Amoebae-resisting Bacteria Isolated from Human Nasal Swabs by Amoebal Coculture

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Amoebae feed on bacteria, and few bacteria can resist their microbicidal ability. Amoebal coculture could therefore be used to selectively grow these amoebae-resisting bacteria (ARB), which may be human pathogens. To isolate new ARB, we performed amoebal coculture from 444 nasal samples. We recovered 7 (1.6%) ARB from 444 nasal swabs, including 4 new species provisionally named *Candidatus Roseomonas massiliae*, *C. Rhizobium massiliae*, *C. Chryseobacterium massiliae*, and *C. Amoebinatus massiliae*. The remaining isolates were closely related to *Methylobacterium extorquens*, *Bosea vestrii*, and *Achromobacter xylosoxidans*. Thus, amoebal coculture allows the recovery of new bacterial species from heavily contaminated samples and might be a valuable approach for the recovery of as-yet unrecognized emerging pathogens from clinical specimens.

Free-living amoebae have been isolated worldwide from soil, water, air (1), and from the noses of healthy persons (2,3). Most amoebae feed on bacteria, and few bacteria can resist digestion by the amoebae after being internalized. Amoebal coculture could therefore be used to selectively grow these amoebae-resisting bacteria (ARB) and clean the samples of other more rapidly growing species that generally overwhelm the agar plates. ARB include established agents of pneumonia such as *Legionella* spp. (4) and *Chlamydophila pneumoniae* (5), and potential human pathogens, such as *Parachlamydia* spp. (6,7) and *Legionella*-like amoebal pathogens (LLAPs) (8).

Human exposure to *Parachlamydia* spp. has been especially well demonstrated. Indeed, two of the eight *Parachlamydiaceae* strains isolated to date were found within amoebae recovered from the nasal mucosa (3). However, the prevalence of this potential human pathogen, of other ARB, and of their amoebal host on the nasal mucosa remains to be determined. The fact that *Parachlamydia* is endosymbiotic at 30°C and lytic at 37°C suggests that the bacteria present within amoebae in the cooler upper respiratory tract may be liberated when the amoebae reach the warmer lower respiratory tract (9). This simultaneous liberation of bacteria may overwhelm the human immune response and explain the role of *Parachlamydia* as an agent of community-acquired pneumonia (6) and of aspiration pneumonia (10).

We hypothesized that, for any ARB, nasal colonization may be a first step towards lower respiratory tract infection. Moreover, ARB may use free-living amoebae as a training ground for the selection of virulence traits, as demonstrated for *Cryptococcus neoformans* (11). Thus, virulent capsular strains were phagocytosed by and replicated in *Acanthamoeba castellanii*, leading to amoebal death, while the nonvirulent acapsular strains were killed (11). The fact that free-living amoebae may promote the expression of virulence traits was also well demonstrated for *L. pneumophila* (12,13) and *Mycobacterium avium* (14). A growing body of evidence support the hypothesis that free-living amoebae play a role in the adaptation of *L. pneumophila* to life within other phagolysosomal cells, such as human macrophages (15–18). Moreover, the growth of *M. avium* in amoebae, which results in enhanced entry into both amoebae and macrophages (14), further supports the hypothesis that adaptation to life within human macrophages may be acquired after exposure to environmental predators such as free-living amoebae. Thus, free-living amoebae may be considered as an evolutionary crib for potential emerging human pathogens.

We intended to isolate new ARB from nasal swabs and to compare the prevalence of free-living amoebae and of ARB, such as *Parachlamydia acanthamoebae*, in healthy participants, homeless persons, and hospitalized patients. We tried to recover ARB and free-living amoebae by using amoebal coculture and amoebal enrichment, respectively, with any recovered amoebae subsequently examined for the presence of ARB.

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Methods

Patients and Study Design

Nasal swabs were taken from 100 patients hospitalized in the infectious diseases unit of the Hôpital Nord of Marseille, France, and from 244 homeless persons. In addition, nasal swabs were taken from 100 healthy participants. All participants took part freely in the study and gave their written informed consent. Amoebal coculture was performed for all 444 samples, while amoebal enrichment on nonnutrient agar was restricted to the samples taken from the 100 healthy volunteers and the 100 hospitalized patients.

Broth and Media

Broth and media were slightly modified from the method of T.J. Rowbotham (19). Peptone yeast-extract glucose (PYG): 100 g proteose peptone (Difco, Sparks, MD), 10 g yeast-extract (Merck, Darmstadt, Germany), 4.9 g $MgSO_4.7H_2O$, 5 g sodium citrate.$2H_2O$, 0.1 g $Fe(NH_4)_2(SO_4)_2.6H_2O$, 1.7 g KH_2PO_4, 1.97 g $Na_2HPO_4.7H_20$, 45 g glucose, and 0.295 g $CaCl_2$ in 5 L of distilled water. Page's modified Neff's amoebae saline (PAS): 120 mg NaCl, 4 mg $MgSO_4.7H_2O$, 4 mg $CaCl_2.2H_2O$, 142 mg Na_2HPO_4, and 136 mg KH_2PO_4 in 1 L of distilled water. Nonnutritive agar plates: 1.5 g agar (Research Organics, Cleveland, OH) was diluted in 100 mL of PAS. All media were autoclaved 15 min at 121°C, and broths were also filtered through a 0.22-μm pore membrane (Corning, New York, NY).

Amoebal Microplates

An *Acanthamoeba polyphaga* strain, Linc-AP1, was grown at 28°C in 150-cm² cell culture flasks (Corning) with 30 mL PYG. After 5 days, the amoebae were harvested and centrifuged at 179 x g (1,000 rpm) for 10 minutes. The supernatant was removed, and the amoebal pellet was resuspended in 50 mL of PAS. Centrifugation and resuspension steps were repeated twice. After the last centrifugation, the amoebae were resuspended in PAS, and 1 mL of 5x10⁵ *A. polyphaga*/mL was distributed in each well of a 12-well Costar microplate (Corning).

Nasal Samples

Nasal swabs were vortexed for 30 seconds in 1 mL of PAS in individual sterile tubes. The suspension was centrifuged at 179 x g (1,000 rpm) for 10 minutes. Two hundred and fifty microliters of supernatant was spread onto amoebal microplates. The pellet was spread on nonnutritive agar plates previously flooded with a turbid suspension of viable *Enterobacter aerogenes* in PAS, drained, and incubated at 28°C for a few hours.

Amoebal Coculture

The microplates were centrifuged at 2,879 x g (4,000 rpm) for 30 minutes and incubated at 32°C in a humidified CO_2 atmosphere. The amoebal cocultures were subcultured on fresh amoebae on days 4 and 7 (samples from controls and patients) or on day 6 (samples from homeless persons). Amoebal cocultures were examined regularly for amoebal lysis. When amoebal lysis was observed and at the time of subculture, the cultures were screened for intra-amoebal bacteria. This screening was achieved by gently shaking the microplates to suspend the amoebae. Then 150 μL of the suspension was cytocentrifuged at 150 x g (800 rpm) for 10 minutes, and slides were stained with Gimenez (20). Fuchsin-stained bacteria easily could be seen within the malachite green-stained amoebae. When Gimenez-stained bacteria were observed, the culture was subcultured on BCYE agar (BioMérieux, Marcy L'Etoile, France) and sheep blood agar (BioMérieux) and on amoebal microplates without antibiotics or with vancomycin (10 μg/mL) or colistin (500 U/mL).

Amoebal Enrichment

Nonnutritive agar plates not supplemented with amphotericin B were incubated at 28°C in a humidified atmosphere and examined daily for amoebae-like cells. When positive, these amoebae-like cells were subcultured on nonnutritive agar plates seeded with viable or UV-inactivated *Enterobacter aerogenes*. After several subcultures on inactivated *E. aerogenes*, recovered amoebae were tentatively grown in axenic PYG broth with or without antimicrobials.

Phenotypic Characterization

Microscopic properties of the isolated bacteria were studied by Gram and Gimenez staining. To confirm that the isolated strains were the Gimenez-stained bacteria, 5 x 10⁵ *A. polyphaga*/mL suspensions were injected with the isolated strain and incubated at 32°C. After 4 days' incubation, culture broth was cytocentrifuged and stained with Gimenez and Gram, as described above.

Genotypic and Phylogenetic Characterization

DNA was extracted according to the manufacturer's instructions by using Chelex Resin (BioRad, Hercules, CA) or FastDNA kit (Bio101, Carlsbad, CA) and FastPrep120 grinder (Bio101), depending on the time of strain recovery. Polymerase chain reaction (PCR) amplification of the 16S rDNA gene was performed by using the fD1 and rP2 primers (21) and Taq DNA polymerase (GibcoBRL, Life technologies, Rockville, MD), according to manufacturer instructions. The success of the amplification was determined by electrophoresis in 1% agarose gel of PCR products stained with ethidium bromide. PCR

products were purified by using the QIAquick PCR purification kit (Qiagen, Courtaboeuf, France). Sequencing was performed with the dRhodamine Terminator Cycle sequencing Ready Reaction with one of nine different primers and with AmpliTaq DNA (Perkin-Elmer Biosystems, Warrington, England), according to the manufacturer's instructions. Sequences were determined on a 3100 ABI Prism automated sequencer (Applied Biosystems, Courtaboeuf, France), according to the manufacturer's instructions. Sequences derived from each primer were aligned, compared, and combined in a single 16S rDNA sequence, using the Autoassembler software version 2.1 (Applied Biosystems). The validity of the sequence obtained was assessed by comparison with two additional sequences obtained similarly but from two other PCRs of the same template DNA. The sequences were compared with sequences available in the GenBank database in October 2002 by using the BLAST 2.2.2 program available on the NCBI Web site (www.ncbi.nlm.nih.gov) (22). When the closest sequence was that of an as-yet unpublished bacterial species, the 16S rDNA sequence homology of the prototype strain of the closest published bacterial species was also identified (Table). The 16S rDNA sequences of the isolates were aligned with the sequences of the best blast hits and with those of the closest relative type strains of each isolate. Sequences were edited by removal of the longer 5′ and 3′ ends so that their lengths matched that of the shortest sequence. The 16S rDNA homology of the edited sequences was then analyzed by the distance matrix program of the MEGA software (32). We considered 16S rDNA sequence similarity to be that of the closest sequence in GenBank of more than 99% and 97% for identification at the species and genus level, respectively (33). With these sequences, neighbor-joining (Kimura 2-parameter), minimum evolution (Kimura 2-parameter), and parsimony (standard parsimony) trees were constructed with the MEGA software (32).

Electron Microscopy

For some strains, reinoculation procedure was also performed onto 5×10^5 *A. polyphaga*/mL suspension in 50-cm³ flasks (Corning). Four days' coculture was harvested, washed in monophosphate buffer (pH 7.2), and fixed in 4% glutaraldehyde. Fixed samples were washed overnight in monophosphate buffer (pH 7.2), and then fixed for 1 hour at 4°C with 1% osmium tetroxide. Dehydration was performed by successive washes in increasing acetone concentrations (25%–100%). After incubating the preparations for 1 hour in a vol/vol suspension of acetone/epon and then overnight in epon, they were embedded in Araldite (Fluka, St Quentin Fallavier, France). Thin sections were cut from embedded blocks with an Ultracut microtome (Reichert-Leica, Marseille, France), deposited on copper grids coated with formvar (Sigma-Aldrich, Taufkirchen, Germany), and stained 10 minutes with a solution of methanol-uranyl acetate and lead nitrate with sodium citrate in water. Grids were examined with a Morgagni 268D electron microscope (Philips, Eindhoven, the Netherlands).

Table. Species and family names of 7 Gimenez-positive, gram-negative bacteria isolated from human noses by amoebal coculture[a]

Nb.	Taxonomy of the isolated strain[b]	16S rDNA homology of closest GenBank sequence	16S rDNA homology of the closest published species	Species name	Reference
	Alphaproteobacteria				
	Methylobacteriaceae				
1	*Candidatus Roseomonas massiliae* (AF531769)	99.9% (AF538712)	98.8% (AF533352)	*Roseomonas gilardii*	(23)
2	*Methylobacterium extorquens* (AF531770)	100% (AF293375)	100% (AF293375)	*M. extorquens*	(24–26)
	Bradyrhizobiaceae				
3	*Bosea* sp. strain 7F (AF531764)	99.2% (AF288308)	99.2% (AF288308)	*Bosea vestrii*	(27)
	Rhizobiaceae				
4	*C. Rhizobium massiliae* (AF531767)	100% (AJ389908)	98.7% (Z30542)	*R. larrymoorei*	(28,29)
	Betaproteobacteria				
	Alcaligenaceae				
5	*Achromobacter xylosoxidans* (AF531768)	99.5% (AF394171)	99.5% (AF411021)	*Achromobacter xylosoxidans*	(30)
	Bacteroidetes				
	Flavobacteriaceae				
6	*C. Amoebinatus massiliae* (AF 531765)	99.2% (AB035150)	95.3 % (AJ271009)	*Chryseobacterium scophtalmum*	(31)
7	*C. Chryseobacterium massiliae* (AF531766)	98.0% (AY043370)	96.3 % (AJ271009)	*Chryseobacterium scophtalmum*	(31)

[a]The 16S rDNA homology of the closest GenBank sequence (BLAST analysis) and that of the closest published species with its name are also included. GenBank accession no. are shown in parenthesis. Nb., isolate number.
[b]Order and family are given.

Statistical Analysis

The mean number of strains isolated from ill hospitalized patients was compared to the mean number of strains isolated from the other participants (healthy participants and homeless cohort) by using the student t test (STATA software version 7.0., Stata Corporation, College Station, TX).

Results

Amoebal Enrichment

We observed trophozoites growing on nonnutrient agar injected with 1 of 200 samples but failed in propagating them due to fungal contamination, despite subcultures on nonnutrient agar not supplemented and supplemented with amphotericin B (2 μg/mL). Thus, no free-living amoebae could be successfully recovered. Fungal contamination was frequent, occurring in 61% of cultures from patients and 41% of cultures from control samples. These fungi were not identified.

Amoebal Coculture

By amoebal coculture, we recovered seven (1.6%) strains of ARB from nasal swabs of 4 of 444 participants. Five isolates were recovered from 100 patients hospitalized in the infectious diseases unit of the Hôpital Nord of Marseilles, whereas only two isolates were recovered from the 344 other participants (244 homeless and 100 healthy volunteers; p = 0.017). All ARB were gram-negative bacilli. No amoebae-resisting fungi were detected. On the basis of 16S rDNA sequence analysis, the isolated ARB were assigned to alphaproteobacteria (n = 4), betaproteobacteria (n = 1), and the Bacteroides-Cytophaga-Flexibacter group (n = 2). The Table shows the taxonomy and the 16S rDNA homology with GenBank sequences of the seven isolated ARB. When we used a 16S rDNA sequence similarity of more than 99% and 97% for identification at the species and genus level, respectively (33), four (57%) ARB belonged to new species (isolates 1, 4, 6, and 7), including one that also corresponded to a new genus (isolate 6).

Isolate 1, *Candidatus Roseomonas massiliae*, was recovered from a 44-year-old healthy homeless man. The 16S rDNA sequence of isolate 1 shared on BLAST analysis 99.9% homology with a *Roseomonas* sp. implicated in a catheter-related bacteremia (X.Y. Han, unpub. data) and 98.8% with *R. gilardii* strain ATCC 49956[T] (Table). Genetic analysis confirmed that isolate 1 belonged to the genus *Roseomonas*, as it shares 93.9%–98.9% 16S rDNA sequence homology with *Roseomonas* spp. and 94.3%–84.6% with other *Methylobacteriaceae*, respectively. Isolate 1 clustered with *Roseomonas* spp. by phylogenetic analysis of the 16S rDNA gene (Figure 1). Thus, bootstrap values of 98%, 99%, and 90% in neighbor-join-

ing, minimum evolution, and parsimony analyses, respectively, supported the fork separating *Roseomonas* spp. (including isolate 1) from their closest relatives, *Craurococcus roseus* and *Paracraurococcus ruber*. More importantly, the isolate was clearly different from the type strain neighbor (*R. gilardii*), with bootstrap values of 100% in neighbor-joining, minimum evolution, and parsimony trees. Isolate 1 has been deposited in the Collection de l'Institut Pasteur, Paris, France, as *Candidatus Roseomonas massiliae* strain CIP 107751[T].

Isolate 2 was recovered from a 27-year-old homeless man with chronic cough, headache, and polylymphadenopathy. The 16S rDNA sequence of isolate 2 shared on BLAST analysis 99.3% homology with *Methylobacterium extorquens* strain JCM 2802[T] (Table). Genetic analysis confirmed that isolate 2 is an additional strain of *M. extorquens* as it shared 99.3% 16S rDNA sequence homology with *M. extorquens* strain JCM 2802[T] and 94.6%–99% with the other *Methylobacterium* spp. Moreover, isolate 2 clustered with *Methylobacterium* spp. by phylogenetic analysis of the 16S rDNA gene (Figure 1).

Isolate 3 was recovered from a 48-year-old HIV-positive man with cerebral toxoplasmosis and severe pneumo-
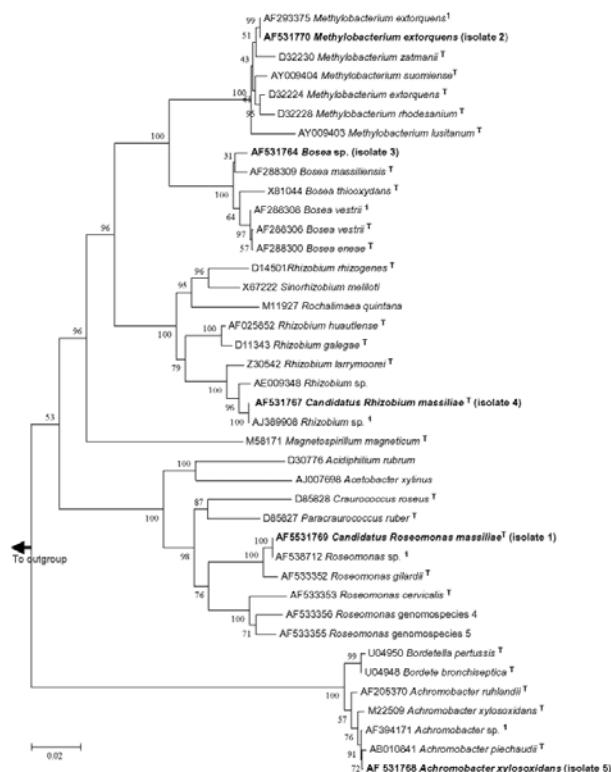

Figure 1. 16S rDNA tree showing relationship of isolates 1 to 5 with related alpha- and beta-proteobacteria. The tree was constructed by using the neighbor-joining method, based on the nearly complete sequence (1,289 nt) of the 16S rDNA gene. Bootstrap values resulting from 100 replications are at branch points. *Staphylococcus aureus* was used as an outgroup.

nia attributed to *Streptococcus pneumoniae*. The 16S rDNA sequence of isolate 3 shared on BLAST analysis 99.2% homology with *Bosea vestrii* strain 34635^T (Table). Genetic analysis confirmed that isolate 3 belonged to the genus *Bosea*, as it shares 98%–99% 16S rDNA sequence homology with *Bosea* spp. Isolate 3 clustered with *Bosea* spp. by phylogenetic analysis of the 16S rDNA gene (Figure 1). However, it clustered with *B. massiliensis*, with bootstrap values separating them (isolate 3 and *B. massiliensis*) from the other *Bosea* spp. of 100%, 56%, and 100% in neighbor-joining, minimum evolution, and parsimony analyses, respectively. The morphology of isolate 3 within *A. polyphaga*, as seen by electron microscopy, is shown in Figure 2.

Isolate 4, *Candidatus Rhizobium massiliae,* was recovered from an 80-year-old woman with pacemaker-associated endocarditis. The 16S rDNA sequence of isolate 4 shared on BLAST analysis 98.7% homology with *Rhizobium larrymoorei* strain 3–10 (Table). Although the isolate shared greater homology with sequences annotated as *Rhizobium* sp., *Agrobacterium* sp., or *A. tumefaciens* (*A. tumefaciens* was renamed *R. radiobacter* [34]), it shared 92.4% sequence homology with *R. radiobacter* strain IFO 13257^T (accession no. D14501). Isolate 4 clustered with two as yet unclassified strains of *Rhizobium* sp. by phylogenetic analysis of the 16S rDNA gene with a node separating them from the closest type strain neighbor, *R. larrymoorei* supported by bootstrap values of 100%, 50%, and 100% in neighbor-joining, minimum evolution and parsimony trees, respectively. Isolate 4 has been deposited in the Collection de l'Institut Pasteur, Paris, France, as *Candidatus Rhizobium massiliae* strain CIP 107749^T.

Isolate 5 (like isolates 4 and 7) was recovered from an 80-year-old woman with a pacemaker-associated endocarditis. The 16S rDNA sequence of isolate 5 shared on BLAST analysis 99.5% homology with *Achromobacter xylosoxidans* strain ATCC 9220^T (Table). Genetic analysis confirmed that isolate 5 belonged to the genus *Achromobacter*, as it shares a 98.4%–99.5% 16S rDNA sequence homology with *Achromobacter* spp. and 97.6%–97.9% with *Bordetella* spp. Isolate 5 clustered with *Achromobacter* spp. by phylogenetic analysis of the 16S rDNA gene (Figure 1). Thus, bootstrap values of 100% in neighbor-joining, minimum evolution, and parsimony trees supported the fork's separating the *Achromobacter* spp. (including isolate 5) from *Bordetella* spp.

Isolate 6, *Candidatus Amoebinatus massiliae,* (like isolate 3) was recovered from a 48-year-old HIV-positive man with cerebral toxoplasmosis and severe pneumonia attributed to *S. pneumoniae*. The 16S rDNA sequence of isolate 6 shared on BLAST analysis 99.2% homology with that of a pathogenic strain belonging to an unpublished new genus and 95.3% with *Chryseobacterium scophtalmum* strain
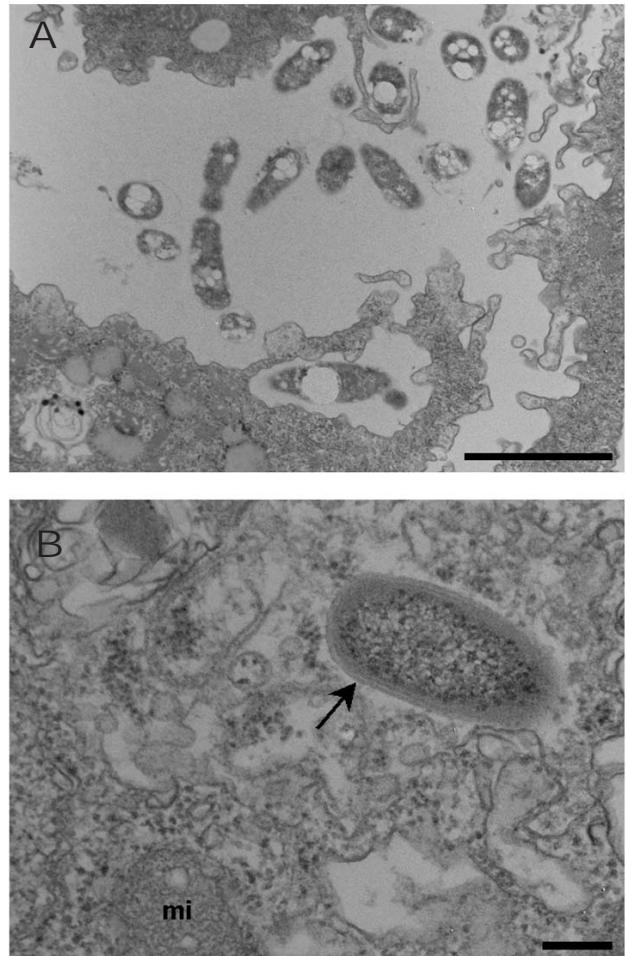

Figure 2A. *Bosea* sp. (isolate 3) in the process of being phagocytized by *Acanthamoeba polyphaga* and in the extracellular media, as seen on electron microscopy. 8,900x magnification. Bar represents 200 nm. B. *Chryseobacterium*-like rod (isolate 7) within *A. polyphaga* (arrows), as seen on electron microscopy. Arrow show the trilammelar membrane. mi = amoebal mitochondria. 36,000 x magnification. Bar represents 200 nm.

LMG 13028^T (Table). On genetic analysis, isolate 6 shared 93.1%–96.0% 16S rDNA sequence homology with *Chryseobacterium* spp. and 81.5%–90.0% with other *Flavobacteriaceae*, respectively. Phylogenetic analysis of the 16S rDNA gene also suggested that isolate 6 belonged to a new genus, related to the *Chryseobacterium* spp. (Figure 3). Thus, bootstrap values of 100%, 100%, and 34% in neighbor-joining, minimum evolution, and parsimony analyses, respectively, supported the fork's separating both *Chryseobacterium*-like bacteria (including isolate 6) from their closest relatives. Isolate 6 has been deposited in the Collection de l'Institut Pasteur, Paris, France, as *Candidatus Amoebinatus massiliae* strain CIP 107750^T.

Isolate 7, *Candidatus Chryseobacterium massiliae,* (like isolates 4 and 5) was recovered from an 80-year-old woman
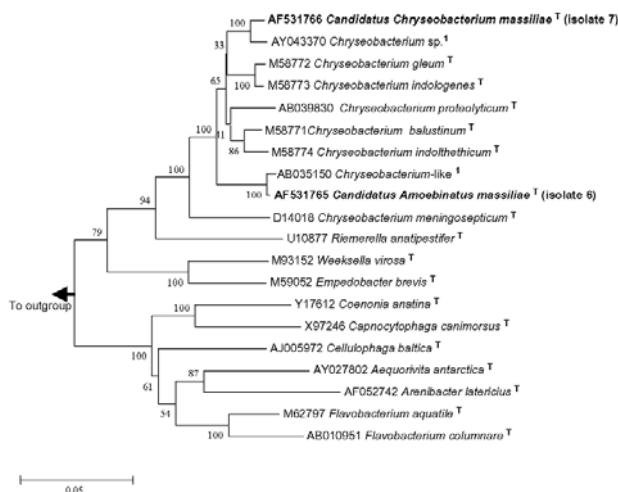
Figure 3. 16S rDNA tree showing relationship of isolates 6 and 7 with related *Flavobacteriaceae*. The tree was constructed by using the neighbor-joining method, based on the nearly complete sequence (1,283 nt) of the 16S rDNA gene. Bootstrap values resulting from 100 replications are at branch points. *Staphylococcus aureus* was used as an outgroup.

with pacemaker-associated endocarditis. The 16S rDNA sequence of isolate 7 shared on BLAST analysis 98.0% homology with that of a strain isolated from the lower respiratory tract of a cystic fibrosis patient (35) and 96.3% with *Chryseobacterium scophtalmum* strain LMG 13028[T] (Table). On genetic analysis, isolate 7 shared 92.5%–97.1% 16S rDNA sequence homology with *Chryseobacterium* spp. and 81.8%–90.3% with other *Flavobacteriaceae*, respectively. Phylogenetic analysis of the 16S rDNA gene (Figure 3) suggested that isolate 7 represented a new species of *Chryseobacterium* (Figure 3). Thus, bootstrap values of 100%, 100%, and 97% in neighbor-joining, minimum evolution, and parsimony analyses, respectively, supported the fork's separating isolate 6 from its closest neighbor, an unpublished species of *Chryseobacterium*. The morphologic features of isolate 7 within *A. polyphaga*, as seen by electron microscopy, is shown in Figure 2. Isolate 7 has been deposited in the Collection de l'Institut Pasteur, Paris, France, as *Candidatus Chryseobacterium massiliae* strain CIP 107752[T].

Discussion

Recovery of *Methylobacteriaceae*, *Rhizobiaceae, Alcaligenaceae*, and *Flavobacteriacae* by amoebal coculture shows that these clades should be added to the growing number of ARB. Our study also shows that humans are exposed to unknown ARB and that the amoebal coculture is an effective tool for the recovery of new species from contaminated samples.

Until this study, the ARB included *Legionella* spp. (4,36), *Chlamydiales* (3,5), *Bradyrhizobiaceae* (37),

Rickettsiales (38), *Listeria monocytogenes* (39), *M. avium* (40), *Procabacter acanthamoeba*, a betaproteobacteria (41), and members of the Bacteroides-Cytophaga-Flexibacter group (*Flavobacterium* spp. and *Amoebophilus asiaticus*) (42). In this study, we recovered additional alphaproteobacteria including a strain of *Methylobacterium extorquans,* a *Bosea* sp., a new *Rhizobium* sp., provisionally named *Candidatus Rhizobium massiliae,* and a new *Roseomonas* sp., named *C. Roseomonas massiliae*. We also recovered a strain of *Achromobacter xylosoxidans* (betaproteobacteria) and two new *Flavobacteriacae* related to the *Chryseobacterium* spp., provisionally named *C. Chryseobacterium massiliae* and *C. Amoebinatus massiliae*. Whether these species use the free-living amoebae as a reservoir and whether they play a role as human pathogens remain to be defined.

ARB in nasal passages of patients and homeless persons are important because they demonstrate the exposure of humans to these bacteria. From the nasal mucosa, ARB may spread to the lower respiratory tract, where they might cause bronchitis and pneumonia, being somewhat adapted to intracellular life. The fact that ARB were more likely to be recovered from patients hospitalized in the infectious disease department than from the other patients sampled is of interest. Further studies will be needed to confirm this statistically significant association and to determine whether patients were colonized with ARB during their hospitalization, whether colonization with ARB may be a marker of exposition to another pathogen involved in the infectious process that lead to hospitalization, or whether the isolated ARB may itself be the etiologic agent of the infectious process that led to hospitalization.

Our study also shows that amoebal coculture is a cell culture system that may be used to recover new bacterial species from heavily contaminated clinical or environmental samples. Indeed, by grazing on bacteria, the free-living amoebae will clean the samples from most rapid growing species that generally overwhelm the agar plates (for example *Enterobacteriaceae, Staphylococci, Streptococci,* and *Neisseriaceae* in a nasal sample). Thus, using that technique, Rowbotham was able to grow *L. pneumophila* from human feces (43). Moreover, the amoebal coculture is a cell culture system that may be performed in the absence of antimicrobials and is thus especially suitable for recovery of new bacterial species of unknown antimicrobial susceptibility. Relative to a broad amplification strategy for microbial rDNA found in nasal washings, amoebal coculture has several advantages. First, it makes a strain available; its phenotype, genotype, antibiotic susceptibility, and virulence may then be further characterized. Second, a broad amplification strategy is unlikely to be successful in obtaining a clear sequence from such heavily contaminated sample. As amoebal resistance may

be associated with virulence (11–16), the amoebal coculture might have the additional advantage of selectively growing potential human pathogens. The main limitations of this technique are the decreased viability of the amoebal cells and their encystment at high incubation temperature, which do not allow the recovery of bacteria requiring a temperature ≥37°C. Another limitation is that, contrary to amoebal enrichment, amoebal coculture did not recover the amoebal host, if any, present in the sample. In addition, this method, like other cell cultures, is associated with a substantial laboratory workload.

We did not isolate *Parachlamydia* spp., which may reflect the scarcity of sources or the low or nonexistent exposure to this potential human pathogen in the area where the survey was performed. This finding may also explain the low prevalence of *Parachlamydia*-associated community-acquired pneumonia that we observed in a recent study (44).

In conclusion, amoebal coculture allows new bacterial species to be isolated from heavily contaminated human samples and might be a valuable approach in identifying potential, as-yet unrecognized human pathogens from non-sterile clinical samples, such as upper and lower respiratory tract specimen.

Acknowledgments

Dr. Greub is a specialist in medical microbiology and infectious disease. He currently works at the Institute of Microbiology of the University of Lausanne, Switzerland. His research focuses on *Parachlamydia acanthamoebae* and other emerging intracellular bacteria.

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