Applied and Environmental Microbiology, Aug. 2011, p. 5697–5706 Vol. 77, No. 16
0099-2240/11/$12.00 doi:10.1128/AEM.00579-11

Diversity and Antibiotic Resistance Patterns of *Sphingomonadaceae* Isolates from Drinking Water[▽]

Ivone Vaz-Moreira,[1,2] Olga C. Nunes,[2] and Célia M. Manaia[1]*

CBQF/Escola Superior de Biotecnologia, Universidade Católica Portuguesa, 4200-072 Porto,[1] and LEPAE—Departamento de Engenharia Química, Faculdade de Engenharia, Universidade do Porto, 4200-465 Porto,[2] Portugal

Received 14 March 2011/Accepted 14 June 2011

Sphingomonadaceae **($n = 86$) were isolated from a drinking water treatment plant ($n = 6$), tap water ($n = 55$), cup fillers for dental chairs ($n = 21$), and a water demineralization filter ($n = 4$). The bacterial isolates were identified based on analysis of the 16S rRNA gene sequence, and intraspecies variation was assessed on the basis of *atpD* gene sequence analysis. The isolates were identified as members of the genera *Sphingomonas* ($n = 27$), *Sphingobium* ($n = 28$), *Novosphingobium* ($n = 12$), *Sphingopyxis* ($n = 7$), and *Blastomonas* ($n = 12$). The patterns of susceptibility to five classes of antibiotics were analyzed and compared for the different sites of isolation and taxonomic groups. Colistin resistance was observed to be intrinsic (92%). The highest antibiotic resistance prevalence values were observed in members of the genera *Sphingomonas* and *Sphingobium* and for beta-lactams, ciprofloxacin, and cotrimoxazole. In tap water and in water from dental chairs, antibiotic resistance was more prevalent than in the other samples, mainly due to the predominance of isolates of the genera *Sphingomonas* and *Sphingobium*. These two genera presented distinct patterns of association with antibiotic resistance, suggesting different paths of resistance development. Antibiotic resistance patterns were often related to the species rather than to the site or strain, suggesting the importance of vertical resistance transmission in these bacteria. This is the first study demonstrating that members of the family *Sphingomonadaceae* are potential reservoirs of antibiotic resistance in drinking water.**

At this writing, the family *Sphingomonadaceae*, within the class *Alphaproteobacteria*, comprises 11 genera: *Blastomonas*, *Erythromonas*, *Novosphingobium*, *Sandaracinobacter*, *Sandarakinorhabdus*, *Sphingobium*, *Sphingomonas*, *Sphingopyxis*, *Sphingosinicella*, *Stakelama*, and *Zymomonas* (http://www.bacterio.cict.fr; last full update, 24 November 2010) (12). Members of this family are strictly aerobic chemoheterotrophs with a characteristic yellow pigmentation (43). In spite of their frequently observed oligotrophic character, members of this family are widespread in nature, occurring in soils, corals, eutrophic waters, plant surfaces, and clinical samples (1, 5, 41, 43). The capacity of sphingomonads (a common designation that includes the genera *Novosphingobium*, *Sphingobium*, *Sphingomonas*, and *Sphingopyxis*) to cope with man-made environments is also relevant. For instance, the ability to degrade xenobiotic compounds is one of the most remarkable properties of these bacteria (1, 37). The capacity to survive in chlorinated waters, allegedly due to the oligotrophic character of these bacteria and their production of biofilms (14, 19, 25, 44), is another demonstration of their plasticity in man-made environments. Sphingomonads are, thus, truly ubiquitous bacteria frequently found in aquatic environments, such as drinking water (bulk water and biofilms formed on pipes, reservoirs, and bathtubs), distilled water, hemodialysis fluids, or supposedly sterile drug solutions (14, 17, 24, 25, 31, 34, 38). The fact that sphingomonads are recognized opportunistic pathogens (6, 23, 24, 28) makes such ubiquity potentially hazardous, mainly in habitats such as tap water. This hazardous potential is exacerbated by the fact that sphingomonads are among the most relevant unappreciated reservoirs of the natural resistome (9). In spite of this, the diversity of *Sphingomonadaceae* in oligotrophic waters and the respective antibiotic resistance patterns are poorly characterized (14, 25). This study aimed at helping to fill this gap, characterizing the diversity and antibiotic resistance patterns of *Sphingomonadaceae* isolated from drinking water. Bacteria isolated from a water treatment plant (WTP), taps, cup fillers for dental chairs, and a water demineralization filter of the same drinking water network were analyzed. The study was intended to (i) assess the diversity of genera and species in the different sites, (ii) determine whether some genera or species were particularly relevant as antibiotic resistance reservoirs in waters, and (iii) compare the patterns of antibiotic resistance in different genera and sites of isolation, inferring possible modes of resistance dissemination.

MATERIALS AND METHODS

Sampling. The bacterial isolates examined in this study were collected from (i) a water treatment plant (both raw and treated water samples), (ii) tap water, (iii) cup fillers for dental chairs, and (iv) biofilm from a water demineralization filter installed in a research laboratory. WTP samples, both raw (groundwater and surface water) and treated (after sand filtration, ozonation, flocculation, activated-carbon treatment, and chlorination), were collected in two sampling periods, in November 2007 and September 2009 (sampling periods A and B, respectively) (see the legend to Fig. 2). These samples were collected at the sampling points used in routine monitoring analysis in the WTP. Tap water samples were collected in three sampling periods (April, July, and October 2009 [sampling periods A, B, and C, respectively]) (see the legend to Fig. 2), from 11 household taps used 1 to 4 times a month and from a tap of a health care unit (T1 to T12 in Fig. 2). Forty-five-liter volumes of water were collected from the WTP or taps, using sterile 5-liter containers (these samples were also processed for other analyses; hence the large volume of water sampled). In the laboratory, a composite sample was prepared by mixing equal volumes of water from each

* Corresponding author. Mailing address: Escola Superior de Biotecnologia, Universidade Católica Portuguesa, 4200-072 Porto, Portugal. Phone: 351 22 5580059. Fax: 351 22 5090351. E-mail: cmmanaia@esb.ucp.pt.

▽ Published ahead of print on 24 June 2011.

container. Samples from the cup fillers of nine dental chairs were collected between February and April 2008 (sampling periods A, B, and C, respectively) (see the legend to Fig. 2) in a university dental school clinic where the chairs are in use for more than 10 years (33). Water was allowed to run for about 1 min before its collection into a 100-ml sterile flask. The biofilm was collected in October 2008 (sampling period A in Fig. 2) from a pleated filter (responsible for the removal of suspended solids) of a laboratory water demineralization system fed with tap water during approximately 4 months of frequent use at a maximal flow rate of 90 liters h^{-1}. The biofilm was collected with a sterile swab and was suspended in 20 ml of sterile saline solution (0.85% [wt/vol] NaCl), and cells were homogenized by vigorous shaking and 15 min of sonication in a water bath. All water and biofilm samples were processed within 4 h after collection. In order to neutralize the activity of disinfectants, 0.1 mg $liter^{-1}$ of sodium thiosulfate was added to the samples of treated water collected in the WTP and from the taps. All the taps, all the dental chairs, and the lab demineralization system were supplied by the same WTP examined in this study.

Bacterial isolation and characterization. Three culture media widely used for microbiological quality control of water were employed: R2A (Difco), *Pseudomonas* isolation agar (PIA; Difco), and Tergitol-7 agar (TTC; Oxoid). R2A agar is a nonselective medium recommended for the examination of total heterotrophic bacteria; PIA and TTC are recommended for the enumeration of *Pseudomonas* bacteria and presumptive coliforms (11, 21), respectively. One hundred-milliliter volumes of water or of decimal serial dilutions thereof were filtered through cellulose nitrate membranes (pore size, 0.45 μm; diameter, 47 mm; Albet), which were placed on the three different culture media and were incubated at 30°C (for R2A and PIA) or 37°C (for TTC) for as long as 7 days. Decimal serial dilutions and filtrations were carried out in triplicate, and after the incubation period, the number and morphology of CFU on filtering membranes with as many as 80 colonies were registered. About 50% of the colonies with a morphotype represented by >10 CFU, and all the colonies with a morphotype represented by ≤10 CFU, were isolated. The colonies isolated on R2A were purified on the same medium, and those isolated on culture media with higher nutrient contents (PIA or TTC) were purified on plate count agar (PCA; Pronadisa). Pure cultures were preserved at −80°C in nutritive broth supplemented with 15% (vol/vol) glycerol. Colony and cellular morphologies, Gram stain reactions, and cytochrome *c* oxidase test results were characterized as described by Smibert and Krieg (35). After this preliminary characterization, 30 to 40% of the cultures comprising Gram-negative rods forming yellow colonies on R2A or PCA, isolated from each type of habitat (WTP, taps, cup fillers, or biofilm) in different sampling periods and on different culture media, were selected for further studies.

Bacterial identification and typing. Bacterial isolates were identified to the species level on the basis of analysis of the 16S rRNA gene sequence. The 16S rRNA gene was amplified and sequenced with primers 27F and 1492R (26) according to the method of Vaz-Moreira et al. (40). The nucleotide sequences were used to query the EzTaxon library (7).

In an attempt to assess intraspecies variability, the nucleotide sequence of the housekeeping gene *atpD* (the beta subunit of membrane ATP synthase) was analyzed (16). *atpD* and 16S rRNA gene sequences were aligned using ClustalW from MEGA software, version 4.0 (39). Nucleotide sequence relatedness was estimated based on the model of Jukes and Cantor (22), and dendrograms were created using the neighbor-joining method. Other methods, namely, maximum parsimony and maximum likelihood, were used to assess the tree stability, and the branches recovered by these three methods are indicated in Fig. 3 (black circles). The type species of the closest neighbors were added to the dendrogram in order to allow the identification of the isolates under study. Nonhomologous and ambiguous nucleotide positions were excluded from the calculations, and bootstrap values, generated from 1,000 resamplings, at or above 50% are indicated at the branch points. A total of 1,229 and 350 nucleotide positions were included in the analysis of the 16S rRNA and *atpD* sequences, respectively. 16S rRNA and *atpD* gene sequences were compared for each pair of isolates belonging to the same species. Strains differing in at least one nucleotide position in any of those gene sequences were classified as belonging to a distinct sequence type (ST).

Antibiotic resistance phenotype. The 86 isolates identified as members of the family *Sphingomonadaceae*—6 from the WTP (3 from raw and 3 from treated water), 4 from the biofilm, 55 from taps, and 21 from the cup fillers—were studied further for their antibiotic resistance phenotypes, determined by using the ATB PSE5 panel (bioMérieux) according to the manufacturer's instructions. Five classes of antibiotics were tested: beta-lactams, aminoglycosides, a fluoroquinolone, a polymyxin, and a sulfonamide. The beta-lactams tested were ampicillin-sulbactam (FAM) (tested at 8 and 4 mg $liter^{-1}$ and 16 and 8 mg $liter^{-1}$, respectively), ticarcillin (TIC) (16 mg $liter^{-1}$), ticarcillin-pyocyanin (TICP) (64 mg $liter^{-1}$), ticarcillin-clavulanic acid (TCC) (16 and 2 mg $liter^{-1}$, respectively), ticarcillin-clavulanic acid-pyocyanin (TCCP) (64 and 2 mg $liter^{-1}$, respectively), piperacillin (PIC) (16 mg $liter^{-1}$), piperacillin-pyocyanin (PICP) (64 mg $liter^{-1}$), piperacillin plus tazobactam (TZP) (16 and 4 mg $liter^{-1}$, respectively), piperacillin plus tazobactam-pyocyanin (TZPP) (64 and 4 mg $liter^{-1}$, respectively), imipenem (IMI) (4 and 8 mg $liter^{-1}$), meropenem (MEM) (4 and 8 mg $liter^{-1}$), ceftazidime (CAZ) (8 and 16 mg $liter^{-1}$), and cefepime (FEP) (8 and 16 mg $liter^{-1}$). The aminoglycosides tested were amikacin (AKN) (16 and 32 mg $liter^{-1}$), gentamicin (GEN) (4 and 8 mg $liter^{-1}$), and tobramycin (TOB) (4 and 8 mg $liter^{-1}$). Also tested were the fluoroquinolone ciprofloxacin (CIP) (1 and 2 mg $liter^{-1}$), the polymyxin colistin (COL) (2 mg $liter^{-1}$), and the sulfonamide cotrimoxazole (TSU) (2 and 38 mg $liter^{-1}$). Phenotypes were defined as resistant, intermediary, or sensitive according to the manufacturer's instructions.

Statistical analyses. The chi-square test was used to compare the antibiotic resistance prevalences among groups of isolates from the different types of water, or to compare resistant and susceptible bacteria, at a significance level (P) of <0.05. A matrix with nominal variables was constructed based on the resistant, intermediary, and sensitive phenotypes, defined according to the instructions of the manufacturer of the ATB PSE5 panel. In order to establish groups of isolates with similar profiles of resistance to the different antibiotics tested, hierarchical ascendant cluster analysis was carried out using Euclidean distance and the Ward method for the aggregation criterion. Other methods, namely, between-groups linkage, nearest group, and farthest group, were applied to assess the stability of the dendrogram. To define the groups of resistance profiles that were significantly different, the R^2 criterion was used, at a significance level (P) of <0.05 (see Fig. 3). The SPSS software package, version 18.0 (SPSS Inc., Chicago, IL), was used for these analyses.

Nucleotide sequence accession numbers. The *atpD* and 16S rRNA gene sequences have been submitted to under accession numbers JF459878 to JF459930 and JF459931 to JF459988, respectively.

RESULTS

Abundance and diversity of *Sphingomonadaceae*. The cultivable *Sphingomonadaceae* were present at a density of 10^0 CFU ml^{-1} in the raw WTP water and at 10^1 CFU ml^{-1} after treatment. In the taps and cup fillers, the densities of *Sphingomonadaceae* were slightly higher, ranging from 10^1 to 10^3 CFU ml^{-1}. The higher counts of *Sphingomonadaceae* observed in the treated water from the WTP may be due to decreases in the levels of other bacteria, which were eliminated more extensively by the disinfection process. The *Sphingomonadaceae* isolates analyzed in this study were identified as members of the genera *Sphingomonas*, *Sphingobium*, *Novosphingobium*, *Sphingopyxis*, and *Blastomonas* (Fig. 1). Most of the isolates ($n = 82$) presented 16S rRNA gene sequence similarity values higher than 97.4% with the type strain of a validly named species and were thus considered members of that species. Four strains, three isolated from the health care unit tap water and one from the WTP (raw groundwater), could not be affiliated with any validly named species. In these cases, 16S rRNA gene sequence similarity values lower than 97.0%, the threshold value recommended for species definition (36), were observed. The closest neighbors were the type strains of the species *Sphingomonas sanxanigenens* (96.3 and 96.9% similarities), *Sphingomonas changbaiensis* (96.8%), and *Blastomonas natatoria* (96.3%).

Apparently, the culture media and temperature did not influence the diversity of genera recovered (Fig. 1A). The only exception was that *Sphingopyxis* spp. and *Blastomonas* spp. were isolated only on R2A and PIA, which were incubated at 30°C, but not on TTC, which was incubated at 37°C.

The distributions of genera were not identical for the different sites of isolation (Fig. 1B). In this respect it is noteworthy

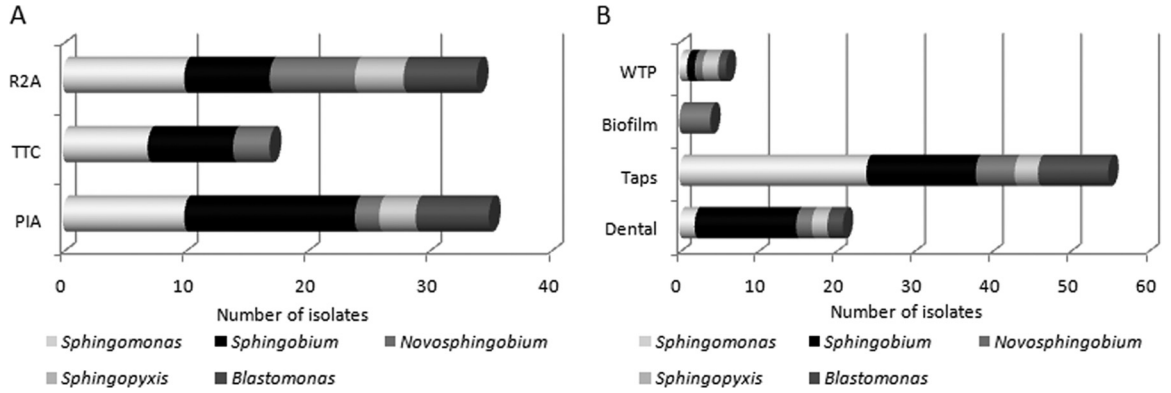

FIG. 1. Summary of the numbers of isolates examined in this study according to isolation conditions (A) and site (B).

that all five genera identified in this study were represented in the group of six isolates from the WTP. Three isolates, identified as *Sphingomonas melonis*, *Novosphingobium subterraneum*, and *Sphingopyxis ginsengisoli*, were recovered from WTP treated water in 2007. Bacteria isolated in 2009 from raw groundwater and surface water from the WTP were identified as *Sphingopyxis taejonensis*, *Blastomonas natatoria*, and *Sphingobium yanoikuyae*, respectively. Tap and cup filler water samples also contained isolates belonging to the different genera, although *Sphingomonas* spp. and *Sphingobium* spp., respectively, were prevalent in these samples (Fig. 1B). The biofilm of the pleated filter was observed to contain only members of the species *Novosphingobium subterraneum.* Considering the whole set of isolates, it was observed that members of the genera *Sphingomonas* and *Sphingobium* were prevalent, constituting 64% of the total (31.4 and 32.6%, respectively).

The diversity of *Sphingomonadaceae* species observed in the water samples examined in this study represented only a small part of the validly named species within the different genera (http://www.bacterio.cict.fr) (12): only 10 of the 44 in the genus *Sphingomonas* (*Sphingomonas panni*, *Sphingomonas yunnanensis*, *Sphingomonas dokdonensis*, *Sphingomonas mucosissima*, *S. sanxanigenens*, *Sphingomonas wittichii*, *S. changbaiensis*, *Sphingomonas koreensis*, *S. melonis*, and *Sphingomonas pituitosa*), 4 of the 24 in the genus *Sphingobium* (*Sphingobium amiense*, *Sb. yanoikuyae*, *Sphingobium xenophagum*, and *Sphingobium rhizovicinum*), 4 of the 15 in the genus *Sphingopyxis* (*Sp. taejonensis*, *Sphingopyxis witflariensis*, *Sp. ginsengisoli*, and *Sphingopyxis chilensis*), 3 of the 18 in the genus *Novosphingobium* (*N. subterraneum*, *Novosphingobium aromaticivorans*, and *Novosphingobium panipatense*), and 1 of the 2 in the genus *Blastomonas* (*B. natatoria*) were identified in this study (Fig. 2).

In an attempt to assess intraspecies variability, the nucleotide sequence of the *atpD* gene was analyzed, as recommended by Gaunt et al. (16). This procedure allowed the differentiation of the 86 isolates into 56 sequence types (Fig. 2). Of the total, 42 isolates could be distinguished at the strain level. The other 44 isolates shared the same sequence type with at least one other isolate and were divided into 14 STs (with 2 to 6 isolates each) (boldface in Fig. 2). These 44 isolates were recovered from the biofilm, cup fillers, and taps and belonged to the genera *Novosphingobium* (all three species), *Sphingobium* (*Sb. amiense*, *Sb. yanoikuyae*, *Sb. xenophagum*), *Sphingomonas* (*S. yunnanensis*, *S. mucosissima*, *S. panni*), *Sphingopyxis* (*Sp. witflariensis*), and *Blastomonas* (*B. natatoria*). Five of the 14 STs comprising more than one isolate (ST8, ST9, ST24, ST25, and ST26) included 12 bacteria from the same site, sampling time, and culture medium, suggesting that they were repetitions (i.e., the same isolate recovered more than once). In other cases, the isolates ($n = 5$) yielding the same ST were recovered on different culture media, though from the same site and sampling date (ST1 isolates on R2A and TTC; ST46 isolates on TTC and PIA), suggesting that they were possibly clones. The other seven STs common to more than one isolate grouped 27 bacteria isolated from different sites, on different sampling dates, and/or on different culture media (ST7, ST37, and ST53), or isolated from the same site but on different sampling dates and/or on different culture media (ST21, ST23, and ST33). ST43 clustered bacteria of different origins, isolated on different dates and on different culture media. The presence of the same ST at different sites on different dates may suggest a common origin of those bacteria but most probably results from the poor resolution of the method used, since only two gene sequences were examined. Nevertheless, in general, it is possible to conclude that the alleles examined in this study allowed good differentiation of the isolates and showed that the same strain is rarely, if ever, observed in different taps, in different cup fillers, or in samples from the WTP. The impressive diversity of species was also observed at the strain level, evidencing the widespread distribution of members of the family *Sphingomonadaceae* in waters.

Antibiotic resistance phenotypes. Antibiotic resistance patterns were analyzed as a function of the site and conditions of isolation or bacterial genus (Table 1). The susceptibility phenotype was determined with respect to 19 antibiotics belonging to five different classes. None of the isolates presented resistance to the aminoglycoside AKN. In contrast, colistin resistance was observed in 91.9% of the isolates, suggesting that it is an intrinsic phenotype in *Sphingomonadaceae.* On average, simultaneous resistance to antibiotics belonging to three different classes (excluding colistin) was observed in 10.5% of the isolates. Most of the multiresistance phenotypes included resistance to at least one of the beta-lactams tested. Actually, resistance to beta-lactams, mainly TIC, PIC, PICP, and

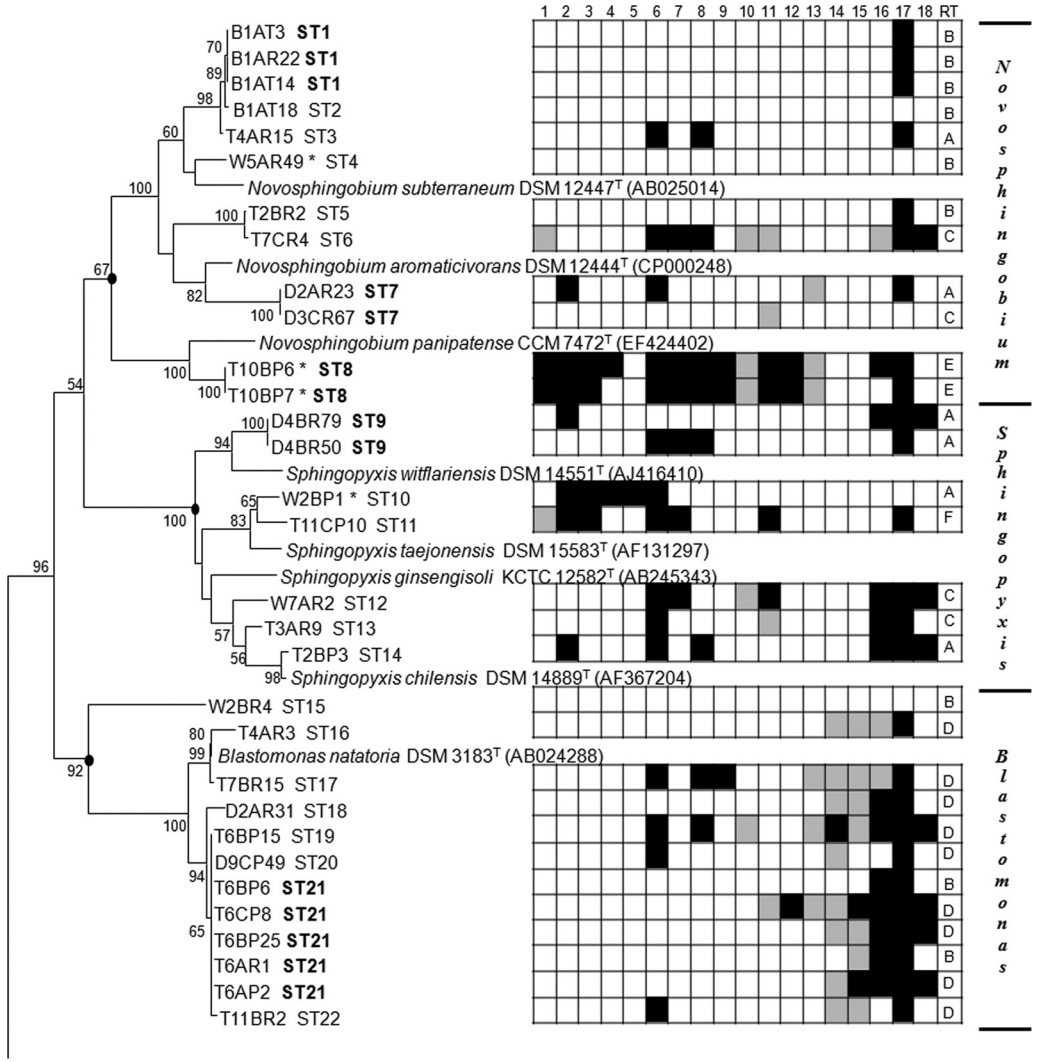

FIG. 2. (Left) Dendrogram constructed on the basis of 16S rRNA gene sequences (1,229 bp). The format of the isolate designations is SxCMn, where "S" stands for the site (W, WTP; T, tap water; D, cup fillers for dental chairs; B, biofilm), "x" for the number of the site, "C" for the sampling period (A, B, or C), "M" for the culture medium (R, R2A; P, *Pseudomonas* isolation agar; T, Tergitol-7 agar), and "n" for the number of the isolate. For the WTP, W1 is the raw surface water, W2 is the raw groundwater, and W3 to W5 are treated-water samples. ST, sequence type. Bar, 1 substitution per 200 nucleotide positions. Strains in which it was not possible to amplify the *atpD* gene are asterisked. (Right) Antibiotic resistance profiles. Black squares indicate resistance; gray squares, an intermediary phenotype; white squares, susceptibility. RT, resistance type. Antibiotics are represented by numbers above the chart as follows: 1, ampicillin-sulbactam; 2, ticarcillin; 3, ticarcillin-pyocyanin; 4, ticarcillin-clavulanic acid; 5, ticarcillin-clavulanic acid-pyocyanin; 6, piperacillin; 7, piperacillin-pyocyanin; 8, piperacillin-tazobactam; 9, piperacillin plus tazobactam-pyocyanin; 10, imipenem; 11, meropenem; 12, ceftazidime; 13, cefepime; 14, gentamicin; 15, tobramycin; 16, ciprofloxacin; 17, colistin; 18, cotrimoxazole.

TZP, was the most prevalent resistance phenotype, with resistance rates above 50%. For the other classes tested, fluoroquinolone resistance and sulfonamide resistance were the second most prevalent, with rates of 25.6 and 20.9%, respectively (Table 1).

According to our results, the isolation conditions (culture medium/temperature) can have some influence on the resistance phenotypes observed. For instance, isolates recovered on PIA or TTC presented significantly ($P < 0.05$) higher percentages of resistance to the beta-lactams TIC, TICP, PIC, PICP, TZP, and TZPP and the sulfonamide TSU than isolates recovered on the nonselective medium R2A. Among the TTC isolates, a significantly higher percentage of resistance was observed for the aminoglycoside TOB, and resistance to the beta-lactams TCC and TCCP or to the aminoglycoside GEN was not observed (Table 1).

Given the low number of isolates from WTP and biofilm samples, it was impossible to compare the resistance prevalence values accurately across all the sites sampled. However, taps and cup fillers could be compared. These sites showed similar percentages of resistance, except for the beta-lactams MEM, TCC, TCCP, and IMI and the aminoglycoside GEN. For MEM, significantly lower resistance rates were observed in the cup fillers than in the taps, whereas the other resistance phenotypes were not detected in the dental chairs (Table 1).

Comparison of the patterns of antibiotic resistance in the different genera of *Sphingomonadaceae* highlighted some distinctive features. For instance, members of the genus *Sphin-*

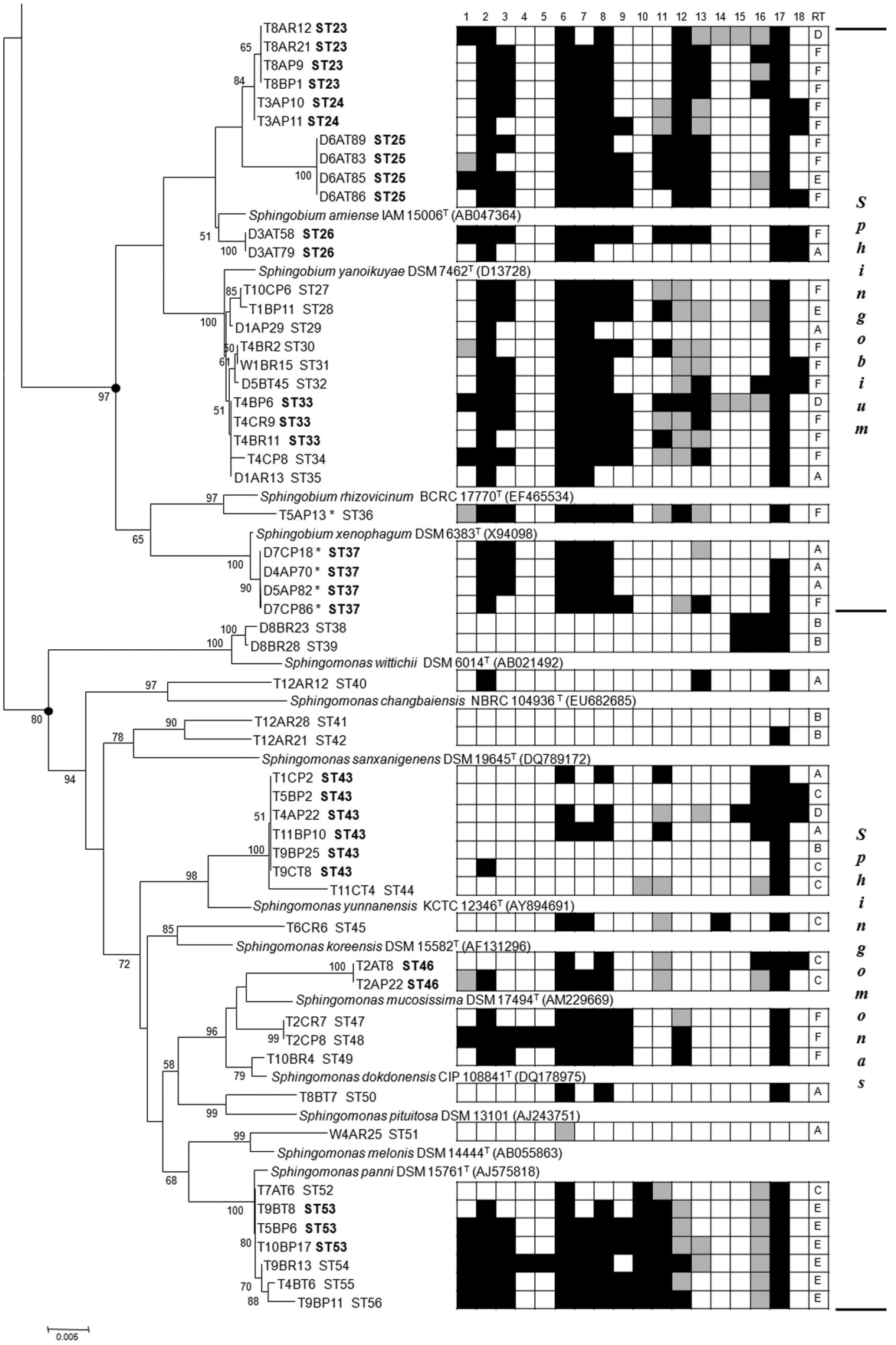

FIG. 2—*Continued*.

TABLE 1. Percentages and types of antibiotic resistance per culture medium, sampling site, and genus

Culture medium, sampling site,[a] or genus (no. of isolates)	% of isolates resistant to:												
	Beta-lactams												
	FAM	TIC	TICP	TCC	TCCP	PIC	PICP	TZP	TZPP	IMI	MEM	CAZ	FEP
Culture media													
R2A (34)	5.9	38.2	14.7	2.9	2.9	55.9	38.2	38.2	17.7	2.9	11.8	11.8	8.8
TTC (17)	17.7	58.8	35.3	0	0	70.6	47.1	58.8	29.4	17.7	35.3	29.4	35.3
PIA (35)	20.0	68.6	51.4	8.6	5.7	82.9	65.7	71.4	37.1	8.6	28.6	31.4	14.3
Sampling sites													
WTP (6)	0	33.3	33.3	16.7	16.7	50.0	33.3	16.7	0	0	16.7	0	0
Biofilm (4)	0	0	0	0	0	0	0	0	0	0	0	0	0
Taps (55)	18.2	54.6	34.6	5.5	3.6	74.6	50.9	65.5	34.6	12.7	27.3	27.3	12.7
Dental cup fillers (21)	9.5	71.4	38.1	0	0	76.2	66.7	52.4	23.8	0	19.1	23.8	33.3
Genera													
Sphingomonas (27)	18.5	44.4	25.9	7.4	7.4	63.0	40.7	55.6	25.9	25.9	29.6	14.8	3.7
Sphingobium (28)	17.9	100	60.7	0	0	100	96.4	89.3	50.0	0	28.6	46.4	46.4
Novosphingobium (12)	16.7	25.0	16.7	8.3	0	41.7	25.0	33.3	16.7	0	16.7	16.7	0
Sphingopyxis (7)	0	57.1	28.6	14.3	14.3	85.7	42.9	28.6	0	0	28.6	0	0
Blastomonas (12)	0	0	0	0	0	33.3	0	16.7	8.3	0	0	8.3	0
Total *Sphingomonadaceae*	14.0	54.7	33.7	4.7	3.5	69.8	51.2	55.8	27.9	8.1	23.3	23.3	16.3

[a] WTP, water treatment plant; R2A, R2A medium; TTC, tergitol-7-agar; PIA, *Pseudomonas* isolation agar.
[b] Amikacin resistance was not observed. FAM, ampicillin-sulbactam; TIC, ticarcillin; TICP, ticarcillin-pyocyanin; TCC, ticarcillin-clavulanic acid; TCCP, ticarcillin-clavulanic acid-pyocyanin; PIC, piperacillin; PICP, piperacillin-pyocyanin; TZP, piperacillin plus tazobactam; TZPP, piperacillin plus tazobactam-pyocyanin; IMI, imipenem; MEM, meropenem; CAZ, ceftazidime; FEP, cefepime; GEN, gentamicin; TOB, tobramycin; CIP, ciprofloxacin; COL, colistin; TSU, cotrimoxazole.

gomonas presented the widest range of resistance phenotypes, comprising resistance to 18 of the 19 antibiotics tested. Members of the genus *Sphingobium*, which presented resistance to 13 of the 19 antibiotics tested, yielded significantly higher percentages of resistance to eight beta-lactams (>46%) than members of the other genera. Members of the genus *Sphingobium* differed from members of the genus *Sphingomonas* by showing no resistance to the beta-lactams TCC, TCCP, and IMI and the aminoglycosides GEN and TOB, and a lower rate of resistance to the fluoroquinolone CIP (Table 1).

Differences in antibiotic resistance patterns can result from the ecology and physiology of the bacteria and may suggest distinct modes and mechanisms of resistance acquisition. The antibiotic resistance patterns of isolates belonging to the genera *Sphingomonas* and *Sphingobium* (which had more than 20 isolates and thus supported such an analysis) were compared on the basis of cluster analysis (Fig. 3). This comparison showed different patterns of antibiotic resistance and of resistance associations in these two genera. In the genus *Sphingomonas*, it was possible to distinguish four significant clusters (S1 to S4). Cluster S3 included the antibiotics for which resistance rates were highest, with prevalences of resistance to PIC, TZP, and COL above 55%. In contrast, the antibiotics for which resistance rates were lowest (4 to 11%), comprising beta-lactams (TCC, TCCP, FEP), a sulfonamide (TSU), and aminoglycosides (GEN and TOB), were clustered in S1. Cluster S4 included the antibiotics for which resistance rates ranged from 15 to 44%: all beta-lactams, including the carbapenem imipenem. Resistance to this carbapenem was observed only in the genus *Sphingomonas* and was associated with CAZ resistance. The other carbapenem tested, MEM, was included in another cluster and was not associated with a phenotype of resistance to any other beta-lactam. In the genus *Sphingobium*, different clusters and patterns of association were observed. In this case, the high-resistance cluster (cluster Sp3, with resistance rates above 46%) included the beta-lactams TIC, PICP, FEP, and CAZ, in addition to those grouped in the equivalent cluster (S3) of *Sphingomonas* spp. Similarly, the low-resistance cluster (Sp1), with resistance rates ranging 0 to 25%, included TICP, CIP, and IMI, in addition to the antibiotics observed in the corresponding cluster (S1) of the genus *Sphingomonas*. In contrast to the pattern for the genus *Sphingomonas*, in the genus *Sphingobium* resistance to the carbapenem MEM was associated with resistance to other beta-lactams. This observation may suggest distinct mechanisms or paths of acquisition of resistance to this carbapenem in the two genera.

The distinctive patterns of resistance observed for the genera *Sphingomonas* and *Sphingobium* supported the hypothesis that antibiotic resistance profiles may also differ for different species. Alternatively, it could be hypothesized that the provenience of the isolates was the most important explanatory factor for the different patterns of resistance observed. Two complementary approaches were used to test these hypotheses: (i) cluster analysis of the isolates based on their respective antibiotic resistance patterns and (ii) analysis of the antibiotic resistance patterns as a function of taxonomic classification (on the basis of 16S rRNA and *atpD* gene sequence analyses) and site of isolation (Fig. 2).

Cluster analysis of the antibiotic resistance patterns led to the definition of six significant clusters, designated resistance type A (RT-A) to RT-F (Fig. 2 and Table 1). The RT-A cluster ($n = 17$) comprised isolates with a core phenotype of resistance to TIC and/or PIC, besides COL. The RT-B cluster ($n = 14$) included mostly isolates susceptible to all antibiotics or

TABLE 1—*Continued*

% of samples resistant to:						
Aminoglycosides[b]		Fluoroquinolone (CIP)	Polymyxin (COL)	Sulfonamide (TSU)	>3 different classes (multiresistance)	Resistance types (no. of isolates)
GEN	TOB					
2.9	5.9	23.5	88.2	11.8	5.9	A (7), B (9), C (5), D (5), E (1), F (7)
0	22.7	11.8	94.2	29.4	11.8	A (2), B (3), C (4), E (3), F (5)
2.9	8.6	34.3	94.3	25.7	14.3	A (8), B (2), C (2), D (7), E (6), F (10)
0	0	16.7	33.3	33.3	16.7	A (2), B (2), C (1), F (1)
0	0	0	75.0	0	0	B (4)
3.6	3.6	29.1	98.2	20.0	10.9	A (6), B (6), C (9), D (10), E (9), F (15)
0	9.5	23.8	90.5	23.8	9.5	A (9), B (2), C (1), D (2), E (1), F (6)
3.7	11.1	25.9	92.6	11.1	7.4	A (5), B (5), C (7), D (1), E (6), F (3)
0	0	10.7	96.4	25.0	3.6	A (6), D (2), E (2), F (18)
0	0	8.3	75.0	8.3	0	A (2), B (6), C (2), E (2)
0	0	57.1	85.7	42.9	42.9	A (4), C (2), F (1)
8.3	16.7	58.3	91.7	33.3	25.0	B (3), D (9)
2.3	11.0	25.6	91.9	20.9	10.5	A (17), B (14), C (11), D (12), E (10), F (22)

resistant only to COL. The RT-C cluster ($n = 11$) consisted of carbapenem-resistant isolates. The RT-D cluster ($n = 12$) comprised isolates with a core phenotype of resistance to CIP and PIC. The RT-E ($n = 10$) and RT-F ($n = 22$) clusters included the isolates with the heaviest multiresistance phenotype, mainly to beta-lactams. RT-E differed from RT-F by including resistance to IMI and CIP.

This analysis showed that most of the time, resistance patterns were similar within the same species, though they often differed among species of the same genus. Within the genus *Sphingomonas*, the majority of species represented by a single isolate (*S. melonis*, *S. pituitosa*, *S. changbaiensis*) were included in RT-A, while those with two isolates were included in RT-B (*S. wittichii*, *S. sanxanigenens*) or RT-C (*S. mucosissima*). All the *S. dokdonensis* isolates belonged to RT-F, while 6 of the 10 *S. panni* isolates clustered in RT-E. In contrast, the *S. yunnanensis* isolates ($n = 7$) were distributed among the RT-A to RT-D clusters. Within the genera *Novosphingobium* and

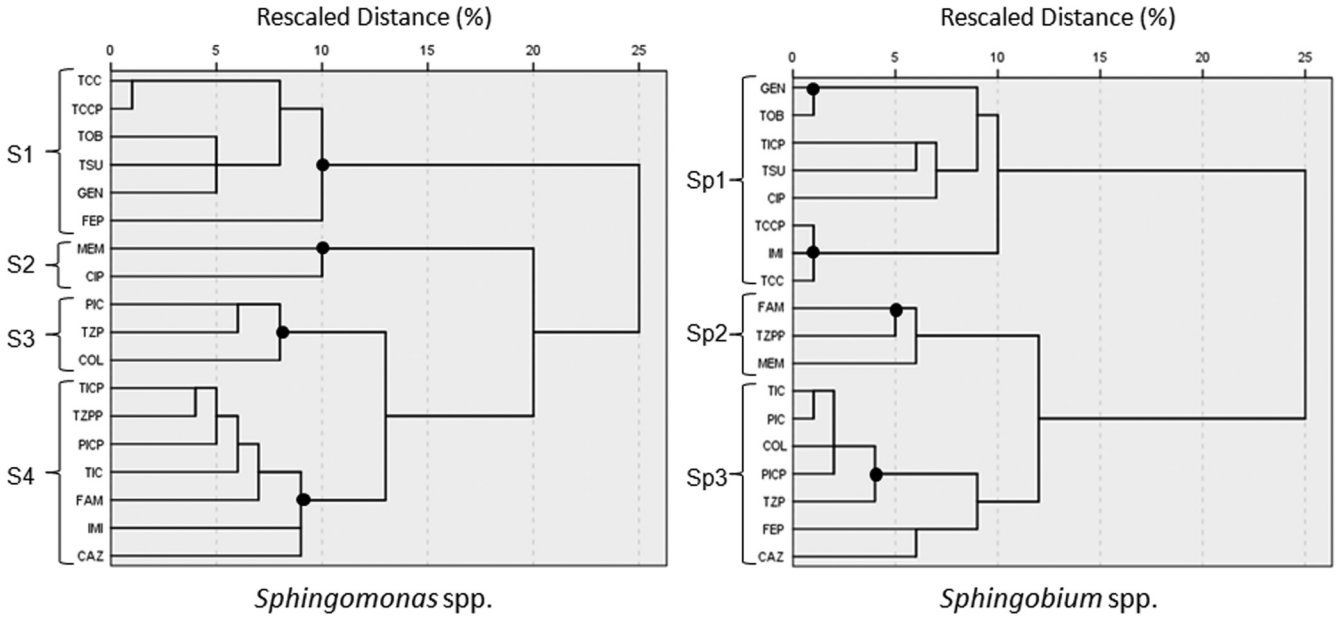

FIG. 3. Cluster analysis based on the antibiotic resistance profiles observed for *Sphingomonas* and *Sphingobium* spp. using Euclidean distance and the Ward method for the aggregation criterion. Groups of resistance profiles that differed significantly from each other were determined by the R^2 criterion, at a significance level (P) of <0.05. Black circles indicate branches recovered by other methods.

Sphingopyxis also, it was possible to observe different resistance patterns for different species. While the majority of the *N. subterraneum* isolates (5 of 6), recovered mainly from the biofilm, belonged to RT-B, both *N. panipatense* isolates, recovered from tap water, were included in RT-E. The four species of the genus *Sphingopyxis* were distributed in three RTs: RT-A (*Sp. witflariensis*, *Sp. taejonensis*, *Sp. chilensis*), RT-C (*Sp. chilensis*, *Sp. ginsengisoli*), and RT-F (*Sp. taejonensis*). In contrast to the other genera, in the genus *Sphingobium* the antibiotic resistance patterns were similar for different species. The majority of isolates in the species *Sb. amiense* (9/12), *Sb. yanoikuyae* (7/11), and *Sb. rhizovicinum* (1/1) belonged to RT-F. *Sb. xenophagum* ($n = 4$) constituted the only exception, in that three of its members were included in RT-A.

Analysis of the antibiotic resistance patterns as a function of the taxonomic classification (on the basis of 16S rRNA and *atpD* gene sequence analyses) and site of isolation found, in general, similar patterns of antibiotic resistance for members of the same species. Nevertheless, different species of the same genus often exhibited distinct resistance types, and sometimes (in 25% of the cases) isolates with the same sequence type showed distinct antibiotic resistance types. In some species it was possible to identify a core of resistance phenotypes, present in every isolate, or in most of the isolates, irrespective of the site, conditions of isolation, or sequence type. This was particularly evident in the genus *Sphingomonas*. In this genus, the species *S. dokdonensis* and *S. panni* were characterized by the multiresistance phenotypes RT-F and RT-E, respectively. The same resistance phenotype was common to most, though not all, the species members irrespective of the site of isolation or sequence type. Imipenem resistance was observed only in the species *Sphingomonas panni*, in isolates with different sequence types and sites of isolation. The resistance type observed for *S. dokdonensis* (RT-F) was found in other taxa, predominantly in the genus *Sphingobium* (Fig. 2). In this genus, *Sb. xenophagum*, with RT-A, differed from the other species by virtue of its general susceptibility to antibiotics. In contrast, *N. panipatense*, characterized by multiresistance phenotypes (RT-E), differed from other *Novosphingobium* species, which were susceptible to most antibiotics (RT-B) (Fig. 2).

This analysis demonstrated that antibiotic resistance profiles could easily be related to the species, or even the genus, suggesting the relevance of population dynamics for the hypothetical dissemination of resistance. Nevertheless, on rare occasions, different isolates of the same species, isolated from different sites, yielded distinct antibiotic resistance patterns, suggesting a possible process of resistance acquisition or loss. Examples could be observed in the different genera (Fig. 2). For instance, within the species *N. subterraneum*, the isolate from the WTP exhibited no resistance phenotype, the biofilm isolates were resistant to COL, and the tap water isolate was resistant to COL, PIC, and TZP. Another example was observed in *Sp. taejonensis*, in which the tap water isolate had resistance to COL, MEM, PICP, and FAM, which was not detected in the WTP strain. In *Sb. yanoikuyae*, two cup filler isolates were susceptible to more antibiotics (RT-A) than the other isolates, isolated from taps, the WTP, and cup fillers. In *S. panni* and *S. yunnanensis* also, distinct patterns could be observed for isolates from different taps. For example, *S. panni* strain T7AT6, isolated from a distinct tap and on a sampling date different from those for all the other *S. panni* isolates, belonged to RT-C, whereas all the other isolates belonged to RT-E. These examples suggest that both vertical and horizontal gene transfer processes are relevant in antibiotic resistance proliferation among *Sphingomonadaceae*.

DISCUSSION

The presence of members of the family *Sphingomonadaceae* in drinking water and even the observation of higher counts in tap water and in dental chairs than in the WTP can be associated both with resistance to chlorination (14, 25) and with the capacity to form biofilms (19, 34). Nevertheless, the possibility that external sources of *Sphingomonadaceae*, other than the water supply, exist in the water distribution network cannot be discarded. In this study it was possible to identify 22 species of the family *Sphingomonadaceae.* Among these, to our knowledge, the species *S. koreensis*, *S. pituitosa*, *S. wittichii*, *Sb. xenophagum*, *Sp. taejonensis*, *Sp. witflariensis*, *N. subterraneum*, and *N. aromaticivorans* were previously referred to as inhabitants of rivers, streams, aquifers, natural mineral water, and tap water (1, 14, 25). *Blastomonas natatoria* was also referred to as a common inhabitant of disinfected water, namely, chlorinated waters (1, 14).

The observed levels of antibiotic resistance can be considered high, in particular for colistin and beta-lactams. Although high prevalences of resistance to a polymyxin (polymyxin B) and to beta-lactams were reported previously for clinical isolates of the species *Sphingomonas paucimobilis* (32), it is relevant that the same findings are extended to other genera and species of the family *Sphingomonadaceae* isolated from water. In general, it was observed that members of the same species shared common antibiotic resistance profiles, suggesting that due to their ecology and physiology, these bacteria tend to acquire and/or develop similar patterns of resistance. Although it was not possible to establish a relationship between the resistance phenotype and the site of isolation, the data analyses demonstrated that multiresistance is widely found in *Sphingomonadaceae* in tap water.

Some of the species that presented the highest rates of (multi)resistance in the current study (RT-E and RT-F) were previously isolated from habitats with human-derived contamination, such as activated sludge (*S. dokdonensis* and *Sp. yanoikuyae*), pet-associated environments (*S. panni* and *N. panipatense*), or potato cultures (*Sp. yanoikuyae*) (3, 4, 10, 15). On the other hand, some species were distinguished by susceptibility to most of the antibiotics. Some of these species are common inhabitants of environments with low human impact, such as soil (*S. sanxanigenens* and *S. melonis*), sea, river, and lake water and sediments (*S. melonis*, *N. subterraneum*, and *N. aromaticivorans*), or freshwater biofilms (*S. melonis*). However, *S. melonis* and *N. subterraneum* are also found in polluted environments, such as gasoline-contaminated soils or mines (2, 8, 13, 20, 30).

Antibiotic resistance in this family of bacteria has not been studied much. Consequently, information on the mechanisms of resistance is scant. Additionally, the complete genome sequences, available for only a few strains, are poorly annotated. These facts represented serious limitations to the detection of

antibiotic resistance genes in these bacteria. The current study demonstrates the need for a better understanding of the ecology of antibiotic resistance acquisition and development in these bacteria.

With this study, some major conclusions were reached. Different genera of the *Sphingomonadaceae* were prevalent in distinct parts of the drinking water network. In about one-half of the isolates, the combination of the 16S rRNA and *atpD* housekeeping genes allowed the differentiation of the *Sphingomonadaceae* to the strain level, emphasizing the diversity of these bacteria in drinking water.

No amikacin resistance was observed, although colistin resistance was intrinsic, mainly in tap water and cup filler isolates. Beta-lactam resistance was also observed to be highly prevalent in members of this family. The use of selective culture media favored the growth of multiresistant bacteria. Distinct patterns of resistance association were observed in *Sphingomonas* and *Sphingobium*. Additionally, within each genus, antibiotic resistance patterns were observed to be mainly species related. These observations may suggest distinct mechanisms or paths of resistance acquisition for the different taxa.

Sphingomonadaceae are common inhabitants of drinking water worldwide (19, 27, 29, 42) and have a remarkable capacity to cope with stress conditions and to adapt to new habitats (14, 19, 25, 37, 44). The current study provides the first evidence that these bacteria can play an important role as antibiotic resistance reservoirs in drinking water. Further studies on the ecology and genome dynamics (mutation and gene transfer) of these bacteria will make important contributions to the elucidation of their role in the propagation of antibiotic resistance.

ACKNOWLEDGMENTS

We gratefully acknowledge the workers from the water treatment plant for their kind collaboration in sample collection and the persons who kindly allowed the sampling of water from their taps.

This study was financed by the Fundação para a Ciência e a Tecnologia (project PTDC/AMB/70825/2006, IVM grant SFRH/BD/27978/2006, Associate Laboratory LA50016).

REFERENCES

1. **Balkwill, D. L., J. K. Fredrickson, and M. F. Romine.** 2006. *Sphingomonas* and related genera, p. 605–629. *In* M. Dworkin et al. (ed.), The Prokaryotes: an evolving electronic resource for the microbiological community, vol. 7. Springer-Verlag, New York, NY. doi:10.1007/0-387-30747-8_23. Accessed 7 June 2011.
2. **Boden, R., E. Thomas, P. Savani, D. P. Kelly, and A. P. Wood.** 2008. Novel methylotrophic bacteria isolated from the River Thames (London, UK). Environ. Microbiol. **10:**3225–3236.
3. **Busse, H.-J., E. Hauser, and P. Kämpfer.** 2005. Description of two novel species, *Sphingomonas abaci* sp. nov. and *Sphingomonas panni* sp. nov. Int. J. Syst. Evol. Microbiol. **55:**2565–2569.
4. **Cardinali-Rezende, J., et al.** 2011. Phylogenetic and physiological characterization of organic waste-degrading bacterial communities. World J. Microbiol. Biotechnol. **27:**245–252.
5. **Cavicchioli, R., F. Fegatella, M. Ostrowski, M. Eguchi, and J. Gottschal.** 1999. Sphingomonads from marine environments. J. Ind. Microbiol. Biotechnol. **23:**268–272.
6. **Charity, R. M., and A. F. Foukas.** 2005. Osteomyelitis and secondary septic arthritis caused by *Sphingomonas paucimobilis*. Infection **33:**93–95.
7. **Chun, J., et al.** 2007. EzTaxon: a web-based tool for the identification of prokaryotes based on 16S rRNA gene sequences. Int. J. Syst. Evol. Microbiol. **57:**2259–2261.
8. **Cui, Z., Q. Lai, C. Dong, and Z. Shao.** 2008. Biodiversity of polycyclic aromatic hydrocarbon-degrading bacteria from deep sea sediments of the Middle Atlantic Ridge. Environ. Microbiol. **10:**2138–2149.
9. **Dantas, G., M. O. A. Sommer, R. D. Oluwasegun, and G. M. Church.** 2008. Bacteria subsisting in antibiotics. Science **320:**100–103.
10. **De Gelder, L., F. P. Vandecasteele, C. J. Brown, L. J. Forney, and E. M. Top.** 2005. Plasmid donor affects host range of promiscuous IncP-1β plasmid pB10 in an activated-sludge microbial community. Appl. Environ. Microbiol. **71:**5309–5317.
11. **Eaton, A. D., L. S. Clesceri, E. W. Rice, and A. E. Greenberg.** 2005. Standard methods for the examination of water and wastewater, 21st ed. American Public Health Association, Washington, DC.
12. **Euzéby, J. P.** 1997. List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet. Int. J. Syst. Bacteriol. **47:**590–592.
13. **Fredrickson, J. K., et al.** 1995. Aromatic-degrading *Sphingomonas* isolates from the deep subsurface. Appl. Environ. Microbiol. **61:**1917–1922.
14. **Furuhata, K., et al.** 2007. Identification of yellow-pigmented bacteria isolated from hospital tap water in Japan and their chlorine resistance. Biocontrol Sci. **12**(2):39–46.
15. **Garbeva, P., L. S. van Overbeek, J. W. L. van Vuurde, and J. D. van Elsas.** 2001. Analysis of endophytic bacterial communities of potato by plating and denaturing gradient gel electrophoresis (DGGE) of 16S rDNA based PCR fragments. Microb. Ecol. **41:**369–383.
16. **Gaunt, M. W., S. L. Turner, L. Rigottier-Gois, S. A. Lloyd-Macgilps, and J. P. W. Young.** 2001. Phylogenies of *atpD* and *recA* support the small subunit rRNA-based classification of rhizobia. Int. J. Syst. Evol. Microbiol. **51:**2037–2048.
17. **Gomila, M., et al.** 2005. Identification of culturable bacteria present in haemodialysis water and fluid. FEMS Microbiol. Ecol. **52:**101–114.
18. Reference deleted.
19. **Hong, P.-Y., et al.** 2010. Pyrosequencing analysis of bacterial biofilm communities in water meters of a drinking water distribution system. Appl. Environ. Microbiol. **76:**5631–5635.
20. **Huang, H. D., et al.** 2009. *Sphingomonas sanxanigenens* sp. nov., isolated from soil. Int. J. Syst. Evol. Microbiol. **5:**719–723.
21. **International Organization for Standardization.** 2000. Water quality: detection and enumeration of *E. coli* and coliform bacteria. Part 1. Membrane filtration method. ISO 9308-1:2000. International Organization for Standardization, Geneva, Switzerland.
22. **Jukes, T. H., and C. R. Cantor.** 1969. Evolution of protein molecules, p. 21–132. *In* H. N. Munro (ed.), Mammalian protein metabolism, vol. 3. Academic Press, New York, NY.
23. **Källman, O., C. Lundberg, B. Wretlind,and Å. Örtqvist.** 2006. Gram-negative bacteria from patients seeking medical advice in Stockholm after the tsunami catastrophe. Scand. J. Infect. Dis. **38:**448–450.
24. **Kilic, A., et al.** 2007. Nosocomial outbreak of *Sphingomonas paucimobilis* bacteremia in a hemato/oncology unit. Jpn. J. Infect. Dis. **60:**394–396.
25. **Koskinen, R., et al.** 2000. Characterization of *Sphingomonas* isolates from Finnish and Swedish drinking water distribution systems. J. Appl. Microbiol. **89:**687–696.
26. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. Wiley, Chichester, England.
27. **Li, D., et al.** 2010. Characterization of bacterial community structure in a drinking water distribution system during an occurrence of red water. Appl. Environ. Microbiol. **76:**7171–7180.
28. **Lin, J. N., et al.** 2010. *Sphingomonas paucimobilis* bacteremia in humans: 16 case reports and a literature review. J. Microbiol. Immunol. Infect. **43:**35–42.
29. **Revetta, R. P., A. Pemberton, R. Lamendella, B. Iker, and J. W. Santo Domingo.** 2010. Identification of bacterial populations in drinking water using 16S rRNA-based sequence analyses. Water Res. **44:**1353–1360.
30. **Rickard, A. H., A. J. McBain, A. T. Stead, and P. Gilbert.** 2004. Shear rate moderates community diversity in freshwater biofilms. Appl. Environ. Microbiol. **70:**7426–7435.
31. **Ryan, M. P., and C. C. Adley.** 2010. *Sphingomonas paucimobilis*: a persistent Gram-negative nosocomial infectious organism. J. Hosp. Infect. **75:**153–157.
32. **Sader, H. S., and R. N. Jones.** 2005. Antimicrobial susceptibility of uncommonly isolated non-enteric Gram-negative bacilli. Int. J. Antimicrob. Agents **25:**95–109.
33. **Silva, V., V. Figueira, H. Figueiral, and C. M. Manaia.** A survey of the bacterial diversity in the cup filler of dental chair units. Braz. J. Microbiol., in press.
34. **Singh, R., et al.** 2003. Microbial diversity of biofilms in dental unit water systems. Appl. Environ. Microbiol. **69:**3412–3420.
35. **Smibert, R. M., and N. R. Krieg.** 1994. Phenotypic characterization, p. 611–651. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, DC.
36. **Stackebrandt, E., and B. M. Goebel.** 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44:**846–849.
37. **Stolz, A.** 2009. Molecular characteristics of xenobiotic-degrading sphingomonads. Appl. Microbiol. Biotechnol. **81:**793–811.
38. **Szymańska, J.** 2007. Bacterial contamination of water in dental unit reservoirs. Ann. Agric. Environ. Med. **14:**137–140.
39. **Tamura, K., J. Dudley, M. Nei, and S. Kumar.** 2007. MEGA4: Molecular

Evolutionary Genetics Analysis (MEGA) software, version 4.0. Mol. Biol. Evol. **24:**1596–1599.
40. **Vaz-Moreira, I., et al.** 2009. *Sphingobium vermicomposti* sp. nov., isolated from vermicompost. Int. J. Syst. Environ. Microbiol. **59:**3145–3149.
41. **White, D. C., S. Sutton, and D. Ringelberg.** 1996. The genus *Sphingomonas*: physiology and ecology. Curr. Opin. Biotechnol. **7:**301–306.
42. **Williams, M. M., J. W. Santo Domingo, M. C. Meckes, C. A. Kelty, and H. S. Rochon.** 2004. Phylogenetic diversity of drinking water bacteria in a distribution system simulator. J. Appl. Microbiol. **96:**954–964.
43. **Yabuuchi, E., and Y. Kosako.** 2005. Family I. *Sphingomonadaceae* Kosako, Yabuuchi, Naka, Fijiwara and Kobayashi 2000b, 1953^{VP} (effective publication: Kosako, Yabuuchi, Naka, Fijiwara and Kobayashi 2000a, 563), p. 233–286. *In* D. J. Brenner, N. R. Krieg, J. T. Staley, and G. M. Garrity (ed.), Bergey's manual of systematic bacteriology, 2nd ed., vol. 2. The *Proteobacteria*, part C: the *Alpha-*, *Beta-*, *Delta-*, and *Epsilonproteobacteria*. Springer, New York, NY.
44. **Yim, M.-S., et al.** 2010. A novel selective growth medium-PCR assay to isolate and detect *Sphingomonas* in environmental samples. J. Microbiol. Methods **82:**19–27.