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Microbiome in cystic fibrosis: Shaping polymicrobial interactions for advances in antibiotic therapy

Susana P. Lopes[1], Nuno F. Azevedo[2], and Maria O. Pereira[1]

[1]IBB-CEB, Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, University of Minho, Campus de Gualtar, Braga, Portugal
[2]LEPABE, Department of Chemical Engineering, Faculty of Engineering, University of Porto, Porto, Portugal

Abstract

Recent molecular methodologies have demonstrated a complex microbial ecosystem in cystic fibrosis (CF) airways, with a wide array of uncommon microorganisms co-existing with the traditional pathogens. Although there are lines of evidence supporting the contribution of some of those emergent species for lung disease chronicity, clinical significance remains uncertain for most cases. A possible contribution for disease is likely to be related with the dynamic interactions established between microorganisms within the microbial community and with the host. If this is the case, management of CF will only be successful upon suitable and exhaustive modulation of such mixed ecological processes, which will also be useful to predict the effects of new therapeutic interventions.

Keywords: Cystic fibrosis; microbial diversity; polymicrobial biofilms; microbial interactions; antibiotic therapy

CF lung environment – the key for microbial diversity

Pulmonary infections caused by bacterial species are recognized as the major cause of morbidity and mortality of cystic fibrosis (CF) patients, leading to premature death in 90% of cases (Rajan and Saiman, 2002). The respiratory tract of CF patients is a compartmentalized niche, which is spatially and temporally heterogeneous according to the anatomic site and to the period of disease evolution (Hélène et al, 2012). The viscous and dehydrated mucus formed on the epithelial-cell surface of the CF airways is composed of heterogeneous availabilities of antibiotics and nutrients (e.g. products of inflammatory cell death, such as DNA and actin polymers), as well as steep oxygen gradients (with zones ranging from aerobic to completely anaerobic) (Yang et al, 2011), which altogether constitute selective forces that may drive the selection and evolution of microbes. Therefore, CF airways offer a favorable environment for the colonization and proliferation of a large variety of microbes, contributing to the persistence of the infection. It is suggested that the microbiome composition may be a great predictor of disease progression, *i.e.* on severity and outcome (Klepac-Ceraj et al, 2010; Delhaes et al, 2012; Peters et al, 2012).

The polymicrobial communities in CF may be defined as a varied collection of organisms (bacteria, fungi, and viruses), with bacterial species being probably the most frequently isolated microbes and presenting a wide number of phylogenetically diverse bacterial genera already detected (Guss et al, 2011). Within these highly diverse bacterial communities, *Pseudomonas aeruginosa* is recognized as the most significant and the most commonly isolated pathogen (Lipuma, 2010). However, the recent use of efficient microbiological diagnostic tools, particularly molecular technologies, has facilitated the identification of a wide spectrum of atypical microorganisms, evidencing a polymicrobial nature of the CF airways (Bittar et al, 2008; Guss et al., 2011).

Although the full pathogenic potential of most unusual species remains unclear, relevant recent research on microbial interactions between atypical and conventional CF-species might provide knowledge concerning the real role on the pathogenicity associated to those unusual pathogens and in parallel in the advance of novel therapeutic strategies for the management of the disease.

With this review, it is intended to provide a general outline of the main aspects about CF lung disease, carefully emphasising the microbiome composition in CF airways, including the major pathogens and the emergent microorganisms. Lastly, several relevant microbial interactions recently reported and the significance that such ecological and evolutionary processes shaping the CF microbial communities may have on CF antibiotic treatment will also be assessed.

Pathophysiology of the CF lung disease

Cystic fibrosis was first described in 1938, as a result of the observation by Dorothy Andersen of scar (fibrosis) tissue and formation of cysts within the pancreas of a human patient (Andersen, 1938). CF is caused by mutations on the CF transmembrane conductance regulator (CFTR) gene (230 kb) encoding a protein with 1480 aminoacids. Over than 1900 mutations have been identified (http://www.genet.sickkids.on.ca/cftr/app/, accessed June 19th, 2013) to date in CFTR. The most prevalent of those mutations (ΔF508) is the deletion of three nucleotides, at the position 508 of the protein sequence, which corresponds to the loss of the aminoacid phenylalanine (Figure 1). CFTR acts as a chloride channel in apical cell membranes of multiple organs (e.g. respiratory, digestive, reproductive and sweat glands) epithelia. Therefore, its malfunction may lead to serious complications in varied organs

(Radlovic, 2012), of which the respiratory system is affected with higher frequency and severity, with major cause of morbidity and mortality in CF patients (Heijerman, 2005). In the lungs, the defective chloride ion transport across epithelial cell surface often results in the decrease of the volume of the periciliary fluid in the lower respiratory tract, compromising the mucociliary clearance (Boucher, 2004a). Thus, the CF lung disease results from the overproduction of dehydrated and viscous mucus that chronically blocks the airways and hampers the respiration of the patients (Figure 2). This often encourages the persistent colonization of bacteria in the lungs, resulting in the subsequent intermittent cycles of bacterial infections and persistent inflammatory responses, which ultimately lead to progressive lung injury (Lubamba et al, 2012).

CF affects different racial and ethnical groups, but is more common among Caucasians (white people) (Cystic Fibrosis Foundation, Patient Registry, Annual Data Report 2010). It is an autosomal recessive disease, since the effect of CF is hidden by the presence of a working copy of the CFTR gene (i.e. CF only develops when neither of the two copies of the CFTR gene present in the body cells works normally).

The CF airways microbiome

Traditionally, the detection and identification of microbial species from CF respiratory samples has relied on culture-based techniques. However, it is well accepted that culture only detects a limited number of microbes and occasionally misidentifies emergent microorganisms (Bittar and Rolain, 2010). Over the past decades, a significant progress on the development of molecular approaches, including polymerase chain reaction – PCR, electrophoretic profiling (*e.g.* terminal restriction fragment length polymorphism - T-RFLP; denaturing/temperature gradient gel electrophoresis – DGGE/TGGE), microarrays, high-throughput parallel sequencing,

16S, 18S or ITS (Internal transcribed spacer) gene sequencing, has led to the detection and identification of a far more diverse microbial community in the CF airways, revealing the polymicrobial nature of CF-associated infections (Rogers et al, 2003; Rogers et al, 2004; Sibley et al, 2006; Bittar et al., 2008; Guss et al., 2011; Delhaes et al., 2012; Willner et al, 2012; Zhao et al, 2012). These polymicrobial communities are not static populations, and contain specific groups of microbes highly associated with disease-derived factors (*e.g.* antibiotic selective pressure) and/or other perturbations (*e.g.* changes in pH, temperature oxygen) (Conrad et al, 2013; Lynch and Bruce, 2013). Substantial shifts in the airway microbiome composition, namely on the community richness (*i.e.* absolute counts of different types of microbes), evenness (*i.e.* relative distribution/abundance of community members) and diversity (*i.e.* the index estimated by into account the richness and evenness, giving information about the rarity and commonness of species in the community) are very likely to occur in CF. In general, these parameters have a significant negative correlation with patient age (reducing for older patients) and in parallel with the decline in pulmonary health (Cox et al, 2010). Furthermore, shifts from clinically stability to episodes of exacerbations may lead to alterations in the relative abundance of species within the community (Carmody et al, 2013). A deep characterization of these polymicrobial communities in CF will certainly provide a better understanding of the relationship between the lung microbiome, disease pathogenesis and treatment outcome.

In this section, we will first focus on the traditional pathogens, which are herein defined as those species that are recurrently recovered from CF respiratory secretions and with undisputed pathogenic potential. An exhaustive list with the emergent organisms, many of them considered atypical, for which pathogenic potential and clinical significance still remains to be determined, will then be assessed.

Traditional bacterial pathogens

A limited number of species are increasingly recognized to significantly contribute for CF lung disease, with prevalence dependent from patient-age (Figure 3). *Staphylococcus aureus* and *Haemophilus influenzae* are the most common pathogens in younger CF patients (Burns et al, 1998; Lambiase et al, 2006), with *S. aureus* being the first to infect and colonize children (Saiman and Siegel, 2004), reaching a prevalence rate of nearly 50 % by the age of 10 years. This organism has been well recognized as a potential pathogen, causing epithelial damage (Lyczak et al, 2002) and worsening the inflammatory response when co-colonized with *P. aeruginosa* (Sagel et al, 2009). Hypermutability and formation of robust biofilms (Hauser et al, 2011) has significantly contributed to the adaptability of *S. aureus* to CF lung environment. Additionally, the incidence of the small colony variant phenotype and methicillin-resistant *S. aureus* is progressively increasing in the CF lung, showing potential threats for adult patients (Spicuzza et al, 2009). *Haemophilus influenzae* also presents high prevalence rates within pediatric patients (~20 %), and is capable to form biofilms in the epithelial surface, persisting and causing disease pathogenesis (Starner et al, 2006). The high prevalence of hypermutable strains of *H. influenza* is likely to benefit the species by promoting a faster adaptation to the changing CF lung environment, for instance when an antibiotic therapy is started (Watson et al, 2004).

By 18 years of age, 80 % of patients are colonized with *P. aeruginosa,* whereas 3.5 % harbor bacteria from the *Burkholderia cepacia* complex (BCC) group (Hoiby, 2011). *P. aeruginosa* is considered the key CF pathogen, both in terms of prevalence and pathogenicity, and is clearly associated with the reduced life expectancy in CF patients (Lyczak et al., 2002). The ability of *P. aeruginosa* to develop a biofilm in CF airways

155 is well recognized (Worlitzsch et al, 2002; Boucher, 2004b). Hassett and colleagues
156 (Hassett et al, 2002) proposed two models for biofilm formation by *P. aeruginosa*
157 (Figure 4), among which one that is supposed to better represent mono-species biofilms
158 formed in CF airway *in vivo*, with *P. aeruginosa* embedded in the dehydrated viscous
159 mucus. *P. aeruginosa* early colonization in the mucus often results in acute persistent
160 infection, with the pathogen in the non-mucoid form. The adaptive evolution of *P.*
161 *aeruginosa* to the CF mucus environment rapidly evolves throughout a series of genetic
162 and phenotypic mutations, by conversion to a mucoid phenotype (due to alginate
163 overproduction) and formation of biofilm (Hoiby et al, 2010a). The alginate allows
164 protection of *P. aeruginosa* biofilm against stressful conditions such as the action of
165 the immune cell system (Hoiby et al, 2001; Hoiby et al, 2010b), osmotic and oxidation
166 stresses and eradication by antibiotic treatment (Yang et al, 2008; Hoiby et al., 2010a).
167 Thus, the mucoid phenotype of *P. aeruginosa* is often correlated with the decline of CF
168 lung function and increased tissue damage. Also, the high rate of hypermutability
169 (Kenna et al, 2007), and intrinsic antibiotic resistance mechanisms (*e.g* multi-drug
170 efflux pumps and an impermeable outer membrane) have considerably contributed to
171 the well-adaptation of *P. aeruginosa* to the CF environment (Worlitzsch et al., 2002;
172 Yoon et al, 2002). The survival of *P. aeruginosa* into anaerobic mucus layers is also
173 recognized (Worlitzsch et al., 2002; Yoon et al., 2002), conferring the organism
174 enhanced tolerance to many antibiotics (Borriello et al, 2004).
175 The 17 members of the BCC group are phenotypically indistinguishable but some of
176 them (*B. multivorans*, *B. cenocepacia*, *B. cepacia*, and *B. dolosa*) are highly
177 transmissible, have pathogenic potential, are very resistant to antibiotic therapy (Miller
178 and Gilligan, 2003) and may lead to a fatal pneumonia known as “cepacia syndrome”
179 (Vandamme et al, 2003). *B. cenocepacia,* initially the member most commonly isolated

from CF patients, accounts for the majority of CF infections caused by the BCC group, presenting a high arsenal of virulence traits (*e.g.* biofilm formation-ability, production of secretion systems, formation of colony variants, presence of lipopolysaccharide and other cell envelope structures) that has been associated to an almost pandrug-resistance of the species (Loutet and Valvano, 2010; Suppiger et al, 2013).

Emergent microorganisms

In addition to the bacterial species documented as CF pathogens, culture-independent approaches have revealed a far greater microbial diversity than the one previously recognized (Table 1). These CF dynamic communities, containing as many as 100 to 1000 bacterial species (Harris et al, 2007; Klepac-Ceraj et al., 2010), still involve many other microbial species, which remain to be characterized. This complex diversity suggests that the microbiome of the CF airways niche is far from being fully described. Among the bacteria increasingly identified in the sputum of patients with CF are anaerobes, which numbers are comparable to those of the typical aerobic pathogens (Bittar et al., 2008; Tunney et al, 2008; Guss et al., 2011), refuting the hypothesis of contamination from the oral cavity. Since the 1990s, the ubiquitous environmental organism nontuberculous mycobacteria has been increasingly isolated from the sputum of patients with CF (Torrens et al, 1998; Valenza et al, 2008), and has now a recognized clinical significance, with a role in the transition of the infection from acute to chronic and lifelong (Pierre-Audigier et al, 2005).

Likewise, there is increasing evidence of diverse fungi having an impact in CF. These include species from *Aspergillus* (Bakare et al, 2003), *Candida* (Chotirmall et al, 2010) and *Scedosporium* genera (Bouchara et al, 2009; Blyth et al, 2010). The majority of these fungal infections are caused by opportunistic molds, with different fungal species

205 presenting variable rates of prevalence, reflecting variations in the geographic
206 distributions and/or lacking of standardization of the mycological examination methods
207 (Bouchara et al., 2009). *A. fumigatus* and *C. albicans* are the most commonly recovered
208 fungi from CF patients (Cimon et al, 2000; Bakare et al., 2003), with the first species
209 being responsible for various diseases in CF patients, the most common being allergic
210 bronchopulmonary aspergillosis (de Almeida et al, 2006). More recently, *Pneumocystis*
211 *jirovecii*, an opportunistic fungus that causes pneumonia in immunosuppressed
212 individuals, has emerged in Brazilian and European CF patients (Gal et al, 2010;
213 Pederiva et al, 2012).
214 Also, viral populations (*e.g.* adenovirus, influenza A and B, respiratory syncytial virus
215 – RSV, rhinovirus) are present in CF polymicrobial communities, with rhinovirus
216 showing high prevalence in a number of studies (Olesen et al, 2006; de Almeida et al,
217 2010; Kieninger et al, 2013). If initially the impact of respiratory viruses could have
218 been underestimated because of the low detection rate by conventional laboratory
219 techniques (usually tissue culture), the advent of new viral detection techniques have
220 further enhanced the awareness of respiratory viruses in CF exacerbations. Respiratory
221 viruses such as the RSV, influenza and rhinovirus have been linked to an increased risk
222 of exacerbations, leading to the deterioration in clinical status in CF (Wat et al, 2008;
223 Wark et al, 2012; Kieninger et al., 2013). However, the presence of viral communities
224 seems not to affect the type or frequency of bacterial infection (Olesen et al., 2006).
225 Although there are already some preliminary data about the implication of some
226 unusual species in the pathophysiology of CF (Waters et al, 2007; Ulrich et al, 2010;
227 Costello et al, 2011), even with such findings, the pathogenesis and clinical relevance
228 of these emergent microorganisms remain unclear. In effect, the adaptation to the CF

airways niche, the interactions between organisms, the impact on the respiratory status of CF patients and even the antimicrobial susceptibility pattern still is to be determined.

Host-microbe and microbe-microbe interactions in CF polymicrobial communities

Social interactions among microorganisms are central to the functioning of any microbial community (Hansen et al, 2007). The large variety and concentration of microbes present within polymicrobial communities, living in close proximity, drive for species-specific physical and chemical interactions that have been developed over thousands of years of coevolution (Peters et al., 2012). In CF, microbiome diversity leads to potential interactions between microbes, which may influence the behaviour of the individual species, the activities of the community as a whole, and the relationships between the host and microbial population. While a few studies have provided information on interactions between the typical CF-associated bacteria (Tomlin et al, 2001; Hoffman et al, 2006; Palmer et al, 2007), only a limited number of studies have demonstrated interactions displayed by some emergent organisms in CF context (Figure 5). Some studies have shown that the virulence of known CF pathogens, such as *P. aeruginosa,* is clearly stimulated by the presence of several species (including anaerobes) previously thought to be as clinically insignificant (Duan et al, 2003; Sibley et al, 2008). Also, *Stenotrophomonas maltophilia* leads to altered biofilm formation and increased resistance to antibiotics by *P. aeruginosa* (Twomey et al, 2012). Modulation of *P. aeruginosa* gene expression is presumably influenced by interactions between bacterial species mediated by intercellular signalling molecules. Fungal-bacterial interactions are also well recognized in diverse contexts (Frey-Klett et al, 2011). In CF, antagonistic relationships were found between *P. aeruginosa* and the fungal species

Aspergillus fumigatus and *Candida albicans*, with the small diffusive molecules secreted by *P. aeruginosa* inhibiting the filamentation and the subsequent biofilm formation of those fungal populations (Holcombe et al, 2010; Mowat et al, 2010). Additionally, relationships between bacteria and viruses are becoming well-explored in literature. A good example is the control of bacterial populations such as *P. aeruginosa*, *B. cenocepacia* and *S. aureus* by bacteriophages – viruses that infect bacteria (Carmody et al, 2010; Hsieh et al, 2011; Morello et al, 2011), with phages producing hydrolases that degrade bacterial exopolysaccharides (Donlan, 2009; Glonti et al, 2010). Conversely, bacteriophages may act as vehicles for bacterial resistance in CF airways. The higher abundance of phage communities present in the respiratory tract of CF patients (Willner et al, 2009), encompassing a reservoir of mobile genes associated to antimicrobial resistance often result in the spread of virulence among bacteria. The consequence is the alteration of the bacterial genome, resulting in adaptation and in the emergence of multi-drug resistant bacteria in the CF airways (Rolain et al, 2009; Fancello et al, 2011; Rolain et al, 2011). Likewise, the presence of other viruses in CF airway stimulate the bacterial adherence by major pathogens such as *S. aureus*, *H. influenza* and *Streptococcus pneumoniae* (Smith et al, 1976).

Recently, the atypical bacteria *Inquilinus limosus* and *Dolosigranulum pigrum* were showed to interact synergistically with the traditional pathogen *P. aeruginosa,* by displaying ability to develop dual-species consortia with increased tolerance to a wide range of antibiotics under *in vitro* aerobic conditions (Figure 6). Although not fully understood, these cooperative relationships were suggested to be the result of a more diverse ecosystem, leading to a higher number of cells in the biofilm, to different spatial arrangements in biofilm-encased cells within the overall consortia and also to more diverse types of matrix composition, which may result in different responses towards

antibiotics (Lopes et al, 2012). Therefore, this may suggest that both species may influence the behavior of the individual species or even the activities of the polymicrobial communities residing in the CF airways.

With the constant challenges that CF polymicrobial communities undergo during the course of infection, there is a potential to exploit the relationship between the resident microbes, as well as on how these multispecies interactions govern the scope and the progression (severity or outcome) of the disease and ultimately how the host responds to polymicrobial infections.

CF antibiotic treatment – importance of shaping polymicrobial interactions

Current therapy for CF focuses on minimizing the microbial community and the host's immune response through the aggressive use of several therapeutics, including antibiotics, bronchodilators, anti-inflammatory drugs, mucolytic agents and airway clearance techniques (Touw et al, 1995). Antibiotic therapy is currently the central therapeutic strategy in CF, and has important advances over the past 50 years in the treatment of the infection have been achieved (Doring et al, 2012; Chmiel et al, 2013). It is often employed as a maintenance therapy and/or to treat infectious exacerbations, attempting to reduce the sputum bacterial load and improving pulmonary symptoms. The selection of antibiotics is based upon the estimation of the *in vitro* antimicrobial sensitivities of a limited number of species cultured from sputum, and are generally directed to the most isolated pathogen *P. aeruginosa* (Balfour-Lynn and Elborn, 2007; Rogers et al, 2010b). Although there may be a convincing correlation between *in vitro* and *in vivo* susceptibilities for acute exacerbations by *P. aeruginosa*, with early colonization being successfully suppressed by aggressive antibiotic therapy

(Schelstraete et al, 2013)), the correlation between results of conventional antibiotic susceptibility testing and treatment outcome is dramatically reduced for chronic infections (Smith et al, 2003), with infection typically persisting for life. In here, the microorganism is well-adapted to the *in vivo* CF environment (see sections above) demonstrating a distinct behavior than that observed under *in vitro* conditions (Rogers et al, 2010a).

Because CF infection is no longer viewed as being caused by a single pathogen, antibiotics used to target a small group of species recognized as key CF pathogens may not have similar effect when other atypical species are present (Lopes et al. submitted for publication). For example, several studies have demonstrated that the anaerobic community found in CF airway is highly resistant to intravenous antibiotics usually applied to treat *P. aeruginosa* infection, with insignificant reduction in cell numbers (Worlitzsch et al., 2002; Tunney et al., 2008).

In parallel, dynamic compositional changes within microbial populations which are dependent from the environmental heterogeneity conditions found in CF (Hauser et al., 2011) as well as social interactions between microorganisms within polymicrobial communities should not be dismissed. This ecological perspective is believed to have important impact for CF therapeutics, offering the prospect of novel approaches to antibiotic treatment. The control of chronic airway infections by, for instance, disturbing some factor within the lung that regulates the microbial community stability and function (e.g. presence of another community member, a nutrient, or an environmental attribute) would be an interesting alternative to antibiotics targeting only a specific pathogen (Conrad et al., 2013). Longer longitudinal studies of composition and dynamics of microbial communities and simultaneously checking for the clinical status of patients and treatment outcomes would be also necessary.

Therefore, a more deep appreciation of the ecological and evolutionary nature that shape the airway communities as well as their effects on lung disease is critically important for the optimal use of current therapies and the development of newer breakthroughs on CF therapy.

Concluding Remarks

Advances in culture-independent molecular assays have enabled to detect a diverse array of microbial species, in addition to those recognized as clinically important for CF pathophysiology. These polymicrobial infections are increasingly viewed as complex communities of interacting organisms, with dynamic processes key to their pathogenicity. Hence, moving the focus of the management from an individual species to the polymicrobial infections and modeling interactions between such traditional and atypical microorganisms will be helpful to predict the effects of new therapeutic interventions, thus dismissing much of the current antibiotic therapy empiricism and increasing the effectiveness of CF therapies.

Declaration of interest

The authors report no declarations of interest.

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747 **Table 1.** Atypical microorganisms emerging in the respiratory tracts of patients with CF

Genus	Examples of identified species	Detection and/or identification method(s)[a]	References
Gram-negative bacteria			
Acinetobacter	*A. baumanii*	Biochemical and molecular approaches	(Coenye et al., 2002)
Achromobacter	*A. xylosoxidans*	Culture, 16S rRNA gene sequencing	(Harris et al., 2007; Bittar et al., 2008)
Agrobacterium	*A. radiobacter*	Culture, 16S rRNA gene sequencing	(Bittar et al., 2008)
Bergeyella		16S rRNA gene sequencing	(Bittar et al., 2008)
Bordetella	*B. hinzii*	Biochemical and molecular approaches	(Bittar et al., 2008; Guss et al., 2011)
Brevundimonas	*B. diminuta*	16S rRNA gene sequencing	(Coenye et al., 2002; Menuet et al., 2008)
Chryseobacterium	C. *indologenes, C. miningosepticum*	Biochemical and molecular approaches	(Coenye et al., 2002)
Comamonas	*C. testosteroni*	Biochemical and molecular approaches	(Coenye et al., 2002; Bittar et al., 2008)
Coxiellaceae		rRNA gene sequencing	(Coenye et al., 2002; Guss et al., 2011)
Craurococcus	*C. roseus*	T-RFLP	(Harris et al., 2007)
Chromobacterium	*C. violaceum*	Biochemical and molecular approaches	(Rogers et al., 2004)

Cuprivadius		16S rRNA gene sequencing	(Coenye et al., 2002)
Eikenella	*E. corrodens*	16S rRNA gene sequencing	(Kalka-Moll et al., 2009)
Escherichia	*E. coli*	Culture, biochemical and molecular approaches	(Harris et al., 2007; Bittar et al., 2008; Guss et al., 2011)
Gemella	*G. haemolysans*	16S rRNA gene sequencing	(Coenye et al., 2002; Bittar et al., 2008; Tunney et al., 2008)
Herbaspirillum		Biochemical and molecular approaches	(Bittar et al., 2008)
Inquilinus	*I. limosus*	Biochemical and molecular approaches	(Coenye et al., 2002)
Kingella	*K. denitrificans, K. oralis*	16S rRNA gene sequencing	(Coenye et al., 2002; Bittar et al., 2008)
Klebsiella	*K. pneumoniae*	Culture, biochemical approaches	(Bittar et al., 2008)
Lysobacter	*L. enzymogenes*	rRNA gene sequencing	(Steinkamp et al., 1989; Khanbabaee et al., 2012)
Moraxella	*M. osloensis, M. catarrhalis*	Biochemical and molecular approaches	(Harris et al., 2007)
Morganella		Biochemical and molecular approaches	(Coenye et al., 2002; Bittar et al., 2008)
Neisseria		16S rRNA gene sequencing	(Coenye et al., 2002)
Ochrobactrum	*O. anthropic*	16S rRNA gene sequencing	(Tunney et al., 2008; Guss et al., 2011)

Paenibacillus	*P. cineris*	Culture	(Menuet et al., 2008)
Pandoreae		Biochemical and molecular approaches	(Leao et al., 2010)
Paracoccus	*P. halodenitrificans*	T-RFLP	(Coenye et al., 2002)
Pseudomonas	*P. huttiensis, stutzeri*	Culture, Biochemical and molecular approaches	(Rogers et al., 2004)
Ralstonia	*R. gilardii, R. mannitolytica*	Biochemical and molecular approaches	(Coenye et al., 2002; Bittar et al., 2008)
Rhizobium	*R. radiobacter*	Biochemical and molecular approaches	(Coenye et al., 2002)
Rickettsiales		rRNA gene sequencing	(Coenye et al., 2002)
Serratia	*S. marcescens*	Culture, Biochemical and molecular approaches	(Harris et al., 2007)
Sphingomonas	*S. paucimobilis*	Culture, Biochemical and molecular approaches	(Burns et al., 1998; Coenye et al., 2002; Tunney et al., 2008; Guss et al., 2011)
Stenotrophomonas	*S. maltophilia*	Culture, rRNA gene sequencing	(Coenye et al., 2002)
Xantomonas	*X. hyacinthi*	Biochemical and molecular approaches	(Coenye et al., 2002)
Gram-positive bacteria			
Carnobacterium		16S rRNA gene sequencing	(Bittar et al., 2008)
Corynebacterium	*C. pseudodiphtheriticum*	Mass spectrometry and molecular methods	(Bittar et al., 2008; Guss et al., 2011)

Dolosigranulum	*D. pigrum*	16S rRNA gene sequencing	(Bittar et al., 2008)
Ganulicatella	*G. adiacens, G. elegans*	16S rRNA gene sequencing	(Harris et al., 2007; Bittar et al., 2008; Guss et al., 2011)
Lactobacillus	*L. delbrueckii*	16S rRNA gene sequencing	(Bittar et al., 2008; Guss et al., 2011)
Mycobacterium[b]	*M. avium, M. abscessus*	16S rRNA gene sequencing	(Harris et al., 2007; Bittar et al., 2008)
Mycrococcus		16S rRNA gene sequencing	(Tunney et al., 2008; Guss et al., 2011)
Nocardia	*N. asteroides*	Culture	(Lumb et al., 2002)
Rothia	*R. mucilaginosa*	16S rRNA gene sequencing	(Bittar et al., 2008; Tunney et al., 2008)
Staphylococcus	*S. epidermidis, S. hominis*	16S rRNA gene sequencing	(Tunney et al., 2008)
Streptococcus	*S. constellatus, S. iniae, S. intermedius* etc.	Culture, T-RFLP, 16S rRNA gene sequencing	(Harris et al., 2007; Bittar et al., 2008; Tunney et al., 2008; Sibley et al., 2009)
Tropheryma	*T. wippley*	rRNA gene sequencing	(Harris et al., 2007)
Anaerobic bacteria			
Actinomyces	*A. odontolyticus*	Culture, Biochemical and molecular approaches	(Tunney et al., 2008; Worlitzsch et al., 2009; Guss et al., 2011)

Bacteroides		Culture, Biochemical and molecular approaches	(Worlitzsch et al., 2009; Guss et al., 2011)
Bifidobacterium		16S rRNA gene sequencing	(Tunney et al., 2008)
Bulleidia		16S rRNA gene sequencing	(Tunney et al., 2008; Guss et al., 2011)
Capnocytophaga	*C. leadbetteri*	Culture, Biochemical and molecular approaches	(Harris et al., 2007; Worlitzsch et al., 2009; Guss et al., 2011)
Clostidrium		Culture, Biochemical and molecular approaches	(Tunney et al., 2008; Worlitzsch et al., 2009)
Dialister	*D. pneumosintes*	16S rRNA gene sequencing	(Bittar et al., 2008; Worlitzsch et al., 2009; Guss et al., 2011)
Eubacterium		Culture, Biochemical approaches	(Worlitzsch et al., 2009)
Fusobacterium	*F. necrophorum, F. nucleatum*	Culture, Biochemical and molecular approaches	(Harris et al., 2007; Tunney et al., 2008; Worlitzsch et al., 2009; Guss et al., 2011)
Gemella	*G. morbillorum*	Culture, Biochemical and molecular approaches	(Bittar et al., 2008; Worlitzsch et al., 2009; Guss et al., 2011)
Lachnospiraceae		16S rRNA gene sequencing	(Bittar et al., 2008)
Lactobacillus		Culture, Biochemical and molecular approaches	(Tunney et al., 2008; Worlitzsch et al., 2009)

Mobiluncus		Culture, Biochemical approaches	(Worlitzsch et al., 2009)
Peptostreptococcus		Culture, Biochemical and molecular approaches	(Bittar et al., 2008; Tunney et al., 2008; Worlitzsch et al., 2009)
Porphyromonas		rRNA gene sequencing	(Harris et al., 2007; Guss et al., 2011)
Prevotella	*P. denticola, P. melaninogenica, P. salivae* etc.	Culture, Biochemical and molecular approaches	(Harris et al., 2007; Tunney et al., 2008; Worlitzsch et al., 2009; Guss et al., 2011)
Propionibacterium		Culture, Biochemical and molecular approaches	(Tunney et al., 2008; Worlitzsch et al., 2009)
Selemonas	*S. noxia, S. infelix*	16S rRNA gene sequencing	(Bittar et al., 2008)
Staphylococcus	*S. saccarolyticus*	Culture, Biochemical and molecular approaches	(Tunney et al., 2008; Worlitzsch et al., 2009)
Streptococcus	*S. pneumoniae, S. salivarius, S. thermphilus* etc.	Culture, Biochemical and molecular approaches	(Bittar et al., 2008; Tunney et al., 2008; Worlitzsch et al., 2009; Khanbabaee et al., 2012)
Tannerella	*T. forsythensis*	16S rRNA gene sequencing	(Bittar et al., 2008)
Veilonella	*V. atypica, V. dispar*	16S rRNA gene sequencing	(Tunney et al., 2008; Worlitzsch et al., 2009; Guss et al., 2011)
Wolinella		Culture, Biochemical approaches	(Worlitzsch et al., 2009)

Fungi			
Acrophialophora	*A. fusispora*	Culture	(Cimon et al., 2005)
Alternaria		Culture	(Nagano et al., 2007)
Aspergillus	*A. fumigatus, A. flavus, A. nidulans, A. terreus, A. niger*	Culture, PCR, ITS gene sequencing and galactomannan enzyme immunoassay	(Bakare et al., 2003; Bouchara et al., 2009; Delhaes et al., 2012; Warren et al, 2012)
Candida	*C. albicans, C. parapsilosis, C. dubliniensis*	Culture, PCR and ITS gene sequencing	(Bakare et al., 2003; Bouchara et al., 2009; Chotirmall et al., 2010; Delhaes et al., 2012)
Cladosporium		Culture	(Nagano et al., 2007)
Cryptococcus		Culture, PCR and ITS gene sequencing	(Delhaes et al., 2012)
Exophiala	*E. dermatidis*	Culture, PCR and ITS gene sequencing	(Kusenbach et al., 1992; Diemert et al., 2001; Horre et al., 2004; Griffard et al., 2010; Delhaes et al., 2012)
Geosmithia	*G. argillacea*	Culture, microscopy, PCR and ITS gene sequencing	(Barton et al., 2010; Giraud et al., 2010)
Malassezia		Culture, PCR and ITS gene sequencing	(Delhaes et al., 2012)
Neosartoria		Culture, PCR and ITS gene sequencing	(Delhaes et al., 2012)
Paecilomyces	*P. variotii*	Culture	(Nagano et al., 2007)

Penicillium	*P. emersonii*	Culture	(Cimon et al., 1999)
Physalospora		Culture, PCR and ITS gene sequencing	(Delhaes et al., 2012)
Pneumocystis	*P. jirovecii*	PCR	(Gal et al., 2010; Delhaes et al., 2012; Pederiva et al., 2012)
Scedosporium	*S. apiospermum, S. prolificans*	Culture, PCR and ITS gene sequencing	(Defontaine et al., 2002; Blyth et al., 2010; Delhaes et al., 2012)
Trichosporon	*T. mycotoxinivorans*	Culture	(Hickey et al., 2009)
Viruses			
Mastadenovirus	*Adenovirus*		(Smyth et al., 1995; Punch et al., 2005; Olesen et al., 2006)
Metapneumovirus	*Human metapneumovirus*	PCR	(Olesen et al., 2006)
Influenza virus (A and B)	*Influenza (A* and *B) viruses*	PCR and immunological methods	(Smyth et al., 1995; Punch et al., 2005; Olesen et al., 2006)
Respirovirus/Rubalavirus	*Human arainfluenza viruses*	PCR and immunological methods	(Smyth et al., 1995; Punch et al., 2005; Olesen et al., 2006)
Pneumovirus	*Respiratory syncytial virus*	PCR and immunological methods	(Smyth et al., 1995; Punch et al., 2005; Olesen et al., 2006)

Enterovirus	*Rhinovirus*	PCR and immunological methods	(Smyth et al., 1995; Punch et al., 2005; Olesen et al., 2006)

748 [a] Legends for abbreviated methods: T-RFLP: terminal restriction fragment length polymorphism; PCR: polymerase chain reaction; ITS: Internal
749 transcribed spacer
750 [b] Although *Mycobacterium* is included as a gram-positive in this table, indeed this bacterial genera is exceptionally impervious to gram staining due to a
751 waxy-coated cell surface
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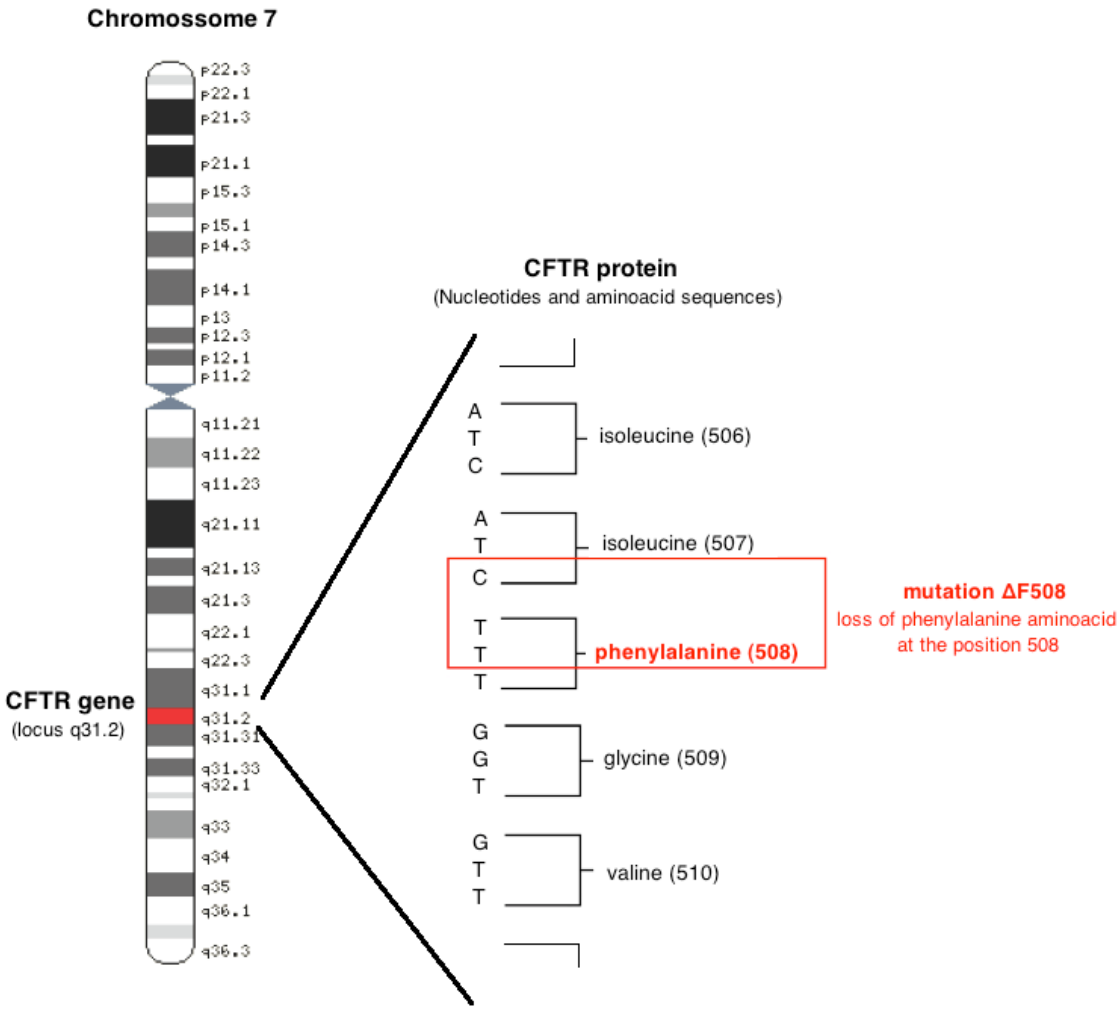
762 **Figures**

763 **Figure 1:**


Chromossome 7
p22.3
p22.1
p21.3
p21.1
p15.3
p15.1
p14.3
p14.1
p13
p12.3
p12.1
p11.2
q11.21
q11.22
q11.23
q21.11
q21.13
q21.3
q22.1
q22.3
q31.1
q31.2
q31.31
q31.33
q32.1
q33
q34
q35
q36.1
q36.3
CFTR gene
(locus q31.2)
CFTR protein
(Nucleotides and aminoacid sequences)
A
T
C
isoleucine (506)
A
T
C
isoleucine (507)
T
T
T
phenylalanine (508)
mutation ΔF508
loss of phenylalanine aminoacid
at the position 508
G
G
T
glycine (509)
G
T
T
valine (510)

764

765 **Figure 2:**

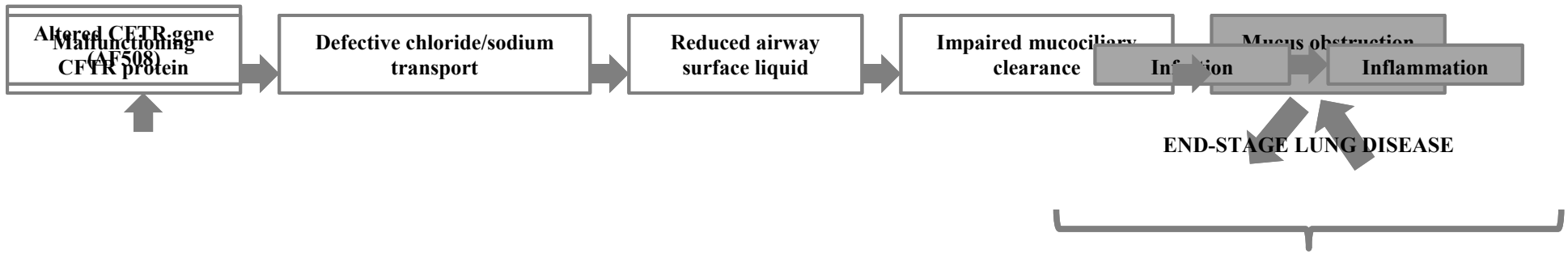


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Figure 3:

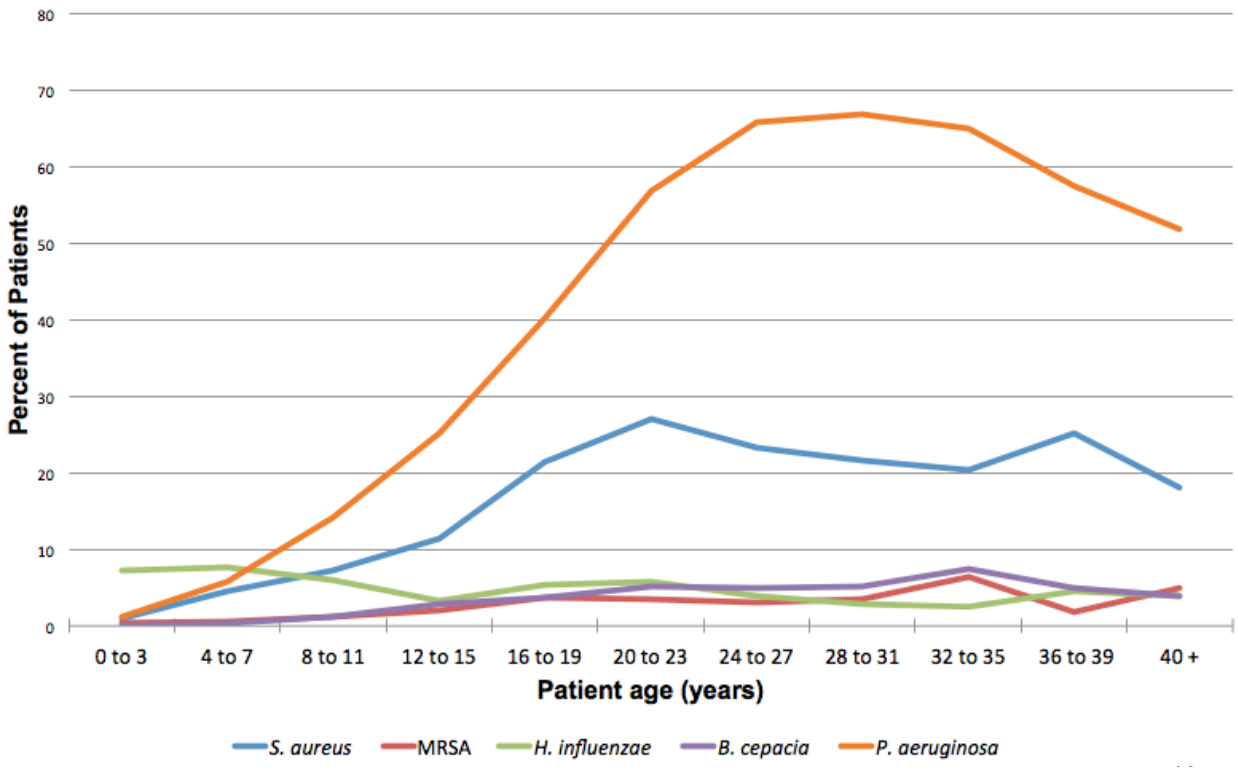
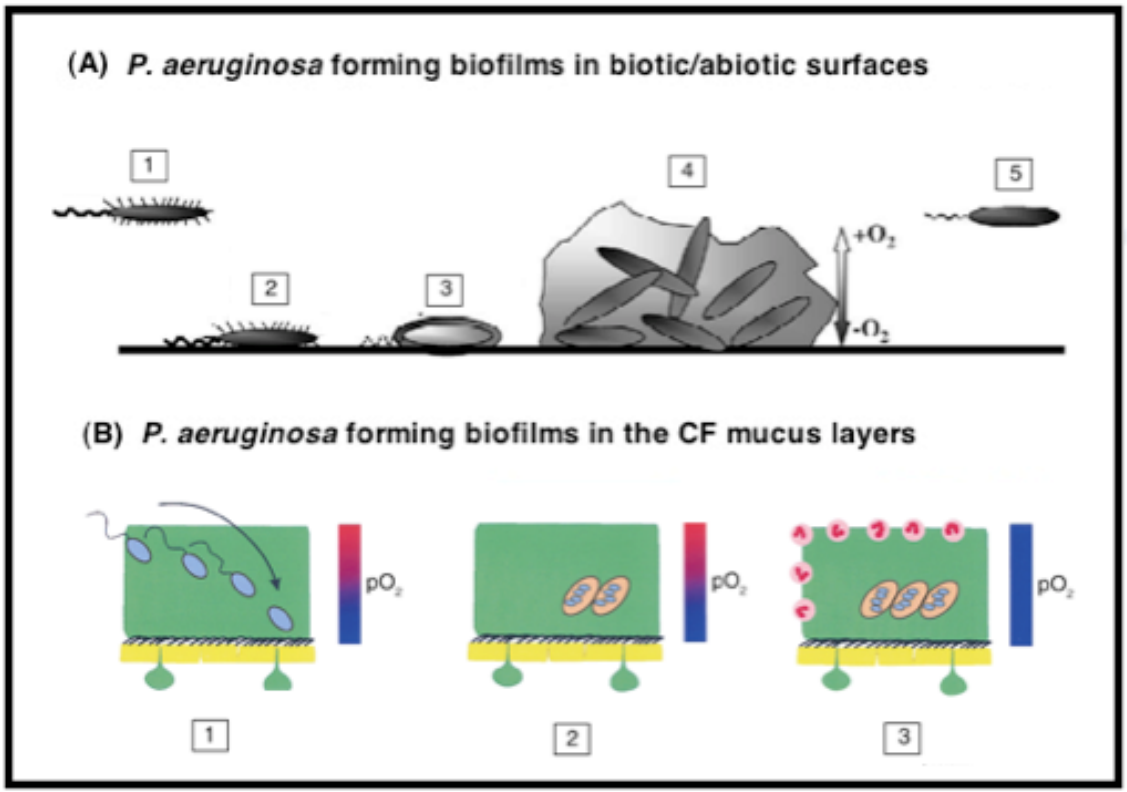

80
70
60
50
40
30
20
10
0
Percent of Patients
0 to 3
4 to 7
8 to 11
12 to 15
16 to 19
20 to 23
24 to 27
28 to 31
32 to 35
36 to 39
40 +
Patient age (years)
S. aureus
MRSA
H. influenzae
B. cepacia
P. aeruginosa

Figure 4:


(A) P. aeruginosa forming biofilms in biotic/abiotic surfaces
1
2
3
4
5
+O2
-O2
(B) P. aeruginosa forming biofilms in the CF mucus layers
pO2
pO2
pO2
1
2
3

811 **Figure 5:**

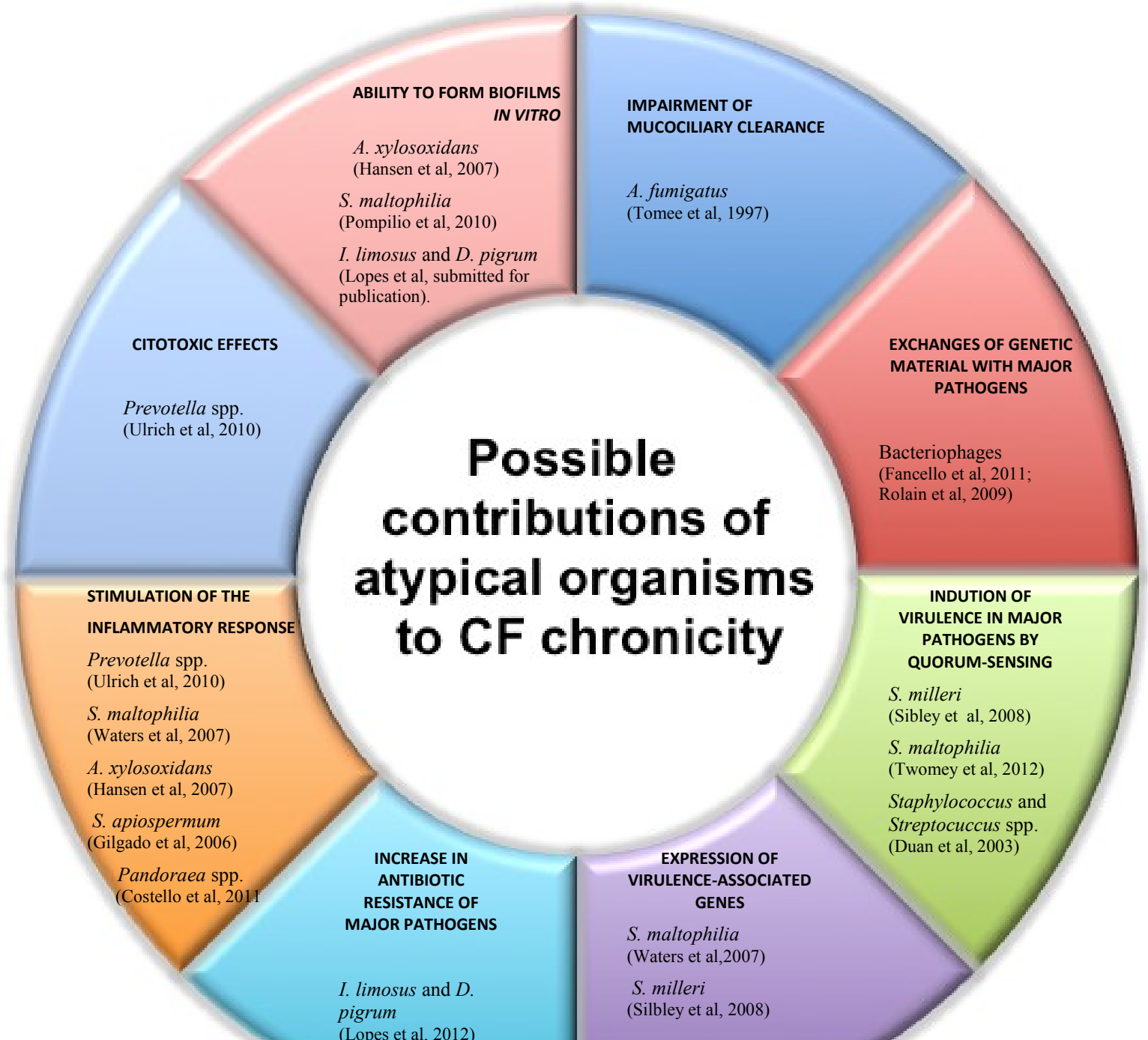
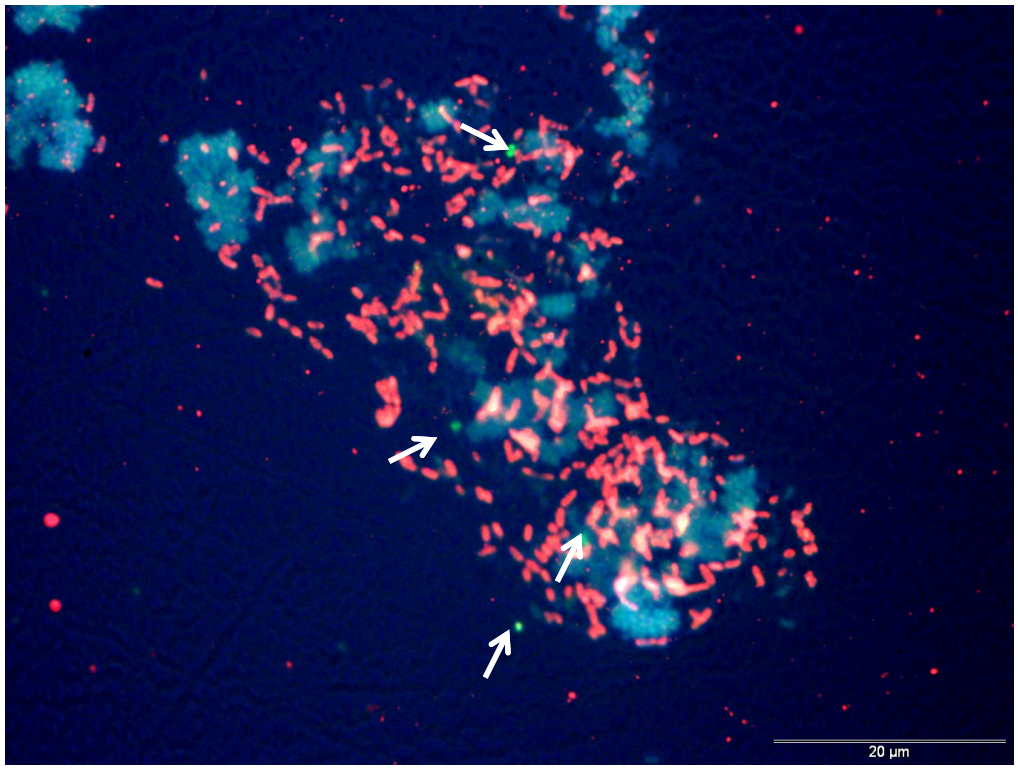

Possible contributions of atypical organisms to CF chronicity
ABILITY TO FORM BIOFILMS IN VITRO
A. xylosoxidans (Hansen et al, 2007)
S. maltophilia (Pompilio et al, 2010)
I. limosus and D. pigrum (Lopes et al, submitted for publication).
IMPAIRMENT OF MUCOCILIARY CLEARANCE
A. fumigatus (Tomee et al, 1997)
EXCHANGES OF GENETIC MATERIAL WITH MAJOR PATHOGENS
Bacteriophages (Fancello et al, 2011; Rolain et al, 2009)
INDUTION OF VIRULENCE IN MAJOR PATHOGENS BY QUORUM-SENSING
S. milleri (Sibley et al, 2008)
S. maltophilia (Twomey et al, 2012)
Staphylococcus and Streptocuccus spp. (Duan et al, 2003)
EXPRESSION OF VIRULENCE-ASSOCIATED GENES
S. maltophilia (Waters et al,2007)
S. milleri (Silbley et al, 2008)
INCREASE IN ANTIBIOTIC RESISTANCE OF MAJOR PATHOGENS
I. limosus and D. pigrum (Lopes et al, 2012)
STIMULATION OF THE INFLAMMATORY RESPONSE
Prevotella spp. (Ulrich et al, 2010)
S. maltophilia (Waters et al, 2007)
A. xylosoxidans (Hansen et al, 2007)
S. apiospermum (Gilgado et al, 2006)
Pandoraea spp. (Costello et al, 2011
CITOTOXIC EFFECTS
Prevotella spp. (Ulrich et al, 2010)

Figure 6:



Legend for figures:

844 **Figure 1.** Location of the CFTR gene in the long (q) arm of the chromosome 7 and mutation **Δ**F508, corresponding to the deletion of
845 phenylalanine aminoacid in the position 508 of the CFTR protein.
846
847 **Figure 2.** Cascade of events that characterizes the pathophysiology of the CF lung disease. Adapted from Lubamba et al, 2012.
848
849 **Figure 3.** Age-specific prevalence of the traditional pathogens recovered from CF respiratory samples. Based on UK CF Registry annual data
850 Report 2009.
851
852 **Figure 4.** Models proposed by Hassett et al, (2002) for *P. aeruginosa* biofilm formation on biofilm formation on: **A)** biotic or abiotic surfaces
853 and within **B)** CF airway mucu**s.** In A) biofilm is formed in 5 developmental stages: (1) free-swimming (planktonic) bacteria; (2) attachment
854 of planktonic bacteria to the surface mediated by flagella and type IV pili; (3) bacteria lose their surface-appendages and forms
855 "microcolonies"; (3) as the cell propagation increases, these "microcolonies" mature into "macrocolonies" and start to produce
856 exopolysachharides; (4) formation of a mature biofilm self-producing a thick and protective exopolysaccharide matrix, development of oxygen
857 gradients; (5) initiation of biofilm detachment/dispersion; In B) the development of the biofilm starts with *P. aeruginosa* readily penetrating
858 into the mucus (1), followed by adaptation of bacteria to the anaerobic environment by losing their surface-appendages, converting to the

mucoid form and forming microcolonies within alginate coats (2) and finally macrocolonies resisting to immune system defenses, setting the stage for chronic infection (3). Images were adapted from Hassett et al. (2002) and from Worlitzsch et al. (2002), respectively.

Figure 5. Lines of evidence that may facilitate adaptation of some unusual microorganisms to the *in vivo* CF environment and may support their contributions to the lung disease chronicity.

Figure 6. Multiplex PNA assay applied to the three-species 24-h-old *in vitro* biofilms formed by *P. aeruginosa*, *I. limosus* and *D. pigrum* formed on polystyrene coupons. *P. aeruginosa* (red cells) seem to dominate the consortium, together with *D. pigrum* (bluish cells). On its turn, the low number of *I. limosus* cells (green, indicated by arrows) mean that this species could be outcompeted by the other species present in the consortium. Similar interactions between other microorganisms of different species have also been visualized.