Amoeba co-culture of soil specimens recovered 33 different bacteria, including four new species and *Streptococcus pneumoniae*

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Amoeba-resistant bacteria (ARB), such as *Legionella* spp., are currently regarded as potential human pathogens that live in the natural environment, and thus their habitat is regarded as a reservoir of human pathogens. To detect ARB in human and environmental samples, co-culture with amoebae has been demonstrated to be an efficient tool. However, to date, only water samples from cooling towers and hospital water supplies have been investigated as possible reservoirs of ARB using this procedure. In the present study, we studied the ARB population of 11 diverse soil and sand sources in proximity to human environments; these sources included the university, the station, hospitals, the square, parks and public beaches in the city of Marseilles, France. As a result, a total of 33 different species of ARB were identified. The ability to grow within and/or lyse amoebae was demonstrated, for what is believed to be the first time, for several species; moreover, 20 of the isolates (61 %), including *Streptococcus pneumoniae*, have been described as human pathogens. However, *Legionella* spp. were not isolated. Four isolates are likely to be the members of new or uncharacterized genera or species, and their capability to be human pathogens needs to be determined. This preliminary work demonstrates that soils and sands in the vicinity of humans are reservoirs of human pathogenic ARB.

Received 5 August 2008
Revised 6 October 2008
Accepted 13 October 2008

INTRODUCTION

Free-living amoebae are important predators that control microbial communities. They are ubiquitous, and have been isolated from various natural sources, such as soil, freshwater, salt water, dust and air. These organisms are also frequently isolated from anthropogenic ecosystems, such as tap water, air-conditioning units and cooling towers, where they feed on the microbial biofilm present in the systems. However, several bacteria have developed mechanisms for surviving phagocytosis by free-living amoebae, and are able to exploit the amoebae as hosts (Molmeret *et al.*, 2005).

Some of these bacteria are pathogenic in humans, and amoebae have been suggested as representative environmental reservoirs that act as ‘Trojan horses’ of the microbial world (Barker & Brown, 1994). As a result of this role, amoebae have attracted the attention of clinical microbiologists during recent decades. In general, the current knowledge of the roles played by free-living amoebae, in addition to the role described above, can be presented in the following terms: they can aid in

transmission, selection of virulence traits, and adaptation of the microbes to macrophages (Greub & Raoult, 2004).

Free-living amoebae may be used as a tool for isolation of intracellular micro-organisms from soil microbial communities, where complex ecological interactions exist between bacteria, viruses, plants, nematodes, microarthropods and protozoa (Brown & Barker, 1999). Until recently, the ability to monitor the microbial community structure was limited by the lack of a suitable cultivation strategy to define the composition of species, and the relative abundance of specific populations, in microbial communities (Crawford, 2005). The method of amoebal co-culture has been used to discover many previously unknown micro-organisms (Adekambi *et al.*, 2004; Adeleke *et al.*, 2001; Birtles *et al.*, 2000; Greub & Raoult, 2003; La Scola *et al.*, 2000, 2003, 2004a, b), as well as to observe a new niche for survival of pathogenic micro-organisms in water (Abd *et al.*, 2005; Greub & Raoult, 2002; La Scola & Raoult, 2001; Landers *et al.*, 2000). We use the term amoeba-resistant bacteria (ARB) instead of endosymbionts, since many of the bacteria able to resist destruction by free-living amoebae do not represent true endosymbionts, and it is also the case that endosymbionts

Abbreviation: ARB, amoeba-resistant bacteria.

may be endosymbiotic or lytic in a given amoeba, depending on the environmental conditions.

Here, we present data from what we believe is the first study to use amoeba co-culture to test soil and sand for the presence of ARB. With the objective of identifying new potential human pathogens, we focused our research on soil and sand sources in human environments.

METHODS

Sample collection and preparation. Altogether, 11 samples were studied: eight soil samples and three sand samples, which were all collected in the city of Marseilles, France. Composite 0.5 kg samples were collected from the top layer (0–10 cm) of nine urban soils and three sands (Table 1). All samples were homogenized, and then passed through a 2 mm sieve prior to analysis. Samples were prepared by dilution in sterile distilled water at a ratio of one to five, with the following three-step filtration procedure: first, they were passed through a 40 µm Falcon cell strainer, then through paper filters, with a final filtration through a membrane with 0.22 µm pore size. Each membrane was shaken in 2 ml Page's modified Neff's amoeba saline (PAS). This suspension was used for inoculation of amoebal microplates.

Amoeba suspension inoculation. Sample extracts were co-cultivated with a PAS suspension of *Acanthamoeba polyphaga* (strain Linc-API) at a concentration of 5×10^5 amoebae ml⁻¹ in 12-well Costar microplates (Corning) (La Scola *et al.*, 2000). Each well contained 1 ml amoeba suspension, and was inoculated with 100 µl of each prepared specimen. After inoculation, microplates were centrifuged at 2500 r.p.m. (500 g) for 30 min, and then incubated at 32 °C in a humidified atmosphere.

For each specimen, three different series (each containing a negative control) of co-culture were performed: (i) without antibiotics; (ii) with 10 µl colistin (Aventis Pharma) and 10 µl vancomycin (vancomycin chlorhydrate; Merck) at concentrations of 500 units ml⁻¹ and 10 µg ml⁻¹, respectively; and (iii) with 10 µl erythromycin (Dakota Pharm) at 4 µg ml⁻¹, in addition to the concentrations of colistin and vancomycin described above. Samples without antibiotics were identified by using numbers. The letters CV were added to identify samples containing colistin and vancomycin, and the letter E was added to identify samples containing colistin, vancomycin and erythromycin. The antibiotics were used to help prevent the excessive growth of extracellular micro-organisms, without suppressing the growth of intracellular micro-organisms (La Scola *et al.*, 2000). The addition of erythromycin inhibits the activity of *Legionella* spp.; when these organisms are present in a sample, their lytic activity does not allow other species of micro-organisms to multiply.

Detection of bacterial growth. The amoebal co-cultures were subcultured on day 3 into wells containing fresh amoebae, and then examined daily for the appearance of amoebal lysis, which suggested the presence of an amoeba-resistant micro-organism. Each well was also screened for the presence of intra-amoebal bacteria. On day 8, the co-culture suspension (100 µl co-culture, and 200 µl PAS) was cytocentrifuged, and slides were stained by using Gram, Gimenez and Ziehl-Neelsen stains. At the same time, a 50 µl suspension of co-culture from each well was subcultured onto agar media. We used three different media: (i) buffered charcoal yeast extract (BCYE) agar (bioMérieux) enriched with *Legionellae* supplement (provided by the manufacturer) for the specimens containing antibiotics, (ii) BMPA (BCYE with cefamandole, polymyxin B and anisomycin) agar plates (Oxoid) for the specimens from wells that did not contain antibiotics,

and (iii) Columbia agar plates with 5% sheep blood (trypticase soy agar; bioMérieux) for specimens from wells with and without antibiotics. All agar plates were incubated at a temperature of 32 °C, and they were screened for growing bacteria every 2 days for up to 10 days. Incubation of BCYE agar plates was carried out in a CO₂ atmosphere of 3.5–9.5% (GENbag CO₂; bioMérieux). When growth appeared, each different colony was subcultured onto BCYE, BMPA or Columbia agar, as appropriate, and incubated under the appropriate conditions in order to obtain visible colonies. Slides for Gimenez and Gram staining (which also served as verification that the colonies contained a single strain only), and bacterial suspensions (in sterile water) for molecular identification, were prepared from each colony. The remainder of each specimen, and all the strains isolated, were preserved at –80 °C.

Cytopathogenic testing of isolates. The lytic ability of each isolate towards amoebae was then checked to verify that the agar-grown bacterium was responsible for amoebal lysis. One colony of each strain was suspended in 1 ml PAS, then 50 µl of this suspension was added to 1 ml amoebal suspension in a 12-well Costar microplate. The behaviour of the amoebae was examined every day using an inverted microscope. On days 2 and 6, the suspension (150 µl bacteria–amoeba co-culture and 150 µl PAS) was cytocentrifuged, and slides were treated with Gimenez stain.

Molecular identification of isolates. Bacterial DNA was extracted from five colonies suspended in 300 µl sterile distilled water, using the QIAamp DNA Minikit (Qiagen), according to the manufacturer's recommendations. For all strains, 16S rRNA gene fragments were amplified by PCR using oligonucleotide primers fD1 and rP2, and sequenced using the internal primers 536F and 1050R, under previously described conditions (La Scola *et al.*, 1997). For isolates that were not expected to be isolated from environmental locations, and for which 16S rRNA sequencing was not distinctive enough for species identification, a portion of the coding region of the *rpoB* gene was also sequenced. For *Klebsiella variicola*, we used CM7–CM31b and CM 81b–CM 32b primer pairs (amplicon size 1000 bp) (Mollet *et al.*, 1997); for *Streptococcus pneumoniae*, we used the rpoBpneumoR–rpoBpneumoF primer pair (amplicon size 740 bp) (Drancourt *et al.*, 2004). Sequences were compared with those available in the GenBank database using the BLAST program (<http://www.ncbi.nlm.nih.gov/BLAST/>).

RESULTS

Diversity of ARB

More than 200 strains of ARB were isolated from all the sources studied. After excluding repeat isolates of the same strain on the basis of 16S rRNA gene sequence, and checking of each strain for the occurrence of amoebal lysis, a total of 33 different species of ARB were isolated from soil and sand samples (Table 1). The species belonged to four phyla, seven classes and 14 orders. Soil and sand samples harboured approximately the same numerical compositions of ARB (approx. 4.3 species per sample versus 5 per sample, respectively).

Amoebal lysis of specimens, and cytopathogenic effect of isolates

Among 11 strains inoculated into amoebae, one was characterized by complete amoebal lysis after 3 days, seven

Table 1. List and taxonomic classification of isolated ARB according to 16S rRNA gene sequencing

Species reported to be human pathogens are in bold. Lytic ability is indicated as: C, complete lysis after 24 h; C3, complete lysis after 3 days; C5, complete lysis after 5 days; P, partial lysis after 5 days; G, intra-amoebal growth. Samples are as follows: 1, soil Université de la Méditerranée, Faculté de Médecine; 2, soil Hôpital de la Timone; 3, soil Hôpital de la Conception; 4, soil Jardin Guy Azais; 5 and 6, Parc Borély sand and soil, respectively; 7, sand Plage Borély; 8, sand Plage de la Pointe Rouge; 9, soil Ferme Pédagogique; 10, soil Parc du XXVI Centenaire; 11, soil Gare St Charles. CV, colistin and vancomycin; E, colistin, vancomycin and erythromycin.

Class or order	Genus/species	Percentage 16S rRNA gene similarity with the most similar GenBank sequence (accession no.)	Percentage 16S rRNA gene similarity with the most similar officially described species (accession no.)	Most similar officially described species	Lytic ability	Sample no.
<i>Alphaproteobacteria</i>						
Sphingomonadales	<i>Sphingomonas</i> sp.	99 (DQ337548)	98 (AF331660)	<i>Caulobacter leidy</i>	G	7
Rhizobiales	<i>Agrobacterium tumefaciens</i>	99 (DQ468100)			G	1
	<i>Bradyrhizobium japonicum</i>	97 (AY117677)			P	9E
Caulobacterales	<i>Brevundimonas diminuta</i>	99 (DQ857897)			G	8
<i>Betaproteobacteria</i>						
Burkholderiales	<i>Comamonas aquatica</i>	99 (DQ187385)			G	5
	<i>Delftia</i> sp.	100 (EF061135)	99 (DQ356901)	<i>Delftia tsuruhatensis</i>	G	4
	<i>Delftia acidovorans</i>	99 (DQ530074)			G	4
	<i>Delftia tsuruhatensis</i>	99 (AY302438)			G	2E
<i>Gammaproteobacteria</i>						
Aeromonadales	<i>Aeromonas eucrenophila</i>	99 (AJ458411)			P	6
	<i>Aeromonas salmonicida</i>	99 (AM296510)			G	5
Pseudomonadales	<i>Acinetobacter junii</i>	98-100 (AM184279)			G	4CV, 9CV
	<i>Acinetobacter calcoaceticus</i>	99 (AJ888984)			G	1CV, 5
	<i>Pseudomonas aeruginosa</i>	99-100 (EF051248)			C	5CV, 6E, 9
	<i>Pseudomonas alcaligenes</i>	99 (AF390747)			G	3E, 4CV
	<i>Pseudomonas fulva</i>	99 (AM411071)			C5	2
	<i>Pseudomonas mendocina</i>	100 (DQ641475)			G	6CV
	<i>Pseudomonas plecoglossicida</i>	100 (DQ140383)			G	10, 11
	<i>Pseudomonas putida</i>	99-100 (AF094745)			C3	1, 5, 6CV
Enterobacteriales	<i>Enterobacter cloacae</i>	99 (Y17665)			G	2, 3E
	<i>Klebsiella variicola</i>	99 (AJ783916)			C5	4E
	<i>Serratia plymuthica</i>	100 (DQ365586)			G	10CV
Xanthomonadales	<i>Stenotrophomonas maltophilia</i>	99 (AJ293470)			P	3E, 4E, 11
<i>Actinobacteria</i>						
Actinomycetales	<i>Microbacterium oxydans</i>	100 (AM286269)			C5	7
	<i>Rhodococcus equi</i>	99 (AY741716)			C5	1
	<i>Rothia dentocariosa</i>	99 (AY546095)			G	8CV
Unclassified	<i>Actinobacterium</i> sp.	99 (AF348727)	98 (AY785738)	<i>Microbacterium oxydans</i>	C5	8
Actinobacteria						
Sphingobacteria/Flavobacteria						
Sphingobacteriales	<i>Flexibacter canadensis</i>	99 (AB078046)			P	4E
	<i>Sphingobacterium multivorum</i>	98 (AB100738)			G	10
	<i>Sphingobacterium</i> sp.	99 (DQ530064)	98 (AB100739)	<i>Sphingobacterium multivorum</i>	G	2E, 7E, 8E, 9
Flavobacteriales	<i>Flavobacterium johnsoniae</i>	97 (DQ530073)		<i>Flexibacter aurantiacus</i>	G	11CV
<i>Bacilli</i>						
Bacillales	<i>Bacillus cereus</i>	99 (AF155958)			P	2
	<i>Staphylococcus pasteuri</i>	99 (AJ717376)			G	7
Lactobacillales	<i>Streptococcus pneumoniae</i>	99 (AY525795)			G	8CV

were characterized by complete or partial amoebal lysis after 8 days, and three were characterized by co-existence of amoebae and bacteria. Co-existence was said to have occurred when there was no noticeable temporal change in the number of bacteria or amoeba compared with intra-amoebal growth for which we were able to observe increasing quantities of bacteria.

Among 33 isolated strains, 12 (~36%) provoked amoebal lysis: one after 24 h, one after 3 days, and five after 5 days, while five strains produced partial amoebal lysis (>50% amoebae were lysed) after 5 days. All the 12 lytic bacterial strains were isolated from the nine soil or sand suspensions for which complete or partial lysis was recorded. The other 21 strains of ARB (~64%) demonstrated intra-amoebal growth.

Identification of isolates

The bacteria were mostly Gram-negative. Three isolates were Gram-positive: *Staphylococcus pasteurii*, *Rothia dentocariosa* and *Str. pneumoniae*. The 16S rRNA gene sequences allowed good identification of 29 isolates that showed ≥99% similarity to known species. However, four isolates exhibited ≤98% 16S rRNA gene similarity to known species, even after determination of almost the complete 16S rRNA gene sequence (Table 2, Fig. 1). The identification of *Str. pneumoniae* was confirmed by use of *rpoB* gene partial sequencing (720 bp with a similarity of 99%). Definitive identification of *Klebsiella variicola* and *Klebsiella singaporensis* (not distinguishable from *Klebsiella pneumoniae* using 16S rRNA sequencing) was also performed using *rpoB* partial gene sequencing (Fig. 2). The number of ARB isolates that have been described as human pathogens was approximately equal for soil and sand samples (2.8 species per soil sample versus 2.3 per sand sample). The highest number of pathogens (four species) was isolated from samples 1, 4 and 6, which were collected from near the university, in the square and in the park, respectively.

DISCUSSION

Until now, the majority of bacteria–protozoa interaction models have focused on cooling tower and hospital water supplies. However, as noticed by Snelling *et al.* (2006), 16S and 18S rRNA studies are urgently required to catalogue the population diversity of bacteria and protozoa in different water types, as well as in different types of soils. Soil is believed to be one of the most complex environments for microbial life, as it contains about 10^9 bacterial cells g^{-1} , and an estimated 10^4 distinct species (Crawford, 2005). Culturing the total diversity of species currently seems to be an unachievable goal, given that such large numbers of isolates are not routinely cultured and identified (Janssen, 2006). We believe that ARB, by their capability to survive within phagocytes, are potential human pathogens. This hypothesis is supported by the fact that *Legionella* have similar cytotoxic defects and intracellular replication in mammalian macrophages and protozoa, suggesting a common adaptive mechanism to the intracellular environment. Moreover, strains of *Acanthamoeba* spp. have been found to produce *Legionella*-containing vesicles, which may be agents of transmission of legionellosis. While *Legionella* has been the most studied ARB to date, we have demonstrated that several previously unidentified ARB are also human pathogens (Greub *et al.*, 2004; La Scola *et al.*, 2001, 2003).

The aim of our project was to identify ARB in the natural environment surrounding humans, and thus detect possible emerging human pathogens or possible new reservoirs of known pathogens. In order to do this we tested soil and sand sources, and, from the results described herein, we can confirm that amoebal co-culture is a pertinent tool to achieve this goal. In this work, the ARB isolates belonged to the phyla Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes. These four phyla, together with five others (Acidobacteria, Verrucomicrobia, Chloroflexi, Planctomycetes and Gemmatimonadetes), have been demonstrated to be dominant in bacterial

Table 2. Proposed names and 16S rRNA gene sequence accession numbers of isolates proposed as new species

Phylum	Class	Order	Genus and species	16S rRNA gene similarity (%)	Proposed name	GenBank accession no.
Proteobacteria	Alphaproteobacteria	Rhizobiales	<i>Bradyrhizobium japonicum</i>	97	' <i>Candidatus</i> Bradyrhizobium massiliensis'	EF203409
	Gammaproteobacteria	Pseudomonadales	<i>Acinetobacter junii</i>	98	' <i>Candidatus</i> Acinetobacter timonensis'	EF203410
Bacteroidetes	Flavobacteria		<i>Flavobacterium johnsoniae</i>	97	' <i>Candidatus</i> Flavobacterium massiliensis'	EF203412
	Sphingobacteria		<i>Sphingobacterium multivorum</i>	98	' <i>Candidatus</i> Sphingobacterium massiliensis'	EF203411

Fig. 1. Phylogenetic tree showing the position of all ARB isolated in this study. The tree was constructed using the neighbour-joining method, based on the partial 16S rRNA gene coding sequence. Taxonomic names or proposed names for *Candidatus* species are indicated at the ends of the branches. Reference strains with accession numbers are in bold.

communities in soil. Members of these phyla make up 92 % of soil inhabitants, on average (Janssen, 2006). The majority of isolates in this study had ≥ 99 % 16S rRNA gene similarity with previously characterized species, but four strains exhibited ≤ 98 % 16S rRNA gene similarity with

known species or genera; these strains belonged to the species *Acinetobacter junii*, *Bradyrhizobium japonicum*, *Flavobacterium johnsoniae* and *Sphingobacterium multivorum*. These four isolates are likely to be new species or genera (Stackebrandt & Goebel, 1994), and polyphasic

Fig. 2. Phylogenetic tree showing the position of our *Klebsiella* isolate among other *Klebsiella* spp. and members of the related genus *Raoultella*; the bootstraps are marked. The tree was constructed using the neighbour-joining method, based on the partial *rpoB* gene coding sequence.

characterization of other isolates will have to be performed for definitive descriptions of these strains. In contrast to analysis of soil libraries of 16S rRNA genes, culture methods open the way for characterization of such isolates.

In our survey, *Pseudomonas aeruginosa* was able to lyse amoebae more quickly than other isolates (Table 1). The ability of *P. aeruginosa* to resist amoeba predation has been described by others (Wang & Ahearn, 1997), and it has been demonstrated recently that this lysis is related to necrosis and apoptosis, and mediated by the effect of type III secretion system effector proteins (Abd *et al.*, 2008). The pathogenicity of *P. aeruginosa* in humans has been demonstrated to be associated with resistance to its lytic effect on amoebae (Fenner *et al.*, 2006). *Pseudomonas putida* also provoked complete amoebal lysis in our study, and this is thought to be the first report of such activity. *P. putida* is rarely pathogenic for humans, but it has been found associated with empyema, urinary tract infections, septicæmia and various other episodes.

Pseudomonas fulva, *Microbacterium oxydans*, *Rhodococcus equi*, *Actinobacterium* sp. and *K. variicola* (or *K. singaporensis*) induced complete, but slower, amoebal lysis. Members of the *Rhodococcus* genus, especially *R. equi*, are known to be pathogens in immunocompromised patients. *K. variicola* is a recently described species that seems to be genetically isolated from *K. pneumoniae* strains (Rosenblueth *et al.*, 2004). Here, we report what we believe is the first culture of *K. variicola* from soil, and the discovery of its lytic ability towards amoebae. It is known that strains of *K. variicola* are abundant in plants, and that they represent less than 10% of the clinical *Klebsiella* isolates that have, in the past, been considered to be *K. pneumoniae*. *K. variicola* seems to be better adapted to the inanimate environment than *K. pneumoniae*, and strains of *K. variicola* from plants can occasionally infect humans (Martinez *et al.*, 2004). Our strain showed equal similarity to *K. variicola* and to *K. singaporensis* (Li *et al.*, 2004).

Aeromonas eucrenophila, *Bacillus cereus*, *Flexibacter canadensis*, *Stenotrophomonas maltophilia*, and the isolate with 97% similarity to *Bradyrhizobium japonicum*, caused partial amoebal lysis. We believe that this is the first report

of the lytic ability of these bacteria towards amoebae. However, many *Bradyrhizobium* spp. have been shown to be ARB in our previous work (Pagnier *et al.*, 2008). Three of the other isolates in the present study could be regarded as potential pathogens. The pathogenicity of *B. cereus* for humans is well-known. *Ste. maltophilia* (previously known as *Pseudomonas maltophilia* and *Xanthomonas maltophilia*) has been associated with various (mostly nosocomial) infections, including bacteraemia, meningitis, wound infections, urinary tract infections and pneumonia. In 2000, there was the first report of the isolation of *A. eucrenophila* from a human source (Albert *et al.*, 2000). Survival of *Aeromonas salmonicida* has been observed in protozoa of the species *Tetrahymena pyriformis* (King & Shotts, 1988), and recently it has been demonstrated that free-living amoeba might play a role as a reservoir of *Aeromonas* spp. (Rahman *et al.*, 2008).

It is remarkable that we did not isolate *Legionella* spp. The species of this genus have been mostly isolated from water, but some, especially *Legionella longbeachae*, have also been obtained from soil samples (Koide *et al.*, 2001; Steele *et al.*, 1990). We do not think that this absence is due to a bias introduced by the technique used, as we have isolated many *Legionella* spp. from hospital water supplies using our technique. Also, in our soil and sand samples, we did not find ARB, such as *Afipia* or *Bosea* spp., that are known to be common in hospital water supplies.

The isolation of *Str. pneumoniae* from sand samples (Plage de la Pointe Rouge) was a surprising result. In order to verify the result, we repeated the culture using the remainder of the sample that had been frozen, and the same *Str. pneumoniae* strain was isolated. This confirmed that isolation of the strain was not a result of contamination at the time of inoculation; however, contamination occurring at the time of sampling, or during preparation of sample for inoculation, cannot be excluded. Until recently, *Str. pneumoniae* was not considered to be an environmental species; however, its isolation from environmental sources has been recently reported (Harakeh *et al.*, 2006). Nevertheless, the results reported by Harakeh *et al.* (2006) are highly questionable, not only because that was the first study to report that *Str. pneumoniae* was very

common in water, but also because all isolates tested were resistant to vancomycin; the latter feature has not been reported for human isolates (Daoud *et al.*, 2006; Haas *et al.*, 2005), except by Harakeh *et al.* (2006). Our isolate was susceptible to vancomycin. It was isolated in a well containing colistin and vancomycin, and this confirms that the strain grew as an intracellular bacterium within amoebae, as it would have been killed by vancomycin if it had been growing extracellularly.

To summarize, this preliminary work reports that several known human opportunistic pathogens act as ARBs when isolated from soil and sand, and that the soil and sand in the vicinity of humans contain some ARB that are believed to be new species. A limitation of the present work is that we used a single species of amoeba. For example, in the case of members of the genus *Legionella*, not all species are able to grow in the protozoa that support the growth of the species *Legionella pneumophila*. It is likely that use of protozoa other than the species *Acanthamoeba polyphaga* would lead to a slightly different panel of isolates. In future, it may be interesting to study soils of other origins, including samples from regions that are more humid than our city (Marseilles has a dry Mediterranean climate), to investigate the ARB populations of soil in different climatic conditions. Use of different protozoan hosts to perform co-culture would also be of interest to give a more accurate evaluation of the panel of ARBs in soil.

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Edited by: G. Muyzer