Isolation and characterization of *Reyranella massiliensis* gen. nov., sp. nov. from freshwater samples by using an amoeba co-culture procedure

Isabelle Pagnier, Didier Raoult and Bernard La Scola

Correspondence

Bernard La Scola
bernard.lascola@univmed.fr

URMITE, CNRS UMR 6236, Faculté de Médecine de Marseille, 13385 Marseille Cedex 05, France

The analysis of three water samples from two cooling towers and one river allowed us to isolate three strains of a novel species of the class *Alphaproteobacteria* which is phylogenetically related to uncultured alphaproteobacteria. Based upon 16S rRNA gene sequence analysis and phenotypic characterization, we propose to name this novel species *Reyranella massiliensis* gen. nov., sp. nov., type strain 521^T (=CSUR P115^T =DSM 23428^T). The most closely related cultivable micro-organism to this novel bacterium is a member of the genus *Magnetospirillum*.

Although most free-living amoebae feed on bacteria (Rodríguez-Zaragoza, 1994), amoebae can also act as reservoirs for some bacteria and can play a role in their transmission and pathogenicity and in protection against biocides (Greub & Raoult, 2004). As has been previously demonstrated for *Legionella pneumophila* (Gao *et al.*, 1997), some micro-organisms use their amoeba-resisting characteristics to evade macrophage anti-microbial activity, thus increasing their pathogenicity in humans. These bacteria are able to resist digestion by amoebae after internalization, and several groups of micro-organisms use this same property as a survival strategy (Fritsche *et al.*, 1993; Hall & Voelz, 1985; Preer *et al.*, 1974). Several alphaproteobacteria, such as *Afipia* sp., *Bosea* sp., *Bradyrhizobium* sp., Rasbo bacterium, *Methylobacterium* sp. and '*Nordella oligomobilis*' are capable of growing within amoebae (La Scola *et al.*, 2004). Some of these species are known human pathogens and can cause pneumonia (Brenner *et al.*, 1991; La Scola *et al.*, 2000, 2002a, b, 2003). Bacterial co-culturing with amoebae can be an efficient tool for the isolation of several amoeba-resistant intracellular micro-organisms. We isolated three strains of a novel alphaproteobacterium from three independent freshwater samples by using an amoeba co-culture system. Based upon the phenotype and genotype, we determined that our isolates do not belong to a known genus and have therefore assigned them to the genus *Reyranella* gen. nov. as *Reyranella massiliensis* gen. nov., sp. nov. In this report, we describe the characteristics of this novel bacterium.

Strain 521^T was isolated from a river sample (Le Reyran, Frejus, France), and strains 3B26 and S181 were isolated from two different cooling towers. All of the strains were

isolated from an amoeba co-culture with *Acanthamoeba polyphaga* (strain Linc AP-1). Water samples were inoculated onto an *A. polyphaga* monolayer, without antibiotics or with several antibiotics (Pagnier *et al.*, 2008; La Scola *et al.*, 2001). Co-cultures were screened at day three for a cytopathogenic effect, and subcultured onto a fresh amoebal monolayer. After eight days, co-cultures were inoculated on BCYE (Oxoid) and Columbia sheep blood agar (bioMérieux). When a bacterial growth was observed on agar plates, colonies were picked up and subcultured onto BCYE and Columbia sheep blood agar in order to obtain pure culture.

Gram and Gimenez staining were used to morphologically characterize the three isolated strains. Oxidase activity was analysed with a dimethyl-para-phenylenediamine oxalate disc (Sanofi Diagnostic Pasteur), and catalase activity was studied by immersing a colony in an ID-ASE solution (bioMérieux) and checking for the presence of microscopic bubbles. Other biochemical tests were performed by inoculation of API 20 NE strips (bioMérieux) according to the manufacturer's instructions. The strips were incubated at 30 °C for either 24 h or 7 days. Bacterial growth was tested on Columbia agar plates with 5 % sheep blood, BCYE medium and chocolate agar (bioMérieux). The cultures were tested for growth at 4, 18, 22, 30, 32, 35 and 37 °C under O₂, CO₂ (Becton Dickinson) and microaerophilic conditions (bioMérieux). Growth was also tested on Schaedler broth with 0.2 % agar (bioMérieux), Müller–Hinton broth (bioMérieux) and PYG nutrient medium (proteose peptone–yeast extract–glucose; La Scola *et al.*, 2000). Intra-amoebal growth was analysed by Gimenez staining of a bacterial and amoebal co-culture. Bacterial motility was examined by microscopic observation of a strain that was grown in PYG medium at 32 °C. The presence of flagella was assessed by negative staining with a molybdenum suspension that was filtered through a

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 521^T and 3B26 are EF394922 and HM048834, respectively.

0.22 µm membrane, and observation by electronic microscopy. Cell size was assessed by the same method.

The three isolates were identified via complete 16S rRNA gene amplification and sequencing as described previously (La Scola *et al.*, 1998). The sequences were compared to sequences that are available in the GenBank database by using the BLAST program (<http://www.ncbi.nlm.nih.gov/>). The sequences obtained from each of the three strains were aligned and compared to each other. The sequences we deposited in GenBank were EF394922 for strains 521^T and S181 and HM048834 for strain 3B26. The phylogenetic relationship between strain 521^T, strain 3B26 and other closely related bacteria was determined by reconstructing a phylogenetic tree. (Fig. 1). Sequences were aligned using CLUSTAL W software (Thompson *et al.*, 1994). The tree was calculated using MEGA4 software (Tamura *et al.*, 2007), using a neighbour-joining method.

All three of the isolates were able to develop rapidly after a 24 h co-culture within amoebae. Gimenez staining and electron microscopy confirmed that the cultured bacteria were contained within the amoeba cells. A cytopathic effect was detected after 4 days of co-culturing. The infected amoebal suspension was used to inoculate BCYE and Columbia agar with 5 % sheep blood, and bacterial colonies developed after 4 days. All three of the isolated strains were Gram-stain-negative, Gimenez-positive, oxidase-positive and catalase-negative. Growth testing on Schaedler broth with 0.2 % agar under various environmental conditions showed that strains were non-motile and microaerophilic. All of the strains grew slowly for 4 days in Müller–Hinton broth and PYG medium. In

liquid medium, strains 521^T and S181 had a homogeneous suspended growth, whereas strain 3B26 had a tendency to agglutinate. Visible colonies were typically observed on agar plates after 4 or 5 days. All three of the strains grew better on BCYE agar than on Columbia agar with 5 % sheep blood or chocolate agar. When the bacterial growth was tested at various temperatures, strains 521^T and S181 grew at all of the temperatures tested, except 4 °C. Strain 3B26 grew weakly at 18 °C, grew normally from 22 to 35 °C, and did not grow at 4 °C or 37 °C. The colonies were small, grey–white in colour, opaque and had a convex morphology. Colony size appeared to decrease with increasing incubation temperature. According to the results from the API 20 NE strips, strains 521^T and S181 are positive for NO₃ reduction, negative for aesculin hydrolysis, and have weak β-galactosidase activity. Strain 3B26 has no NO₃ reduction activity, weak aesculin hydrolysis activity, and normal β-galactosidase activity. While all of the strains have weak urease activity, none of the strains are positive for indole formation, glucose fermentation, arginine dihydrolase activity or gelatin hydrolysis. No flagella were observed when cells were negatively stained and examined by electron microscopy (Fig. 2). Cell size and other characteristics that differentiate the novel isolates from the genus *Magnetospirillum* are shown in Table 1.

By sequencing the complete 16S rRNA gene of the isolated species, we obtained a total of 1458 base pairs, 1450 base pairs and 1452 base pairs for strains 521^T, S181 and 3B26, respectively. The sequences for the three isolates were aligned, and there was 100 % identity between strains 521^T and S181; both strains shared 99.6 % sequence similarity

Fig. 1. Dendrogram representing the phylogenetic relationships between strains 3B26 and 521^T and other members of the alphaproteobacteria. The tree was derived by comparing 16S rRNA gene sequences. GenBank numbers are given in parentheses. The support of each branch, as determined from 100 bootstrap samples, is indicated by the value (%) at each node. The gammaproteobacterium *Legionella pneumophila* was used as an outgroup. Bar, 0.02 substitutions per site.

Fig. 2. Negative electronic picture showing three cells of strain 521^T including one in a division process within a vacuole of *Acanthamoeba castellanii*. Bar, 500 nm.

with strain 3B26. Based upon the sequence similarity values, the bacterium that is the most similar to strain 521^T is alphaproteobacterium KC-IT-F1, which has 99.71 % similarity. The closest isolated bacterium is *Magnetospirillum* sp. CF19 (GenBank accession number AJ863152), which has 89.01 % similarity. In the GenBank database, three uncultured bacteria are most closely related to our isolates (Fig. 1): uncultured bacterium clone m18 from biofilms grown on membranes transferring oxygen (AY444980), alphaproteobacterium KC-IT-F1 from a carbonate cave in Southern Arizona (FJ711195) and uncultured marine bacterium clone SJC1.37 from environmental marine samples (DQ071109).

Analysis of the bacterial 16S rRNA gene sequences showed that the genus closest to the novel isolates is *Magnetospirillum*, which belongs to the order *Rhodospirillales* and

the class *Alphaproteobacteria*. Almost all members of the order *Rhodospirillales* are microaerophilic, similar to our three isolated strains, and specifically the genus *Magnetospirillum* contains a strain that is a freshwater organism (*Magnetospirillum gryphiswaldense*). Currently, species of the genus *Magnetospirillum* have no known pathogenic activity and are mostly studied for their magnetotactic properties.

Description of *Reyranella* gen. nov.

Reyranella (Rey.ra' nell.a. N.L. fem. dim. n. *Reyranella* from Reyran, the river where the type strain was isolated).

Gram-stain-negative, microaerophilic rods that are positive for oxidase activity and negative for catalase activity. Can be grown on Columbia agar with 5 % sheep blood, BCYE agar, PYG nutrient broth and in an amoebal co-culture with *A. polyphaga*. Grow well between 30 and 35 °C. Colonies are small, grey-white in colour, opaque and have a convex morphology. Major phenotypic data that differentiate this genus from the closest genetically related genus, *Magnetospirillum*, are presented in Table 1. Phylogenetic analysis based on 16S rRNA gene sequence comparison shows that the genus belongs to the class *Alphaproteobacteria*. The type species is *Reyranella massiliensis*.

Description of *Reyranella massiliensis* sp. nov.

Reyranella massiliensis (mas.si.li.en'sis. L. fem. adj. *massiliensis* referring to Massilia, Latin name of Marseille, where the strain was characterized).

Exhibits all of the characteristics of the genus and was the first species isolated.

The strain type is 521^T (=CSUR P115^T =DSM 23428^T) and was isolated from the Reyran river (France, south-east). Strains S181 (=CSUR P116 =DSM 23430) and 3B26 (=CSUR P117 =DSM 23429) are also available.

Table 1. Differential characteristics of the three strains of *Reyranella massiliensis* gen. nov. sp. nov. and the closely related genus *Magnetospirillum*

+, Positive; –, negative; ND, not determined. Data for *Magnetospirillum* from Schüler & Schleifer (2005).

Characteristic	Strain 521 ^T	Strain S181	Strain 3B26	<i>Magnetospirillum</i>
Growth temperature (°C)	18, 22, 30, 32, 35, 37	18, 22, 30, 32, 35, 37	18, 22, 30, 32, 35	30
Catalase	–	–	–	Variable
Oxidase	+	+	+	Variable
NO ₃ reduction	+	+	–	+
β-Galactosidase activity	Weak	Weak	+	ND
β-Glucosidase hydrolysis (aesculin)	–	–	Weak	–
Urease activity	Weak	Weak	Weak	–
Cell length (μm)	1.42 ± 0.26	1.41 ± 0.26	1.59 ± 0.32	3–4
Cell width (μm)	0.76 ± 0.08	0.82 ± 0.09	0.83 ± 0.09	0.2–0.7
Motility (flagella)	–	–	–	+

References

- Brenner, D. J., Hollis, D. G., Moss, C. W., English, C. K., Hall, G. S., Vincent, J., Radošević, J., Birkness, K. A., Bibb, W. F. & other authors (1991). Proposal of *Afipia* gen. nov., with *Afipia felis* sp. nov. (formerly the cat scratch disease bacillus), *Afipia clevelandensis* sp. nov. (formerly the Cleveland Clinic Foundation strain), *Afipia broomeae* sp. nov., and three unnamed genospecies. *J Clin Microbiol* **29**, 2450–2460.
- Fritzsche, T. R., Gautom, R. K., Seyedirashti, S., Bergeron, D. L. & Lindquist, T. D. (1993). Occurrence of bacterial endosymbionts in *Acanthamoeba* spp. isolated from corneal and environmental specimens and contact lenses. *J Clin Microbiol* **31**, 1122–1126.
- Gao, L.-Y., Harb, O. S. & Abu Kwaik, Y. (1997). Utilization of similar mechanisms by *Legionella pneumophila* to parasitize two evolutionarily distant host cells, mammalian macrophages and protozoa. *Infect Immun* **65**, 4738–4746.
- Greub, G. & Raoult, D. (2004). Microorganisms resistant to free-living amoebae. *Clin Microbiol Rev* **17**, 413–433.
- Hall, J. & Voelz, H. (1985). Bacterial endosymbionts of *Acanthamoeba* sp. *J Parasitol* **71**, 89–95.
- La Scola, B., Birtles, R. J., Mallet, M.-N. & Raoult, D. (1998). *Massilia timonae* gen. nov., sp. nov., isolated from blood of an immunocompromised patient with cerebellar lesions. *J Clin Microbiol* **36**, 2847–2852.
- La Scola, B., Barrassi, L. & Raoult, D. (2000). Isolation of new fastidious α Proteobacteria and *Afipia felis* from hospital water supplies by direct plating and amoebal co-culture procedures. *FEMS Microbiol Ecol* **34**, 129–137.
- La Scola, B., Mezi, L., Weiller, P. J. & Raoult, D. (2001). Isolation of *Legionella anisa* using an amoebic coculture procedure. *J Clin Microbiol* **39**, 365–366.
- La Scola, B., Mallet, M.-N., Grimont, P. A. D. & Raoult, D. (2002a). Description of *Afipia birgiae* sp. nov. and *Afipia massiliensis* sp. nov. and recognition of *Afipia felis* genospecies A. *Int J Syst Evol Microbiol* **52**, 1773–1782.
- La Scola, B., Mezi, L., Auffray, J.-P., Berland, Y. & Raoult, D. (2002b). Patients in the intensive care unit are exposed to amoeba-associated pathogens. *Infect Control Hosp Epidemiol* **23**, 462–465.
- La Scola, B., Boyadjiev, I., Greub, G., Khamis, A., Martin, C. & Raoult, D. (2003). Amoeba-resisting bacteria and ventilator-associated pneumonia. *Emerg Infect Dis* **9**, 815–821.
- La Scola, B., Barrassi, L. & Raoult, D. (2004). A novel alpha-Proteobacterium, *Nordella oligomobilis* gen. nov., sp. nov., isolated by using amoebal co-cultures. *Res Microbiol* **155**, 47–51.
- Pagnier, I., Raoult, D. & La Scola, B. (2008). Isolation and identification of amoeba-resisting bacteria from water in human environment by using an *Acanthamoeba polyphaga* co-culture procedure. *Environ Microbiol* **10**, 1135–1144.
- Preer, J.-R., Jr, Preer, L. B. & Jurand, A. (1974). Kappa and other endosymbionts in *Paramecium aurelia*. *Bacteriol Rev* **38**, 113–163.
- Rodríguez-Zaragoza, S. (1994). Ecology of free-living amoebae. *Crit Rev Microbiol* **20**, 225–241.
- Schüler, D. & Schleifer, K. H. (2005). Genus IV, *Magnetospirillum* Scheifer, Schüler, and Ludwig 1992, 291^{VP}. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol 2C, pp. 28–31. Edited by D. J. Brenner, N. R. Krieg, J. T. Staley & G. M. Garrity. New York: Springer.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.