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Patterns of virulence factor expression and antimicrobial resistance in *Achromobacter xylosoxidans* and *Achromobacter ruhlandii* isolates from patients with cystic fibrosis

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SUMMARY

Achromobacter spp. are opportunistic pathogens increasingly recovered from adult patients with cystic fibrosis (CF). We report the characterization of 122 *Achromobacter* spp. isolates recovered from 39 CF patients by multilocus sequence typing, virulence traits, and susceptibility to antimicrobials. Two species, *A. xylosoxidans* (77%) and *A. ruhlandii* (23%) were identified. All isolates showed a similar biofilm formation ability, and a positive swimming phenotype. By contrast, 4·3% and 44·4% of *A. xylosoxidans* and *A. ruhlandii*, respectively, exhibited a negative swarming phenotype, making the swimming and swarming abilities of *A. xylosoxidans* significantly higher than those of *A. ruhlandii. A. xylosoxidans* isolates from an outbreak clone also exhibited significantly higher motility. Both species were generally susceptible to ceftazidime, ciprofloxacin, imipenem and trimethoprim/sulphamethoxazole and there was no significant difference in susceptibility between isolates from chronic or sporadic infection. However, *A. xylosoxidans* isolates from chronic and sporadic cases were significantly more resistant to imipenem and ceftazidime than isolates of the outbreak clone.

Key words: *Achromobacter*, clinical microbiology, cystic fibrosis.

INTRODUCTION

Achromobacter spp. are opportunistic pathogens increasingly recovered from adult patients with cystic fibrosis (CF) [1]. However, owing to difficulties of accurate species identification, most clinical *Achromobacter* isolates are often reported as *A. xylosoxidans*. The reported prevalence of airway colonization/infection by *A. xylosoxidans* in CF centres ranges from 2% to 20% [2, 3] but their clinical significance remains unclear. Nevertheless, a retrospective case-control study [4] showed a greater decline in lung function in patients chronically infected with *A. xylosoxidans*, compared to non-infected patients. The clinical course of CF lung disease is widely considered to be dependent on the nature and degree of the inflammatory response to bacterial infection and

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A. xylosoxidans has been shown to be associated with levels of inflammation similar to *Pseudomonas aeruginosa* in chronically infected CF patients [5].

Studies documenting the pathogenic properties of *Achromobacter* spp. are scarce and relatively little is known of the mechanisms which promote survival, colonization and progression to infection of the CF lung. In general, it is believed that the capability of bacteria to initiate and persist in chronic infections is due to their biofilm-forming ability, which renders them tolerant towards antimicrobial agents and host defence mechanisms [6, 7]. In addition, acquisition of antibiotic resistance and bacterial motility have been repeatedly cited as interdependent mechanisms which favour persistence of bacteria in host tissues [8–11].

On sequencing the genome of the *A. xylosoxidans* NH44784-1996 strain, Jakobsen *et al.* [12] identified the presence of an operon encoding an adhesin which had earlier been implicated in biofilm development by *Escherichia coli* [13]. Despite this suggestion of a mechanism that could promote the persistence of *A. xylosoxidans* in the CF airway, few studies have addressed the ability of *Achromobacter* CF isolates to form biofilms [14, 15].

We have previously reported a high prevalence (21·8%) of airway colonization/infection by *A. xylosoxidans* in 179 patients treated in two CF centres in Brazil, and that more than half of the patients were classed as having chronic infection [16]. Isolates were genetically heterogeneous but chronicity was associated with a relatively few of several clones identified by pulsed-field gel electrophoresis (PFGE). We also found evidence of cross-infection with the same clone in over half of all *A. xylosoxidans*-positive patients [16].

A. xylosoxidans is the type species of the *Achromobacter* genus which comprises 15 named species and multiple genogroups (http://www.bacterio.cict.fr/). Recently, new laboratory technologies have allowed the recognition of species other than *A. xylosoxidans* in CF patients, with *A. ruhlandii* being identified as the second most prevalent *Achromobacter* spp. in the United States [17], but by contrast, *A. ruhlandii* has rarely been reported from CF patients in Europe [18, 19].

In this study we investigated the distribution of *A. xylosoxidans* and *A. ruhlandii* in *Achromobacter* spp. recovered from patients treated in CF centres in Rio de Janeiro, as well as the presence of virulence traits associated with bacterial colonization of the host respiratory mucosa, such as biofilm formation, bacterial mobility and antimicrobial resistance.

METHODS

A total of 122 archived *Achromobacter* spp. isolates recovered over a 5-year period from 39 patients treated in two Brazilian CF centres were included in the study. All isolates had been recovered from respiratory secretions and grouped into 22 clonal types defined by PFGE [16].

Isolates were identified to species level by amplification and sequencing of gene $bla_{\text{OXA-114-like}}$, and by multilocus sequence type (MLST) as described previously [20]. Allelic profiles and sequence types (STs) were assigned according to the PubMLST website (http://pubmlst.org/achromobacter/).

The minimum inhibitory concentrations (MICs) of ceftazidime, ciprofloxacin, imipenem and trimethoprim/sulphamethoxazole were determined with E-test strips (AB Biodisk, Sweden) using recommended CLSI breakpoints for non-Enterobacteriaceae [21]. *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27 853 were used as quality control strains for each run of MIC determination.

Approximately 10^7 c.f.u. of each isolate in 200 μl Mueller–Hinton broth supplemented with 0·75% glucose were inoculated into three wells of 96-well microplates. After 20 h at 35 ± 2 °C under mild agitation, the wells were washed three times with distilled water to remove non-adherent bacteria and the biofilms were stained with 200 μl of 0·1% Violet Crystal solution for 15 min, washed, and air dried for 2 h. The biofilm-bound stain was released with 200 μl of 95% ethanol and the optical density (OD) at 595 nm of the obtained solutions was determined with a microplate spectrophotometric reader. Microplate wells without bacteria served as negative controls.

To assess the swimming and swarming abilities of isolates, 3 μl of a fresh nutrient broth (Oxoid, UK) culture were spotted onto the centre of plates containing 0·3% (w/v) nutrient agar, or 0·8% (w/v) nutrient broth with 0·5% (w/v) agar containing 0·5% (w/v) glucose [22]. After 24 h at 30 ± 1 °C, the diameters of the bacterial concentric growths from the inoculation site were measured.

Data were analysed with Graph Pad Prism v. 5·0 (GraphPad Software, USA). Because two or more isolates were recovered from some individuals, we utilized median values of OD_{595} (biofilm) and ring

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diameters (swimming and swarming), of the different isolates from each patient. Statistical significance ($P < 0{\cdot}05$) was determined by the Mann–Whitney test or by Fisher's exact test to compare antimicrobial susceptibility data.

RESULTS

Amplification and sequencing of the $bla_{\text{OXA-114-like}}$ gene and MLST of all 122 *Achromobacter* isolates identified 28 (23·0%) isolates as *A. ruhlandii* and the remaining 94 (77·0%), as *A. xylosoxidans*. Five PFGE clonal groups were identified in 22 *A. ruhlandii* isolates and 17 PFGE clonal groups in the 94 *A. xylosoxidans* isolates. Seven (18·4%) of the 39 patients harboured *A. ruhlandii* alone and 30 (76·9%) grew only *A. xylosoxidans*; the remaining patient yielded both *Achromobacter* species. Four (13·3%) of the 30 patients who harboured exclusively *A. xylosoxidans* isolates developed chronic infection which was defined as the presence of at least three positive cultures for *Achromobacter* spp. during a 1-year period, with a minimum 1-month interval between cultures; chronic infection was also noted in four of the seven patients harbouring *A. ruhlandii*.

The predominant 'outbreak' clone G previously defined by PFGE [16] was recovered from 22 patients and all were identified as *A. xylosoxidans*.

MLST analysis identified 13 STs in 106 isolates; 10 STs in *A. xylosoxidans* isolates ($n = 78$) and three STs in *A. ruhlandii* isolates ($n = 28$). We could not determine STs in 16 *A. xylosoxidans* isolates [PFGE clonal group F ($n = 11$), J ($n = 2$), N ($n = 2$), and S ($n = 1$)]. Three isolates of PFGE clonal group A were further discriminated by MLST: ST13 ($n = 2$), and ST198 ($n = 1$). All clone G isolates fell in ST200.

Most isolates of both species were susceptible to the four antimicrobials tested and no significant differences were found in susceptibility between the species to imipenem, sulphamethoxazole/trimethoprim, and ceftazidime but *A. ruhlandii* isolates were significantly less susceptible to ciprofloxacin ($P = 0{\cdot}002$, Table 1). Moreover, although *A. ruhlandii* isolates from chronic infection also showed a similar susceptibility to three antimicrobials compared to sporadic isolates, there was a strong tendency for the former group towards resistance to ciprofloxacin ($P = 0{\cdot}057$). The outbreak clone of *A. xylosoxidans* exhibited significantly more resistance to ceftazidime than other isolates ($P = 0{\cdot}009$, Table 1) and there was a strong tendency towards resistance to imipenem for *A. xylosoxidans* isolates from chronic infection ($P = 0{\cdot}054$).

All isolates of both species formed biofilms on microplate wells and there was no significant difference in the degree of biofilm formation between *A. xylosoxidans* and *A. ruhlandii* [median OD_{595} (range) = 0·910 (0·310–1·858) and 0·667 (0·300–1·500), respectively]. Similarly, for *A. xylosoxidans*, isolates from chronic or sporadic infection exhibited comparable biofilm-forming capacity [median OD_{595} (range) = 0·858 (0·684–0·944) and 0·915 (0·300–1·858), respectively], as well as isolates of the outbreak clone compared to either chronic or sporadic infection [median OD_{595} (range) = 1·013 (0·693–1·217) and 0·858 (0·310–1·858), respectively]. Further, no significant difference in biofilm-forming capacity was evident for *A. ruhlandii* isolates from chronic or sporadic infection (median OD_{595} (range) = 0·895 (0·300–1·500) and 0·591 (0·545–1·106), respectively All isolates of both species showed a swimming phenotype but 4·3% and 44·4% of *A. xylosoxidans* and *A. ruhlandii*, respectively, were negative in tests for the swarming phenotype. Figure 1 shows that the swimming and swarming abilities of *A. xylosoxidans* were significantly higher than those of *A. ruhlandii.* Similarly, the mobility of isolates of the *A. xylosoxidans* outbreak clone was significantly higher than for other isolates of this species.

DISCUSSION

Prevalence rates of *Achromobacter* spp. recovered from CF respiratory secretions have increased in recent years. This may be due to the generally extended lifespan of these patients and the selective pressure of prolonged and multiple antimicrobial therapy. Similarly, the prevalence may be simply a consequence of increased ascertainment due to the use of improved microbiological isolation and molecular identification techniques [23] which have allowed the recognition of species other than *A. xylosoxidans* in the CF airway.

In an attempt to understand better the epidemiology, prevalence and level of expression of virulence traits, and antibiotic susceptibility of *Achromobacter* spp., 122 archived isolates from 398 patients attending two CF centers in Rio de Janeiro [16] were further investigated. Spilker *et al.* [17] have shown through *nrdA* gene sequences of isolates recovered from 341 CF patients that *A. ruhlandii* accounted for 23·5% of all *Achromobacter* isolates, a proportion markedly

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Table 1. *Distribution, MIC and rates of susceptibility to four antimicrobials in* Achromobacter *spp. recovered from cystic fibrosis patients*

Species (no. of isolates)	Antimicrobial agent	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	Range (μg/ml)	Susceptible (%)	Resistant (%)
A. xylosoxidans	IMP	27	2	0·25–⩾32	90·4	9·6
(n = 94)	SUT	19	1	0·032–256	90·4	9·6
	CIP	1	2	0·38–4	75·5	24·5
	CAZ	2	3	0·032–256	88·2	11·8
A. ruhlandii	IMP	0·50	3	0·125–1	100	0
(n = 28)	SUT	0·064	32	0·023–32	78·6	21·4
	CIP	2	4	0·38–6	42·9	57·1*
	CAZ	3	4	0·75–12	100	0
A. ruhlandii (chronic)	IMP	0·50	0·75	0·125–1	100	0
(n = 22)	SUT	0·064	1	0·032–⩾32	86·4	13·6
	CIP	2	4	0·75–6	31·8	68·2**
	CAZ	3	4	0·75–12	100	0
A. ruhlandii (sporadic)	IMP	0·50	0·5	0·38–1	100	0
(n = 6)	SUT	0·032	⩾32	0·023–⩾32	40	60
	CIP	0·38	2	0·38–2	80	20
	CAZ	1·5	4	1–4	100	0
A. xylosoxidans (outbreak)	IMP	0·75	0·75	0·25–1·5	100	0
(n = 28)	SUT	0·064	0·19	0·032–10	96·4	3·6
	CIP	1·5	2	0·75–3	67·0	32·1
	CAZ	1·5	4	0·032–3	100	0
A. xylosoxidans (chronic plus sporadic)	IMP	0·75	⩾32	0·25–⩾32	86·4	13·6***
(n = 66)	SUT	0·125	⩾32	0·002–⩾32	87·9	12·1
	CIP	1	2	0·38–4	78·8	21·2
	CAZ	2	⩾256	0·50–⩾256	83·3	16·7****

MIC, Minimum inhibitory concentration; IMP, imipenem; SUT, sulphamethoxazole/trimethoprim; CIP, ciprofloxacin; CAZ, ceftazidime.
*P = 0·002: *A. ruhlandii* × *A. xylosoxidans* (CIP); **P = 0·057: *A. ruhlandii* chronic × *A. ruhlandii* sporadic (CIP); ***P = 0·054: *A. xylosoxidans* (chronic plus sporadic × *A. xylosoxidans* (outbreak) (IMP); ****P = 0·009: *A. xylosoxidans* (chronic plus sporadic × *A. xylosoxidans* (outbreak) (CAZ).

similar to our finding of 23·0% in the isolates reported here, and represented 21·0% of our patient cohort. Interestingly, over half (57·1%) of the patients harbouring *A. ruhlandii* isolates developed chronic infection compared to 13·3% in patients who grew *A. xylosoxidans* alone. However, *Staphylococcus aureus* and *P. aeruginosa* were isolated sporadically from three patients, but in two (who were siblings), *A. ruhlandii* of the same ST was the sole organism isolated before death. Although not statistically significant owing to the small number of patients studied, our findings possibly suggest that patients infected/colonized by *A. ruhlandii* are at a higher risk of developing chronic infection. It is noteworthy that a clone of *Achromobacter* spp. designated the Danish epidemic strain which was reported to have chronically infected 13 patients from two Danish CF centres [4, 24], was subsequently identified as *A. ruhlandii* [25]. Moreover, the species has also been implicated in causing cross-infection between CF patients even after limited and indirect contact between them [26], and further supports the view regarding its possible concern in CF communities.

Achromobacter spp. are intrinsically resistant to many antimicrobials agents, and the development of acquired resistance is common during the course of chronic infection, especially for β-lactams [27, 28]. Moreover, variability in antimicrobial resistance of CF *Achromobacter* strains has been reported, although almost exclusively for *A. xylosoxidans* [14, 29]. Nevertheless, most *Achromobacter* isolates from our collection proved susceptible to all four antibiotics tested; the agents were chosen according to the recommendation of the CF Consensus Study Group [30]. No significant difference in susceptibility was noted between species and infection status except for the

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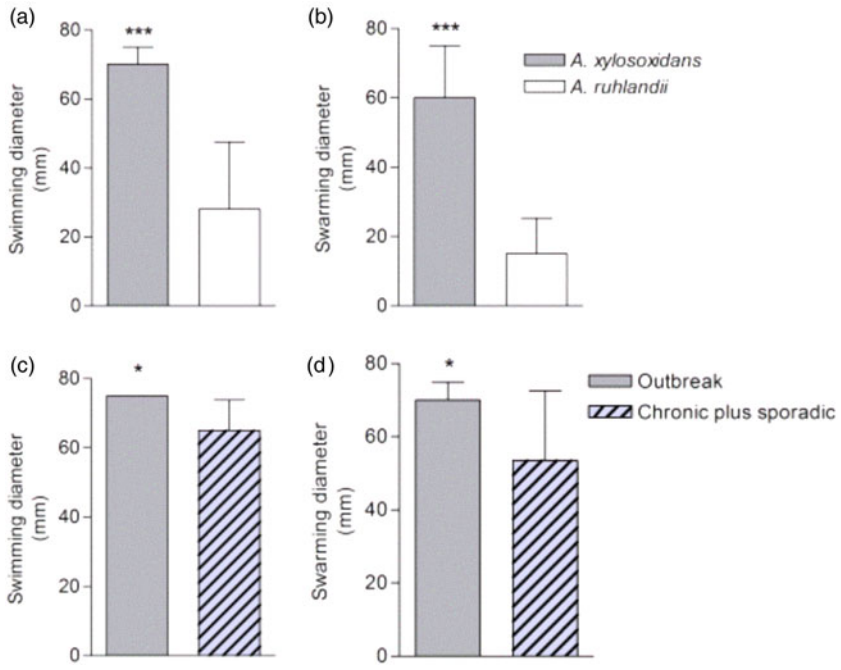

Fig. 1. (*a*) Swimming and (*b*) swarming motility of *Achromobacter xylosoxidans* and *A. ruhlandii*. (*c*) Swimming and (*d*) swarming motility of *A. xylosoxidans* isolates of the outbreak clone and patients with chronic or sporadic infection. Data are medians, and bars represent the interquartile range. * $P < 0{\cdot}05$, *** $P < 0{\cdot}001$.

outbreak clone of *A. xylosoxidans* which showed significantly increased resistance to imipenem and ceftazidime.

Adaptive mechanisms of bacteria probably explain why relatively few pathogens are able to survive and persist in the respiratory tract of CF patients despite an augmented host defence and intensive antibiotic therapy. It is well known that *P. aeruginosa*, and several other species are able to adapt to the CF host lung environment by switching to a biofilm mode of growth [31] and this property is highly correlated with the establishment of chronic infections in these patients. This present study has shown that all *A. xylosoxidans* and *A. ruhlandii* isolates produced large, and similar amounts of biofilm on an abiotic plastic surface. Although biofilm formation by *A. xylosoxidans* from CF patients has been previously reported, both *in vivo* [32] and *in vitro* [14], our study extends this property to *A. ruhlandii*.

Horizontal exchange of genetic material in bacteria occurs with enhanced efficiency within biofilms [8], where the dense population structure promotes plasmid dispersal through conjugation which may stimulate biofilm development. Released DNA stabilizes biofilm structure and mediates genetic exchange through transformation. Since *A. xylosoxidans* can serve as a reservoir of horizontal genetic transfer elements commonly involved in spreading antibiotic resistance [33], the biofilm mode of growth is likely to favour the development of multidrug resistance in CF *Achromobacter*.

Swimming and swarming are flagella-dependent types of bacterial motility in low-viscosity liquid and viscous environments, respectively. Most *A. xylosoxidans* and *A. ruhlandii* isolates studied here were shown to exhibit the swarming motility phenotype. This is in contrast to the findings of Trancassini *et al.* [14] who were unable to detect a swarming phenotype in 57 strains of *A. xylosoxidans*. Swarming has been proposed to contribute to bacterial virulence as it facilitates movement of the bacteria through the mucous layer of host epithelia and is considered to be an important means of surface epithelial colonization [34]. In some species, such as *P. aeruginosa*, swarming cells were shown to exhibit overexpression of a large number of virulence-related genes [35], whereas bacterial mutants with altered swarming motility were also defective in biofilm formation [35, 36]. Therefore, swarming is likely to be involved in early biofilm development. Finally, bacterial cells under swarming conditions have also been shown to be more resistant to the action of antibiotics than their non-swarming counterparts due to a type of adaptive resistance, and not the result of mutant selection

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[35, 37, 38]. This is particularly important because like other types of adaptive resistance, swarming would contribute to antimicrobial therapy failure even if the strains appeared susceptible by routine *in vitro* analysis [34]. In this study, *A. xylosoxidans* isolates, and particularly of the outbreak clone, proved to be better swarmers than chronic or sporadic isolates. This also held true for isolates of *A. ruhlandii.* It is therefore tempting to speculate that swarming mobility may be a key determinant of the ability of strains to spread through cross-infection between patients, but further research in this area is clearly warranted.

In conclusion, this investigation has provided some novel and interesting data on the relative prevalence of *A. xylosoxidans* and *A. ruhlandii,* in CF patients and the susceptibility of such isolates to antimicrobials commonly used for the treatment of these patients in our centre. Although biofilm formation and mobility mechanisms were largely similar in the isolates, an outbreak strain of *A. xylosoxidans* exhibited enhanced swarming ability and there was some correlation between this and isolates from chronic as opposed to sporadic infections. We believe that our study has given us better insight into the infectious disease process of *Achromobacter* in the CF lung and further investigations to clarify the relationship between the expression of bacterial virulence traits and ability to colonize/infect CF patients are justified.

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DECLARATION OF INTEREST

None.

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