported (11, 14).

The four patients represented here had no common predisposing condition. Two were men and two were women, aged between 29 and 49 years. One patient had longstanding diabetes. No underlying conditions are known for the other three. From the cases described previously and in this study, we find that *M. timonae* infects both the previously healthy and the immunocompromised patient (11, 14). These patients' sources of infection are as yet unknown. Perhaps *M. timonae* is an environmental organism like its close relatives *T. mixta* and *D. zoogloeoides* (11). Abscessed teeth were suspected to be the source of infection with strain 97A4424. If so, this species may occur as part of the transient normal oral flora.

Prior to this report, only two strains of *M. timonae* had been described in the literature. We have characterized three additional strains of *M. timonae* sensu stricto along with the type strain by conventional test methods for determining their phenotypic properties. It was recently recommended that proprietary kits not be used as a basis for the phenotypic description of a species (15). Included in our findings were that this species is oxidase positive and oxidizes carbohydrates, classically two basic characteristics used in the identification of gram-negative bacteria. In light of our expanded characterization of these strains, we have written an emended description of this species.

Emended description of *Massilia timonae* (La Scola, Birtles, Mallet, and Raoult 1998). This description is based on four strains, including the type strain. Cells are gram-negative medium straight rods. They are motile, predominantly by means of a single polar flagellum. Lateral flagella may also occur. Tests for oxidase and catalase are positive. Acid is produced oxidatively from levulose, xylose, maltose, and, less avidly, glucose. Lactose, sucrose, and mannitol are not oxidized. Starch and esculin are hydrolyzed. Citrate is used as a sole source of carbon. Gelatin is digested at 25°C; proteolysis is more avid at this lower temperature. Growth occurs at 25 and 35°C, on MacConkey agar, and in nutrient broth with 0% NaCl. Growth does not occur at 42°C, on SS agar, or in nutrient broth with 6% NaCl. The species is sensitive to polymyxin B. A straw-colored growth pigment is produced on HI agar. Reduction of nitrate is variable. Indole production, urea hydrolysis, nitrite reduction, lysine and ornithine decarboxylase, and arginine dihydrolase are negative. H_2S is not produced in the butt of triple sugar iron agar. Other test results are included in Table 2. CFAs are shown in Table 3.

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