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Porphyromonas somerae sp. nov., a Pathogen Isolated from Humans and Distinct from *Porphyromonas levii*

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***Porphyromonas levii* is an anaerobic, pigmented gram-negative bacillus originally isolated from bovine rumen. We describe 58 human clinical strains of *P. levii*-like organisms, isolated from various human clinical specimens that are phenotypically similar to the type strain of *P. levii*, a rumen isolate (ATCC 29147). Our biochemical, comparative 16S rRNA sequence analyses, and DNA-DNA relatedness studies indicate that the human *P. levii*-like organisms are similar to each other but genetically different from the *P. levii* type strain isolated from bovine rumen. We therefore propose the name *Porphyromonas somerae* to encompass the human *P. levii*-like organisms. *P. somerae* was predominantly isolated from patients with chronic skin and soft tissue or bone infections, especially in the lower extremities.**

The species *Bacteroides levii* (11) was excluded from the *Bacteroides* genus (18) and subsequently included in the genus *Porphyromonas*, as *Porphyromonas levii*, on the basis of biochemical, chemical, and comparative 16S rRNA sequence analyses (19). *P. levii* is an anaerobic, pigmented gram-negative bacillus originally isolated from bovine rumen. Recently, *P. levii* was found to cause an outbreak of bovine necrotizing vulvovaginitis and the authors concluded that *P. levii* as an etiologic agent of bovine necrotizing vulvovaginitis may be underdiagnosed (6). *P. levii*-like organisms have been described from human sources, such as the vagina (8), from patients with chronic otitis media (9), and a case of bacterial vaginosis (1). We previously described *B. levii*-like organisms isolated from various human clinical specimens (14). These strains were biochemically characterized as part of a comprehensive reevaluation of pigmented gram-negative anaerobic rods (5). The strains were phenotypically similar to the type strain of *P. levii* from bovine rumen (ATCC 29147) and therefore are referred to as *P. levii*-like organisms (PLLO).

The PLLO isolated from human sources are phenotypically similar to other human *Porphyromonas* species in that they produce pigmented colonies on blood agar and are susceptible to the special-potency vancomycin disk. Unlike other human *Porphyromonas* species, however, PLLO are weakly saccharolytic and indole negative. Because of this, the identification of PLLO in the clinical laboratories has been problematic; they may have been misidentified as pigmented *Prevotella* species or reported as unusual, pigmented gram-negative rods. Therefore, the clinical importance of PLLO maybe underestimated due to the lack of a sound classification and identification scheme.

The 16S rRNA gene is the most widely accepted gene used for bacterial classification and identification (23). Signature nucleotides of 16S rRNA genes allow classification even if a particular sequence has no match in the database, since otherwise unrecognizable isolates can be assigned to phylogenetic branches at the class, family, genus, or subgenus levels. This has contributed greatly to the taxonomy of anaerobic bacteria.

The aim of this study was to characterize the PLLO isolated from humans, determine its taxonomic position compared to other *Porphyromonas* species, and document the need for a new species to encompass it. We also describe the types of infections involving PLLO and the phenotypic tests useful in distinguishing it from other *Porphyromonas* species. Part of the biochemical characterization and clinical information has been presented previously (5, 14).

MATERIALS AND METHODS

Bacterial strains. Fifty-eight PLLO strains were isolated from 53 clinical specimens obtained at the VA Wadsworth Medical Center. The specimen sources included lower extremity skin and soft tissue or bone specimens ($n = 38$); inguinal or sacral area abscess ($n = 5$); intra-abdominal abscess ($n = 2$); transtracheal aspirate ($n = 2$); axillary abscess ($n = 2$); and mastoiditis, blood culture, brain tissue, and infected scalp (1 specimen each). All strains were characterized biochemically, unless otherwise noted. The American Type Culture Collection strain (ATCC 29147) of *P. levii* (a rumen isolate) was included in the biochemical and molecular characterization.

The strains were maintained at −70°C in double-strength skim milk before characterization. All the testing was done from 24- to 72-h pure cultures on brucella (Anaerobe Systems, Morgan Hill, CA) or CDC agar (Becton Dickinson Microbiology Systems, Sparks, MD), unless otherwise noted.

Identification by conventional biochemical methods. The strains were characterized by routine biochemical tests (5, 12, 13), including susceptibility to special-potency vancomycin disk (5 μg/ml) (13), fermentation of prereduced, anaerobi-

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cally sterilized biochemicals (Anaerobe Systems), gas-liquid chromatography of metabolic end products, API ZYM (BioMerieux, Marcy l'Etoile, France), and Rosco diagnostic tablets (Rosco, Taastrup, Denmark). Pigment production was tested on brucella agar (Difco Laboratories, Detroit, MI) supplemented with 5% laked rabbit blood (RLB).

Antimicrobial susceptibility. The production of β-lactamase was detected by the nitrocefin disk test (Cefinase; Becton Dickinson Microbiology Systems). Antimicrobial susceptibility studies were done on 18 clinical PLLO strains and *P. levii* ATCC 29147 using the NCCLS-approved Wadsworth agar dilution method (16).

Cellular fatty acid analysis. Eighteen clinical PLLO strains and *P. levii* ATCC 29147 were included in this study. Cells were grown for 48 h in peptone-yeast-glucose broth with Tween 80 (PYG-Tween; Anaerobe Systems). Growth was harvested by centrifugation (5,000 × *g* for 20 min), and the pellets were processed immediately or kept frozen at −20°C. Methyl ester extraction was carried out by the manufacturer's procedure (MIDI; Microbial ID, Inc., Newark, DE), and analysis was done in an H-P Microbial Identification System (HP 5898A MIS) gas chromatograph, with software from MIDI. The samples were clustered according to the Euclidian distance of principal components accounting for the greatest variance of the organisms: in general, a distance of ≤10 included the same species (MIDI; Microbial ID, Inc.). The corresponding library (ANAEROBE, version Moore 5.0) was used in successive analyses.

16S rRNA sequencing. Eleven strains (WAL 6690, 10252, 11006, 11104, 11122, 11196, 16712, 16734, 16788, 17564, and 17568) were studied by 16S rRNA sequencing. Genomic DNA was extracted and purified from bacterial cells in the mid-logarithmic growth phase, using a QIAamp DNA Mini kit (QIAGEN, Inc., Chatsworth, CA). The 16S rRNA gene fragments were amplified by PCR. Briefly, two subregions of the16S rRNA gene were amplified by using two pairs of primers: region A was defined as 899-bp sequences between primers 8UA (5′-AGAGTTTGATCCTGGCTCAG-3′) and 907B (5′-CCGTCAATTCMTTTRAGTTT-3′), and region B was defined as 711-bp sequences between primers 774A (5′-GTAGTCCACGCTGTAAACGATG-3′) and 1485B (5′-TACGGTTACCTTACGAC-3′). PCR was performed for 35 cycles of 30 s at 95°C, 30 s at 45°C, and 1 min at 72°C, with a final extension at 72°C for 5 min. The PCR products were excised from a 1% agarose gel after electrophoresis and purified using a QIAquick gel extraction kit (QIAGEN). The purified PCR products were sequenced directly with a Biotech Diagnostic Big Dye sequencing kit (Biotech Diagnostics, Laguna Niguel, CA) on an ABI 377 sequencer (Applied Biosystems, Foster City, CA).

Sequence data analysis. The sequencing data were analyzed by comparison of the consensus sequences with GenBank sequences by using Ribosomal Database Project (RDP-II; Michigan State University, East Lansing) (15) and Basic Local Alignment Search Tool (BLAST) software (2), and the percent similarity to other sequences was determined. Closely related sequences were retrieved from GenBank and were aligned with the newly determined sequences by using the program CLUSTALW (21). The resulting multiple sequence alignment was corrected manually using the program GeneDoc (17).

DNA-DNA hybridization. Six clinical strains (WAL 6690, 10252, 11006, 11104, 11122, and 11196) and *P. levii* ATCC 29147, *Porphyromonas asaccharolytica* ATCC 25260, *Porphyromonas cangingivalis* NCTC 12856, *Porphyromonas endodontalis* ATCC 35406, and *Porphyromonas macacae* ATCC 33141 were studied. DNA was isolated using a French pressure cell (Thermo Spectronic, Shelton, CT) and purified by chromatography on hydroxyapatite as described by Cashion et al. (3). DNA-DNA reassociation experiments were done by the spectrophotometric method of De Ley et al. (4) with a Gilford system model 2600 spectrophotometer equipped with a Gilford model 2527-R thermal programmer.

Nucleotide sequence accession number. Type strain WAL 6690 has been assigned ATCC accession no. BAA-1230, and the GenBank accession number for the 16S rRNA gene of *P. somerae* WAL 6690 is AY968205.

RESULTS

Bacterial strains. Fifty-eight PLLO strains were isolated from 53 clinical specimens at the VA Wadsworth Medical Center between the years 1981 and 2003. The medical records were available for 26 patients. Eight of these patients, all of whom presented with skin and soft tissue or bone infection, had diabetes mellitus as a predisposing condition; five of them had neuropathy and peripheral vascular disease. Eleven patients with lower extremity skin and soft tissue infections had underlying osteomyelitis; in one case, a preceding fracture was recorded. PLLO were always isolated together with other anaerobes, such as anaerobic cocci, *Bacteroides fragilis* group, and *Prevotella* sp. The mean number of accompanying anaerobes was 3.9 per specimen. The PLLO was usually also isolated with aerobes (82%). The most common accompanying aerobes were *Enterobacteriaceae*, *Staphylococcus* sp., and *Enterococcus* sp., and the mean number of the accompanying aerobes was 3.1. In the majority of cultures (56%), the PLLO growth was heavy (corresponding to a score of 3+ to 4+).

Identification by conventional biochemical methods. The colonial morphology of PLLO on anaerobic blood agar plates was often poorly defined; it frequently exhibited a patchy growth pattern ("satellitism" around its own colonies). At an early stage of growth, the colonies seldom showed distinct pigmentation or fluorescence under long-wave UV light (366 nm) on RLB or brucella agar; the color of the colonies ranged from whitish yellow to tan. After more prolonged incubation (≥4 days), brown pigment production on RLB agar was apparent. The PLLO strains were all sensitive to the vancomycin special-potency identification disk and resistant to colistin, and all but one were resistant to the kanamycin disk. All isolates studied were indole negative; lipase, catalase, and nitrate negative; fermented glucose, lactose, and mannose weakly (pH 5.3 to 5.4 after 5 days); and produced acetic, propionic, isobutyric, butyric, and isovaleric acid and small amounts of succinic acid as metabolic end products. All strains were negative for lipase, cystine arylaminidase, α-fucosidase, α-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase, β-xylosidase, esculin, and trypsin but positive for alkaline phosphatase, acid phosphatase, naphthol-AS-Bl-phosphohydrolase, *N*-acetyl-β-glucosaminidase, β-galactosidase, and chymotrypsin by the API ZYM system. Most strains (>90%) were positive for esterase, esterase lipase, and leucine arylamidase; valine arylamidase and α-glucosidase activity was variable with the API ZYM tests. Using Rosco diagnostic tablets, the strains were negative for α-fucosidase, β-xylosidase, trypsin, and esculin but positive for *N*-acetyl-β-glucosaminidase and ONPG (*o*-nitrophenyl-β-D-galactopyranoside).

The biochemical tests useful in differentiating PLLO from other species and genera are listed in Table 1.

Antimicrobial susceptibility. β-Lactamase was produced by 21% (10/48) of the strains. All the strains were susceptible to amoxicillin-clavulanic acid (MIC, <0.5 μg/ml), cefoxitin (MIC, <4 μg/ml), imipenem (MIC, <0.5 μg/ml), and metronidazole (MIC, <1 μg/ml). Some strains were resistant to amoxicillin. Most strains were susceptible to clindamycin (MIC, <4 μg/ml); however, resistance to clindamycin was observed in four strains (MIC, 33 to 129 μg/ml).

Cellular fatty acid analysis. The main cellular fatty acids detected in both PLLO and *P. levii* ATCC 29147 were iso-$C_{15:0}$ and anteiso-$C_{15:0}$ (25 to 39% and 16 to 22% of the total fatty acids, respectively). Other cellular fatty acids detected at levels greater than 5% of the total fatty acids included iso-$C_{17:0}$, 3 OH iso-$C_{15:0}$, iso-$C_{13:0}$, $C_{14:0}$, 3 OH iso-$C_{17:0}$, and $C_{16:0}$. The cluster analysis of principal components showed a Euclidian distance of <10 between the PLLO strains, thus indicating that they belong to the same species. *P. levii* ATCC 29147 clustered separately from the PLLO; the Euclidian distance of the *P. levii* type strain from the PLLO was >15.

TABLE 1. Differential characteristics of *Porphyronionas somerae* sp. nov.[a]

Organism	Test result								Source
	Vancomycin special-potency disk	Pigment	Indole	Catalase	Glucose fermentation	α-Fucosidase	β-NAG	Metabolic end products from PYG	
P. asaccharolytica	S	+	+	−	−	+	−	A,P,Ib,B,IV,s	Nonoral
P. catoniae	S	−	−	−	W	+	+	a,P,iv,l,S	Oral
P. endodontalis	S	+	+	−	−	−	−	A,P,Ib,B,IV,s	Oral
P. gingivalis	S	+	+	−	−	−	+	A,P,Ib,B,IV,s,pa	Oral
P. levii	S	+	−	−	W	−	+	A,P,Ib,B,IV,s	Bovine
P. somerae sp. nov.	S	+	−	−	W	−	+	A,P,Ib,B,IV,s	Nonoral
P. uenonis	S	+	+	−	−	−	−	A,P,Ib,B,IV,s	Nonoral
Animal *Porphyromonas* spp.[b]	S	+	+	+	−[w]	−	V	A,P,Ib,B,IV,s (pa)	Cat, dog, monkey
Pigmented *Prevotella* spp.[c]	R	+	V	−	+	+[d]	V	A,S (p,ib,iv,l)	Oral, nonoral

[a] Data were compiled from references 4, 6, and 9–12 and the authors' own determination. β-NAG, *N*-acetyl-β-glucosaminidase; PYG, peptone-yeast-glucose; S, sensitive; R, resistant; +, positive; −, negative; V, variable reaction; W, weak positive reaction; −[w], most strains negative, occasional strains weak-positive reaction; a, acetic; p, propionic; ib, isobutyric; b, butyric; iv, isovaleric; s, succinic; l, lactic; pa, phenylacetic acid. Capital letters indicate major metabolic products, lowercase letters indicate minor products, and parentheses indicate variable reaction from PYG.

[b] Includes *Porphyromonas* species *P. canoris*, *P. cangingivalis*, *P. cansulci*, *P. circumdentaria*, *P. crevioricanis*, *P. gingivicanis*, *P. gulae*, and *P. macacae*. These organisms may be isolated from animal bite infections. *P. crevioricanis* is catalase negative.

[c] Includes *Prevotella* species *P. corporis*, *P. denticola*, *P. intermedia*, *P. loescheii*, *P. melaninogenica*, *P. nigrescens*, *P. pallens*, and *P. tannerae*.

[d] *Prevotella corporis* is α-fucosidase negative.

16S rRNA gene sequencing and sequence data analysis. The 11 PLLO strains had >99% sequence similarity to each other. When comparing the PLLO sequences with the sequences of other reference species in the GenBank library, the PLLO clustered with the *Porphyromonas* genus and the closest related species was *P. levii* ATCC 29147 at 94% sequence similarity (Fig. 1).

DNA-DNA hybridization. The DNA-DNA reassociation studies indicated a low-level affinity of 44 to 53% between PLLO and *P. levii* ATCC 29147. Likewise, the DNA-DNA relatedness between PLLO and *P. asaccharolytica* ATCC 25260, *P. cangingivalis* NCTC 12856, *P. endodontalis* ATCC 35406, or *P. macacae* ATCC 33141 was less than 57%.

DISCUSSION

P. levii was originally included in the genus *Bacteroides*, but biochemical and 16S rRNA sequence studies indicated that it belongs in the genus *Porphyromonas* (19). A 98% 16S rRNA sequence similarity is generally accepted as an indication of the same species (20), and in DNA-DNA hybridization studies, a threshold of 70% or greater similarity indicates relationship at the species level (22). In our study, the PLLO strains were genetically highly related to each other at >99% sequence similarity but not related to *P. levii* ATCC 29147 (94% similarity). Similarly, the DNA-DNA reassociation studies showed a low-level affinity between PLLO and *P. levii* ATCC 29147 (44 to 53% similarity), indicating that they do not belong to the same species. The 16S rRNA sequence similarity and DNA-DNA hybridization results were consistent with the PLLO belonging to the *Porphyromonas* genus, which was also supported by the biochemical data. Our biochemical characterization of PLLO showed that it was phenotypically similar to *P. levii* (19). The PLLO produced pigmented colonies on blood agar and were sensitive to the special-potency vancomycin disk and produced complex metabolic end product profiles that included acetic, propionic, isobutyric, butyric, and isovaleric acids; the major cellular fatty acid was iso-$C_{15:0}$, similar to the *Porphyromonas* genus (10, 19).

A satellite-like growth pattern, negative indole and α-fucosidase, and positive *N*-acetyl-β-glucosaminidase were the key characteristics in differentiating PLLO from other *Porphyromonas* species (Table 1). Unlike most other human *Porphyromonas* species, PLLO was weakly saccharolytic; however, it was easily differentiated from the saccharolytic pigmented *Prevotella* species by biochemical tests and sensitivity to the special-potency vancomycin disk (Table 1). Demonstration of pigment production by PLLO was useful in differentiating it from *Porphyromonas catoniae*, which is also indole negative and weakly saccharolytic but nonpigmented. Human PLLO were genetically distant from *P. levii*; however, using phenotypic tests these two organisms could only be differentiated by cluster analysis of the cellular fatty acid profiles (Euclidian distance of principal components accounting for the greatest variance of the organisms). *P. levii* clustered distantly (>15) from the PLLO strains.

Due to the negative indole reaction and weakly saccharolytic nature of PLLO, it may have commonly been misidentified as *Prevotella* species or another gram-negative rod in clinical laboratories. Using an accurate identification scheme, it may be found to be more common in clinical specimens than has been appreciated. After we became more aware of the PLLO group during our study of comprehensive biochemical reevaluation of pigmented gram-negative anaerobic rods (5), we isolated PLLO more frequently; 17 specimens yielded PLLO between 1981 and 1992, whereas PLLO was isolated from 36 specimens between 1993 and 2003. Even though *P. levii*-like organisms were isolated from vaginosis and chronic otitis media (1, 8, 9), we found that PLLO was predominantly isolated from patients with chronic skin and soft tissue or bone infections in the lower extremities (70%) or the inguinal/sacral area (10%). The most common predisposing condition in the patients with skin and soft tissue or bone infections was diabetes mellitus. This dis-

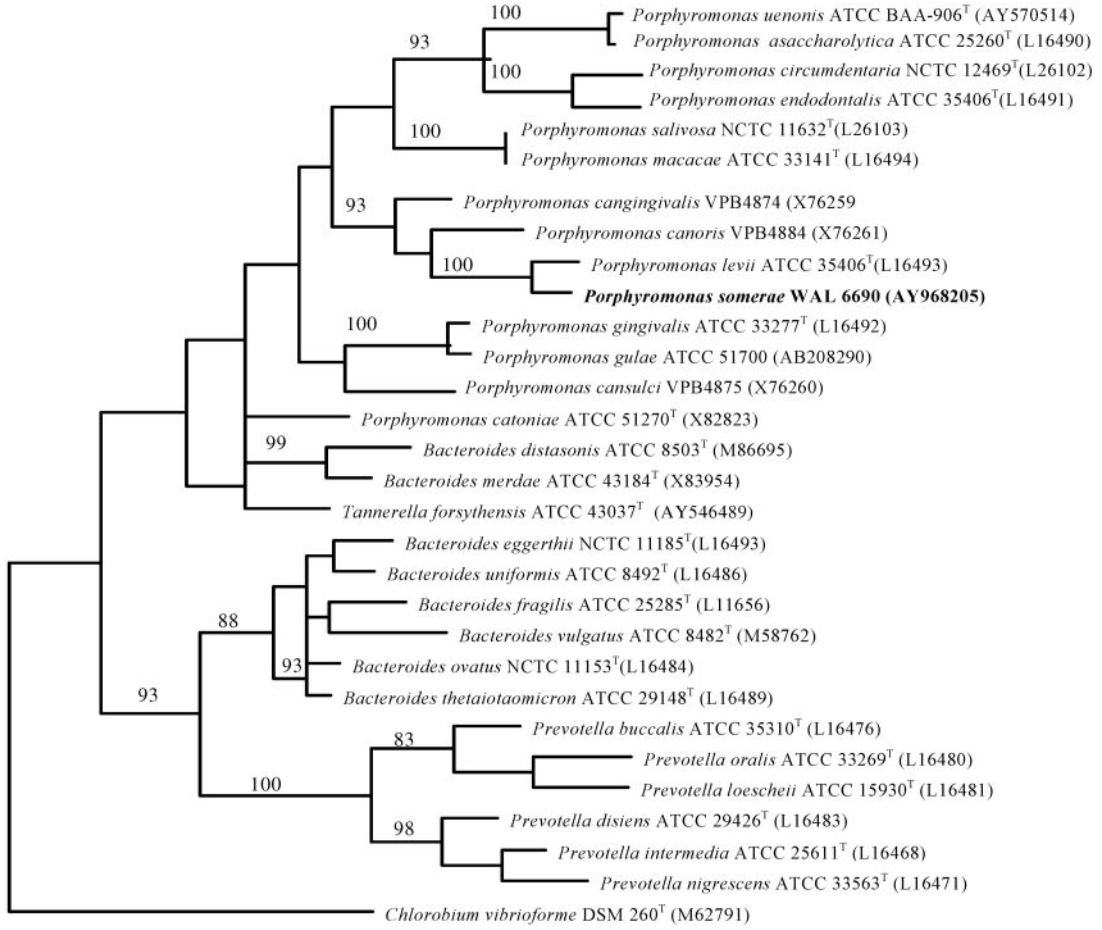

FIG. 1. Unrooted tree showing the position of *P. somerae* sp. nov. within the *Bacteroides* subgroup of the *Cytophaga-Flavobacter-Bacteroides* phylum. The tree was constructed by the maximum parsimony method and is based on a comparison of approximately 1,400 nucleotides. Bootstrap values, expressed as a percentage of 1,000 replications, are given at the branching points.

tribution of types of infection may be related to our patient population and the association with diabetes mellitus. There were several serious infections in which PLLOs were part of the infecting flora. The full extent of the virulence of PLLO remains to be determined, as it was always isolated in mixed culture with accompanying organisms indicative of colonic flora. However, it is notable that 21% of the PLLO strains were β-lactamase producers, a trait among *Porphyromonas* species shared only with the recently described *Porphyromonas uenonis* (7).

Our studies indicate that the human PLLO are phenotypically similar, but genetically different, from the *P. levii* type strain isolated from bovine rumen. We therefore propose the name *Porphyromonas somerae* to encompass the human *P. levii*-like organisms.

Description of *P. somerae* sp. nov. *Porphyromonas somerae* (so′mer.ae, N.L. gen. n. *somerae*, of Somer, in honor of the late Finnish microbiologist Hannele Jousimies-Somer, who has contributed significantly to our knowledge of pigmented anaerobic gram-negative rods and to the identification of clinically important anaerobic bacteria in general) is a gram-negative, obligately anaerobic, nonsporeforming, nonmotile rod. Colonies incubated on blood agar for 2 days often exhibit a "patchy" growth pattern, with larger colonies surrounded by smaller colonies; they are circular, entire, and convex. The colonies on laked rabbit blood agar are white-yellow to tan; after 4 days of incubation, the colonies are pigmented (light brown to dark brown) and show no or occasionally weak red fluorescence under long-wave UV light. The organisms are indole, nitrate, lipase, and catalase negative. They are weakly saccharolytic, and the pH of glucose, lactose, and maltose cultures is 5.3 to 5.4 after 5 days of incubation. Major fermentation products from PYG metabolism are acetic, propionic, isobutyric, butyric, and isovaleric acids. The major cellular fatty acids are iso-$C_{15:0}$ and anteiso-$C_{15:0}$. Organisms are negative for lipase, cystine arylaminidase, α-fucosidase, α-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase, β-xylosidase, esculin, and trypsin; positive for alkaline phosphatase, acid phosphatase, naphthol-AS-Bl-phosphohydrolase, *N*-acetyl-β-glucosaminidase, β-galactosidase, and chymotrypsin; and variable for esterase, esterase lipase, leucine arylamidase; valine arylamidase, and α-glucosidase activities by the API ZYM system. They are negative for α-fucosidase, β-xylosidase, trypsin, and esculin and positive for *N*-acetyl-β-glucosaminidase and ONPG (*o*-nitrophenyl-β-D-galactopyranoside) in the Rosco diagnostic tablets system. β-Lactamase is produced by

21% of the strains. Some strains are resistant to amoxicillin and to clindamycin. They are isolated in mixed culture from various clinical specimens of nonoral origin, mainly from chronic foot infections of diabetics or other patients with vascular insufficiency. The type strain (WAL 6690) has been submitted to CCUG.

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