Pseudomonas aeruginosa and Achromobacter sp. Clonal Selection Leads to Successive Waves of Contamination of Water in Dental Care Units

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Dental care unit waterlines (DCUWs) consist of complex networks of thin tubes that facilitate the formation of microbial biofilms. Due to the predilection toward a wet environment, strong adhesion, biofilm formation, and resistance to biocides, Pseudomonas aeruginosa, a major human opportunistic pathogen, is adapted to DCUW colonization. Other nonfermentative Gram-negative bacilli, such as members of the genus Achromobacter, are emerging pathogens found in water networks. We reported the 6.5-year dynamics of bacterial contamination of waterlines in a dental health care center with 61 dental care units (DCUs) connected to the same water supply system. The conditions allowed the selection and the emergence of clones of Achromobacter sp. and P. aeruginosa characterized by multilocus sequence typing, multiplex repetitive elements-based PCR, and restriction fragment length polymorphism in pulsed-field gel electrophoresis, biofilm formation, and antimicrobial susceptibility. One clone of P. aeruginosa and 2 clones of Achromobacter sp. colonized successively all of the DCUWs: the last colonization by P. aeruginosa ST309 led to the closing of the dental care center. Successive dominance of species and clones was linked to biocide treatments. Achromobacter strains were weak biofilm producers compared to P. aeruginosa ST309, but the coculture of P. aeruginosa and Achromobacter enhanced P. aeruginosa ST309 biofilm formation. Intraclonal genomic microevolution was observed in the isolates of P. aeruginosa ST309 collected chronologically and in Achromobacter sp. clone A. The contamination control was achieved by a complete reorganization of the dental health care center by removing the connecting tubes between DCUs.

Dental instruments on dental care units (DCUs), such as ultrasonic scalers, air scalers, and high-speed turbine dental handpieces, are cooled by DCU-supplied water, which also irrigates and cools the tooth surface during dental treatment (1). Water is also supplied to the DCU cup filler outlet used by patients for oral rinsing and to the bowl rinse outlet rinsing the DCU spitter. Therefore, DCU waterlines (DCUWs) consist of a complex network formed by meters of narrow-bore plastic tubing of mostly 2- to 3-mm internal diameter. In narrow-bore tubes, a thin immobile layer of fluid, called the hydrodynamic boundary layer, exists at the interface of the lumen wall and the moving water (2). This hydrodynamic phenomenon is associated with water stagnation when the DCU is not used, and heating of water to at least 20°C enhances the development of microbial biofilm in the tubes (3).

The DCUWs appear to be a technological niche in which complex interactions occur among members of the microbial community (4–6). Regarding bacteria, DCUWs have been shown to promote the formation of biofilm, predominantly seeded from environmental bacteria in the water supply (4–6). More rarely, human bacteria such as staphylococci or oral bacteria have been detected, indicating a retraction of oral fluids from the surgical devices to the waterlines (7, 8). Routine follow-up for water microbial contamination in the dental health care center (DCC) is performed in order to avoid massive microbial contamination and to detect the presence of pathogenic bacteria. The majority of microbial species described in massive DCUW contamination are Gram-negative, aerobic, heterotrophic environmental species with low pathogenicity (4, 6). However, known pathogens, including Pseudomonas aeruginosa (9, 10), Legionella pneumophila (11, 12), and nontuberculous mycobacterial species (13, 14), have been detected in DCUWs. For these pathogenic bacteria, the role of DCUW contamination in patient’s or dentist’s infections or allergic disorders has been suggested by several published cases (11, 15–17).

To avoid backflow from the oral cavity to waterlines, handpieces are equipped with antiretraction devices, but evidence of antiretraction valve failures has been well documented (18, 19). Consequently, guidelines for infection control in dental health care recommend that dental handpieces be cleaned and sterilized between patients and that the waterlines be flushed for 20 to 30 s after each patient for any device connected to the waterlines in order to elute water and air before treating the next patient (20).
Beside retro-contamination, the DCUW can be contaminated by the water supply, which is either drinking-quality water or water from reservoir bottles integrated in the DCU. Generally, the microbial communities in water present a high capacity for biofilm formation and therefore can easily colonize the DCUW (8, 21).

Purging and intensive decontamination are recommended to prevent microbial colonization (21), but some bacteria display both strong adhesion ability to surfaces and high biocide resistance that lead to persistent and refractory colonization. This behavior is particularly displayed by *P. aeruginosa*, a major human opportunistic pathogen that preferentially thrives in aquatic habitats (22, 23). Adaptability to diverse environments and life conditions is a major trait of *P. aeruginosa* determined by the ability of individual strains to acquire genomic segments, giving rise to strains with customized genomic repertoires (24). Moreover, rapid intraclonal microevolution has been described and related to habitat adaptation (25). Similar adaptive behavior has been suggested by genomics in the genus *Achromobacter* (26).

We studied herein the 6.5-year dynamics of bacterial contamination of DCUWs in a dental health care center (DCC) comprising 61 DCUs connected by a short tube on a feeder pipe supplying softened water. The conditions allowed the successive selection of *P. aeruginosa* and *Achromobacter* sp., followed by the emergence and dominance of a clone of *P. aeruginosa*. Contamination control was achieved by a reorganization of the dental center and changes in hygiene procedures.

MATERIALS AND METHODS

**DCC settings and water treatment.** The DCC of Montpellier University Hospital (France) hosted a general dentistry patient population but also included at-risk patients. It comprised 61 DCUs branched by a short connection on the same water plumbing pipe located just under the floor of the care rooms. The organization of the water treatment and positions of rooms on the water supply system are represented in Fig. 1. The 13 care rooms of the DCC contained 3 to 5 DCUs. Each DCU was provided with a valve to avoid reflux from its DCUW to another DCU. The system was supplied by water containing 6×10⁻⁴ mol/liter Ca²⁺ and Mg²⁺ obtained by mixing water from the municipal water system and softened/deionized water. Water was then re-chlorinated in a mixing bottle at 1 ppm. A preventive biocide (Sterispray) recommended for dental care waterlines was added with an automatic injector (Gamasonic) (“BI” in Fig. 1) located at the beginning of the pipe for water feeding of the DCU. The water supply network was not looped, and an automatic system at the network end performed a 2-min daily purge of the feeding pipe. Water from the purge went directly to the sewer. Biennial decontamination of the DCC network was performed by shock chlorination at 12.36 ppm in water softener and the network after the terminal water supply valve of each DCU had been closed in order to avoid deleterious oxidation of internal DCU devices. After the shock chlorination, when the chlorinate concentration decreased at 3 ppm, the DCU terminal water supply valves were opened, and a prolonged purge was performed at each DCU in order to allow chlorination and rinse of the DCUW.

**Water control and microbiological analysis.** The study material included water sampled from DCUWs, from the main water supply, and from water softener. A routine microbiological follow-up by total mesophiles bacteria counting and *P. aeruginosa* specific detection and counting was performed once a month on one DCU of each of the 13 DCC rooms, with all DCUs being analyzed once a year according to hospital guidelines. Results are reported as CFU 100 ml⁻¹ water. Except for *P. aeruginosa*, only the majority of colonial morphotypes were identified to the species or genus level. Additional analyses were performed after major corrective
measures in order to evaluate their effects and during periods of heavy contamination, such as September 2012 and the year 2013.

All samples were taken at the beginning of a working day before the first patient care. DCU water samples were obtained after 2 min of purging of the air/water lines equipped with sterile syringes. Water samples were collected in 250-ml sterile bottles containing sodium thiosulfate (20 mg liter−1) for chloride inhibition and passed through a 0.45-μm pore filter system. For water containing the preventive biocide (Sterispray), the filters were treated by neutralizer buffer (3 g liter−1) plus sodium bicarbonate (50 g liter−1) plus polysorbate 80) to block the inhibitory effect of biocide on bacterial growth. The filters were then incubated on plate count agar (bioMérieux) and pseudomonad selective agar containing cetrimide (200 mg liter−1) and nalidixic acid (15 mg liter−1) (Oxoid). Incubation was performed at 30°C during 48 h. The bacteria were identified by Gram staining, oxidase reaction, production of pigments onto King A and King B media (Bio-Rad Laboratories) completed by the API 20NE system (bioMérieux), matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), and/or 16S rRNA gene sequencing when necessary. Isolates were stored at −80°C in a preservative medium (Bacterial Preservers; Technical Service Consultant, Ltd.).

Gene amplification and sequencing. Genomic DNA was obtained using the MasterPure DNA purification kit (Epiprise, Madison, WI). Multilocus sequence typing (MLST) (aexA, aexE, guaA, mutL, nuoD, ppsA, and trpE for P. aeruginosa and lepA, ndaA, usaA, nuoL, gltB, rpoB, and eno for Achromobacter) was performed as previously described (27, 28). Amplification products were separated on an ABI 3730xl automated sequencer (Cogenics, France). Sequence comparison and allele affiliation were performed using http://www.pubmlst.org. The combination of allele numbers for each isolate defined the sequence type (ST). The sequences were deposited into the GenBank database, and the undescribed ST was deposited in the pubMLST (P. aeruginosa) database as ST1753.

PFGE and genome comparison. For P. aeruginosa, restriction fragment length polymorphism (RFLP) in PFGE after digestion by Spel was performed according to Corne et al. (29). The PFGE patterns were photographed under UV light and visually compared. The Lambda concatamer ladder (Bio-Rad) was used as a molecular size marker. The PFGE patterns of P. aeruginosa ST309 DCIC isolates were compared in silico with the complete genome sequence of P. aeruginosa strains BI09 and BL20 (accession no. GCA_000480965.1 and GCF_000480745.1, respectively).

Multiplex rep-PCR. DNAs were extracted using the MasterPure DNA purification kit (Epiprise, Madison, WI) as recommended by the supplier. The amplification of replicative extragenic palindromic (REP) sequences, the enterobacterial repetitive intergenic consensus (ERIC), and BOX was done with the primers previously described (30, 31). The rep-PCR approach was performed in a 50-μl mixture containing 160 mM each primers, 200 mM each deoxynucleotide triphosphate (dNTP), 2.5 U Taq polymerase (Promega) in the appropriate buffer, 50 ng of genomic DNA, 2 mM MgCl2, and 3.5% dimethylsulfoxide. The conditions for amplification were an initial denaturation (95°C, 3 min) followed by 30 cycles of denaturation (90°C, 30 s), annealing (40°C, 1 min), extension (72°C, 4 min), and a final extension step (16 min at 72°C). Amplification products were separated on a 1.5% agarose gel in 0.5× Tris-borate-EDTA buffer containing 500 mM ethidium bromide, and the gel was photographed under UV light. A 1-klb DNA ladder (Sigma-Aldrich) was used as a molecular size marker.

Antimicrobial susceptibility testing. Antibiotic susceptibility was tested by disk diffusion assay on Mueller-Hinton agar and interpreted according to the recommendations of the Antibiogram Committee of the French Microbiology Society (CA-SFM) (http://www.sfm-microbiologie.org). Susceptibility to topical antimicrobial agents and biocides was tested by agar well diffusion assay—the so-called “biocidogram” (32). The biocides tested were chlorhexidine in alcoholic solution, 10% povidone-iodine in alcohol solution (Betadine), H2O2, Sterispray (benzalkonium chloride 1% plus chloramine plus EDTA), Aniosurf (didecyl(dimethylammonium chloride plus 1,1 hexamethylene bis (5-(4-chlorophenyl) bignanide)]), and Oxyfloor. Briefly, the surface of 5-mm-thick Mueller-Hinton agar plates was inoculated with a bacterial suspension visually adjusted to a 0.5 McFarland standard (106 CFU ml−1) and then diluted 100-fold. Then 8-mm-diameter holes were made in agar plates with a sterile punch, and the wells were loaded with 150 μl of each compound. The inhibition diameters were measured after 24 h of incubation at 37°C for P. aeruginosa and after 48 h of incubation at 37°C for Achromobacter sp. using the Antibiotic Zone Reader apparatus (Fisher Lilly). Diameters were compared to those of P. aeruginosa ATCC 9027, a strain with the wild-type susceptibility profile used as a quality control in assays of antimicrobial preservatives and efficacy testing. The type strain of Achromobacter xylocosidans was also tested.

Study of biofilm formation. The study of biofilm formation was conducted in flat-bottom 96-well polystyrene microplates (Nunc, Roskilde, Denmark) in tryptic soy broth (TSB). The wells were inoculated by serial dilution of overnight cultures (10−6 CFU ml−1). The ability of strains to form biofilms was evaluated as described by O’Toole and Kolter (33), with modifications. Briefly, 100 μl of cultures was transferred into wells of microplates (4 wells/strain) and incubated for 48 h (72 h for Achromobacter sp.) at 25, 30, and 37°C. After incubation, the growth was evaluated by determination of the optical density at 570 nm (OD570) (GEnios; Tecan). The plates were rinsed thoroughly with sterile water to remove planktonic bacteria, and 150 μl of crystal violet (0.3%) was added to each well. After 15 min at room temperature, the plates were rinsed again and air dried, and the dye was dissolved with 95% ethanol (200 μl). Crystal violet absorbance was determined at 570 nm. The biofilm assay was performed three times. The averages and standard deviations were calculated for all repetitions of the experiment. The cutoff OD (ODc) for the microtiter plate test corresponded to 3 standard deviations above the mean OD of the negative control. In all experiments, the ratio of OD to ODc was calculated. Strains were categorized as nonadherent or weakly, moderately, or strongly adherent according Stepanovic et al. (34). For the biofilm formation with cocultured Achromobacter sp. and P. aeruginosa, we mixed diluted cultures equally, and we transferred this culture into the microplates for 48 h. The number of adhering bacteria within the biofilm was determined according to López et al. (35), with modifications.

Nucleotide sequence accession numbers. The sequences of the isolates determined in this study have been deposited into the GenBank database under accession no. KJ863569 to KJ863645 and KJ867037 to KJ867050.

RESULTS

Synopsis of DCC bacterial contamination. Figure 2 shows that members of the genus Bacillus were the major water contaminants in the DCC from 2009 to 2012, with a decrease in the total inoculum in 2010 followed by a reincrease in 2011. In 2012, P. aeruginosa and Achromobacter appeared and then persistently colonized the DCUWs. From January 2009 to June 2013, water was preventively treated by the Gamasonic system (BI in Fig. 1), which automatically provided 10 ml of Sterispray solution per liter of water in the network, after water softening (“WS” in Fig. 1) and rechlorination (CI and CB in Fig. 1). Additional curative decontaminations were performed regularly by shock chlorination of the softened water supply. Because of massive contaminations in the water softer (WS) and the water before and after Sterispray treatment (BI) in 2012 and 2013, these devices were shutted, the DCUWs being fed by untreated municipal water from June 2013. From June to September, this procedure did not decrease the level of DCUW contamination (>104 CFU/100 ml in 68 samples), despite better microbiological quality of the supplied water: 20 CFU/100 ml after chlorination, <1 CFU/100 ml after chlorination, and absence of Achromobacter sp. and P. aeruginosa. This observation suggested that the downstream colonization of the DCC...
persisted whatever the quality of supplied water. The closing of the DCC in September 2013 allowed the complete reorganization of the DCC water system. Internal DCU tubes were changed, and decontamination of each DCU was performed by oxidative shock with H$_2$O$_2$ at 6 ppm. From October 2013, each DCU was unplugged from the main water feeding tube, and sterile pharmaceutical water with 0.03% H$_2$O$_2$ was provided from a bottle attached to each DCU. Only 1 CFU of *P. aeruginosa* was detected in October and November 2013. Since then, no bacteria were detected in the DCUWs (Fig. 2).

**Molecular typing of **P. aeruginosa** isolates.** Sixty-eight isolates of *P. aeruginosa* collected between June 2012 and November 2013 were compared by multiplex rep-PCR (Table 1). A low level of diversity was observed in the population, with only 5 rep-PCR types (0.073 type per sample). Representative patterns of the 5 rep-PCR types are shown in Fig. 3. A large majority of isolates (n = 58 [85%]) displayed rep-PCR pattern 2, which was detected throughout the study period (28 June 2012 to November 2013) in DCUWs and once in water before BI (Fig. 1). Isolates displaying rep-PCR pattern 1 (n = 4) were detected only once in two care rooms together with isolates of rep-PCR type 2. Isolates of rep-PCR type 3 (n = 4) were mainly detected in the water softener (WS) and only once in DCUWs. Isolates with rep-PCR type 4 (n = 1) and type 5 (n = 1) were recovered only once in DCUWs and water softener (WS), respectively. A subpopulation of 11 isolates representative of the 5 rep-PCR types and of different dates of sampling for rep-PCR type 2 was submitted to MLST (Table 1). All isolates of rep-PCR type 2 belonged to ST309, whereas isolates of rep-PCR types 1, 4, and 5 belonged to ST253, ST312, and ST252, respectively. Isolates of rep-PCR type 3 had an ST previously undescribed in the pubMLST database and now deposited as ST1753. Molecular typing showed that massive and persistent colonization of the DCUWs involved a *P. aeruginosa* clone of rep-PCR type 2 and ST309.

**Molecular identification and typing of **Achromobacter** isolates.** A total of 78 *Achromobacter* isolates were recovered from DCUWs between July 2012 and July 2013. Molecular typing by multiplex rep-PCR of 17 representatives showed that colonization of the DCUW involved only 2 clones. The rep-PCR type A was observed for isolates recovered from July 2012 to July 2013, while the rep-PCR type B was observed only once for strains isolated in 5 different rooms in November 2012 (Table 1 and Fig. 4). MALDI-TOF MS analysis showed the best match (score of >2 for all strains) with *Achromobacter* sp. genomospecies 3. The 16S rRNA gene sequencing showed that both type A and B isolates were most closely related to *Achromobacter denitrificans* (accession no. AJ278451), with 98.7% sequence identity. Type A and B isolates shared 99.6% of their 16S rRNA gene sequences. Multilocus sequencing confirmed that the isolates CYS1 (type A) and DMG18 (type B) belonged to a not yet described species most...
| Isolate or strain | DCC room/site of sampling | Sample type | Date of sampling (day/mo/yr) | Profile by: | ST no. |
|------------------|---------------------------|-------------|-----------------------------|------------|-------|
| T45.9            | Montpellier hospital      | Clinical    | 01/11/10                    |            | 308   |
| PAPE 6           | Montpellier city          | Water main  | 2011                        | 2          | P3 309|
| ATCC 9027 (reference strain) | Clinical (ear) |             |                             |            |       |
| CUZ 1            | 1                         | DCU water, 2nd flow | 28/06/12                   | 2          | P1 309|
| CUZ 2            | 4                         | DCU water, 2nd flow | 28/06/12                   | 1          | 253   |
| CUZ 3            | 10                        | DCU water, 2nd flow | 28/06/12                   | 1          | 253d  |
| CUZ 4            | 10                        | DCU water, 2nd flow | 28/06/12                   | 1          | 253d  |
| CUZ 5            | 5                         | DCU water, 2nd flow | 28/06/12                   | 1          | 253   |
| CUZ 6            | 9                         | DCU water, 2nd flow | 28/06/12                   | 2          | P1 309d|
| CUZ 7            | 6                         | DCU water, 2nd flow | 28/06/12                   | 2          | 309d  |
| CXD 2            | 9                         | DCU water, 2nd flow | 04/07/12                   | 2          | 309d  |
| CXD 3            | 10                        | DCU water, 2nd flow | 04/07/12                   | 2          | P1 309|
| CXD 4            | 9                         | DCU water, 2nd flow | 04/07/12                   | 2          | 309d  |
| CXE 2            | 9                         | DCU water, 2nd flow | 04/07/12                   | 2          | P1 309|
| CXE 3            | 9                         | DCU water, 2nd flow | 04/07/12                   | 2          | 309d  |
| DKJ 4            | 2                         | DCU water, 2nd flow | 22/10/12                   | 2          | P1 309d|
| DKJ 6            | 3                         | DCU water, 2nd flow | 22/10/12                   | 2          | P1 309d|
| DXF 2            | Level – 1                 | Water in softener | 11/01/13                   | 5          | 252   |
| ETC 3            | 12                        | DCU water, 1st flow | 29/04/13                   | 2          | 309d  |
| ETC 7            | 11                        | DCU water, 1st flow | 29/04/13                   | 2          | 309d  |
| FBO 1            | 9                         | DCU water, 1st flow | 06/06/13                   | 2          | 309d  |
| FBO 2            | 9                         | DCU water, 2nd flow | 06/06/13                   | 2          | 309d  |
| FRM 3            | 1                         | DCU water, 2nd flow | 09/08/13                   | 2          | P1 309d|
| FRM 6            | 13                        | Spittoon water  | 09/08/13                   | 2          | P1 309d|
| FRM 11           | 11                        | DCU water, 2nd flow | 09/08/13                   | 2          | 309d  |
| FRM 12           | 4                         | DCU water, 2nd flow | 09/08/13                   | 2          | 309d  |
| FRM 17           | 8                         | DCU water, 2nd flow | 09/08/13                   | 3          | P4 1753|
| FRM 18           | Level – 1                 | Softened water network | 09/08/13                   | 3          | P5 1753|
| FSN 1            | 1                         | DCU water, 2nd flow | 23/08/13                   | 2          | 309d  |
| FSR 2            | 2                         | DCU water, 2nd flow | 27/08/13                   | 2          | 309d  |
| FSR 5            | 3                         | DCU water, 2nd flow | 27/08/13                   | 2          | 309d  |
| FSR 9            | 2                         | DCU water, 2nd flow | 27/08/13                   | 2          | 309d  |
| FST 1            | 12                        | DCU water, 2nd flow | 27/08/13                   | 2          | 309d  |
| FST 2            | 12                        | DCU water, 2nd flow | 27/08/13                   | 2          | 309d  |
| FST 3            | 11                        | DCU water, 2nd flow | 27/08/13                   | 2          | 309d  |
| FST 4            | 11                        | DCU water, 2nd flow | 27/08/13                   | 2          | 309d  |
| FWM 1            | 1                         | DCU water, 2nd flow | 05/09/13                   | 2          | 309d  |
| FWM 5            | 2                         | DCU water, 2nd flow | 05/09/13                   | 2          | 309d  |
| FWM 8            | 3                         | DCU water, 2nd flow | 05/09/13                   | 2          | 309d  |
| FWM 10           | 4                         | DCU water, 2nd flow | 05/09/13                   | 2          | 309d  |
| FWM 12           | 5                         | DCU water, 2nd flow | 05/09/13                   | 2          | 309d  |
| FWM 14           | 6                         | DCU water, 2nd flow | 05/09/13                   | 2          | 309d  |
| FWM 16           | 7                         | DCU water, 2nd flow | 05/09/13                   | 2          | 309d  |
| FWN 2            | Level – 1                 | DCC water entrance | 05/09/13                   | 3          | P6 1753|
| FUO 1            | 1                         | DCU water, 2nd flow | 03/09/13                   | 2          | 309d  |
| FUO 3            | 2                         | DCU water, 2nd flow | 03/09/13                   | 2          | 309d  |
| FUO 5            | 3                         | DCU water, 2nd flow | 03/09/13                   | 2          | 309d  |
| FUO 8            | 4                         | DCU water, 2nd flow | 03/09/13                   | 2          | 309d  |
| FUO 10           | 5                         | DCU water, 2nd flow | 03/09/13                   | 2          | 309d  |
| FUO 13           | 6                         | DCU water, 2nd flow | 03/09/13                   | 2          | 309d  |
| FUU 1            | 1                         | DCU water, 2nd flow | 06/09/13                   | 2          | 309d  |
| FUU 4            | 2                         | DCU water, 2nd flow | 06/09/13                   | 2          | 309d  |
| FUU 6            | 3                         | DCU water, 2nd flow | 06/09/13                   | 2          | 309d  |
| FUU 10           | 10                        | DCU water, 2nd flow | 06/09/13                   | 2          | 309d  |
| FUU 12           | 9                         | DCU water, 2nd flow | 06/09/13                   | 2          | 309d  |
| FUU 13           | 6                         | DCU water, 2nd flow | 06/09/13                   | 2          | 309d  |
closely related to *Achromobacter dolens* and *Achromobacter ruhl-
landii*.

**Kinetics and success of *P. aeruginosa* and *Achromobacter* clones in DCUWs.** Bacterial identification and typing data were integrated into the calendar of microbiological follow-up and decontamination measures of the DCUWs from July 2012 to April 2014 (Fig. 5). In July 2012, a general chlorination and purging were performed in order to eliminate *P. aeruginosa* ST309 that had been detected in 4 rooms in June 2012 (Fig. 5). The procedure allowed the elimination of *P. aeruginosa* from the DCUW tested and, thereafter, *Achromobacter* sp. clone A colonized the center endemically. Due to both the high inoculum of *Achromo-
bacter* sp. clone A and the large number of colonized rooms in October 2012, new chlorination and prolonged purging were performed, leading to the disappearance of *Achromobacter* sp. clone A. A sporadic reemergence of *P. aeruginosa* as the sole bac-
terium isolated in rooms 2 and 3 (Fig. 5 and Table 1) was noted on October, and each DCU in these two rooms was treated by an oxidative shock using H2O2 at 6 ppm. After this treatment, a transient colonization of DCU in 4 rooms by *Achromobacter* sp. clone B was noted, followed by a novel episode of contamination of all of the DCUWs by *Achromobacter* sp. clone A. Network decontami-
nation and purging were performed during the 2012 Christmas closure with transient efficacy since *Achromobacter* sp. clone A reemerged in March 2013. *P. aeruginosa* ST309 and ST1753 persisted in a few rooms besides *Achromobacter* sp. clone A. A new attempt at general decontamination of the main network of DCUWs was performed in August 2013 during the summer clo-
sure of the DCC by the use of H2O2 at 6 ppm. It led to the dramatic decrease of *Achromobacter* sp. clone A contamination concomi-
tant with the extensive and massive colonization of all DCUs in each room of the DCC by *P. aeruginosa* ST309, the inoculum of this clone in most samples exceeding 10⁵ CFU 100 ml⁻¹. *Achro-
mobacter* sp. clone A persisted in few samples and then became undetectable. Of note, *P. aeruginosa* had been detected up-
stream from DCUs in the water treatment room (level /H11002/1) on 5 July 2013 before its clonal success in DCUWs in August 2013 (Fig. 5 and Table 1), suggesting the upstream origin of the contamina-
tion.

In conclusion, successive waves of dominant bacterial popula-
tions in the DCUW seemed to be related to the decontamination procedures.

**Biofilm formation.** Under the experimental conditions, *P. aeruginosa* strains produced more biofilm than *Achromobacter* sp. strains (Fig. 6 and 7). According the criteria of Stepanovic (21–34), *P. aeruginosa* strains appeared to be high biofilm producers, whereas *Achromobacter* sp. strains were weak producers or non-
producers under all conditions tested. Among *P. aeruginosa*
strains, ST309 was the stronger biofilm producer (Fig. 6). The temperature influenced biofilm formation mainly for P. aeruginosa ST309, for which the optimum temperature was 25°C (the average temperature in the DCUW). The biofilm formation decreased markedly at 30°C and further at 37°C.

P. aeruginosa biofilm formation was enhanced when P. aeruginosa was cocultured with Achromobacter sp. (Fig. 7). Bacterial numbering in the biofilm obtained after coculture showed that Achromobacter sp. disappeared from the mature biofilm, suggesting that the biofilm overgrowth observed for P. aeruginosa was due to predation against Achromobacter sp. rather to a real synergy between populations. Figure 6 indicates the evolution of biofilm formation of strains according the chronology of their isolation in DCUWs. Variations in biofilm formation were not significant among P. aeruginosa or Achromobacter strains of the same ST or genotype.

Susceptibility of dominant P. aeruginosa and Achromobacter sp. clones to antimicrobial agents. The effect of the biocide compounds used for water network decontamination (H2O2, Sterispray, and chlorine), for surface and surgical material cleaning (Aniosurf and Oxyfloor), and for topical effect in patients of the DCC (chlorhexidine and Betadine) was tested on selected P. aeruginosa and Achromobacter sp. strains. The 17 P. aeruginosa and 9 Achromobacter sp. isolates submitted to the biocidogram were selected on the basis of different sampling dates or sites and belonged to different genotypes. Inhibition zone diameters were compared to those of P. aeruginosa ATCC 9027 and A. xylosoxidans CECT927T, with no growth inhibition being observed for all Achromobacter sp. strains (Table 2). These 2 biocides contained cationic surface-acting agents belonging to the quaternary ammonium compounds (QAC), suggesting the resistance of all isolates to this group of biocides. Because Sterispray was used for water preservation until April 2013, this result may explain the selection of the dominant populations detected in the DCC. The 4 P. aeruginosa strains DXF2, FRM17, FRM18, and FWU showing inhibition zone diameters similar to that of P. aeruginosa ATCC 9027 were isolated from WS samples upstream from BI in the network (Fig. 1), i.e., before Sterispray injection, or from one DCU in August 2013 after stopping the use of Sterispray. It was noteworthy that P. aeruginosa isolate FWN2 sampled at the water supply entrance before WS and BI was tolerant to Sterispray, despite the fact that QAC was not used for municipal water treatment. However, chloramine, which also belongs to the Sterispray formula, was used for municipal water treatment.

For Achromobacter sp., a significant difference in behavior against biocides was observed for rep-PCR type A and type B isolates (Table 2). The growth rates also differed notably between the two groups of strains, with type A isolates showing visible growth on agar plates after 24 h of incubation at 37°C, while for type B isolates, a 48-h incubation time was required (data not shown). The rep-PCR type B clone represented by isolate DMG18, which transiently colonized DCUWs in November 2012, displayed small inhibition zone diameters for H2O2 and Oxyfloor, two agents acting by production of reactive oxygen species (ROS). This was also observed for 3 strains of type A (CXX1, CYS1, and ETC) isolated...
just after H₂O₂ shock procedures in July 2012 and April 2013, whereas other strains of type A, isolated remotely from the H₂O₂ treatment, displayed large inhibition zone diameters around H₂O₂-filled wells (Table 2). Tolerance to ROS of isolate DMG18 could explain the selection of the type B clone just after decontamination of the DCUWs by H₂O₂ shocking, and its low growth rate could explain its rapid replacement by the rep-PCR type A clone in the absence of H₂O₂ in November and December 2012 (Fig. 5 and Table 2). Variation in susceptibility to H₂O₂ was also observed among P. aeruginosa isolates but seemed unrelated to the emergence of P. aeruginosa genotypes and H₂O₂ treatments (Table 2).

Concerning antimicrobial agents used in human medicine, P. aeruginosa isolates displayed inhibition zone diameters equivalent to those obtained for the standard for chlorhexidine and Betadine. The 2 clones of Achromobacter sp. were less susceptible to chlorhexidine and more susceptible to Betadine than the P. aeruginosa isolates. Resistance to antibiotics appeared unrelated to resistance to biocides. Indeed, all strains of P. aeruginosa studied presented the same wild-type antibiotic resistance phenotype in spite of their various levels of tolerance toward biocides. The high level of resistance to H₂O₂ in November and December 2012 (Fig. 5 and Table 2). Variation in susceptibility to H₂O₂ was also observed among P. aeruginosa isolates but seemed unrelated to the emergence of P. aeruginosa genotypes and H₂O₂ treatments (Table 2).

Genomic survey of P. aeruginosa and Achromobacter isolates. The chronological collection of P. aeruginosa ST309/rep-PCR type 2 and Achromobacter sp. clone A isolates from June 2012 to September 2013 allowed the survey of the intraclonal genomic microevolution by PFGE. Figures 8 and 9 show representative genomic patterns obtained for P. aeruginosa and Achromobacter sp. isolates, respectively. The P. aeruginosa ST309 isolates clearly differed from the control strain T45.9 (ST308) representative of one of the most prevalent clones found in the water network of the university hospital of Montpellier, where the DCC is hosted (personal unpublished data). Almost all ST309 isolates from the DCC displayed an identical PFGE profile (pulsotype 1), but the FKR1 isolate displayed pulsotype 2, differing from pulsotype 1 by 2 DNA fragments and revealing the occurrence of one genomic modification between strains (Fig. 8). Of note, this difference between pulsotypes was not observed in the multiplex rep-PCR experiment. This observation suggested that the genomic modification involved either a large rearrangement or a punctual mutation in the SpeI site, both mechanisms being detected by SpeI RFLP in PFGE but not by rep-PCR. The availability of complete genome sequences for P. aeruginosa ST309 allowed the design of specific primers flanking the SpeI restriction site concerned with the genomic modification. Amplification and sequencing of the corresponding region (300 bp) demonstrated that the SpeI site was present in both pulsotype 1 and 2 isolates. The pulsotype modification was therefore attributed to a genomic rearrangement that involved at least 350 kbp. The FKR1 strain was isolated from the water supply in the technical room near the water entrance in the DCC and just before the injector/analyzer of preventive biocide (Sterispray) (BI in Fig. 1). The FKR1 strain was isolated before the curative decontamination undertaken in August 2013, whereas pulsotype I isolates became dominant in the DCUW just after the curative procedure. Another ST309 strain, P. aeruginosa isolate PAPE 6, presented rep-PCR type 2 and a pulsotype related to pulsotype 1. Again a genomic rearrangement involving the higher-SpeI fragment was observed in PFGE patterns (Fig. 8B). This strain had been collected 2 years before the DCC contamination during the routine control of the municipal water supply of Montpellier city, thereby confirming the likely upstream origin of the contamination.
DCC contamination by *P. aeruginosa* originating from the environment outside the hospital. Isolates of ST1753, described herein for the first time (rep-PCR type 3), also displayed intraclonal modifications of PFGE profiles (data not shown) suggestive of genomic rearrangement, as demonstrated for strains of ST309. For *Achromobacter* sp., all clone B isolates displayed the same pulsotype. In contrast, clone A isolates that succeeded in prolonged colonization displayed 4 pulsotypes mostly differing in the 4 higher-DNA fragments of the PFGE patterns (Fig. 9). Isolates CYS and CXX differed in PFGE but displayed the same biocidalgram with reduced susceptibility to H2O2 and Oxyfloor. Isolate ETC showed reduced susceptibility to H2O2 and Oxyfloor but shared its pulsotype with other isolates displaying full susceptibility to H2O2. These results suggested that PFGE profile modifications were not directly linked to the behavior against biocides. The last strain of *Achromobacter* sp. colonizing the DCC (FRM10) that was isolated after the oxidative shock in August 2013 before disappearing displayed a PFGE modification compared with strains isolated from March to July 2013.

**DISCUSSION**

The originality of this study comes from the description of the bacterial population dynamics at the species and genotype level in a complex water system containing 61 dental chairs during a long period (6.5 years). To our knowledge, molecular follow-up had never been performed to investigate DCU contamination. By this means, the dynamics of contamination has been precisely followed, and equilibrium between bacterial species and genotypes has been studied in relation to environmental conditions.

We demonstrated and characterized herein the colonization of DCUs in the DCC by an undescribed species of the genus *Achromobacter* and by *P. aeruginosa* that emerged in DCC after years of persistent and increasing contamination with *Bacillus* sp. These 3 bacterial species were previously described in DCUWs (4, 10). For *Achromobacter* and *P. aeruginosa*, major clones colonized DCUWs during successive waves of clonal success. The *P. aeruginosa* clone ST309 was detected upstream from the DCUWs in the technical room for water treatment and 2 years before in the main water of the city of Montpellier. These results were in favor of a water contamination.
supply origin of the contamination rather than retraction from surgical devices. Indirect insight in favor of the environmental origin of major clones was the wild phenotype of DCC _P. aeruginosa_ regarding antibiotics and antimicrobial topical agents used in human medicine (36,37). The nonhuman origin of _Achromobacter_ sp. was not demonstrated herein, and the isolates presented a potential tolerance to chlorhexidine, which is the most used antiseptic in odontology. Resistance of _Achromobacter xylosoxidans_ to chlorhexidine has been previously demonstrated in two cases of infections caused by the use of contaminated chlorhexidine solution to disinfect the patient’s skin or medical devices (38–40).

Considering the dynamics of microbial colonization of the DCC, _Achromobacter_ sp. was the most prevalent and persistent bacterium in the water system. Particularly, it was present in the water system in July 2012 just after the elimination of _P. aeruginosa_. _Achromobacter_ sp. became dominant in the ecosystem even when _P. aeruginosa_ ST309 was sporadically detected from October 2012 to July 2013. Then the decontamination procedure in July 2013 eradicated _Achromobacter_ sp. and selected _P. aeruginosa_ ST309, which further succeeded in DCUWs. Because the colonization dynamics showing alternative dominancy of _Achromobacter_ sp. and _P. aeruginosa_ ST309 may have been driven by differential resistance/tolerance to biocides, we performed a biocidogram, a method previously shown to be valuable for investigation of susceptibility to antimicrobial topical agents of a large population of bacterial strains isolated during an outbreak period in a burn unit (32). We showed that all of the clones of _Achromobacter_ and _P. aeruginosa_ that succeeded in the DCC presented reduced inhibition zone diameters to Sterispray, the biocide used for daily preservation of the DCUWs. Cross-resistance or tolerance was observed with Aniosurf used for surface cleaning and surgical material rinsing before sterilization. These commercial biocides contained QAC, suggesting a resistance of DCUW bacterial strains to QAC. The gene _qacF_ (quaternary ammonium compound resistance gene) has been described in _P. aeruginosa_ on a class 1 integron containing gene cassettes for resistance to carbapenem, aminoglycosides, chloramphenicol, and extended-spectrum β-lactams (41). Because the tolerance to QAC observed in this study was not associated with multiresistance to antibiotics, another mechanism of resistance to QAC than _qacF_ in class 1 integron is probable.

For some strains, biocidograms showed a tolerance to 3% H₂O₂ and to Oxyfloor, a biocide containing peracetic acid and thus acting as H₂O₂ by ROS liberation. This tolerance appeared mostly for isolates recovered just after the decontamination procedure by H₂O₂ shock. ROS are classically described as cy-
totoxic by nonspecific cell structure oxidization, against which it is unlikely that bacteria would develop resistance (42). However, recent studies showed that *P. aeruginosa* possesses a number of antioxidant defense enzymes under the control of multiple regulatory systems (43). Particularly, *P. aeruginosa* PAO1 adapted to a novel antimicrobial agent (zinc Schiff-base) by prolonged contact showed increased tolerance against hydrogen peroxide (44). Tolerance to H$_2$O$_2$ with various expressions related to bacterial adaptation was also described in *A. xylosoxidans* recovered from polluted environments (45).

Altogether, the results from biocidograms were similar for biocides displaying the same mechanism of action (H$_2$O$_2$ and Oxylower and Sterispray and Aniosurf) and supported a reasonable interpretation explaining the success of the different clones identified in this study. Based on these results, the main hypothesis supporting the occurrence of the successive colonization waves was that Sterispray treatment led to selective dominancy of *Achromobacter* sp. over *P. aeruginosa* because *Achromobacter* sp. appeared more tolerant to this biocide than *P. aeruginosa*. After Sterispray use was stopped in April 2013, the decontamination shock in August 2013 reduced the biomasses of both bacteria. Then the population of *P. aeruginosa* succeeded against *Achromobacter* sp. in the absence of Sterispray treatment.

The biocidogram is not a validated assay for determination of biocide effect. However, this study demonstrated that biocide diffusion assays rapidly give valuable results that helped in the investigation of outbreaks or contaminations when a large population of bacterial strains has to be tested, as previously shown for antimicrobial topical agents (32). In contrast with antibiograms, biocidograms should be interpreted by comparison of strain behavior with reference strains or within a population because standardized data linking inhibition zone diameters to susceptibility or resistance are not available.

Biofilm formation is a major mechanism explaining the frequent contamination of technological and medical water-connected devices such as DCUs (1–4). We showed that the *P. aeruginosa* ST309 strain that succeeded in the DCC was considered a high biofilm producer. However, *Achromobacter* sp. that presented low or no biofilm formation capacities also succeeded in DCC colonization during some periods. Finally, the present study that chronologically follows the DCC colonization at the strain and genotype level according to biocide decontamination events suggested that the clonal successes were mainly due to the resistance to biocide treatments rather to the high level of biofilm formation. The main argument is the success of the high-biofilm-forming strain of *P. aeruginosa* ST309 in colonizing the DCC that occurred after the elimination of the low-biofilm-forming *Achromobacter* sp. strain by changes in biocide treatments (5 decontaminations and after the stopping of Sterispray use). As observed in experimental biofilm formation after cocultures, the previous col-

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**FIG 7** Study of biofilm formation of *P. aeruginosa* and *Achromobacter* sp. strains alone or combined at three temperatures. Error bars represent the standard deviation. ODc, 3 standard deviations above the mean optical density of the negative control.
## TABLE 2 Results of biocidogram and antibiotyping assays for Achromobacter sp. and *P. aeruginosa* DCC isolates

| Isolate decontamination procedure | Date (day/mo/yr) | ST/ type | Room of sampling | Antibiotype | Inhibition zone diam (mm) with: | Sterispray | 3% H$_2$O$_2$ | Oxysfloor | Aniosurf | Dakin | 10% povidone–iodine | Chlorhexidine | Chlorine |
|----------------------------------|------------------|----------|-----------------|-------------|-------------------------------|-----------|---------------|------------|----------|-------|--------------------------|--------------|--------|
| *P. aeruginosa* ATCC 9027 (standard for antimicrobial assays) | 10/07/12 A 1 | CAF (R5) | 8 16.9 19.9 8 | 30 30.2 16 28 | 10% povidone–iodine | Chlorhexidine | Chlorine |
| A. xylosoxidans CECT 927T (standard for antimicrobial assays) | 07/01/13 A 2 | AzF (R3) | 8 38 34 | 8 29 34 | 16.6 19 | 16.6 19 | 16.6 19 |
| *P. aeruginosa* CUZ 1 | 28/06/12 309 1 | wt | 11.2 26 30 | 8 20 15.2 | 24.2 21 | 15.2 24.2 | 15.2 24.2 |
| *P. aeruginosa* CUZ 2 | 28/06/12 253 4 | wt | 8 26 50 | 8 20 12.4 | 24.5 20 | 24.5 20 | 24.5 20 |
| *P. aeruginosa* CXD 3 | 04/07/12 309 10 | wt | 11.2 26 30 | 8 20 15.2 | 24.2 21 | 15.2 24.2 | 15.2 24.2 |

The inhibition zone diameter is given in millimeters for each biocide tested (where 8 mm is the hole diameter) on representative isolates selected according to genotype and sampling date and site. The isolates are chronologically presented, and the major decontamination procedures are indicated at the corresponding date. Diameters and antibiotypes in boldface corresponded to particular tolerance or resistance against biocides/antibiotics in the population tested.

注: 用于每个抗生素测试的抑制圈直径（孔直径为8 mm）是按照根据基因型和采样日期和地点选择的代表性菌株。这些菌株按照时间顺序呈现，并在相应日期指示了主要的除污染程序。带粗体的直径和抗生素类型对应于在所测试的生物体中对生物ocide/抗生素的特定耐受或抗性。

*See Fig. 1 for room denomination.*

*The abbreviations for the antibiotic resistance profile are as follows: wt, wild type (i.e., resistance to cefotaxime only in *P. aeruginosa*); CAF, resistance to cefotaxime, aminoglycosides, and fluoroquinolones; AzF, resistance to aztreonam and fluoroquinolones; C, resistance to cefotaxime; R2, multidrug resistance to 2 classes of antibiotics, R3, multidrug resistance to 3 classes of antibiotics, R5, multidrug resistance to 5 classes of antibiotics.*
ionization by Achromobacter sp. genotype A could lead to P. aeruginosa colonization because of enhanced biofilm formation mainly if the previous Achromobacter sp. genotype A biofilm has been deteriorated by biocide treatments. Depending on the sampling site, the P. aeruginosa clone ST309 displayed different but related pulsotypes, suggesting intraclonal genomic rearrangements. Comparative genomics of P. aeruginosa presents a picture of a mosaic genome with strain-specific segments that interrupt the core genome (24, 46). As for other bacteria, genome composition and plasticity appear to be related to niche adaptation (24). Some large inversions described by comparative genomics of strains PAO1, PACS2, and PA2192 could probably have been detected by the PFGE genome survey performed herein (11–24). This is also the case for movements of genomic islands of the pKLC102/PAGI-2 family that represent hot spots of inter- and intraclonal genomic diversity in P. aeruginosa (46). Because the two ST309 genomotypes colonized two different niches, the water in the main pipe before biocide treatment and water in DCUWs, we reasonably assumed that the genomic changes observed by PFGE were related to niche adaptation. Moreover, the genomic sequences of strains BL09 and BL20 that belonged to ST309 suggested that the fragments that underwent rearrangements carried phage sequences and other mobile elements well known to promote such genomic rearrangements. Genomic variations in related strains were also described by PFGE for members of the genus Achromobacter that chronically colonized the respiratory tract of patients with cystic fibrosis (47, 48), a particular niche where different forces and selective pressures are exerted on the respiratory tract microbiota and drive bacterial selection and adaptation. Here, the Achromobacter sp. strain FRM10 showing genomic variation was detected during a major change in the DCUW microbial community when the decreasing population of Achromobacter was replaced by P. aeruginosa ST309, also suggesting that genome modifications might correspond to adaptive processes in the Achromobacter sp. population.

The Centers for Disease Control and Prevention and the American Dental Association agreed that the maximum allowable contamination of dental treatment water should not exceed 500 CFU ml⁻¹ (7), an inoculum that roughly corresponds to potable-quality water in European Union countries (ISO/EN 6222). This high threshold is thought to be related to low infectious risk (13), but it appears rather lax considering the microbial dynamics in the DCU niche. From our experience, tolerating 500 CFU ml⁻¹ of water will inevitably lead to uncontrollable waves of bacterial contamination. A high load of pathogenic bacteria as described herein with more than 5.10⁴ CFU 100 ml⁻¹ of P. aeruginosa, certainly presented a local risk because dental surgery is becoming more and more invasive (e.g., gingival grafting, dental implant surgery, etc.)
and because the aerosolization during dental care can introduce the pathogen both high and deep in the respiratory tract. Moreover, it is conceivable that the frequency of infection could be underestimated for several reasons: (i) most patients in odontology are outpatients without available clinical records, (ii) local infectious complications after dental care or surgery are generally not investigated for microbiology, (iii) the impact of transient colonization of patient’s tissues remains even more unknown, (iv) epidemiological links between dental care and sporadic respiratory tract or eye infections are rarely evoked because they have been poorly described in medical literature, and (v) for mycobacterial respiratory tract infections, the clinical manifestations develop months after exposure, rendering the link with dental care impossible to establish. Besides direct pathogenicity, massive bacterial contamination increases the level of endotoxins in the water used for dental care. Endotoxins in DCUWs have been described to adversely affect healing after gingival surgery (16) and to promote occupational asthma in dentists (17).

The massive and persistent contamination of all DCUs by at least $5 \times 10^4$ CFU 100 ml$^{-1}$ of P. aeruginosa and the long-term inefficacy of the different control measures led the reorganization of the DCC. Each DCU is now independent from the others, limiting the risk of bacterial diffusion to the whole center from a localized contaminated site and facilitating the control measures in case of contamination. A bottle of sterile pharmaceutical water with 0.03% H$_2$O$_2$ is used for the care water supply of each DCU. Since our results suggested adaptive process that led to bacterial H$_2$O$_2$ tolerance, it has been proposed to alternate biocides monthly or weekly. After 6 months of new conditions and procedures, less than 1 CFU of mesophilic bacteria per 100 ml of water was detected in 56 samples from all CDD rooms. The contamination of the DCC of Montpellier hospital led to 3 main practical lessons in

![PFGE of SpeI-digested genomic DNA from Achromobacter sp. isolates. Isolate names, dates of sampling, and profile names are indicated according to Table 1. The correspondence with multiplex rep-PCR profiles (Fig. 4) is given at the bottom of the gel. The Lambda concatemer was used as a molecular size marker.](image-url)
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