AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Clinical Microbiology®

BACTERIOLOGY

Impact of High Diversity of *Achromobacter* Populations within Cystic Fibrosis Sputum Samples on Antimicrobial Susceptibility Testing

Chloé Dupont,[a] Estelle Jumas-Bilak,[a,b] Anne-Laure Michon,[a,c] Raphaël Chiron,[d] Hélène Marchandin[a,c]

Université Montpellier, UMR5569 Hydrosciences Montpellier, Equipe Pathogènes Hydriques, Santé, Environnements, UFR des Sciences Pharmaceutiques et Biologiques, Montpellier, France[a]; Centre Hospitalier Régional Universitaire de Montpellier, Hôpital Saint-Eloi, Laboratoire d'Hygiène hospitalière, Montpellier, France[b]; Centre Hospitalier Régional Universitaire de Montpellier, Hôpital Arnaud de Villeneuve, Laboratoire de Bactériologie, Montpellier, France[c]; Centre Hospitalier Régional Universitaire de Montpellier, Hôpital Arnaud de Villeneuve, Centre de Ressources et de Compétences pour la Mucoviscidose, Montpellier, France[d]

ABSTRACT Chronic colonization by opportunistic environmental bacteria is frequent in the airways of cystic fibrosis (CF) patients. Studies of *Pseudomonas aeruginosa* evolution during persistence have highlighted the emergence of pathoadaptive genotypes and phenotypes, leading to complex and diversified inpatient colonizing populations also observed at the intraspecimen level. Such diversity, including heterogeneity in resistance profiles, has been considered an adaptive strategy devoted to host persistence. Longitudinal genomic diversity has been shown for the emergent opportunistic pathogen *Achromobacter*, but phenotypic and genomic diversity has not yet been studied within a simple CF sputum sample. Here, we studied the genomic diversity and antimicrobial resistance heterogeneity of 132 *Achromobacter* species strains (8 to 27 strains of identical or distinct colonial morphotypes per specimen) recovered from the sputum samples of 9 chronically colonized CF patients. We highlighted the high within-sample and within-morphotype diversity of antimicrobial resistance (disk diffusion) and genomic (pulsed-field gel electrophoresis) profiles. No sputum sample included strains with identical pulsotypes or antibiotic susceptibility patterns. Differences in clinical categorization were observed for the 9 patients and concerned 3 to 11 antibiotics, including antibiotics recommended for use against *Achromobacter*. Within-sample antimicrobial resistance heterogeneity, not predictable from colonial morphology, suggested that it may represent a selective advantage against antibiotics in an *Achromobacter* persisting population and potentially compromise the antibiotic management of CF airway infections.

KEYWORDS *Achromobacter*, antimicrobial susceptibility, cystic fibrosis, diversity, genome

Received 18 September 2016 **Returned for modification** 30 September 2016 **Accepted** 25 October 2016

Accepted manuscript posted online 2 November 2016

Citation Dupont C, Jumas-Bilak E, Michon A-L, Chiron R, Marchandin H. 2017. Impact of high diversity of *Achromobacter* populations within cystic fibrosis sputum samples on antimicrobial susceptibility testing. J Clin Microbiol 55:206–215. https://doi.org/10.1128/JCM.01843-16.

Editor Robin Patel, Mayo Clinic

Address correspondence to Hélène Marchandin, helene.marchandin@umontpellier.fr.

Bacterial adaptation during persistence in the respiratory tract of cystic fibrosis (CF) patients has mainly been studied for *Pseudomonas aeruginosa* showing that pathoadaptation mechanisms are particularly efficient, leading to rare and hardly achievable *P. aeruginosa* eradication. Many studies have revealed a variety of phenotypic pathoadaptative traits during long-term colonization, such as auxotrophy and metabolic modifications, quorum-sensing alterations, biofilm production, loss of motility and virulence factors, emergence of mucoid colonies, and an increase of antibiotic resistance (for a review, see reference 1). Moreover, numerous genomic modifications, which were either directly related to phenotype or not, were observed (1, 2). Other species, such as the *Burkholderia cepacia* complex, *Stenotrophomonas maltophilia*, and

Staphylococcus aureus, also displayed adaptive features during lung persistence that were not observed in non-CF patients (3). Most studies that focused on sequential isolates gave results that were interpreted in terms of the longitudinal adaptive evolution of the colonizing strain based on the "assumption that bacterial populations at any given time are genetically uniform" (4). The standard approach in routine clinical microbiology has for a long time been based on the same lasting dogma, i.e., analysis (identification and antimicrobial susceptibility testing) of a single "pure" colony for each potential pathogen. However, CF *P. aeruginosa* populations were shown to be highly diverse. Indeed, in the same sputum sample, colonies that were assayed for a range of phenotypic traits, known to be altered during evolutionary adaptation, showed significant within-specimen diversity (4–7). The resulting coexistence of variant lineages in the respiratory tract of CF patients suggested that they arose from diverse selection pressures in the lung and then developed distinct evolutionary pathways (5, 6, 8). This diversity is considered in French microbiological procedures for CF samples, which recommend the performance of antimicrobial susceptibility testing on each *P. aeruginosa* colonial morphotype detected by visual inspection of agar medium plates (9). However, morphotypes were previously shown to be unreliable predictors of antimicrobial susceptibility (10), suggesting that, depending on the choice of colonies to be submitted for *in vitro* antimicrobial susceptibility testing, results may not reflect the overall resistance exhibited by the *P. aeruginosa* population in the CF lung.

Members of the genus *Achromobacter* are recognized as emerging opportunistic pathogens in CF (11) with variable prevalence depending on CF centers and on the occurrence of outbreaks (12). *Achromobacter xylosoxidans* is the most prevalent species that chronically colonizes CF airways. Its ability to induce local inflammation, as done by *P. aeruginosa*, suggests its pathogenicity (13). Previous studies reporting longitudinal genetic and phenotypic variations of *A. xylosoxidans* during chronic colonization (14–16) prompt the assessment of the intraspecimen diversity of *Achromobacter* species populations by studying the genome fingerprint (pulsed-field gel electrophoresis [PFGE]) and antibiotic susceptibility profile variability.

RESULTS

Patients, morphotype diversity, and studied strains. At the time of sampling, the 9 patients were 11 to 52 years old and were chronically colonized by *Achromobacter* sp. for 5 to 18 years. We observed 3 to 11 different colony morphotypes per sputum sample (Table 1) (an average of 5.5 morphotypes per specimen) and studied a total of 50 colonial morphotypes defined at the intraspecimen level. For some morphotypes, only 1 (3 samples, 6 morphotypes) or 2 (5 samples, 6 morphotypes) colonies could be picked for analysis. In total, we studied 132 colonies (8 to 27 colonies per specimen).

Genomic diversity of *Achromobacter* sp. at intrasputum level. For each patient, colonies corresponded to identical or clonally related strains in PFGE. According to the results of a previous study, including strains from these 9 patients (15), no cross-transmission between patients was observed (Fig. 1). In addition, identical pulsotypes were observed during both previous and current studies, thereby showing that the 9 patients are colonized by persisting strains.

No sputum sample harbored colonies with identical PFGE profiles. We found 2 to 8 different pulsotypes per sputum sample (Fig. 1; Table 1), with an average of 3.5 PFGE profiles per specimen. Pulsotypes were mostly unrelated to the colonial morphotype (Fig. 1). Indeed, within 45 morphotypes (90%), at least one colony shared an identical pulsotype with at least one colony of a distinct morphotype. Contrarily, 16 morphotypes of the 44 for which more than one colony was studied (36.4%) included strains of different pulsotypes, and in all of the samples studied, at least one colonial morphotype gathered colonies with different PFGE profiles. The number of morphotypes was not related to the number of distinct PFGE profiles in a sample (Fig. 1; Table 1).

Heterogeneity of antibiotic susceptibility patterns. The coefficient of variation (CV) ranged from 38% to 68% for the 16 antimicrobial agents and the 132 strains tested, with imipenem and colistin presenting the lowest zone of inhibition (ZOI) measure

TABLE 1 Summarized data for the 9 patients and samples included in the study and main results of intrasample and intramorphotype diversity study

Patient designation	Age (yr)	*Achromobacter* colonization period (yr)	Sputum sample data		Pulsotype diversity		Antimicrobial susceptibility diversity[a]				
							Antibiotype diversity per sample		Antibiotype diversity per morphotype		
			No. of morphotypes	No. of strains studied	Pulsotype no. per sample	Pulsotype no. per morphotype	CV (%)	No. (%) of antimicrobial agents with distinct clinical categorization (S/R)	CV (%)	No. (%) of antimicrobial agents with distinct clinical categorization (S/R)	Antimicrobial agents with different clinical categorization (S/R)[b]
P1	52	18	7	16	3	1 to 2	15	7 (44)	0–10	0–3 (0–19)	AMC, PIP, TPZ, CAZ, C, LEV, SXT
P2	17	10	11	27	2	1 to 2	21	11 (69)	3–27	1–6 (6–37.5)	AMX, AMC, TIC, TCC, CAZ, IMP, CS, C, CIP, LEV, SXT
P3	20	9	4	12	2	1 to 2	9	3 (19)	6–9	0–1 (0–6)	AMX, ETM, C
P4	15	13	3	9	4	1 to 2	21	4 (25)	7–18	1–3 (6–19)	TPZ, CS, C, SXT
P5	19	9	5	15	6	1 to 3	19	6 (37.5)	2–12	0–3 (0–19)	CAZ, CS, C, LEV, SXT, TET
P6	13	9	6	13	8	1 to 3	20	5 (31)	7–14	0–2 (0–12.5)	PIP, TPZ, CAZ, CIP, LEV
P8	20	11	7	21	3	1 to 3	20	6 (37.5)	2–17	0–3 (0–19)	AMC, TIC, CAZ, MEM, CS, C
P11	25	6	4	11	2	1 to 2	11	3 (19)	5–11	0–2 (0–12.5)	ETM, CS, SXT
P12	11	5	3	8	2	1 to 2	21	7 (44)	13–27	2–5 (12.5–31)	AMX, AMC, CAZ, MEM, CS, C, SXT

[a]CV, coefficient of variation of zones of inhibition measured in millimeters; S, susceptible; R, resistant (includes resistant and intermediate clinical categorizations).

[b]AMX, amoxicillin; AMC, amoxicillin-clavulanic acid; PIP, piperacillin; TPZ, piperacillin-tazobactam; TIC, ticarcillin; TCC, ticarcillin-clavulanic acid; CAZ, ceftazidime; IMP, imipenem; ETM, ertapenem; MEM, meropenem; CS, colistin; C, chloramphenicol; LEV, levofloxacin; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline. Underlined antimicrobial agent names indicate that clinical categorization varied at the intrasample level only while identical at the intramorphotype level.

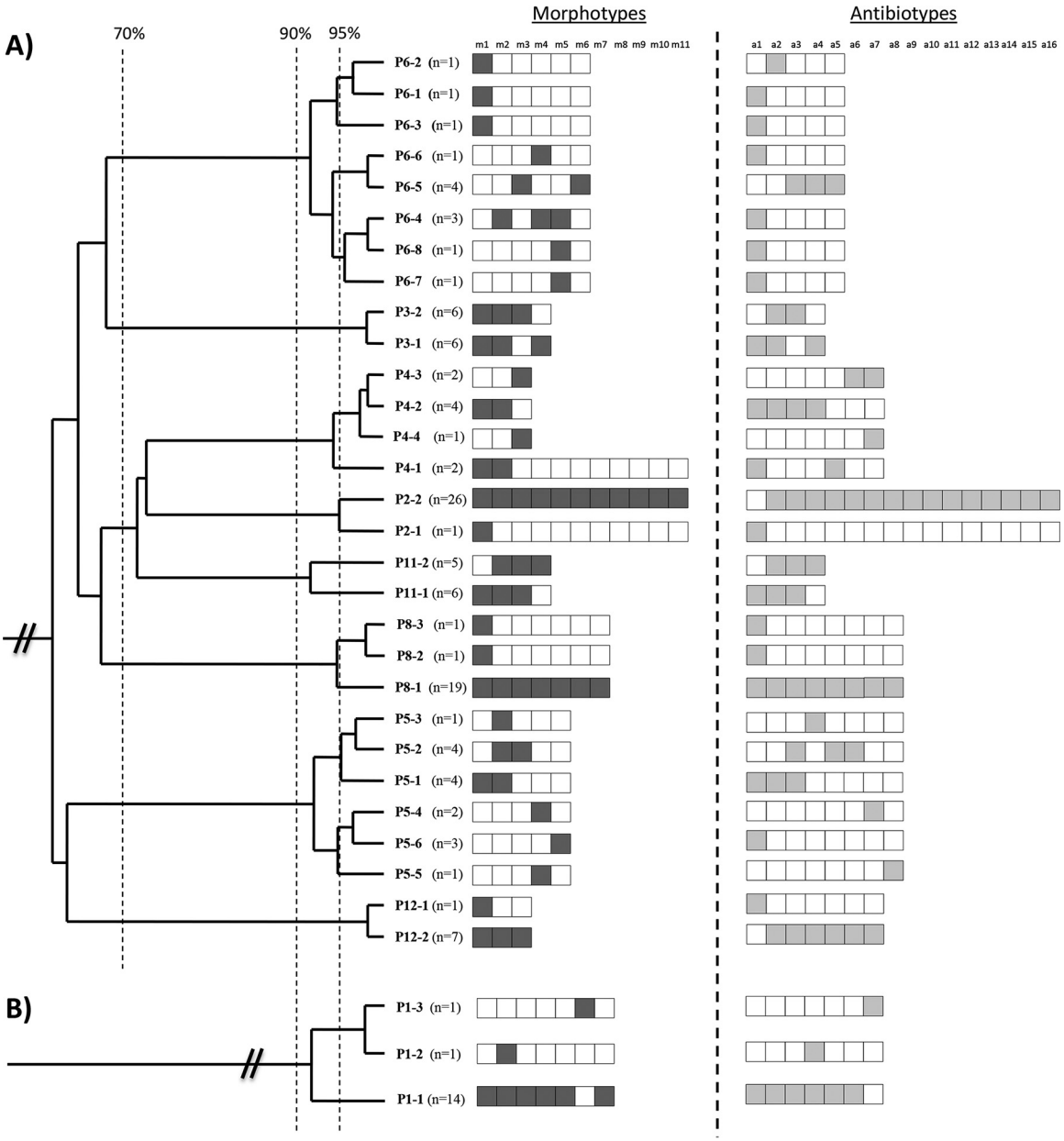

FIG 1 Relation between genomic and phenotypic variability among 132 strains recovered from sputum samples in 9 patients chronically colonized by *Achromobacter* spp. (A) *A. xylosoxidans*; (B) *A. insuavis*. (Left) Genomic diversity among SpeI-generated pulsotypes at the interpatient level and at the intrasample level. Dendrogram was obtained by clustering analysis based on the Dice coefficient and unweighted pair group method with arithmetic means (UPGMA). The Dice coefficient scale is shown at the top of the dendrogram. Pulsotypes were named after the patient number (P1, P2, etc.) and a unique number for each pulsotype observed. The number of strains showing a pulsotype is indicated after the pulsotype name. (Right) Diversity of colonial morphotypes and of antibiomicrobial susceptibility patterns in relation to pulsotypes. The different colonial morphotypes and antibiotypes were defined at the intraspecimen level and were designated by m1, m2, etc. (up to 3 colonies were studied per colonial morphotype) and a1, a2, etc., respectively. The dark gray boxes represent the different morphotypes observed for strains with the opposite PFGE profile. The light gray boxes represent the different antibiotypes presented by strains with the opposite PFGE profile.

dispersion and tetracycline and ertapenem presenting the highest ZOI measure dispersion (Tables 2 and 3). Within a specimen, global CV varied from 9% to 21% with different situations observed depending on the sample; variability was observed for the 16 antimicrobial agents in 2 cases, and variability was detected for 7 to 15 agents in other cases. The antimicrobial agent showing the highest variability in ZOI measures varied according to the sample but was co-trimoxazole in 5 samples. Within a morphotype, CVs of up to 27% were observed (Table 1).

TABLE 2 Breakpoints considered in this study

Species	Antibiotic breakpoint (mm)[a]															
	AMX	AMC	TIC	TCC	PIP	TPZ	CAZ	ETM	IPM	MEM	CS	C	CIP	LEV	SXT	TET
Enterobacteriaceae	19	19	23	23	17–20	17–20	19–22	22–25	16–22	16–22		17	19–22	19–22	13–16	
P. aeruginosa			18	18	18	18	16		17–20	18–24			22–25	17–20		
Acinetobacter spp.			15–20	15–20	18–21	18–21	15–18		17–23	15–21			21	18–21	13–16	12–15
S. maltophilia														12–17	16	
B. cepacia							18–21			16–20					11–16	

[a]Breakpoints are those edited by the Antibiogram Committee of the French Society for Microbiology in 2016 (CA-SFM 2016) for *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *Acinetobacter* spp., *Stenotrophomonas maltophilia*, and *Burkholderia cepacia*. AMX, amoxicillin (25 μg); AMC, amoxicillin-clavulanic acid (20 μg/10 μg); PIP, piperacillin (75 μg); TPZ, piperacillin-tazobactam (75 μg/10 μg); TIC, ticarcillin (75 μg); TCC, ticarcillin-clavulanic acid (75 μg/10 μg); CAZ, ceftazidime (30 μg); IMP, imipenem (10 μg); ETM, ertapenem (10 μg); MEM, meropenem (10 μg); CS, colistin (50 μg); C, chloramphenicol (30 μg); LEV, levofloxacin (5 μg); CIP, ciprofloxacin (5 μg); SXT, trimethoprim-sulfamethoxazole (1.25 μg/23.75 μg); TET, tetracycline (30 μg).

TABLE 3 Intrasample diversity of *in vitro* activity of 16 antimicrobial agents and impact of breakpoints used for clinical categorization

Patient	ZOI	Antibiotic[b]															
		AMX	AMC	TIC	TCC	PIP	TPZ	CAZ	ETM	IPM	MEM	CS[c]	C	CIP	LEV	SXT	TET
1	Minimum (mm)	8	14	30	33	18	20	14	32	30	27	23	9	12	15	15	25
	Maximum (mm)	15	25	40	44	44	44	24	40	38	38	28	23	20	23	30	30
	CV[a] (%)	17	14	13	8	**26**	**26**	16	8	8	10	5	25	17	14	20	8
2	Minimum (mm)	10	15	15	18	34	32	6	11	17	23	6	6	6	9	6	6
	Maximum (mm)	30	25	36	32	43	42	24	24	40	34	28	18	23	21	22	6
	CV (%)	30	16	23	18	7	8	34	20	17	10	34	28	21	21	**46**	0
3	Minimum (mm)	18	19	32	30	36	36	21	20	26	24	16	10	9	9	20	6
	Maximum (mm)	23	25	36	35	40	40	26	34	36	34	23	19	12	13	28	6
	CV (%)	6	8	4	4	5	5	7	19	8	9	12	**20**	8	11	13	0
4	Minimum (mm)	6	6	6	6	10	10	6	6	32	12	6	15	6	10	6	6
	Maximum (mm)	6	14	10	12	20	21	12	6	36	18	26	20	20	20	23	9
	CV (%)	0	27	14	21	19	18	25	0	3	16	44	13	37	30	**61**	9
5	Minimum (mm)	6	6	6	6	6	6	6	6	6	6	10	6	6	6	6	6
	Maximum (mm)	6	6	6	6	6	6	18	6	6	6	33	30	12	23	40	23
	CV (%)	0	0	0	0	0	0	42	0	0	0	29	38	19	55	**69**	51
6	Minimum (mm)	6	6	6	6	6	9	6	6	6	6	13	22	13	11	30	6
	Maximum (mm)	6	6	6	6	23	24	30	20	18	6	23	34	27	33	40	11
	CV (%)	0	0	0	0	41	35	**51**	44	27	0	21	16	24	30	13	11
8	Minimum (mm)	6	6	14	21	40	40	6	6	32	6	6	9	6	6	6	6
	Maximum (mm)	16	25	44	45	45	45	45	12	40	28	12	25	10	12	6	6
	CV (%)	26	31	22	19	5	5	**40**	26	8	47	23	35	11	21	0	0
11	Minimum (mm)	6	24	6	27	6	6	6	12	30	29	10	6	6	6	6	6
	Maximum (mm)	18	28	6	35	6	6	6	32	34	32	20	12	10	10	25	6
	CV (%)	23	6	0	7	0	0	0	25	5	3	30	22	12	12	**32**	0
12	Minimum (mm)	6	10	30	25	30	30	11	6	24	16	10	10	6	6	6	6
	Maximum (mm)	30	19	40	38	40	40	22	10	40	28	23	28	10	10	30	10
	CV (%)	36	25	11	14	8	8	29	14	16	19	34	34	18	19	**43**	15
Total	Minimum (mm)	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
	Maximum (mm)	30	28	44	45	45	45	45	40	40	38	33	34	27	33	40	30
	CV (%)	47	41	58	50	51	50	58	65	38	47	39	39	41	45	60	**68**

[a]Coefficient of variation of zones of inhibition measured in millimeters.
[b]Antibiotic name abbreviations are given in the footnote to Table 2.
[c]Susceptibility breakpoint of 12 mm was applied according to Biswas et al. (26).

Eighteen discrepant categorizations, mainly affecting levofloxacin ($n = 5$ samples) and ciprofloxacin susceptibility results ($n = 3$ samples), were observed when the different thresholds available for other Gram-negative bacilli were applied (Table 2). In the absence of recommended thresholds for *Achromobacter* spp., these observations highlighted the impact of the choice of breakpoints edited for other bacilli on *Achromobacter* susceptibility results.

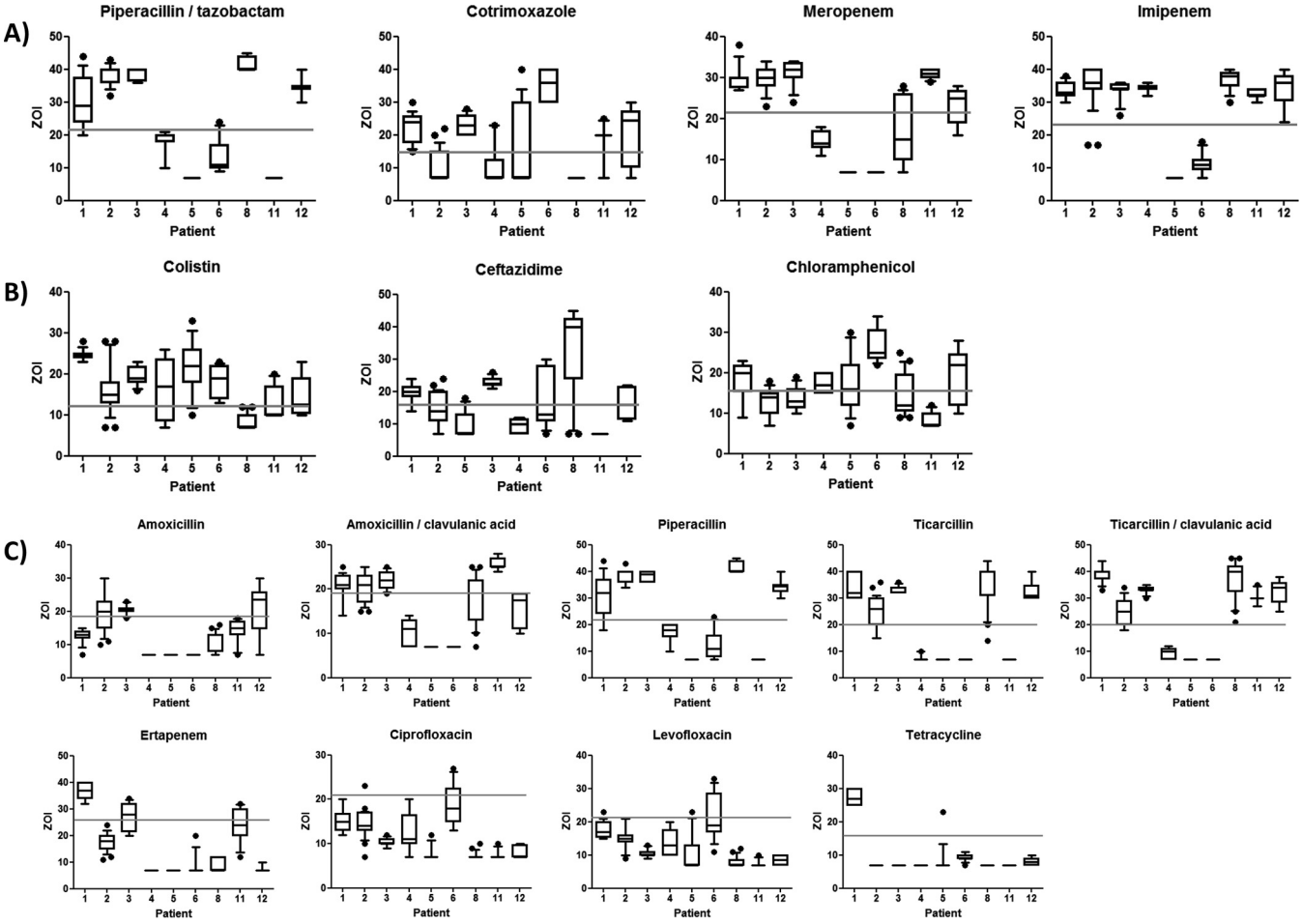

FIG 2 Intrasample variations in antimicrobial susceptibilities toward 16 antimicrobial agents of *Achromobacter* spp. recovered from sputum specimens in 9 patients. (A) First-line antibiotics; (B) second-line antibiotics; (C) other antimicrobial agents. Whiskers plots illustrate the spread of zone of inhibition (ZOI) measures in millimeters for multiple strains within a sample in 9 patients chronically colonized with *Achromobacter insuavis* (patient 1) or *Achromobacter xylosoxidans* (other patients) for the 16 antimicrobial agents studied. For each patient, 8 to 27 strains were analyzed. The gray line indicates the susceptibility cutoff point edited for *Acinetobacter* sp. by the Antibiogram Committee of the French Society for Microbiology in 2016 or, when not available, that edited for *Enterobacteriaceae*. For colistin, the cutoff was that defined by Biswas et al. (26).

Differences in clinical categorization were observed for the 9 patients and concerned 3 to 11 antibiotics (Fig. 2), with chloramphenicol, ceftazidime, colistin, and co-trimoxazole being the most affected agents and ticarcillin-clavulanic acid, imipenem, and tetracycline being the less affected agents (Table 1). Divergent categorization was mainly noted within a morphotype (Table 1) and was observed for one to six antimicrobial agents within 34 of the 44 morphotypes for which more than one colony was studied (77%).

Altogether, our results showed that depending on the patient, the antimicrobial agent, and the strain morphotype, antimicrobial susceptibility patterns may be incompletely described when only one colony of each morphotype in a sputum sample is selected for antimicrobial susceptibility testing.

Finally, no clear relation between antibiotypes and pulsotypes was observed because we found that among the 17 pulsotypes, including several strains, the majority ($n = 14$; 82%) included strains presenting distinct antibiotypes (Fig. 2), but conversely, among the 24 antibiotypes presented by several strains in a sample, strains mostly had identical pulsotypes ($n = 14$; 58%).

DISCUSSION

The few longitudinal studies available in the literature reported genetic, genomic, and phenotypic variations in CF *Achromobacter* strains over time. Dynamics of pheno-

type (biofilm formation, motility, antimicrobial resistance), genotype (studied by PFGE, PCR on bacterial repetitive sequences [rep-PCR], randomly amplified polymorphic DNA [RAPD]), and genome (whole-genome sequencing [WGS]) suggested within-host evolution of clonal lineages linked to adaptation or pathoadaptation mechanisms. For example, WGS comparing longitudinally collected isolates of *A. xylosoxidans* and *Achromobacter insuavis* that were isolated from CF patients identified strain-specific mutations in genes mostly involved in general metabolism but also in virulence and antimicrobial resistance (17). Moreover, genome fingerprint diversity was a frequent finding during *Achromobacter* persistence (12 out of the 13 patients in the study by Dupont et al. [15] and 10 out of the 14 patients in the study by Amoureux et al. [14]).

Although not designed to study within-sample diversity, these studies suggested that genomic variants may coexist within samples. In a previous study, PFGE and/or multiplex rep-PCR detected genomic diversity at the intraspecimen level in 11 out of 22 samples containing multiple *Achromobacter* strains and in 6 out of the 9 patients studied (15). Intraspecimen diversity in *P. aeruginosa* populations was recently demonstrated with heterogeneous populations composed of multiple coexisting variants derived from a single ancestor in a CF lung (7). Regarding phenotype, a study investigating up to 15 characteristics for up to 40 colonies of *P. aeruginosa* cultured from a single CF specimen showed a high and dynamic diversity, including pathoadaptive traits, such as antibiotic resistance, making accurate diagnosis and treatment challenging (4). *P. aeruginosa* population genomics revealed extensive within-patient genetic diversity (4). In our study, dedicated to intraspecimen *A. xylosoxidans* diversity, each patient was colonized by at least two related strains in PFGE. Despite the fact that it is likely that strain diversity, which is a dynamic process highly dependent on niche conditions, may have evolved since the previous study by Dupont et al. (15), we compared the pulsotype diversity observed in both studies. For *A. xylosoxidans*, diversity was either the same (5 patients) or greater (3 patients) within a unique sample in the present study than that observed for strains collected over 13 to 38 months. The most striking example was noted for strains in patient 6 because no genomic variation was previously observed during a 3-year period of colonization (2009 to 2012), while 9 genomic profiles were found for strains recovered from a unique specimen in 2015. For patient 1, who was colonized by *A. insuavis*, we previously found an exceptionally high genomic diversity over the 5-year period of colonization studied (15), contrasting with the low intraspecimen diversity observed here, suggesting that strain behavior and persistence strategy may differ according to the species.

The main consequence of within-specimen diversity on the therapeutic management of CF airway infections may be the incomplete evaluation of population resistance.

Treatment options for such infections are often limited by the innate resistance of *Achromobacter* spp. to most cephalosporins except ceftazidime and to the aminoglycosides. *In vitro* studies showed that the most active drugs against *A. xylosoxidans* were carbapenems, piperacillin-tazobactam, minocycline, and co-trimoxazole (18–20). The most active additive or synergistic combinations were chloramphenicol plus minocycline, ciprofloxacin plus imipenem, and ciprofloxacin plus meropenem (21). Consequently, first-line treatment options against *A. xylosoxidans* included piperacillin-tazobactam, carbapenems (imipenem and meropenem), and trimethoprim-sulfamethoxazole, while second-line drugs are ceftazidime, minocycline, colistin, and chloramphenicol (22). In addition, acquired resistance in *Achromobacter* spp., leading to multidrug resistance and pan-resistance, has been reported for CF strains (23, 24). Therefore, it has been recommended that antimicrobial therapy should be directed by susceptibility testing (25). In this study, we used a disk diffusion assay, a method that has been frequently used in routine practice and, despite the fact that it is not a reference method, has been shown to be able to predict the MIC values for some antibiotics that are active against *Achromobacter* spp. like carbapenems, co-trimoxazole, and colistin in comparative studies of resistance test methods (19, 26). In the absence of specific recommendations to perform antimicrobial susceptibility test-

ing and to interpret results for *Achromobacter* spp., most laboratories currently applied, by default, recommendations edited for other Gram-negative bacilli in order to provide interpreted antibiograms to clinicians. For example, some authors used thresholds defined for *P. aeruginosa* (27) despite incomplete transposability to *Achromobacter* spp., notably due to distinct intrinsic resistances. Here, we showed that clinical categorization may vary according to the choice made for result interpretation. In addition, as previously described for *P. aeruginosa* (28), we highlighted the existence of heterogeneous *Achromobacter* populations in all of the the sputum samples analyzed with the coexistence of susceptible and resistant strains. The different antibiotic resistance profiles were observed within sputum samples independently of the colonial morphotype and pulsotype. First described here for *Achromobacter* spp., similar results were previously obtained during a survey of 101 sputum samples studying 4 *P. aeruginosa* colonies per morphotype and showing an average of 3 resistance profiles per morphotype (29). Our study thus showed that *Achromobacter* spp. are CF pathogens, for which the intrasample and the intramorphotype antimicrobial susceptibility diversity leads to an underestimation of antibiotic resistance and impairs current strategies of antimicrobial susceptibility testing on each colonial morphotype (10, 30). Consequently, some authors have suggested that performing direct sputum susceptibility testing may be more accurate and may overcome the impact of resistance phenotype diversity (30, 31). However, such approaches likely underestimate resistances harbored by minor subpopulations. Exploring the overall intrasample *Achromobacter* diversity remains challenging, labor-intensive, and expensive. Moreover, bacterial population diversity is a highly dynamic process, during which minor variants may emerge and become predominant under specific selection pressure conditions (32). Hence, changes in antimicrobial resistance during exacerbation periods have been previously demonstrated for *P. aeruginosa* populations (28).

In conclusion, our results showed that *Achromobacter* spp. display a highly diversified population during chronic colonization in CF patients and that this diversity is significantly undersampled during the routine processing of CF sputum samples, even if one colony of each colonial morphotype is considered in the analyses, as recommended for *P. aeruginosa*. Together with the absence of specific breakpoints for *Achromobacter* spp., our results questioned the clinical relevance of antimicrobial susceptibility testing on persisting *Achromobacter* in CF.

Although all of the underlying processes promoting diversity remain unelucidated (33), by conferring an adaptive ability to diverse environmental conditions, diversity is usually considered a selective advantage for a bacterial population (34). Beside antimicrobial resistance, the population diversity probably concerns other pathoadaptive features that enhance the persistence of *Achromobacter* in the CF lung, like biofilm formation and motility (16).

MATERIALS AND METHODS

Patients, samples, and strains. Nine patients chronically colonized by *A. xylosoxidans* ($n = 8$) or *A. insuavis* ($n = 1$) and attending the CF center of the Montpellier University Hospital, a large regional French CF center caring for more than 200 children and adults each year, were included. Patients were previously included in a study on within-patient genomic diversity of *Achromobacter* spp. involved in chronic colonization defined according to Pereira et al. (35). During this previous study, *Achromobacter* strains that were recovered over colonization periods of 13 to 51 months between November 2008 and September 2013 were analyzed (15). Patients who attended the CF center in 2015 were included in this study, and their numbering is identical in reference 15 and herein: P1 to P6, P8, P11, and P12.

Specimens were routine sputum samples collected in 2015 for diagnostic purposes in the nine patients. At the time of sampling, patients P1, P5, and P6 experienced an episode of pulmonary exacerbation while clinical status was stable for other patients. Fifty microliters of sputum was cultured for 48 h at 37°C on chocolate agar PolyViteX (bioMérieux). The primary culture was collected and diluted in physiological serum. Dilutions from 10^{-6} to 10^{-8} were cultured on chocolate agar plates to obtain a maximum of 200 visually distinct colonies. The genus identification of colonies was performed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) using a Microflex LT mass spectrometer and a MALDI BioTyper DB 5627 MSP list database (Bruker Daltonics). Every time it was possible, three *Achromobacter* colonies of each morphotype were selected per sputum sample with the help of a magnifying glass and were subcultured on Trypticase soy agar (BD).

Morphotypes were defined by three different parameters: the size, the color, and the form of the colonies.

Pulsed-field gel electrophoresis of SpeI-digested DNA. Pulsed-field gel electrophoresis (PFGE) was performed after DNA macrorestriction by SpeI as previously described (15). The profiles were visually analyzed and annotated with a letter corresponding to the patient and a number corresponding to the pulsotype. PFGE bands were measured and scored as present (1) or absent (0) in a binary table, and the Dice coefficient and unweighted pair group method with arithmetic means (UPGMA) (http://genomes.urv.cat/UPGMA/) were used for pulsotype clustering analysis.

Determination of antibiotic susceptibility. All selected colonies were individually tested for antimicrobial susceptibility by disk diffusion assay on Mueller-Hinton agar (BD). The 16 antimicrobial agents tested and disk (Bio-Rad) load are indicated in the Table 2 and 3 footnotes. Bacterial inoculum was adjusted to a 0.5 McFarland standard. Colonies from the same specimen were assayed the same day by the same operator using the same batch of antibiotic disks and Mueller-Hinton agar plates. Measures of zones of inhibition (ZOI) and coefficient of variation (CV) were reported at both the intraspecimen and the intramorphotype levels. Data were analyzed with GraphPad Prism using box and whiskers. The whiskers were drawn down to the 10th percentile and up to the 90th. Points below and above the whiskers were drawn as individual dots.

In the absence of specific breakpoints for *Achromobacter* spp., results were compared to the different breakpoints edited by the Antibiogram Committee of the French Society for Microbiology in 2016 (CA-SFM 2016) (http://www.sfm-microbiologie.org/) for defining susceptible, intermediate, and resistant categories for different Gram-negative bacilli (*P. aeruginosa*, *Acinetobacter*, *Stenotrophomonas maltophilia*, and *Enterobacteriaceae*). For colistin, we used the diameter breakpoint of 12 mm proposed by Biswas et al. because of its good correlation with the MICs obtained by the Etest on *Achromobacter* in the context of CF (26).

For antibiotype definition and further analysis of the impact of intraspecimen diversity on antimicrobial susceptibility results, both the intermediate and resistant categories were pooled in the resistant category and results were interpreted according to the breakpoints of CA-SFM 2016 for *Acinetobacter* or, when not available, according to those for *Enterobacteriaceae*.

ACKNOWLEDGMENTS

We gratefully acknowledge Linda Aleyrangues, Agnès Masnou, and Monique Alvado for their excellent technical assistance.

REFERENCES

1. Hauser AR, Jain M, Bar-Meir M, McColley SA. 2011. Clinical significance of microbial infection and adaptation in cystic fibrosis. Clin Microbiol Rev 24:29–70. https://doi.org/10.1128/CMR.00036-10.
2. Hogardt M, Heesemann J. 2010. Adaptation of *Pseudomonas aeruginosa* during persistence in the cystic fibrosis lung. Int J Med Microbiol 300:557–562. https://doi.org/10.1016/j.ijmm.2010.08.008.
3. Goerke C, Wolz C. 2004. Regulatory and genomic plasticity of *Staphylococcus aureus* during persistent colonization and infection. Int J Med Microbiol 294:195–202. https://doi.org/10.1016/j.ijmm.2004.06.013.
4. Winstanley C, O'Brien S, Brockhurst MA. 2016. *Pseudomonas aeruginosa* evolutionary adaptation and diversification in cystic fibrosis chronic lung infections. Trends Microbiol 24:327–337. https://doi.org/10.1016/j.tim.2016.01.008.
5. Wilder CN, Allada G, Schuster M. 2009. Instantaneous within-patient diversity of *Pseudomonas aeruginosa* quorum-sensing populations from cystic fibrosis lung infections. Infect Immun 77:5631–5639. https://doi.org/10.1128/IAI.00755-09.
6. Marvig RL, Johansen HK, Molin S, Jelsbak L. 2013. Genome analysis of a transmissible lineage of *Pseudomonas aeruginosa* reveals pathoadaptive mutations and distinct evolutionary paths of hypermutators. PLoS Genet 9:e1003741. https://doi.org/10.1371/journal.pgen.1003741.
7. Workentine ML, Sibley CD, Glezerson B, Purighalla S, Norgaard-Gron JC, Parkins MD, Rabin HR, Surette MG. 2013. Phenotypic heterogeneity of *Pseudomonas aeruginosa* populations in a cystic fibrosis patient. PLoS One 8:e60225. https://doi.org/10.1371/journal.pone.0060225.
8. Jorth P, Staudinger BJ, Wu X, Hisert KB, Hayden H, Garudathri J, Harding CL, Radey MC, Rezayat A, Bautista G, Berrington WR, Goddard AF, Zheng C, Angermeyer A, Brittnacher MJ, Kitzman J, Shendure J, Fligner CL, Mittler J, Aitken ML, Manoil C, Bruce JE, Yahr TL, Singh PK. 2015. Regional isolation drives bacterial diversification within cystic fibrosis lungs. Cell Host Microbe 18:307–319. https://doi.org/10.1016/j.chom.2015.07.006.
9. Société Française de Microbiologie. 2015. Référentiel en microbiologie médicale, 5.1 ed, p 193–200. Société Française de Microbiologie, Paris, France.
10. Clark ST, Diaz Caballero J, Cheang M, Coburn B, Wang PW, Donaldson SL, Zhang Y, Liu M, Keshavjee S, Yau YC, Waters VJ, Elizabeth Tullis D, Guttman DS, Hwang DM. 2015. Phenotypic diversity within a *Pseudomonas aeruginosa* population infecting an adult with cystic fibrosis. Sci Rep 5:10932. https://doi.org/10.1038/srep10932.
11. Mahenthiralingam E. 2014. Emerging cystic fibrosis pathogens and the microbiome. Paediatr Respir Rev 15(Suppl):13–15.
12. Cools P, Ho E, Vranckx K, Schelstraete P, Wurth B, Franckx H, Ieven G, Van Simaey L, Van Daele S, Verhulst S, De Baets F, Vaneechoutte M. 2016. Epidemic *Achromobacter xylosoxidans* strain among Belgian cystic fibrosis patients and review of literature. BMC Microbiol 16:122. https://doi.org/10.1186/s12866-016-0736-1.
13. Hansen CR, Pressler T, Nielsen KG, Jensen PØ, Bjarnsholt T, Høiby N. 2010. Inflammation in *Achromobacter xylosoxidans* infected cystic fibrosis patients. J Cyst Fibros 9:51–58. https://doi.org/10.1016/j.jcf.2009.10.005.
14. Amoureux L, Bador J, Siebor E, Taillefumier N, Fanton A, Neuwirth C. 2013. Epidemiology and resistance of *Achromobacter xylosoxidans* from cystic fibrosis patients in Dijon, Burgundy: first French data. J Cyst Fibros 12:170–176. https://doi.org/10.1016/j.jcf.2012.08.005.
15. Dupont C, Michon AL, Jumas-Bilak E, Nørskov-Lauritsen N, Chiron R, Marchandin H. 2015. Intrapatient diversity of *Achromobacter* spp. involved in chronic colonization of cystic fibrosis airways. Infect Genet Evol 32:214–223. https://doi.org/10.1016/j.meegid.2015.03.012.
16. Trancassini M, Iebba V, Citerà N, Tuccio V, Magni A, Varesi P, De Biase RV, Totino V, Santangelo F, Gagliardi A, Schippa S. 2014. Outbreak of *Achromobacter xylosoxidans* in an Italian cystic fibrosis center: genome variability, biofilm production, antibiotic resistance, and motility in isolated strains. Front Microbiol 5:138.
17. Ridderberg W, Nielsen SM, Nørskov-Lauritsen N. 2015. Genetic adaptation of *Achromobacter* sp. during persistence in the lungs of cystic fibrosis patients. PLoS One 10:e0136790. https://doi.org/10.1371/journal.pone.0136790.
18. Saiman L, Chen Y, Tabibi S, San Gabriel P, Zhou J, Liu Z, Lai L, Whittier S. 2001. Identification and antimicrobial susceptibility of *Alcaligenes xy-*

losoxidans isolated from patients with cystic fibrosis. J Clin Microbiol 39:3942–3945. https://doi.org/10.1128/JCM.39.11.3942-3945.2001.
19. Almuzara M, Limansky A, Ballerini V, Galanternik L, Famiglietti A, Vay C. 2010. *In vitro* susceptibility of *Achromobacter* spp. isolates: comparison of disk diffusion, Etest and agar dilution methods. Int J Antimicrob Agents 35:68–71. https://doi.org/10.1016/j.ijantimicag.2009.08.015.
20. Llorca Otero L, Girón Moreno R, Buendía Moreno B, Valenzuela C, Guiu Martínez A, Alarcón Cavero T. 2016. *Achromobacter xylosoxidans* infection in an adult cystic fibrosis unit in Madrid. Enferm Infecc Microbiol Clin 34:184–187. https://doi.org/10.1016/j.eimc.2015.05.006.
21. Goss CH, Burns JL. 2007. Exacerbations in cystic fibrosis. 1. Epidemiology and pathogenesis. Thorax 62:360–367. https://doi.org/10.1136/thx.2006.060889.
22. Abbott IJ, Peleg AY. 2015. *Stenotrophomonas*, *Achromobacter*, and nonmelioid *Burkholderia* species: antimicrobial resistance and therapeutic strategies. Semin Respir Crit Care Med 36:99–110. https://doi.org/10.1055/s-0034-1396929.
23. Yamamoto M, Nagao M, Hotta G, Matsumura Y, Matsushima A, Ito Y, Takakura S, Ichiyama S. 2012. Molecular characterization of IMP-type metallo-β-lactamases among multidrug-resistant *Achromobacter xylosoxidans*. J Antimicrob Chemother 67:2110–2113. https://doi.org/10.1093/jac/dks179.
24. Lobo LJ, Tulu Z, Aris RM, Noone PG. 2015. Pan-resistant *Achromobacter xylosoxidans* and *Stenotrophomonas maltophilia* infection in cystic fibrosis does not reduce survival after lung transplantation. Transplantation 99:2196–2202. https://doi.org/10.1097/TP.0000000000000709.
25. Gibson RL, Burns JL, Ramsey BW. 2003. Pathophysiology and management of pulmonary infections in cystic fibrosis. Am J Respir Crit Care Med 168:918–951. https://doi.org/10.1164/rccm.200304-505SO.
26. Biswas S, Dubus JC, Reynaud-Gaubert M, Stremler N, Rolain JM. 2013. Evaluation of colistin susceptibility in multidrug-resistant clinical isolates from cystic fibrosis, France. Eur J Clin Microbiol Infect Dis 32:1461–1464. https://doi.org/10.1007/s10096-013-1898-5.
27. Wang M, Ridderberg W, Hansen CR, Høiby N, Jensen-Fangel S, Olesen HV, Skov M, Lemming LE, Pressler T, Johansen HK, Nørskov-Lauritsen N. 2013. Early treatment with inhaled antibiotics postpones next occurrence of *Achromobacter* in cystic fibrosis. J Cyst Fibros 12:638–643. https://doi.org/10.1016/j.jcf.2013.04.013.
28. Fothergill JL, Mowat E, Ledson MJ, Walshaw MJ, Winstanley C. 2010. Fluctuations in phenotypes and genotypes within populations of *Pseudomonas aeruginosa* in the cystic fibrosis lung during pulmonary exacerbations. J Med Microbiol 59:472–481. https://doi.org/10.1099/jmm.0.015875-0.
29. Foweraker JE, Laughton CR, Brown DF, Bilton D. 2005. Phenotypic variability of *Pseudomonas aeruginosa* in sputa from patients with acute infective exacerbation of cystic fibrosis and its impact on the validity of antimicrobial susceptibility testing. J Antimicrob Chemother 55:921–927. https://doi.org/10.1093/jac/dki146.
30. Serisier DJ, Tuck A, Matley D, Carroll MP, Jones G. 2012. Antimicrobial susceptibility and synergy studies of cystic fibrosis sputum by direct sputum sensitivity testing. Eur J Clin Microbiol Infect Dis 31:3211–3216. https://doi.org/10.1007/s10096-012-1687-6.
31. Zebouh M, Thomas C, Honderlick P, Lemee L, Segonds C, Wallet F, Husson MO. 2008. Direct antimicrobial susceptibility testing method for analysis of sputum collected from patients with cystic fibrosis. J Cyst Fibros 7:238–243. https://doi.org/10.1016/j.jcf.2007.10.002.
32. Mowat E, Paterson S, Fothergill JL, Wright EA, Ledson MJ, Walshaw MJ, Brockhurst MA, Winstanley C. 2011. *Pseudomonas aeruginosa* population diversity and turnover in cystic fibrosis chronic infections. Am J Respir Crit Care Med 183:1674–1679. https://doi.org/10.1164/rccm.201009-1430OC.
33. Markussen T, Marvig RL, Gómez-Lozano M, Aanæs K, Burleigh AE, Høiby N, Johansen HK, Molin S, Jelsbak L. 2014. Environmental heterogeneity drives within-host diversification and evolution of *Pseudomonas aeruginosa*. mBio 5:e01592-14.
34. Lieberman TD, Flett KB, Yelin I, Martin TR, McAdam AJ, Priebe GP, Kishony R. 2014. Genetic variation of a bacterial pathogen within individuals with cystic fibrosis provides a record of selective pressures. Nat Genet 46:82–87.
35. Pereira RH, Carvalho-Assef AP, Albano RM, Folescu TW, Jones MC, Leão RS, Marques EA. 2011. *Achromobacter xylosoxidans*: characterization of strains in Brazilian cystic fibrosis patients. J Clin Microbiol 49:3649–3651. https://doi.org/10.1128/JCM.05283-11.