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Achromobacter spp. in Cystic Fibrosis Patients: A Genomic-Based Approach to Unravel Microbe-Host Adaptation

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"Imagination is more important than knowledge."

Albert Einstein

ABSTRACT

Bacteria belonging to the genus *Achromobacter* are widely distributed in natural environments and have been recognized as emerging nosocomial pathogens for their contribution to a wide range of human infections. *Achromobacter* spp. can establish chronic infections associated with inflammation, produce biofilm, resist common disinfectants, readily acquire antibiotic resistance and outcompete resident microbiota. In particular, cystic fibrosis (CF) patients with lung disease are the most frequently colonized and infected by *Achromobacter* species usually developing persistent respiratory tract infections.

In the last five years the number of publications regarding these pathogens has doubled in comparison to the preceding five-year period and their whole genome sequencing data availability has seen a steep increase, underlining both the growing research interest for these microorganisms as well as their emergence in the clinical setting. Nonetheless, many clinical aspects and pathogenic mechanisms still remain to be elucidated.

The main focus of this thesis has been to unravel underlying key processes and to investigate the adaptive mechanisms exploited by these microorganisms during lung infection in CF patients. This has been pursued by analysing both genomic and phenotypic data of 103 *Achromobacter* spp. clinical isolates from 40 CF patients followed at the CF centres in Verona (Italy), Rome (Italy), and Copenhagen (Denmark).

The work presented in this thesis provides new knowledge on the onset of *Achromobacter* spp. infections and their adaptation to the CF lung environment. With further genomic and phenotypic studies it will be possible to translate these results into the clinical setting, leading to better predictions of the infection course and improvement of treatment strategies to the benefit of CF patients.

SOMMARIO

I batteri appartenenti al genere *Achromobacter* sono ampiamente distribuiti negli ambienti naturali e sono stati riconosciuti come patogeni nosocomiali emergenti per il loro contributo ad un'ampia gamma di infezioni umane. *Achromobacter* spp. possono stabilire infezioni croniche associate ad infiammazione, produrre biofilm, resistere ai comuni disinfettanti, acquisire prontamente resistenza agli antibiotici e competere con il microbiota residente. In particolare, i pazienti affetti da fibrosi cistica (FC) presentanti malattia polmonare sono più frequentemente colonizzati da *Achromobacter* spp. e sviluppano infezioni persistenti del tratto respiratorio.

Negli ultimi cinque anni il numero di pubblicazioni riguardanti questi patogeni è raddoppiato rispetto al quinquennio precedente, con un conseguente aumento dei dati di *whole genome sequencing* disponibili in rete. Questo sottolinea il crescente interesse di ricerca per tali microrganismi e la loro insorgenza in ambito clinico. Tuttavia, molti aspetti clinici e meccanismi patogenetici restano ancora da chiarire.

L'obiettivo principale di questa tesi è quello di studiare i processi chiave e di indagare i meccanismi adattativi attuati da questi microrganismi durante l'infezione polmonare nei pazienti FC. Ciò è stato svolto analizzando sia dati genomici che fenotipici di 103 isolati clinici di *Achromobacter* spp. provenienti da 40 pazienti FC in cura nei centri FC di Verona (Italia), Roma (Italia) e Copenaghen (Danimarca).

Il lavoro presentato in questa tesi fornisce nuove conoscenze sull'insorgenza di infezioni di *Achromobacter* spp. e sul loro adattamento all'ambiente polmonare di pazienti FC. Con ulteriori studi genomici e fenotipici sarà possibile traslare questi risultati in ambito clinico, portando a migliori previsioni del decorso dell'infezione e al miglioramento delle strategie di trattamento a beneficio dei pazienti FC.

PUBLISHED WORK INCLUDED IN THIS THESIS

The work described in this thesis has been a multi-step process that has seen the publication of the following peer-reviewed papers on international journals (* denotes equal contribution):

- **Veschetti L.***, Sandri A.*, Johansen H.K., Lleò M.M., Malerba G. *Hypermutation as an Evolutionary Mechanism for Achromobacter xylosoxidans in Cystic Fibrosis Lung Infection.* Pathogens 2020, 9(2), 72. DOI:10.3390/pathogens9020072 PMID: 31973169.
- **Veschetti L.***, Sandri A.*, Patuzzo C., Melotti P., Malerba G., Lleò M.M. *Genomic Characterization of Achromobacter spp. Isolates from Chronic and Occasional Lung Infection in Cystic Fibrosis Patients.* Microbial Genomics 2021; 7:000606. DOI: 10.1099/mgen.0.000606 PMID: 34292148.
- **Veschetti L.***, Sandri A.*, Patuzzo C., Melotti P., Malerba G., Lleò M.M. *Mobilome Analysis of Achromobacter spp. Isolates from Chronic and Occasional Lung Infection in Cystic Fibrosis Patients.* Microorganisms 2021, 9(1), 130. DOI: 10.3390/microorganisms9010130 PMID: 33430044.
- Sandri A.*, **Veschetti L.***, Saitta G.M., Passarelli Mantovani R., Carelli M., Burlacchini G., Preato S., Sorio C., Melotti P., Montemari A.L., Fiscarelli E.V., Patuzzo C., Signoretto C., Boaretti M., Lleò M.M., Malerba G. *Achromobacter spp. Adaptation in Cystic Fibrosis Infection and Candidate Biomarkers of Antimicrobial Resistance.* International Journal of Molecular Sciences 2022, 23, 9265. DOI: 10.3390/ijms23169265 PMID: 36012535.
- Sandri A., Haagensen J.A.J., **Veschetti L.**, Johansen H.K., Molin S., Malerba G., Signoretto C., Boaretti M., Lleò M.M. *Adaptive Interactions of Achromobacter spp. with Pseudomonas aeruginosa in Cystic Fibrosis Chronic Lung Co-infection.* Pathogens 2021, 10, 978. DOI:10.3390/ pathogens10080978 PMID:4451442.

PUBLISHED WORK NOT INCLUDED IN THIS THESIS

- Pomari E., Malerba G., **Veschetti L.**, Franceschi A, Moron Dalla Tor L., Deiana M., Degani M., Mistretta M., Patuzzo C., Ragusa A., Mori A., Bisoffi Z., Buonfrate D. *Identification of miRNAs of Strongyloides stercoralis L1 and iL3 larvae isolated from human stool.* Sci Rep. 2022 Jun 15;12(1):9957. DOI: 10.1038/s41598-022-14185-y PMID: 35705621.
- Zago V., **Veschetti L.**, Patuzzo C., Malerba G., Lleò M.M. *Shewanella algae and Vibrio spp. Strains Isolated in Italian Aquaculture Farms Are Reservoirs of Antibiotic Resistant Genes That Might Constitute a Risk for Human Health.* Marine Pollution Bulletin 154 (2020) 111057. DOI: 10.1016/j.marpolbul.2020.111057 PMID: 32174504.
- Zago V., **Veschetti L**., Patuzzo C., Malerba G., Lleò M.M. *Resistome, Mobilome and Virulome Analysis of Shewanella algae and Vibrio spp. Strains Isolated in Italian Aquaculture Centers.* Microorganisms 2020, 8(4), 572. DOI:10.3390/microorganisms8040572 PMID:32326629.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

CF = cystic fibrosis

DNA = deoxyribonucleic acid

ESBL = extended-spectrum β-lactamase

ICE = integrative and conjugative element

IL = interleukin

IME = integrative and mobilizable element

IS = insertion sequences

LPS = lipopolysaccharide

MBL = metallo-β-lactamase

MGE = mobile genetic element

MMR = mismatch repair

NG = non-affiliated genogroup

ORF = open reading frame

RND = resistance-nodulation-cell division

T2SS = type II secretion system

T3SS = type III secretion system

T6SS = type VI secretion system

T7SS = type VII secretion system

TNF = tumour necrosis factor

WGS = whole genome sequencing

1.1 OVERVIEW

This dissertation is meant to critically introduce the current literature regarding *Achromobacter* spp. role in cystic fibrosis (CF) and examine their adaptation strategies - including modifications of metabolism and virulence, acquisition of antibiotic resistance, development of hypermutation and exchange of mobile genetic elements - in light of the findings of the work included in this thesis.

1.2 STRUCTURE OF THESIS

This thesis is organised in four chapters:

Chapter 1 - the current chapter - is a general introduction to the thesis as a whole.

Chapter 2 presents the theoretical background regarding the role of *Achromobacter* spp. in CF.

Chapter 3 reports the state of the art of *Achromobacter* spp. adaptation knowledge as well as the findings of the studies included in this thesis.

Chapter 4 encloses the overall conclusions and discusses future perspectives.

Finally, the appendix includes the full-length research articles included in this thesis.

2.1 INTRODUCTION

Achromobacter spp. are non-lactose fermenting, catalase and oxidase positive Gram-negative bacilli widely distributed in the environment, mainly in moist soil and water sources but also in plants[1]. These motile opportunistic pathogens are mainly found in wet environments but are increasingly isolated also in nosocomial settings. Nosocomial outbreaks are often caused by contamined disinfectant solutions, dialysis fluids, saline solutions and deionized water[2]. *Achromobacter* spp. strains isolated from these sources are usually resistant to a variety of antibiotics, due to both innate and adaptive antimicrobial resistance[1].

Achromobacter spp. colonization events have been associated to a variety of infections such as bacteraemia, meningitis, pneumonia, peritonitis and urinary tract infections[3]. These usually occur in subjects with underlying immunodeficiency, and subjects that underwent surgical procedures. In particular, *Achromobacter* strains are primarily isolated from the respiratory tract of cystic fibrosis (CF) patients[1], where these microorganisms can persist for a long time in both lower and upper airways[4].

CF is a monogenic autosomal recessive disorder that is strictly linked with chronic bacterial respiratory infections: persistent airways infections and the ensuing prolonged lung inflammation lead to lung insufficiency, which accounts for the majority of CF morbidity and mortality[5]. Many opportunistic pathogens can cause lung infections in CF patients, among them: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Burkholderia cepacia* complex, *Stenotrophomonas maltophilia*, *Achromobacter* spp., non-typeable *Haemophilus influenzae*, *Aspergillus* and nontuberculous mycobacteria[6]. The impact of *Achromobacter* spp. infection on lung function is still debated[1,7–10]; nonetheless, it has been reported that infection with the type species *Achromobacter xylosoxidans* results in a heightened host inflammatory response[4]. Moreover, chronic infections with

A. xylosoxidans lead to a greater number of pulmonary exacerbation events[1] and annual hospitalizations[11,12], thus highlighting the growing importance of this opportunistic pathogen in CF.

2.2 *ACHROMOBACTER* SPP. AS EMERGING OPPORTUNISTIC PATHOGENS

As mentioned above, although primarily isolated from the airways of CF patients, *Achromobacter* spp. can cause a broad range of infections in hosts with other underlying conditions. Not only these bacteria are able to establish chronic infections associated with lung inflammation in CF patients[4,8], they also produce biofilm, resist common disinfectants[2,13], readily acquire antibiotic resistance[14] and outcompete resident microbiota[15,16]. These could be some of the reasons causing an increase in research interest regarding *Achromobacter* spp. in the last 20 years (Figure 1). Notably, in the last 10 years the number of publications regarding *Achromobacter* spp. has more than tripled in comparison to the preceding decade (Figure 1), underlining both the increasing research interest for these microorganisms as well as their emergence in the clinical setting, especially in CF.

Thanks to the advent of next-generation sequencing technologies, the rise in number of publications regarding *Achromobacter* spp. has been followed by a steep increase in whole genome sequencing (WGS) data availability[17,18]. This has allowed scientists to make great progress in understanding these emerging pathogens, nonetheless, many clinical aspects and pathogenic mechanisms concerning *Achromobacter* spp. still remain to be elucidated.

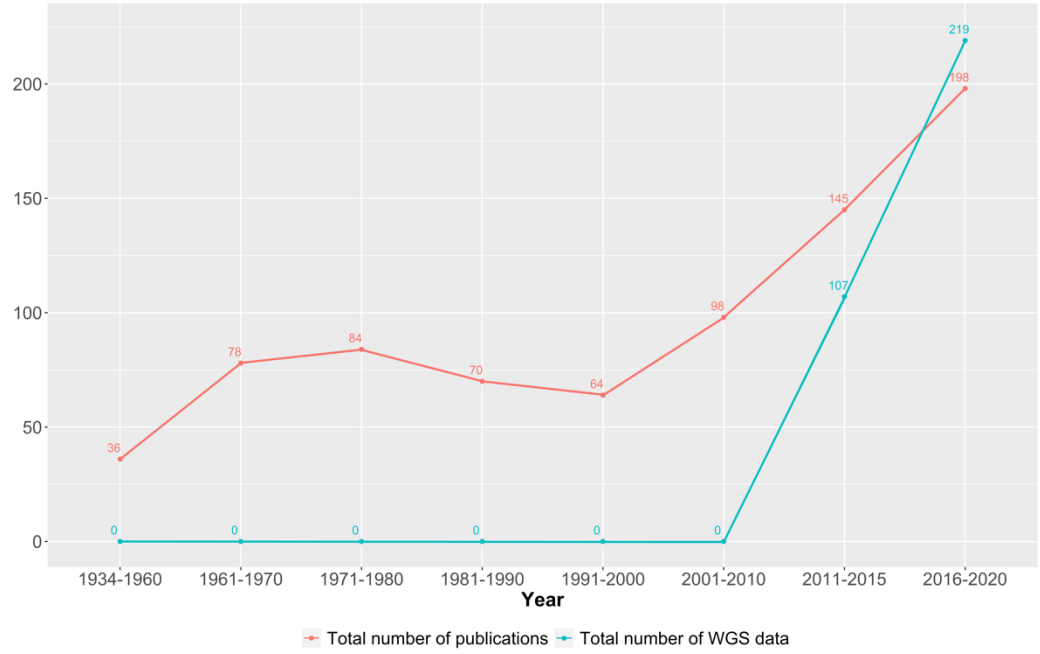

Figure 1 *Achromobacter* spp. research interest. The number of publications is based on a literature search using PubMed (https://pubmed.ncbi.nlm.nih.gov, consulted on 01-01-2021) with Title=*Achromobacter*. The number of available whole genome sequencing (WGS) data is based on the content of NCBI (https://www.ncbi.nlm.nih.gov/Traces/wgs/) with Term=*Achromobacter* and Project type=WGS. This figure was generated with R version 4.0.4[19].

2.3 TAXONOMY, GENOME AND PAN-GENOME

Achromobacter spp. are classified as members of the β-proteobacteria and belong to the order of *Burkholderiales*, which also includes the *Burkholderia* genus. The family name of *Achromobacter* is *Alcaligenaceae,* which is the same family *Bordetella* and *Alcaligenes* belong to[2]. In particular, most members of the genus *Bordetella* are human pathogens involved in respiratory infections and a common origin of *Achromobacter* and *Bordetella* members has been suggested through phylogenetic analyses[20–22].

The genus *Achromobacter* is continuously evolving, with recently named novel species being described since 2016[23–26]. Recent studies have resulted in the reclassification of previously described species: *A. spiritinus* has been classified as *A. marplatensis,* and *A. sediminum* has been assigned to the novel genus *Verticia*[27,28].

Achromobacter spp. ever-changing taxonomy coupled with their biochemical similarities with other Gram-negative bacilli often result in

misidentification[29–31], despite the fact that recent efforts to improve the accuracy of identification methods have been made. This problem has repercussions on both clinicians and researchers, in fact it hinders an accurate diagnosis and affects the reliability of already published works as well as that of deposited genomes. Indeed, we found that some of the publicly available *Achromobacter* sp. genomes were wrongly classified as belonging to a different species (Milestone 2[32]), which was also confirmed by another study[33]. The increasing use of WGS may assist the correct identification of the different species through bioinformatic analyses such as dDDH (digital DNA-DNA hybridization) and ANI (average nucleotide identity) calculations, or even the identification of the sequence type, with analyses like in silico MLST (multi locus sequence typing).

Table 1 *Achromobacter* child taxa with a validly published name and indication of the availability of complete reference genomes.

Child taxa	Reference of publication	Taxonomic status	Complete reference genome
Achromobacter aegrifaciens	Vandamme, 2013 [34]	Correct name	Not available
Achromobacter agilis	Vandamme, 2016 [23]	Correct name	Not available
Achromobacter aloeverae	Kuncharoen, 2017 [24]	Correct name	Not available
Achromobacter animicus	Vandamme, 2013 [35]	Correct name	Not available
Achromobacter anxifer	Vandamme, 2013 [34]	Correct name	Not available
Achromobacter deleyi	Vandamme, 2016 [23]	Correct name	Available
Achromobacter denitrificans	Coenye, 2003 [36]	Correct name	Available
Achromobacter dolens	Vandamme, 2013 [34]	Correct name	Not available
Achromobacter insolitus	Coenye, 2003 [37]	Correct name	Available
Achromobacter insuavis	Vandamme, 2013 [34]	Correct name	Not available
Achromobacter kerstersii	Vandamme, 2016 [23]	Correct name	Not available
Achromobacter marplatensis	Gomila, 2011 [38]	Correct name	Not available
Achromobacter mucicolens	Vandamme, 2013 [35]	Correct name	Not available
Achromobacter pestifer	Vandamme, 2016 [23]	Correct name	Available
Achromobacter piechaudii	Yabuuchi, 1998 [39]	Correct name	Not available
Achromobacter pulmonis	Vandamme, 2013 [35]	Correct name	Not available
Achromobacter ruhlandii	Yabuuchi, 1998 [39]	Correct name	Available
Achromobacter sediminum	Zhang, 2014 [40]	Synonym (*Verticia* spp.)	Not available
Achromobacter spanius	Coenye, 2003 [37]	Correct name	Available
Achromobacter spiritinus	Vandamme, 2013 [35]	Synonym (*A. marplatensis*)	Not available
Achromobacter veterisilvae	Dumolin, 2020 [41]	Correct name	Not available
Achromobacter xylosoxidans	Yabuuchi, 1981 [42]	Correct name	Available

The first published *Achromobacter* spp. complete genome sequence was that of an environmental strain isolated from soil[43], whereas the first complete genome assembly of a clinical isolate from a CF patient was published two years later, in 2013[44]. To date, the genus *Achromobacter* comprises 22 named species and multiple genogroups[45]; WGS data is available for all 22 species but complete reference genomes are available only for *Achromobacter deleyi, Achromobacter denitrificans, Achromobacter insolitus, Achromobacter pestifer, Achromobacter ruhlandii, Achromobacter spanius,* and *Achromobacter xylosoxidans*. Detailed information regarding *Achromobacter* child taxa with a validly published name is reported in Table 1.

The *Achromobacter* sp. genome[43,44,46–52] consists of a single chromosome comprising an average of 6.5 Mbp (range=5,876,039-7,013,095 bp) and presents a relatively high GC content (mean=65.5%, range=63.8-67.72%). A mean of 5,978 (range=5,328-6,459) open reading frames (ORFs) have been predicted with a coding density of ~90%; nonetheless a mean of 19% (range=10-29%) of ORFs still remain classified as having hypothetical function. This contributes to the difficult understanding of *Achromobacter* spp. metabolism, pathogenic potential and adaptation mechanisms.

Pan-genome analysis, which considers the set of all the genes present in a given species, revealed that *Achromobacter* sp. has an open pan-genome and its conserved core genome – i.e. the set of homologous genes that are present in all genomes of an analysed dataset - consists of ~30% of the genes carried in an average genome of this genus[16,20]. This means that a great part of the pan-genome is categorized as accessory genome, which comprises genes that are not conserved among strains. Typically, these features coupled with such a large genome size characterize species living in a community with frequent lateral gene transfer and high adaptability to diverse environmental conditions[53,54]. The remarkable plasticity of *Achromobacter* spp. genomes and their adaptive potential were also confirmed by our findings (Milestone 1-5[32,55–58]), which will be discussed in detail in Chapter 3.

2.4 CONTRIBUTION TO CYSTIC FIBROSIS INFECTIONS: *ACHROMOBACTER* SPP. PREVALENCE AND SPECIES HETEROGENEITY

Prevalence data from the latest European CF patients annual registry (2019)[59] showed a high number of *Achromobacter* spp. infections in Denmark, Belgium and Greece (prevalence: 15.56%, 11.01% and 10.40% respectively), while other countries presented a prevalence around or lower than 10%. Moreover, a higher percentage of adults with *Achromobacter* infection has been reported when compared with data regarding paediatric patients. Similarly to European data, the latest US annual registry (2020) showed that the prevalence of *Achromobacter* spp. varies by age group, with an increase of infections in adult patients[60]. While a general trend analysis of comprehensive European data is not possible (*Achromobacter* spp. infection data are available starting from 2018), the prevalence data from the US annual registry reported that *Achromobacter* infections rose from 1.9% in 2005 to around 7% in 2011 and have since remained stable[26]. Overall, the prevalence of *Achromobacter* species in CF patients is less than 10% at the majority of centres worldwide.

Table 2 *Achromobacter* spp. CF prevalence in different countries. The number of *Achromobacter* infected CF patients included in each study is reported in the last row. a=6 healthcare centers in Argentina were involved in the study; b= study by the UK national reference laboratory; c=86 CF treatment centers in the US were involved in the study.

	Country						
***Achromobacter* species**	Argentina[a] (%)[61]	Denmark - Aarhus (%)[62]	Denmark – Copenhagen (%)[63]	France – Dijon (%)[64]	Italy - Verona (%)[32]	UK[b] (%)[65]	USA[c] (%)[66]
A. aegrifaciens	-	5	2	15	12	-	-
A. dolens	10	2	-	-	3	8	17
A. insolitus	-	-	-	4	12	-	4
A. insuavis	5	24	20	19	8	12	-
A. marplatensis	2	2	-	-	-	2	-
A. pulmonis	2	-	-	-	-	-	-

A. ruhlandii	17	19	25	-	-	3	24
A. xylosoxidans	63	36	52	57	65	61	43
Patients included in the study	41	43	51	47	26	96	341

Table 2 reports an overview of *Achromobacter* spp. CF prevalence in different countries based on prevalence studies published in the literature. In particular, as regards Italy, we reported the prevalence of *Achromobacter* species in CF patients followed at the CF centre of Verona (Italy), even though the number of clinical isolates analysed in our study (Milestone 2[32]) is limited, since it constitutes the first Italian *Achromobacter* species prevalence report.

Overall, the type species *A. xylosoxidans* is the most often isolated *Achromobacter* species among CF patients in all countries[32,61–66], while *A. marplatensis* and *A. pulmonis* show the lowest prevalence. Nonetheless, the distribution of *Achromobacter* species in CF patients appears to be different among countries (Table 2). *A. insuavis* infections are reported with a similar frequency in Denmark and France (20-24% and 19%, respectively), and at a lower rate in UK (12%), Italy (8%) and Argentina (5%). *A. dolens* is most prevalent in US (17%) followed by Argentina (10%), while it has a prevalence <10% in UK, Italy and Denmark (8%, 3% and 0-2%). Furthermore, *A. ruhlandii* prevalence is 17-25% in Argentina, US and in Denmark, where an outbreak was reported at two CF centres[12,67]; *A. insolitus* seems to have a higher prevalence in Italy (12%) than in France and US (both 4%), and *A. aegrifaciens* has a prevalence of 12-15% in Italy and France while in Denmark is <5%.

Even though more than half of CF patients with airway colonization by *A. xylosoxidans* develop chronic infections, usually associated with decline in respiratory function and lung inflammation[4,8,11,62,64,68], the clinical impact of different *Achromobacter* species is still not well characterized. The high prevalence of *A. xylosoxidans*, *A. ruhlandii*, *A. dolens* and *A. insuavis* among clinical isolates, coupled with the phylogenetic clustering of these species

and their ability to develop chronic infections[64,69,70], might indicate that they could be better adapted to cause opportunistic chronic infections[16].

Overall, the majority of CF patients seem to harbour a unique strain of *Achromobacter* spp., nonetheless cases of cross-infection among patients have been reported, even by indirect person-to-person transmission[12,71]. For example, the *A. ruhlandii* Danish epidemic strain has been identified in multiple patients attending the same CF centre and, more recently, patient-to-patient transmission was verified also for *A. xylosoxidans* and *A. insuavis* strains. In some cases, clear epidemiological connections (e.g. sibling pairs, visit-based) were found, but in other instances no epidemiological connection to support cross-infection could be identified[26,63]. In all the reported cases, WGS proved to be essential for *Achromobacter* species typing and identification of patient-to-patient transmission.

It is important to underline that although prevalence data is available in the literature, the limited number of CF patients included in some of the studies and the still not optimal species-level identification techniques[29–31,72,73] coupled with the changing nomenclature hinder an accurate prevalence estimation.

3.1 ENVIRONMENTAL AND CLINICAL STRAINS: GENOMIC DETERMINANTS OF PATHOGENICITY AND ADAPTATION

The increase in number of sequenced *Achromobacter* spp. genomes enabled researchers to focus at first on the genomic differences among clinical and environmental isolates and afterwards on the genomic determinants of pathogenicity and adaptation during persistent infections, allowing to identify a variety of adaptation mechanisms in the CF lung environment.

A phylogenetic study aimed at evaluating differences among environmental and clinical strains showed that in the latter 35 genes involved in metabolism, regulation, and efflux pumps were positively selected, and that this group of isolates carried a greater number of antibiotic resistance genes, namely for resistance against aminoglycosides, β-lactams, chloramphenicol and sulphonamides[16]. Interestingly, it was shown that the most frequently mutated genes were involved in general metabolism, which is the key to adapt to host conditions and outcompete the resident microbiota[74]. Indeed, during CF lung colonization, bacteria survive under the selective pressure imposed by the host immune system and antibiotic therapies by increasing the efficiency in nutrient acquisition, developing the ability to avoid toxic compounds, and promoting the colonization of new areas [75]. An overview of *Achromobacter* spp. adaptation strategies – which will be discussed in detail in the following sections - is represented in Figure 2.

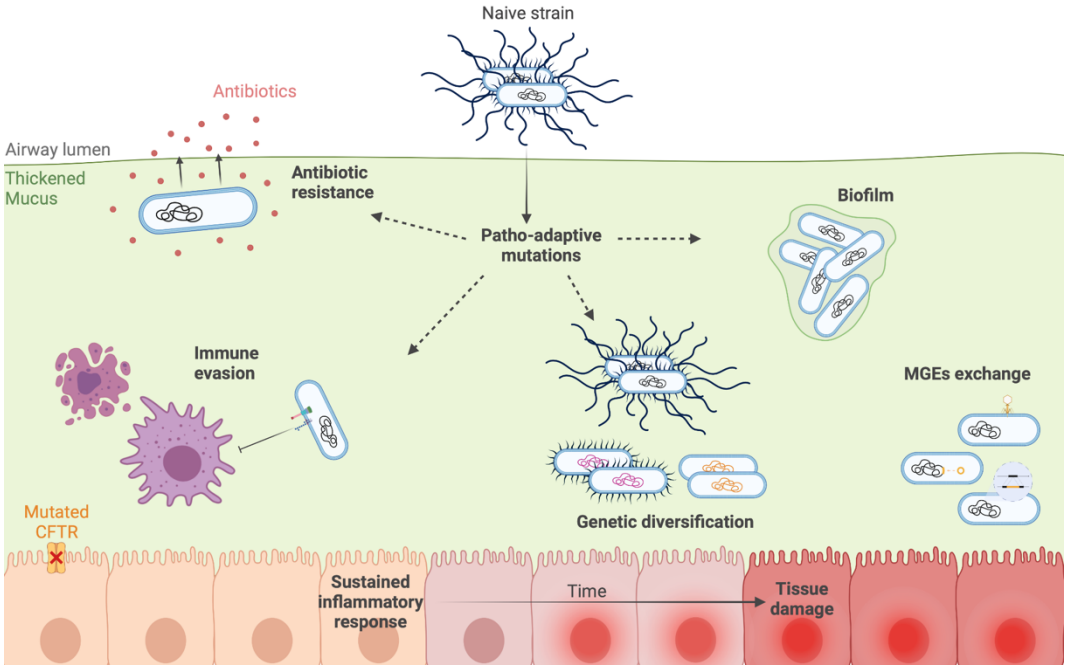

Figure 2 *Achromobacter* spp. adaptation strategies. Proposed *Achromobacter* spp. adaptation strategies: antibiotic resistance, immune evasion, genetic diversification, biofilm production and exchange of mobile genetic elements. MGEs=mobile genetic elements, CFTR=cystic fibrosis conductance transmembrane regulator. This figure was created on BioRender.com.

3.2 SUMMARY OF THE WORK INCLUDED IN THIS THESIS

In the light of what has been reported, we decided to investigate with genomic-based and phenotypic-based approaches the adaptive mechanisms exploited by *Achromobacter* spp. during lung infection in CF patients.

The work described in this thesis has been a multi-step process that encompassed different aspects of adaptation. In particular, in the first phase of the study the focus was on a small number of clinical isolates in order to explore whether different adaptation strategies could be employed by *Achromobacter* spp. (Milestone 1[55]). Once the occurrence of hypermutation as well as longitudinal microevolution was observed, the sample collection was widened including clinical isolates collected at the Verona CF centre. This allowed a comprehensive genomic characterization of chronic and occasional lung infection isolates. Indeed, the genomic analysis of chromosomal content (Milestone 2[32]) was complemented by the investigation of *Achromobacter* spp. mobilome (Milestone 3[56]).

In order to integrate the obtained results and to identify possible markers of persistence, the number of analysed samples was increased by including *Achromobacter* spp. clinical isolates collected at Rome CF centre, and a phenotypic characterization of the Verona and Rome collections was performed (Milestone 4[57]).

Finally, the possible inter-species dynamics that might take place in co-infection and co-colonization events were explored by analysing adaptive interactions of *Achromobacter* spp. with *Pseudomonas aeruginosa* – one of the major CF pathogens (Milestone 5[58]).

A more detailed summary of each study milestone is reported in the following sub-sections, whereas an overview of the main findings of the research articles published at each milestone is reported in Table 3.

Table 3 Milestones overview. The main findings of the research articles included in this thesis are reported in the table together with the number of isolates analysed.

Milestone	Number of analysed isolates	Main findings
1	4	• Occurrence of hypermutation as an adaptive mechanism in *Achromobacter xylosoxidans* chronic lung infection.
2	54	• Most of the occasional isolates lacked functional genes involved in invasiveness, chemotaxis, type III secretion system and anaerobic growth, whereas the great majority of chronic isolates had these genomic features. • Almost all late chronic isolates lacked functional genes involved in lipopolysaccharide production. • Species-specific distribution of bla_{OXA} genes. • No occasional isolate had hypermutator characteristics, while 60% of early chronic (<1 year from first colonization) and 78% of late chronic (>1 year from first colonization) isolates were classified as hypermutators.

		• Some isolates presented two different *mutS* genes, these seem to have a complementary rather than compensatory function.
3	54	• Most of the detected phages were previously described in other pathogens and carried type II toxin-antitoxin systems as well as other pathogenic genes. • The partial sequence of phage Bcep176 was found in all the analysed *Achromobacter xylosoxidans* genome sequences, suggesting the integration of this phage in an ancestor strain. • ICEs carrying pathogenic genes were found to be widespread among our isolates and seemed to be involved in transfer events within the CF lung.
4	95	• Virulence testing showed that isolates from occasionally infected patients induced significantly higher mortality in *G. mellonella* larvae than chronic infection isolates. • Antibiotic susceptibility testing showed that isolates from chronically infected patients were significantly more resistant to sulfonamide and meropenem than occasional isolates. • No significant difference was observed in biofilm production among the two groups.
5	4	• *Achromobacter* sp. isolate secreted exoproducts interfering with the adhesion ability of a co-isolated *P. aeruginosa* strain and affected its biofilm formation. • Both inter-species competition and cohabitation are represented during chronic co-infections in CF airways, and evolution of these interplays can happen even at the late stages of chronic infection.

3.2.1 MILESTONE 1: Hypermutation as an Evolutionary Mechanism for Achromobacter xylosoxidans in Cystic Fibrosis Lung Infection [55]

Achromobacter xylosoxidans can cause chronic infections in the lungs of patients with cystic fibrosis (CF) by adapting to the specific environment. The study of longitudinal isolates allows to investigate its within-host evolution to unravel the adaptive mechanisms contributing to successful colonization. In this study, four clinical isolates longitudinally collected from two chronically infected CF patients underwent whole genome sequencing (WGS), de novo assembly and sequence analysis. Phenotypic assays were also performed. The isolates coming from one of the patients (patient A) presented a greater number of genetic variants, diverse integrative and conjugative elements, and different protease secretion. In the first of these isolates (strain A1), we also found a large deletion in the *mutS* gene, involved in DNA mismatch repair (MMR). In contrast, isolates from patient B showed a lower number of variants, only one integrative and mobilizable element, no phenotypic changes, and no mutations in the MMR system. These results suggest that in the two patients the establishment of a chronic infection was mediated by different adaptive mechanisms. While the strains isolated from patient B showed a longitudinal microevolution, strain A1 could be clearly classified as a hypermutator, confirming the occurrence and importance of this adaptive mechanism in *Achromobacter xylosoxidans* infection.

3.2.2 MILESTONE 2: Genomic Characterization of Achromobacter spp. Isolates from Chronic and Occasional Lung Infection in Cystic Fibrosis Patients [32]

To better understand the mechanisms contributing to a successful colonisation by *Achromobacter* species, we sequenced the whole genome of 54 isolates from 26 patients with occasional and chronic lung infection. We performed a phylogenetic analysis and compared virulence and resistance genes, genetic variants and mutations, and hypermutability mechanisms between chronic and occasional isolates. We identified five *Achromobacter*

species as well as two non-affiliated genogroups (NGs). Among them were the frequently isolated *Achromobacter xylosoxidans* and four other species whose clinical importance is not yet clear: *Achromobacter insuavis*, *Achromobacter dolens*, *Achromobacter insolitus* and *Achromobacter aegrifaciens*. While *A. insuav*is and *A. dolens* were isolated only from chronically infected patients and *A. aegrifaciens* only from occasionally infected patients, the other species were found in both groups. Most of the occasional isolates lacked functional genes involved in invasiveness, chemotaxis, type III secretion system and anaerobic growth, whereas the great majority (>60%) of chronic isolates had these genomic features. Interestingly, almost all (n=22/23) late chronic isolates lacked functional genes involved in lipopolysaccharide production. Regarding antibiotic resistance, we observed a species-specific distribution of bla_{OXA} genes, confirming what has been reported in the literature and additionally identifying $bla_{OXA\text{-}2}$ in some *A. insolitus* isolates and observing no bla_{OXA} genes in *A. aegrifaciens* or NGs. No significant difference in resistance genes was found between chronic and occasional isolates. The results of the mutator genes analysis showed that no occasional isolate had hypermutator characteristics, while 60% of early chronic (<1 year from first colonization) and 78% of late chronic (>1 year from first colonization) isolates were classified as hypermutators. Although all *A. dolens*, *A. insuavis* and NG isolates presented two different *mutS* genes, these seem to have a complementary rather than compensatory function. In conclusion, our results show that *Achromobacter* species can exhibit different adaptive mechanisms and some of these mechanisms might be more useful than others in establishing a chronic infection in CF patients, highlighting their importance for the clinical setting and the need for further studies on the less clinically characterized *Achromobacter* species.

3.2.3 MILESTONE 3: Mobilome Analysis of Achromobacter spp. Isolates From Chronic and Occasional Lung Infection in Cystic Fibrosis Patients [56]

Although a variety of mobile genetic elements (MGEs) carrying antimicrobial resistance genes have been identified in clinical isolates, little

is known about the contribution of *Achromobacter* spp. mobilome to its pathogenicity. To provide new insights, we performed bioinformatic analyses of 54 whole genome sequences and investigated the presence of phages, insertion sequences (ISs), and integrative and conjugative elements (ICEs). Most of the detected phages were previously described in other pathogens and carried type II toxin-antitoxin systems as well as other pathogenic genes. Interestingly, the partial sequence of phage Bcep176 was found in all the analysed *Achromobacter xylosoxidans* genome sequences, suggesting the integration of this phage in an ancestor strain. A wide variety of IS was also identified either inside of or in proximity to pathogenicity islands. Finally, ICEs carrying pathogenic genes were found to be widespread among our isolates and seemed to be involved in transfer events within the CF lung. These results highlight the contribution of MGEs to the pathogenicity of *Achromobacter* species, their potential to become antimicrobial targets, and the need for further studies to better elucidate their clinical impact.

3.2.4 MILESTONE 4: Achromobacter spp. Adaptation in Cystic Fibrosis Infection and Candidate Biomarkers of Antimicrobial Resistance [57]

Achromobacter spp. can establish occasional or chronic lung infections in patients with cystic fibrosis (CF). Chronic colonization has been associated with worse prognosis highlighting the need to identify markers of bacterial persistence. To this purpose, we analyzed phenotypic features of 95 *Achromobacter* spp. isolates from 38 patients presenting chronic or occasional infection. Virulence was tested in *Galleria mellonella* larvae, cytotoxicity was tested in human bronchial epithelial cells, biofilm production in static conditions was measured by crystal violet staining and susceptibility to selected antibiotics was tested by disk diffusion method. The presence of genetic loci associated to the analyzed phenotypic features was evaluated by genome wide association study. Isolates from occasionally infected patients induced significantly higher mortality of *G. mellonella* larvae and showed a trend for lower cytotoxicity than chronic infection isolates. No significant difference was observed in biofilm production among the two groups. Additionally, antibiotic susceptibility testing showed that

isolates from chronically infected patients were significantly more resistant to sulfonamides and meropenem than occasional isolates. Candidate genetic biomarkers of persistence associated with antibiotic resistance or sensitivity were identified. *Achromobacter* spp. isolates from chronic and occasional lung infection exhibit different virulence and antibiotic resistance characteristics, some of which might be linked to persistence in CF lungs. This highlights the potential to identify predictive markers of persistence that could be translated into the clinical setting.

3.2.5 MILESTONE 5: Adaptive Interactions of Achromobacter spp. with Pseudomonas aeruginosa in Cystic Fibrosis Chronic Lung Co-Infection [58]

In the lungs of patients with CF the main pathogen *Pseudomonas aeruginosa* is often co-isolated with other microbes, likely engaging in inter-species interactions. In the case of chronic co-infections, this cohabitation can last for a long time and evolve over time, potentially contributing to the clinical outcome. Interactions involving the emerging pathogens *Achromobacter* spp. have only rarely been studied, reporting inhibition of *P. aeruginosa* biofilm formation. To evaluate the possible evolution of such interplay, we assessed the ability of *Achromobacter* spp. isolates to affect the biofilm formation of co-isolated *P. aeruginosa* strains during long-term chronic co-infections. We observed both competition and cohabitation. An *Achromobacter* sp. isolate secreted exoproducts interfering with the adhesion ability of a co-isolated *P. aeruginosa* strain and affected its biofilm formation. Conversely, a clonal *Achromobacter* sp. strain later isolated from the same patient, as well as two longitudinal strains from another patient, did not show similar competitive behaviour against its *P. aeruginosa* co-isolates. Genetic variants supporting the higher virulence of the competitive *Achromobacter* sp. isolate were found in its genome. Our results confirm that both inter-species competition and cohabitation are represented during chronic co-infections in CF airways, and evolution of these interplays can happen even at the late stages of chronic infection.

3.3 CANDIDATE GENOMIC MECHANISMS AFFECTING THE MICROBE-HOST INTERACTION IN CLINICAL STRAINS ADAPTATION

The findings of the work included in this thesis highlighted a plethora of adaptation strategies which include modifications of metabolism and virulence, acquisition of antibiotic resistance, development of hypermutation and exchange of mobile genetic elements. Each of these aspects will be further discussed in the following sub-sections by reporting the state of the art of *Achromobacter* spp. adaptation knowledge in the light of the findings of the work included in this thesis.

3.3.1 METABOLISM

Studies on within-host evolution of *Achromobacter* spp. in CF patients have found that the most mutated genes during adaptation were involved in general metabolism, virulence and antimicrobial resistance[76]. In particular, the ability to survive with limited oxygen has been identified as one of the possible mechanisms favouring persistence of *Achromobacter* in the CF airways environment[16]. Indeed, in our study (Milestone 2[32]) we found that the majority of *Achromobacter* spp. occasional isolates showed a lack of functional genes related to anaerobic growth, whereas the great majority of chronic isolates had this potential. Anaerobic growth ability might favour microorganisms persistence by conferring them the possibility to locate deeper within the mucous layer and within biofilm structures or in more hypoxic regions of the lung, where antibiotics can be dramatically less effective due to anaerobic conditions[77] and where they are able to evade immune response (Figure 3).

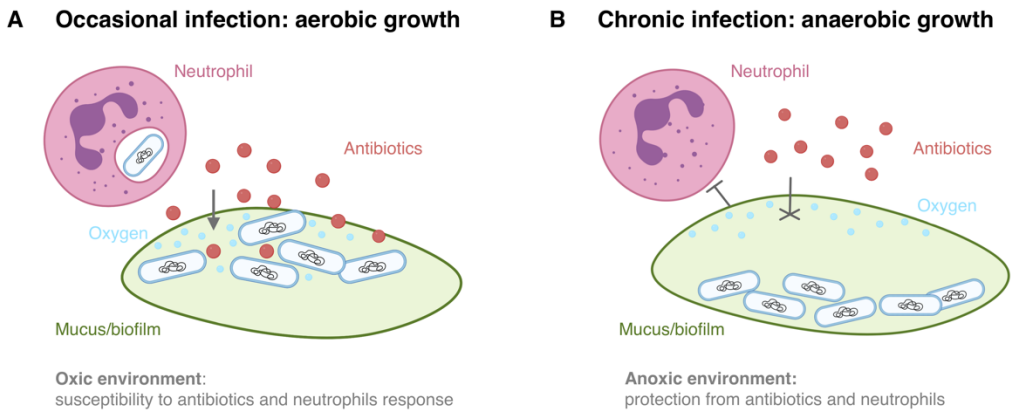

Figure 3 Occasional and chronic infection adaptation: oxic and anoxic environment. **A** Occasional infection isolates grow aerobically in the superficial mucous or biofilm layer, leaving them susceptible to antibiotic therapies and immune response. **B** Chronic infection isolates grow anaerobically locating deeper within the mucous layer and biofilm stuctures, where antibiotics are less effective and they are able to evade immune response. This figure was created on BioRender.com.

In addition, the use of denitrification for energy production in oxygen depleted environments, such as CF mucus, has been demonstrated for *Pseusomonas aeruginosa* - another CF pathogen[78] - and there is evidence that molybdenum uptake, upon which denitrification depends, is essential for anaerobic proliferation and influences virulence in this pathogen[79,80]. Results suggesting that *Achromobacter* and *P. aeruginosa* may share this adaptive mechanism have been reported in a study regarding CF clinical isolates[16].

3.3.2 VIRULENCE

As already mentioned in section 2.3, the *Achromobacter* genus has been found to be phylogenetically related to *Bordetella* and this strong link highlights the potentially pathogenic nature of this CF emerging pathogen from the phylogenetic perspective[20–22]. The mechanisms underlying *Achromobacter* spp. ability to colonize the respiratory tract as well as other sites of the human body are still not fully clear, but a number of virulence factors have been described that likely support their invasiveness and survival in hostile environments. Similarly to other Gram-negative pathogens, *Achromobacter* spp. express membrane-bound virulence factors. Among them, the Vi capsular polysaccharide, which enables surface adhesion and protection from environmental toxins; the O-antigen, involved

in eliciting the host immune response[20]; and lipopolysaccharide (LPS), which induces the production of key inflammatory cytokines such as IL-6, IL-8 and TNF-α[81]. Additionally, a heat-stable cytotoxic factor has been identified and associated with increase of pro-inflammatory cytokines in vitro[81]. All members of *Achromobacter* genus also present peritrichous flagella that enable swimming motility, contributing to biofilm formation and host cell invasion[82].

Biofilms consist of bacterial microcolonies encased in a matrix of polysaccharides, DNA and proteins and can have variable morphology, growing attached to a surface or as unattached aggregates in mucus or in sputum[81,83]. Biofilm formation is a growth phenotype that many bacteria causing CF infections use for survival and proliferation in hostile environment: this ability allows to shield pathogens against environmental stress and increase tolerance towards antibiotics and host defences[83,84]. The presence of genes linked to biofilm formation in other bacteria has also been reported in *Achromobacter* spp. genomes. Among them, the *flgB* gene, which encodes a flagellar basal body rod protein implicated in *Bordetella bronchiseptica* and *P. aeruginosa* biofilm formation[85,86], and the pgaABCD operon, which encodes the polysaccharide β-1,6-GlcNAc involved in both cell-cell adherence and cell-surface adherence in other CF pathogens[44].

Among cell membrane components, secretion systems have an important function in bacterial pathogenicity since they are involved in the release of toxins, proteases and other virulence factors. Numerous genes encoding different types of secretion systems have been identified in *Achromobacter* spp. genome, including: type II secretion system (T2SS), which is widely conserved among γ-proteobacteria and is involved in the release of extracellular toxins and proteases; type III secretion system (T3SS) that delivers virulence factors directly into the host cell; type VI secretion system (T6SS) that mediates the transport by direct contact with the target cells; and type VII secretion system (T7SS) which includes sigma-fimbriae encoding genes[87]. In particular, T3SS is known to enhance the bacterial

ability to infect host cells with effector proteins and to contribute in immune response evasion[20,44,82].

As concerns secreted virulence factors, genomic studies reported the presence of genes encoding colicin V, a cytotoxic protein that likely gives *Achromobacter* spp. environmental advantages by eliminating competing flora and enabling tissue invasion, and AepA, which facilitates the production of cellulases and proteases enabling mucosal invasion[44]. In addition, production of phospholipase C was observed, which allows hydrolysis of phospholipids of the alveolar surfactants and tissue disruption[88].

Studies focusing on *Achromobacter* adaptive characteristics investigated a variety of virulence features considered important for *in vivo* colonization and infection, such as biofilm production, motility and secretion.

A study of 52 *A. xylosoxidans* strains isolated during an outbreak at the CF Centre in Rome (Italy) found that the great majority of strains were motile and biofilm producers, and a significant prevalence of strong biofilm-producing strains was found in patients with severely impaired lung function[14]. In order to understand whether biofilm production played an important role not only during acute infections but also in bacterial persistence, we analysed 95 *Achromobacter* spp. isolates from occasional and chronic infection (Milestone 4[57]). Our results showed no significant difference in biofilm production among the two groups, nonetheless a reduced biofilm formation has been observed in chronic strains over time of infection, which may result from within-host adaptation to the CF lung during chronic colonization[84]. Indeed, it has been reported that gene expression profiles at biofilm stage differ from planktonic cells[89], and that in biofilm stage genes associated with anaerobic respiration are upregulated, suggesting an adaptation to the microaerobic and anaerobic conditions prevalent in the late stage of CF chronic infections[90].

Another virulence trait, the presence of T3SS, which pathogenic bacteria use to inject effectors into host cells[91], has been considered as a key discriminant among clinical and environmental *Achromobacter* spp. strains.

Comparative genomics analyses showed that virulence genes related to T3SS are more common in *Achromobacter* spp. CF isolates rather than in environmental strains[16,20], linking this feature with the infection ability of these microorganisms. Furthermore, we found that the presence of functional T3SS genes seems to be associated with the establishment of chronic infections in the CF lung, while occasional infection isolates lack functional T3SS genes, leaving them susceptible to the immune response (Figure 4A and 4B; Milestone 2[32]).

Recently, the pathogenic and inflammatory role of *Achromobacter* spp. has been investigated and their cytotoxicity in macrophages has been reported[92]. In our study (Milestone 4[57]), we found that these pathogens determined cytotoxicity also in human bronchial epithelial cells and that chronic infection isolates induced greater cytotoxicity than occasional isolates, even though no statistically significant difference was found.

An additional mechanism that might be involved in *Achromobacter* spp. persistence in CF lungs is the accumulation of mutations in genes involved in LPS production, probably leading to a reduced recognition by the host defence system (Figure 4C and 4D; Milestone 2[32]). Indeed, a reduction in the number of LPS lipid A acyl chains by other bacteria was shown to modulate the recognition of LPS by toll-like receptors[93].

This trend indicating that *Achromobacter* spp. exhibit higher virulence during occasional infection was also confirmed by our virulence testing results (Milestone 4[57]); indeed, isolates from occasionally infected CF patients induced significantly higher mortality of *G. mellonella* larvae than chronic infection isolates (Figure 4E and 4F).

Another key factor to evaluate in microbial persistence is the presence in the same niche of co-colonising or co-infecting microbes, giving rise to inter-species interactions. In the case of chronic co-infections, this cohabitation can last for a long time and evolve over time, potentially contributing to the clinical outcome. Interestingly, it was found that some *Achromobacter* spp. strains are able to inactivate *P. aeruginosa* quinolone signal, which could

give them an advantage over this and maybe other CF pathogens during co-habitation in the lung environment[94]. Interactions involving *Achromobacter* spp. have only rarely been studied, reporting inhibition of *P. aeruginosa* biofilm formation[95]. To evaluate the possible evolution of such interplay, we assessed the ability of *Achromobacter* spp. isolates to affect the biofilm formation of co-isolated *P. aeruginosa* strains during long-term chronic co-infections (Milestone 5[58]). We also recently assessed the presence of secreted proteases finding that they could play a role in inter-species competition (Milestone 1[55]). We confirmed that both inter-species competition and cohabitation are represented during chronic co-infections in CF airways, and evolution of these interplays can happen even at the late stages of chronic infection (Milestone 5[58]).

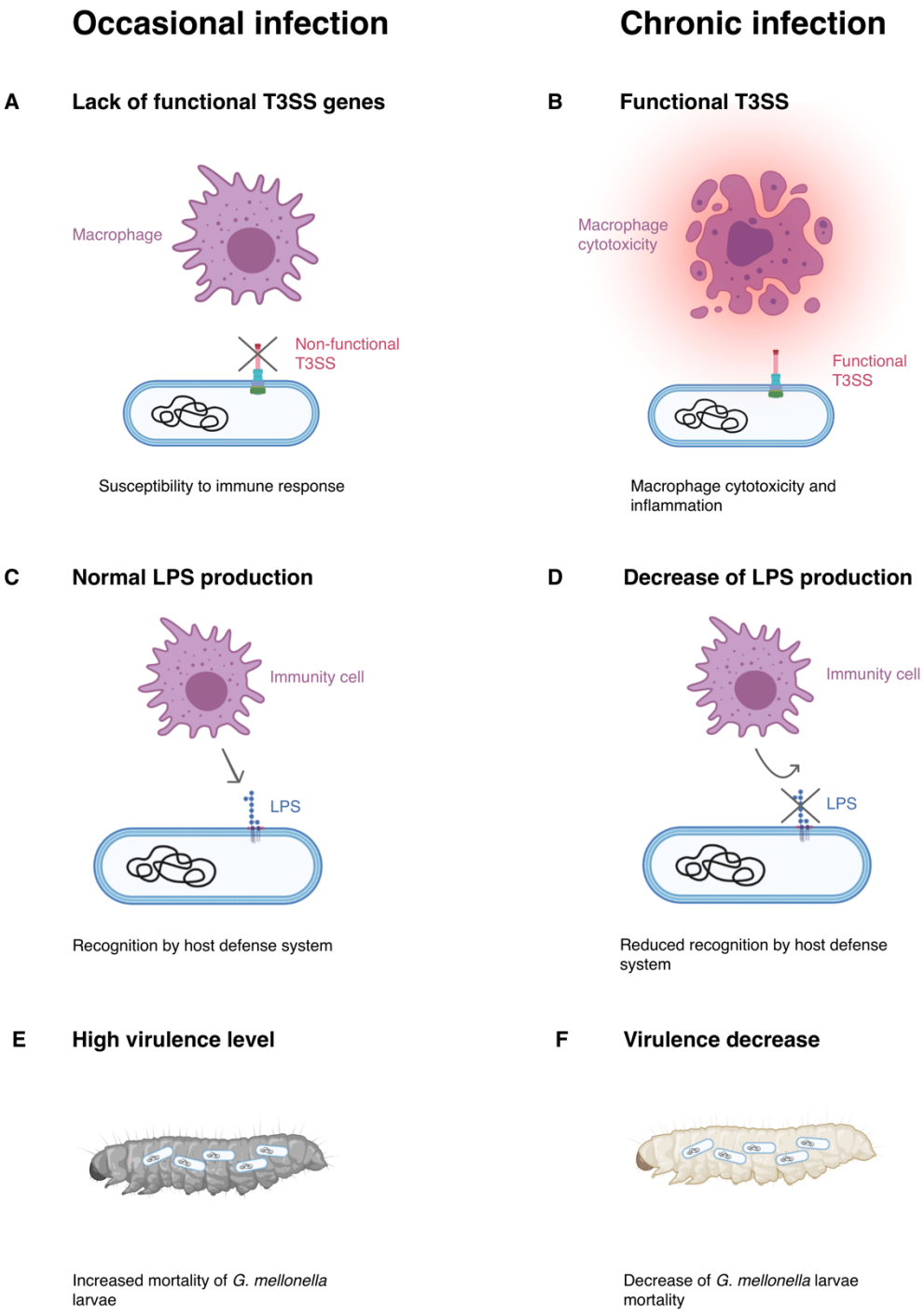

Figure 4 Occasional and chronic infection adaptation: virulence. Occasional infection isolates lack functional T3SS genes (**A**), produce LPS (**C**) and show a high virulence in *G. mellonella* larvae (**E**). Chronic infection isolates have functional T3SS genes (**B**), decreased LPS production (**D**) and show a decrease in *G. mellonella* mortality (**F**). T3SS=type III secretion system; LPS=lipopolysaccharide. This figure was created on BioRender.com.

3.3.3 ANTIMICROBIAL RESISTANCE

An important factor for the survival of *Achromobacter* spp. within the host is its resistance to antibiotics, mediated by naturally occurring and acquired systems of defence, rendering infections particularly hard to eradicate. A variety of mechanisms contribute to the resistance patterns of bacteria such as production of degrading enzymes, efflux pump system or changes in the antibiotic target.

Achromobacter spp. show an innate resistance to many classes of antibiotics, especially to aminoglycosides, aztreonam, tetracyclines, penicillins and cephalosporins, which include antibiotics relevant to CF lung infection treatment[14,82,96]. In particular, trimethoprim-sulfamethoxazole, ceftazidime, piperacillin, and carbapenems have been reported as the most active agents against *Achromobacter* spp. isolates[31]. Among carbapenems, several studies showed imipenem to be more active than meropenem against *Achromobacter* isolates[97–99].

The most conserved genes conferring antibiotic resistance among *Achromobacter* spp. can be classified in 5 groups: class A and class B β-lactamase, group B chloramphenicol acetyltransferase, rRNA methylases, and resistance-nodulation-cell division (RND) efflux pump[100]. Particularly, many members belonging to the RND efflux pump group are associated with intrinsic resistance. Among them, AxyABM, which is able to extrude most cephalosporins, fluoroquinolones, aztreonam and chloramphenicol, and AxyXY-Opr, which extrudes aminoglycosides but can also accommodate cefepime, tetracyclines and carbapenems[82,101,102].

Another important resistance mechanism is the production of OXA-114-like enzymes, which belong to class D of β-lactamases and show great activity against penicillin G, early cephalosporins, piperacillin, and ticarcillin[31]. Moreover, extended-spectrum β-lactamase (ESBLs), AmpC type β-lactamase, and metallo-β-lactamase (MBLs) have been detected in *Achromobacter* isolates and appear to contribute to resistance to β-lactams including carbapenems[103–110].

Many studies focused on the role of antimicrobial resistance in *Achromobacter* CF infections. In addition to carrying a greater number of genes involved in resistance against aminoglycosides, beta-lactams, chloramphenicol and sulphonamides, clinical strains showed positive selection of three genes encoding efflux pump components: *emrA*, *macA* and *mexW*[16]. The protein products of these genes are elements of a major facilitator superfamily multidrug export complex, an ABC efflux pump that exports macrolides and an RND-type efflux pump, respectively. Positive selection of these genes suggest that efflux pumps represent another key mechanisms for adaptation to a pathogenic lifestyle as they are implicated in bacterial virulence[111] and show a tendency to favour loss of specificity, which translates into multi-drug resistance[112,113].

Another recent study[63] of 101 *Achromobacter* clinical isolates showed that development of antibiotic resistance is associated with chronic infections; in particular, late isolates were statistically significantly less susceptible than early and single isolates. Moreover, nearly all isolates were resistant or intermediate resistant to aztreonam, ceftriaxone, cefuroxime, ciprofloxacin, moxifloxacin, penicillin, rifampicin, tobramycin and trimethoprim. Interestingly, it was also reported that isolates belonging to the Danish epidemic strain (*A. ruhlandii*) were resistant or intermediate resistant to a median of 20 antibiotics, while the median was 14 for other *Achromobacter* isolates (*A. xylosoxidans* and *A. insuavis*), which could be one of the reasons this strain has become so widespread among CF patients in Denmark.

A k-mer based genome-wide association study[33] with antibiotic resistance phenotype was also performed on the same isolates and showed that inorganic ion transport genes contribute to antibiotic resistance development in all tested antibiotics (amoxicillin-clavulanate, ceftazidime, chloramphenicol, colistin, imipenem, meropenem, piperacillin-tazobactam, sulfamethizole, tigecycline and trimethoprim-sulfamethoxazole). An enrichment of transcription genes associated with resistance to β-lactams,

and of translation, ribosomal structure genes and energy production genes in the trimethoprim/sulfonamide resistant group was also observed.

Our bioinformatic analysis of 54 *Achromobacter* genomes from CF patients (Milestone 2[32]) found that 54% of isolates presenting deleterious variants in antibiotic resistance genes carried mutations in at least one *bla* gene. In particular, bla_{OXA} genes follow a species-specific distribution in *Achromobacter* species: while the specificity of $bla_{OXA\text{-}114}$, $bla_{OXA\text{-}243}$, $bla_{OXA\text{-}364}$ for *A. xylosoxidans*, *A. insuavis* and *A. dolens* respectively was already reported in literature[114], we found no bla_{OXA} genes for *A. aegrifaciens* and some *A. insolitus* isolates.

Additionally, antibiotic susceptibility testing of 95 *Achromobacter* spp. clinical isolates (Milestone 4[57]) showed that isolates from chronically infected patients were significantly more resistant to sulfonamides and meropenem than occasional isolates.

Differences in antibiotic susceptibility during adaptation between occasional and chronic infection isolates are summarised in Figure 5.

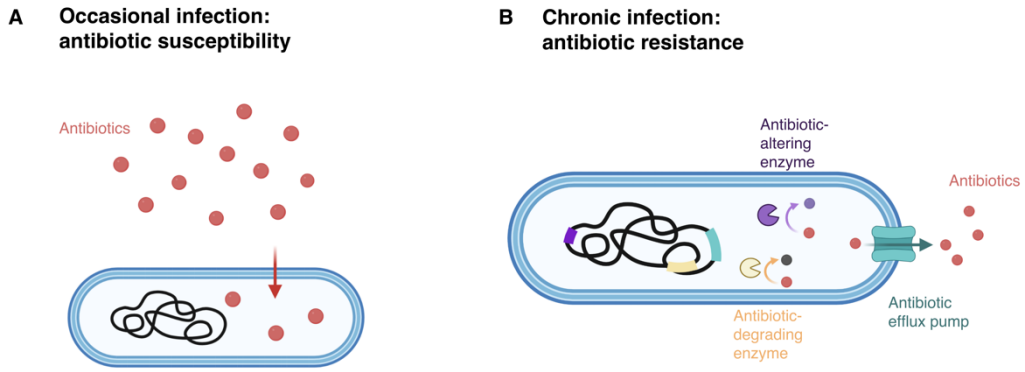

Figure 5 Occasional and chronic infection adaptation: antibiotic resistance. A Occasional infection isolates are susceptible to antibiotic therapies. **B** Chronic infection isolates are resistant to antibiotics through mechanism such as antibiotic-altering enzymes, antibiotic-degrading enzymes and efflux pumps. This figure was created on BioRender.com.

3.3.4 HYPERMUTATION

Among the variety of adaptation mechanisms identified in the CF lung environment, another relevant aspect is the high-rate accumulation of pathoadaptive mutations leading to hypermutation[115], which is a key feature for the clonal diversification observed in CF chronic infections. Typically, short-term adjustments are likely to be the result of regulatory alterations in gene expression whereas long-term adaptation is the result of the accumulation of pathoadaptive mutations[76]. Interestingly, the generation rate of mutations can be accelerated due to defects in DNA repair or error avoidance systems in hypermutable strains, thus giving rise to clonal diversification within the host[116]. Some of the genes involved in this phenomenon, also referred to as mutator genes, are *mutL*, *mutS*, *pfpI*, *superoxide dismutase*, *radA*, *radC*, *rad50*, *uvrA*, *uvrB*, *uvrC*, and *uvrD*[117]. The occurrence of hypermutation has been demonstrated for various CF pathogens, such as *Pseudomonas aeruginosa*[116,118–120] and *Burkholderia cepacia* complex[121], and more recently we reported the occurrence of this phenomenon also in *Achromobacter* spp. (Milestone 1[55]) [117]. In particular, *Achromobacter* spp. hypermutation events appear to be observed frequently in strains isolated from the lungs of chronically infected patients while no occasional infection isolate showed hypermutator characteristics to date (Milestone 1[55], Milestone 2[32])[63,117], thus suggesting that hypermutation might constitute an advantageous adaptive mechanism in the lung environment (Figure 6). Interestingly, in our study (Milestone 2[32]) we reported the presence of two copies of *mutS* gene in *A. dolens* genome and a variable copy number of this gene in *A. insuavis*. Moreover, we identified *A. dolens* hypermutator isolates carrying mutations in a single *mutS* gene, thus suggesting that both genes are needed for effective mismatch repair in this species.

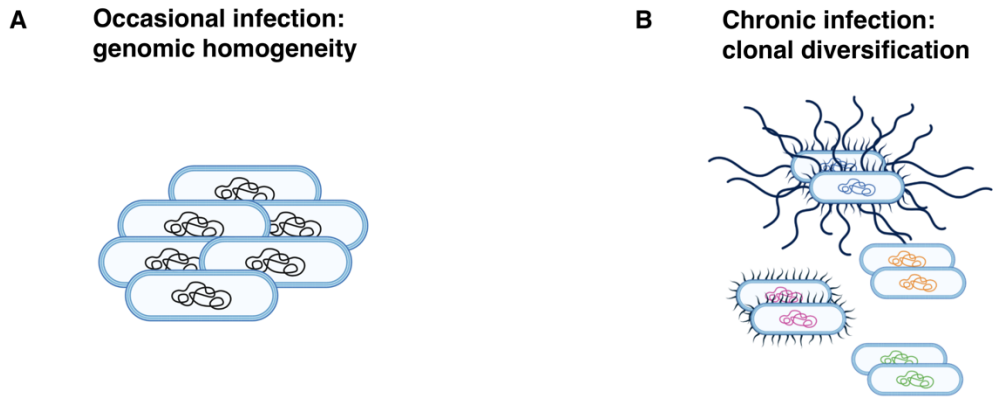

Figure 6 Occasional and chronic infection adaptation: hypermutation. A Occasional infection isolates show genetic homogeneity. **B** Chronic infection isolates show clonal diversification linked to hypermutation events. This figure was created on BioRender.com.

3.3.5 MOBILOME

Horizontal gene transfer is the transfer of genetic elements among organisms by means other than vertical transmission and is a well-described mechanism that has been increasingly studied due to its role in the rapid dissemination of genetic elements among bacteria[122–129]. Mobile genetic elements (MGEs) are segments of DNA that encode proteins mediating their own movement and have an important role in bacterial adaptation[130–135]. In particular, the acquisition of MGEs harbouring genes related to virulence and antimicrobial resistance can enable their microbial host to synthesize products that affect the fitness of resident microbiota and co-infecting pathogens or confer antibiotic resistance[136–138]. MGEs have been detected in the great majority of prokaryotic organisms and a variety of MGEs have been identified in *A. xylosoxidans* clinical isolates, such as plasmids, IS26, IS440, and class I and class II integrons[100,103]. Nevertheless, literature about the scale and importance of mobilome is still scarce for *Achromobacter* species.

A bioinformatic study proposed that the most likely candidates to be involved in horizontal transfer with *Achromobacter* spp. were *Sinorhizobium* sp. as well as *Ralstonia*, *Pseudomonas* and *Burkholderia* sp.[16], which share a similar GC content with *Achromobacter* and are soil organisms that are also responsible for CF opportunistic infections[139,140]. These results have been confirmed in our study (Milestone 3[56]) describing the mobilome - phages,

insertion sequences (IS), integrative and conjugative elements (ICEs), and integrative and mobilizable elements (IMEs) – of 54 *Achromobacter* spp. clinical isolates from occasional and chronic CF lung infection.

Among MGEs, phages are the most abundant in the biosphere. They are viruses which infect bacteria and have been of interest to scientists as vectors of horizontal gene transfer and drivers of bacterial evolution[141]. In our study (Milestone 3[56]), most of the phages detected in all *Achromobacter* species were previously described in other pathogens and carried genes related to MGEs stability, biofilm formation and stress responses, highlighting the importance of MGEs role in *Achromobacter* pathogenicity. Moreover, type II toxin-antitoxin systems, which have been reported to occur more often in pathogenic bacteria and have been evaluated as antimicrobial targets[142–149], were identified in the analysed isolates. Additionally, we proposed for *A. xylosoxidans* an ancestral uptake of the phage Bcep176 from *Burkholderia*.

IS elements, another type of MGEs, are segments of DNA shorter than 2.5 kbp capable of inserting at multiple sites in the genome[150]. They show a simple genetic organization and usually cluster in islands within genomes. In our study (Milestone 3[56]), different classes of ISs, which are frequently associated to antimicrobial resistance genes and to class I and II integrons[100,103], were also detected either inside of or in proximity to pathogenicity islands. In particular, we identified ISs from a wide variety of microorganisms, especially from species of clinical interest including *B. cepacia* complex, *P. aeruginosa*, and *S. maltophilia*.

ICEs are self-transmissible MGEs that usually mediate the transfer of diverse properties to enable the host to better adapt to hostile conditions[151]. We found a great number of ICEs carrying genes related to a variety of functions such as secretion, motility, quorum sensing, metabolism, mismatch repair, and resistance to different classes of antimicrobial molecules (Milestone 3[56]). In particular, the most represented antibiotic resistance genes were the sulphonamide resistance gene *sul1* and the *aac(6')* family aminoglycoside acetyltransferase, which are frequently found within MGEs

such as integrons, plasmids and transposons carried by other Gram-negative opportunistic pathogens[152]. As concerns adaptation to the lung environment, phages and ISs have shown high consistency in longitudinal isolates, whereas variations in the presence and pathogenic content of ICEs over time were observed, indicating a frequent exchange of MGEs within the CF lungs.

Plasmids, one of the most widespread types of MGEs among microorganisms, are circular or linear extrachromosomal DNA molecules that can replicate independently from chromosomal DNA[153]. Little is still known about *Achromobacter* spp. plasmid content, especially regarding strains isolated from CF patients. Indeed, the majority of the available literature concerns environmental strains from aquacultures and soil. In particular, plasmids that have been reported in *Achromobacter* spp. are the wide host range IncP plasmids[103], a 27 kbp plasmid coding for nitrite reductase and nitrous oxide reductase[154], the 70 kbp 2,4-dichlorophenoxyacetic acid-degradative pEST4011 plasmid[155], and the 98 kbp plasmid pA81 harbouring genes encoding heavy metal resistance determinants[156].

Overall, our findings underline MGEs contribution to the genomic plasticity of *Achromobacter* isolates and support that MGEs might play an important role in pathogenesis and adaptation during chronic infections, highlighting the need for further studies.

4.1 CONCLUSIONS

Achromobacter spp. are emerging opportunistic pathogens that are being increasingly isolated from the airways of CF patients and are able to colonise and persist in this niche thanks to their high adaptability to diverse environmental conditions.

In order to unravel underlying key processes and to investigate the adaptive mechanisms exploited by these microorganisms during lung infection, we analysed genomic and phenotypic data of 103 clinical isolates from 40 CF patients followed at the CF centres in Verona (Italy), Rome (Italy), and Copenhagen (Denmark).

Our studies results gave us insights into many aspects of colonisation and adaptation, among them: metabolism, virulence, antibiotic resistance, hypermutation events and MGEs.

Occasional infection isolates showed genomic homogeneity (no hypermutation events occurred), presented the genomic potential to grow aerobically and produce LPS, and phenotypically demonstrated to be highly virulent and susceptible to more classes of antibiotics when compared to chronic infection isolates. On the other hand, chronic infection isolates presented the genomic potential to grow anaerobically and produce reduced quantities or a mutated form of LPS, and were phenotypically less virulent but resistant to more classes of antibiotics compared to occasional infection isolates.

These results suggest that occasional infection isolates are more susceptible to antibiotic therapies and immune response, thus being easier to eradicate. Conversely, chronic infection isolates show greater resistance to antibiotics and are better at evading immunity. In particular, attenuation of virulence is one of the mechanisms related to immune evasion and persistence. Indeed, phenotypic studies results indicate that *Achromobacter* spp. isolates from

chronic lung infection are less virulent than occasional ones and that the observed attenuation of virulence might also be an advantage in establishing a persistent infection. These findings support the adaptive trade-off evolution hypothesis that virulence genes are not required or are selected against in chronic infections[157], thus highlighting the potential to identify predictive markers of persistence that could be translated into the clinical setting.

Overall, the work presented in this thesis provide new knowledge on *Achromobacter* spp. adaptation and evolution in the CF lung environment, present for the first time a bioinformatic analysis of MGEs and their contribution to *Achromobacter* spp. pathogenicity, and give insights into inter-species interaction evolution with *P. aeruginosa*. With further studies it will be possible to translate these results into clinically relevant information, leading to better predictions of the infection course and improvement of treatment strategies to the benefit of CF patients.

4.2 DISCUSSION OF CHALLENGES AND AREAS OF FURTHER RESEARCH

The work presented in this thesis is a starting point to promote further research on *Achromobacter* spp., in fact, many aspects remain to be elucidated.

Firstly, this genus still suffers from difficulties in diagnosis due to misidentification caused by its biochemical similarity to other Gram-negative bacilli and continuously evolving taxonomy. Some advances have already been made regarding the identification of *Achromobacter* species by the introduction of *nrdA* gene analysis, multilocus sequence typing[66,158] and the creation of databases for matrix-assisted laser desorption ionization-time of flight mass spectrometry[29,30], which is the most employed technique in routine clinical microbiology laboratories for this aim. In the near future, the expanding number of collected isolates could lead to the creation of new or updated comprehensive databases that will allow a more accurate routine identification of this opportunistic pathogen.

Additionally, we still have a limited understanding of *Achromobacter* adaptation to the CF lungs environment attributable to the restricted number of comparative studies, especially involving isolates from occasionally infected patients. In fact, the comparison between chronic and occasional infection isolates may allow the identification of genetic markers of persistence, while studies of less clinically characterized *Achromobacter* species could help in understanding whether some species are more likely to establish a chronic colonization of the CF airways.

Another problem contributing to the difficult understanding of *Achromobacter* spp. adaptation is the large number of ORFs classified as having hypothetical function. Bioinformatic and functional studies regarding the product of these genes could give further insights on their role and contribution to *Achromobacter* metabolism, pathogenic potential and adaptation mechanisms.

Noteworthily, the variety of MGEs identified in *Achromobacter* genomes and their diverse virulence and antibiotic resistance profiles have confirmed *Achromobacter* spp. as a reservoir of MGEs. Not only they contribute to genomic plasticity, but some of these elements can also even become a constitutive part of the bacterial genome, thus highlighting the need for further studies to better elucidate MGEs clinical impact and their potential to become antimicrobial targets in treatment regimens.

Finally, co-infection and co-colonisation of multiple species or multiple clones can happen in CF airways and *Achromobacter* sp. needs to be studied not as a single entity but as part of complex systems governed by inter-species dynamics. Further studies regarding different species interplay or metagenomic analyses of CF airways could help in unravelling *Achromobacter* spp. role in the lung microbiota.

Overall, the increasing number of studies focusing on this emerging pathogen and the continuous refinement of research techniques will likely allow a deeper knowledge of *Achromobacter* species that could successfully be translated into the clinical setting to the benefit of the patients.

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Article

Hypermutation as an Evolutionary Mechanism for *Achromobacter xylosoxidans* in Cystic Fibrosis Lung Infection

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Abstract: *Achromobacter xylosoxidans* can cause chronic infections in the lungs of patients with cystic fibrosis (CF) by adapting to the specific environment. The study of longitudinal isolates allows to investigate its within-host evolution to unravel the adaptive mechanisms contributing to successful colonization. In this study, four clinical isolates longitudinally collected from two chronically infected patients underwent whole genome sequencing, de novo assembly and sequence analysis. Phenotypic assays were also performed. The isolates coming from one of the patients (patient A) presented a greater number of genetic variants, diverse integrative and conjugative elements, and different protease secretion. In the first of these isolates (strain A1), we also found a large deletion in the *mutS* gene, involved in DNA mismatch repair (MMR). In contrast, isolates from patient B showed a lower number of variants, only one integrative and mobilizable element, no phenotypic changes, and no mutations in the MMR system. These results suggest that in the two patients the establishment of a chronic infection was mediated by different adaptive mechanisms. While the strains isolated from patient B showed a longitudinal microevolution, strain A1 can be clearly classified as a hypermutator, confirming the occurrence and importance of this adaptive mechanism in *A. xylosoxidans* infection.

Keywords: lung infection; opportunistic pathogen; bacterial evolution; comparative genomics; clonal diversification

1. Introduction

Cystic fibrosis (CF) is strictly linked with chronic bacterial respiratory infections; in fact, airways infection and the ensuing inflammation account for the majority of morbidity and mortality of CF patients [1]. *Achromobacter xylosoxidans* is an opportunistic pathogen in patients with CF, where this microorganism can survive for a long time in both lower and upper airways [2]. Infection results either from acquisition of bacteria present in the environment or from direct or indirect transmission [3,4] and is usually complicated by the intrinsic and acquired multidrug resistance traits carried by this microorganism. Chronic lung infection by *A. xylosoxidans* has been associated with decline in

respiratory function, increased frequency of exacerbations and lung inflammation [3,5,6]. Nevertheless, very little is known about the pathogenic mechanisms allowing *A. xylosoxidans* to colonize and persist in CF airways.

Pathogenic mechanisms of other CF pathogens are well-known; in particular, *Pseudomonas aeruginosa* has been extensively studied, due to its high incidence in CF patients and the difficult—in many cases impossible—eradication. One of the main mechanisms favouring the persistence of *P. aeruginosa* in the airways of CF patients is the ability to genetically adapt during chronic infection. In particular, short-term adjustments are believed to be the result of regulatory alterations in gene expression whereas long-term adaptation is the result of the accumulation of pathoadaptive mutations [7]. Consequently, the potential and speed for bacterial pathogens to genetically adapt to the host immune system and drug therapies may be determined by the within-host mutation rate [8]. Interestingly, the generation rate of mutations can be accelerated due to mutations in the DNA mismatch repair system, giving rise to hypermutation events and to clonal diversification within the host [9]. The adaptation of bacteria to a heterogeneous and changing environment can promote selection of hypermutable strains. Since *A. xylosoxidans* is an emerging pathogen, its pathoadaptive evolution and its impact in relation to chronic infections is still not clear. In this work we studied longitudinally collected clinical isolates of *A. xylosoxidans* to unravel the adaptive mechanisms contributing to its successful colonization of CF lungs.

2. Results

We sequenced the genome of four *A. xylosoxidans* isolates longitudinally collected from sputum samples of two CF patients. Two isolates were collected from each patient: in 2005 and 2008 from patient A, and in 2008 and 2014 from patient B. Genotypic relatedness was verified by checking core genome similarities: Isolates coming from each patient showed 87% similarity with the respective longitudinal isolate. Both patients had been chronically infected for 9 years: The year of first *A. xylosoxidans* isolation was 1996 and 1999 for patients A and B, respectively. The general information on de novo assembly of each genome is presented in Table 1. The assembly lengths varied between 6.6–6.9 Mbp and the GC-content varied between 67.63–68.09%, which is comparable to the published reference genome NH44784-1996 [10].

Table 1. Information on the de novo assembly of each genome. Longitudinal isolates are numbered (1, 2) following the time of isolation.

Patient	Isolate	Genome Size (bp)	GC-Content (%)	No. Contigs	N50	Mean Coverage Depth (x)	No. Coding Sequences	Mapping Reads (%)
A	A1	6913734	68.09	291	78688	66	6359	98.3
A	A2	6879357	68.08	187	78799	50	6339	97.87
B	B1	6634994	67.63	178	100359	49	6041	98.07
B	B2	6628209	67.63	158	93753	40	6050	98.35

Sequence reads were aligned to the reference genome and to the de novo assembly of the other isolate from the same patient (i.e., reads from A1 isolate were aligned to the de novo assembly of A2 and vice versa) to investigate genetic changes over time. The mapping reads percentage is shown in Table 2.

Table 2. Percentage of reads of each isolate mapping against the de novo assembly of the corresponding longitudinal isolate's genome and against NH44784-1996 reference genome.

Isolate Reads	Longitudinal Isolate de novo Assembly	Mapping Reads vs de novo Assembly (%)	Mapping Reads vs NH44784-1996 (%)
A1	A2	96.49	52.37
A2	A1	97.31	46.27
B1	B2	98.78	83.08

B2	B1	96.73	82.98

Since a high number of mapping reads was obtained between the de novo assemblies (range: 96.49–98.78%), this approach allowed us to detect almost all the variants between longitudinal isolates. Differently, carrying out the analysis by only mapping the reads against the reference genome, these variants would not have been considered, as the lower number of mapping reads suggests.

2.1. Variant Analysis

To take into account the possibility of calling false positive variants within the same patient over time, the sequencing reads of A1 and B1 isolates were mapped on the genome assembly of A2 and B2 respectively, and vice versa, and only the intersection of the two groups of variants was taken into consideration. A total of 187 variants was identified in patient A genomes, while a total of only eight was identified in genomes from patient B as shown in Table 3. The majority of genetic alterations were represented by SNPs. Moreover, the greater number of SNPs in patient A genomes (85%) is due to transitions and not transversions (mean ratio: 5.8), which is typical of hypermutators.

Since a great number of variants were identified in strains from patient A, the variant analysis was performed also against the reference genome in order to investigate variants independently. A mean total of 116 variants were found in patient A while a mean total of only 26 were found in patient B. As shown in Table 5, the genome of isolate A1 harboured a greater number of variants (n = 162), particularly SNPs (n = 150), when compared to the other isolates; even twice as many SNPs than its longitudinal isolate A2. Moreover, 73% of A1 SNPs are due to transitions (transitions/transversions ratio: 2.75), supporting the possibility that a hypermutation event occurred.

Table 3. Genetic variants found in each genome, by type (SNPs, indel—transitions, transversions), by translational changes (synonymous, missense, nonsense, other) and by the predicted functional impact (frameshift, disruptive in-frame insertion/deletion, stop gain/loss).

Analysis	**Comparison between Longitudinal Isolates**		**Comparison with Reference Genome**			
Genome	**A**	**B**	**A1**	**A2**	**B1**	**B2**
Total	187	8	162	70	10	42
No. SNPs	150	6	150	68	8	39
No. indel	37	2	12	2	2	3
No. Synonymous SNPs	38	3	87	43	4	24
No. Missense SNPs	89	3	53	14	4	10
No. Nonsense SNPs	5	0	0	0	0	0
No. Other SNPs	18	0	10	11	0	5
Frameshift	13	2	8	0	0	2
Disruptive in-frame insertion	1	0	0	0	0	0
Disruptive in-frame deletion	0	0	0	0	0	1
Stop gain	5	0	1	0	0	0
Stop lost	1	0	0	0	0	0
Transitions	128	2	110	43	5	24
Transversions	22	4	40	25	3	15
Transition/transversion ratio	5.8	0.5	2.75	1.72	1.66	1.6

As shown in Table 3, among the mutations that could cause a protein loss of function, frameshift mutations are the majority followed by stop gain mutations. In particular, genomes of patient A show a higher number of mutations when compared to genomes of patient B. A2 and B1 genomes do not harbour mutations with a predicted high impact on protein function, since the missense SNPs have all been annotated as having a moderate impact; the B2 genome harbours only three mutations, while frameshift mutations are the majority in the A1 genome. Gene products affected by mutations

identified through comparison between longitudinal isolates are summarized in the supplementary material (Table S1).

The list of genes presenting variants, divided by functional classes according to the protein function as reported on UniProt database, is shown in Table 4. In genomes from patient A, in particular in A1 genome, the majority of genes presenting variants are involved in metabolism (39%), followed by genes involved in transcription and translation (12%) and transporter proteins encoding genes (12%).

Table 4. List of gene-presenting variants, grouped by functional class. "Other" includes membrane protein, AsmA family protein, Tol-Pal system protein TolB.

Analysis	Comparison between Longitudinal Isolates			Comparison with Reference Genome				
Functional Category	**A**	**B**	**Total**	**A1**	**A2**	**B1**	**B2**	**Total**
Metabolism	66	0	66	49	18	3	4	74
Transcription and translation	21	1	22	13	3	1	4	21
Virulence, disease and defence	5	0	5	2	4	0	0	6
Hypothetical protein	34	2	36	4	1	2	3	10
Transporter	21	3	24	18	6	0	3	27
Iron acquisition and metabolism	10	0	10	6	1	0	1	8
Stress response	2	0	2	1	1	0	0	2
DNA repair	2	0	2	0	3	0	0	3
Antibiotic resistance	7	0	7	5	3	0	0	8
Mobile genetic elements	0	0	0	0	0	0	0	0
Other	3	0	3	4	0	0	0	4
Total	171	6	177	102	40	6	15	163

2.2. Genetic Basis of Hypermutation

Since we suspected that a hypermutation event occurred in A1 isolate, the occurrence of genes involved in DNA repair was investigated in all four genomes. All clinical isolates differed from the reference genome because they harboured two copies of the *mutL* gene; they lacked superoxide dismutase genes *sodA, sodB, sodC* and DNA repair gene *radC*; and they had only one copy of nucleotide excision repair genes *uvrA* and *uvrB*. Moreover, the isolates coming from patient B presented an extra copy of *uvrD*. Genes involved in DNA repair in each clinical isolate and in the reference strain are summarized in the supplementary material (Table S2).

Furthermore, gene sequences were thoroughly examined and a 95 nucleotides deletion that translated to a 36 amino acids gap was found in A1 *mutS* gene (reference locus tag NH44784_RS30630). No other mutations in DNA repair genes were found in any other genome. The mutation of *mutS* might explain the higher rate of SNPs found in A1, thus defining this isolate as a hypermutator.

2.3. Mobile Genetic Elements

The presence of mobile genetic elements such as phages, plasmids, integrative and conjugative elements (ICEs), integrative and mobilizable elements (IMEs), and *cis*-mobilizable elements (CIMEs), was investigated. For phages research, only complete regions were considered. As shown in Table 5, *Burkholderia sp.* phages (KS9, Bcep176, BcepMu, KS14) were identified in all genomes. In addition, A2 also carries phages 118970 and YMC11/02/R656 from *Salmonella sp*. and *Pseudomonas sp*. respectively.

ICEs were detected in isolates from patient A. In A1, we found one putative ICE containing genes related to Type 4 Secretion System (T4SS), bicyclomycin resistance and iron metabolism. A2 presented one putative ICE-containing gene related to T3SS, multidrug resistance, motility, virulence and metabolism. Both B1 and B2 presented the same putative IME-containing genes related to multidrug resistance.

Although plasmids were not found, the presence and variation of phages, ICEs and IMEs illustrates the potential ability of *A. xylosoxidans* to carry genetic and transferable elements that could contribute to the dissemination/acquisition of microbial functions.

Table 5. Intact prophage regions, ICEs and IMEs found in each genome. Length of the regions is indicated in Kbp.

Mobile Elements	A1	A2	B1	B2
PHAGE_Burkho_KS9_NC_013055	21	21	-	-
PHAGE_Burkho_Bcep176_NC_007497	46.7	39	18.6	24.6
PHAGE_Salmon_118970_sal3_NC_031940	-	31.6	-	-
PHAGE_Pseudo_YMC11/02/R656_NC_028657	-	29.3	-	-
PHAGE_Burkho_BcepMu_NC_005882	-	-	40.2	40.9
PHAGE_Burkho_KS14_NC_015273	-	-	31.7	-
PHAGE_Aeromo_vB_AsaM_56_NC_019527	-	-	-	-
PHAGE_Synech_S_CBS1_NC_016164	-	-	-	-
ICEs	93	227	-	-
IMEs	-	-	15.6	15.6

2.4. Phenotypic Features

To investigate possible phenotypic variations within the same host over time that might be related to hypermutation, we evaluated features such as growth, virulence and antibiotic resistance, which are known to undergo modifications during bacterial adaptation. As shown in Figure 1, no significant changes in terms of growth rate and adhesion ability occurred in the isolates from the two patients. However, we measured a strong protease activity in the culture supernatant of A1 isolate, that was significantly higher in comparison to strain A2. No such variation was observed between the isolates from patient B. As concerns antibiotic susceptibility, only A1 strain showed increased resistance to meropenem (MIC > 8 mg/L) [11].

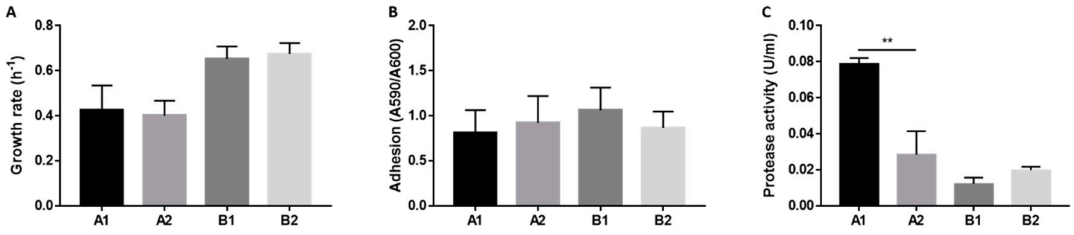

Figure 1. Growth rate (**A**), biofilm formation (**B**) and protease activity (**C**) of *A. xylosoxidans* isolates. Growth rate was calculated from 24 h growth curves in LB medium (**A**). Adhesion was measured by crystal violet staining of surface-attached bacteria divided by A_{600} of non-attached bacteria (B). Protease activity was measured in culture supernatant by azocasein assay. Protease activity is expressed as enzymatic units per ml (C). Each value represents the mean ± SEM of three experiments. Statistical analysis was performed by t test, ** $p < 0.01$.

3. Discussion

By sequencing and analysing the genomes of longitudinal isolates of *A. xylosoxidans* collected from two infected CF patients, we have identified different adaptive mechanisms of *A. xylosoxidans* to survive in a hostile environment like the CF lungs.

Two isolates were longitudinally collected from each patient with an interval of 3 years in patient A and 6 years in patient B. During this time, both patients were chronically infected. In addition to the standard comparison with a reference strain, we compared each *A. xylosoxidans* genome with its longitudinal isolate in order to study genomic variation avoiding biases linked to the choice of the reference genome. In all genomes, the majority of genes presenting variants are involved in metabolism, as previously shown in other studies [7], followed by genes involved in transcription and translation and transporter proteins encoding genes. Metabolic regulation, gene expression and

trade-off are evidently major targets of adaptation, probably enabling a physiological adaptive response through changes in regulatory genes [12,13].

When mutations accumulate at a high rate throughout the genome, we are usually in the presence of so-called hypermutators. Hypermutation arises through mutations that disrupt the methyl-directed mismatch repair (MMR) system, and *mutS* inactivation is the most widespread defect [14]. Although a previous study reported the finding of possible *A. xylosoxidans* hypermutable isolates in a CF patient [7], this conclusion was weakened by the absence of mutations in the MMR genes. On the contrary, in this study we identified an isolate (namely A1) that not only presents a high number of SNPs mainly due to transitions but also carries a large deletion in *mutS* gene, thus clearly defining this isolate as a hypermutator.

While this suggests the co-evolution of sub-populations from an original infecting strain in patient A, isolates of patient B seem to represent a situation of longitudinal microevolution. Both evolutionary mechanisms—hypermutation and microevolution—enabled long-term adaption in CF lungs, although it has been previously suggested that high mutation rates offer bacterial advantages in pathogenesis. In fact, hypermutation has been credited with facilitating the phenotypic changes and clonal diversification characteristic of *P. aeruginosa* adaptation to the CF lung environment [15,16]. Furthermore, MMR-deficient hypermutators are overrepresented in populations of various other pathogenic bacteria such as *Escherichia coli*, *Salmonella* spp., *P. aeruginosa* and *Staphylococcus aureus* [17]. Populations that are rapidly adapting to new or changing environments usually provide opportunities for hypermutable genotypes to rapidly spread beneficial mutations. However, the emergence of hypermutators does not always accelerate adaptive evolution. It has also been shown that hypermutator populations do not always produce greater fitness gains than DNA-repair proficient populations [18]. A mutator might rise transiently to high frequency and then be eliminated if the non-mutator type produces an even more beneficial mutation than that produced by the mutator [19]. For instance, the high protease secretion as well as meropenem resistance observed in the hypermutator isolate A1 could have been a result of adaptation to a stressful condition, but probably did not produce a greater fitness. Indeed, non-mutator strain A2 was isolated 3 years later, suggesting that even a low mutation rate was sufficient to generate beneficial mutations, similarly to the microevolution observed in patient B.

In conclusion, we report the occurrence of hypermutation as a mechanism that could be involved in *A. xylosoxidans* long-term infection of CF lungs. Further studies, possibly on a larger scale, will be needed to understand the frequency and benefits of *A. xylosoxidans* hypermutability.

4. Materials and Methods

4.1. Bacterial Isolates

Four clinical isolates of *A. xylosoxidans* were collected from two patients followed at Rigshospitalet in Copenhagen, Denmark. The use of the stored bacterial isolates was approved by the local ethics committee at the Capital Region of Denmark (Region Hovedstaden) with registration number H-4-2015-FSP. Two isolates were longitudinally collected from each patient: in 2005 and 2008 from patient A, and in 2008 and 2014 from patient B. Susceptibility to the following antibiotics was tested: amoxicillin + clavulanic acid, ampicillin, aztreonam, ceftazidime, ceftriaxone, cefuroxime, chloramphenicol, ciprofloxacin, colistin, imipenem, meropenem, moxifloxacin, penicillin, piperacillin + tazobactam, sulfamethoxazole, sulfamethoxazole + trimethoprim, tetracycline, tobramycin, trimethroprim.

4.2. Library Preparation and Whole-Genome Sequencing

DNA was purified from over-night liquid cultures of single colonies using the DNEasy Blood and Tissue Kit (Qiagen). Libraries were made with Nextera XT and sequenced on an Illumina MiSeq using the v2 250 × 2 kit [20]. This project has been deposited at EMBL under the accession PRJEB35058. Sequence data can be found with the experiment accession numbers ERX3614542 (strain A1), ERX3614543 (strain A2), ERX3614548 (strain B1), and ERX3614549 (strain B2). The pair end

sequencing yielded 3,842,110 reads for isolate A1; 2,947,162 reads for isolate A2; 2,806,776 reads for isolate B1; and 2,847,706 reads for isolate B2. As a reference genome sequence, the annotated genome *A. xylosoxidans* NH44784-1996 was used, which belongs to a strain isolated from sputum of a CF patient followed at Copenhagen CF Center in 1996 [10].

4.3. De Novo Assembly

The quality of the raw reads was assessed using FastQC v0.11.7, and adapter and quality trimming was performed accordingly following the illuminaclip, leading, trailing, slidingwindow and milen steps of Trimmomatic v0.36 [21] (Illuminaclip:adapter_file.fa:2:30:20 leading:3 trailing:3 slidingwindow:4:20 minlen:50). Sequence reads from each isolate were de novo assembled using the SPAdes v3.11.1 [22] assembly toolkit using the careful option. The quality of the de novo assemblies was evaluated using QUAST QC v5.0.0 [23] and by mapping the reads on the corresponding assembly using Bowtie 2 v2.3.4.1 [24] (coverage range: 97.87–98.35%); Samtools v1.9 [25] was used to obtain sorted bam files; Bedtools v2.27.1 [26] was used to obtain the per base sequence coverage; and Qualimap v2.2.1 [27] was used to obtain mapping and coverage statistics. All the de novo assemblies were annotated using Prokka v1.13 [28]. Genotypic relatedness was verified by checking core genome similarities obtained using the Harvest-OSX64-v1.1.2 suite [29].

4.4. Variant Analysis

Two types of variant analysis were carried out by aligning sequence reads to the *A. xylosoxidans* NH44784-1996 reference genome and to the de novo assembly of the longitudinal isolate from the same patient (reads from isolate 1 were aligned to the de novo assembly of isolate 2 from the same patient and vice versa) using Bowtie 2 v2.3.4.1. Sorted bam files were obtained using Samtools v1.9 and the MarkDuplicates tool from Picard v2.17.10 was used to mark duplicates. Finally, HaplotypeCaller of the Genome Analysis Toolkit (GATK) v4.0.6.0 [30] was used with the sample-ploidy option set to 1 to call SNPs and indels. In order to annotate the variants and predict their functional effects, SnpEff v4.3t [31] toolbox was used employing custom-built SnpEff databases obtained starting from fasta sequences and gff annotations of the genomes. Only variants supported by a minimum of 20 reads were retained.

For the analysis performed between the isolates, an ulterior step was performed in order to consider only true variants and discard false positives. Starting from the vcf files, the variants' positions were extracted, and a bed file was created in order to extract from the de novo assembly the sequences containing the variant and a flanking region of 75 bp in each direction using Bedtools v2.27.1, thus obtaining 151 bp sequences. These were then mapped on the de novo assembly of the corresponding longitudinal isolate using Bowtie 2 v2.3.4.1 with the option end-to-end and setting the mismatch penalty to 0. Moreover, only the reads that mapped where a variant was called in the longitudinal isolate were kept. Finally, the occurrences of transitions and transversions were counted and the transitions/transversions ratio was calculated.

4.5. Mutator Genes Analysis

The genetic basis of hypermutation was investigated from whole genome sequencing data by analysing genes involved in this phenomenon [32]. The following genes present in the reference NH44784-1996 were considered: RS17415/*PfpI*, RS20390/*PfpI*, RS30630/*MutS*, RS06175/*MutL*, RS27975/*RadA*, RS21930/*Rad50*, RS11590/*UvrA*, RS27010/*UvrB*, RS31300/*UvrC*, RS27580/*UvrD*, and RS09700/*UvrD*.

4.6. Mobilome Analysis

The Phage Search Tool Enhanced Release (PHASTER) [33] was used in order to identify and annotate prophage sequences. The presence of plasmids was investigated in two ways. First, PlasmidFinder v2.0 [34] was used on the de novo assembled genomes. Moreover, the plasmidSPAdes pipeline was used on the whole genome sequencing dataset, and the DNA sequences of the putative

plasmids were then blasted against the non-redundant (nr) BLAST database [35]. The presence of integrative and conjugative elements (ICEs), integrative and mobilizable elements (IMEs), and *cis*-mobilizable elements (CIMEs) was studied using the ICEfinder tool based on the ICEberg 2.0 database of bacterial integrative and conjugative elements [36].

4.7. Growth Curves

Bacterial strains were plated on LB agar and incubated at 37 °C for 24–48 h. One to two colonies were inoculated in 10 mL LB medium shaking at 37 °C overnight. OD_{600} was measured using a spectrophotometer, cultures were diluted to 0.05 OD/mL in LB medium and 150 µL/well were incubated in a 96-well plate for 20–24 h shaking at 37 °C. Using an automated plate reader, A_{600} was measured every 20 min. Growth rate was calculated using GraphPad Prism software.

4.8. Adhesion Assay

Bacterial strains were plated on LB agar and incubated at 37 °C for 24–48 h. One to two colonies were inoculated in 10 mL LB medium shaking at 37 °C overnight. OD_{600} was measured using a spectrophotometer, cultures were diluted to 0.05 OD/mL in LB medium and 150 µL/well were incubated in a 96-well plate for 20–24 h at 37 °C. After measuring A_{600}, wells were washed twice with water to remove unattached cells, and surface-attached cells were stained with 0.1% crystal violet solution for 15 min. Wells were rinsed and washed three times with water, then dried for 1–2 h. Thirty percent acetic acid was added, incubated at room temperature for 15 min, and A_{590} was measured. Thirty percent acetic acid was used as blank.

4.9. Protease Activity Measurement

A. xylosoxidans strains were plated on LB agar and incubated at 37 °C for 24–48 h. One to two colonies were inoculated in 10 mL LB medium shaking at 37 °C for 16 h. OD_{600} was measured and cultures were diluted to 0.1 OD/mL in 10 mL of LB medium. After shaking at 37 °C for 16 h, cultures were diluted to 1 OD/mL and centrifuged at 7000× *g* for 30 min at 4 °C. Supernatants were collected and sterile-filtered. Protease activity in culture supernatants was determined by azocasein assay as previously described [37]. Briefly, 350 µL reaction mixture containing 0.1 M Tris-HCl, pH 8.0, and 1% azocasein (Sigma-Aldrich, previously resuspended in 0.5% $NaHCO_3$) was added to 150 µL supernatant and incubated at 37 °C for 20 min shaking. After the addition of 1 mL 7% ice-cold perchloric acid, the solution was centrifuged. One-hundred-and-fifty microlitres of 10 N sodium hydroxide were added to the clear supernatant and OD_{430} was measured. One protease unit was calculated as the amount of enzyme producing an increase of 0.1 OD per hour.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: List of gene products affected by loss of function mutations in each genome versus the genome of its longitudinal isolate; Table S2: Presence of genes involved in DNA repair in each clinical isolate and in the reference strain.

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II

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Genomic characterization of *Achromobacter* species isolates from chronic and occasional lung infection in cystic fibrosis patients

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Abstract

Achromobacter species are increasingly being detected in cystic fibrosis (CF) patients, where they can establish chronic infections by adapting to the lower airway environment. To better understand the mechanisms contributing to a successful colonization by *Achromobacter* species, we sequenced the whole genome of 54 isolates from 26 patients with occasional and early/late chronic lung infection. We performed a phylogenetic analysis and compared virulence and resistance genes, genetic variants and mutations, and hypermutability mechanisms between chronic and occasional isolates. We identified five *Achromobacter* species as well as two non-affiliated genogroups (NGs). Among them were the frequently isolated *Achromobacter xylosoxidans* and four other species whose clinical importance is not yet clear: *Achromobacter insuavis, Achromobacter dolens, Achromobacter insolitus* and *Achromobacter aegrifaciens*. While *A. insuavis* and *A. dolens* were isolated only from chronically infected patients and *A. aegrifaciens* only from occasionally infected patients, the other species were found in both groups. Most of the occasional isolates lacked functional genes involved in invasiveness, chemotaxis, type 3 secretion system and anaerobic growth, whereas the great majority (>60%) of chronic isolates had these genomic features. Interestingly, almost all (*n*=22/23) late chronic isolates lacked functional genes involved in lipopolysaccharide production. Regarding antibiotic resistance, we observed a species-specific distribution of bla_{OXA} genes, confirming what has been reported in the literature and additionally identifying bla_{OXA-2} in some *A. insolitus* isolates and observing no bla_{OXA} genes in *A. aegrifaciens* or NGs. No significant difference in resistance genes was found between chronic and occasional isolates. The results of the mutator genes analysis showed that no occasional isolate had hypermutator characteristics, while 60% of early chronic (<1 year from first colonization) and 78% of late chronic (>1 year from first colonization) isolates were classified as hypermutators. Although all *A. dolens, A. insuavis* and NG isolates presented two different *mutS* genes, these seem to have a complementary rather than compensatory function. In conclusion, our results show that *Achromobacter* species can exhibit different adaptive mechanisms and some of these mechanisms might be more useful than others in establishing a chronic infection in CF patients, highlighting their importance for the clinical setting and the need for further studies on the less clinically characterized *Achromobacter* species.

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Keywords: *Achromobacter*; cystic fibrosis; lung infection; bacterial evolution; comparative genomics; virulence factors; antibiotic resistance; hypermutation.

Abbreviations: ANI, average nucleotide identity; CF, cystic fibrosis; LPS, lipopolysaccharide; NG, non-affiliated genogroup; T3SS, type 3 secretion system.

Sequences have been deposited at the NCBI SRA database under project no. PRJEB40979, secondary accession: ERP124698.

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Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files. Seven supplementary tables are available with the online version of this article.

DATA SUMMARY

The authors confirm all supporting data, code and protocols have been provided within the article or through supplementary data files.

Impact Statement

Achromobacter species can develop chronic lung infections in cystic fibrosis (CF) patients, which have been associated with worsening of the clinical condition. Although the frequency of infection by these opportunistic pathogens is increasing, little is still known about the mechanisms that are involved in their pathogenicity and persistence. To better elucidate these features, we analysed the genome of 54 isolates collected from both occasionally and chronically infected patients. This approach represents a novelty in the available literature, as the majority of studies have focused on chronic infection only, while the comparison with occasional isolates may allow us to identify genetic markers of persistence. Moreover, most of the available information regards the type species *Achromobacter xylosoxidans*, while data are lacking about other clinically relevant species. We identified four of them in our collection and additionally performed species-specific analyses. Our results show the occurrence of different adaptive mechanisms in terms of virulence, antibiotic resistance, within-host genomic evolution and hypermutation, suggesting that some features might be more useful than others to establish chronic infections. These findings could support the creation of diversified treatment regimens and improve the management and clinical outcome of *Achromobacter* species lung infections in CF patients.

INTRODUCTION

Achromobacter species are ubiquitous Gram-negative bacilli, widely distributed in aquatic environments and soil, and increasingly found in nosocomial settings. They act as opportunistic pathogens in certain populations, such as subjects with cystic fibrosis (CF), where these microorganisms can survive for a long time in both lower and upper airways [1]. The genus *Achromobacter* comprises 22 named species and multiple genogroups [2]; whole genome sequencing data are available for all 22 species but complete reference genomes are available only for seven of them: *Achromobacter deleyi, Achromobacter denitrificans, Achromobacter insolitus, Achromobacter pestifer, Achromobacter ruhlandii, Achromobacter spanius* and *Achromobacter xylosoxidans*. The type species *A. xylosoxidans* is the most often isolated *Achromobacter* species among CF patients and more than half of CF patients with airway colonization by *A. xylosoxidans* develop chronic infection, which has been associated with a decline in respiratory function and lung inflammation [1, 3–5]. Of note, an outbreak caused by another species, *A. ruhlandii,* was reported at two Danish CF centres [6, 7].

Lung infection caused by *Achromobacter* results either from acquisition from the environment or from direct/indirect transmission [7, 8] and is usually complicated by the innate and acquired multidrug resistance carried by these microorganisms. Approximately 50 drug-resistance-associated genes have been predicted in the *A. xylosoxidans* type strain [9, 10]. *A. xylosoxidans* is intrinsically resistant to aminoglycosides, many cephalosporins and aztreonam [9, 11] but only a few intrinsic resistance mechanisms have been identified to date. The resistance-nodulation-cell division (RND)-type multidrug efflux pump AxyAB-OprM is involved in resistance to cephalosporins (except cefepime), aztreonam, nalidixic acid, fluoroquinolones and chloramphenicol [12], while AxyXY-OprZ and AxyEF-OprN are responsible for resistance to aminoglycosides and levofloxacin, respectively [13, 14]. Moreover, the chromosomally encoded narrow-spectrum class D lactamase $bla_{\text{OXA-114}}$ showed hydrolysis of piperacillin and ticarcillin [15]. In addition, clinical isolates of *A. xylosoxidans* may exhibit acquired resistance, especially for beta-lactams. To date, genes identified include IMP-type carbapenemase genes $bla_{IMP\text{-}1}$, $bla_{IMP\text{-}10}$ and $bla_{IMP\text{-}19}$ [16, 17], VIM-type carbapenemase-encoding genes $bla_{VIM\text{-}1}$ [18] and $bla_{VIM\text{-}2}$ [19], the extended-spectrum beta-lactamase-encoding gene $bla_{VEB\text{-}1}$ [20], and the metallo-lactamase-encoding gene $bla_{TMB\text{-}1}$ [21].

With regard to pathogenic mechanisms, similar to other gram-negative pathogens, *A. xylosoxidans* expresses cell membrane-bound virulence factors such as the Vi capsular polysaccharide, involved in surface adhesion and protection from phagocytosis and toxins, and the O-antigen, which elicits a host immune response [22]. Lipopolysaccharide (LPS) also induces key inflammatory cytokines [23]. *A. xylosoxidans* is also equipped with various secretion systems that mediate the release of molecules to provide capability for invasion of the host cells, but little is known about its exoproducts [24, 25]. Previous *Achromobacter* comparative genomics analyses showed that virulence genes related to the type 3 secretion system (T3SS) are more common in CF isolates than in environmental strains [22, 26]. Genome analysis also showed the presence of genes encoding colicin V, a protein cytotoxic to similar bacteria, and *aepA*, involved in cellulase and protease regulation [24]. In addition, production of phospholipase C and ability to inactivate the *Pseudomonas aeruginosa* quinolone signal molecule were observed [27, 28]. Moreover, a heat-stable cytotoxic factor, associated with an increase of pro-inflammatory cytokines *in vitro*, was identified [29]. Jakobsen and colleagues also investigated the presence of secreted virulence factors known to be important for other CF pathogens such as *P. aeruginosa*, but reported the absence of extracellular proteases, chitinase and rhamnolipids in clinical strains. More recently, we detected protease secretion from *A. xylosoxidans* CF clinical isolates [30]. Regarding the biofilm mode-of-growth, *A. xylosoxidans* showed poor surface adhesion [24] but can form unattached or loosely attached aggregates, held together by polysaccharides forming

a peripheral shell around the bacterial cells [31]. This ability might help bacteria to form complex communities and survive in a hostile environment such as CF lungs.

Another mechanism contributing to the persistence of pathogens in the airways of CF patients is the ability to genetically adapt during chronic infection through accumulation of pathoadaptive mutations [25]. This phenomenon can be accelerated due to mutations in the DNA mismatch repair system, giving rise to hypermutation events and to clonal diversification within the host [32]. The mutator genes whose mutations have been associated with development of hypermutation are *mutL*, *mutS*, *pfpI*, *superoxide dismutase*, *radA*, *radC*, *rad50*, *uvrA*, *uvrB*, *uvrC* and *uvrD* [33].

To better understand the mechanisms underlying CF lung colonization by *Achromobacter* species, we sequenced the whole genome of 54 isolates from patients with both occasional and chronic infection. By using different bioinformatics tools, we performed a phylogenetic analysis and compared virulence and resistance genes, genetic variants and mutations, as well as hypermutability mechanisms between chronic and occasional *Achromobacter* isolates.

METHODS

Sample collection and identification

The samples analysed in this study have already been described in our previous work [34]. Briefly, 54 *Achromobacter* clinical isolates were collected from 53 sputum samples of 26 patients followed at the CF Centre of Verona (Italy) and were identified as *Achromobacter* species by MALDI-TOF-MS (bioMérieux. Informed consent was obtained according to projects CRCFC-CEPPO026 and CRCFC-CEPPO031 approved by the local Ethical Committee. Collection was mainly performed in two time periods: 2014–15 and 2017–18. Sputum samples were collected approximately every 3 months for microbiological analysis. Only one isolate from each sample was included in the study, except for one P09 sample from which two morphologically different isolates were identified and included (9–4 and 9–5).

The classification of occasional and chronic infection was assessed using information regarding all *Achromobacter* isolates identified between 2013 and 2018. According to the European Consensus criteria (ECC), infection was defined as chronic when at least three positive cultures with at least a 1 month interval between the samples were obtained within ≥6 months from the first colonization event [35]. Isolates from chronically infected patients were further classified as early (<1 year from first colonization event) and late isolates (>1 year from first colonization). A minimum of one and a maximum of six successively collected isolates (mean=2.5 isolates per patient) were recovered from chronically infected patients with a mean time delay of 197 days (range=21–1182 days). Only one isolate was recovered from each occasionally infected patient, except for P06 and P12 from which we recovered two isolates with a time delay of 112 and 155 days, respectively.

Genome sequencing

Collected isolates underwent whole genome sequencing at the Technological Platform Centre of the University of Verona as previously described [34]. Sequencing data were submitted to the NCBI SRA database with project number PRJEB40979. Genomic DNA was extracted using a QIAamp DNA Blood Mini Kit (Qiagen) and its quality was assessed using Nanodrop (Thermo Fisher Scientific) and Fragment Analyzer (Agilent Technologies). Libraries were prepared using the KAPA PCR-free kit and sequenced on a NextSeq500 Illumina (Illumina) platform generating 150 bp paired-end reads with mean reads yield of 10978104 and mean coverage of 190×. Read quality controls, *de novo* assembly and genome annotation were performed similarly to our previous work [30]. Details on the sequencing and *de novo* assembly are available in Table S1 (available in the online version of this article).

Phylogenetic analysis

Based on MALDI-TOF identification results, all complete *A. xylosoxidans* genomes stored in the NCBI RefSeq database [36] (*n*=7 – January 2020, accessions: GCF_900475575.1, GCF_001051055.1, GCF_001558755.2, GCF_001558915.1, GCF_001559195.1, GCF_000165835.1 and GCF_001457475.1) were considered as reference genomes for this study.

The average nucleotide identity (ANI) among all available *Achromobacter* species genomes, regardless of their assembly status (*n*=142 – NCBI RefSeq database, January 2020), and all sequenced isolates was calculated using FastANI [37] in order to ensure the correct species classification of the reference and isolate genomes. Isolates were considered to belong to the same species when ANI≥95 %, whereas isolates having ANI<95% with all available *Achromobacter* species genomes were considered as unaffiliated to any species sequenced up to now and referred to as non-affiliated genogroups (NGs) in this work [38]. This was further supported by *in silico* multilocus sequence typing (MLST) scheme analysis (Table S2). After ANI calculation, some reference genomes deposited as *A. xylosoxidans* showed ANI>95% with genomes of other *Achromobacter* species (*A. insuavis, A. aegrifaciens, A. dolens* incomplete genome) suggesting their misclassification. Thus, in this study they were used as reference genomes for isolates having ANI>95%, with the exception of *A. dolens*, whose complete reference genome was not available yet. In order to avoid using incomplete genomes as reference, the *A. ruhlandii* complete reference genome was used because it had the closest phylogenetic relationship with *A. dolens*. In addition, the *A. insolitus* reference genome (accession: GCF_008245125.1) was considered for the analysis, as it showed ANI>95% with some genomes.

The phylogenetic tree was generated performing a core genome alignment using the parsnp tool of the Harvest-OSX64-v1.1.2 suite [39]. The tree file in newick format was used as input in Microreact [40] together with a metadata file for visualization.

Functional annotation of relevant genomic variants

Analysis of variants was performed according to the Bacdist pipeline [41] considering all the genome sequences coming from the same patient and belonging to the same clone type (maximum SNP distance between clonally related isolates was 217) against the closest reference genome. Details on variant analysis results are available in Table S3. The amino acid sequences of genes presenting variants or mutations (here defined as variants that cause gene loss of function) were extracted and, using eggnog-mapper v1 [42], each gene product was classified in one of 25 eggnog functional categories, further grouped into four eggnog main categories: information storage and processing, cellular processes and signalling, metabolism, and poorly characterized.

Virulence factors and antibiotic resistance genes

In order to determine the presence of virulence factor and antibiotic resistance genes, the *de novo* assemblies were analysed using BLAST [43] against the PATRIC database (accessed January 2020) [44] and using Abricate [45] to search against the Resfinder database (accessed January 2020) [46]. Matches were filtered, thus considering matches with at least 50% gene coverage and 95% gene identity.

The presence of mutations was evaluated in two ways. To analyse genes that are present in the reference genomes, the defence mechanism category of the eggnog-mapper output was taken into consideration: the presence of mutations was evaluated, and the functional impact of missense variants was predicted using the Provean protein tool. In order to analyse genes that are present in the isolates genomes but not in the reference, BLAST and Abricate outputs were considered: genes having an identity <100% were analysed by extracting the corresponding amino acid sequence and using BLASTP against the non-redundant (nr) database. The functional effect of each mutation was predicted using the Provean protein tool [47]. Heatmaps were generated for visualization purposes using the pheatmap R package v1.0.8 [48]. Fisher's exact test was performed per clone type to ascertain the significance of results of statistical testing using R v3.5.

Mutator genes

The genetic basis of hypermutation was investigated from whole genome sequencing data by analysing genes involved in this phenomenon. The following list of mutator genes taken from the literature was considered: *mutL*, *mutS*, *pfpI*, *superoxide dismutase*, *radA*, *radC*, *rad50*, *uvrA*, *uvrB*, *uvrC* and *uvrD* [33]. In order to verify the presence of hypermutator strains, a combination of factors such as the occurrence of high-impact mutations and deleterious missense in mutator genes (Table S4), the transition/transversion rate and overall and per-site mean yearly variant rate (Table S3) were taken into consideration. Additional information on the classification of the isolate is reported in Table S5.

RESULTS

Fifty-four *Achromobacter* clinical isolates were longitudinally collected over 5 years from the sputum samples of 26 patients followed at the CF Centre of Verona (Italy). Among them, 17 presented chronic lung infection while nine were occasionally colonized. Fig. 1 shows the sample collection timeline and the first colonization event for each patient (data available since 2013). In 10 patients with chronic infection (P01, P02, P05, P08, P09, P10, P13, P18, P19, P21) we collected isolates identified as early colonisers (<1 year from first colonization); the first isolate collected from four of them (P02, P09, P13, P21) coincides with the first colonization event. Moreover, we collected both early and late (>1 year from first colonization) colonizing strains from four chronically infected patients (P01, P05, P08, P10). In two of these patients (P01, P05) early and late isolates were isolated a few months apart (5 months between isolates 1–1 and 1–2; 2 months between isolates 5–3 and 5–4). From two patients with occasional infection we collected *Achromobacter* species twice during the sampling periods (~3 months apart in P06, ~5 months apart in P12).

Phylogenetic analysis

Fig. 2 shows the phylogenetic tree based on core genome alignment of 54 collected *Achromobacter* species isolates and seven reference genomes. In order to accurately classify the isolates in the genus *Achromobacter*, we calculated the ANI among all available *Achromobacter* species genomes and the genomes of our isolates. As a result of the phylogenetic analysis, five *Achromobacter* species were identified: *A. aegrifaciens, A. dolens, A. insolitus, A. insuavis* and *A. xylosoxidans*. The majority of isolates was identified as *A. xylosoxidans* (67%, *n*=36), followed by *A. insuavis* (13%, *n*=7), *A. dolens* (7%, *n*=4), *A. aegrifaciens* (7%, *n*=4) and *A. insolitus* (6%, *n*=3). Among a total of 26 patients, 17 (65%) were infected by *A. xylosoxidans*, three (12%) by *A. aegrifaciens*, three (12%) by *A. insolitus*, two (8%) by *A. insuavis* and one (3%) by *A. dolens*. Interestingly, four strains [3] isolated from two patients (P03, P16) showed an ANI <95% against all the analysed genomes suggesting that they likely belong to *Achromobacter* species with no genomic assembly available yet. Because solely based on phylogenetic information, in this study we refer to them as non-affiliated genogroups (NGs).

A. xylosoxidans and *A. insolitus* were collected from both patients with chronic and occasional infections. By contrast, *A. aegrifaciens* was only observed in occasionally infected patients while *A. insuavis, A. dolens* and NG isolates established chronic infections. For each of the patients with more than one isolate (10 with chronic and two with occasional infection) the genetic relatedness between genomes was also evaluated. As expected, all strains isolated from each chronic patient were clonally related (maximum SNP distance between clonally related isolates was 217). For the occasionally infected patients with more than one isolate, one of them (P06) harboured clonal isolates while different clones were identified in the other one (P12).

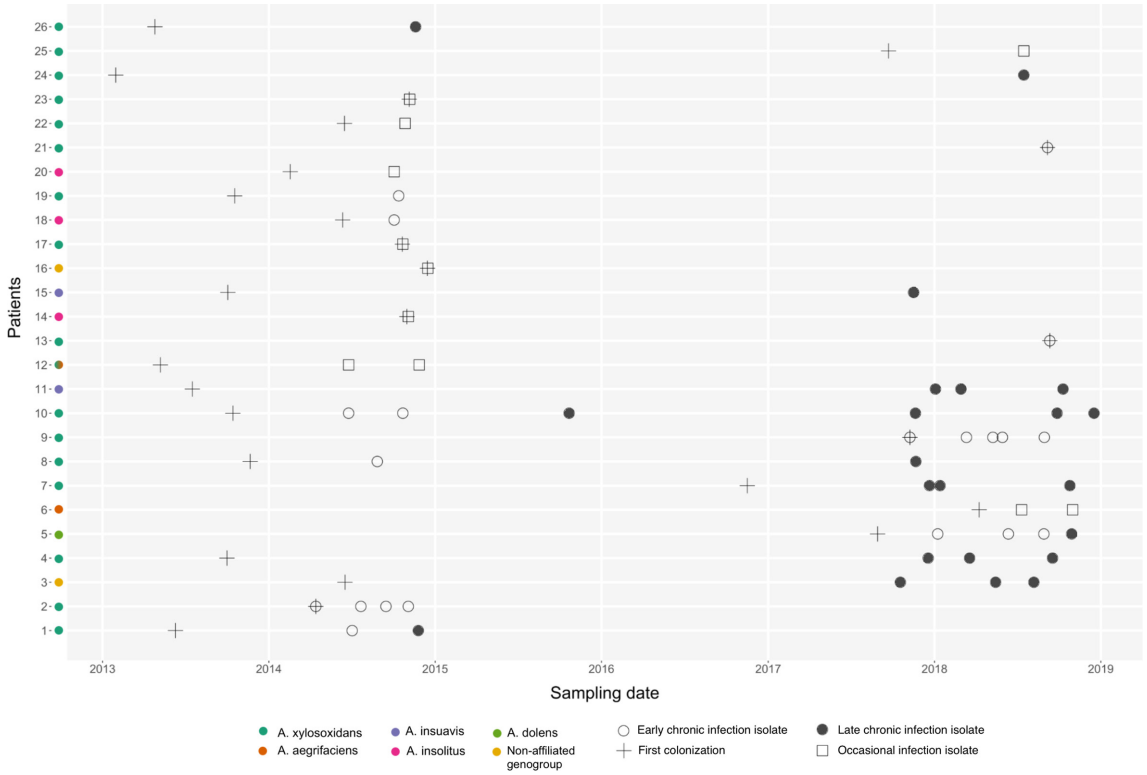

Fig. 1. Isolate collection timeline. Dates of collection of *Achromobacter* species isolates and first colonization event are shown for each patient enrolled in the study. A minimum of one isolate and a maximum of six isolates were collected from 26 patients between 2014 and 2018. +=first colonization event; □=occasional isolates; ○=early chronic isolates; ●=late chronic isolates; in some cases, isolates correspond with the first colonization event (superimposed symbols). In the left column, patient ID numbers are reported; coloured dots indicate the *Achromobacter* species of the isolates collected from each patient; a bicoloured dot indicates that isolates belonging to two species were recovered from the patient.

Virulence factors and antibiotic resistance

The results of the virulence factor analysis (Fig. 3) showed that argininosuccinate synthase, carbamoyl phosphate synthase large chain, *hfq* and large subunit ribosomal protein L36p are present in all collected isolates. These proteins are mainly involved in protein synthesis and stress response. The differences among early chronic infection isolates (n=20, <1 year from first colonization), late chronic infection isolates (n=23, >1 year from first colonization) and occasional infection isolates (n=11) were investigated. To evaluate differences in gene functionality, we took into account the presence of mutations in virulence factor genes (Table S6). All late chronic isolates (n=23/23) and 90% (n=18/20) of early chronic isolates carried functional genes associated with the ability to grow in an anaerobic environment (17 clonetypes, P=0.002), while only 45% (n=5/11, four clone types) of occasional isolates had this potential. Moreover, 96% (n=22/23) of late chronic isolates lacked genes involved in LPS production or carried mutations in these genes (10 clone types, P=0.036). Occasional isolates showed a significantly lower content of functional genes associated with the ability to infect cells (55%, n=6/11, five clone types, P=0.028) and, to a lesser extent, chemotactic movement (55%, n=6/11, five clone types, P=0.194) and T3SS (73%, n=8/11, seven clone types, P=0.11).

Focusing on differences among species, the results suggested that *A. aegrifaciens* (n=3), *A. insolitus* (n=3) and all the NG isolates (n=4) carried a restricted number of genes related to virulence. The analysis showed the absence of respiratory nitrate reductase (anaerobic growth), GDP mannose 4,6 dehydratase (pathogenicity) and *liN* (flagellar protein) genes in *A. aegrifaciens*, *A. insolitus* and one NG isolate. T3SS genes were also absent in these isolates and in one additional *A. xylosoxidans* occasional isolate. *fliP* and *fliC* genes (flagellar proteins) were not present in *A. aegrifaciens* or *A. insolitus* genomes together with the serum resistance-linked gene *yihY*, which was also absent in all NG isolates. Endotoxin-related genes were all found to be present in some *A. xylosoxidans* genomes while a putative oxidoreductase was also detected in all *A. insuavis* isolates (n=4). Moreover, UDP 2 acetamido 3 amino 2,3 dideoxy D-glucuronic acid acetyltransferase gene (O antigen biosynthesis) was also found in *A. dolens* genomes (n=4). Finally, the putative cysteine hydrolase *ycaC* gene was found in *A. aegrifaciens* and in the majority (94%, n=34/36) of

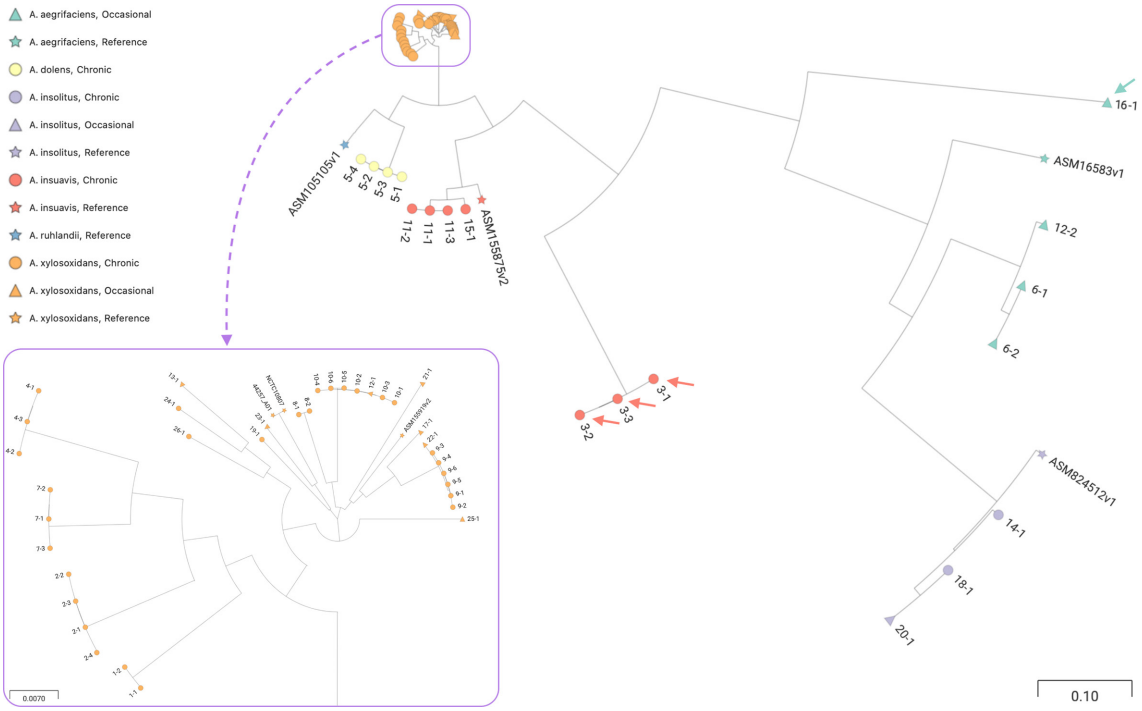

Fig. 2. Phylogenetic tree based on core genome SNPs of 54 collected *Achromobacter* species isolates and eight reference genomes. △ indicates occasional infection isolates, ○ indicates chronic infection isolates, while ☆ indicates a reference genome. The symbols are coloured according to the *Achromobacter* species identified with ANI analysis. Arrows indicate genomes with ANI<95 % against all the analysed reference genomes; in this case, symbols are coloured according to the closest *Achromobacter* species reference genome that was used for variant analysis. Genomic distance scales are reported at the bottom of the figure.

A. xylosoxidans isolates, while *motA* (flagellar motility) was only present in *A. aegrifaciens* genomes.

Differences among chronic and occasional isolates were also investigated by species. In contrast to the chronic isolates, *A. xylosoxidans* occasional isolates (*n*=5/36) all carried *fliP* and NADP-dependent malic enzyme (growth in gluconeogenic conditions) genes while occasional isolates belonging to the NGs (*n*=1/4) lacked *cheB*, *cheW*, *cheY* (chemotaxis), *fliN* (flagellar protein), *yscS* (T3SS) and respiratory nitrate reductase. Conversely, no differences were detected among *A. insolitus* chronic and occasional isolates (*n*=3).

From the results of the antibiotic resistance genes (Fig. 4), the most represented genes are the sulfonamide resistance gene *sul1* and the *aac(6′)* family aminoglycoside acetyltransferase, followed by class D β-lactamase bla_{OXA} genes. The analysis showed a species-specific distribution of bla_{OXA} genes: $bla_{OXA\text{-}114}$ in *A. xylosoxidans*, $bla_{OXA\text{-}243}$ in *A. insuavis*, $bla_{OXA\text{-}364}$ in *A. dolens*, $bla_{OXA\text{-}2}$ in *A. insolitus* (except for isolate 14–1 carrying no bla_{OXA} genes), and no bla_{OXA} genes in *A. aegrifacies* or NG isolates. Moreover, $bla_{CARB\text{-}2}$ was found in isolates from patient P01. In addition to bla_{OXA} genes, 31% of all isolates carried other antibiotic resistance genes. Among the isolates presenting deleterious variants – compromising gene function – in antibiotic resistance genes (Table S7), 54% (*n*=7/13) carried mutations in at least one *bla* gene.

Mutator genes

The genetic basis of hypermutation was investigated by verifying the occurrence of high-impact mutations and deleterious missense within genes involved in this phenomenon: *mutL*, *mutS*, *pfpI*, *superoxide dismutase*, *radA*, *radC*, *rad50*, *uvrA*, *uvrB*, *uvrC* and *uvrD* [33]. Moreover, a combination of factors such as the transition/transversion rate and overall and per-site mean yearly variant rate were taken into consideration. Similarly to other CF pathogens presenting hypermutation events during chronic infection, in this study no occasional isolates of *Achromobacter* species (*n*=11) showed hypermutator characteristics, while 60% of early chronic isolates (*n*=12, 4/10 patients) and 78% of late chronic isolates (*n*=18, 8/11 patients) were classified as hypermutators. In particular, patients from whom we collected both early and late isolates (*n*=4) showed different hypermutation onset patterns: P01 and P05 presented hypermutant strains in early isolates, in P10 hypermutation developed in late isolates and P08 isolates showed no hypermutation-linked phenomena.

The results of the mutator genes analysis, reported in Fig. 5, showed that all *A. dolens* (*n*=4), *A. insuavis* (*n*=4) and NG isolates (*n*=3) presented two different *mutS* genes. Moreover, *A. insolitus* (*n*=3), *A. dolens* and NG isolates lacked *radC* while *A. dolens* and NG isolates also lacked a functional *uvr* system.

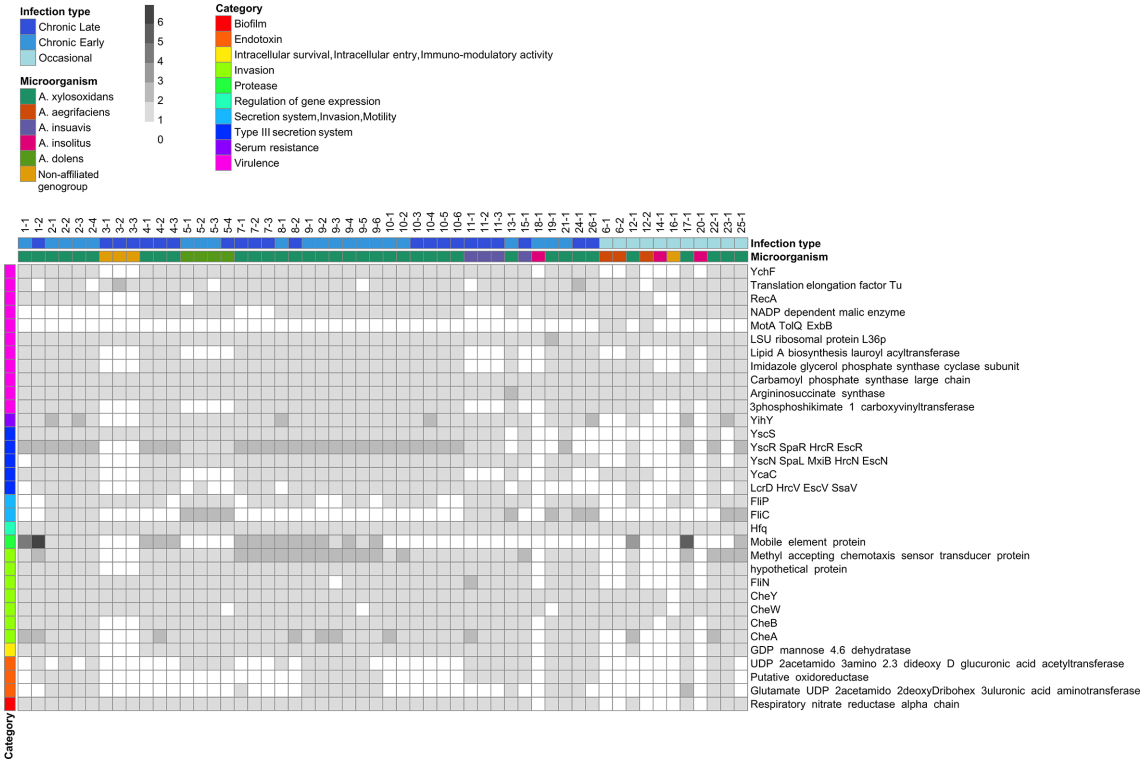

Fig. 3. Virulence factor genes identified in *Achromobacter* species isolates. The heatmap shows the presence and number of virulence factor genes on a grey scale. Additional information regarding the microorganism species, infection type and gene functional annotation (PATRIC categories) is presented in the annotation rows and column.

DISCUSSION

Achromobacter species are opportunistic pathogens that can cause lung infections in CF patients. Although chronic colonization has been associated with a decline in respiratory function, increased frequency of exacerbations and lung inflammation, little is still known about its pathogenic mechanisms. To provide new insights about *Achromobacter* virulence, antibiotic resistance and evolution, we performed bioinformatics analysis of 54 whole genome sequences and compared these features between chronic and occasional isolates, as well as among different *Achromobacter* species. Although patients carrying these isolates were defined as chronically or occasionally infected based on their clinical microbiological history since 2013, clinical isolates included in this study were mainly collected in 2014–15 and 2017–18, thus limiting the continuous observation of adaptation during these 5 years. Nonetheless, they still provide longitudinal data that can support the understanding of pathogenicity and persistence, particularly in poorly characterized microorganisms such as *Achromobacter* species. Moreover, we further compared isolates collected within the first year of chronic colonization (early chronic isolates) with those collected later (late chronic isolates) in order to observe early changes occurring during the very first phase of chronic colonization, when eradication is still likely to be feasible, and identify markers of persistence that might be associated with an enhanced risk of eradication therapy failure.

As expected, the majority of isolates belong to *A. xylosoxidans* (65%) while none was identified as *A. rhulandii*, the second most frequently recovered species in the USA and Argentina and previously reported to be responsible for outbreaks in Danish CF centres [49–51]. We also identified four other species whose clinical importance is less clear: *A. aegrifaciens, A. dolens, A. insolitus* and *A. insuavis*. The prevalence of *A. dolens* (3%) and *A. insolitus* (12%) in our patients was comparable with those reported in previous studies (2–17%) while dissimilarities were observed for *A. aegrifaciens* (12%) with respect to the literature (5%) [49, 50, 52–54]. Also, the prevalence of *A. insuavis* (8%) is similar to that described in Argentina (5%) but differs from Denmark (24%) [49, 50]. Interestingly, four isolates showed a low ANI (<95%) against all the available *Achromobacter* genomes, and thus we were unable to classify them within known species. This suggests that they might belong to *Achromobacter* species not yet sequenced or even to new species. In this study we considered them as non-affiliated genogroups. Predictably, all the longitudinal isolates from chronic patients belonged to the same *Achromobacter* species and were clonally related. Interestingly, the same situation also occurred in one of the occasionally infected patients (P06).

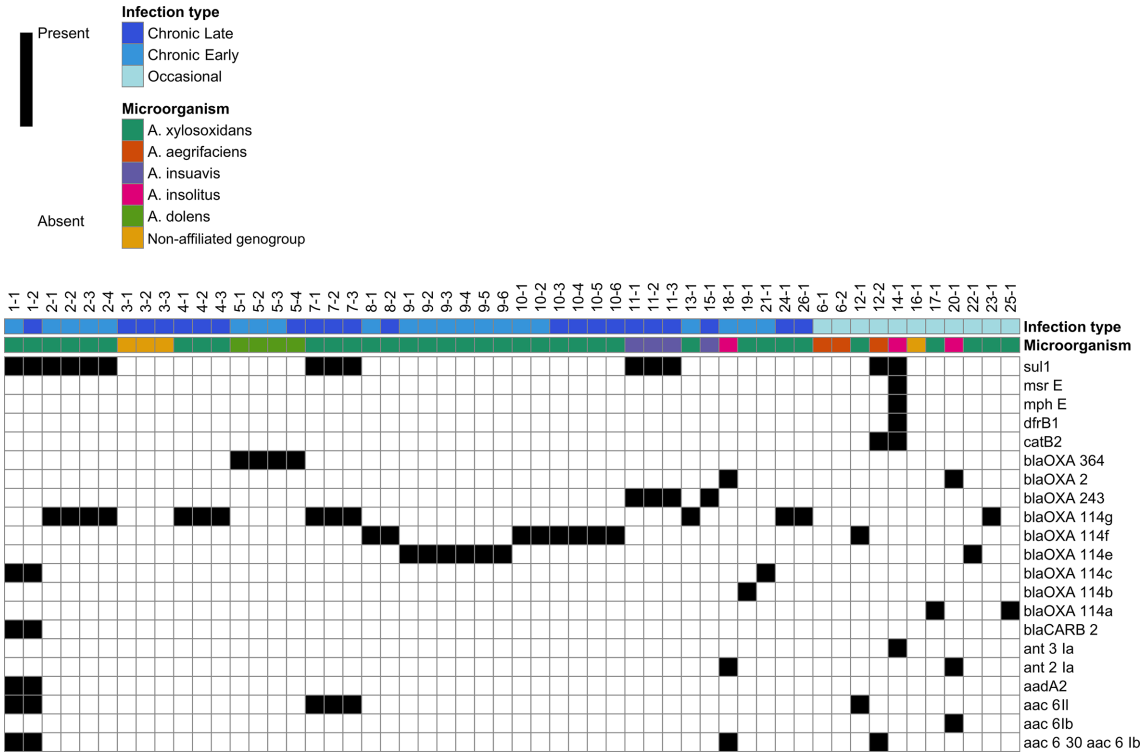

Fig. 4. Antibiotic resistance genes identified in *Achromobacter* species isolates. The heatmap shows the presence of antibiotic resistance genes on a black and white scale. Additional information regarding the microorganism species and the infection type is presented in the annotation rows.

Considering the species affecting chronic and occasional patients, *A. insuavis* and *A. dolens* were isolated only from chronically infected patients, *A. aegrifaciens* only from occasionally infected patients, while all the other species (*A. xylosoxidans*, *A. insolitus* and NGs) were found in both groups. For some species we only have a few isolates, and this may suggest that some *Achromobacter* species might be more likely to establish a chronic infection in CF patients. In particular, *A. aegrifacens* – found in two occasional patients – lacks various genes related to virulence: this could limit its ability to colonize CF lungs. Indeed, *A. agrifaciens* as well as the majority of occasional isolates showed a lack of functional genes related to the ability to infect cells, chemotactic movement, T3SS and anaerobic growth, whereas the great majority of chronic isolates have these potentials. In particular, anaerobic growth ability confers to the cells the ability to locate deeper within the mucous layer or within biofilm structures or in more hypoxic regions of the lung, where antibiotics can be dramatically less effective due to anaerobic conditions [55]. This can favour resistance of bacterial cells to antibiotic therapies and attack from neutrophils. Additionally, almost all (n=22/23) late chronic isolates lacked functional genes involved in LPS production, probably leading to a reduced recognition by the host defence system. For example, a reduction in the number of LPS lipid A acyl chains by other bacteria was shown to modulate the recognition of LPS by toll-like receptors [56]. These might represent evolutionary mechanisms that favour bacterial persistence.

Regarding antibiotic resistance, we observed a species-specific distribution of bla_{OXA} genes. While the specificity of $bla_{OXA\text{-}114}$, $bla_{OXA\text{-}243}$ and $bla_{OXA\text{-}364}$ respectively for *A. xylosoxidans*, *A. insuavis* and *A. dolens* has already been reported in the literature [57], we observed no bla_{OXA} genes in *A. aegrifaciens,* NGs or one *A. insolitus* isolate (out of three). Additionally, we identified $bla_{OXA\text{-}2}$ in two *A. insolitus* isolates. However, we found that $bla_{OXA\text{-}2}$ does not follow a species-specific distribution when analysing all the available genome assemblies belonging to this species (n=16). Among the isolates presenting deleterious variants in antibiotic resistance genes (n=13), 54% carried mutations in at least one *bla* gene. These mutations have been bioinformatically predicted to compromise protein function, probably reverting an antibiotic-resistant phenotype to a sensitive one, potentially influencing the results of antibiotic profiling with implications for therapies.

No significant difference in resistance genes was found between chronic and occasional isolates. Besides bla_{OXA}, the most well-represented antibiotic resistance genes among all isolates are the sulfonamide resistance gene *sul1* and the *aac(6')* family aminoglycoside acetyltransferase, both frequently found within mobile genetic elements such as integrons, plasmids

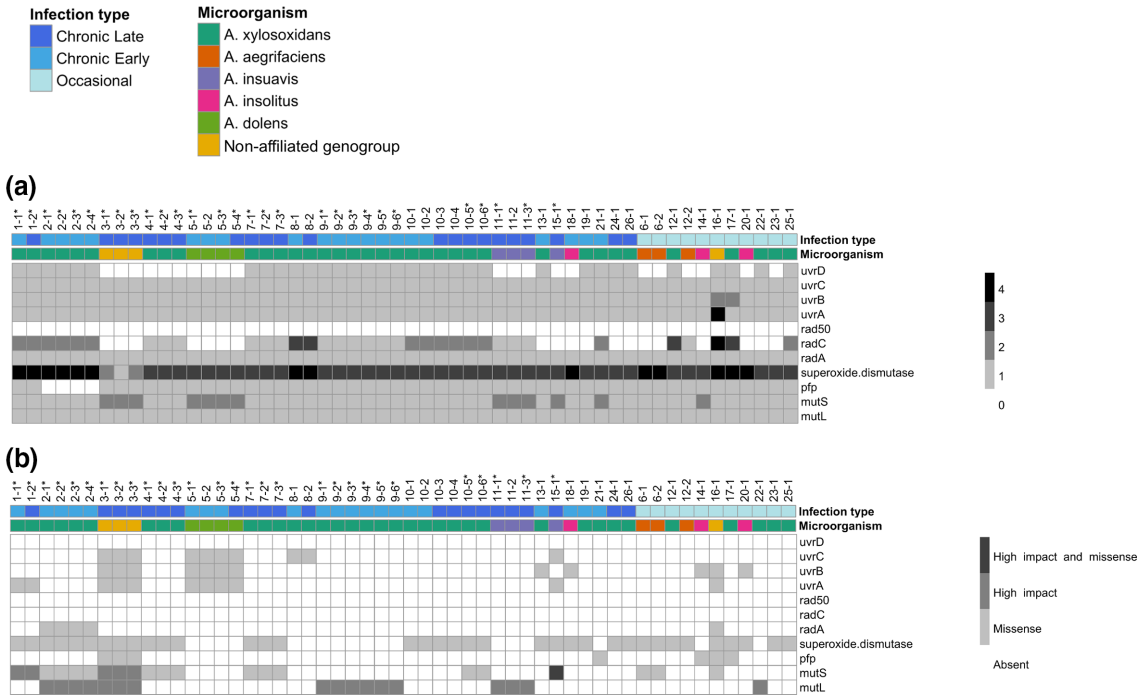

Fig. 5. Mutator genes identified in *Achromobacter* species isolates. Heatmap (a) shows the presence and number of mutator genes on a grey scale. Heatmap (b) shows the presence and type of mutations in these genes on a grey scale. Additional information regarding the microorganism species and the infection type is presented in the annotation rows. *Hypermutable isolates.

and transposons carried by other Gram-negative opportunistic pathogens [58]. Moreover, all the isolates belonging to one chronically infected patient (P01) carried the $bla_{CARB\text{-}2}$ gene, usually found in *P. aeruginosa*, another microorganism frequently involved in CF lung infections. Interestingly, in this patient both $bla_{CARB\text{-}2}$ and *sul1* were found to be carried by an integron. Moreover, we previously observed that these isolates carry various *P. aeruginosa* insertion sequences [34], supporting the possibility of inter-species genetic transfer. Further analysis of *Achromobacter* resistance-related mobile genetic elements could provide additional insights into antibiotic resistance acquired through its interaction with other microorganisms.

Overall, we detected a small number of genes linked to antibiotic resistance while a previous study using a multiple-database-based annotation approach predicted up to 50 drug-resistance associated genes in the *A. xylosoxidans* type strain [9]. However, that study analysed only one genome, using an approach that is hardly applicable to a large number of genome sequences due to the intensive manual effort required. To overcome this limitation, we first evaluated and compared the content of multiple databases to select the one able to ensure the most comprehensive outcome achievable with a more standard single-database-based approach. Nonetheless, the available databases report a restricted number of robustly annotated genes, not specific for *Achromobacter* species, probably leading to an underestimation of their presence. This lack currently limits the possibility of performing a more accurate analysis of the antibiotic resistance genes on a large number of strains belonging to this specific genus and highlights the need of a more specific database.

Regarding bacterial evolution, we focused on the study of hypermutation – in which defects in DNA repair processes promote an increased mutation rate, one of the main mechanisms favouring bacterial persistence in CF airways. The occurrence of hypermutation during chronic colonization of the CF lung has been demonstrated for various microorganisms such as *P. aeruginosa* [32, 59–61], *Burkholderia cepacia* complex [62] and *A. xylosoxidans* [30, 63]. Similarly, no occasional isolates showed hypermutator characteristics while 60% of early chronic and 78% of late chronic isolates were classified as hypermutators. Interestingly, the results of analysis of the mutator genes showed that all *A. dolens*, *A. insuavis* and NG isolates presented two different *mutS* genes (mismatch repair). To our knowledge, this peculiarity has not previously been reported. To validate this finding, we analysed *mutS* genes present in the available reference genomes of *A. dolens* (n=6) and *A. insuavis* (n=3): while the former presented two copies of the gene, in *A. insuavis* its copy number (one or two) was variable among the assemblies. One could hypothesize that the presence of an additional *mutS* could decrease the hypermutability potential. However, we identified hypermutator isolates carrying mutations in a single *mutS* gene, thus suggesting that both genes are needed for effective mismatch repair and indicating that they have a complementary rather

than compensatory function. Further studies are needed to elucidate the role of *mutS* genes in these species, their involvement in hypermutability and their importance for pathogenicity.

The presence of hypermutators in the bacterial populations of the CF airways is often associated with the parallel occurrence of different subpopulations with different phenotypic traits [32]. The study of subpopulations requires the isolation of more colonies – preferably, morphologically different – from the same sample. In this study, we were able to identify morphologically different clones only in one sample (isolates 9–4 and 9–5), while a single isolate was collected from each of all the other samples. However, we cannot exclude that morphologically similar clones were present in the collected samples. Since the design of the isolate collection limited our ability to investigate *Achromobacter* subpopulations in CF airways, the variations that we observed over time within longitudinal isolates may reflect longitudinal evolution as well as intra-patient diversity. The most accurate approach to study intra-patient bacterial adaptation would be to analyse successively recovered clonally related isolates coming from the same patient against the first isolate recovered from that patient. However, due to a technological limit in the sequencing strategy (short-read technology) a complete genome of the first isolates could not be obtained. Thus, analysis of the isolates here was performed using a same-patient same-clone type approach; that is, we analysed all the isolates coming from the same patient and having the same clone type using the closest reference genome. In particular, for the majority of identified species the reference was a complete reference genome of the same species, while for *A. dolens* and NG isolates the reference was the closest complete reference genome available, since no complete reference genomes are available yet (e.g. *A. dolens* isolates were analysed against the *A. ruhlandii* reference genome).

In conclusion, the variety of virulence and antibiotic resistance genetic profiles observed in the different species underlines the importance of accurate species identification to properly manage the infection and apply diverse therapeutic regimens. Lung colonization not only by *A. xylosoxidans* but also by other species has already revealed clinically relevant consequences such as a high risk of pulmonary exacerbation [51] or high transmissibility among patients [6, 7]. Our results support that *Achromobacter* species can exhibit different adaptive mechanisms and suggest that some of them might be more useful to establish a chronic infection in CF patients, highlighting their importance for the clinical setting and the need for further studies of the less clinically characterized *Achromobacter* species.

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Author contributions
Conceptualization: A. S.; Methodology L. V.; Validation L. V., G. M.; Formal analysis L. V., A. S.; Investigation L. V., A. S., C. P.; Resources P. M., G. M., M. M. L.; Data curation L. V.; Writing – Original Draft Preparation L. V., A. S.; Writing – Review and Editing C. P., P. M., G. M., M. M. L.; Visualization L. V., A. S; Supervision G. M., M. M. L.; Project Administration G. M., M. M. L.; Funding Acquisition M. M. L.

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

Ethical statement
Informed consent of all patients was obtained according to projects CRCFC-CEPPO026 and CRCFC-CEPPO031 approved by the local Ethical Committee.

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Mobilome Analysis of *Achromobacter* spp. Isolates from Chronic and Occasional Lung Infection in Cystic Fibrosis Patients

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Abstract: *Achromobacter* spp. is an opportunistic pathogen that can cause lung infections in patients with cystic fibrosis (CF). Although a variety of mobile genetic elements (MGEs) carrying antimicrobial resistance genes have been identified in clinical isolates, little is known about the contribution of *Achromobacter* spp. mobilome to its pathogenicity. To provide new insights, we performed bioinformatic analyses of 54 whole genome sequences and investigated the presence of phages, insertion sequences (ISs), and integrative and conjugative elements (ICEs). Most of the detected phages were previously described in other pathogens and carried type II toxin-antitoxin systems as well as other pathogenic genes. Interestingly, the partial sequence of phage Bcep176 was found in all the analyzed *Achromobacter xylosoxidans* genome sequences, suggesting the integration of this phage in an ancestor strain. A wide variety of IS was also identified either inside of or in proximity to pathogenicity islands. Finally, ICEs carrying pathogenic genes were found to be widespread among our isolates and seemed to be involved in transfer events within the CF lung. These results highlight the contribution of MGEs to the pathogenicity of *Achromobacter* species, their potential to become antimicrobial targets, and the need for further studies to better elucidate their clinical impact.

Keywords: mobilome; *Achromobacter*; lung infection; cystic fibrosis; horizontal gene transfer (HGT); prophages; integrative and conjugative elements (ICEs); insertion sequences (ISs); integrative and mobilizable elements (IMEs); antimicrobial resistance genes; virulence genes

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1. Introduction

Horizontal gene transfer (HGT) is the transfer of genetic elements among organisms by means other than vertical transmission [1,2]. HGT is a well-described mechanism that has been increasingly studied due to its significant role in the rapid dissemination of genetic elements between bacteria [3–9]. Mobile genetic elements (MGEs) are segments of DNA that encode proteins mediating their own movement within intracellular mobility or HGT phenomena [10]. MGEs include a wide variety of different elements that can be horizontally transmitted in three ways: transformation, transduction, or conjugation.

Transformation is the process by which bacteria take up foreign DNA from the local environment [11], while transduction consists of a DNA transfer mediated by phages, also called bacteriophages [10]. These are bacterial viruses that can contain into their capsid elements of host DNA, which can be transferred to a new host and be inherited, thus

allowing the host to express traits carried by phages [12]. Finally, conjugation consists of the transfer of DNA through cell-to-cell contact and requires independently replicating genetic elements such as conjugative plasmids and integrative and conjugative elements (ICEs) [7,13,14]. ICEs can integrate into and excise from genomes using integrase, circularize, replicate, and then transfer via conjugation [13–15]. Another type of MGE that can horizontally transfer through conjugation is integrative mobilizable elements (IMEs), which encode an integrase and circularize like ICEs but have to exploit the conjugative machinery of co-resident ICEs or conjugative plasmids [16]. Another type of MGEs is represented by insertion sequences (ISs), which are small DNA segments that are able to move themselves to new genomic locations within a single cell. ISs are often present in multiple copies in the same genome and can thus facilitate homologous recombination events [17].

MGEs have an important role in antimicrobial resistance and virulence [18–21] as well as in bacterial adaptation. Indeed, they encode genes related to these mechanisms and can enable their host to synthesize products that affect the fitness of co-infecting pathogens, like bacteriocins [22,23], or confer antibiotic resistance [24]. In particular, a strong link between ICEs and the dissemination of antibiotic resistance genes has been demonstrated [13]. It is thus clear that MGEs and HGT events contribute to the pathogenic potential of microbes [19,25,26]. Another mechanism favoring bacterial persistence is the ability to genetically adapt during chronic infection through the accumulation of pathoadaptive mutations [27]. This phenomenon can be accelerated due to mutations in the DNA mismatch repair system (MMR), giving rise to hypermutation events and to clonal diversification within the host [28].

Although MGEs have been detected in the great majority of prokaryotic organisms, the scale and importance of HGT events is still not clear for less characterized bacterial species such as *Achromobacter* spp., an opportunistic pathogen that can cause lung infections in patients with cystic fibrosis (CF). Even though chronic colonization has been associated with a decline in respiratory function, increased frequency of exacerbations, and lung inflammation [25,26,29,30], literature regarding *Achromobacter* mobilome and its contribution to pathogenicity is still scarce. Nonetheless, a rich variety of mobile genetic elements carrying resistance genes in addition to its natural multidrug resistance have been identified in clinical isolates [31].

To better understand the role and impact of MGE on *Achromobacter* pathogenicity potential, we performed whole genome sequencing of 54 clinical isolates and investigated the presence and content of phages, ISs, ICEs, and IMEs through bioinformatic analyses.

2. Materials and Methods

2.1. Samples Collection and Identification

Fifty-four isolates were collected from the sputum samples of 26 patients followed at the CF Center of Verona and were identified as *Achromobacter* spp. by MALDI-TOF-MS (bioMerieux Marcy-l'Étoile, France). Additional information on patients, type of infection (chronic, occasional), and sampling timeline was reported in Table S1. Informed consent was obtained according to projects CRCFC-CEPPO026 and CRCFC-CEPPO031 approved by the local Ethical Committee.

According to the European Consensus criteria (ECC), infection was defined as chronic when at least three positive cultures over $\geq$6 months were obtained with at least a 1-month interval between the samples [32]. Sputum samples were collected approximately every 3 months for microbiological analysis. The classification of occasional and chronic infection was assessed using the information regarding all the *Achromobacter* isolates identified between 2013 and 2018. A minimum of 1 isolate and a maximum of 6 isolates from each infected patient were stored and then used in this study. The collection activity was mainly performed in two time periods: 2014–15 and 2017–18. The average nucleotide identity (ANI) among all available *Achromobacter* spp. genomes (n = 142, NCBI RefSeq database, January 2020) and all sequenced isolates were calculated in order to verify the correct

species classification of the reference and isolates genomes. Isolates were considered to belong to the same species when ANI $\geq$ 95%, whereas isolates with ANI < 95% with all available *Achromobacter* spp. genomes were considered as new genogroups (NG) [33]. Genotypic relatedness among longitudinal isolates was verified by checking core genome similarities obtained using the Harvest-OSX64-v1.1.2 suite [34].

2.2. Genome Sequencing

All the isolates underwent whole genome sequencing at the Technological Platform Centre of the University of Verona. Sequences were submitted to the NCBI SRA database with project number PRJEB40979. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Milan, Italy) and its quality was assessed using NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE, USA) and a Fragment Analyzer System (Agilent Technologies, Santa Clara, CA, USA). Libraries were prepared using the KAPA PCR-free kit (Roche Sequencing Solutions, Pleasanton, CA, USA) and sequenced on a NextSeq500 Illumina platform (Illumina, Hayward, CA, USA) generating 150bp paired end reads with a mean read yield of 10978104 and a mean coverage of 190X. Read quality controls, de novo assembly, and genome annotation were performed similarly to our previous work [35]. Details on the sequencing and de novo assembly are available in Table S2.

2.3. Mobilome Analysis

Phage Search Tool Enhanced Release (PHASTER) [36] was used in order to identify and annotate the prophage sequences based on similarity. Only phages classified as intact by the tool were included into the subsequent analyses. Phylogenetic analysis of Burkho Bcep176 phage was performed by extracting the phage sequence from each *Achromobacter xylosoxidans* isolate and using the Parsnp tool of the Harvest-OSX64-v1.1.2 suite with the options -c -r ! -C 1000 [34]. The tree file in Newick format was used as an input in iTOL [37] for visualization. The presence of ICEs, IMEs, and cis-mobilizable elements was ascertained using the online version of the ICEfinder tool based on the ICEberg [38]. Results were checked to ensure that no ICEs spanning two different contigs were called. Finally, the ISfinder tool [39] was used to evaluate the presence of ISs. The identified mobile genetic elements annotations were manually investigated to evaluate the gene content according to the literature, i.e., presence of antimicrobial resistance genes, virulence factors, and other pathogenic genes. Heatmaps were generated for results visualization purposes using the pheatmap R package v1.0.8.

3. Results

Fifty-four *Achromobacter* spp. clinical isolates were longitudinally collected over 5 years from sputum samples of 26 patients followed at the CF Center of Verona (Italy). Among them, 17 presented chronic lung infection while nine were occasionally colonized. Genomic analysis identified five *Achromobacter* species: *Achromobacter aegrifaciens* (7%, *n* = 4), *Achromobacter dolens* (7%, *n* = 4), *Achromobacter insolitus* (6%, *n* = 3), *Achromobacter insuavis* (13%, *n* = 7), and *A. xylosoxidans* (67%, *n* = 36). Interestingly, four strains isolated from two patients showed an average nucleotide identity of <95% against all the other analyzed genomes, suggesting they likely belonged to *Achromobacter* species with no reference genomes available yet. In this study, we refer to them as new genogroups (NG).

3.1. Phage Analysis

Phage analysis (Figure 1) identified the presence of 12 bacteriophages that were first described in the following six host genera: *Burkholderia*, *Pseudomonas*, *Aeromonas*, *Erwinia*, *Salmonella,* and *Synechococcus*. Interestingly, the most represented ones are *Burkholderia* (six phages) and *Pseudomonas* (two phages), two of the major CF pathogens. In particular, Burkho Bcep176, and Burkho KS9 phages were identified in the isolates of a high number of patients (65% and 42%, respectively). While some phages were identified in different *Achromobacter* species, others were found to be specific for *A. xylosoxidans,* such as Burkho

Bcep176, Aeromo vB AsaM, Pseudo YMC, Pseudo PAJU2, Salmon 118970, and Synech S CBS1. In particular, the tail sequence of the first one was present in all *A. xylosoxidans* isolates. Among the other species, only *A. insuavis* isolates presented Burkho KS14. Except for few cases, phage presence did not seem to be correlated to the type of infection (chronic or occasional). In chronic patients presenting clonal longitudinal isolates, the type and number of phages was consistent over time except for one case (patient 4). Moreover, in a patient with occasional infections, we observed variations between two longitudinal isolates belonging to the same clonetype. These two cases suggest that phage gain or loss might have happened within the CF lung environment.

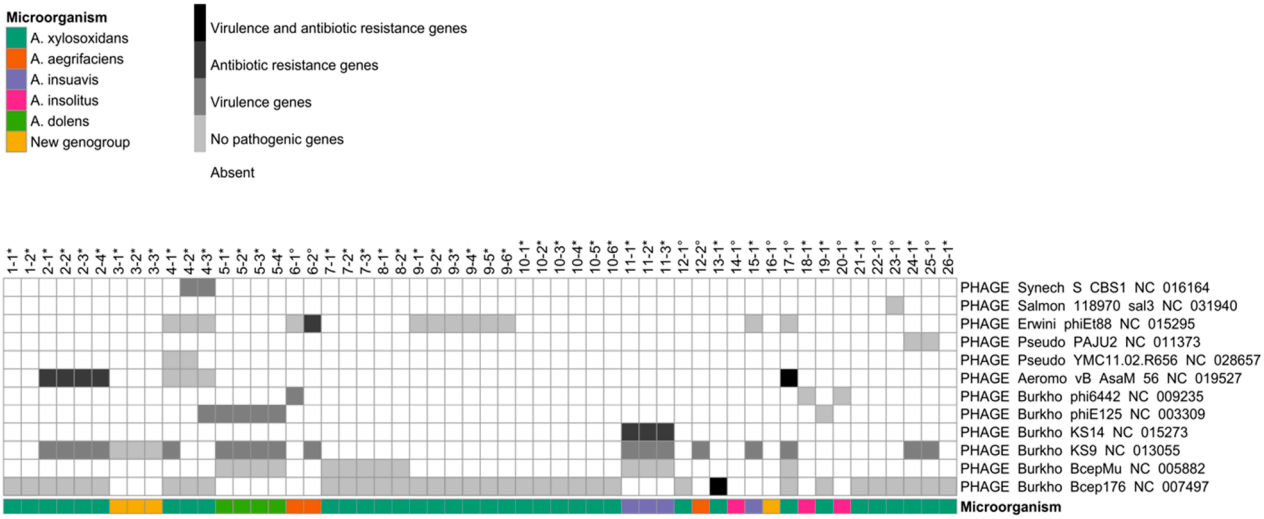

Figure 1. Phage analysis. The heatmap shows the presence of phages and the category of pathogenic genes that they carry (grey scale). The columns represent patients and isolates identification numbers. * indicates chronic infection isolates; ° indicates occasional infection isolates. Additional information regarding the microorganism species is represented in the annotation row (color scale) at the bottom.

Investigating the pathogenic genes carried by the identified phages, we observed that only Burkho Bcep176 and Aeromo vB AsaM carry both virulence and antibiotic resistance genes, while four phages (Burkho BcepMu, Pseudo YMC, Pseudo PAJU2, and Salmon 118970) carried none. Among the phages carrying pathogenic genes, the RelE/ParE and HicA/B type II toxin/antitoxin (TA) systems were identified (Table S3). These systems are involved in MGEs stability, biofilm formation, stress responses, and antibiotic persistence. Interestingly, in a Pseudo PAJU2 phage, we detected the presence of the l*exA* gene, which is involved in the repression of the SOS response to DNA damage, thus enhancing the mutation rate while preventing apoptosis.

3.2. Insertion Sequences (ISs) Analysis

The ISs analysis results (Figure 2) showed the presence of ISs from a wide variety of microorganisms, including opportunistic human pathogens such as *Burkholderia cepacia* complex, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, *Ralstonia* spp., *Sphingomonas paucimobilis*, and *Bordetella parapertussis*, as well as environmental species like *Shewanella* spp., *Salmonella* spp., and *Pseudomonas syringae.* The majority of *A. xylosoxidans* and *A. insuavis* isolates carried more than two types of ISs simultaneously, while the other *Achromobacter* species harbored a maximum of two ISs types. Interestingly, ISs related to plasmid maintenance and rearrangement were found in nine isolates (all isolates from patients 1, 7, 8, 14, and isolate 12–1), indicating the possible presence of plasmids in these strains. Variations in ISs among clonal longitudinal strains were only observed in two chronic patients (10 and 11).

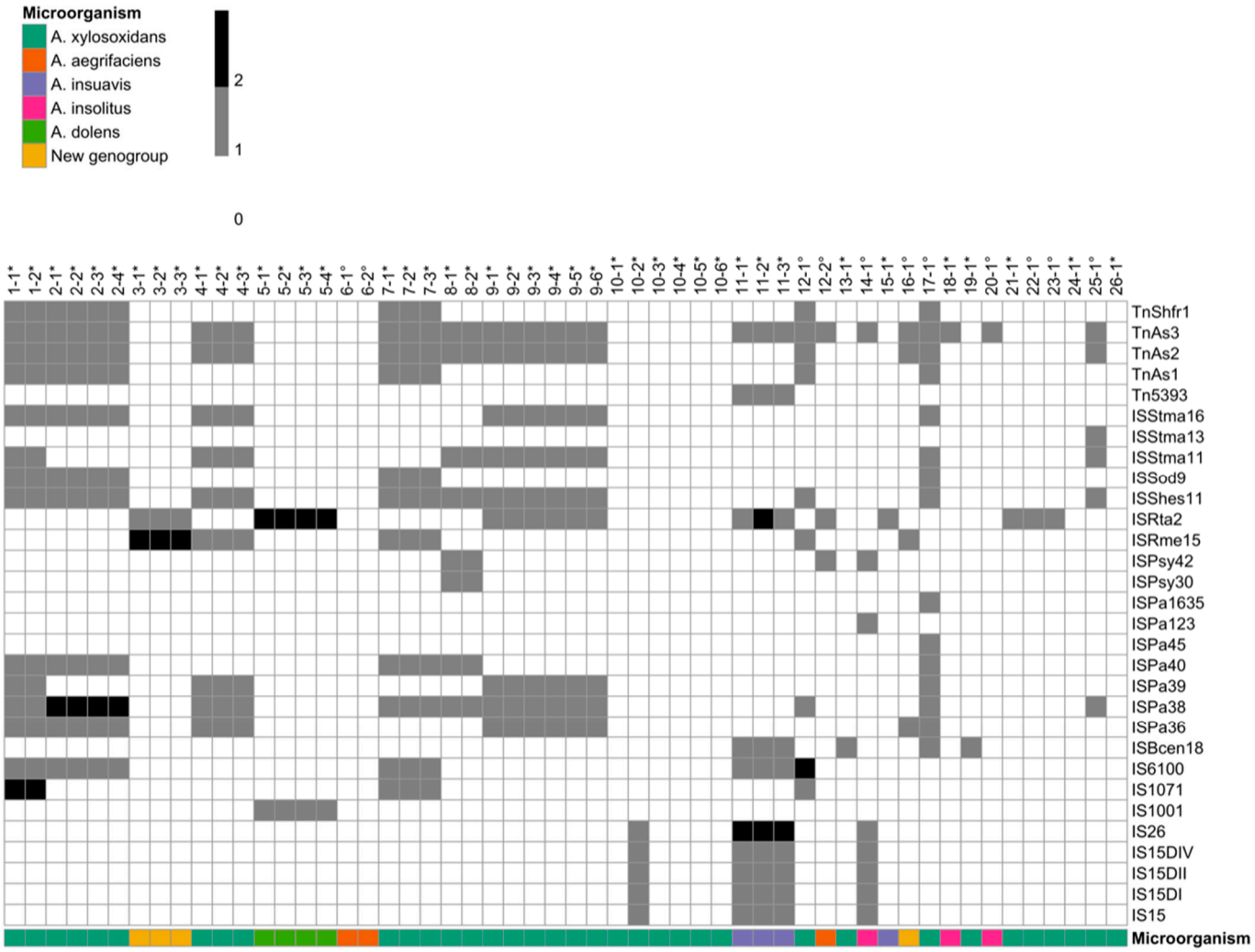

Figure 2. Insertion sequences (ISs) analysis. The heatmap shows the presence and number (grey scale) of ISs. The columns represent patients and isolates identification numbers. * indicates chronic infection isolates; ° indicates occasional infection isolates. Additional information regarding the microorganism species is represented in the annotation row (color scale) at the bottom.

3.3. Integrative and Conjugative Elements (ICEs) and Integrative and Mobilizable Elements (IMEs) Analysis

The presence of ICEs was widespread among our isolates (74%), while IMEs were only found in four strains (Figure 3). Interestingly, ICEs carrying a combination of virulence factors, antibiotic resistance genes, and mismatch repair (MMR) genes were only detected in *A. xylosoxidans*, while no pathogenic genes were identified in *A. insuavis* ICEs. Among the antibiotic resistance genes carried by ICEs, we found genes related to penicillin, beta-lactams, tetracycline, bleomycin, sulfonamide, novobiocin, and deoxycholate resistance in addition to antibiotic efflux systems. Various virulence factors were also identified (Table S4), including genes involved in a Type 4 Secretion System (T4SS), Type 2 Secretion System (T2SS), hemolysin, flagella, pilus, and proteases. Moreover, we frequently observed the presence of LysR-type transcriptional regulators, which control diverse set of genes, including those involved in virulence, metabolism, quorum sensing, and motility. Among MMR genes, *radC* was found in a high number (58%) of *A. xylosoxidans* isolates. Within longitudinal isolates, we observed variations in the presence and pathogenic content of ICEs over time, indicating the possible exchange of these elements within the CF lungs.

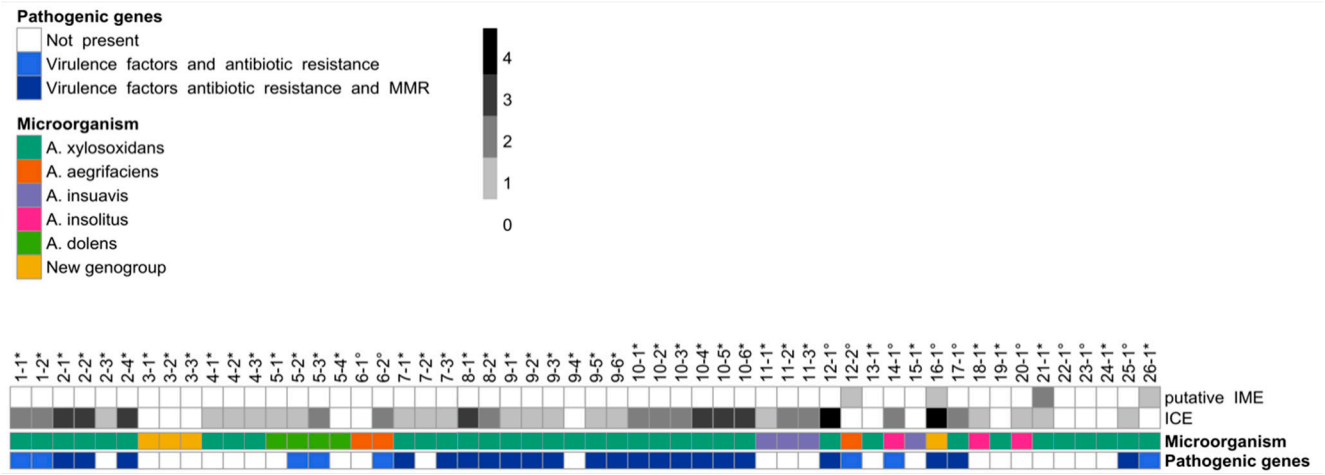

Figure 3. Integrative and conjugative elements (ICEs) and integrative and mobilizable elements (IMEs) analysis. The heatmap shows the presence and number (grey scale) of ICEs and IMEs. The columns represent patients and isolates identification numbers. * indicates chronic infection isolates; ° indicates occasional infection isolates. Additional information regarding the microorganism species and pathogenic genes carried by mobile elements is represented in the annotation row (color scale) at the bottom.

4. Discussion

Achromobacter spp. is an opportunistic pathogen that can cause lung infections in CF patients. Although chronic colonization has been associated with a decline in respiratory function, increased frequency of exacerbations, and lung inflammation, little is known about its pathogenic mechanisms. In particular, virulence features related to its ability to colonize chronically, or only occasionally, the lungs of CF patients are largely unknown. Infections are usually complicated by the innate and acquired multidrug resistance carried by these microorganisms. Moreover, a rich variety of mobile genetic elements carrying resistance genes have been identified in clinical isolates, such as plasmids, IS26, IS440, and class I and class II integrons [31,40]. To provide new insights about *Achromobacter* spp. mobilome and its role in virulence and antibiotic resistance, we performed genomic analyses of 54 clinical isolates mainly collected in 2014–15 and 2017–18. Patients carrying these isolates could be defined as chronically or occasionally infected based on their clinical microbiological history since 2013 and not restricted to the isolates that were included in this study. Although the two-period collection limits the continuous observation of MGEs evolution during these 5 years, it still provides longitudinal data that can support the understanding of MGEs role, particularly in poorly characterized microorganisms such as *Achromobacter* spp.

Well-represented MGEs in *Achromobacter* spp. are phages, which infect bacteria. They are the most abundant organisms in the biosphere [41], and they have been of interest to scientists as vectors of HGT and drivers of bacterial evolution In previous literature, *Sinorhizobium*, *Ralstonia*, *Pseudomonas*, and *Burkholderia* have been suggested to be the most likely to be involved in horizontal transfer with *Achromobacter* spp. [42], most likely because they share a similar GC content. MGEs genetic content can be functional even in evolutionarily distant genomic backgrounds, like in the case of transposable elements carrying "blurry" promoters [43]. Although phages previously reported in other environmental species have also been detected, the majority of identified phages in this study were first described in species of clinical interest. In particular, typical *Burkholderia* phages were the most frequently detected followed by typical *Pseudomonas* phages. Since both are among the main respiratory pathogens in CF patients, we can hypothesize that phages can be exchanged between bacteria through horizontal transfer during lung colonization. Moreover, the tail and host adsorption apparatus sequence of phage Bcep176 from *Burkholderia* was present in all *A. xylosoxidans* isolates. The ubiquity

of this sequence within this specific species suggests an ancestral uptake of the phage, followed by the loss of its structural components and the consequent inability of further transfer. The phylogenetic analysis of this sequence confirmed a genetic relatedness (72% core genome similarity) among all *A. xylosoxidans* isolates (Figure S1). Additionally, its presence was also ascertained in two reference genomes (RefSeq accessions: NZ_LS483395.1 and NZ_LN831029.1), further supporting the hypothesis of integration of this phage in an ancestor strain of *A. xylosoxidans*.

The majority of detected phage sequences carried virulence or antibiotic resistance genes highlighting the importance of mobile genetic elements in the pathogenicity of *Achromobacter* spp. Interestingly, genes involved in MGE stability, biofilm formation, stress responses, and antibiotic persistence such as Type II TA systems were identified. These systems have been reported to occur more often in pathogenic bacteria than in nonpathogenic microorganisms and therefore have been correlated to bacterial pathogenicity [44–50]. Since they are present in bacteria but not in eukaryotic cells, they have also been evaluated as antimicrobial targets [51,52] that can lead to bacterial cell lethality both by the artificial activation of toxins and by targeting the TA operon promoter region. The further study and characterization of Type II TA systems in *Achromobacter* spp. could thus lead to the implementation of novel and alternative treatment regimens in CF therapy. In particular, the RelE/ParE and HicA/B type II TA systems have been identified in our collection of isolates. While the latter is induced by nutrient starvation and might provide a survival advantage in difficult conditions [53], the RelE/ParE system has been well characterized as a genetic element that promotes stable plasmid inheritance [54]. Although we cannot provide evidence of plasmids in these isolates due to a limit in our study design regarding genomic DNA extraction, we neither can exclude their presence in light of these results. Plasmid-related genes were also found within ISs carried by nine isolates. The presence of these systems within both phages and ISs encourages further studies regarding the presence and content of plasmids in *Achromobacter* spp.

IS elements are defined as small (<2.5 kbp) segments of DNA capable of inserting at multiple sites in the genome [55]. They show a simple genetic organization and usually cluster in islands within genomes. They participate in chromosome rearrangements and plasmid integration, and are involved in antibiotic resistance, gene acquisition, and many pathogenic and virulence functions. Indeed, IS sequences in 14 isolates were found to be inside or in close proximity of pathogenicity islands, including ICEs (Table S4). Previous studies also reported that the presence of ISs is frequently associated to antimicrobial resistance genes and to class I and II integrons [31,40].

Similar to phages, ISs from a wide variety of microorganisms were identified, particularly from the species of clinical interest including CF opportunistic pathogens such as *B. cepacia* complex, *P. aeruginosa*, and *S. maltophilia*. This further supported the possible occurrence of interspecies transfer of mobile genetic elements among CF pathogens. A high consistency of both phages and IS in terms of type and carried genes was observed among longitudinal strains, with the exception of a few isolates that showed gain or loss of these elements despite the brief time between isolation events. The transiency of these mobilome profiles might suggest the temporary presence of subpopulations rather than a longitudinal microevolution.

ICEs are self-transmissible mobile genetic elements that usually mediate the transfer of diverse properties to enable the host to better adapt to hostile conditions [14]. Although they have been scarcely reported in literature for *Achromobacter* species, ICEs were found to be widespread among our isolates. We observed the presence of genes involved in a variety of functions such as secretion, motility, quorum sensing, metabolism, MMR, and resistance to different classes of antimicrobial agents. Even though some IMEs have also been identified in our isolates, the distinction of IMEs and incomplete ICEs is still problematic for currently available bioinformatic tools when processing short-read draft assemblies.

While the other mobile genetic elements investigated in this study (phages and IS) showed high consistency in longitudinal isolates, we observed variations in the presence

and pathogenic content of ICEs over time, indicating a frequent exchange of these elements within the CF lungs. This contribution to the genomic plasticity of *Achromobacter* isolates might play an important role in pathogenesis and adaptation during chronic infections.

The variety of MGEs identified in *Achromobacter* genomes and their diverse virulence and antibiotic resistance profiles confirm that *Achromobacter* spp. are a reservoir of HGT elements. Not only do they contribute to genomic plasticity, but some of these elements can even become a constitutive part of the bacterial genome, as supported by the presence of phage Bcep176 in all our *A. xylosoxidans* isolates as well as in the analyzed reference genomes. Our results highlight the contribution of mobile genetic elements to the pathogenic potential of *Achromobacter* species, the need for further studies to better elucidate their clinical impact, and their potential to become antimicrobial targets in treatment regimens.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-2607/9/1/130/s1, Figure S1: Phylogenetic tree of phage Burkho Bcep176 tail sequence, Table S1: Sampling timeline, Table S2: Assembly details, Table S3: Phage content details, Table S4: ICE and IME content details.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of the Centro Ricerca Clinica of Verona (protocols codes CRCFC-CEPPO026 and CRCFC-CEPPO031).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The genomic sequences analyzed in this study are openly available in NCBI SRA database within project number PRJEB40979.

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IV

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Achromobacter spp. Adaptation in Cystic Fibrosis Infection and Candidate Biomarkers of Antimicrobial Resistance

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Abstract: *Achromobacter* spp. can establish occasional or chronic lung infections in patients with cystic fibrosis (CF). Chronic colonization has been associated with worse prognosis highlighting the need to identify markers of bacterial persistence. To this purpose, we analyzed phenotypic features of 95 *Achromobacter* spp. isolates from 38 patients presenting chronic or occasional infection. Virulence was tested in *Galleria mellonella* larvae, cytotoxicity was tested in human bronchial epithelial cells, biofilm production in static conditions was measured by crystal violet staining and susceptibility to selected antibiotics was tested by the disk diffusion method. The presence of genetic loci associated to the analyzed phenotypic features was evaluated by a genome-wide association study. Isolates from occasional infection induced significantly higher mortality of *G. mellonella* larvae and showed a trend for lower cytotoxicity than chronic infection isolates. No significant difference was observed in biofilm production among the two groups. Additionally, antibiotic susceptibility testing showed that isolates from chronically-infected patients were significantly more resistant to sulfonamides and meropenem than occasional isolates. Candidate genetic biomarkers associated with antibiotic resistance or sensitivity were identified. *Achromobacter* spp. strains isolated from people with chronic and occasional lung infection exhibit different virulence and antibiotic susceptibility features, which could be linked to persistence in CF lungs. This underlines the possibility of identifying predictive biomarkers of persistence that could be useful for clinical purposes.

Keywords: *Achromobacter*; cystic fibrosis; drug resistance; virulence; biomarkers; adaptation

1. Introduction

Achromobacter spp. are opportunistic pathogens that can colonize the lungs of patients with cystic fibrosis (CF), causing chronic or occasional infections. In particular, chronic colonization has been associated with a decline in respiratory function, increased frequency of exacerbations and lung inflammation [1]. Although virulence factors supporting invasiveness and survival have been described (e.g., swimming motility, biofilm formation, lipopolysaccharide, type III secretion system, phospholipase C, proteases) [2–13], virulence

features related to the ability of *Achromobacter* spp. to colonize the lungs of CF patients chronically or occasionally are still not fully clear.

Additionally, multidrug resistance strongly contributes to *Achromobacter* spp. persistence in CF patients. These bacteria show an innate resistance to many classes of antibiotics, especially to those relevant to CF lung infection treatment such as aminoglycosides, aztreonam, tetracyclines, penicillins and cephalosporins [2,14,15]. Moreover, clinical isolates exhibit acquired resistance, especially to β-lactams. The most active agents against *Achromobacter* spp. are, among others, trimethoprim–sulfamethoxazole, ceftazidime, piperacillin and carbapenems [16]; however, mutations of genes related to antibiotic resistance may occur, causing resistance to these antibiotics [13]. Indeed, isolates from long-term chronic infection tend to be resistant to more antibiotics than earlier or occasional isolates [16].

The variation in virulence factors and antibiotic resistance among *Achromobacter* spp. isolates highlights the necessity to better understand the involvement of these features in the pathogenic potential and mechanisms of colonization of these microorganisms. To this purpose, we evaluated virulence, cytotoxicity, biofilm formation and antibiotic susceptibility of clinical isolates causing occasional and chronic CF infections.

2. Results

Ninety-five *Achromobacter* spp. isolates were analyzed in this study. Seventy-nine isolates (range = 1–11 successively collected isolates, mean = 3.3 isolates/patient) were recovered from 24 chronically-infected CF patients with a mean time delay of 469 days (range = 21–1825 days). One isolate was recovered from each occasionally-infected CF patient (n = 14), except for two patients, P06 and P12, from whom we recovered 2 isolates with a time delay of 112 days and 155 days, respectively. We compared phenotypic features such as virulence, biofilm formation, cytotoxicity and antibiotic susceptibility between chronic and occasional and between early and late chronic isolates.

2.1. Virulence

Virulence is an important feature for bacterial pathogenicity, invasion and interactions with the host. Virulence testing (Figures1 and S1) in a *G. mellonella* larvae model showed that isolates from occasionally-infected patients induced significantly higher mortality of larvae than chronic infection isolates (Kaplan–Meier survival estimate p-value = 0.02; Cox hazard ratio = 1.32; 95% CI = 1.04–1.66). When comparing early against late chronic isolates, no significant difference was observed.

Biofilm formation plays an important role in the persistence of bacteria in CF chronic lung infections, protecting pathogens against environmental stress and increasing tolerance towards antibiotics and host defenses. No significant difference was observed in biofilm production among chronic and occasional isolates and between early and late chronic isolates (Figure 2A,B).

The ability to cause cytotoxicity could play an important role in tissue inflammation and degeneration. Cytotoxicity was assessed on both WT and CF bronchial epithelial cells. Although no statistically significant difference was found, we observed that chronic infection isolates showed a trend for greater cytotoxicity than occasional isolates in both cell types (Figure 2C,E). Moreover, when comparing early and late chronic isolates we observed an increase (Wilcoxon Mann–Whitney test p-value = 0.05 after 10,000 permutations) of cytotoxicity from early to late isolates in WT cells (Figure 2D).

Virulence, cytotoxicity and biofilm formation results per isolate are shown in Figure S2.

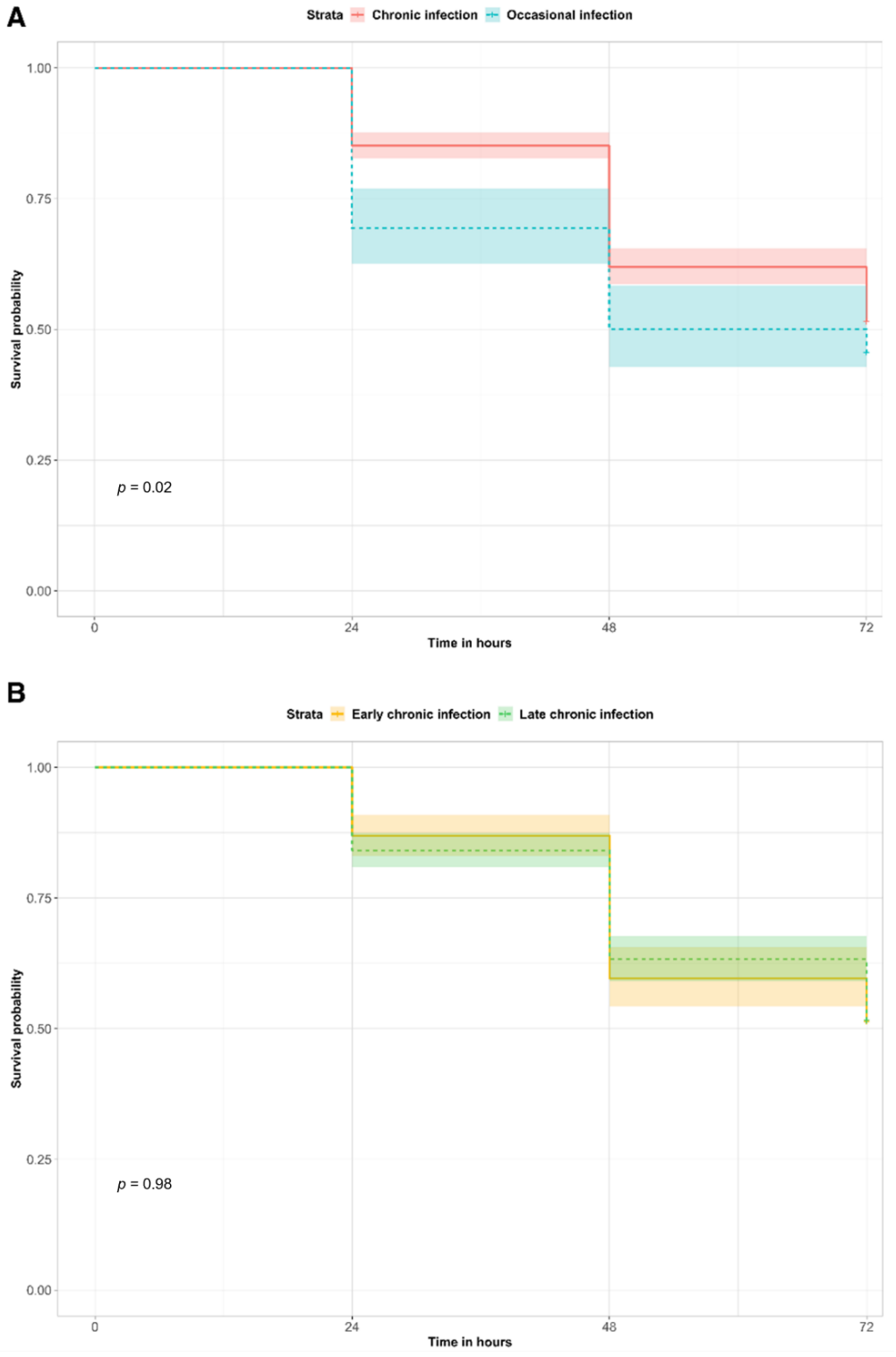

Figure 1. *G. mellonella* survival curve for virulence testing. The survival probabilities of *G. mellonella* larvae infected with chronic or occasional infection isolates (**A**) and with early or late chronic infection isolates (**B**) are reported at each time point. The *p*-values of the survival curve comparisons are indicated with *p*.

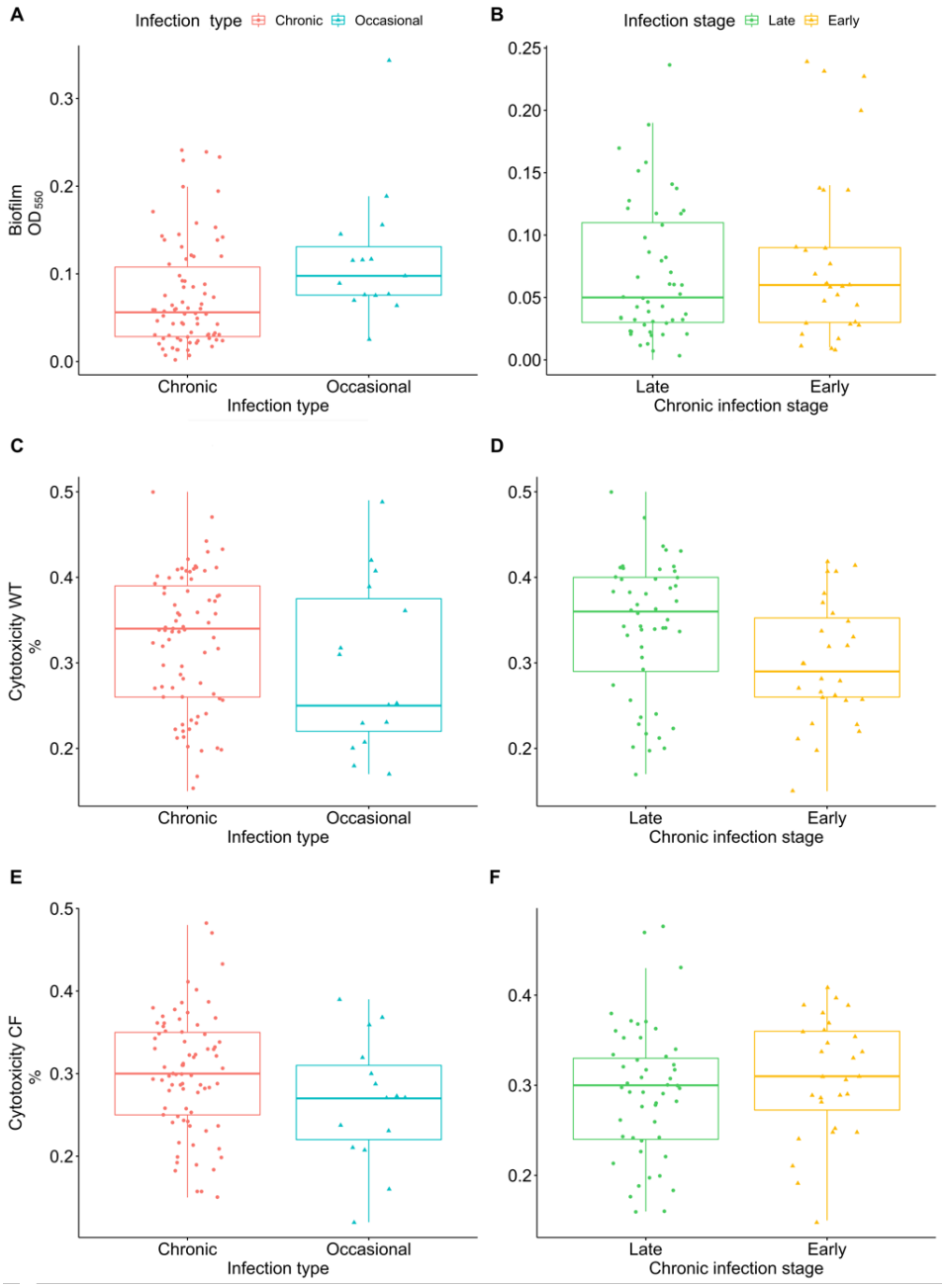

Figure 2. *Achromobacter* spp. biofilm formation and cytotoxicity on WT and CF bronchial epithelial cells. Biofilm formation in occasional and chronic isolates (**A**) and in early and late chronic isolates (**B**) measured by crystal violet staining (OD_{550}). Cytotoxicity of occasional and chronic isolates and of early and late chronic isolates on WT cells (**C**,**D**) and CF cells (**E**,**F**) expressed as percentage of LDH release compared to the maximum value (positive control).

2.2. Antimicrobial Susceptibility

An important factor for the survival of infectious bacteria is their resistance to administered antibiotics, making infections hard to eradicate. We evaluated six antibiotics that have been reported to show variable susceptibility in chronic strains [16]: SXT, TGC, SSS, IPM, TZP and MEM.

The majority of strains were resistant to SXT and TGC and sensitive to IPM and TZP, while different susceptibility between occasional and chronic isolates was observed for SSS and MEM (Figure 3): when compared to occasional isolates, strains from chronically-infected patients were significantly more resistant to SSS (Fisher's exact test *p*-value = 0.04

after 10,000 permutations; CI = 0.042–0.62; odds ratio = 0.17) and MEM (Fisher's exact test *p*-value = 0.01 after 10,000 permutations; CI = 0–0.34; odds ratio = 0). No significant difference in resistance was observed when comparing early and late chronic isolates (Figure 4). Antimicrobial susceptibility results per isolate are shown in Figure S3.

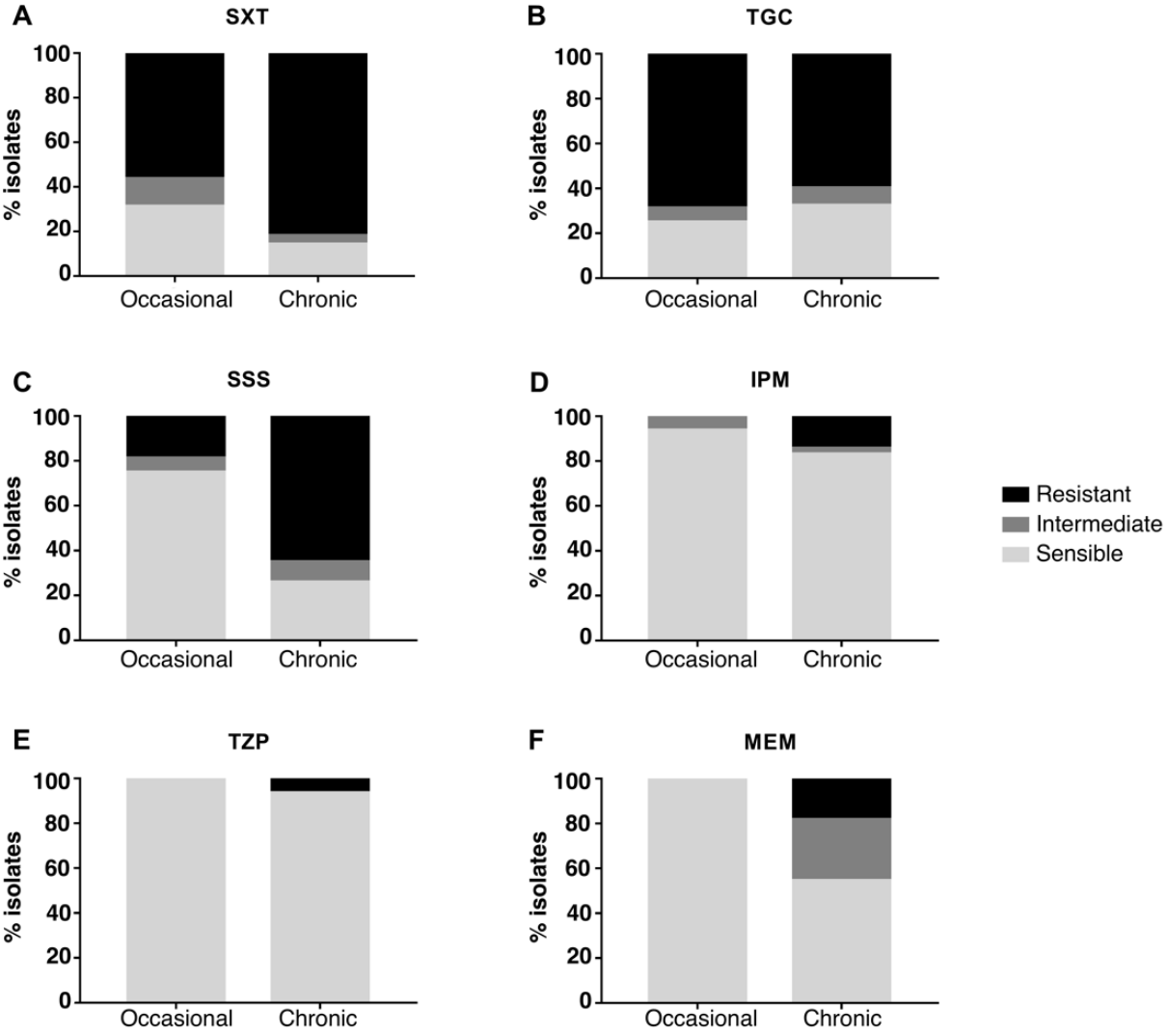

Figure 3. Antimicrobial susceptibility testing of chronic and occasional isolates. The percentage of resistant, intermediate and sensible isolates from chronic and occasional infection are represented for each antibiotic tested. SXT = trimethoprim–sulfamethoxazole (**A**), TGC = tigecycline (**B**), SSS = sulfonamides (**C**), IPM = imipenem (**D**), TZP = piperacillin-tazobactam (**E**), MEM = meropenem (**F**).

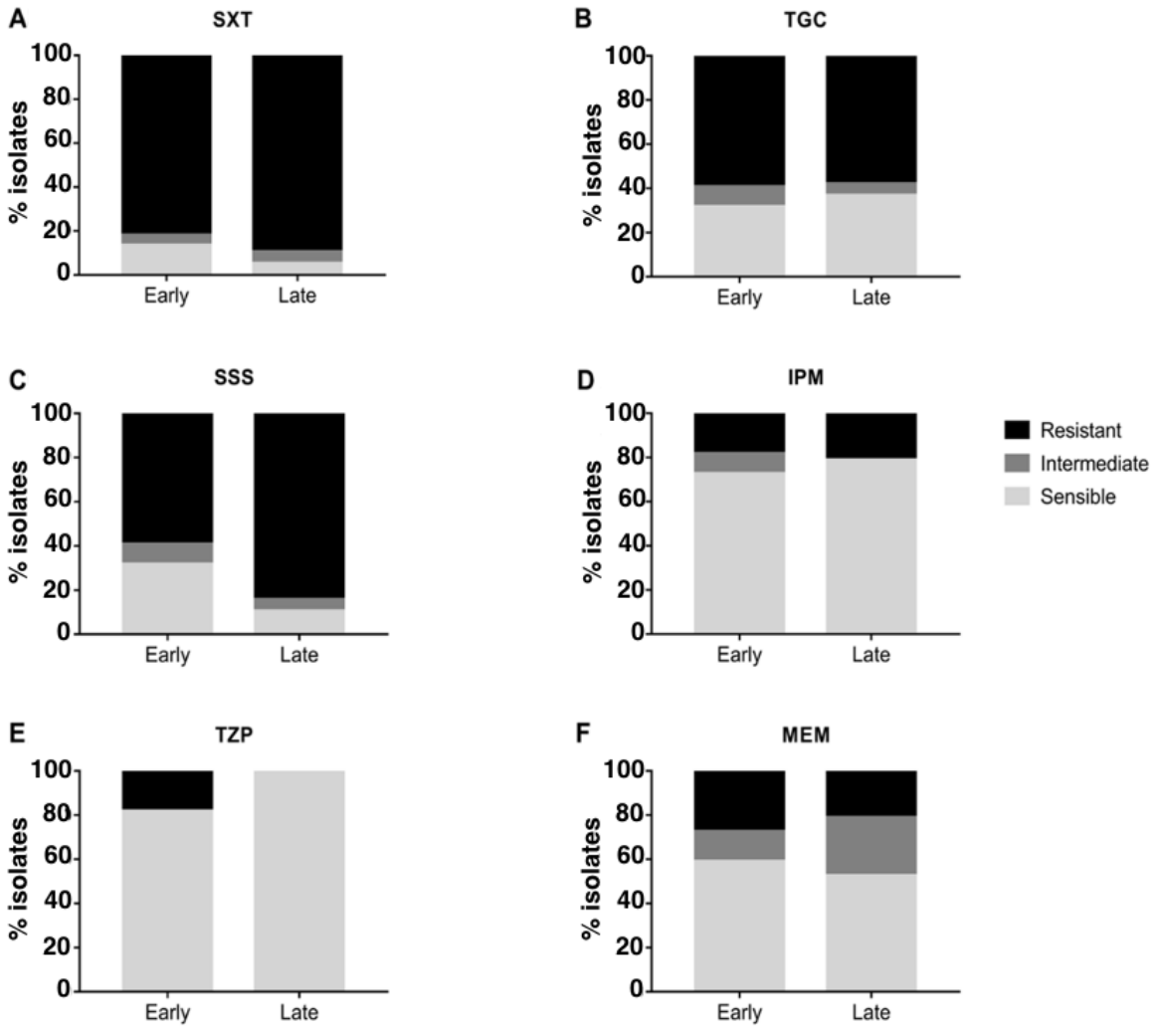

Figure 4. Antimicrobial susceptibility testing of early and late chronic isolates. The percentage of resistant, intermediate and sensible isolates from chronic and occasional infection are represented for each antibiotic tested. SXT = trimethoprim–sulfamethoxazole (**A**), TGC = tigecycline (**B**), SSS = sulfonamides (**C**), IPM = imipenem (**D**), TZP = piperacillin-tazobactam (**E**), MEM = meropenem (**F**).

2.3. Biomarkers of Antimicrobial Resistance

Genome analysis of the 54 *Achromobacter* spp. isolates from the Verona collection was performed to identify genetic components significantly associated with virulence, biofilm, cytotoxicity and antimicrobial resistance. As regards the former three features, no genetic loci were significantly associated to the phenotypic traits. Within the tested antibiotics, we found various candidate biomarkers of resistance or sensitivity linked to transmembrane transporters and efflux pumps (e.g., secretory components, ABC transporters), transcriptional regulators (e.g., AraC) and metabolic enzymes (Tables 1 and S1 for details). In addition, some hypothetical proteins were associated with sensitivity to SSS and resistance to MEM. Some node sequences showed statistically significant correlation (q-value ≤ 0.05) with both IPM and MEM, and with both SSS and SXT (Table S1).

Table 1. Genetic loci significantly associated with antimicrobial resistance. The most significant results of association analysis are reported. IPM = imipenem, SXT = trimethoprim–sulfamethoxazole, SSS = sulfonamides (sulphadiazine, sulphathiazole), MEM = meropenem, *q*-value = *p*-value adjusted for the False Discovery Rate.

Antimicrobial	Associated with	Node ID	Sensitivity (%)	Specificity (%)	*q*-Value	Annotation
IPM	Resistance	n1316743	100.0	100.0	8.4×10^{-23}	Arginine-tRNA ligase
IPM	Resistance	n284357	100.0	100.0	8.4×10^{-23}	Diguanylate cyclase (GGDEF domain) with GAF sensor
IPM	Resistance	n1001723	100.0	100.0	8.4×10^{-23}	ABC transporter
IPM	Resistance	n1228398	100.0	100.0	8.4×10^{-23}	Type II secretory pathway component GspD
IPM	Resistance	n1320154	100.0	100.0	8.4×10^{-23}	AraC family transcriptional regulator
IPM	Resistance	n119911	100.0	100.0	8.4×10^{-23}	NA
IPM	Resistance	n1360642	100.0	100.0	8.4×10^{-23}	General secretion pathway protein GspN
IPM	Resistance	n1607259	100.0	100.0	8.4×10^{-23}	Fe^{2+}-dicitrate sensor, membrane component, FecR
IPM	Resistance	n1496359	100.0	100.0	8.4×10^{-23}	Glutathione S-transferase family protein
MEM	Resistance	n382985	88.9	97.1	1.3×10^{-2}	ABC transporter ATP-binding protein
MEM	Resistance	n776344	88.9	97.1	1.3×10^{-2}	Hypothetical protein
MEM	Resistance	n1454097	88.9	97.1	1.3×10^{-2}	ABC transporter ATP-binding protein
SSS	Susceptibility	n477893	81.3	86.1	2.8×10^{-3}	Hypothetical protein
SSS	Susceptibility	n1248808	81.3	86.1	2.8×10^{-3}	ABC transporter ATP-binding protein
SSS	Susceptibility	n888310	81.3	86.1	2.8×10^{-3}	Aminomethyl-transferring glycine dehydrogenase
SXT	Susceptibility	n1221225	85.7	100.0	7.6×10^{-9}	NA
SXT	Susceptibility	n1593088	85.7	100.0	7.6×10^{-9}	16S rRNA (uracil(1498)-N(3))-methyltransferase
SXT	Susceptibility	n222900	85.7	100.0	7.6×10^{-9}	NA
SXT	Susceptibility	n979447	85.7	100.0	7.6×10^{-9}	DNA mismatch repair endonuclease MutL
SXT	Susceptibility	n120539	85.7	100.0	7.6×10^{-9}	Efflux RND transporter periplasmic adaptor subunit
SXT	Susceptibility	n335885	85.7	100.0	7.6×10^{-9}	Efflux RND transporter periplasmic adaptor subunit
SXT	Susceptibility	n71145	85.7	100.0	7.6×10^{-9}	M61 family metallopeptidase
SXT	Susceptibility	n346933	85.7	100.0	7.6×10^{-9}	Acyl-CoA synthetase
SXT	Susceptibility	n1069885	85.7	100.0	7.6×10^{-9}	Helix-turn-helix domain-containing protein
SXT	Susceptibility	n1157928	85.7	100.0	7.6×10^{-9}	Helix-turn-helix domain-containing protein
SXT	Susceptibility	n527920	85.7	100.0	7.6×10^{-9}	Patatin-like phospholipase family protein

3. Discussion

To identify phenotypic features related to the ability of *Achromobacter* spp. to establish chronic or occasional colonization in CF airways, we evaluated virulence, biofilm formation, cytotoxicity, and antimicrobial susceptibility of 95 clinical isolates and compared results between chronic and occasional isolates and between early and late chronic ones.

Isolates were collected at the CF Center of Verona (Italy) and Bambino Gesù Hospital in Rome (Italy). While the Verona collection included a larger number of occasional isolates, the Rome one comprised many longitudinally-isolated chronic strains encompassing a long period of time. The two collections showed similar phenotypic characteristics (Figures S2 and S3); so, their combination allowed the analysis of a more homogeneous group of strains than using them separately; however, the final collection still included a lower number of occasional (n = 16) than chronic isolates (n = 79, 30 early and 49 late chronic isolates).

To evaluate the virulence of *Achromobacter* spp. isolates, we used the well-characterized *G. mellonella* larvae model [17,18]. Occasional infection isolates caused higher mortality than strains from chronic infection, indicating that *Achromobacter* spp. exhibit higher virulence during occasional infection. This observation suggests that virulence attenuation could be a key factor during the establishment of chronic infection. Differences in virulence between early and late chronic strains of another CF pathogen, *Pseudomonas aeruginosa*, were also previously highlighted [18], leading to the hypothesis that multiple mutations could be responsible for virulence attenuation during late infection. Although we did not observe a significant difference in virulence between early and late chronic isolates of *Achromobacter* spp., a similar mechanism of adaptation could be proposed for occasional and chronic isolates, where selection of strains with an increasing ability to persist may occur.

As for biofilm formation, the majority of our isolates showed a low-moderate production of biofilm, confirming the poor adhesion ability of *Achromobacter* spp. on surfaces [7,9]. Although no significant difference in biofilm production was observed between chronic and occasional isolates nor between early and late chronic isolates, the great majority of strains unable to form biofilm were isolated from chronic infection. This could suggest a mechanism of within-host adaptation in the CF lung; e.g., acquisition of mutations in genes with a role in surface adhesion could lead to decreased biofilm production or to formation of unattached aggregates [10,13,19].

To investigate whether a reduced virulence in the chronic isolates coincided with lower cytotoxicity, we compared the cytotoxic potential of chronic and occasional strains in WT and F508del human bronchial epithelial cultured cells, but no significant difference was found. Although no statistically significant difference was found, we observed that chronic infection isolates induced slightly greater cytotoxicity than occasional isolates—an opposite trend compared to the results of biofilm and virulence testing. We observed an increased cytotoxicity from early to late isolates in cells expressing WT CFTR. No significant difference was observed in CF cells; this could indicate an underlying adaptation of late chronic strains to the CF lung environment leading to a more indolent colonization.

Biomarkers analysis of virulence traits (biofilm, cytotoxicity and virulence in *G. mellonella* larvae) showed no associated genetic loci. This is probably due to the fact these aspects are known to be mainly regulated through RNA modulation [20,21] rather than through the accumulation of genomic mutations.

Achromobacter spp. are reported to increasingly develop resistance to various antibiotics. We tested susceptibility of all isolates to 6 antibiotics that have been reported to show variable susceptibility in chronic strains [16]. *Achromobacter* spp. strains generally displayed resistance to TGC and susceptibility to TZP and IPM, confirming their previously-reported innate resistance to tetracyclines [2] and susceptibility to beta-lactams and carbapenems [16]. Interestingly, an increased resistance to MEM was observed in chronic isolates. Moreover, even though IPM and MEM belong to the same class of antimicrobials, we observed both imipenem resistant but meropenem susceptible (IRMS) and meropenem resistant but imipenem susceptible (MRIS) phenotypes. These phenotypes were previously reported for *P. aeruginosa* and various members of the *Enterobacteriaceae* family [22] as well as for *Achromobacter* spp. clinical isolates [22,23]. In addition, we observed significantly higher resistance to SSS in chronic infection isolates than in occasional ones, in concordance with previous studies [23,24]. Resistance of chronic infection isolates to SSS could be associated with the use of SXT to eradicate CF pathogens. The majority of both occasional and chronic isolates was resistant also to SXT, in contrast with previous investigations; e.g., a recent one found that the majority of *Achromobacter* spp. strains from Danish CF patients were sensitive to SXT [16], considered as one of the most active agents against *Achromobacter* spp. infections [25]. This suggests that the acquisition of resistance to SXT might have occurred among isolates in our collections, e.g., through the spread of mobile genetic elements carrying resistance genes [26] or due to the different antibiotic treatment regimens used.

Candidate biomarkers were identified for SXT, IPM, MEM and SSS by analyzing sequenced isolates. Some of them are known to be involved in antibiotic resistance, such as ABC transporters [27], while the role of other candidates—associated with antibiotic resistance or with sensitivity—should be further investigated. Of particular interest are hypothetical proteins, which could provide additional information on *Achromobacter* spp. resistance mechanisms upon further characterization. These candidate biomarkers could help in the identification of strains that are becoming persistent and support their eradication before chronic infection is fully developed. Finally, in order to assess whether biomarkers presence could be linked to an adaptation mechanism such as clonal expansion or horizontal gene transfer, we further performed genome analysis but neither of these hypotheses was confirmed. In conclusion, our results show that *Achromobacter* spp. isolates from chronic and occasional lung infection exhibit different virulence and antibiotic resistance characteristics, some of which might be linked to persistence in CF lungs. We identified potential predictive markers of persistence such as decreased virulence, higher cytotoxicity, resistance to antibiotics, as well as genetic biomarkers [3], that could be translated into the clinical setting either to help preventing the development of chronic infections or to support therapeutic treatments aimed at eradicating *Achromobacter* spp.

4. Materials and Methods

4.1. Samples Collection

Ninety-five *Achromobacter* spp. isolates were collected from the sputum samples of 38 patients followed at the CF Center of Verona and Bambino Gesù Hospital in Rome (Italy): 54 isolates were recovered from 26 patients in Verona [3,26], while 41 isolates were recovered from 12 patients in Rome. Patients were classified as occasionally- and chronically-infected with *Achromobacter* spp. according to the European Consensus Criteria or Leeds criteria. In the Verona collection, 43 longitudinal isolates were collected from 17 patients with chronic infections while 11 strains were collected from 9 patients with occasional infection. The Rome collection comprised 36 strains collected over time from 7 patients with chronic infection and 5 strains collected from 5 patients with occasional infections. Isolates from chronically-infected patients were further classified as early (<1 year from 1st colonization event) and late isolates (>1 year from 1st colonization). Relatedness of strains was verified to confirm that subsequent isolates from one patient actually represented a single strain. An overview of the isolates included in each collection is reported in Table 2. Informed consent was obtained according to projects CRCFC-CEPPO026 and CRCFC-CEPPO031, approved by the Ethical Committee. All the isolates included in this study were identified as *Achromobacter* spp. by MALDI-TOF-MS (bioMerieux Marcy-l'Étoile, France). Strains were stored in Microbank (Pro-Lab Diagnostics, Neston, UK) at −80 °C. Detailed information regarding the collections is reported in Table S2.

Table 2. *Achromobacter* spp. collections summary. The number of isolates included in each collection is reported in the table; the number of patients from which the strains were collected is indicated in parenthesis. E = early chronic infection isolates; L = late chronic infection isolates.

	Rome-*n* (Patients)		**Verona-*n* (Patients)**	
Chronic infection isolates	36 (7)	E: 6 (4) L: 30 (7)	43 (17)	E: 24 (10) L: 19 (10)
Occasional infection isolates	5 (5)		11 (9)	

4.2. Virulence Testing

Virulence was assessed in *Galleria mellonella* larvae. Ten larvae were inoculated with a 1×10^6 CFU bacterial suspension of each clinical isolate through the last proleg into the haemocoel using a 0.3 mL syringe and incubated in Petri dishes, on filter paper, at 37 °C, in the dark. In the control group, larvae were injected with sterile saline solution. Larvae were

monitored daily up to 72 h and death was assessed by lack of movement after stimulation and blackening.

4.3. Biofilm Formation Assay

Bacterial strains were plated onto LB agar and grown at 37 °C for 24–48 h. A single colony was inoculated in BHI medium and grown for 16 h at 37 °C with shaking. OD_{600} was measured, cultures were diluted to 0.1 OD/mL and 200 µL/well were incubated in a 96-well plate for 24 h at 37 °C. Wells were washed with saline solution and stained with 0.1% crystal violet solution for 15 min, then rinsed, washed with water and air dried. After 30 min of incubation with 30% acetic acid at 37 °C, absorbance at 550 nm was measured.

4.4. Cytotoxicity Testing

Human CF bronchial epithelial cell lines CFBE14o- 4.7 WT-CFTR (WT cells) and DeltaF508-CFTR (CF cells) (Merck, Darmstadt, Germany), overexpressing WT and F508del CFTR cDNA, respectively, were cultured in 200 µL EMEM supplemented with 1% Fetal Bovine Serum, 0.5–2 µg/mL Puromycin and 2 mM L-Glutamine into Fibronectin/Collagen/BSA-coated 96-well plates incubated at 37 °C and 5% CO_2. At 80% confluency, 50 µL/well of 2 OD_{600}/mL bacterial suspension were added to cell cultures and incubated at 37 °C for 4 h. CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) measuring LDH release was used according to manufacturer's instructions. Briefly, 50 µL of cells suspension were added with 50 µL of CytoTox 96 Reagent and incubated for 30 min in the dark. After adding 50 µL of Stop Solution, absorbance at 450 nm was recorded. Cytotoxicity was calculated by dividing for the absorbance of the positive control (treated with Lysis Solution).

4.5. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility was determined by disk diffusion assay. Bacterial suspension at 0.5 McFarland was streaked onto Mueller–Hinton agar plates, antibiotic-containing disks (Oxoid) were placed onto the agar surface, plates were incubated at 37 °C for 48 h and the diameter of the zone-of-inhibition was measured. Disks contained 1.25/23.75 µg trimethoprim–sulfamethoxazole (SXT), 15 µg tigecycline (TGC), 300 µg sulfonamides (sulphadiazine, sulphathiazole) (SSS), 10 µg imipenem (IPM), 100/10 µg piperacillin-tazobactam (TZP) or 10 µg meropenem (MEM). Since no EUCAST or CLSI breakpoint standard is available for *Achromobacter* spp., susceptibility profiles were interpreted as resistant (R), intermediately resistant (I) or sensible (S) based on breakpoints proposed in previous literature [15,16,28].

4.6. Statistical Analysis

Statistical analysis was carried out to compare chronic and occasional isolates and early and late chronic isolates. Virulence results were tested by the Kaplan–Meier method using the log-rank test to compare the overall survival of larvae over an observation period of 72 h. Hazard ratios were computed with a Cox regression model. Cytotoxicity and biofilm formation results were tested using a Wilcoxon Mann–Whitney test. Outlier values were observed in biofilm formation results (n = 2, isolates 7-3 and 12-2) and were excluded from statistical analysis. Fisher's exact test was used to ascertain the significance of antibiotic susceptibility results. Since observations per isolate were not independent due to a longitudinal collection strategy, for each test, p-values were adjusted performing 10,000 permutations of the infection type (chronic, occasional) or infection stage (early chronic, late chronic) stratified by collection (Verona, Rome). R version 4.0.4 (R Foundation for Statistical Computing, Vienna, Austria) [29] was used for statistical analysis and for results visualization. Boxplots were generated using ggpubr v0.4.0, survival curves using survminer v0.4.9, and heatmaps using pheatmap v1.0.8 R libraries.

4.7. Identification of Genetic Loci Associated with Virulence, Biofilm, Cytotoxicity and Antimicrobial Resistance

The DBGWAS 0.5.4 software (bioMerieux, Lyon, France) [30] was used to identify genetic components significantly associated with virulence, biofilm, cytotoxicity and antimicrobial resistance. In previous work [3,26] we sequenced the whole genome of the 54 *Achromobacter* spp. isolates from the Verona collection. A phylogenetic analysis and a comparison of virulence and resistance genes, genetic variants and mutations, and hypermutability mechanisms between chronic and occasional isolates was also performed. The de novo assembled contigs of these genomes were used as input for the association analysis. The following phenotypic cut-offs were used as parameters when running the analysis: biofilm production of 0.115 (absorbance at 550 nm), cytotoxicity of 0.36 (% cytotoxicity vs. positive control), virulence causing 5 dead larvae in 48 h; while the cut-offs for antimicrobial resistance were defined as described in Section 4.5. In particular, DBGWAS accepts continuous phenotypes, so we translated the categorical variables "resistant (R)", "intermediately resistant (I)" and "sensible (S)" to a dummy variable such that S = 0, I = 1 and R = 2. All available annotations of *Achromobacter* genes from the UniProt database (www.uniprot.org, ref. 320,589 genes; accessed on 1 March 2022) were used in the annotation step of the virulence, biofilm and cytotoxicity association analysis, whereas all known bacterial resistance genes from the UniProt database (www.uniprot.org, ref. 36,658 genes; accessed on 1 March 2022) were used for annotation in the antimicrobial resistance association analysis.

Sensitivity and specificity of candidate components having the same order of magnitude of the lowest *q*-value—i.e., *p*-value adjusted for the False Discovery Rate—of each analysis were calculated using the following formulas: for nodes positively associated to the phenotype sensitivity = Pheno1Count/Pheno1TotalCount and specificity = (Pheno0TotCount-Pheno0Count)/Pheno0TotCount; for nodes negatively associated to the phenotype sensitivity = Pheno0Count/Pheno0TotCount and specificity = (Pheno1TotCount-Pheno1Count)/Pheno1TotCount. In particular, Pheno1Count = isolates displaying the phenotype and carrying the allele, Pheno1TotalCount = isolates displaying the phenotype, Pheno0Count = isolates not displaying the phenotype and carrying the allele, Pheno0TotCount = isolates not displaying the phenotype. All candidate components having the highest sensitivity and specificity, down to a threshold of 80%, and lowest *q*-value were further analyzed (tests were considered to be statistically significant if q-value < 0.05). Every k-mer present in only one isolate was discarded.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23169265/s1.

Author Contributions: Conceptualization: A.S.; Methodology: A.S., L.V. and R.P.M.; Validation: A.S., L.V., R.P.M. and M.C.; Formal analysis: A.S. and L.V.; Investigation: A.S., L.V., R.P.M., G.M.S., G.B., S.P. and C.P.; Resources: C.S. (Claudio Sorio), P.M., E.V.F., G.M., C.S. (Caterina Signoretto), M.B. and M.M.L.; Data curation: A.S. and L.V.; Writing—Original Draft Preparation: A.S., L.V. and G.M.S.; Writing—Review & Editing: R.P.M., M.C., C.S. (Claudio Sorio), P.M., E.V.F., A.L.M., C.P., C.S. (Caterina Signoretto), M.B., M.M.L. and G.M.; Visualization: A.S. and L.V.; Supervision: M.B., M.M.L. and G.M.; Project Administration: A.S., M.B. and M.M.L.; Funding Acquisition: M.M.L. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Azienda Ospedaliera Universitaria di Verona (protocol code CRCFC-CEPPO026 and CRCFC-CEPPO031).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Sequences of the 54 *Achromobacter* spp. isolates from the Verona collection have been deposited at the NCBI SRA database under project n. PRJEB40979.

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Conflicts of Interest: The authors declare no conflict of interest.

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Adaptive Interactions of *Achromobacter* spp. with *Pseudomonas aeruginosa* in Cystic Fibrosis Chronic Lung Co-Infection

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Abstract: In the lungs of patients with cystic fibrosis (CF), the main pathogen *Pseudomonas aeruginosa* is often co-isolated with other microbes, likely engaging in inter-species interactions. In the case of chronic co-infections, this cohabitation can last for a long time and evolve over time, potentially contributing to the clinical outcome. Interactions involving the emerging pathogens *Achromobacter* spp. have only rarely been studied, reporting inhibition of *P. aeruginosa* biofilm formation. To evaluate the possible evolution of such interplay, we assessed the ability of *Achromobacter* spp. isolates to affect the biofilm formation of co-isolated *P. aeruginosa* strains during long-term chronic co-infections. We observed both competition and cohabitation. An *Achromobacter* sp. isolate secreted exoproducts interfering with the adhesion ability of a co-isolated *P. aeruginosa* strain and affected its biofilm formation. Conversely, a clonal *Achromobacter* sp. strain later isolated from the same patient, as well as two longitudinal strains from another patient, did not show similar competitive behavior against its *P. aeruginosa* co-isolates. Genetic variants supporting the higher virulence of the competitive *Achromobacter* sp. isolate were found in its genome. Our results confirm that both inter-species competition and cohabitation are represented during chronic co-infections in CF airways, and evolution of these interplays can happen even at the late stages of chronic infection.

Keywords: inter-species interactions; *Achromobacter* spp.; *Pseudomonas aeruginosa*; lung infection; cystic fibrosis

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1. Introduction

Development of chronic lung infections and progressive inflammation is the major cause of morbidity and ultimate mortality for patients with cystic fibrosis (CF) [1]. Colonization with *Pseudomonas aeruginosa*, the most common pathogen isolated from CF airways, is particularly difficult to eradicate and is associated with an accelerated decline in lung function, with a poor prognosis [2]. Other respiratory pathogens play a role at different stages of the lung disease: *Staphylococcus aureus* and *Haemophilus influenzae* are the main pediatric pathogens, while *Burkholderia cepacia* complex, *Achromobacter* spp., *Stenotrophomonas maltophilia* and nontuberculous mycobacteria are mainly found in adults [3]. In particular, in the last decade, *Achromobacter* spp. gained attention as important emerging pathogens that can cause severe chronic infections in CF patients, associated with lung inflammation and decline in respiratory function [4–9] and further complicated by their innate and

acquired multidrug resistance hindering eradication therapies [10,11]. The *Achromobacter* genus comprises 22 species [12]; *Achromobacter xylosoxidans* is the most often isolated species among CF patients, followed by *Achromobacter ruhlandii*, *Achromobacter insuavis*, *Achromobacter insolitus*, *Achromobacter dolens*, *Achromobacter agrifaciens* and *Achromobacter spanius* [7,13–17].

Due to the polymicrobial nature of CF lung infection, it is likely that microbes could engage in inter-species interactions, acting competitively or synergistically with each other to gain an adaptive advantage, thereby influencing the community composition, resistance to antibiotics and the course of airway disease [18,19]. In the case of chronic co-infections, this cohabitation can last for a long time and likely evolve. Interactions are usually favored by microbial proximity promoted by intra- and inter-species co-aggregation in biofilm communities [20,21]. The biofilm mode of growth, typical of CF chronic infections, allows bacteria to form highly organized, structured aggregates attached on the epithelial surface that protect the community from mechanical forces and penetration of chemicals [22,23]. Thus, biofilms decrease bacterial susceptibility to antimicrobial agents, promoting bacterial tolerance and/or resistance and favoring the failure of eradication therapies [24].

P. aeruginosa is often co-isolated with other microbial species sharing the same environment. While its interactions—including both cooperation and competition—with classical pathogens *Burkholderia* spp. and *S. aureus* have been extensively studied [25–32], the available information regarding interactions with emerging pathogens such as *Achromobacter* spp. and *S. maltophilia* is still limited. Despite the reported co-isolation of *P. aeruginosa* and *Achromobacter* spp. from sputum samples and the increasing number of patients becoming chronically infected with the latter [6,9,33,34], thus far, only one recent study evaluated the occurrence of inter-species interactions between these two microorganisms, reporting that *P. aeruginosa* biofilm formation can be affected by *A. xylosoxidans* [35]. To evaluate the possible evolution of such interplay and its underlying mechanisms, in the present study, we assessed the ability of *Achromobacter* spp. isolates to affect the biofilm of *P. aeruginosa* strains sharing the same lung environment during long-term chronic co-infections and searched for genetic features of virulence possibly associated with the competition ability.

2. Results

P. aeruginosa and *Achromobacter* spp. clinical isolates were longitudinally collected from two CF patients chronically co-infected for over 9 years: patient A since 1996, and patient B since 1999. For each patient, the two species were isolated from the same sputum sample twice: in 2005 and 2008 from patient A, and in 2008 and 2014 from patient B. The general information on each isolate is presented in Table 1. The genotypic relatedness of longitudinal isolates was verified by core genome similarity: *P. aeruginosa* isolates from patients A and B showed 82% and 83% similarity, respectively, while *Achromobacter* spp. isolates from both patients showed 87% similarity. While all *Achromobacter* spp. isolates had been initially identified as *A. xylosoxidans* [36], a recent phylogenetic analysis reclassified the isolates from patient A as *A. insuavis* [37]. The first *A. insuavis* strain collected from this patient, named isolate A1, was previously classified as a hypermutator [36]. *P. aeruginosa* isolates from patient A belong to the DK08 clone type, sampled from multiple patients at the Copenhagen CF Center [38].

Table 1. Identification and time of isolation of each clinical strain.

Patient	Isolate	Species	Year of Isolation	Other Characteristics
A	A1	*A. insuavis*	2005	Hypermutator
A	P1	*P. aeruginosa*	2005	DK08 clone type
A	A2	*A. insuavis*	2008	
A	P2	*P. aeruginosa*	2008	DK08 clone type
B	A3 *	*A. xylosoxidans*	2008	
B	P3	*P. aeruginosa*	2008	
B	A4 *	*A. xylosoxidans*	2014	
B	P4	*P. aeruginosa*	2014	

* A3 and A4 isolates were called B1 and B2 in a previous study [36].

2.1. Phenotypic Variations

To investigate possible phenotypic variations within the same host over time, we evaluated features such as growth rate and adhesion, which are known to often undergo modifications during bacterial adaptation into the CF lung. We previously observed that no significant changes in terms of growth rate and adhesion ability occurred over time within the longitudinal *Achromobacter* spp. isolates from the two patients [36]; growth curves are shown in Figure S2. On the contrary, *P. aeruginosa* isolates underwent the phenotypic evolution known to occur during CF chronic infection: the growth rate significantly diminished over time in both patients, while the adhesion ability increased significantly in patient A (Figure 1).

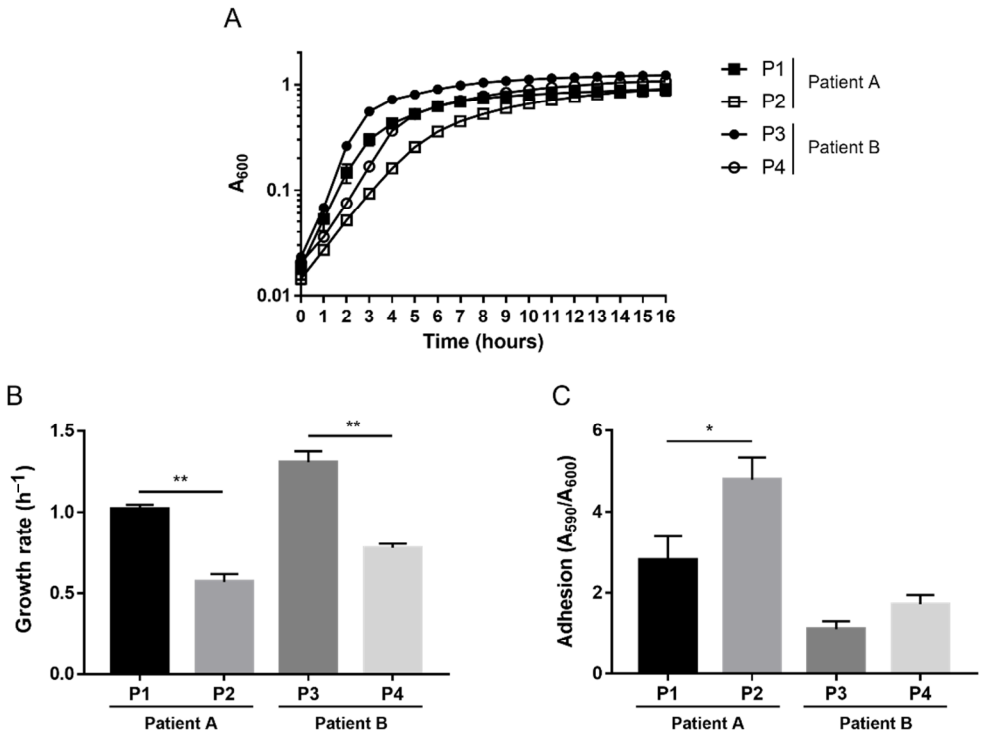

Figure 1. Growth curves (**A**), growth rate (**B**) and adhesion (**C**) of *P. aeruginosa* isolates. For growth curves, absorbance at 600 nm (A_{600}) was measured every hour for the first 16 h (**A**). The growth rate was calculated from the exponential phase of growth curves (**B**). Adhesion was measured by absorbance of crystal violet-stained surface-attached bacteria (A_{590}) divided by absorbance of planktonic bacteria (A_{600}) (**C**). Each value represents the mean ± SEM of three experiments. Statistical analysis was performed by *t*-test, * $p < 0.05$, ** $p < 0.01$.

2.2. Effects of Achromobacter spp. on P. aeruginosa Adhesion

To investigate whether *Achromobacter* spp. isolates exhibited competition against *P. aeruginosa*, we first evaluated the potential effects on its adhesion. This is an essential ability for biofilm formation, which is considered a key feature for the successful colonization of CF lungs by *P. aeruginosa* and other bacterial species. The adhesion of each *P. aeruginosa* strain was measured in the absence and presence of the culture supernatant collected from the co-isolated *Achromobacter* sp. strain. The culture supernatant contained all the exoproducts released during bacterial growth, including virulence factors; e.g., we previously observed a higher protease activity in an A1 culture supernatant than in supernatants from the other *Achromobacter* spp. isolates [36]. The P1 isolate showed a significantly lower adhesion ability when grown in the presence of A1 exoproducts (Figure 2). No such inhibitory effect was exhibited by the culture supernatants of the other *Achromobacter* spp. strains on their *P. aeruginosa* co-isolates, nor on the *P. aeruginosa* laboratory strain PAO1 (Figure S3).

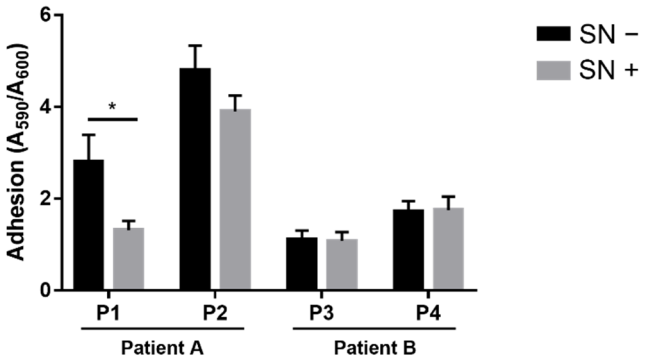

Figure 2. *P. aeruginosa* adhesion in the absence and presence of the co-isolated *Achromobacter* spp. culture supernatant (SN−, SN+). Adhesion was measured by crystal violet staining of surface-attached bacteria divided by A_{600} of planktonic bacteria. Each value represents the mean ± SEM of 3 experiments. Statistical analysis was performed by *t*-test, * $p < 0.05$.

2.3. Effects of Achromobacter sp. on P. aeruginosa Biofilm Formation

To investigate whether the A1 isolate could also inhibit *P. aeruginosa* biofilm formation, mixed biofilm cultures were grown in a flow chamber system for up to 5 days. To distinguish the two species, *P. aeruginosa* strains were tagged with the green fluorescent protein (GFP), and the fluorescence emission was checked (Figure S1). In single-species cultures, as expected, *P. aeruginosa* isolates could form big, stable aggregates firmly attached on the glass surface (Figure 3A,B). On the contrary, *Achromobacter* sp. strains showed a poor adhesion ability on glass, forming sporadic, unstable aggregates characterized by the scattering and dispersal of planktonic cells (Figure 3C). When the two microbes were cultured together, *Achromobacter* sp. could adhere and form mixed biofilms with *P. aeruginosa*. However, the *Achromobacter* sp. A1 isolate interfered with the biofilm formation of the co-isolated *P. aeruginosa* P1 strain. As shown in Figure 3D, P1 aggregates are smaller in the presence of A1, as also confirmed by the *P. aeruginosa* biomass quantification (Figure 3F). Such inhibitory effect was not observed in mixed biofilms formed by their longitudinal co-isolates A2 and P2 (Figure 3E).

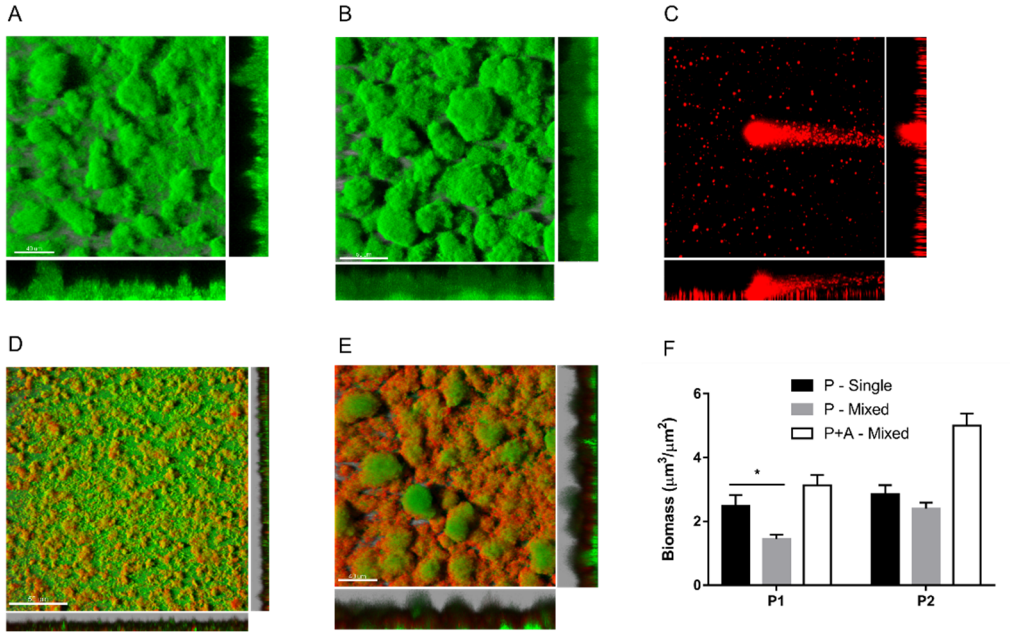

Figure 3. Single-species biofilms formed by *P. aeruginosa* P1 (**A**) and P2 (**B**) isolates, representative image of *Achromobacter* sp. biofilm structures (**C**), mixed biofilms formed by P1 + A1 (**D**) and P2 + A2 (**E**) strains and biomass quantification (**F**). Biofilms were grown in a flow chamber system for 5 days and monitored by confocal microscopy. *P. aeruginosa* isolates were tagged with GFP (green), and *Achromobacter* sp. cells were counterstained with Syto62 (red). *P. aeruginosa* biomass in single and mixed biofilms (P-Single, P-Mixed) and total biomass of mixed biofilms (P+A-Mixed) were calculated using Comstat2 software. Each value represents the mean ± SEM of 3 experiments. Statistical analysis was performed by the Mann–Whitney test, * $p < 0.05$.

2.4. Genetic Variants in Achromobacter sp. Virulence Genes

Previously, we performed variant analysis of *Achromobacter* spp. isolates and observed that the A2, A3 and A4 genomes harbor no or few mutations, with a predicted high impact on protein function, while various frameshift mutations and a stop gain were detected in the A1 genome [36]. To find genetic evidence that could explain the observed inhibitory effect of the A1 isolate on the adhesion and biofilm formation of the P1 strain, we evaluated whether some of the genetic variants in the A1 genome involve genes related to virulence and inter-species competition. Interestingly, in A1, but not in the A2 genome, we detected the presence of a type VI secretion system tip protein, the VgrG gene, whose product is reported to bind antibacterial effectors targeting essential cell structures during inter-species competition between Gram-negative bacteria such as *Acinetobacter baumannii* [39]. Moreover, we found a stop gain in the HlyD family efflux transporter periplasmic adaptor subunit gene, whose product is a component of type I secretion systems involved in the secretion of virulence factors such as toxins and proteases [40]. When compared to the reference genome, this A1 gene results in a slightly shorter protein (−15 amino acids), while for the A2 strain, the predicted length of the same gene product is largely reduced (−129 amino acids). Although no data are available regarding the effect of these variants on the protein function, we can hypothesize that the HlyD protein is more likely functional in the A1 rather than the A2 isolate.

3. Discussion

For a long time, CF lung infection has been studied and treated as a disease caused by a single pathogen, while, nowadays, we are aware of its polymicrobial nature [41,42]. Within microbial communities, intra- and inter-species interactions can take place and potentially influence the course of the infection [18,19]. Interactions involving the emerging pathogens *Achromobacter* spp. have only rarely been studied, probably because the clinical relevance of

this microorganism became evident more recently. Nonetheless, their increased prevalence in CF and their frequent co-isolation with other pathogens such as *P. aeruginosa* suggest that *Achromobacter* spp. likely have to compete for space and nutrients [43]. In the case of chronic co-infections, where cohabitation can last for a long time, the evolution of such interplays might as well be part of the bacterial adaptation processes known to occur in CF airways. In the present investigation, we focused on *Achromobacter* spp.'s behavior towards *P. aeruginosa* within biofilm communities and observed both competition and cohabitation interplays during chronic co-infections.

As regards the biofilm mode of growth, *Achromobacter* spp. are motile (swimming) via long, peritrichous flagella but lack twitching motility [44,45], which can contribute to the development of a surface-attached biofilm as it may help in stabilizing interactions with the surface [46]. Indeed, the poor adhesion ability of this microorganism in vitro (on polymeric surfaces within 48 h) has been reported [45]. A reduction in surface attachment over time during infection was also shown in sequential CF isolates, in association with the acquisition of mutations in genes with a presumptive role in surface adhesion [47]. However, some studies highlighted *Achromobacter* spp.'s ability to adhere on hydrogel contact lenses [48,49] and to form unattached or loosely attached aggregates held together by polysaccharides forming a peripheral shell around the bacterial cells [45,50]. Our current and previous [36] results confirm the poor adhesion ability of *Achromobacter* spp. on polystyrene and glass, and the formation of loosely attached aggregates characterized by the scattering and dispersal of planktonic cells. Interestingly, when cultured with *P. aeruginosa*, the two microbes could form mixed biofilms, suggesting that *Achromobacter* spp.'s adhesion might be enhanced on biotic surfaces.

Concerning inter-species interactions, we observed that only the A1 isolate has inhibitory effects against the co-isolated *P. aeruginosa* strain, interfering with its adhesion ability and affecting its biofilm formation capability. Conversely, the clonal strain A2 later isolated from the same patient, as well as two *Achromobacter* sp. strains longitudinally collected from another patient, did not show similar competitive behavior against their *P. aeruginosa* co-isolates. Thus far, only one recent study evaluated the occurrence of inter-species interactions between *Achromobacter* sp. and *P. aeruginosa* CF isolates, reporting that *P. aeruginosa* biofilm formation can be affected by *A. xylosoxidans* [35]. The isolate showing competition in our study belongs to a different species, *A. insuavis*, suggesting that this behavior might be common to various species of the genus. Interestingly, this isolate was previously classified as a hypermutator [36], and the isolates that Menetrey and colleagues observed to affect the *P. aeruginosa* biofilm were morphologically different clones collected from the same sputum sample of a chronically infected patient [35], a situation often exactly associated with the presence of hypermutators [51]. Although no genomic data are available from their study—limiting the evaluation of the hypermutation contribution—the association of this evolutionary mechanism with *Achromobacter* spp.'s competitive behavior should be further verified.

Investigating the genomic features that could be implicated in the observed competition, in the genome of the A1 isolate, we identified genetic variants supporting its higher virulence. Only in this isolate, we detected a type VI secretion system tip protein, the VgrG gene, whose product is known to be involved in inter-species competition between Gram-negative bacteria [39]. Additionally, *VgrG* paralogues have also been reported to regulate bacterial motility, biofilm formation and protease production in *Aeromonas* sp. [52]. Moreover, the A1 strain harbored a likely functional *HlyD* gene, while a deleterious variant was present in the genome of its clonal late isolate. HlyD is essential for the secretion of the RTX hemolytic toxin HlyA from *Escherichia coli* [53] and seems to be involved in the protease secretion mechanisms of P. aeruginosa [54]. We previously observed a higher protease secretion from the A1 strain in comparison to its clonal late isolate and to isolates from patient B [36], further supporting a higher expression of virulence traits in this isolate that could be involved in the observed competition against *P. aeruginosa*. Additionally, the genes related to the quorum sensing system were checked in all the *P. aeruginosa* isolates for the

presence of variants, but no mutations were detected that could indicate down-regulation of this system.

Interestingly, 3 years after the A1 and P1 strains' isolation, their competitive interplay evolved towards a more indolent cohabitation or even cooperation—similar to the situation observed in patient B—which might represent a survival advantage. Although major adaptations of bacteria causing CF chronic infections are likely to happen during the early stage of chronic infection, while in the late stage, the situation is supposedly more stable, we observed variations in the interplay between two microorganisms in the late stage of a chronic co-infection, suggesting that adaptive mechanisms are still ongoing. In the later stage, isolates A2 and P2 could also grow together in mixed biofilm communities, supporting the possibility that close microbial interactions might occur between them. Indeed, bacterial proximity within biofilm communities can favor social exchanges of signal molecules and genetic elements, influencing many aspects of the community itself such as the microbial composition, nutrient availability, and antibiotic resistance [21]. Although the occurrence of close microbial interactions within the CF airway has not been demonstrated, in this particular case, their relevance should be considered: *Achromobacter* spp. are usually rich in mobile genetic elements carrying antibiotic resistance [11,55], whose exchange with and acquisition by other microbes such as *P. aeruginosa* might influence the course of infection and the outcome of antibiotic therapies.

In conclusion, despite our observations being limited to restricted mechanisms on a small number of selected strains, our results show that both inter-species competition and cohabitation are represented during chronic co-infections in CF airways, and evolution of these interplays can happen at the late stages of chronic infection. Furthermore, we provided insights on virulence mechanisms that could be involved in *Achromobacter* spp.'s competitive abilities. Future studies on a larger scale, involving more strains from more patients, are needed to better understand the interplay between competition and adaptation in the lungs of CF patients. Further mechanisms involved in inter-species interactions should also be explored, such as regulation of quorum sensing and secretion of specific virulence factors or metabolic by-products. In addition, evaluating interactions involving other microbial species would increase insights into the extent and complexity of such interplays and their contribution to the clinical outcome. This highlights the importance and necessity of further studies with a larger number of isolates, encouraging further research on this subject.

4. Materials and Methods

4.1. Bacterial Isolates

Four clinical isolates of *Achromobacter* spp. and *P. aeruginosa* were collected from two CF patients followed in the CF clinic at Rigshospitalet in Copenhagen, Denmark. The use of the stored bacterial isolates was approved by the local ethics committee at the Capital Region of Denmark (Region Hovedstaden) with the registration number H-4-2015-FSP. *P. aeruginosa* and *Achromobacter* spp. were isolated from the same sputum sample twice from each patient: in 2005 and 2008 from patient A, and in 2008 and 2014 from patient B.

4.2. Growth Curves

Bacterial strains were plated on LB agar and incubated at 37 °C for 24–48 h. One colony was picked from the plate and inoculated in 10 mL LB medium, with shaking at 37 °C overnight. Optical density at 600 nm (OD_{600}) was measured using a spectrophotometer, cultures were diluted to 0.05 OD/mL in LB medium and 150 µL/well was incubated in a 96-well plate for 20–24 h, with shaking at 37 °C. Using an automated plate reader, OD_{600} was measured every 20 min. Growth rate was calculated using GraphPad Prism 7.0.

4.3. Culture Supernatant Collection

Achromobacter spp. strains were plated on LB agar and incubated at 37 °C for 24–48 h. One colony was picked from the plate and inoculated in 10 mL LB medium, with shaking

at 37 °C for 16 h. OD_{600} was measured, and cultures were diluted to 0.1 OD/mL in 10 mL of LB medium. After shaking at 37 °C for 16 h, cultures were diluted to 1 OD/mL and centrifuged at 7000× *g* for 30 min at 4 °C. Supernatants were collected and sterile filtered.

4.4. Adhesion Assay

Bacterial strains were plated on LB agar and incubated at 37 °C for 24–48 h. One colony was picked from the plate and inoculated in 10 mL LB medium, with shaking at 37 °C overnight. OD_{600} was measured using a spectrophotometer, cultures were diluted to 0.05 OD/mL in LB medium and 150 µL/well was incubated in a 96-well plate for 20–24 h at 37 °C. After measuring OD_{600}, wells were washed twice with water to remove unattached cells, and surface-attached cells were stained with 0.1% crystal violet solution for 15 min. Wells were rinsed and washed three times with water and then dried for 1–2 h. Thirty percent acetic acid was added, incubated at room temperature for 15 min, and absorbance at 590 nm was measured. Adhesion measured by crystal violet staining (absorbance at 590 nm) was normalized on growth (absorbance at 600 nm). In competition assays, *P. aeruginosa* adhesion was measured in the presence/absence of 10% *Achromobacter* spp. culture supernatant.

4.5. GFP Tagging

P. aeruginosa strains were tagged with a mini-Tn7 construct carrying gentamycin resistance and GFPmut3b genes under the control of the growth-dependent *E. coli* ribosomal promoter *rrnB* P1 [56]. The construct was introduced in *P. aeruginosa* by conjugative transfer as described by Choi and Schweizer [57]. Briefly, recipient *P. aeruginosa* strains were mixed with *E. coli* donor and helper strains (pRK2013 and pNTS2), and a drop of bacterial suspension was placed in the center of an LB agar plate and incubated at 37 °C overnight. Transconjugants were selected by plating bacteria on LB agar containing gentamycin and trimethoprim. Mini-Tn7 insertion was checked by colony PCR using PTn7R and PglmSF primers [57]. To assess the growth-dependent fluorescence emission, *P. aeruginosa* GFP-tagged strains were cultured in a 96-well plate for 12 h, with shaking at 37 °C, while fluorescence (excitation 475 nm, emission 520 nm) and OD_{600} were measured every 20 min using an automated plate reader.

4.6. Biofilm Formation Assay

A flow chamber system was assembled and sterilized following the protocol from Tolker-Nielsen and Sternberg [58]. Briefly, 250 µL of bacterial cultures (0.05 OD/mL) was injected in each flow cell channel. Flow cells were left upside-down for an hour without flow to let bacteria attach on the cover glass and then were turned and incubated at 30 °C for up to 5 days with flow (A10 minimal medium added with $MgCl_2$, $CaCl_2$ and trace metals). Biofilm formation was observed by confocal laser scanning microscopy for GFP-tagged cells and Syto62 staining of total cells. For statistical analysis, at least 7 pictures/channel were taken, homogeneously distributed along the channel. Pictures were visualized and elaborated using Imaris 7.4 software. Biomass was calculated using Comstat2 software [59].

4.7. Genomic Analysis

Whole genome sequencing and assembly were performed as previously described [36]. Sequences have been deposited at EMBL under the projects n. PRJEB35058 (*Achromobacter* spp. sequences) and PRJEB40978 (*P. aeruginosa* sequences). Sequence data can be found with the experiment accession numbers ERX3614542 (strain A1), ERX3614543 (strain A2), ERX3614548 (strain A3, previously called B1), ERX3614549 (strain A4, previously called B2), ERS5248144 (strain P1), ERS5248145 (strain P2), ERS5248146 (strain P3) and ERS5248147 (strain P4). Genotypic relatedness among longitudinal isolates was verified by checking core genome similarities obtained using the Harvest-OSX64-v1.1.2 suite [60]. Variant analysis was performed as previously described [36]. Briefly, two types of variant analysis were carried out: the first by aligning sequence reads to the reference genome, and the

second by aligning them to the de novo assembly of the longitudinal isolate from the same patient. In the first case, the annotated genomes *A. xylosoxidans* NH44784-1996 and *P. aeruginosa* PAO1 (RefSeq accessions: GCF_000967095.2 and GCF_000006765.1) were used as reference genomes. Bowtie 2 v2.3.4.1 [61] was used for performing reads alignment, and the SnpEff v4.3t toolbox [62] was used to annotate variants and predict their functional effects. Only variants supported by a minimum of 20 reads were retained.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pathogens10080978/s1, Figure S1: Growth and fluorescence emission curves of GFP-tagged *P. aeruginosa* isolates; Figure S2: Growth curves of *Achromobacter* spp. isolates, Figure S3: Adhesion of *P. aeruginosa* PAO1 strain in absence and presence of the culture supernatants collected from the clinical *Achromobacter* spp. isolates.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki. The collection and use of the bacterial isolates used in this study were approved by the local Ethics Committee at the Capital Region of Denmark (Region Hovedstaden) with registration number H-4-2015-FSP.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study as approved by the Ethics Committee.

Data Availability Statement: Sequencing data are publicly available at EMBL under the projects n. PRJEB35058 (*Achromobacter* spp. sequences) and PRJEB40978 (*P. aeruginosa* sequences).

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