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New *Afipia* and *Bosea* strains isolated from various water sources by amoebal co-culture

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Abstract

It has been suspected that some species belonging to the alphaproteobacteria might cause pneumonia in humans. It is thus of special interest to isolate new members of this phylum, and to further characterize their pathogenicity. The amoebal co-culture method allowed the isolation of various new bacterial species during the last few years, including fastidious alphaproteobacterial species that were isolated from complex environments. In this work, we isolated new bacterial strains from a drinking water network or from river water using amoebal co-culture with *Acanthamoeba castellanii*. One *Afipia* sp. strain and two *Bosea* sp. strains presented 16SrDNA and partial *rpoB* gene sequences suggesting that they could be representative of new species, and were thus further characterized using phenotypic tests.

Keywords: *Acanthamoeba castellanii*; *Afipia*; *Bosea*; Co-culture; Amoeba-resisting bacteria; River water; Drinking water; *Candidatus*

Introduction

Bosea and *Afipia* are alphaproteobacteria genera that both belong to the family *Bradyrhizobiaceae*. Several *Bosea* species have been isolated from the rhizosphere of various plants and have unique metabolic features: oxidation of thiosulfate [7], the ability to use opines as sole carbon source [19], and the ability to degrade *N*-acyl homoserine lactones [6]. Similarly, strains related to *Afipia felis* have been isolated from Antartic soils and were the first described bacteria being able to use both methanesulfonate and dimethylsulfone as sole carbon source [17]. Some *Bosea* strains were also associated with biofilms occurring in water purification processes employing microfiltration and reverse osmosis membranes [5], and they have recently been shown to resist decontamination treatments used for drinking water purification [20]. *Afipia* spp. accounted for as many as 28% of bacterial species grown from a dental unit biofilm [21]. Most of the species belonging to these genera are fastidious micro-organisms that grow better at 30–35 °C than at 37 °C, and that are difficult to isolate on conventional axenic cultivation media. This could partially explain why *Afipia* and *Bosea* spp. are infrequently isolated from environmental and/or clinical samples. *Afipia* and *Bosea* species are resistant to destruction by amoebae of the genus *Acanthamoeba*, being thus considered as amoebae-resisting bacteria (ARB). The amoebal co-culture method has been successfully used to recover new bacterial species from hospital drinking water networks [14,15]. Resistance to destruction by amoebae could be considered as a virulence trait [10] because a bacterial species able to resist the microbicidal effector mechanisms of free-living

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amoebae might also be able to resist destruction by human macrophages. Given the resistance of these species to amoebae, the amoebal co-culture method seems to be appropriate to isolate new, potentially pathogenic species belonging to these genera. In this work, we describe two new *Bosea* strains and one new *Afipia* strain that were all isolated from water sources using an amoebal co-culture method. According to the recommendations of Murray and Schleifer [18], we used the term "*Candidatus*" for the description of these strains, since this is only a preliminary report and additional studies including DNA–DNA re-association experiments will be necessary for complete species description.

Materials and methods

Samples

From May to August 2004, 200 samples were collected from the water network of the University Hospital in Lausanne, Switzerland [23]. A total of 153 tap swabs, 26 water samples, and 21 shower-head swabs were collected. Additionally, two 1 L Seine river water samples were collected at the entry of the Morsang-sur-Seine (France) drinking water plant every 3 months in 2005 [22]. Approx. 1 kg of sand from the sand filtration unit was also collected at the same time points.

Amoebal microplates

Acanthamoeba castellanii strain ATCC 30010 was grown at 28 °C in 75 cm^2 cell culture flasks (Corning) with 30 mL peptone yeast-extract glucose (PYG) [9]. When cells formed an homogenous monolayer, the amoebae were harvested and washed three times in 50 mL of Page's acanthamoeba saline (PAS) [9] (centrifugations at 2000*g*/10 min to pellet the amoebae). After the last centrifugation, the amoebae were resuspended in PAS, and 1 mL of a 5×10^5 *A. castellanii*/mL suspension was distributed in each well of a 24-well Costar microplate (Corning).

Processing of samples

For water samples, 1 L samples were filtered through a 0.2 μm cellulose nitrate membrane and the membrane was re-suspended in 10 mL sterile water. For sand samples, approx. 100 g sand were transferred to sterile bottles to which sterile PBS was added for a final volume of 200 mL and vigorously vortexed for 30 s.

To recover ARB (see amoebal co-culture), 200 μL water or PBS were spread onto amoebal microplates. Once inoculated, microplates were centrifuged at 1500*g* for 30 min and incubated at 32 °C. Amoebal co-cultures (F0) were subcultured on fresh amoebae on day 6 and subcultures (F1) were incubated for 14 days at 32 °C. Amoebal co-cultures were examined daily for amoebal lysis. When amoebal lysis was observed, at the time of subculture, and after 14 days of subculture, 100 μL co-cultures were seeded onto charcoal yeast extract (CYE) agar plates and incubated for 20 days at 32 °C. Seeding on CYE was also systematically performed from F1 subcultures after 14 days of incubation.

Comparative sequence analysis

To identify the recovered bacteria, PCR amplification and sequencing of the 16S rRNA encoding gene was performed directly from agar-grown bacteria resuspended in sterile PBS using primers fD1 and rP2 (Table 1), producing an approximately 1500 bp fragment [24]. The 16S rRNA encoding gene was sequenced for every *Afipia* or *Bosea* species using previously described primers (Table 1) [1]. The discriminative partial sequence of the *rpoB* gene was also amplified with primers Br3200F and Br3950R (Table 1) [11] and all sequences were compared with sequences available in the GenBank database in October 2006, using the BLAST 2.2.2 program available on the NCBI website (www.ncbi.nlm.nih.gov). *RpoB* sequences were aligned with the complete *rpoB* sequence of *Afipia felis* AY242824 in order to select the hypervariable region corresponding to positions 3380–3800 [11]. The homology of the edited sequences was then analyzed by the distance matrix program of the MEGA3 software [12]. According to Khamis et al. [11], we considered that isolates belonged to the same species when hypervariable region of *rpoB* gene sequence similarity was $\geqslant$98%, whereas they likely represent different species when sequence similarity was $\leqslant$96%. With these 16S rRNA and *rpoB* sequences, neighbor-joining (p-distance), minimum evolution (p-distance), and parsimony (standard parsimony) trees were constructed using the MEGA3 software [12].

Phenotypic tests

The phenotypes of the strains were evaluated as follows. Morphological and tinctorial properties were determined by Gram and Gimenez staining. Growth was tested at 32 °C on Columbia agar with 5% sheep blood, chocolate agar and CYE agar. Growth on CYE agar was attempted at 30 and 37 °C. Oxidase activity was detected using a dimethyl-*p*-phenylenediamine oxalate disk (Pasteur Diagnostic). Catalase activity was detected by emulsifying a colony in 3% hydrogen peroxide and by assessing the presence of microscopic bubbles. Other biochemical tests were performed by