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Stenotrophomonas maltophilia phenotypic and genotypic features through 4-year cystic fibrosis lung colonization

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Abstract

Introduction. *Stenotrophomonas maltophilia* has emerged as one of the most common multi-drug-resistant pathogens isolated from people with cystic fibrosis (CF). However, its adaptation over time to CF lungs has not been fully established.

Hypothesis. Sequential isolates of *S. maltophilia* from a Brazilian adult patient are clonally related and show a pattern of adaptation by loss of virulence factors.

Aim. To investigate antimicrobial susceptibility, clonal relatedness, mutation frequency, quorum sensing (QS) and selected virulence factors in sequential *S. maltophilia* isolates from a Brazilian adult patient attending a CF referral centre in Buenos Aires, Argentina, between May 2014 and May 2018.

Methodology. The antibiotic resistance of 11 S. *maltophilia* isolates recovered from expectorations of an adult female with CF was determined. Clonal relatedness, mutation frequency, QS variants (RpfC–RpfF), QS autoinducer (DSF) and virulence factors were investigated in eight viable isolates.

Results. Seven *S. maltophilia* isolates were resistant to trimethoprim–sulfamethoxazole and five to levofloxacin. All isolates were susceptible to minocycline. Strong, weak and normomutators were detected, with a tendency to decreased mutation rate over time. *XbaI* PFGE revealed that seven isolates belong to two related clones. All isolates were RpfC–RpfF1 variants and DSF producers. Only two isolates produced weak biofilms, but none displayed swimming or twitching motility. Four isolates showed proteolytic activity and amplified *stmPr1* and *stmPr2* genes. Only the first three isolates were siderophore producers. Four isolates showed high resistance to oxidative stress, while the last four showed moderate resistance.

Conclusion. The present study shows the long-time persistence of two related *S. maltophilia* clones in an adult female with CF. During the adaptation of the prevalent clones to the CF lungs over time, we identified a gradual loss of virulence factors that could be associated with the high amounts of DSF produced by the evolved isolates. Further, a decreased mutation rate was observed in the late isolates. The role of all these adaptations over time remains to be elucidated from a clinical perspective, probably focusing on the damage they can cause to CF lungs.

INTRODUCTION

Stenotrophomonas maltophilia, an environmental non-fermentative Gram-negative bacterium, has been classified by the World Health Organization as one of the leading drug-resistant pathogens in hospitals worldwide [1]. Respiratory tract colonization by *S. maltophilia* is commonly seen in patients suffering from cystic fibrosis (CF), the most

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Keywords: *Stenotrophomonas maltophilia*; cystic fibrosis; chronic colonization; quorum sensing; virulence factors; adaptation.

Abbreviations: CAS, chrome azurol S; CF, cystic fibrosis; CLSI, Clinical and Laboratory Standards Institute; DNA, *rpf* (regulation of pathogenicity factors) gene cluster; DSF, diffusible signal factor; LEV, levofloxacin; Nar, membrane-bound nitrate reductase; PFGE, pulsed-field gel electrophoresis; QS, quorum sensing; RAPD-PCR, random amplification of polymorphic; *rpf*, regulation of pathogenicity factors; RpfC–RpfF, variants of the *rpf* cluster; StmPr, extracellular protease; SXT, trimethoprim–sulphamethoxazole; Xcc, *Xanthomonas campestris* pv. *campestris*.

Two supplementary figures are available with the online version of this article.

frequently observed severe autosomal recessive genetic disease in Caucasians [2–4].

Several worldwide CF centres have reported that the prevalence of *S. maltophilia* has increased in recent decades, reaching 10 to 18% of patients with CF [5]. However, its contribution to lung damage has not been fully established [6]. Nevertheless, it was demonstrated that chronic colonization/infection with *S. maltophilia* is an independent risk factor for pulmonary exacerbation and has been associated with a specific immune response and with a threefold increased risk of mortality or lung transplantation in CF patients [7, 8]. Furthermore, the pathogenic role of *S. maltophilia* in patients with CF is suggested by its ability to form biofilms on the CF-derived epithelial monolayer and its contribution to the inflammatory process in a murine model of acute respiratory infection that leads to a compromised respiratory function and death [9].

Factors that have been involved in the virulence of *S. maltophilia* include, among others, biofilm formation, extracellular enzymes such as proteases, siderophores and resistance to oxidative stress [10–14]. The quorum sensing (QS) system of *S. maltophilia* is based on the diffusible signal factor (DSF). DSF production and detection depends on the *rpf* regulation of pathogenicity factors gene cluster, which encodes the synthase RpfF and the RpfC/RpfG two-component system, responsible for perception and transduction of the DSF signal [15]. Huedo *et al*. described two variants of the *rpf* cluster in *S. maltophilia* strains: the RpfC–RpfF1 variant strains produce DSF, while the RpfC–RpfF2 variant strains only do so in the presence of DSF producers [15].

Treatment of *S. maltophilia* infections is difficult because of its intrinsic multi-drug resistance (including carbapenems) and its ability to form biofilms [6, 16]. Even though trimethoprim–sulphamethoxazole (SXT) is the first-line drug against *S. maltophilia* infections [17, 18], worldwide emergence of SXT resistance has been reported [17], and furthermore, high rates of resistance were observed in *S. maltophilia* isolates from patients with CF [19, 20]. Other drugs with activity against *S. maltophilia* are minocycline and levofloxacin [21].

Many pathogens alter their phenotype and genotype during chronic infection in response to selection pressures. Bacterial adaptation to survive in the CF lung has predominantly been studied for *Pseudomonas aeruginosa* and *Staphylococcus aureus* [22–24]. Since the adaptation strategies of *S. maltophilia* isolates have been sparsely examined [2, 3, 9, 25, 26], the longitudinal analysis based on sequential isolates in a patient with CF from our study will provide an opportunity to examine the modifications that *S. maltophilia* may undergo during the course of chronic lung colonization.

The aim of this work was to investigate antimicrobial susceptibility, clonal relatedness, mutation frequency, DSF production, the presence of RpfC–RpfF variants and selected virulence factors in sequential *S. maltophilia* isolates from a Brazilian adult patient attending a CF referral centre in Buenos Aires, Argentina, between May 2014 and May 2018.

METHODS

Bacterial isolates and culture conditions

A total of 11 *S. maltophilia* isolates were recovered from sputum samples of an adult Brazilian female with CF between May 2014 and May 2018 at Instituto de Investigaciones en Microbiología y Parasitología Médica, Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Tecnológicas (IMPaM, UBA-CONICET), Buenos Aires, Argentina (Table 1). Isolates were identified as *S. maltophilia* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). *S. maltophilia* K279a, whose genome has been fully sequenced (GenBank: AM743169.1), was used as a reference strain [27]. *S. maltophilia* D457 (GenBank: HE798556.1) was used as a control for the RpfC–RpfF2 variant [28]. *S. maltophilia* SmEL06, a trimethoprim–sulfamethoxazole-resistant CF isolate recovered from a chronically colonized paediatric patient in Hospital General de Niños 'Pedro de Elizalde', Argentina, positive for *intl1*, *qacEΔ1-sul1* and *sul1* genes, was used as a control for the detection of resistance determinants for SXT [29]. *Xanthomonas campestris* pv. *campestris* (Xcc) 8004 and *X*cc 8523 (*rpfF* mutant) were used in the DSF bioassay [14, 30]. Isolates were kept frozen at −20 °C in 15% glycerol. Before use, bacteria were cultured on tryptone soya agar (TSA; Oxoid Ltd) for 24 h at 35 °C. Unfortunately, bacterial cells could not be recovered from the Sm1324, Sm1331 and Sm1332 samples (Table 1). Unless stated otherwise, all cultures were grown in tryptone soya broth (TSB; Oxoid Ltd) and incubated at 35 °C. When indicated, cultures were aerated during growth on a water bath shaker at 200 r.p.m.

Susceptibility

Antimicrobial susceptibility was assessed using the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) recommendations [31]. The testing was performed on Mueller–Hinton agar plates with minocycline, levofloxacin (LEV) and trimethoprim–sulfamethoxazole (SXT) discs (Britania). *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control strains. The breakpoints used were those recommended by the CLSI for *S. maltophilia*.

Detection of resistance determinants for SXT

The presence of class 1 and class 2 integrons as well as *qacEΔ1-sul1*, *sul1* and *dfrA1* genes was tested by PCR using total DNA (extracted by the boiling method) as a template. Table 2 shows the primers, amplicon sizes and references corresponding to the detection of resistance determinants for SXT. The PCR reactions for detection of class 1 and class 2 integrons were performed in a total volume of 25 ul containing 0.2 mM each dNTP, 1 U of GoTaq DNA polymerase (Promega) in 5× GoTaq reaction buffer, 10 pmol of each primer (Table 2) and 5 µl of template DNA. The thermocycler conditions for the detection of the *intI1* gene were: 5 min at 95 °C, followed by 35 cycles of 45 s at 94 °C, 1 min at 64 °C and 90 s at 72 °C, with a final extension of 10 min at 72 °C. For the detection

Table 1. Characteristics of 11 sequential *S. maltophilia* isolates

CF isolates	Date of sample	Co-isolated micro-organisms	Antibiotic resistance	API 20NE biocode	Mutation frequency (*f*)
Sm1318	26 May 2014	*C. albicans*	SXT	II	(S) $8.10\times10^{-7}\pm0.16$
Sm1321	8 August 2014	*C. albicans*	SXT	II	(N) $3.58\times10^{-8}\pm0.09$
Sm1324	4 November 2014	–	SXT LEV	ND	ND
Sm1326	24 February 2015	*Streptococcus australis*	LEV	I	(W) $4.19\times10^{-8}\pm0.21$
Sm1331	13 November 2015	*C. albicans*	LEV	ND	ND
Sm1332	25 February 2016	*C. albicans* *Streptococcus haemolyticus*	SXT	ND	ND
Sm1336	12 July 2016	*C. albicans* *Klebsiella pneumoniae*	LEV	II	(S) $5.34\times10^{-7}\pm0.11$
Sm1340	1 December 2016	*K. pneumoniae*	LEV	III	(W) $7.55\times10^{-8}\pm0.07$
Sm1342	9 July 2017	*C. albicans*	SXT	I	(S) $8.68\times10^{-7}\pm0.22$
Sm1346	11 December 2017	*C. albicans* *Streptococcus sanguinis* group	SXT	II	(W) $1.40\times10^{-7}\pm0.18$
Sm1347	24 May 2018	–	SXT	III	(N) $3.98\times10^{-8}\pm0.09$

The antimicrobial susceptibility for minocycline, trimethoprim–sulfamethoxazole (SXT) and levofloxacin (LEV) was assessed using the disc diffusion method according to CLSI recommendations. API20NE biocodes were determined based on the production of β-galactosidase and cytochrome oxidase: I, 0452341 (−/−); II, 0452345 (−/+); and III, 0472345 (+/+). Rifampicin resistance mutation frequency (*f*) was determined as described in the Methods section and the isolates were classified as normomutators ($8\times10^{-9}<f<4\times10^{-8}$), weak mutators ($4\times10^{-8}\leq f<4\times10^{-7}$) or strong mutators ($f\geq4\times10^{-7}$). *S. maltophilia* K279a was used as control (N; $f=3.83\times10^{-8}\pm0.04$).
CF, cystic fibrosis; ND, not determined.

of the *intI2* gene, the thermocycler conditions were: 5 min at 95 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C, with a final extension of 10 min at 72 °C. The PCR reactions for the detection of the *qacEΔ1-sul1*, *sul1* and *dfrA1* genes were performed in a 25 µl final volume containing 1 U of GoTaq DNA polymerase (Promega) in 5× GoTaq reaction buffer, dimethyl sulfoxide 10%, 0.4 mM of each dNTP, 10 pmol of the corresponding primers (Table 2) and 3 µl of bacterial DNA. The amplification profile was 5 min at 95 °C, 30 cycles of 1 min at 95 °C, 1 min at 60 °C (for *qacEΔ1-sul1*), 68 °C (for *sul1*) or 55 °C (for *dfrA1*), and 1 min at 72 °C, followed by a final extension of 10 min at 72 °C. For PCR negative controls, DNA template was substituted with sterile distilled water. The strains used as a positive control in the PCR reaction were *Serratia marcescens* SCH909 [32] (positive control *intI1* and *dfrA1*), *Salmonella enterica* serovar Infantis S21 [33] (AJ311891, positive control *sul1* and 3′CS region) and *E. coli* 8157 EU780012.1 [34] (positive control *intI2*). *S. maltophilia* SmEL06, a SXT-resistant CF isolate recovered from a chronically colonized paediatric patient in Argentina that was positive for the *intI1*, *qacEΔ1-sul1* and *sul1* genes, was included in the PCR reaction for the detection of resistance determinants for SXT [29]. K279a, a strain that is sensitive to SXT, was also included [27]. PCR amplifications were carried out in a MyCycler thermal cycler (Bio-Rad). PCR-amplicons were resolved in 1.5% agarose gels containing ethidium bromide and visualized under UV light.

Mutation frequency assay

Rifampicin resistance mutation frequency was determined as described previously [35]. Independent triplicate Luria Bertani (LB) broth cultures of each *S. maltophilia* isolate were grown with agitation. After overnight incubation total viable cell count was determined by plating on LB agar plates and the cell pellet from 1 ml culture was inoculated on LB/rifampicin plates (250 µg ml^{-1}).The spontaneous rifampicin-resistant mutation frequency was calculated by dividing the mean number of rifampicin-resistant colonies by the total viable cell count. *S. maltophilia* isolates were classified into four categories based on their mutation frequencies (*f*): hypomutators ($f\leq8\times10^{-9}$), normomutators ($8\times10^{-9}<f<4\times10^{-8}$), weak mutators ($4\times10^{-8}\leq f<4\times10^{-7}$) and strong mutators ($f\geq4\times10^{-7}$) [35].

Phenotypic and genotypic typing

Phenotypic typing of the isolates was performed using API20NE test kits (bioMérieux, France) according to the manufacturer's instructions. Genotypic typing was assessed by RAPD-PCR as described previously [36]. Briefly, amplification reactions were performed in a 25 µl final volume containing 2 U of GoTaq DNA polymerase (Promega) in 5× GoTaq reaction buffer, 4 mM $MgCl_2$, dimethyl sulfoxide 10%, 0.4 mM of each dNTP, 50 pmol of primer ERIC-2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) and 3 µl of

Table 2. Oligonucleotides used to examine the presence of integrons and virulence factors

Target/gene	Primers	Sequence (5′–3′)	Amplicon size (bp)	Reference
Class 1 integrase/*intI1*	Sulpro3	GCCTGACGATGCGTGGA	Variable region of integrons	[17, 32]
	Sul1r	TTTGAAGGTTCGACAGC		
Class 2 integrase/*intI2*	Inti 2 F	GCAAATGAAGTGCAACGC	495	[17]
	Inti 2 R	ACACGCTTGCTAACGATG		
3'CS region/*qacEΔ1-sul1*	qacEΔ1F	ATCGCAATAGTTGGCGAAGT	798	[33]
	Sul1B	GCAAGGCGGAAACCCGCGCC		
Sul1/*sul1*	Sul1F	CTTCGATGAGAGCCGGCGGC	437	[33]
	Sul1B	GCAAGGCGGAAACCCGCGCC		
DfrA1/*dfrA1*	dfrA1-F	AGCTGTTCACCTTTGGC	180	[17, 32]
	dfrA1-R	CCTGAAATCCCCAGCAA		
NarG/*narG*	narG-F	GGCTTGAGCACGATGCGGGT	623	[36]
	narG-R	GTGGGCAAGGAGCACGAGGC		
StmPr1/*stmPr1*	StmPr1-F	GCCGCAGTGTTGGTTCGATCCA	1621	[12]
	StmPr1-R	CAGTTCTCGGTGCACGGCTCTT		
StmPr2/*stmPr2*	StmPr2-F	CGTGCCAGCTTCTCCAACTA	570	This study
	StmPr2-R	AGGACTGTTGATGGTGCAGG		
RpfC/*rpfC*	RpfC-F	TGTTCGGCTGGCTGCTGT	1019 (*rpfC–rpfF1*)	[28]
	RpfC-R	GGCCAGCACTTCGGTCAT	450 (*rpfC–rpfF2*)	

bacterial DNA. PCR product patterns were considered identical when the positions of all bands matched, regardless of band intensity [37]. Isolates were also genotyped by PFGE using the restriction enzyme *XbaI* (Thermo Fisher Scientific) as reported previously [36]. Gels were stained with ethidium bromide and digitized (Molecular Imager Gel Doc XR, Bio-Rad). The dendrogram of PFGE profiles was constructed using UPGMA cluster analysis with Treecon software (http://bioinformatics.psb.ugent.be). Strains were defined as having a clonal relationship if they possessed ≥85% similarity to the PFGE profiles [38].

Screening of clusters *RpfC–RpfF1* and *RpfC–RpfF2*

The presence of the QS system variants, RpfC–RpfF1 and RpfC–RpfF2, was investigated by PCR. Table 2 shows the primers, amplicon sizes and references corresponding to the detection of these two variants. The amplification reactions were performed in a 25 µl final volume containing 1 U of GoTaq DNA polymerase (Promega) in 5× GoTaq reaction buffer, dimethyl sulfoxide 10%, 0.4 mM of each dNTP, 10 pmol of the each primer (Table 2) and 3 µl of bacterial DNA. The thermocycler conditions were 5 min at 95 °C, 30 cycles of 1 min at 95 °C, 1 min at 60 °C and 90 s at 72 °C, followed by a final extension of 10 min at 72 °C. The strains used as a positive control in the PCR reaction were *S. maltophilia* K279a (positive control *rpfC–rpfF1*) and *S. maltophilia* D456 (positive control *rpfC–rpfF2*) [28]. The length of the amplification products, analysed by electrophoresis in a 1.5% agarose gel, was used to discriminate between variants.

Detection of *narG*, *stmPr1* and *stmPr2* genes

The presence of *narG*, *stmPr1* and *stmPr2* genes was determined by PCR using a simple boiling DNA extraction as a template. Table 2 shows the primers, amplicon sizes and references corresponding to the detection of these virulence factors. All primers were designed based on the genomic sequence of K279a (GenBank: AM743169.1). Amplification reactions were performed in a 25 µl final volume containing 1 U of GoTaq DNA polymerase (Promega) in 5× GoTaq reaction buffer, dimethyl sulfoxide 10%, 0.4 mM of each dNTP, 10 pmol of the corresponding primers (Table 2) and 3 µl of bacterial DNA. The amplification profile was 5 min at 95 °C, 30 cycles of 1 min at 95 °C, 30 s at 50 °C (for *narG*), 55 °C (for *stmPr1*) or 48 °C (for *stmPr2*) and 1 (*stmPr2* and *narG*) or 2 min (*stmPr1*) at 72 °C, followed by a final extension of 10 min at 72 °C. *S. maltophilia* K279a was used as a positive control in he PCR reaction for *narG*, *stmPr1* and *stmPr2* [12, 36]. The amplification products were analysed by electrophoresis in a 1.5% agarose gel.

Nitrate reduction

The ability of the isolates to reduce nitrate was assessed using the API 20NE system.

Biofilm formation

Biofilm formation was evaluated using a static microtitre plate model and standardized cultures as described previously [10]. After 24 and 48 h of incubation in TSB at 35 °C, biofilms were stained with crystal violet (CV) and total biomass (attached cells and extracellular matrix) was quantified by measuring the OD_{546} of ethanol-dissolved CV using a FlexStation 3 plate reader (Molecular Devices). The cut-off (ODc) was defined as three standard deviations above the mean OD of the negative control (uninoculated medium). Each isolate was assayed in octuplicate and classified as strong (OD >4×ODc), moderate (2×ODc<OD≤4×ODc), weak (ODc <OD≤2×ODc) and non-biofilm producer (OD ≤ODc) [39]. The levels of CV staining were also expressed relative to the final culture density measured prior to biofilm assay (OD546 CV/OD546 growth ratio) to avoid variations due to differences in bacterial growth [10].

For the following assays, each *S. maltophilia* isolate was incubated at 35 °C with agitation (200 r.p.m.) in the corresponding media and after the incubation period (specified in the corresponding reference) the final culture density was adjusted with fresh media to an OD_{546} of 1.00 in order to avoid variations due to differences in bacterial growth.

Motility assays

Swimming and twitching motility assays were performed in triplicate according to Rashid and Kornberg [40], with some modifications. Briefly, motility plates were inoculated with bacteria from standardized overnight TSB cultures with a sterile toothpick. Swimming agar was surface-inoculated and after incubation at 30 °C for 24 h, the growth zone was measured in millimetres. Twitching plates were inoculated to the bottom of the Petri plate, incubated at 35 °C for 48 h, and examined for a haze zone of growth at the interface between the agar and the Petri plate. Then, the agar was removed, the plate was stained with CV (0.1%) for 30 min and twitching activity was measured in milimetres.

Protease production

The proteolytic activity was visualized on nutrient agar (Difco) supplemented with 1% skimmed milk powder. Quantification was performed by placing a 40 µl volume of *S. maltophilia* standardized cultures in 7.2 mm diameter wells previously cut into the agar plate. The plates were incubated for 24 h and the diameter of the zones of hydrolysis was measured [10]. The proteolytic activity was also examined after incubation for 48 and 72 h.

Siderophore production

The production of siderophores was determined by a modified chrome azurol S (CAS) agar assay as described previously [13]. Standardized suspensions were stab-inoculated onto CAS agar plates and incubated at 35 °C for 48 h. Siderophore production was recorded as the diameter of the orange halo produced by the colony. Each isolate was classified as a weak (4.0–5.9 mm), moderate (6.0–8.9 mm) or strong siderophore producer (>9 mm) [36].

Sensitivity to oxidative stress

Sensitivity to hydrogen peroxide was determined using the disc assay [10]. Briefly, *S. maltophilia* 24 h TSB cultures were standardized and 100 µl aliquots were spread on TSA plates. Sterile Whatman filter paper discs (7 mm diameter) were impregnated with 3 µl of 12% H_2O_2 and placed in triplicate on each plate. After incubation at 35 °C for 24 h, the diameter of the zone of growth inhibition around the discs was measured. The resistance of each isolate to hydrogen peroxide was classified as: low (>18 mm), moderate (16–18 mm), or high (<16 mm) [36].

DSF production

DSF production was assayed, as described previously, by measuring the restoration of endoglucanase activity to the *rpfF* mutant Xcc 8523 by extracts from culture supernatants [14]. The diameters of zones of carboxymethyl cellulose (CMC; Sigma-Aldrich) hydrolysis, produced by 50 µl of extract, were measured and converted to relative endoglucanase units with a standard curve constructed using dilutions of a standard of cellulase I (Sigma Aldrich). One unit of endoglucanase was defined as the amount that gave a hydrolysis zone with a diameter of 12 mm.

Statistical analysis

Experiments were performed on three different occasions, at least in triplicate. Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, www.graphpad.com). Results were analysed using one-way analysis of variance (ANOVA) with Dunnett's post-test, and differences were considered significant at P values <0.05.

RESULTS AND DISCUSSION

A total of 11 *S. maltophilia* isolates, identified by MALDI-TOF MS, were recovered from he expectorations of an adult female with CF between May 2014 and May 2018. The patient attended the Instituto de Investigaciones en Microbiología y Parasitología Médica (IMPaM, UBA-CONICET), Buenos Aires, Argentina, from December 2006. *P. aeruginosa* and *Burkholderia cepacia* complex were recovered intermittently from the patient between December 2006 and November 2009. *P. aeruginosa* was eradicated in 2010 and *B. cepacia* complex in 2011. Then, negative cultures were observed until May 2014 when *S. maltophilia* was isolated for the first time and showed persistence over a 4-year period. In accordance with previous reports [8], polymicrobial cultures were obtained from 9/11 samples, with *Candida albicans* (7/9, Table 1) being the most frequent co-isolated micro-organism.

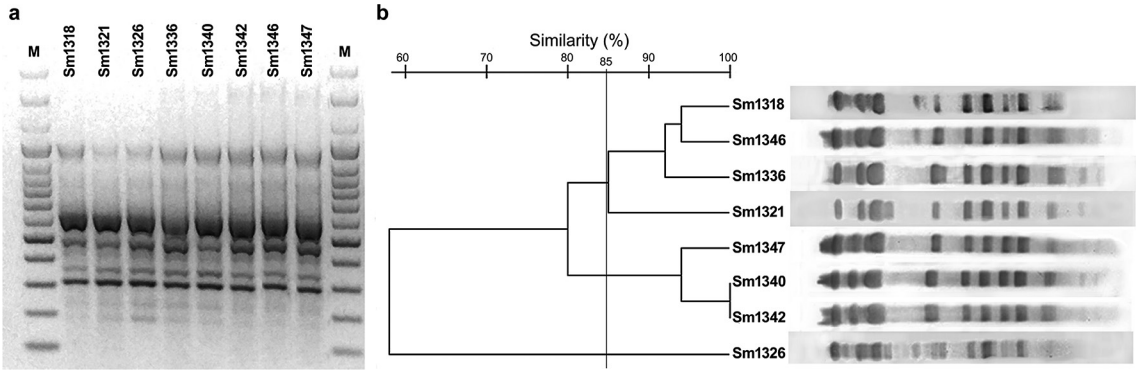

Fig. 1. Genotypic typing of sequential *S. maltophilia* isolates. (a) RAPD-PCR amplification patterns obtained with primer ERIC-2. M, 1 Kb molecular weight marker. (b) Phylogenetic analysis of *XbaI* PFGE profiles. The dendrogram of PFGE profiles of eight *S. maltophilia* isolates was constructed using UPGMA cluster analysis with Treecon software. Isolates were defined as having a clonal relationship if they possessed ≥85% similarity to the PFGE profiles.

Susceptibility

Table 1 summarizes the resistance data. Seven out of 11 *S. maltophilia* isolates showed resistance to SXT and 5 were resistant to levofloxacin, while only Sm1321 had intermediate susceptibility to this fluoroquinolone. In accordance with previous reports, all sequential isolates were susceptible to minocycline [21, 36].

Detection of resistance determinants to SXT

Previously, we had found that class 1 integrons increase minimum inhibitory concentrations (MICs) for SXT in epidemiologically unrelated *S. maltophilia* strains from our country [17]. Further, worldwide emergence of SXT resistance has been reported in *S. maltophilia* isolates from patients with CF [20]. The spread of resistance is related to dihydropteroate synthase (*sul1* and *sul2*) genes and to dihydrofolate reductase (*dfrA*) gene cassettes usually located in the variable region of class 1 and class 2 integrons [17, 18]. In this study, the presence of class 1 and 2 integrons was examined by cartography of integrons as described previously [41] using PCR in the five SXT-resistant *S. maltophilia* isolates (Table 2). None of the isolates, or the SXT-sensitive strain K279a, were positive for *qacEΔ1-sul1*, *sul1*, *dfrA1* and integrase genes (*intI1* and *intI2*) under the described PCR amplification conditions (Fig. S1, available in the online version of this article). Although all primers were designed while taking the conserved regions of the different targets into account, it is important to note that specific genes were examined by PCR, given that negative results can be interpreted not only as the absence of the gene but also as insufficient complementarity to the primer used. However, we have recently reported that two SXT-resistant *S. maltophilia* CF isolates recovered from a chronically colonized paediatric patient in Argentina were positive for class 1 integrase, *qacEΔ1-sul1* and *sul1* genes [29]. One of these isolates, SmEL06, was included in the PCR assays, which accurately detected the three mentioned SXT resistance determinants (Fig. S1). Thus, our results suggest that other antimicrobial resistance mechanisms unrelated to the acquisition of multidrug-resistant integrons are emerging in *S. maltophilia* strains isolated from patients with CF, which should be studied in the near future, since SXT continues to be considered as the antimicrobial therapy of choice for severe *S. maltophilia* infections [18].

Evaluation of mutation frequency

Microbial populations of *S. maltophilia*, as well as other bacterial species in CF lungs, are exposed to a challenging environment and repeated antibiotic treatments that favour the emergence of mutator phenotypes [3, 42]. The emergence and adaptation of strong mutators in *S. maltophilia* within the CF lungs is not well established, since although two groups identified a decreased mutation rate over time [2, 9], another group found a trend toward increased mutation rates in isolates from a patient [3]. We analysed this capacity in our isolates in order to contribute to this controversial role in *S. maltophilia*. Table 1 shows that, based on the spontaneous rifampicin-resistant mutation frequency, three CF *S. maltophilia* isolates were classified as strong mutators (Sm1318, Sm1336 and Sm1342), another three as weak mutators (Sm1326, Sm1340 and Sm1346), and the remaining two (Sm1321 and Sm1347), as well as the control strain K279a, as normomutators [35]. Although our sampling number is small (n=8) the prevalence of strong mutators (37.5%) is close to that described in previous reports [3]. In accordance with a previously reported tendency of most *S. maltophilia* clonally related genotypes to vary their mutation frequency over time and become less mutable as a strategy for persistence in the CF lung [2, 9], we observed a tendency towards a decreased mutation rate over time (Table 1).

Phenotypic and genotypic typing

Biochemical typification of the *S. maltophilia* sequential isolates performed using API20NE showed three biotypes: I (Sm1326 and Sm1342), II (Sm1318, Sm1321, Sm1336 and Sm1346) and III (Sm1340 and Sm1347), based on the production of β-galactosidase and cytochrome oxidase (Table 1).

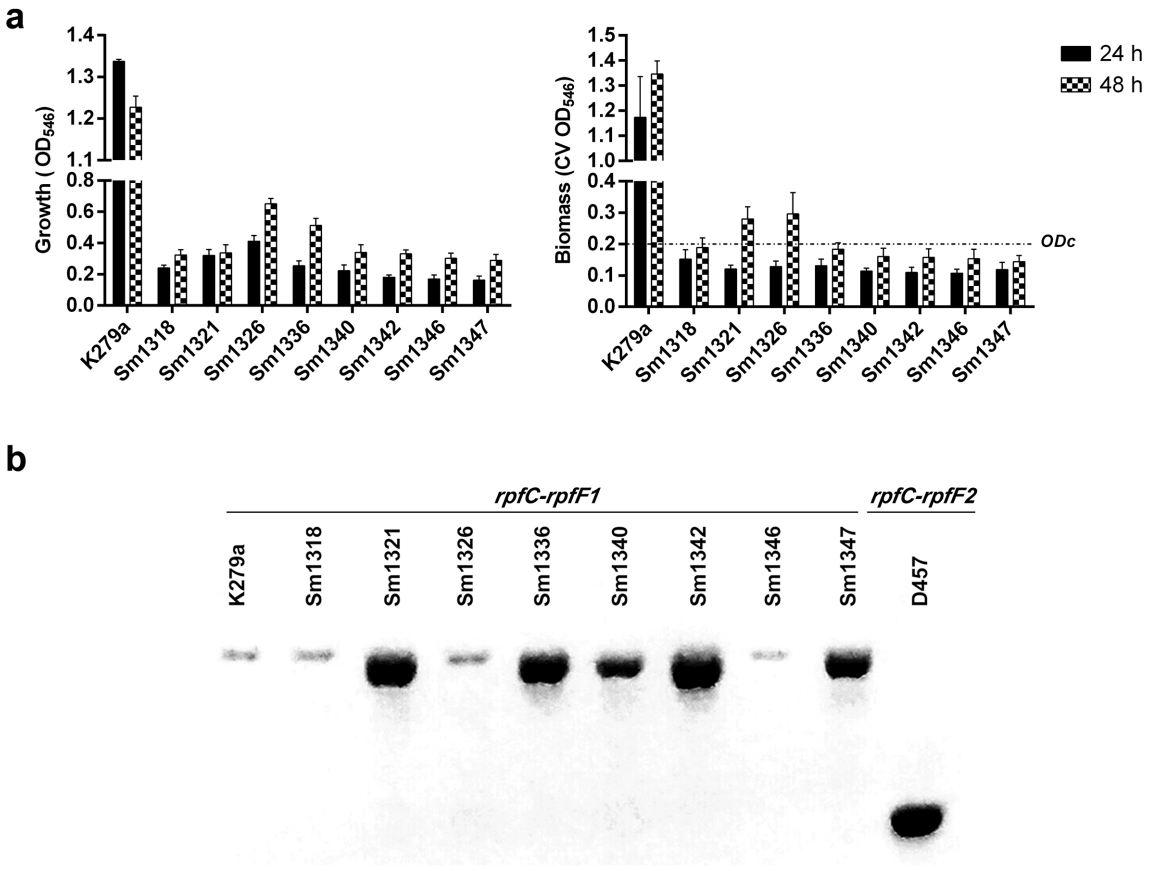

Fig. 2. (a) Bacterial growth (left panel) and biofilm biomass (right panel) of sequential *S. maltophilia* isolates cultured statically in TSB at 35 °C was determined in microtitre plates at 24 and 48 h by measuring the OD_{546} of the culture and of the CV dissolved in ethanol, respectively. ODc, cut-off, defined as three standard deviations above the mean OD of the negative control. Results are expressed as the mean±standard deviation of one of three representative experiments carried out in octuplicate. *S. maltophilia K279a* was used as a control. (b) Screening of RpfC–RpfF1 and RpfC–RpfF2 quorum-sensing variants. *S. maltophilia* K279a (RpfC–RpfF1 variant) and *S. maltophilia* D457 (RpfC–RpfF2 variant) were used as positive controls.

Even though *S. maltophilia* is usually oxidase-negative, analysis of 766 isolates showed that 20% were oxidase-positive. Thus, *S. maltophilia* should be considered to be variable with respect to oxidase activity [43].

Genotypic typing assessed by RAPD PCR, using primer ERIC-2, revealed that all the isolates shared a unique fingerprint (Fig. 1). Then, *XbaI* PFGE was conducted to evaluate the relationship among these indistinguishable isolates. This technique indicated that seven isolates belonged to two related clones, while Sm1326 had a different profile (Fig. 1). Two isolates of one of these clones, Sm1340 and Sm1342, presented indistinguishable PFGE DNA banding patterns, although characterized by different susceptibility to antimicrobials, API20NE biocodes and mutation frequency (Table 1).

Presence of virulence factors

The CF *S. maltophilia* isolates were characterized by the presence and/or expression of selected virulence determinants.

Biofilm formation in microtitre plates was evaluated at 24 and 48 h of incubation. Growth was determined by measuring the OD_{546} prior to evaluating biofilm formation proficiency by CV staining. Fig. 2a (left panel) shows that at 24 h of incubation CF isolates presented poor bacterial growth, with ODs ranging from 0.163 to 0.411, and none of them formed biofilms (Fig. 2a, right panel). Improvement of growth was registered at 48 h, when most isolates reached mean OD_{546} readings of approximately 0.300, except for Sm1326 and Sm1336, which attained an OD_{546} of 0.652 and 0.512, respectively. Only two CF isolates, Sm1321 and Sm1326, formed biofilms after 48 h of incubation. Even though these isolates had different growth proficiency, they were both classified as weak biofilm producers. In contrast, the positive control K279a showed growth ODs of 1.347 and 1.227 at 24 and 48 h, respectively, and formed strong biofilms at both times. In order to avoid variations due to differences in bacterial growth, we also expressed biofilm formation results as OD_{546} CV/OD_{546} growth ratio. At 48 h, the values for Sm1321 and Sm1326 were 0.747±0.110 and 0.452±0.107, respectively, showing a better adherence proficiency for Sm1321 in spite of its poor growth. We do not rule out the possibility that reduced biofilm formation in the studied CF isolates could

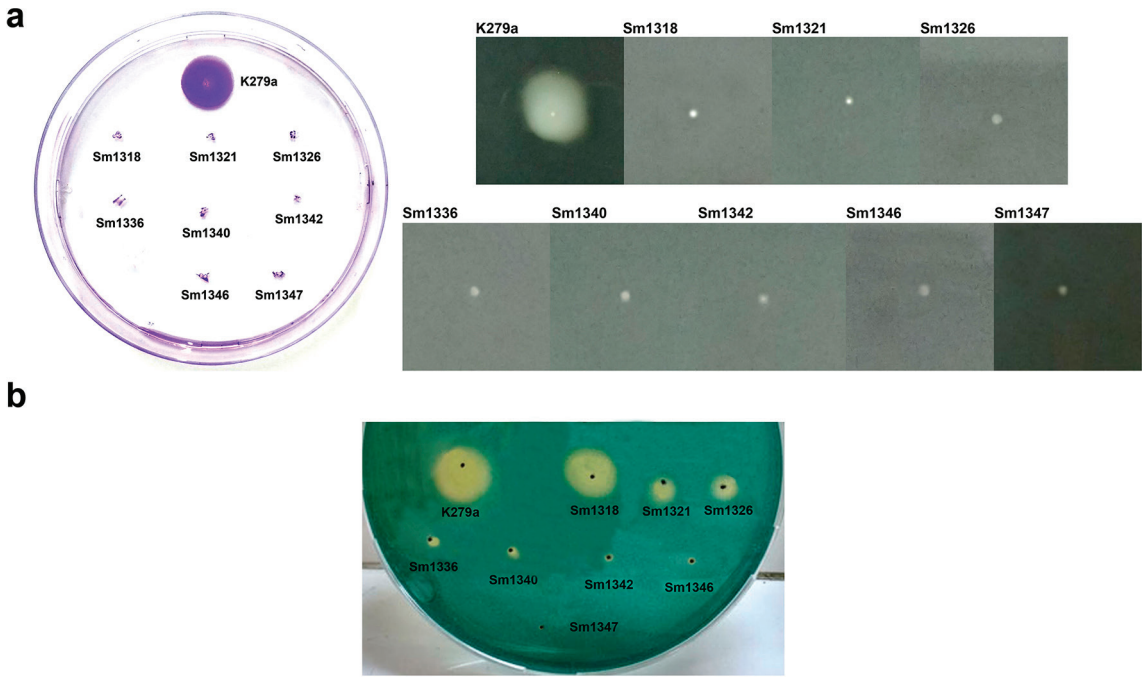

Fig. 3. Motility and siderophores assays of sequential *S. maltophilia* isolates. (a) Twitching motility (left panel). Twitching plates were inoculated to the bottom of the Petri plate and incubated at 35 °C for 48 h. Then, the agar was removed, the plate was stained with CV and twitching activity was measured in millimetres. Swimming motility (right panel). Isolates were surface-inoculated on swimming agar and, after incubation at 30 °C for 24 h, the growth zone was measured in millimetres. (b) Siderophore production was determined by the modified chrome azurol S (CAS) agar assay and recorded as the diameter of the orange halo produced by the colony after incubation at 35 °C for 48 h. *S. maltophilia* K279a was used as a positive control in all assays.

be associated with a growth defect. However, since biofilm formation is a multifactorial process, the superior adherence proficiency of Sm1321 despite its deficiency in bacterial growth suggests that other factors are also involved. It is worth noting that different studies reported that non-CF strains are significantly more efficient than CF strains in forming biofilms [44, 45]

Fig. 3a shows that none of the isolates had the ability to move via swimming or twitching, motilities that have been involved in biofilm formation [10, 11]. The mean diameter of the swimming zones for the positive control K279a was 9.29±0.26 mm. Regarding twitching motility, only K279a formed a hazy growth surrounding the colony and the mean diameter of the twitching zones, visualized by CV staining, was 15.13±0.90 mm. There were also striking differences between the adherence patterns. K279a showed a doughnut-shaped adherent zone, whereas the CF isolates remained adhered near the stab inoculation point.

The absence of swimming and twitching motilities has previously been reported in CF and non-CF *S. maltophilia* clinical isolates [25]. Accordingly, we have recently advised, in a congress oral presentation, that *S. maltophilia* isolates recovered from chronically colonized paediatric patients with CF in Argentina differed in some phenotypic traits, and some of them were non-biofilm producers and did not show the ability to move via swimming or twitching [29, 46].

Some strains of *S. maltophilia* such as K279a have a membrane-bound nitrate reductase, and it has been proposed that growth potential under microoxic conditions increase the ability to form biofilms [27]. In this study K279a was used as a control and presented nitrate reduction activity in the API 20NE system and amplified the 623 bp fragment corresponding to *narG* by PCR. By contrast, no nitrate reduction activity was detected in the studied isolates and, accordingly, the PCR for *narG* rendered negative results (Fig. S2). In a previous study of 63 *S. maltophilia* isolates from patients exposed to invasive devices in a university hospital in Argentina we detected 26 isolates with no nitrate reduction activity and negative PCR for *narG* [36].

The major extracellular protease StmPr1 has been proposed as a relevant virulence factor for *S. maltophilia* [12]. None of the isolates showed proteolytic activity after 24–48 h of incubation, but, in contrast, at 24 h, K279a showed strong proteolytic activity. However, after 72 h of incubation Sm1321, Sm1336, Sm1340 and Sm1342 showed weak exoenzyme activity with zones of proteolysis ranging from 4.99 to 2.89 mm, while the mean value for K279a was 18.03 mm (Table 3). Accordingly, these isolates, as well as K279a, amplified the 1621 bp fragment corresponding to the *stmPr1* gene, which encodes the major alkaline serine protease, and the 570 bp fragment corresponding to the *stmPr2* gene, by PCR (Table 3, Fig. S2). Isolates Sm1318 and Sm1326 also amplified both genes

Table 3. Protease, siderophore and DSF production, and stress oxidative resistance

Isolate	Protease			Siderophore production (mm)	Stress oxidative resistance (mm)	DSF production ($U_{endoglucanase\ activity}$)
	Enzyme activity (mm)	*stmPr1 gene*	*stmPr2 gene*			
K279a	18.03±0.17	+	+	(S) 11.2±0.2	(HR) 13.62±0.28	11.77±0.73
Sm1318	0	+	+	(S) 10.4±0.7	(HR) 15.43±0.49	11.54±0.71
Sm1321	2.89±0.08	+	+	(W) 5.5±0.4	(HR) 14.42±0.29	11.20±1.02
Sm1326	0	+	+	(W) 4.5±0.1	(HR) 14.70±0.48	11.19±0.77
Sm1336	2.97±0.06	+	+	0	(HR) 15.31±0.21	13.42±0.26
Sm1340	2.89±0.07	+	+	0	(MR) 17.42±0.44	13.82±0.42
Sm1342	4.99±0.02	+	+	0	(MR) 16.48±0.30	12.78±0.64
Sm1346	0	–	–	0	(MR) 16.89±0.38	14.33±0.55
Sm1347	0	–	–	0	(MR) 16.67±0.46	16.43±0.68

The proteolytic activity on nutrient agar supplemented with 1% skimmed milk powder was measured after 72 h of incubation. The presence of *stmPr1* and *stmPr2* genes was tested by PCR. Siderophore production was visualized as an orange halo produced by the colony on modified CAS agar. Each isolate was classified as a weak (W, 4.0–5.9 mm), moderate (M, 6.0–8.9 mm) or strong siderophore producer (S,>9 mm). Sensitivity to hydrogen peroxide was determined as zones of growth inhibition surrounding each disc after 24 h of incubation. The resistance of each isolate to hydrogen peroxide was classified as low (LR, >18 mm), moderate (MR, 16–18 mm) or high (HR, <16 mm). DSF production was assayed by measuring the restoration of endoglucanase activity to the *rpfF* mutant Xcc 8523 by extracts from culture supernatants of *S. maltophilia* isolates. Diameters of zones of carboxymethyl cellulose hydrolysis were converted to relative endoglucanase units with a standard of cellulase I. All assays were conducted as described in in the Methods section and *S. maltophilia* K279a was used as a control.

(Fig. S2) but scored negative for protease activity, suggesting the presence of mutations in these genes similar to those previously reported [12]. On the other hand, isolates Sm1346 and Sm1347 were non-proteolytic and failed to amplify the *stmPr1* and *stmPr2* genes (Fig. S2).

Siderophores are important virulence factors, since they allow micro-organisms to survive in the host. When grown on modified CAS agar plates, only the first three sequential *S. maltophilia* isolates were CAS-positive for siderophore production (Table 3). They were classified as moderate (Sm1318) and weak siderophore producers (Sm1321 and Sm1326). As previously reported, the reference strain K279a showed strong siderophore production [13].

Sensitivity to oxidative stress, a relevant determinant for bacterial survival, was evaluated using the hydrogen peroxide disc assay. Based on the diameters of the growth inhibition zones, two different groups were identified among the *S. maltophilia* isolates: one of them (Sm1318, Sm1321, Sm1326 and Sm1336, as well as K279a) exhibited high resistance, while the other (Sm1340, Sm1342, Sm1346 and Sm1347) showed moderate resistance (Table 3). Thus, a sequential evolution from high to moderate resistance over time was detected.

Screening of RpfC–RpfF clusters and DSF production

In this study, the presence of the RpfC–RpfF1 and RpfC–RpfF2 variants was investigated by PCR using the primers shown in Table 2. All of the *S. maltophilia* isolates, as well as K279a, amplified the 1019 bp fragment corresponding to the RpfC–RpfF1 variant (Fig. 2b). Furthermore, extracts from culture supernatants of all isolates restored the endoglucanase activity of the *rpfF* mutant Xcc 8523, indicating DSF production. Interestingly, Table 3 shows that the first three CF isolates (Sm1318, Sm1321 and Sm1326) produced similar concentrations of DSF to those of K279a, within a range of 11.19 to 11.77 endoglucanase units. On the other hand, the last five CF isolates produced significantly higher concentrations of DSF ($P<0.001$), especially Sm1346 and Sm1347 (14.33±0.55 and 16.43±0.68 U, respectively).

Huedo *et al.* suggested that the QS signalling of RpfC–RpfF1 strains has a positive role in biofilm formation [15]. Accordingly, in a recent paper, we presented results that provided evidence for the positive role of DSF from the RpfC–RpfF1 variant K279a in biofilm formation, swimming and twitching motilities, and siderophore production, as well as resistance to oxidative stress [47]. Even though the eight RpfC–RpfF1 CF isolates produced DSF, they were non-motile and only Sm1321 and Sm1326 formed weak biofilms. Furthermore, siderophore production was only detected in the first three isolates and resistance to hydrogen peroxide decreased over time, since the first four isolates exhibited high resistance, while the last four showed moderate resistance. These features could be associated with the high amounts of DSF produced by the last five isolates. Torres *et al.* [48] demonstrated that DSF synthesis in *X. campestris* has to be finely controlled to form structured biofilms, since a DSF overproducer mutant formed only unstructured arrangements of bacteria.

The described virulence phenotypic changes were observed in CF isolates recovered longitudinally, which, with the exception of Sm1326, belong to two related clones (Fig. 1). However, isolates of the same clone did not share the same virulence/resistance pattern. It is known that different populations of the same clone can evolve in the CF lung, given that they are exposed to a difficult environment and repeated antibiotic treatments that favour the emergence of mutant phenotypes [25]. In the present study, based on the spontaneous rifampicin-resistant mutation frequency, CF isolates were classified as strong mutators, weak mutators and normomutators, and a decreased mutation rate tendency was observed over time. Furthermore, Sm1340 and Sm1342 presented indistinguishable PFGE profiles but different API20NE biocodes, mutation frequency and susceptibility to SXT and levofloxacin (Table 1). These findings are in agreement with another report on the small relationship between *S. maltophilia* genotypes and observed phenotypes, including susceptibility to antibiotics, which suggests the existence of complex regulatory mechanisms that need to be investigated [3].

There is currently no consensus on the management of patients with CF and *S. maltophilia*-positive cultures. In some cases of exacerbation or chronic colonization/infection these patients are treated with antibiotics, but without consensus on the optimal regimen [49]. In a recent single-centre US cohort study, 44% of 88 patients who had a positive culture for *S. maltophilia* developed chronic infection, a result in accordance with that described by Barsky *et al.* [50]. Thus, as previously suggested, randomized controlled trials are needed to determine the effectiveness of antibiotic treatment for *S. maltophilia* infection in people with CF. Furthermore, approaches for eradication as well as those recommended for initial *P. aeruginosa* infection might be investigated [49, 50].

The limitations of our study include having considered only one chronically colonized patient and analysed only one colony from each clinical specimen at each different time point, which underestimates the heterogeneity of *S. maltophilia* in the CF airways at each sampling moment. Analysis of the intra-host evolution of multiple isolates of *S. maltophilia* represents a more appropriate way to address the complexity of the adaptation of CF pathogens thoroughly. However, several studies that analyse pathogens in the setting of CF have used the one isolate per sample model [26, 51]. We have initiated the study of *S. maltophilia* isolates from chronically colonized paediatric patients with CF from different hospitals of Argentina in order to evaluate genotypic and phenotypic adaptations involved in the progression from acute to chronic colonization.

CONCLUSION

The present study shows the long-time persistence of two related *S. maltophilia* clones in an adult female with CF. During the adaptation of the prevalent clones to the CF lungs over time, we identified a gradual loss of virulence factors that could be associated with the high quantities of DSF produced by the evolved isolates. Further, a decreased mutation rate was observed in the late isolates. The role of all these adaptations over time remains to be elucidated from a clinical perspective, probably focusing on the damage they can cause to CF lungs.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
This study received ethical approval from the Comité de Ética Independiente of Nexo AC (IRB0005349, prot. no. 2105). Patient data were anonymized and informed consent was obtained.

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