Frontiers in Bioscience Scholar

Front. Biosci. (Schol Ed) **2022**; 14(2): 9
https://doi.org/10.31083/j.fbs1402009

Original Research

Taxonomic position, antibiotic resistance and virulence factors of clinical *Achromobacter* isolates

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Academic Editor: Gustavo Caetano-Anollés
Submitted: 17 December 2021 Revised: 9 February 2022 Accepted: 11 February 2022 Published: 21 March 2022

Abstract

The role of *Achromobacter* species in lung disease remains unclear. The aim of this study was to characterize *Achromobacter* isolated from persons with cystic fibrosis and from other clinical samples. Whole genome sequences from 101 *Achromobacter* isolates were determined (81 from patients with cystic fibrosis and 20 from other patients) and analysed. Taxonomic analysis showed nine species including two putative novel species. Thirty-five novel sequence types were present. The most active agent was co-trimoxazole followed by imipenem, but Minimal Inhibitory Concentrations (MICs) were high. Acquired antibiotic resistance genes were rare. Their presence did not correlate with minimal inhibitory concentrations suggesting that other mechanisms are involved. Genes for proposed virulence factors were present in only some isolates. Two putative novel species were identified. The putative virulence properties of *Achromobacter* involved in infections are variable. Despite the high MICs, acquired resistance genes are uncommon.

Keywords: *Achromobacter*; cystic fibrosis; antibiotic resistance; virulence, taxonomy

1. Introduction

The genus *Achromobacter* currently comprises 20 species. *Achromobacter* species are mostly found in aquatic environments but may also be present among the intestinal microbiota of healthy persons. Furthermore, they may cause a range of human infections, in particular pulmonary infections in persons with cystic fibrosis (CF) [1–4]. In a Canadian and a French study, respectively, 11% and 27% of persons with CF were tested positive for *Achromobacter* by bacterial culture [5,6]. Colonization and/or infection may be persistent in persons with CF, but in some circumstances sputum can be rendered culture negative following antibiotic therapy [6]. Nevertheless, treatment is challenging due to both intrinsic and acquired resistance [2]; *Achromobacter* species encode OXA and AmpC type β-lactamases and efflux pumps [2,7]. Biofilm formation, which appears to be an intrinsic ability of all strains, contributes to antimicrobial resistance and virulence [8].

Several genome assemblies have been reported, but these were limited to one to six isolates, which yields only limited insight in the occurrence of virulence genes and acquired antibiotic resistance [9–12]. In this study we determined the whole genome sequences (WGS) of 101 *Achromobacter* isolates and report the diversity of the isolates, minimal inhibitory concentration for eight antimicrobial agents, and the presence of putative virulence genes. The isolates were obtained from CF, bronchiectasis (BE), and other diseases.

The aim of this study was to characterize the phylogenetics, antibiotic resistance, and virulence factors of clinical *Achromobacter* isolates based on whole genome sequencing.

2. Materials and methods

2.1 Bacterial isolates

A total of 101 *Achromobacter* isolates were analysed. These isolates had been cultured from respiratory samples of persons with CF (n = 81), respiratory samples of patients with other diseases (n = 13), blood cultures (n = 5), a patient with mastoiditis and a patient with otitis me-

dia. The isolates were recovered between 2003 and 2016 in four different countries: United Kingdom (n = 27), Spain (n = 27), the Netherlands (n = 46), and Australia (n = 1) (**Supplementary Table 1**).

Samples and patient data were collected in compliance with the Declaration of Helsinki ICH-GCP, the Declaration of Taipei regarding Health Databases and Biobanks, and with local and European regulations for collection and handling of patient data. Since the study concerned retrospectively collected anonymized patient data and bacterial strains, informed consent at the individual patient level was not required for this study. In addition, the Spanish and UK strains were collected in accordance with their local ethics guidelines and described in prior studies [13,14]. In the Netherlands, use and analysis of bacterial strains with anonymized patient data does not require approval from Institutional Review Boards/Ethics Committees.

Isolates were initially identified by Matrix Assisted Laser Desorption/Ionisation and Time-Of Flight Mass Spectrometry (MALDI-TOF MS) using a Microflex with Biotyper software MBT-BDAL-5627 MSP library (Bruker, Germany) according to the instructions of the manufacturer.

2.2 Whole genome sequencing

Bacterial DNA was purified using the Qiacube with the DNeasy Blood & Tissue kit with the enzymatic lysis protocol (Qiagen, Carlsbad, CA). Library for sequencing with the MiSeq or Nextseq (Illumina, San Diego, CA) platforms were prepared with the Nextera XT library prep kit (Illumina) according to the manufacturers' instructions. Contigs were assembled with SPAdes genome assembler v.3.6.2. with its default parameters and contigs shorter than 500 nucleotides were discarded [15]. Raw read sequences of all 101 isolates were uploaded to the NCBI's SRA database under the BioProject ID PRJNA723829.

2.3 Whole genome sequence analysis

Fast-ANI, developed for fast alignment-free computation of whole-genome Average Nucleotide Identity (ANI), was performed to confirm the species assignments [16,17]. A cut-off of 95% was used to define species [18,19].

Multi-locus Sequence Typing (MLST) was performed using PubMLST with the scheme for *Achromobacter xylosoxidans* [https://pubmlst.org/general.shtml]. Novel alleles and sequence types from MLST were submitted to the PubMLST database [https://pubmlst.org/general.shtml]. The MLST-based Minimum Spanning Tree was generated with PHYLOViZ 2.0 using the GoeBurst algorithm [https://phyloviz.readthedocs.io/en/].

The evolutionary history of the OXA-type β-lactamases was inferred using the Neighbor-Joining method in MEGA X [20,21]. The evolutionary distances were computed using the Poisson correction method and with the uniform variation rate for amino acid substitutions per site. All ambiguous positions were removed for each sequence pair and a bootstrap test with 1000 replicates was performed.

The assembled contigs were analyzed for the presence of acquired resistance genes by ResFinder [last accessed October 28, 2019] from the Center for Genomic Epidemiology (DTU, Copenhagen, Denmark) [22].

2.4 Determination of minimal inhibitory concentrations

Minimum Inhibitory Concentrations (MICs) of antimicrobial agents were determined by the standard ISO broth microdilution method with frozen panels (Trek Diagnostic Systems, Westlake, OH). The following antimicrobial agents (concentration ranges) were tested: ciprofloxacin (0.03–32 mg/L); tobramycin (0.125–128 mg/L); ceftazidime (0.25–256 mg/L); meropenem (0.06–64 mg/L); imipenem (0.125–128 mg/L); aztreonam (0.25–256 mg/L); trimethoprim/sulfamethoxazole (0.06–32 mg/L); and colistin (0.25–16 mg/L). The MIC_{50} and MIC_{90} were determined. The MIC_{50} and MIC_{90} are defined as the MIC, which inhibits 50% or 90% of the isolates, respectively.

3. Results and discussion

WGS yielded an average of 247 contigs per isolate (range 109–714); the average coverage was 51× (range 15–109×), and the total length of the assemblies varied between 5.71–7.16 MB, with a GC content between 64.29 and 68.33% (**Supplementary Table 2**).

MALDI-TOF, which is commonly used to identify isolates in routine diagnostic microbiology, identified 95 isolates as *A. xylosoxidans*, three as *A. insolitus*, and one as *A. spanius*. Two isolates were identified only to the *Achromobacter* genus level. Analysis of the WGS results confirmed 63/95 (66.3%) isolates as *A. xylosoxidans*. This was in agreement with the identification rates reported before with the used default MALDI-TOF database [2]. The remaining 32 *A. xylosoxidans* isolates were *A. insuavis* (n = 11), *A. ruhlandii* (n = 7), *A. deleyi* (n = 5), and two putative novel species, which were designated species1 (n = 8) and species2 (n = 1) in this manuscript. The isolate identified as *A. spanius* by MALDI-TOF was found to be *A. deleyi* by WGS. Two of the three isolates identified as *A. insolitus* by MALDI-TOF were confirmed by WGS, the third isolate was found to be *A. aegrifaciens* by WGS. The two isolates which were identified only to the genus level by MALDI-TOF were *A. spanius* and *A. deleyi* by WGS analysis (**Supplementary Table 1**). With the exception of the two novel species, all *Achromobacter* species have been reported previously in persons with CF [5].

Prior to this study, 485 *Achromobacter* STs had been reported. MLST analysis of this collection yielded 53 novel alleles and 35 novel sequence types (STs), indicating that only a portion of the genetic diversity had been assessed previously. A total of 61 STs were present among the

Table 1. ST, disease, MICs (mg/L), presence/absence OXA-family β-lactamase and acquired resistance genes.

ID	Species WGS	ST	Disease	Aztreonam	Ceftazidime	Ciprofloxacin	Colistin	Co-trimoxazole	Imipenem	Meropenem	Tobramycin	OXA β-lactamase	Acquired antibiotic resistance genes
537301	*A. aegrifaciens*	501	CF	>256	16	8	0.5	8	2	0.5	>128	-	sul2
548963	*A. deleyi*	518	CF	>256	8	8	>16	0.25	2	1	>128	-	-
548966	*A. deleyi*	518	CF	>256	8	32	>16	0.5	4	8	>128	-	-
548967	*A. deleyi*	518	CF	>256	16	32	1	0.5	4	8	128	-	-
548969	*A. deleyi*	518	CF	>256	8	32	>16	0.5	4	8	64	-	-
548972	*A. deleyi*	518	CF	128	2	1	0.5	≤0.06	2	0.12	0.5	-	-
548973	*A. deleyi*	518	CF	256	2	0.5	1	≤0.06	2	≤0.06	0.5	-	-
548974	*A. deleyi*	518	CF	256	8	8	0.5	0.25	0.5	2	4	-	-
534814	*A. insolitus*	503	CF	64	1	0.5	1	>32	1	≤0.06	>128	-	aac(3)-IV, aadA11, aph(3")-Ib, aph(4)-Ia, aph (6)-Id, tet(a), dfrA12, sul1
546376	*A. insolitus*	515	CF	64	2	16	4	0.5	1	0.12	>128	-	-
543171	*A. insuavis*	64	other	>256	4	2	1	0.12	0.25	0.12	32	+	-
537313	*A. insuavis*	274	CF	128	4	1	0.5	0.25	2	≤0.06	>128	+	aac(3)-IV, aph(4)-Ia, tet(A)
533618	*A. insuavis*	303	CF	128	4	4	1	0.25	2	0.12	128	+	-
555838	*A. insuavis*	303	CF	>256	8	1	1	1	4	2	16	+	-
537308	*A. insuavis*	491	CF	256	4	8	2	1	1	1	>128	+	-
546381	*A. insuavis*	496	other	256	4	1	0.5	0.5	4	0.12	8	+	-
533616	*A. insuavis*	502	CF	>256	8	32	2	4	2	0.5	32	+	-
539942	*A. insuavis*	512	CF	128	4	2	1	≤0.06	2	2	64	+	-
539939	*A. insuavis*	513	other	256	4	2	1	0.12	2	≤0.06	64	+	-
537305	*A. insuavis*	514	CF	>256	32	16	1	4	2	4	>128	+	-
543523	*A. insuavis*	520	other	128	64	1	>16	≤0.06	2	32	32	+	-
546373	*A. ruhlandii*	41	CF	>256	>256	>32	0.5	1	4	32	>128	+	-
546375	*A. ruhlandii*	41	CF	>256	>256	>32	1	0.5	4	32	>128	+	-
548953	*A. ruhlandii*	41	CF	>256	>256	32	2	0.5	4	32	128	+	-
548956	*A. ruhlandii*	497	CF	>256	>256	>32	>16	4	16	>64	>128	+	-
533619	*A. ruhlandii*	509	CF	32	128	8	>16	>32	32	>64	1	+	-
533627	*A. ruhlandii*	511	CF	128	2	1	2	0.12	2	0.12	32	+	-
543530	*A. ruhlandii*	519	CF	>256	4	8	2	8	4	32	2	+	-
551600	*A. spanius*	516	CF	256	4	2	0.5	0.25	8	1	2	-	-
537315	*A. xylosoxidans*	2	CF	>256	16	32	16	32	2	1	>128	+	aac(6')-Ib3, aac(6')-Ib-cr, sul1
533625	*A. xylosoxidans*	20	CF	>256	16	2	1	0.25	2	4	64	+	-
533630	*A. xylosoxidans*	20	CF	>256	128	4	0.5	0.25	2	32	128	+	-
542589	*A. xylosoxidans*	22	other	256	4	2	2	0.5	2	0.25	128	+	-
543228	*A. xylosoxidans*	22	CF	>256	8	2	8	1	2	4	32	+	-

Table 1. Continued.

ID	Species WGS	ST	Disease	Aztreonam	Ceftazidime	Ciprofloxacin	Colistin	Co-trimoxazole	Imipenem	Meropenem	Tobramycin	OXA β-lactamase	Acquired antibiotic resistance genes
537297	*A. xylosoxidans*	27	CF	>256	16	8	>16	8	16	>64	2	+	aadA1, sul1
537316	*A. xylosoxidans*	27	CF	>256	>256	8	>16	2	64	>64	>128	+	-
537320	*A. xylosoxidans*	27	CF	256	4	4	2	0.25	8	0.25	32	+	-
551605	*A. xylosoxidans*	27	BE	256	8	2	0.5	1	4	0.12	64	+	-
533615	*A. xylosoxidans*	28	CF	>256	16	4	2	0.25	16	>64	64	+	-
533617	*A. xylosoxidans*	28	CF	>256	128	16	2	0.5	32	32	64	+	-
533623	*A. xylosoxidans*	28	CF	>256	64	8	8	8	64	32	64	+	-
537310	*A. xylosoxidans*	28	CF	256	4	8	1	≤0.06	2	0.12	>128	+	ant(2")-Ia
537314	*A. xylosoxidans*	28	CF	128	2	2	2	0.5	8	0.5	16	+	-
533620	*A. xylosoxidans*	175	CF	256	4	2	1	≤0.06	2	0.12	16	+	-
543529	*A. xylosoxidans*	175	other	128	4	2	1	≤0.06	2	0.12	32	+	-
551604	*A. xylosoxidans*	175	BE	256	16	8	2	0.5	2	2	128	+	-
551607	*A. xylosoxidans*	175	CF	>256	32	8	>16	0.12	4	4	>128	+	-
551608	*A. xylosoxidans*	175	BE	>256	32	8	1	0.12	2	0.5	>128	+	-
537303	*A. xylosoxidans*	180	CF	>256	128	32	1	1	2	4	>128	+	-
546371	*A. xylosoxidans*	180	CF	256	8	16	4	0.5	4	16	>128	+	-
537298	*A. xylosoxidans*	182	CF	128	2	2	8	1	8	0.25	128	+	-
537302	*A. xylosoxidans*	182	CF	128	1	2	2	0.25	8	0.25	32	+	-
537309	*A. xylosoxidans*	184	CF	>256	16	16	4	>32	2	2	>128	+	aada2, ereA, tet(g), dfrA1, sul1
537312	*A. xylosoxidans*	184	CF	>256	32	16	2	16	8	8	>128	+	sul1
546380	*A. xylosoxidans*	226	CF	256	2	4	4	0.25	2	0.25	128	+	-
537323	*A. xylosoxidans*	237	CF	>256	32	16	1	32	4	8	>128	+	aac(6')-IIc, ant(2")-Ia, sul1
533614	*A. xylosoxidans*	272	CF	>256	64	4	>16	2	4	16	64	+	-
533622	*A. xylosoxidans*	272	CF	>256	32	4	>16	8	4	0.5	32	+	-
537304	*A. xylosoxidans*	273	CF	>256	8	4	1	16	2	1	>128	+	aac(6')-Ib3,aac(6')-Ib-cr
537324	*A. xylosoxidans*	273	CF	256	4	8	2	0.25	2	1	>128	+	aac(6')-Ib-Hangzhou, aac(6')-Ib-cr
543527	*A. xylosoxidans*	277	other	>256	>256	2	1	16	32	32	>128	+	OXA2, GES, aac(6')-Ib3, ant(2")-Ia, aph(3")-Ib, aph(6)-Id, aac(6')-cr, ereA, sul1
543233	*A. xylosoxidans*	281	CF	128	2	2	4	≤0.06	2	0.12	32	+	-
533621	*A. xylosoxidans*	290	CF	>256	8	8	>16	1	2	2	64	+	-
543532	*A. xylosoxidans*	290	other	256	4	2	1	≤0.06	2	0.12	32	+	-
537300	*A. xylosoxidans*	323	CF	256	16	8	1	0.5	1	1	>128	+	-
533624	*A. xylosoxidans*	426	CF	128	8	8	2	0.12	8	1	>128	+	-
539940	*A. xylosoxidans*	426	BE*	>256	8	0.5	≤0.25	≤0.06	2	≤0.06	8	+	-
543229	*A. xylosoxidans*	426	CF	256	4	2	4	2	4	2	64	+	-

Table 1. Continued.

ID	Species WGS	ST	Disease	Aztreonam	Ceftazidime	Ciprofloxacin	Colistin	Co-trimoxazole	Imipenem	Meropenem	Tobramycin	OXA β-lactamase	Acquired antibiotic resistance genes
533631	*A. xylosoxidans*	486	CF	128	4	2	1	0.12	2	0.12	128	+	-
533632	*A. xylosoxidans*	486	CF	128	2	2	1	0.12	2	0.12	128	+	-
533633	*A. xylosoxidans*	486	CF	128	4	2	1	≤0.06	2	0.12	64	+	-
533628	*A. xylosoxidans*	487	CF	128	2	2	2	0.12	1	0.12	128	+	-
537322	*A. xylosoxidans*	488	CF	128	8	16	1	≤0.06	1	1	>128	+	-
539941	*A. xylosoxidans*	488	other	256	8	4	2	≤0.06	2	0.12	128	+	-
537317	*A. xylosoxidans*	489	CF	256	4	>32	1	8	2	4	8	+	-
537319	*A. xylosoxidans*	489	CF	256	8	8	2	≤0.06	2	2	>128	+	-
551609	*A. xylosoxidans*	492	CF	256	2	8	0.5	0.5	1	0.25	8	+	-
546369	*A. xylosoxidans*	494	CF	>256	8	16	1	0.5	2	0.5	128	+	-
546379	*A. xylosoxidans*	495	CF	>256	16	16	>16	2	2	4	>128	+	-
546382	*A. xylosoxidans*	495	CF	>256	8	8	4	0.5	2	1	128	+	-
533613	*A. xylosoxidans*	498	CF	>256	4	16	≤0.25	2	1	1	8	-	-
551602	*A. xylosoxidans*	499	other	>256	4	4	4	1	8	0.25	128	+	-
539943	*A. xylosoxidans*	500	other	128	8	>32	2	0.25	1	0.5	>128	+	-
539944	*A. xylosoxidans*	500	BE	128	2	2	1	0.12	2	0.12	32	+	-
551610	*A. xylosoxidans*	504	BE	>256	64	4	1	0.5	128	>64	128	+	aac(6')-Ib3, aac(6')-Ib-cr, sul1
539938	*A. xylosoxidans*	505	other	256	8	8	1	1	16	1	128	+	-
537299	*A. xylosoxidans*	506	CF	>256	32	8	>16	1	2	16	32	+	-
537311	*A. xylosoxidans*	507	CF	256	8	32	2	4	1	8	128	+	-
537296	*A. xylosoxidans*	508	CF	>256	128	16	4	4	4	16	64	+	-
543230	*A. xylosoxidans*	510	CF	128	2	2	16	0.25	2	0.12	8	-	-
537318	*A. xylosoxidans*	517	CF	>256	8	4	16	0.12	1	1	>128	+	-
543526	*A. xylosoxidans*	521	other	256	4	4	2	0.5	2	0.25	64	+	-
546367	*species1*	57	CF	>256	128	16	2	8	>128	>64	>128	+	sul1
533626	*species1*	144	CF	128	2	16	1	1	1	1	2	+	-
548957	*species1*	144	CF	128	2	1	1	1	2	0.12	16	+	-
548959	*species1*	144	CF	>256	16	>32	>16	1	4	8	>128	+	-
548961	*species1*	144	CF	128	4	2	1	0.5	2	0.12	16	+	-
551603	*species1*	144	other	256	8	4	4	0.25	4	0.25	32	+	-
555840	*species1*	144	CF	>256	16	8	1	>32	2	1	128	+	-
533629	*species1*	428	CF	128	2	2	1	0.12	1	0.12	32	+	-
533612	*species2*	493	CF	>256	64	4	>16	4	1	8	16	-	-

*bronchiectasis.

isolates in this study. Novel alleles and STs were submitted to the PubMLST database [https://pubmlst.org/general.shtml]. A MLST-based Minimum Spanning Tree showed that the isolates clustered by species. The isolates of the different species differed by at least 6 or 7 loci. The majority of the *A. xylosoxidans* isolates were not closely related as only six pairs of single-locus variants were present, whereas 18 the other sequence types diverged by 2 or more loci (Fig. 1).

The five *A. deleyi* isolates were all recovered in the UK and belonged to a single ST; based on sequence alignments it is possible that they belonged to a single outbreak. Six of the eight novel species1 isolates belonged to ST144 and also appeared to be closely related; a definitive conclusion would require more knowledge of the population structure and mutation rates of this species. The two other isolates belonged to ST57and ST428.

OXA-family β-lactamases were detected in *A. deleyi*, *A. dolens*, *A. insuavis*, *A. ruhlandii*, in 61 of the 63 *A. xylosoxidans*, and in putative novel species1 (Table 1); these were presumably chromosomally located [5]. The sequences of the OXA-family β-lactamases showed variability, as has been reported before [5]. The evolutionary history of the OXA-type β-lactamases was inferred using the Neighbor-Joining method and the OXA-β-lactamases clustered with the respective type strains of their species (Fig. 2) [5]. No relevant differences were observed between isolates with and without OXA-family β-lactamases regarding the MIC_{50}/MIC_{90} values for the β-lactam antibiotics ceftazidime (respectively 4 mg/L and 128 mg/L vs 4 mg/L and 16 mg/L), aztreonam (both 256 mg/L and >256 mg/L), imipenem (2 mg/L and 16 mg/L vs 2 mg/L and 4 mg/L) or meropenem (1 mg/L and 32 mg/L vs 1 mg/L and 8 mg/L) (Table 1). This indicates that other mechanisms, such as efflux pumps, must be involved in resistance against these antimicrobials [2].

MICs for individual isolates are reported in Table 1. The most active agent was co-trimoxazole followed by imipenem. The MIC-distribution of the isolates in this study has been reported previously [23].

Only 14 isolates carried known acquired antibiotic resistance genes. One *A. xylosoxidans* isolate carried nine resistance genes including a GES-type Extended-Spectrum β-Lactamase (ESBL) and five aminoglycoside resistance genes; one of these aminoglycoside resistance genes was *aac-(6')-Ib-cr*, which also confers resistance to several fluoroquinolones and which has been described in *Achromobacter* before [24]. Multiple aminoglycoside resistance genes were found in five isolates. Nine isolates had the *sul1* gene, which is associated with class 1 integrons. Integrons have been described in *Achromobacter* previously [25,26]; however, in three of these isolates (537297, 537315, 551610) the class 1 integron integrase was not detected. The complete integrons could not be reconstructed due to the use of short-read sequencing. Other detected resistance genes were *dfrA1*, *dfrA12*, *ereA*, *tet(A)*, *tet(G)*, bla_{OXA-2}, and *sul2*, which encode resistance to trimethoprim (*dfr*), macrolides (*ere*), tetracycline (*tet*), β-lactam antibiotics (*bla*), and sulfonamide (*sul*), respectively (Table 1). Since most isolates did not have acquired resistance genes, high MICs must be the result of other mechanisms; a likely explanation is the presence of efflux mechanisms possibly combined with reduced porin expression.

Despite infections by *Achromobacter* spp. being mostly limited to persons with CF and immunocompromised hosts, many virulence factors have been proposed for these species [12,27]. The role of many of these (putative) virulence factors in infection remains to be elucidated. Some of these factors, such as O-antigens and capsules, may play a role in disease, but are also just basic bacterial structures. For our analysis, based on the encoding sequences, the following virulence factors were studied: *hlyA*, *yqfA1*, and *yfqA2*, and regions 1, 6, 23, and 24, defined by Li *et al*. [26]. Proposed factors without a defined role in virulence were excluded from the analysis [12].

Region 1, encoding a type III secretion system that has been implied in pathogenicity in other bacteria [27], was absent in some *A. xylosoxidans* isolates (n = 12, 19.0%), one *A. ruhlandii* isolate, and all isolates of *A. aegrifaciens*, *A. insolitus* and *A. spanius*. The region 1 sequences in the other isolates were species-specific, but in *A. xylosoxidans* two variants were present. A ~5 kb region of variant 1 was replaced by a ~6.5 kb sequence. Annotation showed that the assigned gene functions of both sequences were similar.

Region 6, encoding genes for dTDP-rhamnose synthesis, an O-antigen component, was present in only 19 isolates and 6 of these belonged to the same ST. The sequences could be divided into seven variants, which clustered into four groups. One variant, present in four *A. insuavis* isolates, lacks the gene for CDP-glucose 4,6-dehydratase. An additional 25 isolates encoded only the glucose-1-phosphate cytidylyltransferase (Table 2, Ref. [12]).

Region 13, involved in activation of hemolysin, was present in 49 *A. xylosoxidans* isolates as well as all *A. insuavis* and species1 isolates and absent in nine *A. xylosoxidans* isolates including four from ST27, two from ST22 and ST496 each. The two *A. insolitus* isolates and the single *A. aegrifaciens* isolate also lacked this region. Five *A. xylosoxidans* isolates lacked the gene for the MSF transporter and the LysR family regulator. This was also the case for the *A. ruhlandii*, *A. spanius*, and the species2 isolate. Interestingly, the *A. deleyi* isolates encoded only the MSF transporter and the LysR family regulator (Table 2).

The annotation of the *A. xylosoxidans* type strain (accession number NZ_LN831029) showed the presence of three different hemolysins encoded by the *hlyA*, *aqfA1* and *aqfA2* genes. The first encoded a 3296 amino acid protein. However, this region has also been annotated as an agglutinin with additional hypothetical proteins. The opposite strand encoded a putative isopropyldehydratase large subunit. Of note, *A. xylosoxidans* isolates do not show

Table 2. ST, disease, presence and absence of specific virulence genes.

ID	Species WGS	ST	Region1[a,b]	Region6[c]	Region13[d]	hlyA	yqfA1	yqfA2	Region23	Region24
537301	*A. aegrifaciens*	501	-	-	-	-	+	+	-	-
548963	*A. deleyi*	518	+	+/-	MSF, LysR	+	+	+	-	-
548966	*A. deleyi*	518	+	+/-	MSF, LysR	+	+	+	-	-
548967	*A. deleyi*	518	+	+/-	MSF, LysR	-	+	+	-	-
548969	*A. deleyi*	518	+	+/-	MSF, LysR	-	+	+	-	-
548972	*A. deleyi*	518	+	+	MSF, LysR	+	+	+	-	-
548973	*A. deleyi*	518	+	-	MSF, LysR	+	+	+	-	-
548974	*A. deleyi*	518	+	+/-	MSF, LysR	-	+	+	-	-
534814	*A. insolitus*	503	-	-	-	-	+	+	-	-
546376	*A. insolitus*	515	-	+/-	-	+	+	+	-	-
543171	*A. insuavis*	64	+	+	+	-	+	+	+	+
537313	*A. insuavis*	274	+	+	+	-	+	+	+	+
533618	*A. insuavis*	303	+	+	+	-	+	+	-	+
555838	*A. insuavis*	303	+	-	+	-	+	+	-	+
537308	*A. insuavis*	491	+	+	+	-	+	+	+	+
546381	*A. insuavis*	496	+	-	+	-	+	+	-	+
533616	*A. insuavis*	502	+	-	+	-	+	+	-	+
539942	*A. insuavis*	512	+	-	+	-	+	+	-	+
539939	*A. insuavis*	513	+	+	+	-	+	+	+	+
537305	*A. insuavis*	514	+	+	+	-	+	+	+	+
543523	*A. insuavis*	520	+	+/-	+	-	+	+	+	+
546373	*A. ruhlandii*	41	+	+/-	no MSF & LysR	+	+	+	-	-
546375	*A. ruhlandii*	41	+	+/-	no MSF & LysR	+	+	+	-	-
548953	*A. ruhlandii*	41	+	+/-	no MSF & LysR	+	+	+	-	-
548956	*A. ruhlandii*	497	+	+/-	no MSF & LysR	+	+	+	-	-
533619	*A. ruhlandii*	509	+	+	no MSF & LysR	+	+	+	-	-
533627	*A. ruhlandii*	511	+	+	no MSF& LysR	+	+	+	-	-
543530	*A. ruhlandii*	519	-	+/-	no MSF & LysR	+	+	+	-	-
551600	*A. spanius*	516	-	+/-	no MSF & LysR	+	+	+	-	-
537315	*A. xylosoxidans*	2	+/-	-	+	+	+	+	-	-
533625	*A. xylosoxidans*	20	-	-	+	+	+	+	-	-
533630	*A. xylosoxidans*	20	-	-	+	+	+	+	-	-
542589	*A. xylosoxidans*	22	+/-	-	-	+	+	+	-	-
543228	*A. xylosoxidans*	22	+/-	-	-	+	+	+	-	-
537297	*A. xylosoxidans*	27	+	-	+	+	+	+	+truncated	-
537316	*A. xylosoxidans*	27	+	-	-	+	+	+	+	-
537320	*A. xylosoxidans*	27	+	-	-	+	+	+	+	-
551605	*A. xylosoxidans*	27	+	-	-	+	+	+	+	-
533615	*A. xylosoxidans*	28	+/-	-	+	+	+	+	-	-
533617	*A. xylosoxidans*	28	+/-	-	+	+	+	+	-	-
533623	*A. xylosoxidans*	28	+/-	-	+	+	+	+	-	-
537310	*A. xylosoxidans*	28	+/-	-	+	+	+	+	-	-
537314	*A. xylosoxidans*	28	+/-	-	+	+	+	+	-	-
533620	*A. xylosoxidans*	175	+/-	-	+	+	+	+	-	-
543529	*A. xylosoxidans*	175	+/-	-	+	+	+	+	-	-
551604	*A. xylosoxidans*	175	+/-	-	+	+	+	+	-	-
551607	*A. xylosoxidans*	175	+/-	-	+	+	+	+	-	-
551608	*A. xylosoxidans*	175	+/-	-	+	+	+	+	-	-
537303	*A. xylosoxidans*	180	-	-	+	+	+	+	-	-

hemolysis on sheep blood agar. The *hlyA* region was absent from *A. insolitus*, *A. insuavis*, *A. spanius*, species1, and *A. deleyi* with one exception (Table 2). The region, which was also present in *A. ruhlandii*, appeared to be variable;

Table 2. Continued.

ID	Species WGS	ST	Region1[a,b]	Region6[c]	Region13[d]	hlyA	yqfA1	yqfA2	Region23	Region24
546371	*A. xylosoxidans*	180	-	+/-	+	+	+	+	-	-
537298	*A. xylosoxidans*	182	+/-	-	+	+	+	+	-	-
537302	*A. xylosoxidans*	182	+/-	-	+	+	+	+	-	-
537309	*A. xylosoxidans*	184	+/-	+/-	+	+	+	-	-	-
537312	*A. xylosoxidans*	184	+/-	+/-	+	+	+	+	-	-
546380	*A. xylosoxidans*	226	+	-	+	+	+	+	-	-
537323	*A. xylosoxidans*	237	+/-	-	+	+	+	+	-	-
533614	*A. xylosoxidans*	272	+/-	-	+	+	+	+	-	-
533622	*A. xylosoxidans*	272	+/-	-	+	+	+	+	-	-
537304	*A. xylosoxidans*	273	+/-	-	+	+	+	+	-	-
537324	*A. xylosoxidans*	273	+/-	-	+	+	+	+	-	-
543527	*A. xylosoxidans*	277	+	-	no MSF & LysR	+	+	+	+	-
543233	*A. xylosoxidans*	281	+/-	-	+	+	+	+	-	-
533621	*A. xylosoxidans*	290	+	+/-	+	+	+	+	-	-
543532	*A. xylosoxidans*	290	+	+/-	+	+	+	+	-	-
537300	*A. xylosoxidans*	323	+/-	-	+	+	+	+	-	-
533624	*A. xylosoxidans*	426	+/-	-	+	+	+	+	-	-
539940	*A. xylosoxidans*	426	+/-	+/-	+	-	+	+	-	-
543229	*A. xylosoxidans*	426	+/-	-	+	+	+	+	-	-
533631	*A. xylosoxidans*	486	+/-	-	+	+	+	+	-	-
533632	*A. xylosoxidans*	486	+/-	-	+	+	+	+	-	-
533633	*A. xylosoxidans*	486	+/-	-	+	+	+	+	-	-
533628	*A. xylosoxidans*	487	-	-	+	+	+	+	-	-
537322	*A. xylosoxidans*	488	-	-	+	+	+	+	-	-
539941	*A. xylosoxidans*	488	-	+/-	+	+	+	+	-	-
537317	*A. xylosoxidans*	489	-	+/-	+	+	+	+	-	-
537319	*A. xylosoxidans*	489	-	+	+	+	+	+	-	-
551609	*A. xylosoxidans*	492	+/-	+/-	+	+	+	+	+	-
546369	*A. xylosoxidans*	494	+	-	no MSF & LysR	+	+	+	+	-
546379	*A. xylosoxidans*	495	+/-	-	-	+	+	+	-	-
546382	*A. xylosoxidans*	495	+/-	-	-	+	-	+	-	-
533613	*A. xylosoxidans*	498	-	-	-	+	+	+	-	-
551602	*A. xylosoxidans*	499	+/-	-	+	+	+	+	+	-
539943	*A. xylosoxidans*	500	+/-	-	+	+	+	+	-	-
539944	*A. xylosoxidans*	500	+/-	-	+	+	+	+	-	-
551610	*A. xylosoxidans*	504	+	+	+	+	+	+	-	-
539938	*A. xylosoxidans*	505	+	+	+	+	+	+	-	-
537299	*A. xylosoxidans*	506	+	-	no MSF& LysR	+	+	+	+	-
537311	*A. xylosoxidans*	507	+	+	no MSF & LysR	+	+	+	-	-
537296	*A. xylosoxidans*	508	+	-	no MSF & LysR	+	+	+	-	-
543230	*A. xylosoxidans*	510	-	-	-	+	+	+	-	-
537318	*A. xylosoxidans*	517	+	+/-	+	+	+	+	-	-
543526	*A. xylosoxidans*	521	-	+/-	+	+	+	+	-	
546367	*species1*	57	+/-	-	+	-	+	+	-	+
533626	*species1*	144	+/-	+	+	-	+	+	-	+
548957	*species1*	144	+/-	+	+	-	+	+	-	+
548959	*species1*	144	+/-	+	+	-	+	+	-	+
548961	*species1*	144	+/-	+	+	-	+	+	-	+
551603	*species1*	144	+/-	-	+	-	+	+	-	+

a thorough assessment of this variability was not possible, because the assembly of these sequences was highly fragmented for most isolates (despite good genome coverage for sequencing). The *aqfA1* gene was present in all isolates, ex-

Table 2. Continued.

ID	Species WGS	ST	Region1[a,b]	Region6[c]	Region13[d]	hlyA	yqfA1	yqfA2	Region23	Region24
555840	*species1*	144	+/-	+/-	+	-	+	+	-	+
533629	*species1*	428	+/-	+	+	-	+	+	-	+
533612	*species2*	493	+	+	no MSF & LysR	+	+	+	-	-

[a]+/- lack putative outer protein B, D, D; 2 secreted protein, a regulatory protein; 2 hypothetical proteins.

[b]region definitions: Li *et al*., [12].

[c]+/- only glucosec-phosphate cytidylyltransferase.

[d]MSF is a transporter protein; LysR a transcriptional regulator.

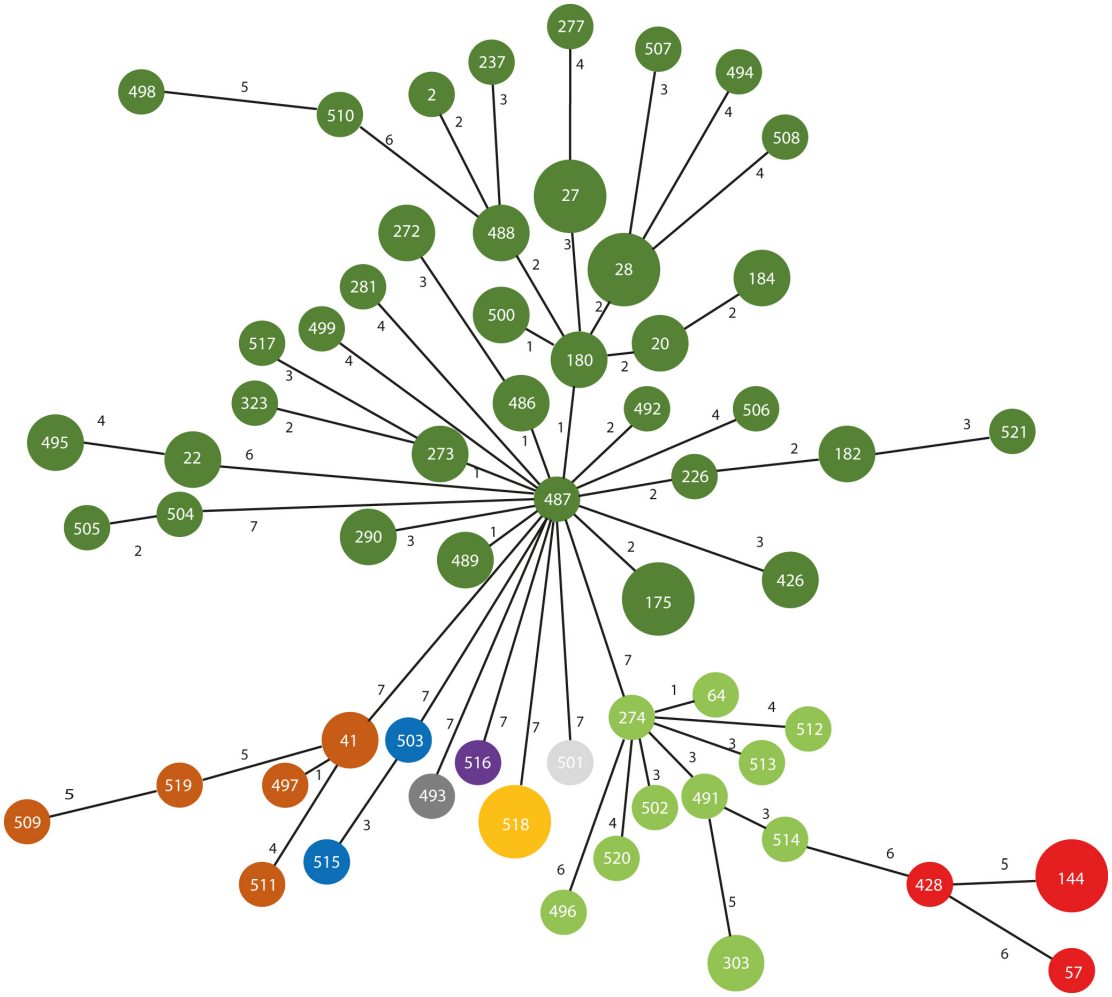

Fig. 1. MLST-based Minimum Spanning Tree of 101 *Achromobacter* isolates. Sequence types (STs) were based on seven housekeeping genes as determined by whole genome sequencing. The numbers in the nodes indicate the STs assigned by PubMLST (www.pubmlst.org). The numbers on the lines between the nodes indicate the number of loci differences between two STs. Distances between the nodes are not drawn to scale. Dark green: *A. xylosoxidans*; light green: *A. insuavis*; dark red: species 1; orange: *A. deleyi*; brown: *A. ruhlandii*; blue: *A. insolitus*; purple: *A. spanius*: dark grey: species2; light grey: *A. aegrifaciens*.

cept for one *A. xylosoxidans* isolate, and also the *aqf2* gene was present in all isolates, except for one *A. xylosoxidans*. The presence or absence of the hemolysin activating region did not match with the presence or absence of any of the putative hemolysin genes; this suggests either issues with the annotation or a complex regulation of expression.

Region 23, involved in lipopolysaccharide biosynthesis, was present in 15 isolates including six *A. insuavis* isolates and with 3 isolates belonging to *A. xylosoxidans* ST27. The nearly 22 kb region was truncated after approximately 15.6 kb. The function of the lacking part is unknown. Region 24 encoding capsule production was present in all *A. insuavis* and species1 isolates, but not in any of the other species (Table 2).

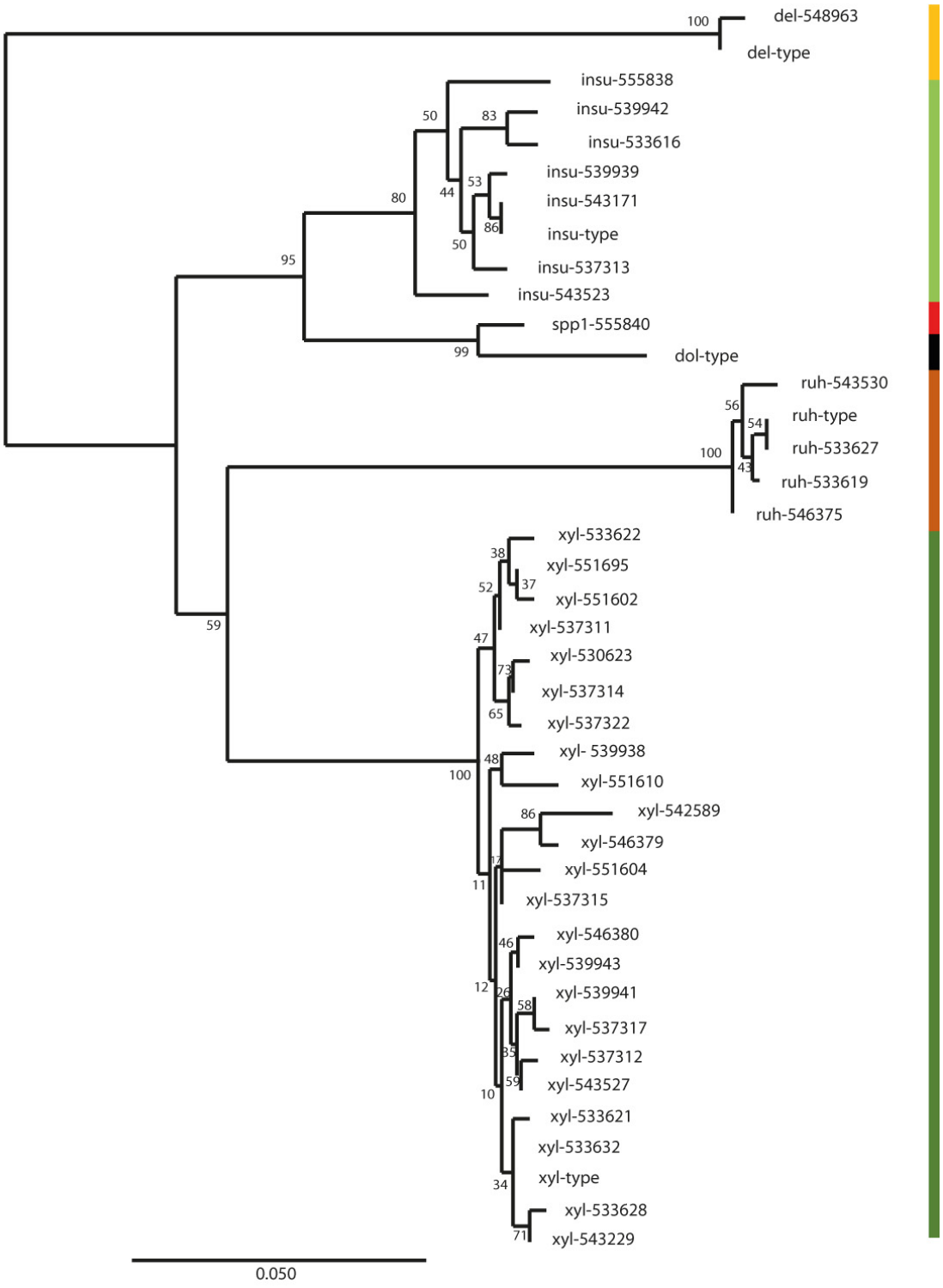

Fig. 2. The evolutionary history of the OXA-type β-lactamases was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.70431316 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. There were a total of 281 positions in the final dataset. The isolate identifications were given a 3 or 4 letter abbreviation for the species name followed by the isolate identification (Table 1). Type strains are indicated by a 3 or 4 letter abbreviation for the species name followed by –type. Abbreviations used: del: *A. deleyi*; dol: *A. dolens*; insu: *A. insuavis*; ruh: *A. ruhlandii*; xyl: *A. xylosoxidans*. The colors in the bars indicate the species. The same color scheme as for Fig. 1 was used. Dark green: *A. xylosoxidans*; light green: *A. insuavis*; dark red: species1; orange: *A. deleyi*; brown: *A. ruhlandii*; black: *A. dolens*.

It should be noted that expression of virulence factors was not confirmed *in vitro* or *in vivo* and that virulence features are only predicted on the bases of sequence analysis.

The 20 non-CF isolates consisted of 15 *A. xylosoxidans* (75%), four *A. insuavis* (20%) and one species1 isolate (5%) (Table 1). The CF-isolates appeared to be more diverse: 48 *A. xylosoxidans* (59%), seven *A. insuavis*, *A. deleyi*, *A. ruhlandii* and species1 (each 8.6%), two *A. insolitus* (2.5%), and one *A. aegrifaciens*, *A. spanius* and species2 (each 1.2%). Comparison of the average MICs for CF isolates and non-CF isolates showed that the MICs for aztreonam, imipenem and tobramycin were approximately two times higher for non-CF isolates (non-CF vs CF:153.6 and 84.1 g/L, 11.0 and 5.5 mg/L, and 58.4 and 34.5 mg/L, respectively), whereas the MICs for ceftazidime, ciprofloxacin, colistin, co-trimoxazole, and meropenem were 1.4-2.9 higher for CF isolates (CF vs non-CF: 19.3 and 12.9 mg/L, 8.9 and 3.1 mg/L, 2.0 and 1.4 mg/L, 2.4 and 1.0 mg/L, 5.0 and 3.4 mg/L) (Table 1). No relevant differences were observed in the presence of putative virulence factors.

4. Conclusions

In conclusion, only 63/95 (66.3%) of the isolates were correctly identified using routine MALDI_-TOF identification probably indicating a lack of well-typed *Achromobacter* isolates in the standard database. Two putative novel species were identified. Isolates from persons with CF appeared to be more diverse. Despite the high MICs the presence of acquired resistance genes is uncommon, although some isolates harbored several acquired resistance genes. The average MICs for CF isolates were lower for aztreonam imipenem, and tobramycin, but higher for ceftazidime, ciprofloxacin, colistin, co-trimoxazole, and meropenem. The putative virulence genes of *Achromobacter* involved in infections or colonization are variable, but no difference in putative virulence factors were observed.

Abbreviations

CF, cystic fibrosis; ESBL, Extended-Spectrum β-Lactamase; MALDI-TOF, matrix assisted laser desorption/ionisation time-of-flight analyzer, MIC, minimal inhibitory concentration; MLST, Multi-Locus Sequence Typing; ST, sequence type.

Author contributions

ACF, MDA, MMT, JSE RC and MBE designed the research study. BB-T, MvW, JFM performed the research. ACF and JRB analyzed the data. ACF, JRB, MBE wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Acknowledgment

Not applicable.

Funding

The research leading to these results has received support from the Innovative Medicines Initiative Joint Undertaking under grant agreement number [115721-2], resources of which are composed of financial contribution from the European Union's Seventh Framework Programme (FP7/2007-2013) and EFPIA companies in kind contribution. Spanish CF isolates were recovered from projects supported by Instituto de Salud Carlos III of Spain (grants PI12/00743 and PI15/00466) and cofinanced by the European Development Regional Fund (A Way to Achieve Europe program; Spanish Network for Research in Infectious Diseases grant REIPI RD12/0015). MD-A was partially supported by Fundación Francisco Soria Melguizo (Madrid, Spain).

Conflict of interest

The authors declare no conflict of interest.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbs1402009.

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