Stenotrophomonas maltophilia in cystic fibrosis

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Dedicated to my dear family, my partner Sebastian

and friends

“Thus,

the task is not so much to see what no one yet has seen,

but to think what nobody yet has thought about that which everybody sees.”

Arthur Schopenhauer, 1860

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Abbreviations

AAC	N-acetyltransferases
ATP	adenosine triphosphate
AR	adjusted Rand coefficient
BMI	body mass index
cAMP	cyclic adenosine monophosphate
CF	cystic fibrosis
CFU	colony-forming unit
C. elegans	*Caenorhabditis elegans*
CFDR	cystic fibrosis-related diabetes
CFTR	cystic fibrosis conductance regulator gene
CI	confidence intervals
CLSI	Clinical and Laboratory Standards Institute
CLSM	confocal laser scanning microscopy
COL	colistin
COPD	chronic obstructive pulmonary disease
CVC	central venous catheter
DL	Diversilab®
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DSF	diffusible signal factor
EDTA	ethylenediaminetetraacetic acid
EGCg	epigallocatechin-3-gallate
EPS	extracellular polymeric substances

ERIC-PCR	enterobacterial repetitive intergenic consensus - polymerase chain reaction
FAME	fatty acid methyl esters
FEV_1	forced expiratory volume in 1 second
GC	gas chromatography
H_2O_2	hydrogen peroxide
HSCT	hematopoietic stem cell transplantation
ICU	intensive care unit
IFA	immunofluorescence assay
IgG	immunoglobulin G
IL-8	Interleukin- 8
IL-10	Interleukin-10
LB	Luria-Bertani
LPS	lipopolysaccharide
MALDI-TOF MS	matrix-assisted laser desorption ionisation-time of flight mass spectrometry
MDR	multiple-drug resistant
MDRO	multiple-drug resistant organism
MBC	minimum bactericidal concentration
MHA	Müller Hinton agar
MHB	Müller Hinton broth
MIC	minimum inhibitory concentration
MLST	multilocus sequence typing
m/z	mass divided by charge number of ions
NFGN	non-fermenting Gram-negative
NGM	nematode growth medium

PAI	peak area index
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCA	principal component analysis
PFGE	pulsed-field gel electrophoresis
PI	propidium iodide
P. aeruginosa	*Pseudomonas aeruginosa*
RAPD	random amplified polymorphic DNA
rep-PCR	repetitive-sequence-based polymerase chain reaction
ROC	receiver operating curve
rRNA	ribosomal ribonucleic acid
SEM	scanning electron microscope
SID	Simpson's index of diversity
SMA	Steno medium agar
SmeABC	*Stenotrophomonas maltophilia* multidrug efflux system SmeR (smeR), SmeS (smeS), SmeA (smeA), SmeB (smeB), and SmeC (smeC) genes
SMF-1	*S. maltophilia* frimbriae 1
S. maltophilia	*Stenotrophomonas maltophilia*
Syto9	green fluorescent nucleic acid stain
TEB	Tris/boric/EDTA buffer
TEM-1	type of lactamase
TNFα	tumor necrosis factor alpha
TSA	trypticase soya agar
TSB	tryptone soya broth
VIA agar	vancomycin, imipenem, and amphotericin B agar

XMSM	selective medium for the isolation of *S. maltophilia*
XTT	2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt
W	Wallace’s coefficient

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1 Introduction

1.1 Nature of Gram-negative bacterial infections

Most microorganisms do not cause disease, but those capable of provoking disease in nearly any susceptible host, are truly considered pathogens (NCBI, 2007). Thus, clinical microbiologists have long recognised the relevance of identifying infectious microbial pathogens, since they are generally responsible for inducing diseases in humans (Fournier *et al*., 2013).

In the last decades, non-fermenting Gram-negative (NFGN) bacteria have emerged as important opportunistic pathogens, despite efforts made to optimize the surveillance and treatment of infectious diseases (Enoch *et al*., 2007). This particular group of bacteria is found widespread in the environment (soil and water) and primarily affects critically ill or immunocompromised patients (Enoch *et al*., 2007; McGowan Jr, 2006). Indeed, they represent a challenge for health care due to their propensity for multiple, intrinsic or acquired drug resistance; features that characterise them as multiple-drug resistant (MDR) organisms (MDROs) (Bhattacharya, 2013). As a consequence, MDROs have great impact on morbidity and mortality rates in patients (Slama, 2008).

For a long time *Pseudomonas aeruginosa* has played a dominant role as a pathogen among the non-fermenting Gram-negative pathogens, as it is often the cause of respiratory infections in patients, especially those suffering from chronic lung diseases and cystic fibrosis (CF) (Hogardt and Heesemann, 2010). However, this predominantly *P. aeruginosa*-centred view has been altered by the ever-growing role of *Stenotrophomonas maltophilia*, a global emerging Gram-negative bacteria mostly associated with human infections, especially those of the respiratory tract (Brooke, 2012; de Vrankrijker *et al*., 2010; Barchitta *et al*., 2009).

1.2 History and clinical significance of *S. maltophilia*

In the early 1940s, *Stenotrophomonas maltophilia* was initially described as *Bacterium booker* and later named *Pseudomonas maltophilia* (Hugh and Leifson, 1963). However, subsequent allocation of this species to the genus *Xanthomonas* was supported by results obtained from rRNA (ribosomal ribonucleic acid) cistron analysis (Swings *et al*., 1983). A large study involving *Xanthomonas* strains, which analysed 295 phenotypic

features, revealed the identity of seven strains as *X. maltophilia*, although two of them were type strains *Pseudomonas betle* and *Pseudomonas hibiscicola* (Van den Mooter and Swings, 1990). Due to intense debates regarding the transfer of *P. maltophilia* to the genus *Xanthomonas*, the creation of a genetic genus which would include only a single species was proposed and accepted, *Stenotrophomonas maltophilia* (Palleroni and Bradbury, 1993). *Stenotrophomonas*' (Gr. adj. *stenus* = narrow, Gr. n. *trophus* = one who feeds, Gr.n. *monas*= unit) designation would then reflect the observed characteristic of its limited spectrum of nutrition (Stanier *et al*., 1966). Data based on DNA (deoxyribonucleic acid)-rRNA hybridization studies, sequencing and mapping investigations of PCR (polymerase chain reaction)-amplified 16S rRNA genes, eventually resulted in a consensus classification of *X. maltophilia* as *S. maltophilia* (Denton and Kerr, 1998; Nesme *et al*., 1995).

S. maltophilia is usually considered as a pathogen with reduced virulence, but it can cause a broad spectrum of nosocomial infection complications with a considerable mortality rate of up to 37.5 % (Falagas *et al*., 2009). The most common clinical manifestations caused by this bacterium include respiratory tract infections (Nicodemo and Paez, 2007; Weber *et al*., 2007; Nseir *et al*., 2006); bacteraemia (Chang *et al*., 2012; Lai et al., 2004), biliary sepsis (Papadakis *et al*., 1995); infections of the bones and joints (Bin Abdulhak *et al*., 2009; Landrum *et al*., 2005), urinary tract (Vartivarian *et al*., 1996) and soft tissues (Sakhnini *et al*., 2002); eye infections (Penland and Wilhelmus, 1996); endocarditis (Wladis, 2011; Takigawa *et al*., 2008) and meningitis (Rojas *et al*., 2009).

Respiratory tract infections are often a clinical sign observed as consequence of stenotrophomonad infection (Brooke, 2012). Since it is quite difficult to distinguish between a simple colonisation and infection caused by this pathogen, diagnosis should be based on the association of physical examination, medical record and diverse clinical tests (results from radiograph, laboratory tests, image findings and microbiology assays) (Nicodemo and Paez, 2007). Since most of these findings are frequently associated with polymicrobial infections and the role played by individual species is still not clearly defined, such described clinical situations can be even more complex. However, there are no doubts that *S. maltophilia* is able to cause nosocomial pneumonia as shown in previous investigations (Nseir *et al*., 2006; Weber *et al*., 2007). A four-year study

examined the prevalence, risk factors and effect on outcome of MDR bacteria in patients diagnosed with severe acute exacerbation of chronic obstructive pulmonary disease (COPD). It was shown that COPD patients with severe acute exacerbation were commonly infected with MDRO's, and *S. maltophilia* was isolated from 3.0 % of this cohort. Further, it has been demonstrated that this pathogen accounts for 6.75 % and 1.11 % of the cases of ventilator-associated pneumonia and hospital-acquired pneumonia, respectively (Weber *et al*., 2007). It is important to remember that the mortality rate as a consequence of *S. maltophilia* pulmonary infections is high, especially when there are complications such as bacteraemia and airway obstruction (Nicodemo and Paez, 2007; Fujita *et al*., 1996).

Bloodstream infections represent a serious threat to the patient's health (Vidal *et al*., 2003) and indwelling medical devices, such as central venous catheters (CVC), are common sources of NFGN bacteraemia (Hanna *et al*., 2004). *S. maltophilia* is a flagellated and fimbriated bacterium. These external appendages enable the bacteria to efficiently adhere to a variety of surfaces, both biotic and abiotic (Pompilio *et al*., 2010b). As a consequence of this, *S. maltophilia* readily forms biofilms on medical devices, resulting in eventual patient infection (Yeshurun *et al*., 2010; Lai *et al*., 2006). A retrospective cohort study carried out at the Chaim Sheba Medical Center in Israel examined 570 adult patients that underwent hematopoietic stem cell transplantation (HSCT) (Yeshurun *et al*., 2010). Through this four-year investigation, results showed that 19 (3.3 %) of the HSCT patients had *S. maltophilia* isolated from their blood cultures. During the time of infection, 17 (90.0 %) of these patients had CVC, from which 15 (88.0 %) of them were bacteraemic and the remaining two (12.0 %) showed signs of other invasive infections. Another study has observed that haematological and oncological patients were also susceptible to CVC-related *S. maltophilia* systemic infections and recurrent bacteraemia (Lai *et al*., 2006). The major clinical characteristics observed in these patients were nosocomial bacteraemia, preceding antibiotic therapy and neutropenia. Univariate analysis characterised long-lasting (> 10 days) neutropenia and initial failure to remove the CVC as risk factors. Relapse cases were, in fact, not reinfection episodes, as the same strain was identified through random amplified polymorphic (RAPD) DNA. Therefore, it has been strongly suggested that CVC removal

is crucial for successful treatment of CVC-related *S. maltophilia* bacteraemia as well as for prevention of relapses.

Akin to respiratory infection, differentiation between colonisation and infection in the urinary tract due to *S. maltophilia* can also be challenging (Falagas *et al*., 2009; Vartivarian *et al*., 1996). Long-term urinary bladder catheterisation, urinary tract abnormalities and genitourinary malignancies are usually associated with this type of infection. There is an increased risk of developing severe infection in immunocompromised patients, especially if the therapy involves antibiotics ineffective against this pathogen (Vartivarian *et al*., 1996).

Infection syndromes of intact skin and soft tissues are frequent and predominantly affect immunocompromised hosts suffering from haematologic malignancies, neutropenia, undergoing chemotherapy, use of CVC or exposed to broad-spectrum antibiotic therapy (including carbapenems) (Bin Abdulhak *et al*., 2009). If the *S. maltophilia* skin infections are properly identified and treated, the outcomes can be favourable. Several reports have also described *S. maltophilia* endocarditis. Most cases were related to intravenous drug abusers or complications relating to prosthetic valve implantation surgery (Rojas *et al*., 2009; Khan and Mehta, 2002; Denton and Kerr, 1998).

Rare infections due to *S. maltophlia* include bone and eye infections, as well as meningitis. In a case report, a month after a discectomy procedure, osteomyelitis was described and successful treatment was achieved through administration of a dual therapy, consisting of use of trimethoprim-sulfamethoxazole with another antimicrobial agent to which the isolate was susceptible *in vitro* (Landrum *et al*., 2005). Further, a retrospective review of laboratorial records showed that corneal transplantation, hospital-acquired postkeratoplasty, soft contact lens wear, herpes simplex virus keratitis, Stevens-Johnson syndrome, and toxic epidermal necrolysis were predisposing factors for an eye infection (conjunctivitis, keratitis, and endophthalmitis) (Penland and Wilhelmus, 1996). Meningitis has only been reported in 15 cases, which seem to be strongly related to neurosurgical procedures (Yemisen *et al*., 2008).

Conversely, *S. maltophilia* is not exclusively associated with nosocomial infections. A systematic review indicated several reports dealing with community-acquired infections (determined as infections that occur between 48 h and 72 h prior to hospitalisation) in children and adult patients who, in most cases, possessed some type of comorbidity

(COPD, trauma, CVC, prior antibiotic use, malignancy, prior hospitalisation, human immunodeficiency virus (HIV) infection, or other immune suppression) (Falagas *et al*., 2009). Similarly to nosocomial infections, common symptoms observed in these patients were: bacteraemia; ocular, respiratory tract and wound/tissue infections; otitis and cellulitis.

Interestingly, it has been suggested that *S. maltophilia* is associated with polymicrobial infections (Davies and Rubin, 2007). Recently, a six-year study conducted in a university hospital in Greece analysed data from 68 non-CF patients. Indeed, it was observed that 33.8 % of *S. maltophilia* infections were associated with polymicrobial infections (Samonis *et al*., 2012). Whether other more virulent pathogens may play a more pivotal role than *S. maltophilia* remains unclear, but there is evidence linking poor prognosis with polymicrobial infections (Araoka *et al*., 2010).

1.3 Microbiology

S. maltophilia consist of straight or slightly curved non-sporulating Gram-negative, rod-shaped cells, which are 0.5 to 1.5µm long (Hugh and Ryschenkow, 1961) (Figure 1). They can be found as single cells or in pairs and are motile due to the presence of a few polar flagella (Palleroni and Bradbury, 1993). It can also form small-colony variants, considered an adapted survival form in chronic infections, which can be difficult to detect in clinical specimens (Anderson *et al*., 2007). When inoculated in Columbia blood agar, it forms small greyish, slightly mucous colonies on blood agar. However, some strains may cause a brownish discolouration in clear media, most likely due to secondary chemical reactions among extracellular products. This microorganism lives strictly aerobically and its optimal growth temperature varies between 30-35 °C (Denton and Kerr, 1998).

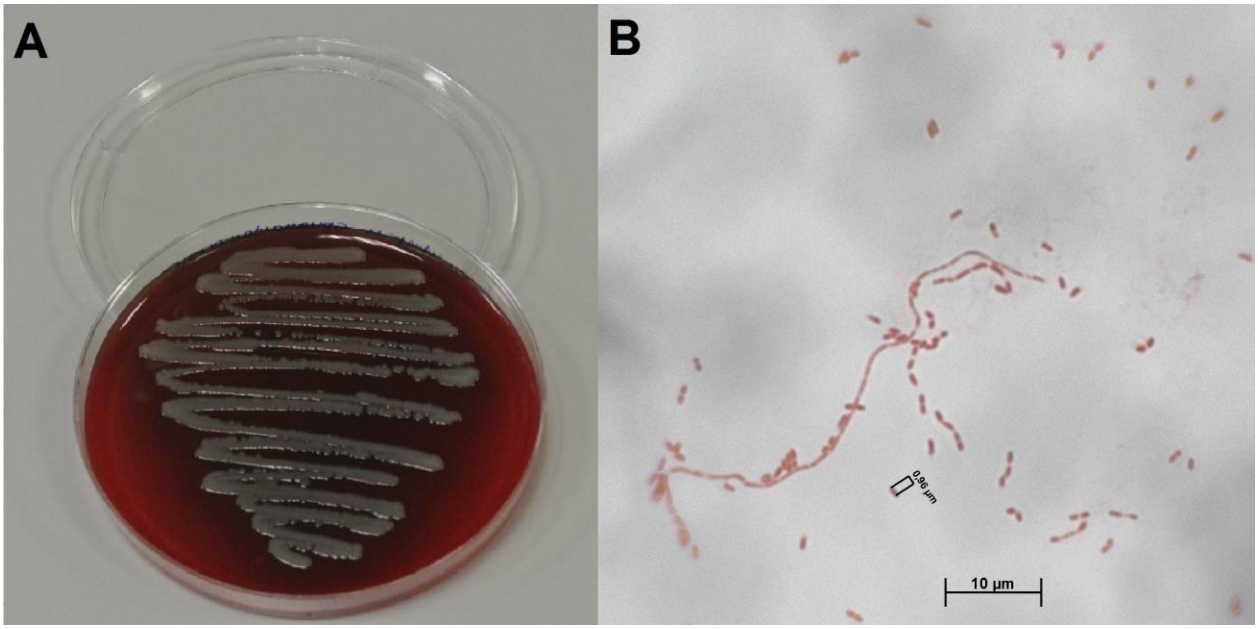

Figure 1 ***Stenotrophomonas maltophilia.*** A) *Stenotrophomonas maltophilia* culture on Columbia blood agar incubated at 35 °C for a period of 24 h. B) Gram-staining of *S. maltophilia*.

Although, standard microbiological data refer to *S. maltophilia* as an oxidase-negative bacterium, recent data analysis of a collection of 766 isolates indicated that approximately 20.0 % of these strains were actually oxidase positive (Carmody *et al*., 2011).

Misidentification of *S. maltophilia* could have major clinical implications. For example, a study observed that 3 (9.0 %) out of 32 clinical isolates obtained from CF patients were incorrectly identified as *Burkholderia cepacia*, due to delayed analysis of oxidase test results (Burdge *et al*., 1995). In this particular case, as *B. cepacia* is a significant pathogen in CF patients (CFFPR, 2012), misinterpretation of test results raises particular concern. Further, a recent study observed that API-20NE, a method typically used for the identification of clinical isolates, has only correctly identified 3.0 % of *S. maltophilia* isolates, recognising most of them as either *B. cepacia* or *Pseudomonas luteola* (Pinot *et al*., 2011).

Identification can also be challenging and complex because *S. maltophilia* can be coisolated with other microorganisms, such as bacteria or yeast (e.g. *P. aeruginosa*, *Staphylococcus aureus*, *Acinetobacter baumanii*, *Escherichia coli*, *Enterobacter* species, *Candida albicans*) in samples recovered from patients (Araoka *et al*., 2010; Gülmez and Hasçelik, 2005; Lai *et al*., 2004). Thus, to improve the isolation of this pathogen from

polymicrobial cultures or environmental specimens, a number of selective media have been designed (Denton *et al*., 2000; Kerr *et al*., 1996; Juhnke and des Jardin, 1989). Despite this, there is currently no selective medium approved by international guidelines, nor is such a medium commercially available.

1.4 Pathogenicity

1.4.1 Extracellular enzymes

Several factors may contribute to *S. maltophilia* colonisation or infection of hosts, such as production of proteinase, lipase, and elastase. It is believed that the primary function of proteinases is to provide a source of free amino acids or simple sugars for bacterial survival and growth (Travis *et al*., 1995). However, bacterial proteinases during an infection are also virtually unregulated by the host proteinase inhibitors, and therefore, are capable of destroying host proteins (Windhorst *et al*., 2002). For example, the extracellular protease *StmPr1* from *S. maltophilia* degrades several human proteins from serum (fibronectin and fibrinogen) and connective tissue (collagen), contributing to local tissue damage and hemorrhage (Windhorst *et al*., 2002).

1.4.2 Lipopolysaccharide

Lipopolysaccharide (LPS) is a major component of the outer membrane of most Gram-negative bacteria (Wang and Quinn, 2010). The LPS comprises three structure components: O-antigen repeats, core polysaccharides, and lipid A, the last being responsible for the toxic effects experienced during infections caused by Gram-negative bacteria (Wang and Quinn, 2010). Early reports have described the contribution of LPS regarding its role in the development and maintenance of colonies (DeShazer *et al*., 1998; Goldberg *et al*., 1995; Rahmati-Bahram *et al*., 1996). A loss of O-polysaccharide production in mutant *P. aeruginosa* will produce avirulent isolates (Goldberg *et al*., 1995). In *S. maltophilia*, the phosphoglucomutase (*spgM*) gene encodes an enzyme for the biosynthesis of phosphoglucomutase, which is correlated to LPS synthesis. Consequently, *spgM* mutants exhibit a modest increase in susceptibility to diverse antimicrobials and proved to be completely avirulent in infection experiments performed with animal host models. Therefore the outer membrane polysaccharide is a virulence

factor involved not only in colonisation, but also resistance to complement mediated cell killing.

1.4.3. Immunostimulatory effects

Another feature that supports the development of infectious manifestations is the immunostimulant effect of *S. maltophilia*, especially upon interleukin- 8 (IL-8) and tumor necrosis factor alpha expression (TNFα) (Waters *et al*., 2007; Vickers and Smikle, 2006). IL-8 and TNFα are defined as anti-inflammatory cytokines that activate neutrophils and macrophages (Opal and DePalo, 2000). Lipid A stimulation in peripheral-blood monocytes and alveolar macrophages induces the production of TNFα, which is in part responsible for the pathogenesis of airway inflammation (Waters *et al*., 2007; Vickers and Smikle, 2006). *S. maltophilia* substantially induces IL-8 expression and recruitment of polymorphonuclear leukocytes into the lungs (Waters *et al*., 2007). A prolonged exposure to these cytokines might disrupt pulmonary functions, and developed into pneumonia-like conditions (Miller *et al*., 2005).

1.4.4. Adherence and biofilms

An essential step for a successful colonisation, and ultimately, induction of disease, resides in the ability of pathogens to adhere to host surfaces (Finlay and Falkow, 1997). This phenomenon is usually mediated by fibrillar structures known as fimbrae or pili (Klemm and Schembri, 2000). It is believed that bacterial colonisation will be favoured either through direct binding of the bacteria to the host target cell mediated by fimbrae or pili or through indirect pilus cross-linking among bacteria (De Oliveira-Garcia *et al*., 2003; Finlay and Falkow, 1997). A positively charged surface and flagella, together with production of fimbrial adhesions, have been associated with biofilm formation. A bacterial biofilm consists of a microbial community embedded in an extra-cellular polysaccharide matrix or extracellular polymeric substances (EPS) (Flemming and Wingender, 2010). EPS are composed by polysaccharides (Ma *et al*., 2009; Zogaj *et al*., 2001), extracellular DNA (Mann *et al*., 2009; Rice *et al*., 2007) and other macromolecules, as well as biosurfactants (Pamp and Tolker-Nielsen, 2007; Davey *et*

al., 2003), flagella and pili (Harmsen *et al*., 2010), lipids (Matsuyama *et al*., 1990) and proteins (Borlee *et al*., 2010; Fexby *et al*., 2007).

S. maltophilia is recognized by its ability to form biofilms on abiotic surfaces including glass and plastics like polystyrene (Brooke, 2012), as well as on host tissues such as bronchial epithelial cells (Pompilio *et al*., 2010b) (Figure 2).

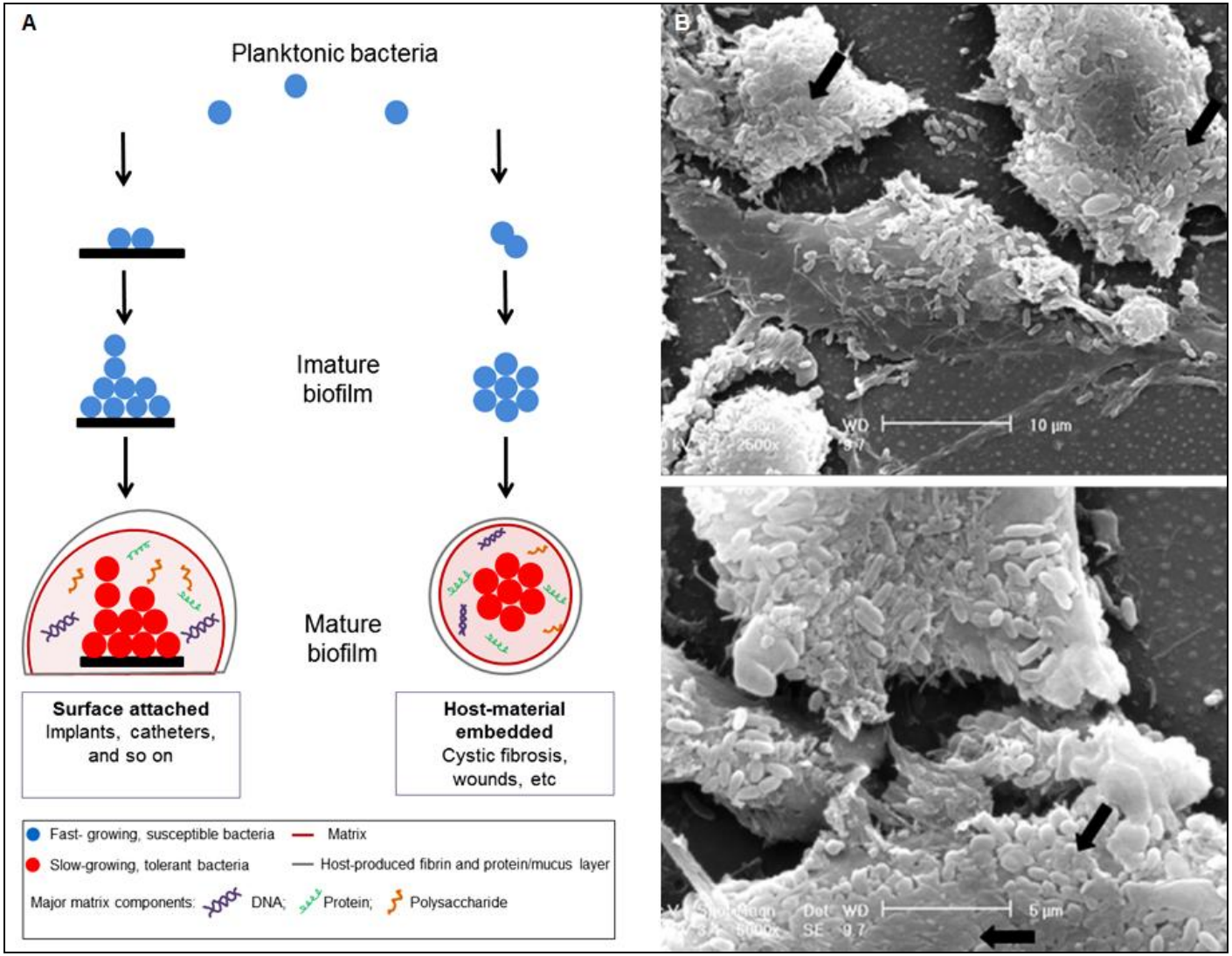

Figure 2 Bacterial biofilm formation. A) The pathways of bacterial biofilm development; surface-attached (right) and non- surface attached (left) (modified from Bjarnsholt *et al*., 2013). B) Scanning electron microscope (SEM) observation of 24 h-old-biofilm formed by clinical isolate *S. maltophilia* OBGTC9 on IB3-1 bronchial epithelia cell monolayer (Reprint from Pompilio *et al*., 2010b with permission from the corresponding author).

In vitro tissue culture assays have demonstrated that adherence to epithelial cells (HEp-2 monolayer cells) and also to inner surfaces was linked to a specific protein, the *S. maltophilia* frimbriae 1 (SMF-1) (De Oliveira-Garcia *et al*., 2003). In the presence of anti-SMF-1 antibodies, the adherence of this microbial pathogen to eukaryotic cells was inhibited. Interestingly, inhibition of adherence and biofilm formation occurred in an anti-SMF-1 dose-dependent manner, suggesting that fimbriae assist in interactions between the *S. maltophilia* cell surface and host cell/abiotic surfaces.

Bacterial cells are not physically aware of the density of other microorganisms around them, but through a signalling system involving the production of specific molecules they can, indeed, indirectly detect this (Bjarnsholt, 2013). This process is recognised as quorum sensing. Contrary to other Gram-negative bacteria, instead of using the usual autoinducer molecules (LuxIR system), *S. maltophilia* engages in a type of cell-to-cell signalling highly related to the diffusible signal factor (DSF)-dependent system of the phytopathogen *Xanthomonas campestris* (Fouhy *et al*., 2007). It was shown in a nematode model (*Caenorhabditis elegans*) that DSF plays an important role in functions attributed to antibiotic resistance and virulence. For example, interruption of DSF signalling in *S. maltophilia* will lead to a decrease in biofilm formation, loss of motility, and reduced production of extracellular proteases. Additionally, it was also demonstrated that DSF signalling of *S. maltophilia* can affect *P. aeruginosa* behaviour by modifying its biofilm formation and polymyxin- tolerance characteristics (Ryan *et al*., 2008).

Confocal microscopy has shown that a *S. maltophilia* clinical isolate (obtained from a CF patient) formed microcolonies in the presence of an extracellular matrix on CF sputum-derived bronchial epithelial IB3-1 cell monolayers. In addition, the degree of adherence of clinical isolates to the bronchial epithelial cells varied among the clinical isolates, and there was no correlation between biofilm formation on polystyrene and on lung cells. This indicates that biofilm formation on abiotic surface does not necessarily reflect the same characteristics observed on biotic surfaces in animal models or human patients, as previously suggested (Worlitzsch *et al*., 2002).

Environmental factors, such as presence of sodium phosphate, chloride concentration, pH, temperature, aerobic and anaerobic conditions, and the presence of copper and silver ions might also modulate *S. maltophilia*. Studies have shown that biofilm formation

has been favoured by the following conditions: presence of sodium phosphate (Brooke, 2007); temperature growth of 32 °C, aerobic conditions in 6.0 % CO_2 atmosphere (Di Bonaventura *et al*., 2007). Conversely, high concentrations of chloride seem to block important exopolysaccharide groups (Critchley *et al*., 2003), whereas copper-silver ions might bind to important biological molecules responsible for electrostatic and hydrophobic interactions (Shih and Lin, 2010). Both conditions were linked to a reduction of biofilm formation.

1.5 Epidemiology and risk factors

The clinical importance of *S. maltophilia* is enhanced by the emergence of multiple-drug resistance. This bacterium can be recovered from amply diverse sources, such as soil, plant roots, animals, invertebrate insects, water treatment and distribution system, sinkholes, lakes, rivers, washed salads, faucets, tap water, bottled water, hand-washing soap, contact lens solutions, ice machines, contaminated topical antiseptic and sink drains (Brooke, 2012; Zanetti *et al*., 2009; Denton and Kerr, 1998).

Moreover, it is also able to form biofilms in water distribution systems, representing a potential risk for immunocompromised individuals (Sacchetti *et al*., 2009; Brooke, 2008). Once drains consist of aqueous environments, gradual deposition of bacteria might occur, leading to formation of biofilms. A study conducted in an U.S. teaching hospital collected samples from below the sink drainer, which not only revealed the presence of MDROs, including *S. maltophilia*, but also uncovered existence of thick biofilms (Brooke, 2008). After testing the antimicrobial effects of 10.0 % peracetic acid and 3.0 % hydrogen peroxide (H_2O_2) to prevent contamination of a microfiltered water dispenser, it was noticed that 40 min post-treatment with H_2O_2 was most effective and reduced the number of *S. maltophilia* cells to <1 log CFU/100 mL (Sacchetti *et al*., 2009). Consequently, identification of these environmental sources is necessary to prevent and/or control bacterial contamination.

The carriage of *S. maltophilia* in humans was also investigated. Interestingly, a faecal carriage rate of 6.0 % (14 out of 218 stool samples) was detected in outpatients with diarrheal illness or people they have come into contact with (Von Graevenitz and Bucher, 1983). In a small series, four out of 12 haematologic malignancy patients from

whom faecal samples were obtained were considered colonised by the bacterium. In contrast, the organism in question was only isolated in 2.9 % (n=2) of individuals from a control group (n=69 individuals) (Kerr *et al*., 1991).

Multiple continents have documented an increasing isolation rate of *S. maltophilia*. For example, a study conducted in a range of U.S. hospitals from 1993 to 2004 revealed that *S. maltophilia* was, during this period, the most frequently isolated Gram-negative bacillus obtained from clinical isolates from patients in the intensive care unit (ICU) (4.3 % of total of 74,394 isolates) (Lockhart *et al*., 2007). A German surveillance program conducted in ICUs indicated *S. maltophilia* as one of the 13 most frequent microorganisms responsible for nosocomial infections. Over a two-year period of investigation, the number of infections caused by *S. maltophilia* per 1,000 patient-days was 1.4 (range 0-7.6) (Meyer *et al*., 2006). In a tertiary care hospital in Taiwan, over a five-year period, increases of 49.0 % and 85.0 %, respectively, were noted in all types of nosocomial infection and nosocomial bloodstream infection caused by this pathogen (Tan *et al*., 2008). Another study conducted by SENTRY Antimicrobial Surveillance Program from 1997 to 2008 showed that the recovery rate of *S. maltophilia* from hospitalised patients with pneumonia varies (range 2.3 % - 3.3 %) between hospitals and geographic regions (Jones, 2010). In 2004, over 3,000 paediatric clinical isolates from three continents (North America, Latin America and Europe) were analysed by the SENTRY Antimicrobial Surveillance Program. The results indicated that *S. maltophilia* was among the top 15 isolated pathogens in North America and Latin America, but not in Europe (Fedler *et al*., 2006).

In most cases *S. maltophilia* acquisition is nosocomial (Brooke, 2012; Falagas *et al*., 2009). Molecular typing of isolates from hospitalised individuals or CF patients by pulsed-field gel electrophoresis, Enterobacterial Repetitive Intergenic Consensus - Polymerase Chain Reaction (ERIC-PCR), and semi-automated repetitive-sequence-based polymerase chain reaction (rep-PCR) assay (Diversilab®) uncovered a high genetic diversity between most strains, with occasional small clusters of close related isolates (Wu *et al*., 2011; Gülmez and Hasçelik, 2005; Valdezate *et al*., 2004; Denton *et al*.,1998). This finding indicates that *S. maltophilia* is acquired from an independent source, probably even prior to hospital admission, and the bacterium is then selected, during antimicrobial exposure, from the commensal flora. Further, a higher rate of

mutation is observed in clinical *S. maltophilia* isolates than in environmental ones. This fact suggests a possible adaptation mechanism to new or challenging environments, such as the CF airway (Taddei *et al*., 1997).

Transmission of *S. maltophilia* infection to susceptible individuals can be avoided if important criteria concerning risk factors are considered. Currently, a number of risk factors associated with this pathogen have been identified, including underlying malignancy (Calza *et al*., 2003), the presence of indwelling medical devices (Metan *et al*., 2006), prolonged hospitalisation, ICU stay, chronic respiratory disease (Waters *et al*., 2013; Waters *et al*., 2011), and compromised host immune system (Calza *et al*., 2003). Long-term therapy involving use of broad-spectrum antibiotics was also found to be an independent risk factor for stenotrophomonad infection (Paez and Costa, 2008).

1.6 Antibiotic resistance

S. maltophilia exhibits high-level intrinsic antibiotic resistance and several molecular mechanisms contribute to its drug resistance (Brooke, 2012).

Beta-lactamases are a group of enzymes produced by some bacteria, which confer upon them resistance to the action of beta-lactam antibiotics (Abraham and Chain, 1988). Lactamases are the main protagonists underpinning natural resistance to β-lactams. This arises from the expression of two inducible β-lactamases, L1 and L2. L1 belongs to Ambler class-B zinc dependent metalloenzymes, which hydrolyse all classes of β-lactams, but not monobactams (Avison *et al*., 2002). L2 is an Ambler class-A serine active-site β-lactamase that hydrolyses cephalosporins and monobactams, but it is inhibited by clavulanic acid and other β-lactamase inhibitors (Walsh *et al*., 1997). In general, these two chromosomal β-lactamases are induced when cells are exposed to β-lactams. Although the production of both β-lactamases is controlled by the same regulator (AmpR- ampicillin resistant), the expression of both enzymes is variable and is linked to chromosomal genes that are polymorphic even interspecies (Avison *et al*., 2002). TEM1-type lactamases (first reported in 1965 from an *Escherichia coli* isolate from a patient in Temoneira), a plasmid-encoded constitutive broad-spectrum 2-lactamase, can be related to mobile genetic elements and can exchange genetic material with other bacteria (Avison *et al*., 2000).

The active efflux of toxic substances and antimicrobial agents out of the bacterial cell has long been recognised as a resistance mechanism (Li *et al*., 1994). A membrane fusion protein, an energy-dependent transporter, and outer membrane proteins compose the efflux pump of *S. maltophilia* (Nicodemo and Paez, 2007; Alonso and Martínez, 2000). In 2000, for the first time a multi-drug efflux pump of *S. maltophilia* was cloned and characterised. The system received the name SmeDEF (Alonso and Martínez, 2000). Later on, it was verified that the SmeDEF multi-drug efflux pump was overexpressed in 33.0 % of clinical isolates, and it was correlated to the increase of tetracycline, choramphenicol, erythromycin, norfloxacin and ofloxacin minimum inhibitory concentrations (MICs) (Alonso and Martínez, 2001). Additionally, the SmeABC system, having SmeC as an alternative outer membrane multiple drug efflux protein of *S. maltophilia*, was described (Li *et al*., 2002). Recently, it has been shown that approximately 63.0 % of clinical *S. maltophilia* overexpressed SmeABC and exhibited high resistance to ciprofloxacin. These isolates were also typed using multilocus sequence typing, and a high degree of genetic diversity was observed among them (Cho *et al*., 2012).

It has been suggested that aminoglycoside-modifying enzymes, temperature-dependent resistance due to modifications of the outer membrane, and efflux pumps are involved in aminoglycoside resistance (Brooke, 2012; Nicodemo and Paez, 2007). Enzymatic alterations of the aminoglycosides are correlated to a certain family of enzymes, which includes O-nucleotidyltransferases, O-phosphotransferases, and N-acetyltransferase (Looney *et al*., 2009). The strains producing the enzyme *aac(6')-Iz* showed higher resistance to gentamicin (Lambert *et al*., 1999). Further, deletion of the *aac(6')-Iz* acetyltransferase gene in a wild type *S. maltophilia* strain induced increased susceptibility to aminoglycoside antibiotics, including netilmicin, sisomicin, tobramycin, neomycin, and gentamicin (Li *et al*., 2003). Susceptibility variation to aminoglycosides and polymyxin was also associated to outer-membrane LPS features. *S. maltophilia* strains resistant to the aforementioned antibiotics tended to exhibit changes in LPS structure by surface expression of a high molecular weight polysaccharide component (McKay *et al*., 2003; Rahmati-Bahram *et al*., 1996).

These, among other mechanisms (Table 1), are responsible for *S. maltophilia* antibiotic resistance.

Table 1 Summary of molecular mechanisms of antimicrobial resistance in *S. maltophilia* (modified from Brooke, 2012)

Mechanisms
• *β*-Lactamases chromosomally and plasmid encoded and on mobile elements
• Multidrug efflux pumps, e.g. SmeDEF, SmeABC, associated with resistance to quinolones, tetracycline, chloramphenicol, erythromycin, aminoglycosides, and *β*-lactams
• Class 1 integrons and insertion element common region (ISCR) elements associated with resistance to trimethoprim-sulfamethoxazole
• Phosphoglucomutase (SpgM) associated with resistance to polymyxin B, polymyxin E, nalidixic acid, gentamicin, vancomycin, ceftazidime, ticarcillin-clavulanic acid, and piperacillin-tazobactam
• Reduction in outer membrane permeability
• Quinolone protection protein (SmQnr) determinant associated with resistance to quinolones
• Modification of antibiotics
• Mutation of bacterial topoisomerase and gyrase genes

1.7 Treatment of infections

Adoption of an adequate antimicrobial regimen to treat *S. maltophilia* infection remains a challenge due to the high-level intrinsic resistance, increasing resistance prevalence of this bacterium and doubts about data regarding *in vitro* susceptibility testing characteristics (Looney *et al*., 2009).

The use of trimethoprim-sulfamethoxazole (also known as co-trimoxazole) alone, or in combination with other antimicrobial agents, is considered the first line of treatments for suspected or culture positive *S. maltophilia* infections. *In vitro* data indicates that trimethoprim-sulfamethoxazole acts as a bacteriostatic agent against this pathogen (Zelenitsky *et al*., 2005). However, patient hypersensitivity to this agent, due to the production of nitroso metabolites of sulfomethoxazole, might limit its use (Cheng *et al*., 2008).

β-lactam antibiotics show low activity, and given the previously mentioned resistance mechanisms, the use of penicillins and cephalosporins, particularly carbapenems, is

limited against *S. maltophilia*. In some instances, β-lactamase inhibitors, such as clavulanic acid, could actually increase the susceptibility of this bacterium to such agents (Muñoz Bellido *et al*., 1997). For example, for patients that cannot be treated with trimethoprim-sulfamethoxazole, ticarcillin-clavulanic acid could be considered a second therapeutic option (Falagas *et al*., 2008; Denton and Kerr, 1998). Although some *in vitro* activity is shown by the higher-class cephalosporins, such as ceftazidime, cefoperazone, and cefepime, there are increasing reports of strengthened resistance and low activity, a fact explained by the diversity of *S. maltophilia* isolates and the variable occurrence of inducible lactamases (Falagas *et al*., 2008). Similarly, aminoglycosides also show poor activity against *S. maltophilia* due to high intrinsic resistance and, consequently, they play no role in monotherapy (Looney *et al*., 2009; Nicodemo and Paez, 2007).

Conversely, the new fluoroquinolones (clinafloxacin, gatifloxacin, levofloxacin, moxifloxacin and sitafloxacin) exhibit superior *in vitro* activity than earlier quinolones (Nicodemo *et al*., 2004; Weiss *et al*., 2000; Valdezate *et al*., 2001a). Further, the tetracyclines doxycycline, minocycline and especially tigecycline have demonstrated good *in vitro* activity against clinical isolates of *S. maltophilia* (Sader *et al*., 2013; Tekçe *et al*., 2012; Looney *et al*., 2009), but clinical experience with this compound is limited (Samonis *et al*., 2012).

Polymyxins are a type of antibiotic that have a polycationic peptide ring that interacts with anionic LPS molecules from the outer membrane of Gram-negative bacteria and, consequently, causes increase in cell-envelope permeability, leakage of cell contents and finally cell death (Schindler and Osborn, 1979; Newton, 1956). In the past decade, polymyxins have gained a role in the treatment of infections caused by multiresistant Gram-negative bacilli (Landman *et al*., 2008). By contrast, the real value of *in vitro* susceptibility testing data and toxicity effects with these drugs remain yet to be clarified (Looney *et al*., 2009; Nicodemo and Paez, 2007).

Chloramphenicol is a bacteriostatic antimicrobial able to inhibit protein synthesis (Kasten, 1999). It has been reported that chloramphenicol combination therapy was successful in the treatment of three *S. maltophilia* meningitis cases (Feder, 1986). Unfortunately, clinical experience with this drug is quite limited and toxicity side effects should be kept in mind (Looney *et al*., 2009; Nicodemo and Paez, 2007).

1.8 Cystic Fibrosis

Cystic fibrosis or mucoviscidosis is the most common fatal autosomal recessive disorder in the white population (O'Sullivan and Freedman, 2009). The incidence of CF varies around the world, but estimates show that 1 in 2,000-3,000 new borns in the European Union is affected by this genetic disorder (WHO, 2014).

This disease is characterised by more than 1,700 different mutations in the cystic fibrosis conductance regulator (CFTR) gene, which encodes a small conductance ATP- and cAMP-dependent chloride channel that is mainly expressed in the apical border of epithelial cells lining exocrine glands (Rowe *et al*., 2005). Imbalance of ion concentration across the cell membrane due to absence of this channel will result in secretion of viscous fluids that eventually may result in plugged and atrophic ducts (Vankeerberghen *et al*., 2002).

In the airways of CF patients, chloride secretion is decreased while sodium absorption is increased. Thus, the secreted mucous is thick, viscous and difficult to clear (O'Sullivan and Freedman, 2009). In addition, increased inflammatory response in the affected lung may be detectable through the occurrence of: downregulation of epithelial interleukin 10 (IL-10) production (an anti-inflammatory factor) (Bonfield *et al*., 1995); and increased degradation of annexin 1 (an anti-inflammatory protein present in bronchioalveolar lavage fluid) (Tsao *et al*., 1998). As a result, the CF lung provides a favourable environment for the development of chronic colonisation by diverse microorganisms (de Vrankrijker *et al*., 2010; Rowe *et al*., 2005).

In past decades, due to the development of better antimicrobial therapies, nutritional support and lung transplantation, life expectancy for CF patients has increased to around 40 years (CFFPR, 2012). Other studies suggested a median survival age of up to 50 years to be a realistic scenario for these individuals (Dodge *et al*., 2007).

Generally, CF patients reveal a progressive microbiological history. Typically, infants are infected by a limited spectrum of bacteria, mainly *Staphylococcus aureus* and *Haemophilus influenzae*. However, chronic colonisation by *Pseudomonas aeruginosa* becomes notable with age, and transient detection of other Gram-negative organisms also occurs (CFFPR, 2012; Hauser *et al*., 2011) (Figure 3).

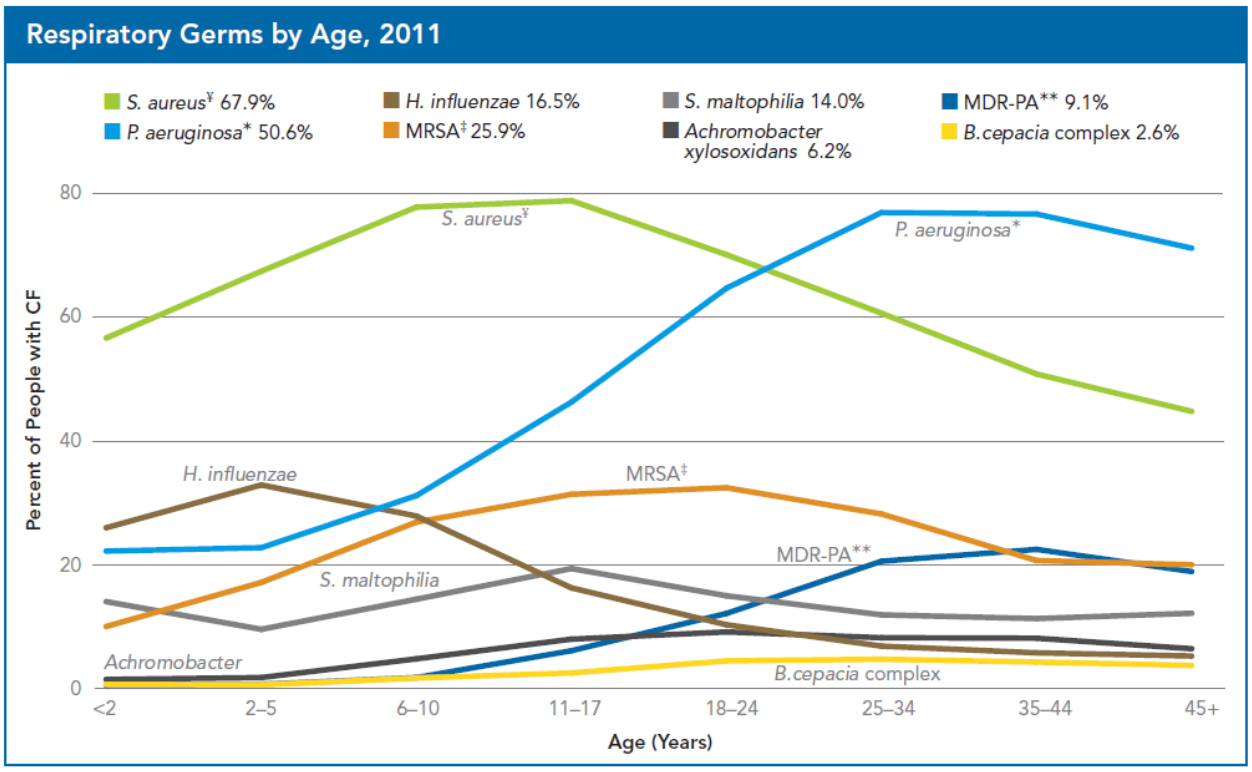

Figure 3 Prevalence of respiratory pathogens in CF patients by age. Data source from Cystic Fibrosis Foundation Patient Registry (CFFPR), 2012.

Chronic microbial infections lead to progressive lung function depression and pulmonary exacerbations, considered major causes of death in CF patients (Buzzetti *et al*., 2009; FitzSimmons, 1993). Pulmonary exacerbations are referred as intermittent episodes of acute worsening of symptoms, which may include clinical features such as increased cough and sputum production, shortness of breath, chest pain, loss of appetite, loss of weight and lung function decline (Goss and Burns, 2007).

Pulmonary exacerbations in CF patients are related to diverse microorganisms, and one of them is *S. maltophilia*. The first report of *S. maltophilia* in a CF patient only occurred later in 1979 (Blessing *et al*., 1979). It is considered a ubiquitous organism, which can also be widely isolated from different nosocomial sources (Brooke, 2012; Denton and Kerr, 1998).

Even though *S. maltophilia* commonly infects the respiratory tract of CF patients, its importance in the pathophysiology of CF lung disease remains unclear. Several worldwide CF centers have observed an increased prevalence of *S. maltophilia* (de Vrankrijker *et al*., 2010). Recently, it has been suggested that chronic colonisation by *S.*

maltophilia might be a risk for developing pulmonary exacerbation, death or lung transplant in CF patients (Waters *et al*., 2013; Waters *et al*., 2011). The question whether *S. maltophilia* is casuality related to the disease progression or a marker of the disease's severity is still not defined.

Therefore, isolation of this microorganism in CF patients becomes a cause for concern within the CF community, as previously described, due to the emergence of strains that might be resistant to current available antibiotic therapeutics suitable for treating CF.

1.9 Aim of the study

S. maltophilia is a globally emerging environmental Gram-negative rod, whose prevalence has recently increased in CF centres worldwide (de Vrankrijker *et al*., 2010). It has recently been described as an independent risk factor for pulmonary exacerbation and has been associated with increased risk of death or lung transplantation in CF patients (Waters *et al*., 2013, Waters *et al*., 2011). In addition, this pathogen exhibits an inherently resistant profile against a plethora of antibiotic agents and it has the ability to form biofilms on abiotic (e.g. glass and plastics like polystyrene) and biotic (bronchial epithelial cells of host) surfaces (Brooke, 2012; Pompilio *et al*., 2010b). It is known that bacterial populations in CF patients are constantly exposed to a challenging environment and through repeated courses of antibiotic treatment. Therefore, strong diversification over time and the emergence of mutator phenotypes is likely to occur (Tenaillon *et al*., 1999). The significance of chronic and/or persistent colonisation of CF airway by *S. maltophilia* has not been sufficiently clarified, which leads to widely disparate views regarding the relevance of this bacterium in these patients (Goss *et al*., 2004; Valdezate *et al*., 2001b). The efficacy of currently available therapeutics is diminishing due to the ongoing multi-drug-resistant nature of *S. maltophilia*. This, coupled with the lack of in-depth knowledge concerning drug resistance profiles of *S. maltophilia*, makes this microbe difficult to control in the CF population.

This study attempts to investigate the relevance and importance of *S. maltophilia* in the CF-lung environment. Therefore, *S. maltophilia* was characterised and the following issues were investigated:

1. Experiments for isolation and detection of *S. maltophilia* from sputum samples of CF patients - proper identification and antimicrobial susceptibility testing.

Since other microorganisms such as *P. aeruginosa* and *Aspergillus fumigatus* can easily overgrow on solid media, isolation and identification of other potentially pathogenic microorganisms can often be challenging to microbiology laboratories. The first part of this dissertation focuses on the development and utilisation of a selective medium for improved isolation of *S. maltophilia* in comparison to conventional media. We also reported the activity of eight antimicrobial agents against this microorganism.

2. Phenotypic and genotypic characteristics of *S. maltophilia* isolates obtained from CF patients in comparison to other sources.

The second part of this study is dedicated to investigating the genotypic diversity, specific assimilation, whole-cell fatty acid methyl ester profiles and protein composition of CF isolates, in comparison to other non-CF isolates and environmental samples.

3. Evaluation of specific immune response against *S. maltophilia* in CF patients.

In general, chronic exposure to a pathogen leads to a specific immune response. Infection markers for *S. maltophilia*, such as antibodies, may be helpful to determine the state of colonisation/infection by this bacterium in CF patients. Thus, we developed a quantitative immunofluorescence assay for the detection of specific *S. maltophilia* antibodies in serum from CF patients. We also verified if there was a correlation between *S. maltophilia* antibody titres and different categories of colonisation (never *S. maltophilia* or *P. aeruginosa*, intermittent and chronic).

4. Adaptation and dynamics of *S. maltophilia* bacterial population in CF airways during chronic infection.

Some studies have reported high prevalence of *P. aeruginosa* mutators in chronically colonised CF patients. Hypermutation is thought to play a role in the evolution of antibiotic resistance, which consequently results in limitation of antibiotic-therapy options. To expand understanding on the matter of *S. maltophilia* adaptation to CF airways, we examined the genotypic diversity, mutation frequency and antibiotic resistance of *S. maltophilia* isolates from chronically colonised CF patients.

5. Evaluation of EGCg as an antimicrobial agent against *S. maltophilia*

Finally, we evaluated the antimicrobial activity of EGCg, the most abundant polyphenol present in green tea, against CF *S. maltophilia* isolates and *S. maltophilia* acute pulmonary infection induced in wild type and *Cftr* mutant mice. We further determined EGCg effects on biofilms in comparison to those of colistin (COL).

2 Material and methods

2.1 Antimicrobial agents

Table 2 Antimicrobial agents

Antimicrobial agents	Company
Amphotericin B	Sigma-Aldrich, St. Louis, USA
Ampicillin sodium salt	Sigma-Aldrich, St. Louis, USA
Colistin (27655-1G)	Sigma-Aldrich, St. Louis, USA
Gernebcin (tobramycin – 40 mg/mL)	InfectoPharm, Heppenheim
Imipenem	ZIENAM, Merck Sharp&Dohme, India
Rifampicin (R3501-250 mg)	Sigma-Aldrich, St. Louis, USA
Vancomycin	Ratiopharm, Ulm
Colistin, Ceftazidime, Fosfomycin, Levofloxacin, Moxifloxacin, Ticarcillin/clavanulate, Trimethoprim/sulfametaxazole, Tigecycline, Tobramycin (MIC test strips)	Liofilchem, Roseto degli Abruzzi, Italy

2.2 Chemical products

Table 3 Chemical products

Chemical products	Company
Acetic acid	Merck, Darmstadt
Agar	Carl Roth, Karlsruhe
Aqua sterile distilled	B. Braun, Melsungen
Boric acid	Carl Roth, Karlsruhe
Blood agar base (Fa. CM 55)	Oxoid, Wesel
Calcium chloride ($CaCl_2$)	Fluka Chemie AG, Switzerland
Cholesterol/cholesterin	Carl Roth, Karlsruhe
Crystal violet	Sigma-Aldrich, St. Louis, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, St. Louis, USA
Disodium hydrogen phosphatedihydrate (Na_2HPO_4. $2H_2O$)	Merck, Darmstad
Dipotassium hydrogen phosphate (K_2HPO_4)	Merck, Darmstad
Epigallocatechin-3-gallate (EGCg E4143-50mg)	Sigma-Aldrich, St. Louis, USA
Ethanol ≥99.8 %,p.a.	Carl Roth, Karlsruhe
Ethidium bromide	Carl Roth, Karlsruhe
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth, Karlsruhe
Magnesium sulfate heptahydrate ($MgSO_4$. $7H_2O$)	Merck, Darmstad
Menadione	Sigma-Aldrich, St. Louis, USA

Methanol	Baker, USA
n-Hexan	Merck, Darmstad
Phosphate buffer saline pH 7.4 supplement with Tween 20 (PBS/Tween20)	Sigma-Aldrich, St. Louis, USA
Potassium dihydrogen phosphate (KH_2PO_4)	Merck, Darmstad
Saponin	Sigma-Aldrich, St. Louis, USA
Sodium chloride (NaCl)	Carl Roth, Karlsruhe
Sodium hydroxide (NaOH)	Merck, Darmstadt
Sodium hypochlorite (NaClO)	Carl Roth, Karlsruhe
tert-Butylmethylether	Merck, Darmstad
Trimethylsulfonium hydroxide	Macherey-Nagel, Düren
Tris(hydroxymethyl)aminomethane (Tris)	Carl Roth, Karlsruhe
Ultrapure MilliQ water (0.22 µm filter)	Merck, Darmstad
2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT)	Sigma-Aldrich, St. Louis, USA
5-Fluoro-2'- deoxyuridine (FUDR)	Calbiochem, Darmstadt
100 base pair DNA ladder (100 BP Ladder, 100 µg)	GE Healthcare, Little Chalfont, UK

2.3 Buffer solutions, solutions and media (solid and liquid)

Candida chrom agar (Brillance) (Oxoid, Wesel, Germany)

Typical formula
4 g/L peptone
13.6 g/L chromogenic mix
13.6 g/L agar
pH 6.0 ± 0.2

Columbia agar with sheep blood (Oxoid, Wesel, Germany)

Typical formula
23 g/L special peptone
1 g/L starch
5 g/L NaCl
10 g/L agar
pH 7.3 ± 0.2

Luria-Bertani (LB) agar

10 g/L trypton (Becton Dickinson, USA)
5 g/L yeast extract (Becton Dickinson, USA)
10 g/L NaCl (Merck, Darmstadt)
15 g/L agar (Merck, Darmstadt)
Sterilise by autoclaving the solution at 121 °C for 20 min.

Luria-Bertani (LB) broth	10 g/L trypton (Becton Dickinson, USA) 5 g/L yeast extract (Becton Dickinson, USA) 10 g/L NaCl (Merck, Darmstadt) Sterilise by autoclaving the solution at 121 °C for 20 min.
Lysis buffer (*C. elegans* assay)	3.3 mL 5M NaOH 2.8 mL 12 % NaOCl 3.9 mL MilliQ water
Malt extract agar plate (Oxoid, Wesel, Germany)	Typical formula 30 g/L malt extract peptone 5 g/L mycological peptone 15 g/L agar pH 5.4 ± 0.2
MacConkey agar plate (Oxoid, Wesel, Germany)	Typical formula 20 g/L peptone 10 g/L lactose 5 g/L bile salts 5 g/L NaCl 0.075 g/L neutral red 12 g/L agar pH 7.3 ± 0.2
Müller-Hinton agar (MHA) (Oxoid, Wesel, Germany)	Typical formula 300 g/L beef, dehydrated infusion of 17.5 g/L casein hydrolysate 1.5 g/L starch 17 g/L agar pH 7.3 ± 0.2
Müller-Hinton broth (MHB) (Oxoid, Wesel, Germany)	Typical formula 300 g/L beef dehydrated infusion of 17.5 g/L casein hydrolysate 1.5 g/L starch Add 21 g to 1 L of distilled water and autoclave at 121 °C for 20 min. Adjust pH to 7.3.
M9 Buffer (*C. elegans* assay)	3 g/L KH_2PO_4 6 g/L Na_2HPO_4 5 g/L NaCl 1 mL/L 1 M $MgSO_4$ Sterilise by autoclaving at 121 °C for 20 min.

Nematode growth medium (NGM) agar (*C. elegans* assay)	3 g NaCl 17 g agar 2.5 g peptone Dissolved in 975mL of distilled water and autoclave for 121 °C for 20 min. After autoclaving procedure, add under sterile conditions: 1 mL 1M $CaCl_2$ 1 mL of 5 mg/mL cholesterol dissolved in absolute ethanol 25 mL 1M KPO_4 1 mL 1M $MgSO_4$
NGM liquid medium (*C. elegans* assay)	3 g NaCl 2.5 g peptone Dissolved in 975 mL of distilled water and autoclave for 121 °C for 20 min. After autoclaving procedure, add under sterile conditions: 1 mL 1M $CaCl_2$ 1 mL of 5 mg/mL cholesterol dissolved in absolute ethanol 25 mL 1M KPO_4 buffer pH 6.0 (108.3 g KH_2PO_4, 35.6 g K_2HPO_4 to 1 L of H_2O) 1 mL 1M $MgSO_4$
Tris/borate/EDTA 1M solution buffer (electrophoresis)	121.1 g/L tris 61.8 g/L boric acid 7.4 g/L EDTA Sterilise by autoclaving the solution at 121 °C for 20 min. Adjust pH to 8.35-8.4.
Trypticase soya agar (TSA) (Becton Dickinson, USA)	Typical formula 15 g/L pancreatic digest casein 5 g/L papaic digest of soybean 5 g/L NaCl 15 g/L agar pH 7.3 ± 0.2
Tryptone soya broth (Oxoid, Wesel, Germany)	Typical formula 17 g/L pancreatic digest of casein 3 g/L enzymatic digest of soya bean 5 g/L NaCl 2.5 g/L K_2HPO_4 2.5 g/L glucose Add to 1 L of H_2O and sterilise by autoclaving at 121 °C for 20 min. Adjust pH to 7.3.

2.4 Commercial kits and solutions

Table 4 Commercial kits and solutions

Commercial kits and solutions	Company
API 20 NE strips	bioMérieux, Basingstoke, UK
Aqua 10 mL	B. Braun, Melsungen
LIVE/DEAD® BacLight™ bacterial viability and counting kit	life technologies, Darmstadt
DiversiLab® general Fingerprinting bacterial kit (30 samples)	bioMérieux, l'Etoile, France
DiversiLab® DNA chip and reagents	bioMérieux, l'Etoile, France
Fluoline H Immunoglobulin G (IgG) fluorescence (Ref 75 603)	bioMérieux, Nottingham, UK
Propidium iodide 1mg/mL	life technologies, Darmstadt
Taq PCR Core Kit (10× PCR Buffer, 25mM $MgCl_2$, deoxynucleotide triphosphate (dNTP) Mix - 10mM of each)	QIAGEN, Hilden
Matrix Vitek MS-CHCA	bioMérieux, l'Etoile, France
SPUTASOL	Oxoid, Wesel
Target Vitek	bioMérieux, l'Etoile, France
UltraClean™ microbial DNA isolation kit	Mo Bio Laboratories, Carlsbad, USA
Phosphate buffer saline (PBS) 10× solution	Gibco, Grand Island, USA
RPMI medium supplement with HEPES (L-Glutamine + 25mM HEPES) 500 mL	Gibco, Grand Island, USA

Table 5 Equipment

Equipment	Company
Agilent 2100 bioanalyzer	Agilent Technologies, Santa Clara, USA
Agitator (IKAMAG®REO)	Ika Labortechnik, Staufen,
Bioanalyzer Chip Vortexer (MS 3)	Ika Labortechnik, Staufen
Biosafety Cabinet Type II, Category 2, Hera Safe	Thermo Scientific, Waltham, USA
Capillary FID GC system	SRI instruments, Torrance, USA
Central processing unit (connected to Agilent 2100 bioanalyzer)	Hewlett-Packard, Palo Alto, USA
Centrifuge (5415D)	Eppendorf, Hamburg
Centrifuge (Heraus Megafuge 16)	Thermo Scientific, Waltham, USA
Certomat U/H incubator shaker	B. Braun, Melsungen
Computer software	Microsoft Windows XP
Electric pipet-aid (0.1 mL to 200 ml)	Hirschmann Laborgeräte, Eberstadt
Horizontal electrophoresis gel box	Carl Roth, Karlsruhe

Freezer -20 °C (GGPv 6590)	Liebherr, Bulle, Switzerland
Gel photo documentation system	Intas Science Imaging Instruments GmbH, Göttingen
Hewlett Packard 5890 Series II Gas Chromatograph System with Computer/w software	Hewlett Packard, Palo Alto, USA
Incubator	Binder, Tuttlingen
Incubator Heraues Instruments - Function Line	Thermo Scientific, Waltham, USA
Inhalator	Pari Boy ® SX, Starnberg
Inverse confocal laser scanning microscope	Opera system, Perkin-Elmer Cellular Technologies, Waltham, USA
MicroScan® Walkaway system	Siemens, Erlangen
Microscope Axio observer	Zeiss, Jena
Microscope Wild M5A	Heerbrugg, Switzerland
Microtitreplate reader (Sunrise-96-well plate)	Tecan, Männedorf
Monitor 17 inches	Hewlett-Packard, Palo Alto, USA
Multi-channel pipette (8-channel) adjustable volume (30-300 µL)	Thermo Scientific, Waltham, USA
Nanodrop(ND- 1000) spectrophotometer	Peqlab Biotechnologie GmbH, Erlangen
Pipette (adjustable volume, 0.5-50 µL)	Carl Roth, Karlsruhe
Pipette (adjustable volume, 0.5-10 µL)	Carl Roth, Karlsruhe
Pipette (adjustable volume, 2-20 µL)	Eppendorf, Hamburg
Pipette (adjustable volume, 10-100 µL)	Eppendorf, Hamburg
Pipette (adjustable volume, 20-200 µL)	Eppendorf, Hamburg
Pipette (adjustable volume, 100-1000 µL)	Eppendorf, Hamburg
Power supply Vokam (SAE 2761)	Shandon Scientific, London, UK
PTC-100 Programmable Thermal Controller (ERIC-PCR reaction run)	MJ Research, St. Bruno, Canada
pH meter (pH 522)	WTW, Weilheim
Refrigerator (FKU1800)	Liebherr, Bulle, Switzerland
Balance (AW series)	Shimadzu, Kyoto, Japan
Balance (Tuning-Fork Sensor)	Shinko Denshi, Tokyo, Japan
Small Animal Laryngoscope Model LS-2	PennCentury, Wyndmoor, USA
Thermocycler FlexCycler (rep-PCR reaction run)	Analytik Jena, Jena
Vitek® Mass Spectrometer	Axima, Shimadzu Corporation, Kyoto, Japan
Vortex Genie 2™ (G560-E)	Bender & Hobein AG, Baden-Württemberg
Water bath	Köttermann Labortechnik, Hänigsen

2.5 Oligonucleotides

The primers used in DNA amplification for identification and for Enterobacterial repetitive Intergenic Consensus - polymerase chain reaction (ERIC-PCR) were purchased from Tib Molbiol (Berlin, Germany).

Table 6 Oligonucletide primers for amplification of 16S rRNA gene

Oligonucleotide	Sequence	Amplicon size (base pair – bp)
DG74	5′-AGG AGG TGA TCC AAC GCG A-3′	300
Rw01	5′-AAC TGG AGG AAG GTG GGG AT-3′	350

Table 7 Oligonucleotides for amplification priming in Enterobacterial Repetitive Intergenic Consensus - polymerase chain reaction (ERIC-PCR)

Oligonucleotide	Sequence	Annealing temperature (T_A)
Primer ERIC-1	5’-ATG AAG CTC CTG GGG ATT CAC-3’	55 °C
Primer ERIC-2	5’-AGG TAA GTG ACT GGG GTG AGC G-3’	55 °C

2.6 Improved detection of *S. maltophilia* in sputum sample and susceptibility testing profiles

2.6.1 Culture on conventional media

A total of 623 sputum samples from 165 CF patients admitted to the Department of Paediatric Pulmonology from the Children’s Hospital and West German Lung Centre at University Hospital Essen, Essen (Germany) were investigated. Growth of *S. maltophilia* was verified by inoculating 10 µL of sputum onto routinely implemented media. In cases where sputum exhibited a very mucoid consistency, an equal volume of SPUTASOL (LIQUID) was added, and this solution was vigorously homogenized on a vortex mixer at room temperature. The solid media used were Columbia agar with sheep’s blood, chocolate blood agar, MacConkey agar, Candida chrome agar (Brilliance), and malt agar. The incubation period for all media was 48 h at 35 °C, with the exception of malt agar, which was incubated for an additional eight days at room temperature.

2.6.2 Culture on selective media

Steno medium agar (SMA) was formulated as follows: 40 g of blood agar base to 1 L of distilled water, which was autoclaved for 15 min at 121 °C. The medium was allowed to cool to 50-60 °C in a water bath and the pH was adjusted to 7.0±0.2. Freshly filtered sterilised solutions of amphotericin B, imipenem, and vancomycin were added to make final concentrations of 2.5 mg/L, 32 mg/L, and 10 mg/L, respectively. The medium was transferred into petri dishes (diameter, 90 mm) and stored for 3 weeks at 4 °C. Medium validation was carried out by culturing *S. maltophilia* (ATCC 13637) and an imipenem-susceptible *Klebsiella pneumonia* reference strain (ATCC 10031) for as long as 25 days. Through this quality control, it was possible to demonstrate that SMA remains stable for up to 21 days. Imipenem activity is significantly reduced after this period of time. Aliquots from fresh sputa samples (not older than three days and stored at 4 °C) were plated onto SMA and incubated aerobically at 35 °C for up to 48 h.

2.6.3 Identification of *S. maltophilia* clinical isolates

Isolates that grew on SMA agar were identified by standardised biochemical laboratory methods, including MicroScan® Walkaway and API 20 NE strips. Fresh cultures of *S. maltophilia* isolates on Columbia agar with sheep blood were used to carry out DNA extraction. *S. maltophilia* ATCC 13637 reference strain was used as a quality control. Genomic DNA extraction was performed with the UltraClean™ microbial DNA isolation kit in accordance with the manufacturer's instructions. The extracted DNA was stored at -20 °C until amplifications were conducted. All clinical isolates were confirmed to be *S. maltophilia* by amplifying the genomic region that codes for the 16S ribosomal subunit, using the primers described in Table 6. Amplification reactions were conducted in a 50 µL final volume containing 1 µL of 10mM d-NTP mix, 5 µL of 10× PCR buffer, 0.5 µL (100µM) of each primer, 0.25 µL of 1.5 Unit (U) *Taq* DNA polymerase (Qiagen), 37.75 µL double distilled water and 5 µL DNA (5 ng/µL). The PCR program was carried out as follows: 25 cycles of denaturation step at 95 °C for 30 s, annealing of the primer at 50 °C for 60 s, and 2 min of extension at 72 °C. The last cycle extension was at 72 °C for 5 min. Amplicons were sent to LGC Genomics (Berlin, Germany) for further sequencing.

2.6.4 Susceptibility testing against eight antimicrobial agents

Antibiotic susceptibility of 65 *S. maltophilia* isolates obtained from 33 CF patients was analysed. MIC test strips with stable continuous gradient of ceftazidime, colistin, fosfomycin, levofloxacin, moxifloxacin, ticarcillin-clavulanate, trimethoprim-sulfamethoxazole, and tigecycline were used. Minimum inhibitory concentration (MIC) tests were conducted following the Clinical and Laboratory Standards Institute (CLSI) guidelines (M07-A8) (CLSI, 2009). *S. maltophilia* ATCC 16637 reference strain was used for quality assurance purposes. An adjusted suspension of each isolate to the density of 0.5 McFarland standard was plated on Müller-Hinton agar (MHA) in three directions to ensure uniform growth. MIC test strips were applied once the agar surface was completely dry. The plates were incubated for 24 h at 35 °C. The MIC was interpreted (according to the manufacturer's guidelines) as the zone of growth inhibition that intersected the MIC test strip. CLSI has established interpretive criteria for the characterisation of susceptibility breakpoints of *S. maltophilia*, for all of the above antimicrobial agents except for colistin and moxifloxacin. Susceptibility breakpoints established for *P. aeruginosa* were applied, when testing colistin and moxifloxacin (Andrews, 2009; CLSI, 2009).

2.7 Genotyping

All the clinical sputum samples from CF and ICU patients included in this study were collected as part of the routine screening procedure of the University Hospital of Essen, Essen, Germany. A total of 102 *S. maltophilia* isolates (CF=71, ICU=20, environment=11) were identified and genomic DNA from each sample was isolated as previously described. ATCC13637 was used to assure quality control.

2.7.1 Repetitive-Sequence-Based Polymerase Chain Reaction (rep-PCR)

Diversilab® (DL) bacterial DNA fingerprinting kit, which included rep-PCR master mix 1, bacterial primers, and kit-specific positive and negative controls, was used following the protocol recommended by bioMérieux. A total of 35 ng of genomic DNA, 2.5 U of AmpliTaq DNA polymerase, and 1.5 µL of 10× PCR buffer were added to the rep-PCR master mix to achieve a final volume of 25 µL. Thermal cycling parameters were as

follows: initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 70 °C for 90 s, and final extension at 70 °C for 3 min. Analysis of rep-PCR products was conducted by a DL system, in which amplified fragments of various sizes and intensities were separated electrophoretically and detected on a microfluidics Labchip with an Agilent 2100 Bioanalyzer. Loading volume was 1 µL. Electrophoresis data was examined through the internet-based DL software (version 3.4). Dendrograms were based on Pearson correlation coefficients, which generated a scatter plot and a virtual gel image. Isolates with a similarity of ≥ 95 % were considered as clinically related, and isolates with a similarity >98 % were considered as “indistinguishable” from one another.

2.7.2 Enterobacterial Repetitive Intergenic Consensus - Polymerase Chain Reaction (ERIC-PCR)

Amplification reactions were performed in a final volume of 50 µL of PCR mixture prepared for each isolate, containing final concentrations of 1×PCR buffer, 4mM $MgCl_2$, 0.5µM of each primer (Table 7), 80µM dNTP mixture, 1.25 U of *Taq* polymerase, sterile distilled aqua and 10 ng/µL extracted DNA (Finger *et al*., 2006). The Taq PCR Core Kit was used to carry out this DNA amplification. A positive control (ATCC 13637) was used in each run. After 5 min of denaturation at 94 °C, 45 cycles were run as follows: denaturation at 96 °C for 1 min, annealing at 55 °C for 2 min, extension at 72 °C for 1 min, and a final extension step at 72 °C for 5 min. The amplification products were analysed by electrophoresis using 1 % agarose gels. A 100bp DNA ladder was used as a molecular size marker. Band analysis of the amplicons obtained was performed using the Phoretrix 1 D software (TotalLab Ltd, Newcastle upon Tyne, UK, v12.2), whereas cluster analysis was conducted with Phoretrix 1D Pro (v12.2). Band pattern similarity was estimated by means of the Pearson correlation coefficient and clustering of patterns was defined using the Ward algorithm. Only five environmental isolates were suitable for use in this assay due to sample contamination or reading error.

2.8.3 Calculation of discriminatory power

The discriminatory power of DL and ERIC-PCR were calculated using Simpson's index of diversity (SID) and confidence intervals (CI). SID calculates the probability that two unrelated strains sampled from the test population will be placed into different typing groups. The quantitative correspondence between typing methods was assessed based on adjusted Rand (AR) and Wallace (W) coefficients (Severino *et al*., 2011; Pinto *et al*., 2008; Carriço *et al*., 2006). The AR index provides quantification of congruence; whereas the W coefficient assists in the calculation of the probability that two strains classified as the same type by one method would also be classified as the same type by other methods. Since the Rand coefficient leads to an overestimation of agreement amongst typing methods, its use was avoided. Index of diversity and degree of congruence between typing methods were estimated using an online application (http://www.comparingpartitions.info/; accessed on 01/15/2014).

2.8 Profiles of cellular fatty acid methyl esters (FAME) through gas chromatography (GC)

Reference strains ATCC13637 and DSM8573, environmental samples (n=11), as well as clinical isolates obtained from CF (n=71) and ICU (n=20) patients were cultured on TSA and incubated at 28 °C for 24 h. Approximately 100 mg of culture material (wet weight) were used. Experiments were performed as previously described (Miller and Berger, 1985; Müller et al., 1998). Bacterial pellets were saponified in 30.0 % (w/v) sodium hydroxide (NaOH) dissolved in water. Then, cellular fatty acids were methylated (hydrochloric acid in methanol), extracted (n-hexane in methyl *tert*-buthyl-ether), and cleaned (NaOH). The fatty acid methyl esters (FAMEs) were processed on a Hewlett (Avondale, PA) gas chromatographic system; series II gas chromatograph equipped with a split inlet and flame ionisation detector, automatic sampler 6890, and fused-silica capillary column (Ultra 2, HP 19091 B-102; 25m by 0.2 mm with 5.0 % cross-linked phenylmethyl silicone) as the stationary phase. The instruments were coupled with a HP Vectra XU 5/90C Computer loaded with 3365 series II Chemstation (version 3.34) software. The parameters conformed to those recommended by MIDI system (Microbial Identification System, operating manual, version 5.0, 1995, MIDI, Inc.): hydrogen was

used as a carrier gas, sample volume 2 µL, split ratio 1:100, injector temperature of 250 °C (temperature detector 300). Temperature program ramped from 170 °C to 260 °C at 5 °C/min and from 260 °C to 310 °C at 40 °C/min followed by an isotherm phase at 310 °C for 4 min. A ballistic increase to 310 °C allowed column cleaning during a hold of two min. The Sherlock Software Package of MIDI (version 1.06) performed analyses together with Sherlock System Software (Part 1300) and the Library Generation Software (LGS, Part 1303). Sherlock Pattern Recognition algorithm was used to obtain quantitative and qualitative (peak identification) information from the chromatography data. Cluster analysis through unweighted pair matching (Euclidean distance) and principal component analysis of the chromatographic data were performed using the implemented LGS software. The peak area index, which represents the amount of fatty acids produced in the broth culture during bacterial growth, was determined as follows: total peak area/solvent peak area × 10,000 (Müller *et al*., 1998). The unpaired non-parametric Mann-Whitney U test (significance set at $P < 0.05$) was used to address the fatty acid production differences amongst CF, ICU and environment isolates (Prism 5.0 - Graph Pad Software, San Diego, CA, USA).

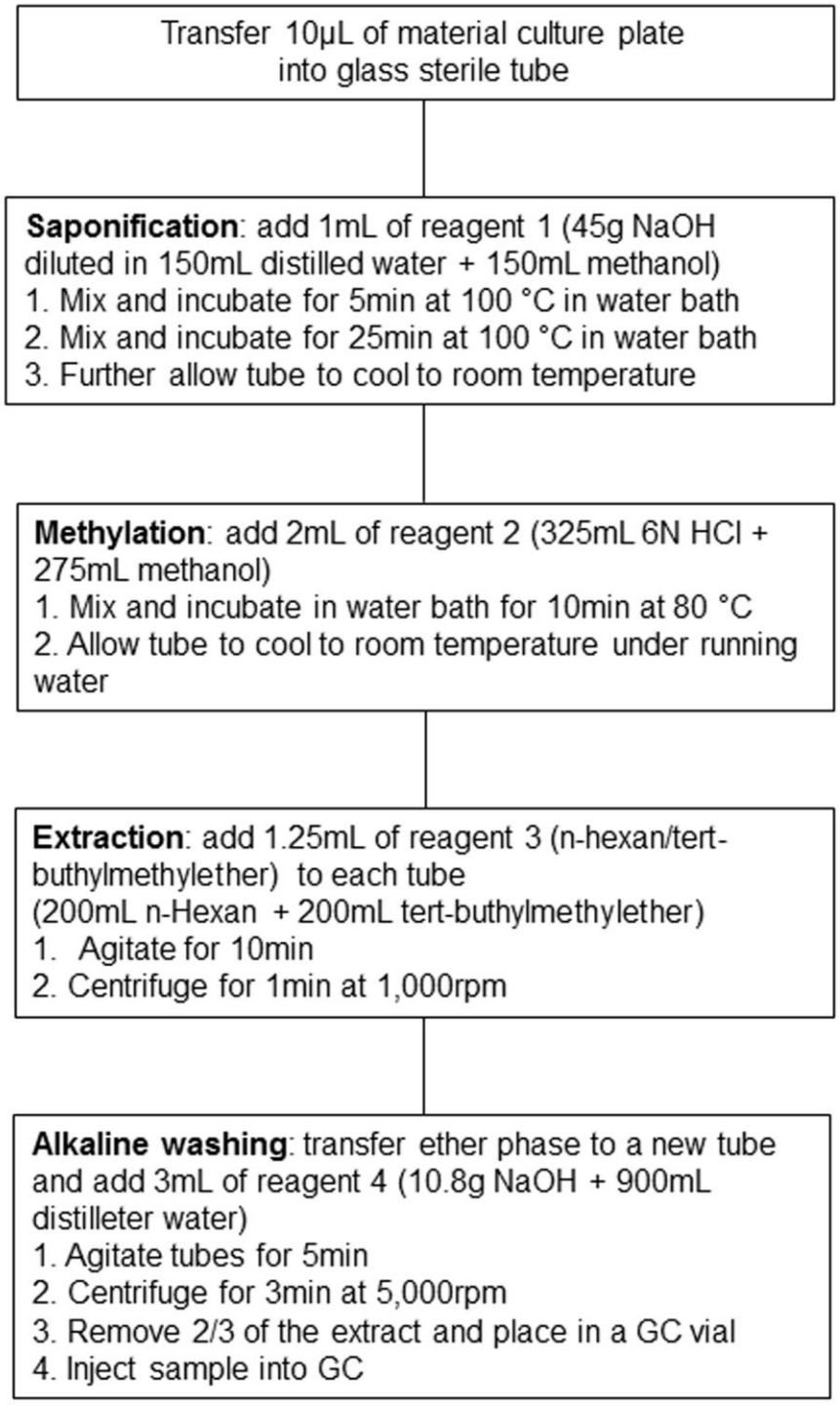

Figure 4 Sample preparation flow chart for GC experiments.

2.9 Matrix-assisted Laser Desorption Ionisation-Time of Flight Mass Spectrometry (MALDI-TOF MS)

Material from fresh colonies from an overnight culture of each of the *S. maltophilia* isolates (CF patient isolates, n=69; ICU patient isolates, n=20; environment isolates, n=11) were transferred to two sample position spots on a disposable MALDI-TOF MS target slide (bioMérieux). Two CF samples and the environmental reference strain were not suitable for use in this assay due to sample contamination or reading error. Subsequently, the bacterial sample was overlaid with 1 µL of α-cyano-4-hydroxy cinnamic acid as organic matrix solution (VITEK MS-CHCA) and allowed to dry at room temperature. Measurements were performed on a Shimadzu AXIMA Assurance ^{TM} mass spectrometer and analysed with the Vitek® MS Plus identification system provided by bioMérieux. The mass spectrometer is equipped with a 20-Hz N_2-LASER and an ion-source with an acceleration voltage of 20 kV and extraction delay time of 200 ns. Spectra were acquired in positive ion mode and within a mass range of 2,000 to 20,000 Da. Data from a minimum of 500 shots per sample spot were collected. The acquired spectra were compared to the Vitek MS RUO® system database. The acquired spectra were compared to the Vitek MS V2.0 Knowledge Base for Clinical Use. The spectra of the tested samples were matched against the SuperSpectra® included in the database. Peak pattern that produced a match with the identification data with a confidence level exceeding 80.0 % were considered significant and were displayed. The reference strain *Escherichia coli* ATCC 8739 was used to calibrate and to validate the mass spectrometer of each used target. All peak lists of MALDI-TOF mass spectra were processed to perform hierarchical cluster analysis (HCA), based on Euclidean distance for Ward's method, in order to calculate the degree of similarity among the isolates. Principal component analysis (PCA) of the processed peak lists was performed as a multivariate analysis to identify the first three principal components and also to uncover differences of the isolates in respect of these components. The data were processed by creating peak lists, ranging from 2,000 to 20,000 Da with intervals of 5 Da, containing either zero values (absence of peak) or the peak´s relative intensity values. After importing the peak lists into the software DataLab®, the data were standardised prior calculation of the distance matrix. The result of the HCA was visualised as a dendrogram. PCA was applied to determine if the dimension of the parameter space

spread up by all possible m/z values may be reduced to fewer dimensions of which the first three most important principle components explain most of the variability of the data. In the three dimensional plot each isolate is represented as a dot in the PC1-3 space. Clustering in respect of PCA is not the same as in HCA because of different mathematical approaches. PCA is more sensitive to reveal differences amongst individuals, whereas HCA, in contrast, is more sensitive to demonstrate similarities.

2.10 Dynamic adaptation of *S. maltophilia* in chronically colonised CF patients

2.10.1 Molecular typing by repetitive-sequence-based-PCR (rep-PCR)

In order to verify the molecular diversity of the isolates (n=90), rep-PCR was performed using the DL bacterial DNA fingerprinting kit, following the manufacturer's recommendations as previously described. We used ATCC 13637 reference strain as a quality control. Subculture stability and reproducibility of rep-PCR results were defined by the average similarity of >98 % of three different colonies obtained at three time points (1, 5 and 10 days).

2.10.2 Mutation frequency assay

Frequency of mutations conferring rifampicin resistance were estimated as described (Turrientes *et al*., 2010). *S. maltophilia* isolates (n=90) obtained from chronically colonised CF patients were cultured on blood agar plates and incubated for 24 h at 35 °C. One independent colony from each blood agar plate was inoculated in three glass tubes containing 2 mL of LB broth, and further incubated for 24 h in an orbital agitation incubator at 35 °C (150 rpm). Subsequently, aliquots (100 µL) of a 10^{-6} dilution from each overnight culture were cultured on LB agar plates. An additional 500 µL of these overnight cultures were seeded into LB agar plates supplemented with rifampicin (250 g/L). Colony counts of LB and LB-rifampicin agar plates were performed after 24 h and 48 h incubation, respectively. The result for each isolate derives from the mean value obtained from three independent experiments. If any of the following conditions were met: no colonies were detected on LB-rifampicin agar plates, suspicion of

contamination, or ten times difference in the standard deviation of the mutation frequencies from the three independent experiments; the experiments were repeated. In these cases, only the new values reported in the three new experiments would be included. *S. maltophilia* ATCC13637 reference strain (mutation frequency $f \approx 1.5 \times 10^{-7}$) was used as a control. The mean number of mutants for each isolate was calculated. Isolates were classified into four categories based on their mutation frequencies: hypomutator ($f \leq 8 \times 10^{-9}$), normomutator ($8 \times 10^{-9} < f < 4 \times 10^{-8}$), weak hypermutator ($4 \times 10^{-8} < f < 4 \times 10^{-7}$) and strong hypermutator ($f \geq 4 \times 10^{-7}$) (Turrientes *et al*., 2010).

2.10.3 Antimicrobial susceptibility testing

In order to verify if the isolates become more resistant over time, *in vitro* activities of ceftazidime, colistin, co-trimoxazole, fosfomycin, levofloxacin, moxifloxacin, tigecycline and tobramycin, were tested against all 90 *S. maltophilia* isolates. Susceptibility testing was performed using MIC test strips and resistance breakpoints were those of CLSI guidelines (M07-A8). For colistin, we used the breakpoints established for *P. aeruginosa*. *S. maltophilia* ATCC 16637 was tested for quality assurance purposes. A suspension with adjusted density of 0.5 McFarland standard was plated on MHA. MIC was read after incubation at 35 °C for 24 h. According to the manufacturer's guidelines, the zone of growth inhibition that intersected the MIC test strip was interpreted as the MIC. Fisher's test was used for comparison of resistance between nonmutator (hypomutator and normomutator) and mutator (weak hypermutator and strong hypermutator) groups.

2.11 Antibody titres against *S. maltophilia* in patients with CF - development of a quantitative immunofluorescence assay (IFA)

Serum samples from CF patients belonging to the University Hospital Essen epidemiological surveillance bank and healthy subjects were randomly selected and analysed. The serum collection consisted of a single specimen per patient. Based on their sputum culture status, CF individuals were classified within the study period as previously described (Döring and Høiby, 1983): *chronic*, two or more positive sputum

cultures for *S. maltophilia* in a given year; *intermittent*, one positive culture for *S. maltophilia* in a given year or previous positive culture; and *never S. maltophilia*, absence of positive culture for *S. maltophilia*. Additional groups were composed of: “CF patients never *S. maltophilia*/*P. aeruginosa*” and “CF patients never *S. maltophilia* but chronic *P. aeruginosa*”. These were included to verify potential cross-reactivity with *P. aeruginosa* antibodies. Serologic response was measured by indirect IFA using *S. maltophilia* (ATCC13637) whole cells as the antigen, which was later visualised by Fluoline H IgG fluorescence. *P. aeruginosa* (ATCC 27853) was added to the serum for absorption of non-*S. maltophilia* specific antibodies. For antigen preparation, reference strain ATCC 13637 was cultured overnight on blood agar plates at 35 °C. Colonies were harvested from the plate with 1 µL inoculation loop and resuspended in 1 mL PBS/Tween 20. The suspension was diluted 1:100 and aliquots of 10 µL were transferred to slide spots, which were air-dried. The slide was immersed on acetone for 15 min. Slide was air-dried and stored at room temperature. For *P. aeruginosa* antibody absorption, four plates of reference strain ATCC 27853 were cultured at 37 °C for 24 h. Colonies were collected after adding 2.5 mL of NaCl 0.9 % (w/v) to each plate. A volume of 300 µL of formalin at final concentration of 1.0 % was then also added and incubated for 48 h at room temperature. This “Pyo suspension” was centrifuged for 5 min at 5,000g. The pellet was washed three times (1:1) with NaCl 0.9 %. A total of 10 µL of serum was mixed with 100 µL of Pyo suspension (1:10 dilution) and 90µL of PBS/Tween 20 and further incubated for 1 h at 37 °C. After incubation, this mixture was centrifuged for 10 min at 5,000 g and 20 µL of supernant was used for IFA (dilution 1:20). Serologic response was observed through immunofluorescence. The slide spots were each coated with 20 µL of sera diluted to varying degrees with the range 1:80 to 1:2,560, from the CF patients and healthy individuals, and incubated for 30 min at 37 °C in a humid chamber. Slide spots were washed with PBS/Tween 20 for 10 min and allowed to dry for 5 min at 37 °C. A total of 20 µL of conjugate Fluoline H (1:500 dilution in PBS/Tween 20) was added to each spot and incubated for 30 min at 37 °C in a humid chamber. Slide-spots were washed in PBS/Tween 20 for 10 min at room temperature and then washed for 10 s with distilled water. Excess liquid was removed by gently tapping the slide on filter paper. Whilst still moist, slides were mounted and examined under the microscope. Results were expressed as a quantitative antibody level (titre). To verify if there were

significant differences in the antibody titres between the groups, unpaired non-parametric Mann Whitney test was used. Receiver operating characteristic (ROC) curve analyses were used to define optical cut-off titres through the highest sensitivity and specificity points (Kelly *et al*., 2008; Fan *et al*., 2006). Sensitivity and specificity values were calculated at 95.0 % confidence intervals (CI) using the Prism 5.0 software package (Graph Pad Software, San Diego, CA, USA).

2.12 Antimicrobial effects of Epigallocatechin-3-gallate (EGCg), a natural compound of green tea as an alternative therapy

2.12.1 Preparation of the antimicrobial agents

EGCg and colistin (COL) were obtained from Sigma (Sigma-Aldrich, St Louis, MO, USA). Stock solutions (1,024 mg/L) of EGCg were freshly prepared and diluted in MHB containing 1.0 % (v/v) DMSO. COL stock solutions (2,048 mg/L) were also dissolved and diluted in MHB including 1.0 % DMSO. COL stock solution was stored at -20 °C until use. For *in vivo* experiments, EGCg and COL were either only dissolved in PBS (wild type mice experiments) or sterile distilled aqua (*Cftr* mutant mice experiments) at room temperature.

2.12.2 Microdilution assay

Susceptibility testing was carried out according to the guidelines of the CLSI (CLSI, 2009). For the broth microtitre dilution tests, 96-well microtitre plates with 50 µL of between 0.5 to 1,024 mg/L EGCg per well were inoculated with 50 µL of MHB containing a bacterial cell suspension of between 1×10^5 to 5×10^5 colony-forming units per mL (CFUs/mL) of *S. maltophilia*. The plates were incubated for 24 h at 35 °C. MIC was determined as the lowest concentration able to inhibit visible growth. MBC was determined by transferring 10 µL aliquots of the cell suspensions that exhibited no signs of growth in broth microtitre dilution plates onto Columbia blood agar with 5.0 % (v/v) sheep blood. The inoculated plates were further incubated for 24 h at 35 °C. After incubation, MBC was defined as the lowest concentration that showed no growth.

2.12.3 Time-kill assay

The kinetics of the bactericidal effect of EGCg on *S. maltophilia* was investigated using reference strain ATCC 13637 and two CF clinical isolates (obtained from an intermittent and a chronically colonised patient, respectively designated as Sm1 and Sm2) in a microtitre plate assay as previously described (Gordon and Wareham, 2010). The clinical isolates Sm1 and Sm2 were both chosen as their MIC's and MBC's (Table 8) were representative for the collection of strains and they are both strong biofilm producers. Samples were cultured on blood agar plates and grown for 24 h at 35 °C. One glass tube per culture, containing 5 mL of TSB, was inoculated with a single colony and was incubated overnight in an orbital shaker at 35 °C with agitation at 145 rpm. A dilution of 1:10 was carried out (approximately 1×10^7 CFU/mL) and aliquots of 100 µL were placed in wells of a sterile flat-bottomed 96-well polystyrene microtitre plate (BRANDplates, BrandTech Scientific, Essex, CT, USA). The plates were incubated for 0, 2.5, 5, 10, or 24 h at 35 °C. At the indicated time points, a mixture of 0.85 % saline supplemented with XTT and menadione (at final concentrations of 100 g/L and 10 g/L, respectively) was added and the plates were further incubated for 2 h in the dark at 35 °C. A set of controls consisted of wells containing only TSB, TSB with inoculum and TSB with EGCg (tested concentrations). The reduction of XTT was used as a marker of cell viability. XTT reduction was measured colorimetrically at 492 nm.

Table 8 Susceptibility of *S. maltophilia* isolates to COL and EGCg as determined by the reference microdilution method of the Clinical and Laboratory Standards Institute.

Compounds	**ATCC 13637**		**Sm1**		**Sm2**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
COL (mg/L)	128	256	256	256	256	256
EGCg (mg/L)	8	32	32	128	32	64

2.12.4 *In vivo* experiments

Caenorhabditis elegans

Caenorhabditis elegans var. Bristol strain N2 was used.

Mouse strains

All mice were maintained in isolated cages to provide a pathogen-free environment at the Central Laboratory Animal Facility of the University Hospital Essen, Essen, Germany. Animal experiments were approved by the ethical committee of Landesumweltamt Nordrhein-Westfalen (Q1299/12), Germany.

C57/BL6

These mice were purchased from Harlan Winkelmann GmbH (Roosdorf, Germany).

B6.192P2 (CF/3)-*Cftr* $^{TgH\ (neoim)Hgu}$

This *Cftr* mutant mouse strain is bred and housed at the University Hospital Essen, Essen, Germany.

2.12.4.1 Cytotoxicity of EGCg on *C. elegans*

All experiments were carried out using age-synchronised young adult animals. Synchronization was performed by bleaching gravid adults with lysis buffer as previously described (Stiergale, 2006) but with the following modifications. Eggs were plated onto lawns of *Escherichia coli* OP50 (food source) on NGM agar media, and allowed to hatch and develop into young adults at 25 °C (approximately 42 h after synchronisation). Synchronous population was collected and washed five times using M9 solution. Final concentrations of EGCg (256 mg/L; 512 mg/L; 1,024 mg/L and 2,048 mg/L) were added to the wells, as indicated. Control consisted of animals exposed only to NGM liquid. The nematodes were incubated for 48 h at 25 °C. Data is expressed as the average of two independent experiments performed in triplicate.

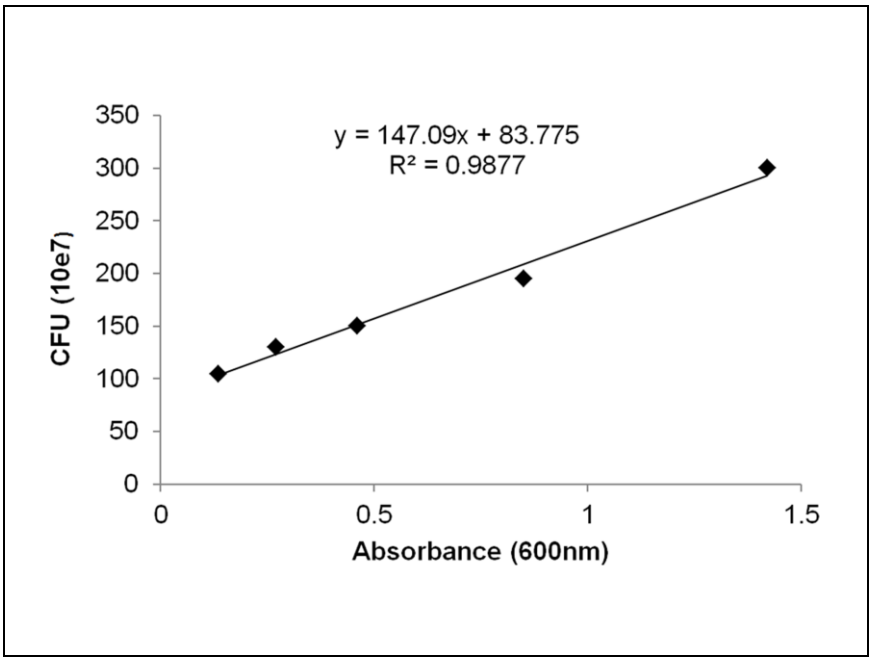

Figure 5 Representative standard curve of clinical isolate Sm1 for A600nm-CFU relationship for *S. maltophilia* (Sm1).

2.12.4.2 Antimicrobial effects of EGCg on *S. maltophilia* during infection in *C. elegans*

A *C. elegans* synchronised population was obtained as described above (2.13.4.1). To infect the nematodes, 20 mL of LB broth were inoculated with a single colony of *S. maltophilia* clinical isolate (Sm1) which was then incubated overnight in an orbital incubator 35 °C with agitation (150 rpm). Aliquots of 1 mL of inoculum adjusted to 0.5×10^7 CFUs/mL were added to each well containing varying concentrations of EGCg (2×MIC= 512 g/L and 4×MIC= 1,024 g/L) diluted in NGM liquid. Control wells consisted of *C. elegans* containing 1 mL NGM and 1 mL of inoculum. A control set containing only NGM medium liquid and *C. elegans* was also used. The assays were carried out in duplicate of three independent experiments with approximately 20 worms per group. The experiments were assessed every 24 h for worm mortality on continuous exposure to pathogen and EGCg under the conditions described above. Worms were considered dead if they did not move or did not exhibit muscle tone. *C. elegans* survival was plotted using Kaplan-Meier survival curves and analysed by the log rank test using GraphPad Prism software. Curves resulting in *P* values < 0.05 relative to control were considered significantly different.

2.12.4.3 Inhalation effects of EGCg *in vivo* pulmonary infection

Antimicrobial effects of EGCg were investigated in comparison to COL in female C57BL/6 specific-pathogen-free mice at age of 6 to 8 weeks. Two hours before infection, C57BL/6 mice were nebulised for 5 min with 1 mL of 1,024 mg/L EGCg (n=11), 128 mg/L COL (n=12), and 1mL 1×PBS (n=13) using an inhalator (Pari Boy® SX, Starnberg). The outside of the mouthpiece of this device was sealed, leaving only a small orifice, in order to fit the mouse's nose. Acute pulmonary infection was performed with clinical isolate Sm1. Bacteria from frozen stock were cultured on LB agar plates overnight and a suspension of these bacteria was adjusted to an OD of 0.225. Bacterial suspension was then cultured at 35 °C for 60 min with gentle agitation (125 rpm) to reach early logarithmic growth phase. Bacteria were washed with RPMI 1640 supplemented with 25mM HEPES (pH 7.4). After definition of the OD (Figure 5), bacteria were resuspended at a density of 1×10^{7} CFU/ 20µL in RPMI 1640. A solution of xylazine (10 mg/Kg) and ketamine (50 mg/Kg) prepared in sterile 1×PBS at final concentrations of 2 g/L and 10 g/L respectively, was used to anesthetise the mice. Intratracheal instillation was achieved using a 20-gauge needle with the assistance of a laryngoscope, allowing the delivery of 20 µL of the inoculum directly into the trachea (Rayamajhi *et al*., 2011). One hour post-infection, mice were induced to inhale EGCg, COL and PBS again under the same conditions detailed above. Clinical conditions of the mice were assessed as described previously (Lloyd and Wolfensohn, 1999). Mice were euthanized by cervical dislocation four hours after the infection. In order to release intracellular bacteria, the lungs of the mice were mechanically homogenised and lysed for 10 min in 5 g/L saponin with gentle shaking (125 rpm) at 35 °C and washed once in RPMI 1640 (HEPES 25mM, pH 7.4). Finally, dilutions of the homogenate lung tissues were cultured on LB agar plates in triplicate. Bacterial load from mouse lungs was determined after 18 h of growth at 35 °C. In *Cftr* mutant mice, both nebulisations (two hours prior infection and one hour post-infection) were performed with 1,024 mg/L EGCg (n=10) and sterile distilled water (n=10) as control. The infection in *Cftr* mutant mice was performed with 20 µL of bacterial suspension adjusted to 1×10^{6} CFU.

2.12.5 Biofilm formation assay

The biofilm assay was performed as previously described (Stepanović *et al*., 2007) but with the following modifications. Overnight cultures of *S. maltophilia* in 5 mL TSB with an optical density 620 equivalent to 1 (OD_{620}) (approximately 1×10^9 CFU/mL) were diluted to 1:10, and 100 µL of the diluted inoculum (approximately 1×10^7 CFU/mL) were transferred to the wells of a sterile flat-bottomed 96-well polystyrene microtitre plate (BRANDplates, BrandTech Scientific, Essex, CT, USA) and incubated for 24 h at 35 °C. Nonadherent cells were subsequently removed by washing two times with 200 µL sterile distilled water. The amount of biofilm biomass was assessed by crystal violet staining. Biofilms were stained with 125 µL of 1.0 % (w/v) crystal violet for 20 min. The dye solution was discarded, and the plate was washed three times with sterile distilled water and allowed to air-dry for 24 h at room temperature. Stained biofilms were exposed to 30.0 % (v/v) acetic acid for 30 min, and the OD_{620} of the extracted dye was subsequently measured.

2.12.6 Effects of EGCg and COL on biofilm formation

The effects of EGCg and COL on *S. maltophilia* biofilm formation were determined as follows. First, biofilms were cultivated as described above (Pompilio *et al*., 2011a; Stepanović *et al*., 2007). After 24 h incubation at 35 °C, the formed biofilms were washed twice with 200 µL of distilled water to remove nonadherent cells. TSB supplemented with EGCg or COL (Table 8) was added to wells containing biofilms and further incubated for 24 h at 35 °C. After the incubation period, biofilm biomass was assessed by crystal violet staining. Biofilms were washed twice with 200 µL of distilled water and stained with 125 µL of 1.0 % (w/v) crystal violet for 20 min. Dye solution was then removed and discarded, and plates were washed three times with sterile distilled water and allowed to air-dry in the dark for 24 h. A volume of 15 µL 30.0 % (v/v) acetic acid was added to stained biofilms and incubated for 30 min at room temperature. The OD_{620} of the extracted dye was subsequently measured.

2.12.7 Effects of EGCg and COL on young and mature *S. maltophilia* biofilms

To evaluate the disruptive effects of EGCg, we cultivated Sm biofilms (ATCC13637, Sm1 and Sm2) in a 96-well microtitre plate assay (Pompilio *et al*., 2011b; Xu *et al*., 2011). Tubes with 10 mL TSB were cultivated with a single colony from each tested sample. Aliquots of 100 µL from 1:10 dilution of the overnight cultures (approximately 1×10^7 CFU/mL) were transferred to the wells of sterile flat-bottomed 96-well polystyrene microtitre plates. The plates were incubated for 24 h at 35 °C. The 24-h-old biofilms were treated with 100 µL of EGCg at 0.25×MIC, 0.5×MIC, or 1×MIC (Table 8) and were incubated at 35 °C for an additional 24 h. After incubation, the culture medium (TSB with EGCg or COL) was removed, and the treated biofilms were washed two times with sterile distilled water. Seven-day-old biofilms were cultivated in parallel. These biofilms were washed with sterile distilled water every 24 h during the incubation-period, and the medium was supplemented with new TSB. After the maturation period, 7-day-old biofilms were treated in the same manner as 24-h-old biofilms. To determine the metabolic activity of the biofilms, we added 100 µL aliquots of saline solution XTT (100 g/L) and menadione (10 g/L). The plates were incubated in the dark for 2 h at 35 °C, and the absorbance was measured at 492 nm. The *in vitro* effect of EGCg on the viability of *S. maltophilia* biofilm was plotted as the ratio of viability (cells with active metabolism) in treated samples to viability in untreated samples. Control wells contained TSB without EGCg. The *in vitro* effect of EGCg on the viability of *S. maltophlia* biofilm was plotted as the ratio of viability (cells with active metabolism) in treated samples to viability in untreated samples. The effects of COL on young and mature biofilms were determined as described for EGCg.

2.12.8 Confocal laser scanning microscopy of *S. maltophilia* biofilms

Qualitative and quantitative microscopic evaluations of the biofilms were carried out through a combination of LIVE/DEAD *Bac*Light viability staining and automated confocal laser scanning microscopy (CLSM), as previously described (Müsken *et al*., 2010). A single colony of each sample was inoculated into individual tubes containing 2 mL of LB medium and incubated in an orbital shaker for approximately 16 h at 35 °C with agitation (180 rpm). Overnight cultures were diluted with fresh LB broth to an OD_{600}

of 0.02 (1× 10^9 CFU/mL), and 100 µL aliquots from these dilutions were transferred to the wells and covered with air-permeable cover foil. The plates were incubated for 24 h at 35 °C in an incubator with humidified atmosphere. The 48-h-old biofilms of strains ATCC 16367, Sm1, and Sm2 were visualised after 24 h exposure to COL or EGCg at various concentrations. For this assay, the DNA-binding dyes Syto9 (green) and propidium iodide (PI; red) were used. This two-colour kit differentially stains living (green) and membrane-compromised/dead (red) bacteria according to differences in membrane permeability. After addition of COL or EGCg, 20 µL of a staining solution containing Syto9 and PI (at final concentrations of 1.4 µM and 8.3 µM, respectively) was added to each well. Biofilm susceptibility to either COL or EGCg was determined on the basis of the fractions of red (including co-localized) and green biovolume (μm^3) calculated from the image stacks with a custom-designed algorithm in the software Developer XD (Definiens). Syto9 was excited with a 488 nm wavelength laser and detected with a 540/75 nm band-pass emission filter, whereas PI was excited at 561 nm and detected with 600/40 band-pass emission filter. The negative controls were biofilms treated with LB broth supplemented with 1.0 % (v/v) DMSO, and the positive control (killing biofilm control) were treated with formalin at final concentration of 1.0 % (v/v). Visualisation of biofilm sections was performed with the software IMARIS version 5.7.2 (Bitplane, South Windsor, USA). Data are expressed as means of two independent experiments. Experiments were carried out in duplicate.

2.12.9 Statistical analysis

All assays were performed in triplicate, and the results are shown as means ± SDs. A one-way analysis of variance (ANOVA), followed by the Dunnett test, was used to detect differences in efficacy against biofilm formation (spectrophotometric OD readings) and biovolume/bioviability of young and mature biofilms between isolates exposed to EGCg or COL for 24 h. The Mann-Whitney U test was used to address the differences among bacterial counts in mice nebulised with EGCg, COL, PBS and aqua. Significance was set at $P < 0.05$.

3 Results

3.1 Improved detection and susceptibility testing of *S. maltophilia* isolates

3.1.1 Steno medium agar (SMA) improved the isolation of *S. maltophilia* from sputum samples

A comparative investigation of a novel selective medium (SMA) with conventional media for the detection of *S. maltophilia* was carried out. Out of 623 sputum samples from 165 CF patients, conventional media revealed that yeasts (52.4 %), *Pseudomonas* spp. (52.0 %), *Staphylococcus aureus* (48.3 %) and *Aspergillus* spp. (31.7 %) were the most frequent isolated microorganisms. The frequency of the most isolated microbes in relation to all samples analysed is shown in Figure 6.

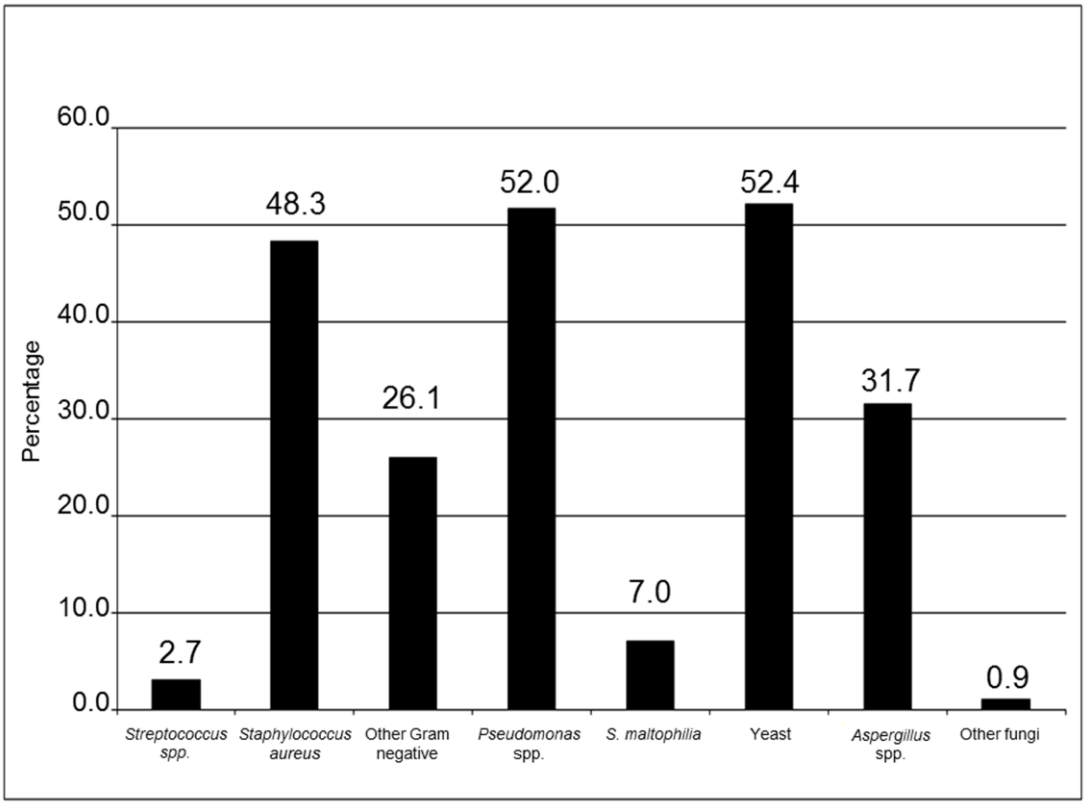

Figure 6 Isolation frequency of microorganisms using conventional media from 623 sputa from 165 CF patients.

A total of 72 sputum samples from 33 CF patients aged 1 to 51 years revealed the presence of *S. maltophilia*. It was observed that conventional media supported the

growth of this pathogen in 7.1 % of the sputum samples examined. Interestingly, the detection rate of this pathogen increased to 11.6 % when a selective medium was used, indicating that SMA supported the growth of 64.0 % more *S. maltophilia* isolates than conventional media. There was no case in which the conventional media allowed detection of *S. maltophilia* and SMA did not. Usually, *S. maltophilia* was isolated alone, as well as with other accompanying facultative pathogenic species, such as *Aspergillus fumigatus* (30.6 %), *Candida albicans* (46.3 %) and *P. aeruginosa* (39.6 %). Patient demographics related to the detection of *Aspergillus* spp., *Pseudomonas* spp., and *S. maltophilia* are shown in Table 9. Contrary to *Aspergillus* spp. and *Pseudomonas* spp., *S. maltophilia* was more frequently isolated from female than from male patients.

Table 9 Demographic characteristics of 165 CF patients according to clinical relevance interest of species isolation

Patient features	*Aspergillus* spp.	*Pseudomonas* spp.	*S. maltophilia*
Mean age ±SD	24.1±6.5	27.4±8.2	22.2±8.8
(range)	(1-51)	(5-79)	(1-51)
Female (%)	36.6	39.5	57.5
Total (*n*)	71	91	33

SD= standard deviation

3.1.2 Tygecycline and trimethoprim-sulfamethoxazole demonstrated the best *in vitro* inhibitory activity against *S. maltophilia* isolates

The MICs at which 50 % (MIC_{50}) and 90 % (MIC_{90}) of *S. maltophilia* isolates were inhibited by various antibiotics and the percentages of susceptible and resistant isolates, according to established breakpoints, are presented in Table 10. Tigecycline and trimethoprim-sulfamethoxazole, amongst all tested agents, revealed the highest *in vitro* activity against *S. maltophilia* (98-100.0 % susceptibility), followed by quinolones (levofloxacin and moxifloxacin) and fosfomycin (89-92.0 % susceptibility). Conversely, colistin, ceftazidime, and ticarcillin-clavulanate acid exhibited limited *in vitro* activity (44-64.0 % susceptibility).

Table 10 *In vitro* activity of eight antimicrobial agents tested against *S. maltophilia* by Etest.

Antimicrobial agent	MIC (µg/mL)			% (n=65) of strains		
	Range (µg/mL)	50 % of strains	90 % of strains	Susceptible	Intermediate	Resistant
Ceftazidimine	0.016-256	3	32	63.0	7.6	29.4
Colistin	0.016-256	3	12	44.6	26.1	29.3
Fosfomycin	0.016-256	6	12	92.3	-	7.7
Levofloxacin	0.002-32	0.625	2	92.3	-	7.7
Moxifloxacin	0.002-32	0.25	1	89.5	7.5	3.0
Ticarcillin-clavulanate acid	0.016-256	4	32	64.1	16.4	19.5
Tigecycline	0.016-256	0.094	0.218	98.4	-	1.6
Trimethoprim-sulfamethoxazole	0.002-32	0.38	1	100	-	-

MIC= minimum inhibitory concentration. MICs at which 50 (MIC_{50}) and 90 % (MIC_{90}) of *S. maltophilia* isolates were inhibited.

3.2 Genotyping methods reveal that *S. maltophilia* strains are highly diverse

Analysis of bacterial pathogens using various typing methods is essential for investigating strains relatedness, tracing geographic dissemination of bacterial clones (cross-transmission), as well as for long-term epidemiological studies (Struelens, 1996). Therefore, molecular analyses of *S. maltophilia* isolates from different sources (sputa from CF and ICU patients; and environment) were performed using the semi-automated rep-PCR Diversilab (DL) system (n=102) and ERIC-PCR (n=97).

The DL system was able to type most of the isolates, except for two clinical CF samples. By using a similarity cut-off of 95 %, DL typing revealed great heterogenicity by distinguishing 100 isolates into 70 DL types, of which 30 were considered singletons or unique patterns. The other clusters represented clonally related isolates compromising two or more isolates. The largest clonally related group was composed of six isolates.

ERIC-PCR also successfully typed all isolates, with a few exceptions that did not exhibit band pattern (n=6; 2 from CF and 4 from ICU patients, respectively). Cluster analysis revealed the presence of eight different profiles. The dendrogram derived from ERIC-PCR is illustrated in Figure 3.2. Among them, four groups were strictly composed of isolates from CF patients (groups A, B, C and D), whereas one group was formed only by isolates obtained from ICU individuals (group E). The other three clusters contained a mixture of isolate types/origins.

Interestingly, statistical analyses showed that DL and ERIC-PCR provided relatively high resolution power with SID values of 0.990 (95 % CI; 0.983 to 0.996) and 0.869 (95 % CI, 0.851 to 0.888), respectively. Clustering analyses of both typing methods revealed a lot of related and distinct genotypes. Overall, AR and W coefficient values (<0.5) demonstrated a weak correlation between the information provided by DL and ERIC-PCR methods.

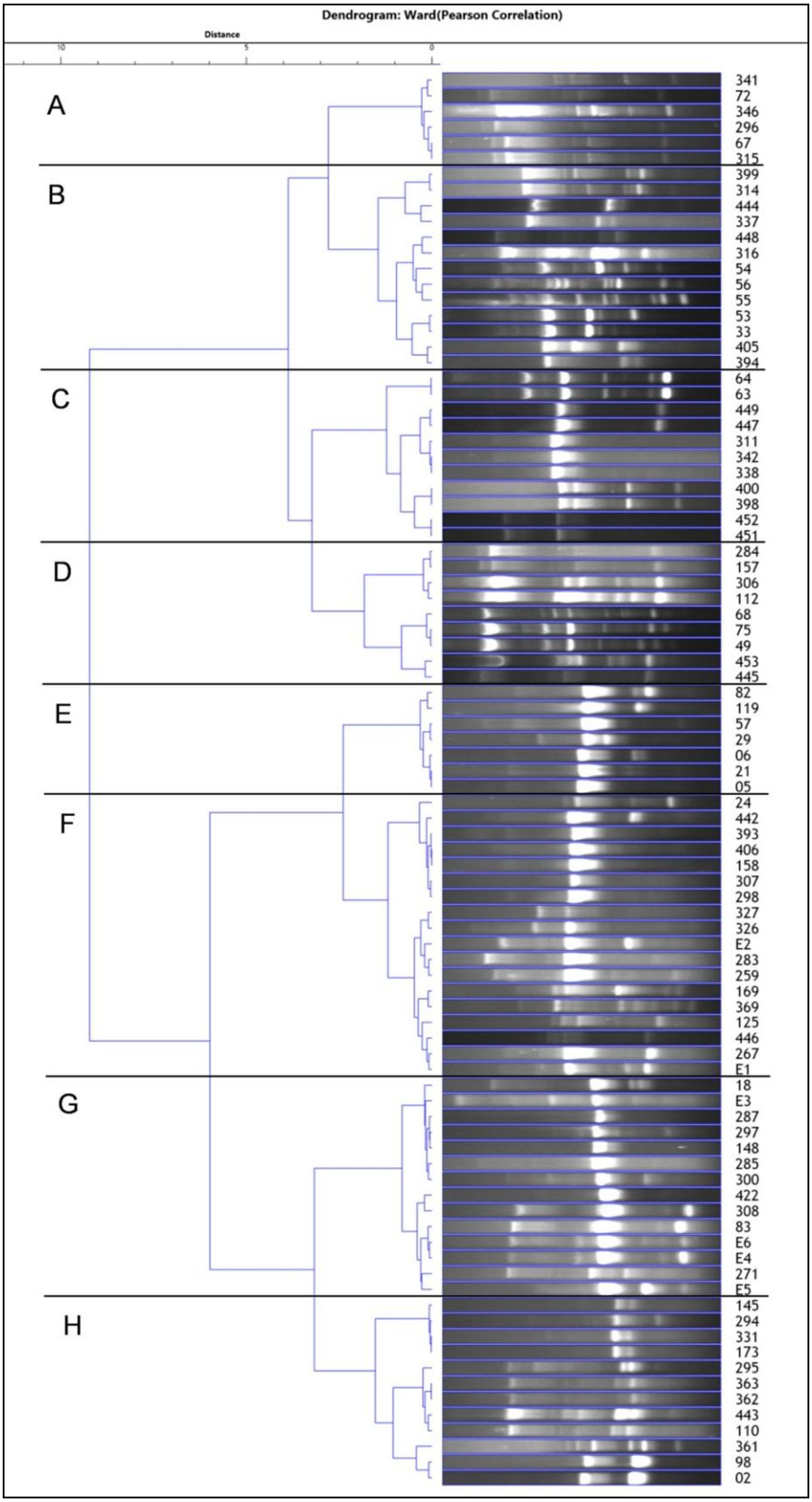

Figure 7 Dendrograms of *S. maltophilia* based on the genomic fingerprints generated by ERIC-PCR. Genetic similarity among samples isolated from CF, ICU and environment were estimated by Ward algorithm.

3.3 *S. maltophilia* CF isolates demonstrated a higher content of fatty acids in comparison to environmental and ICU isolates

Environmental and physiological factors are known to affect the fatty acid composition of bacteria. For that reason, the relationship between the fatty acid profiles of *S. maltophilia* isolates from CF and ICU patients, and also from the environment, was investigated by Microcrobial Identification System (MIS) 2-D plot. The clustering analysis revealed no grouping corresponding to CF, ICU or environmental isolate types (Figure 8). However, clustering was observed within “CF chronic colonised” and “CF non chronic colonised” groups.

Further, the peak area index (PAI), which provides information about the amount of fatty acids present in the broth culture during bacterial growth, was investigated. Isolates of *S. maltophilia* from CF patients, ICU patients and the environment showed mean PAI values of 12.7 (range 4.0-98.8), 9.2 (range 7.2-12.6) and 7.4 (range 5.4-11.2). Surprisingly, specimens from CF patients revealed significantly higher amounts of fatty acids in comparison to samples from ICU patients and the environment. There was also a significant difference in the amount of fatty acids between the ICU patient samples and environment samples (Figure 9).

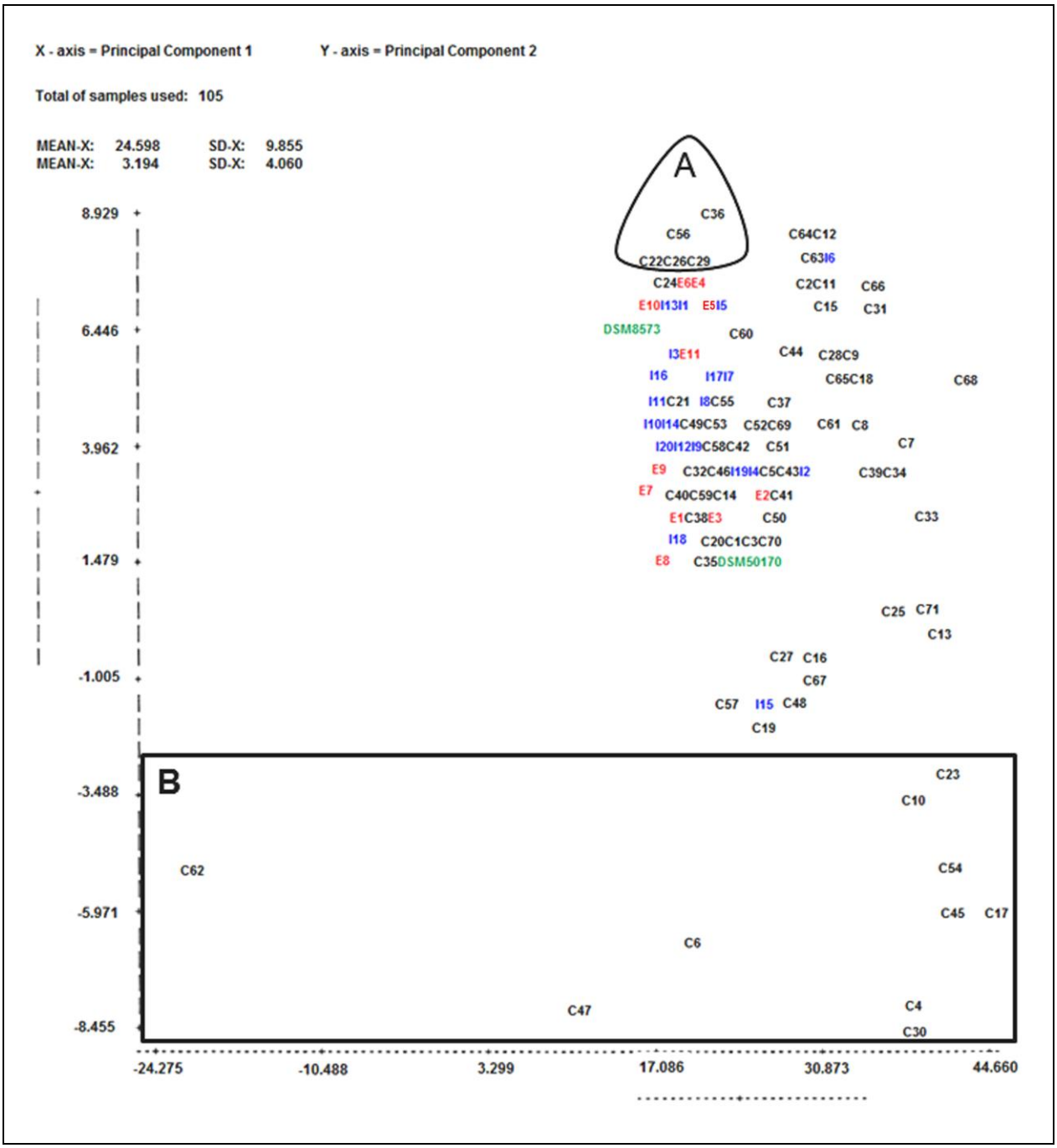

Figure 8 MIS 2-D plot cluster analysis of FAME profiles from *S. maltophilia* isolated from different sources (CF patients=C; ICU patients= I; environmental= E). Group A: *S. maltophilia* isolates from CF patients initially colonised with this bacterium (non-chronic colonised). Group B: *S. maltophilia* isolates from CF patients chronically colonised with this pathogen.

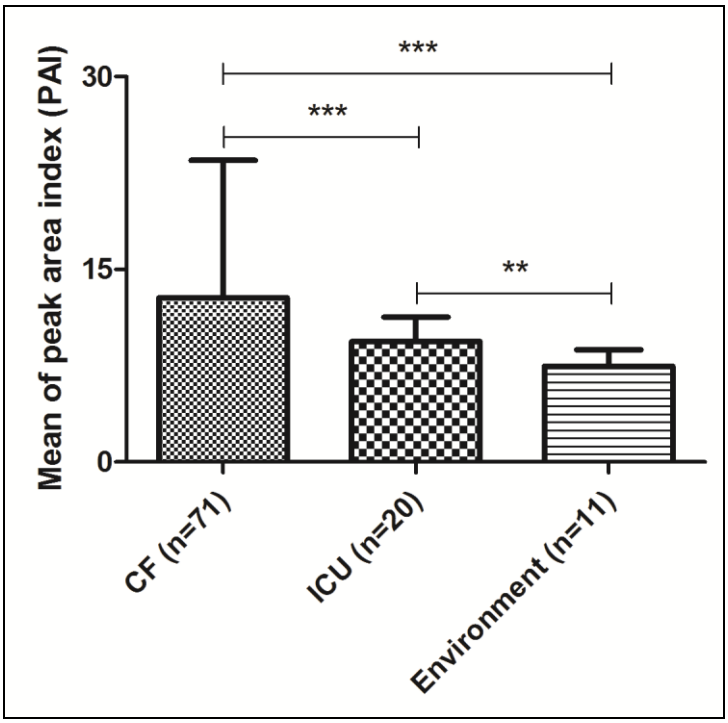

Figure 9 Peak area index of *S. maltophilia* isolates from cystic fibrosis (CF) patients, intensive care unit (ICU) patients and from the environment. **P*<0.05; ***P*<0.01; ****P*<0.0001.

3.4 MALDI-TOF mass spectra revealed that *S. maltophilia* isolates from CF patients are clustered together

Principal component analysis (PCA), based on MALDI-TOF mass spectra, was carried out in an attempt to differentiate *S. maltophilia* isolates based on their origin (CF and ICU patients, and environment). Figure 10 shows the score plot of the first three principal components on a set of mass spectra obtained from 100 *S. maltophilia* isolates. In this plot, *S. maltophilia* isolates obtained from CF patients could be identified as a distinct group, whereas ICU and environmental samples grouped in a mixed manner.

The dendrogram generated by the hierarchical cluster analysis, based on the peak lists derived from MALDI-TOF mass spectra, revealed the presence of five distinct groups (A, B, C, D and E) (Figure 11). Clusters B and D were strictly composed of samples isolated from CF patients.

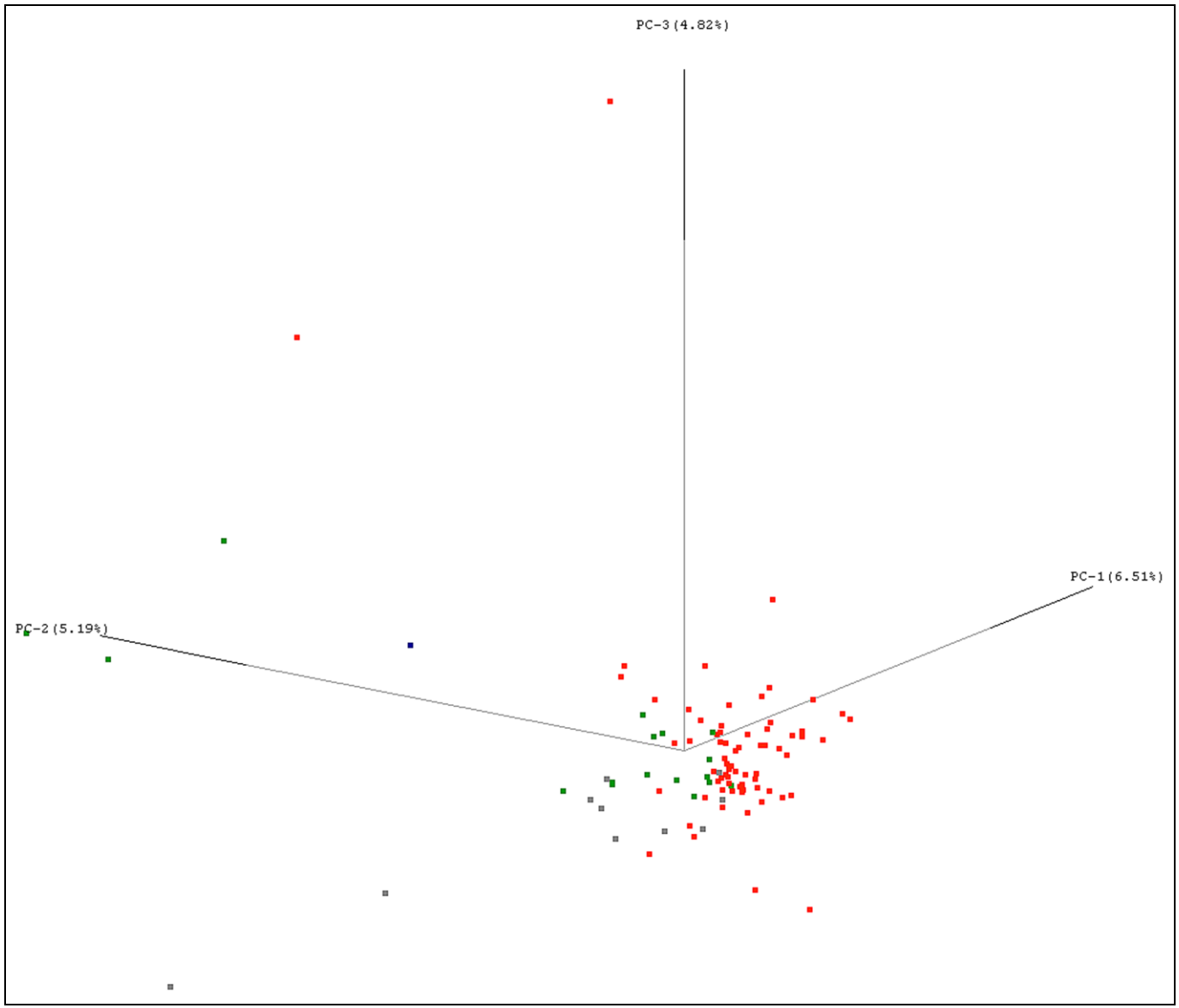

Figure 10 Principal component analysis of 100 *S. maltophilia* isolates obtained from CF and ICU patients and from the environment.

CF, ICU, environmental and ATCC 13637 isolates are respectively represented by the colours red, green, gray and blue.

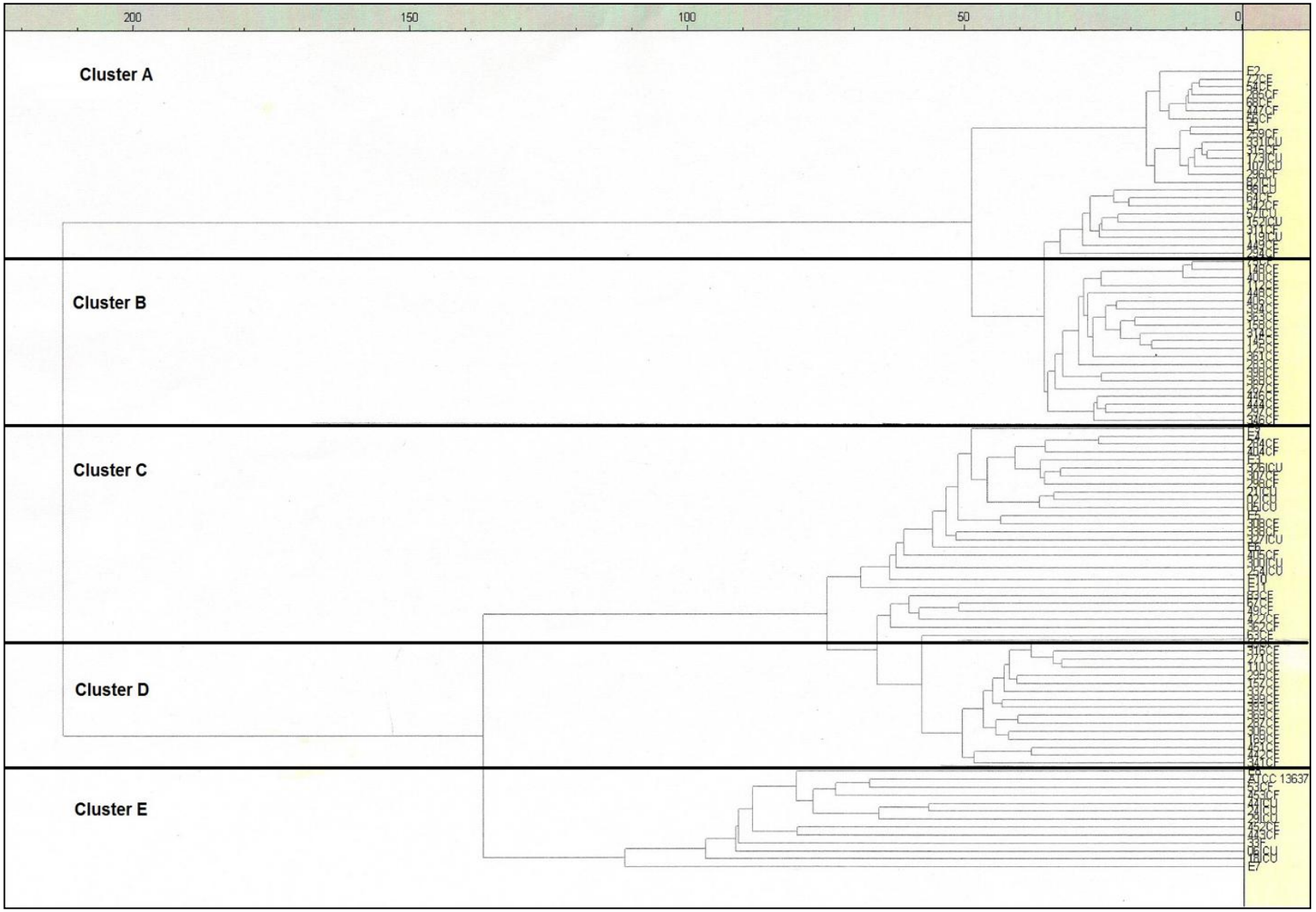

Figure 11 Dendrogram of 100 *S. maltophilia* isolates obtained from CF and ICU patients and from the environment. Distances are displayed in relative units.

A visual comparison of averaged mass spectra of CF isolates in comparison to those of ICU or environmental samples did not show any remarkable difference (Figure 12). Taking this into consideration, this fact emphasizes the necessity to conduct more complex analyses, such as hierarchical cluster analysis and PCA.

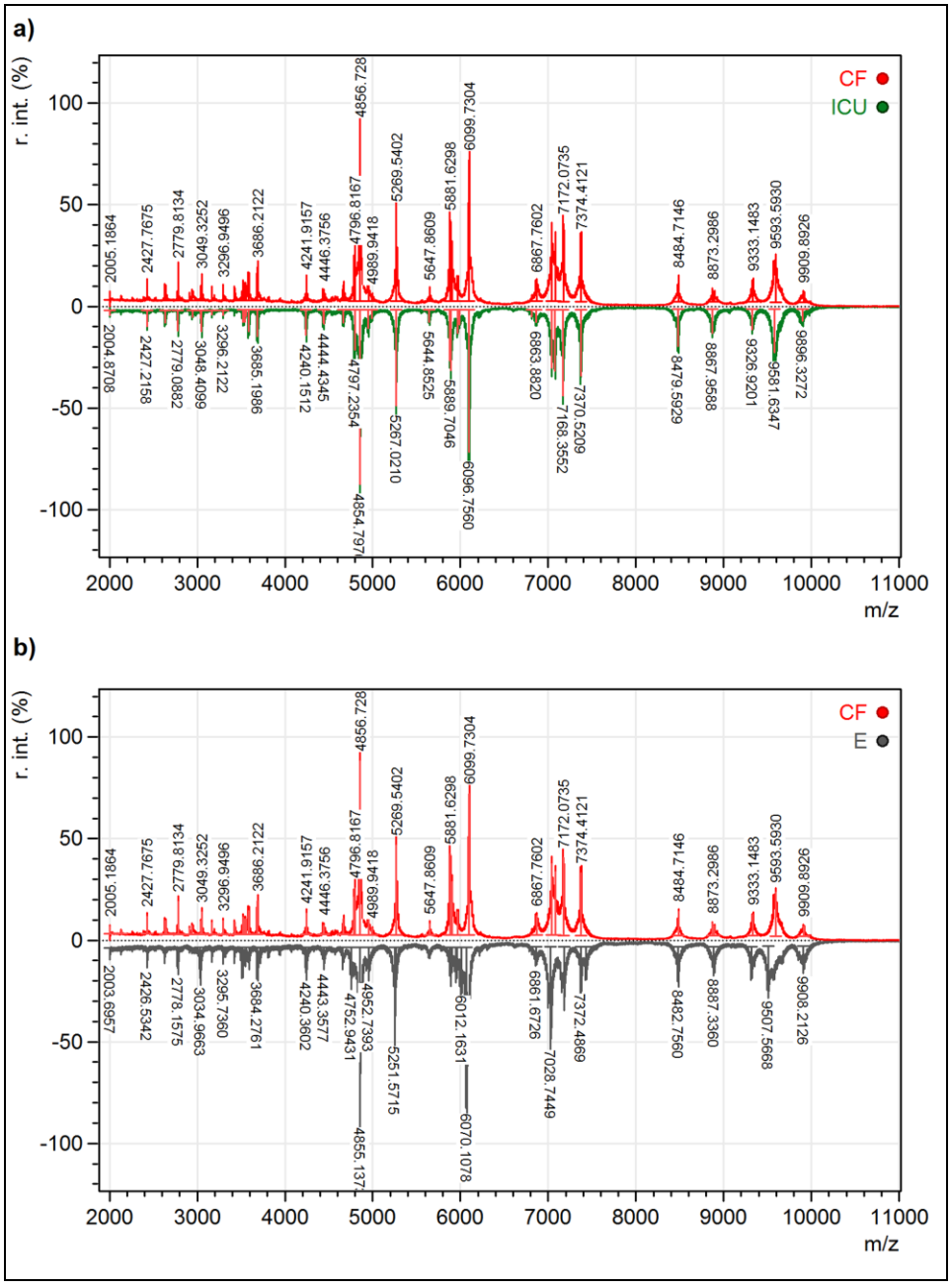

Figure 12 Averaged MALDI-TOF mass spectra of *S. maltophilia* samples. A) Comparison between averaged MALDI-TOF mass spectra of CF (red) and environmental (green) *S. maltophilia* samples. B) Comparison between averaged mass spectra of CF samples (red) and ICU (green) *S. maltophilia* isolates. r. int. = relative intensity.

3.5 Chronic *S. maltophilia* infection in CF patients is associated with a specific immune response

S. maltophilia antibody titres were measured in 100 sera samples from 64 CF patients and 36 healthy individuals by quantitative immunofluorescence assay. Data demonstrated that 28 (43.7 %) out of the 64 patients had chronic "*S. maltophilia*" colonisation, 10 (15.7 %) had intermittent "*S. maltophilia*" colonisation and 11 (17.2 %) never had either *S. maltophilia* or *P. aeruginosa* isolated from their respiratory tract. It was also observed that 15 (23.4 %) of CF patients were chronically colonised with *P. aeruginosa* but never with *S. maltophilia*. Baseline characteristics of the CF patients are summarised in Table 11.

The relationship between antibody titres and forced expiratory volume in 1 s (FEV_1) was examined. A significant inverse correlation between the mean *S. maltophilia* antibody titres and FEV_1 percent prediction was observed (R square=0.2106; P=0.0013) (Figure 13). Therefore, it is plausible to assume that CF patients with high antibody titres consequently tend to exhibit high FEV_1 values. In this particular case, due to the limited number of patients, multivariate regression analysis was not performed to address the associations between *S. maltophilia* antibody titre and markers of clinical outcome.

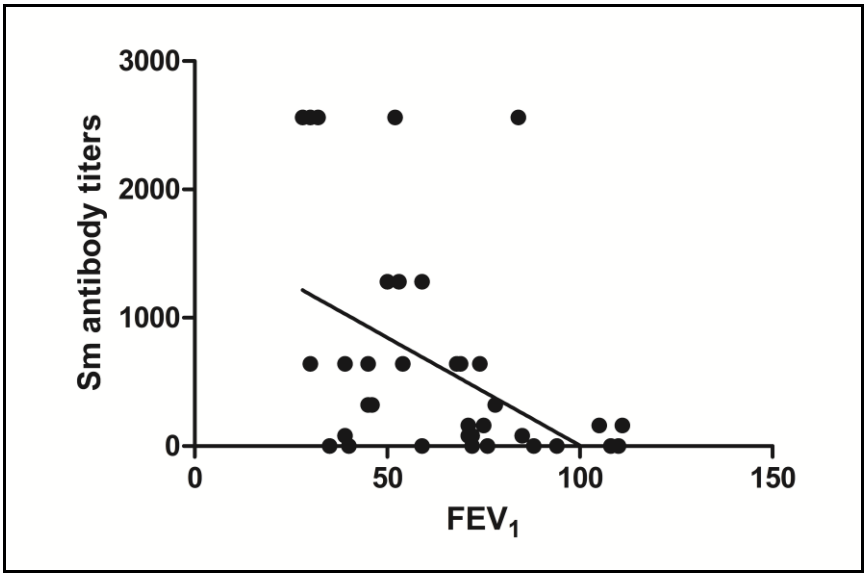

Figure 13 Mean *S. maltophilia* antibody titres and corresponding FEV_1 percent predicted. Each dot represents a single patient. Sm= *S. maltophilia*.

Table 11 Demographic and clinical data regarding healthy subjects and CF patient groups.

Characteristic	Healthy (n=36)	Never Sm/Pa (n=11)	Intermittent Sm (n=10)	Chronic Sm (n=28)	Chronic Pa never Sm (n=15)
Sm antibody titre (geometric mean)	0.91	7.3	22.6	320.1	30.5
Age, mean (SD)	31.9 (±9.7)	14.0 (±7.9)	20.0 (±10.1)	21.2 (±8.9)	26.0 (±7.2)
Sex (male)	18 (50%)	9 (81.9%)	5 (50%)	13 (46%)	6 (40%)
Mutation dF580/dF580	-	18.10%	60%	50%	73.30%
Pancreatic insufficiency	-	90.90%	100%	75%	93.30%
CFDR	-	0%	20%	10.70%	33.30%
BMI (range)	-	18.1 (14.1-22.3)	19.3 (15.1-23.4)	18.0 (14.5-23.8)	19.8 (16.2-30.4)
FEV1 mean (range)	-	84.7 (35-111)	61.7 (40-94)	55.7 (28-108)	56.4 (16-116)
Pseudomonas aeruginosa positive	-	0.00%	90%	50%	100%
Burkolderia cepacia positive	-	0%	0%	0%	0%
Aspergillus positive	-	18.10%	80%	53.50%	86.70%
Lung transplant	-	0%	0%	0%	6.70%

Definitions of abbreviations: Sm= *Stenotrophomonas maltophilia*; BMI= body mass index; CFDR=cystic fibrosis-related diabetes; FEV_1= forced expiratory volume in 1 second.

S. maltophilia antibody titres were substantially different between the three groups of CF patients, except for between "never *S. maltophilia/P. aeruginosa*" and "intermittent *S. maltophilia*" groups (P=0.2688) (Figure 14). Patients with "chronic *S. maltophilia*" exhibited significantly higher *S. maltophilia* antibody titres in comparison to healthy individuals (P<0.0001), CF patients with "intermittent" (P=0.0315) and "never *S. maltophilia/P. aeruginosa*" (P=0.0002). No evidence of *S. maltophilia* antibodies cross-reactivity to *P. aeruginosa* was found due to the fact that *S. maltophilia* antibody titres in patients "never *S.maltophilia/P. aeruginosa*" and "never *S. maltophilia* but chronic *P. aeruginosa"* were not significantly different from each other (P=0.1898) (Figure 14).

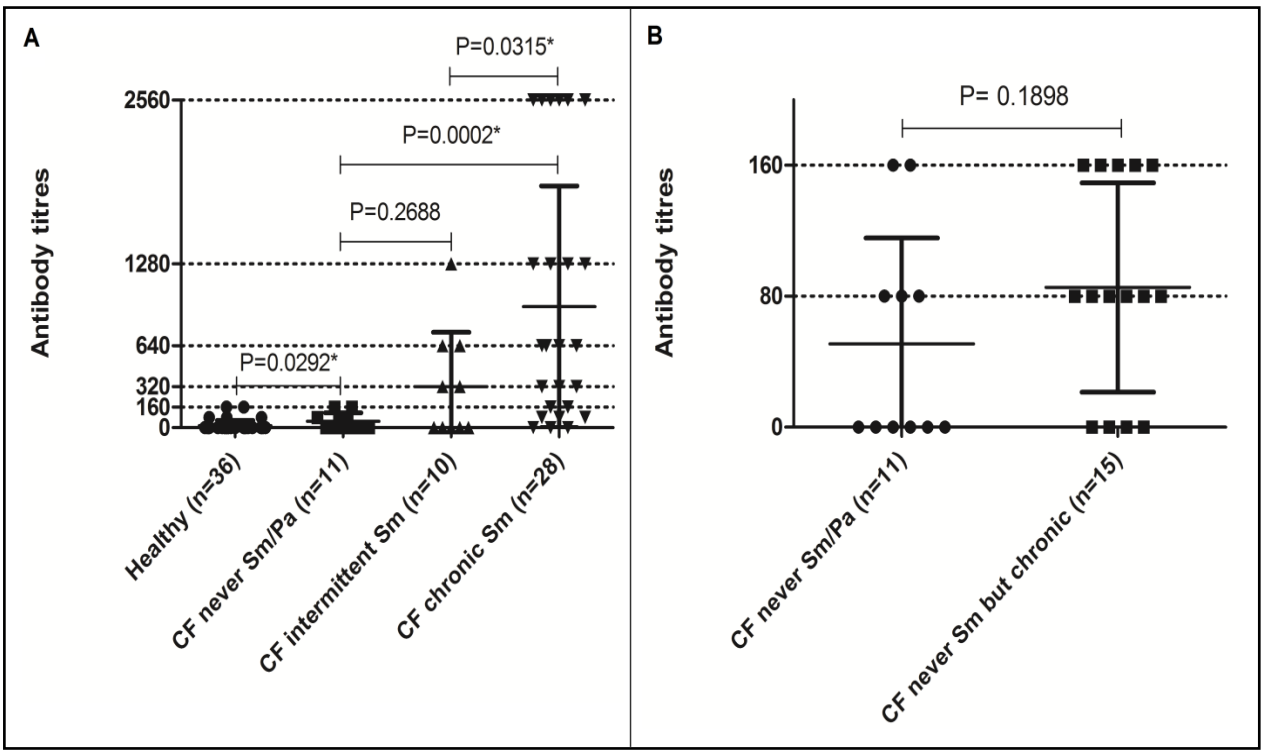

Figure 14 Antibody levels of *S. maltophilia*. A) Antibody levels of *S. maltophilia* (Sm) whole cell in serum samples from healthy subjects (healthy), CF patients with chronic (CF chronic Sm), intermittent (CF intermittent Sm), and never Sm without *P. aeruginosa* (CF never Sm/Pa). Antibody levels are reported as titres. P values <0.0005 by non-parametric test were considered significantly different. B) Comparison of antibody titre between CF "never Sm/Pa" and CF "never Sm but chronic Pa". Antibody titres did not differ between the groups (P=0.1898).

Figure 15 illustrates the ROC curve analysis used to identify CF *S. maltophilia* chronically colonised patients according to titre value. The area under the receiver operating characteristic curve (AUC), a measurement known as the discriminatory power of the diagnostic test, was 0.88 (95 % CI: 0.59-0.91). The titre value of >1:120 was considered the best cut-off, since it has provided both the highest sensitivity and specificity to distinguish between CF "never *S.maltophilia/P.aeruginosa*" and CF "chronic *S. maltophilia*". Based on that, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were 78.5 %, 81.8 %, 70.7 % and 84.7 %, respectively.

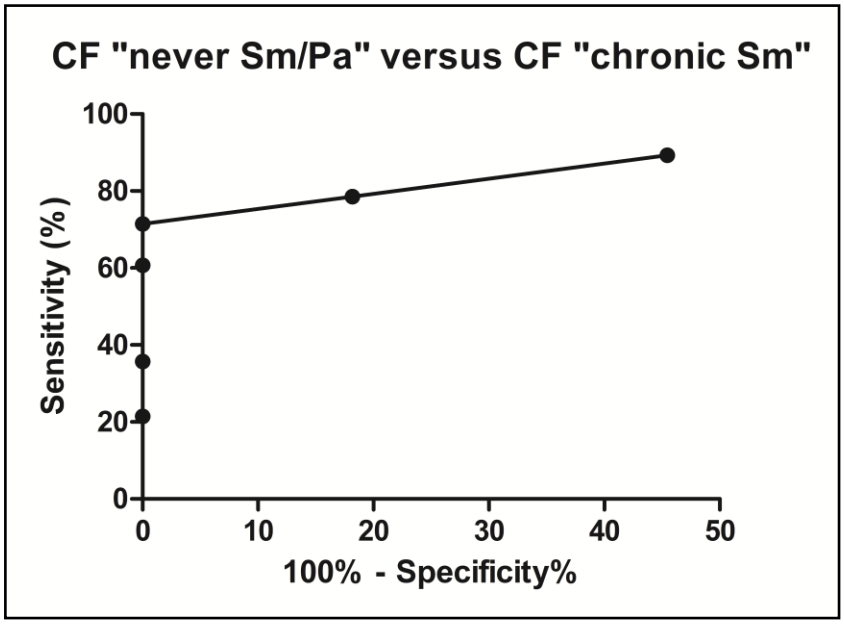

Figure 15 Receiver operating curve (ROC) for IFA detecting serum antibodies against *S. maltophila* (Sm) whole cell. ROC data (AUC value=0.8815; standard error=0.052; P=0.0002) of CF patients never colonised *S.maltophilia*/*P.aeruginosa* (Sm/Pa) versus CF patients with chronic *S. maltophilia*.

3.6 Long-term adaptation of *S. maltophilia* bacterial population in the CF lung: high molecular diversity, hypermutation and antibiotic resistance

To expand the undergoing of the matter of *S. maltophilia* adaptation to the CF airway, we examined the genotypic diversity, mutation frequency and antibiotic resistance of *S. maltophilia* isolates from chronically colonised CF patients. Our long-term *S. maltophilia* sputa collection, over a period of four years, consisted of a total of 90 isolates obtained from 19 CF patients classified as chronically colonised (Waters *et al*.,

2011). An average of 4.8 (±3.3) sputum samples per patient was analysed. Most of the CF patients were females (52.6 %) and the mean age for the onset of chronic colonisation with *S. maltophilia* was 25 years (±12.8). There was no correlation between the mean number of sputa samples collected per patient and the type of mutation in the CFTR gene.

To evaluate the possibility of CF *S. maltophilia* populations being a consequence of a single (or few) clones' dissemination, these bacterial isolates were fingerprinted using the semi-automated, rep-PCR method (DiversiLab® system). Demographic and genotypic characteristics of the CF patients, as well as the genotype features related to the *S. maltophilia* isolates obtained from these patients are summarised in Table 12. Genotyping of the consecutive clinical isolates revealed the existence of 38 genotypes. In addition, a total of 13 (68.4 %) of the CF patients, during the course of the study, harboured diverse *S. maltophilia* genotypes. Patient 3, for example, was the one that harboured the most different genotypes (n=8) within 34 months (Figure 16). Patients 7, 10 and 11 presented three genotypes, while patients 8, 9, 13, 14, 15, and 17 presented only two. It was also noted that 31.6 % (n=6) of these patients were colonised with unique clones (Table 12). Interestingly, data also showed that certain *S. maltophilia* genotypes were shared by 10 patients (1, 3, 4, 7, 10, 11, 13, 15, 16 and 17).

In order to exclude confounding factors that could alter the rep-PCR banding patterns due to culture age and/or clonal expansion during growth of multiple subcultures, rep-PCR pattern stability was examined. At three different time points (1, 5 and 10 days), three distinguishable colonies from a clinical CF isolate were collected and rep-PCR was carried out. Through this approach, it was possible to demonstrate that *S. maltophilia* genotype was subculture stable and rep-PCR was also able to give reproducible results (Figure 17).

Table 12 Demographic and genotypic characteristics of 90 *S. maltophilia* isolates obtained from 19 CF patients.

Patient	Sex	Age (years)	CF mutation	No. of sputa	Genotypes
Patient 1	F	17	dF508/R553X	9	**1**,8,9,22,32
Patient 2	M	12	dF508/dF508	2	30
Patient 3	F	11	dF508/dF508	15	**11**,12,**13**,**14**,**16**,21,23,37
Patient 4	F	28	dF508/327-26-A>G	10	10,**13**,**14**,**16**
Patient 5	M	27	dF508/dF508	5	34
Patient 6	M	24	dF508/dF508	2	6
Patient 7	F	21	not identified	7	**1**,2,**28**
Patient 8	F	36	not identified	3	24,25
Patient 9	F	27	R347P/G542X	3	5,38
Patient 10	M	21	dF508/R31C	4	**13**,**14**,15
Patient 11	M	18	dF508/R553X	3	3,20,**33**
Patient 12	F	12	dF508/dF508	2	29
Patient 13	M	27	G1069R/3007delG	3	**18**,19
Patient 14	M	20	dF508/dF508	6	26,36
Patient 15	M	19	dF508/dF508	2	**11**,**27**
Patient 16	F	14	not identified	6	17,**18**,**27**,31,**33**
Patient 17	M	47	dF508/2789+5G>A	4	**18**,**28**
Patient 18	F	65	dF508/-	3	35
Patient 19	F	31	dF508/dF508	2	4

The numbers in bold refer to the genotypes which are shared by different patients.

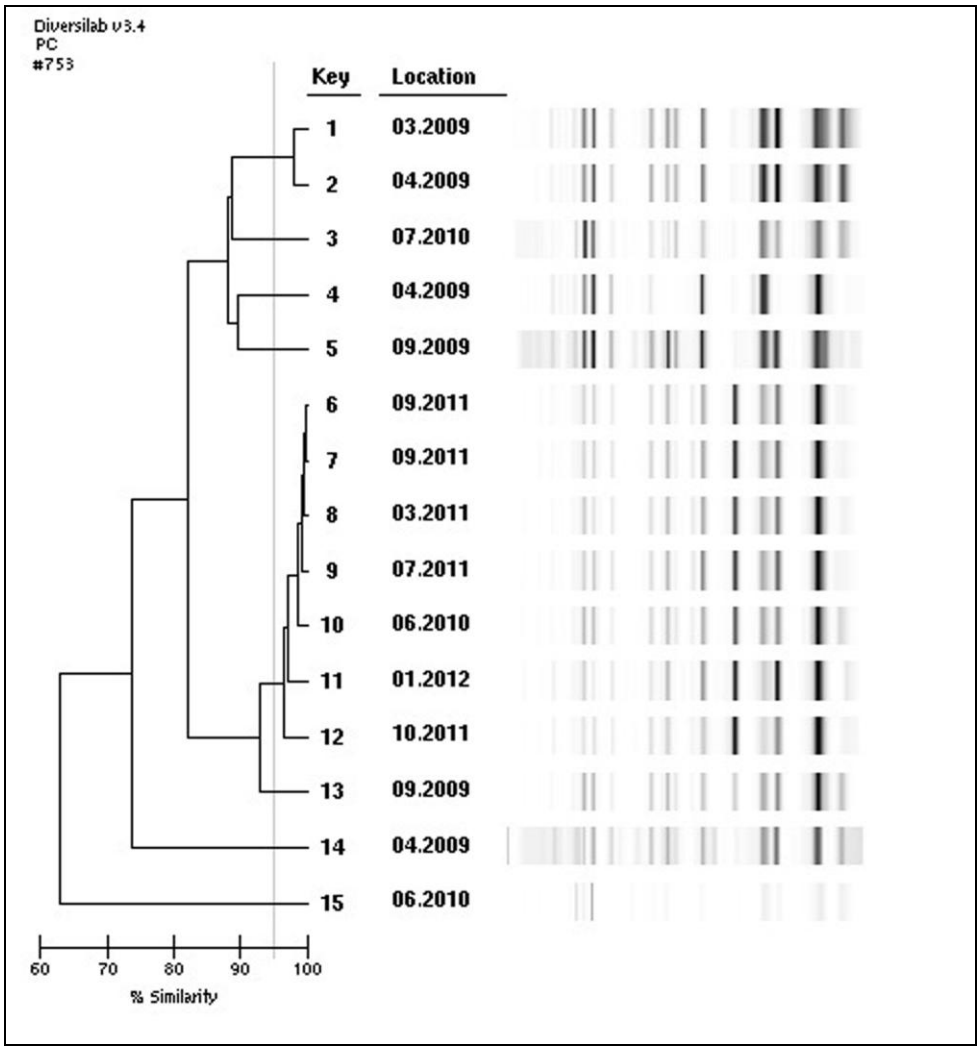

Figure 16 Molecular epidemiology of *S. maltophilia* samples isolated from patient 3. Dendrogram, virtual gel and the similarity matrix generated by the DL software. The gel-like image and similarity matrix exhibit the presence of eight different genotypes.

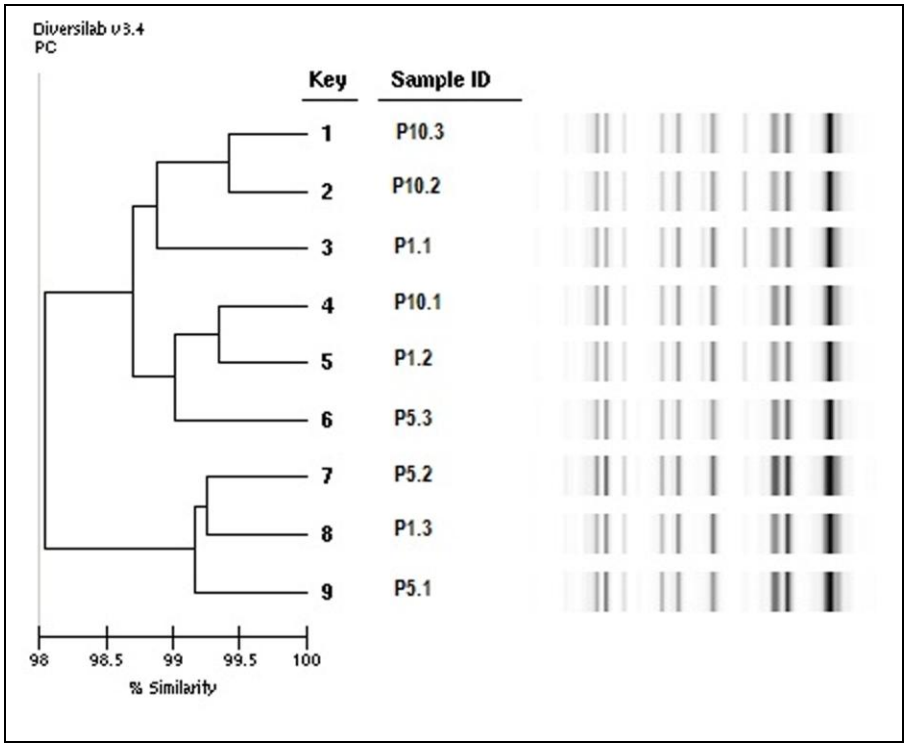

Figure 17 Assessment of subculture stability and reproducibility of rep-PCR. *S. maltophilia* was cultivated for 1, 5 and 10 days (indicated by the first number) and colonies from three sites (indicated by the second number) were investigated by rep-PCR. The horizontal bar at the bottom left shows the percent similarity coefficient of these samples. Subculture stability and reproducibility of *S. maltophilia* are shown by a high index of similarity (>98 %).

Strong diversification over time and emergence of spontaneous mutator phenotypes can occur in bacterial populations colonising CF airways, since they are constantly exposed to a challenging environment and repeated antibiotic therapies (Tenaillon *et al*., 1999). The presence of mutators in CF patients chronically colonised by different bacteria have been supported by other studies (Román *et al*., 2004; Prunier *et al*., 2003; Oliver *et al*., 2000). For that reason, mutator status of *S. maltophilia* isolates obtained from CF chronically colonised patients were examined. Estimation of the mutation frequencies revealed that 31.2 % (n=28) were strong mutators, 23.3 % (n=21) weak mutators, 27.7 % (n=25) normomutators and 17.8 % (n=16) hypomutators. Approximately 58.0 % (n=11) of these patients harboured at least one strong mutator strain, whereas about 37.0 % (n=7) of them harboured at least one hypomutator strain. Interestingly, it was noted that mutation rates of the most clonally related genotypes varied over time (Figure 18) with a tendency to become a weaker mutator, except for genotype 1.

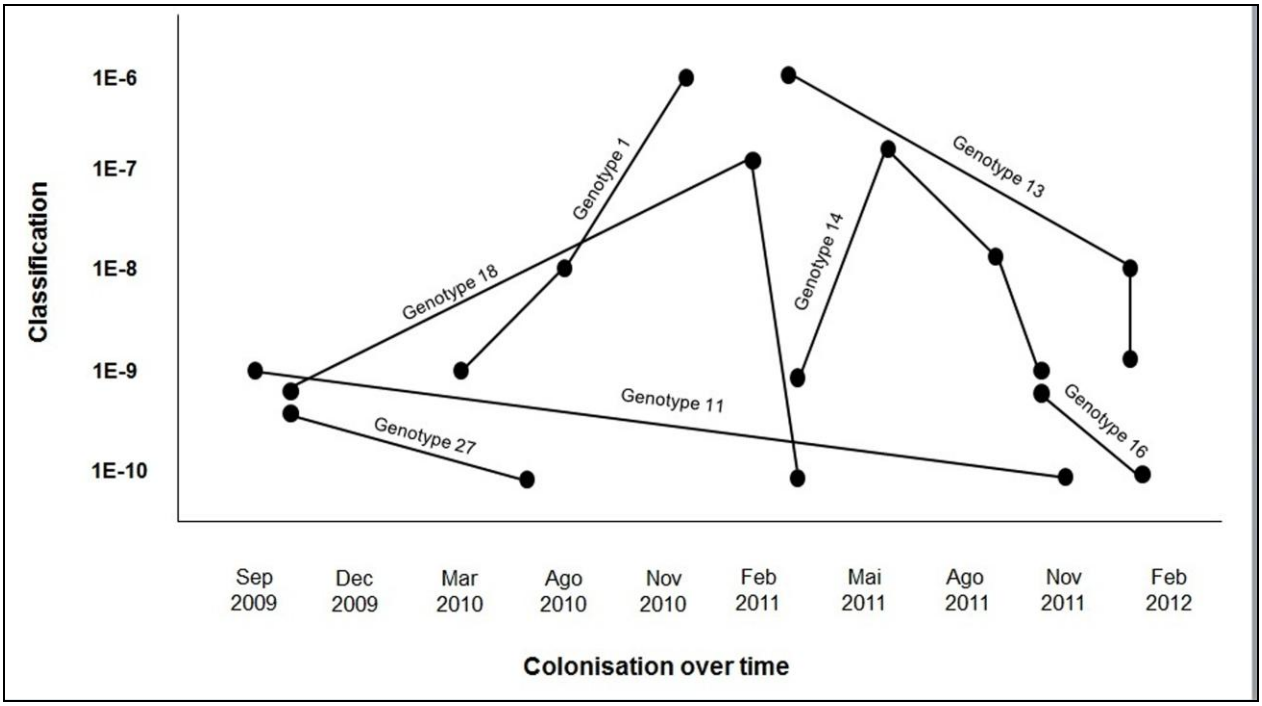

Figure 18 Mutation status of the genotypes clonally related and shared among different CF patients over time. Each black dot represents a single isolate obtained from a patient. The black line links the isolates that share the same genotype pattern according to rep-PCR. The similarity among isolates was established as ≥95 % according to Pearson correlation index.

It has been shown by other studies that mutator *P. aeruginosa* isolates obtained from chronically colonised CF patients tend to be more resistant to antibiotics than nonmutators (Ciofu *et al*., 2005; Oliver *et al*., 2000). To verify the impact of high prevalence of mutator strains on antibiotic resistance, susceptibility testing for nine antimicrobial agents was conducted (Table 13). Similarly as demonstrated for a different sputa collection, tigecycline and trimethoprim-sulfamethoxazole also exhibited the best inhibitory activity against *S. maltophilia* isolates obtained from chronically colonised CF patients. However, mutator isolates were neither found to be more resistant nor showed significantly higher MICs than non-mutator isolates for all antibiotics tested.

Table 13 Comparison of MICs for different antimicrobial agents tested against *S. maltophilia* isolates with nonmutator and mutator phenotypes[a]

	Nonmutator isolates (n=41)		Mutator isolates (n=49)		
Antibiotic	No. (%) resistant	Mean MIC[b]	No. (%) resistant	Mean MIC[b]	Statistical significance (*P*)[c]
Ceftazidime	63.4	26.5	46.9	18.6	0.1402
Colistin	56.0	6.8	59.1	7.3	0.8319
Co-trimoxazole	14.6	0.3	18.3	0.3	0.5634
Fosfomycin	60.9	37.2	67.3	51	0.6589
Levofloxacin	24.3	1.1	32.5	1.3	0.4855
Moxifloxacin	26.8	0.6	32.5	1	0.6463
Tigecycline	2.4	1	4.0	1.5	1.0000
Tobramycin	82.9	82.8	87.7	78.3	0.5593

[a] Non-mutator isolates (hypomutator and normomutator) included isolates with frequencies of $\leq 8 \times 10^{-9}$ and $8 \times 10^{-9} < f < 4 \times 10^{-8}$; whereas rmutator isolates (weak hypermutator and strong hypermutator) had a frequency of $4 \times 10^{-8} < f < 4 \times 10^{-7}$ and $\geq 4 \times 10^{-7}$. [b] Geometric mean of the MIC in mg/L. [c] *P* values (Fisher's test) resulting from comparison of the MICs among non-mutator and mutator isolates.

3.7 EGCg, the main component of green tea, displays antibacterial and anti-biofilm properties against *S. maltophilia*

In vitro characterisation of antibacterial effects of EGCg was carried out against 60 CF *S. maltophilia* isolates. EGCg was shown to be effective in inhibiting the growth of all tested isolates. The MIC values ranged from 64 to 512 mg/L, whereas MBC values ranged from 64 to 1024 mg/L (Figure 19).

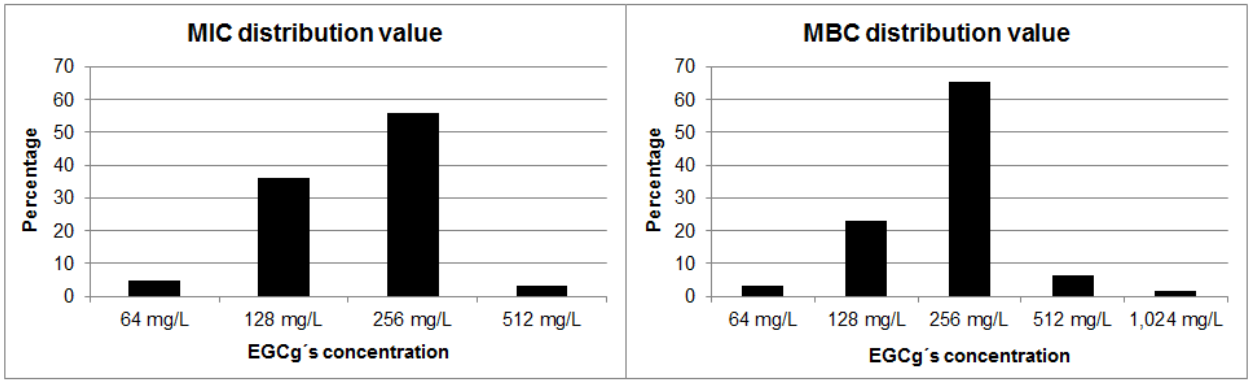

Figure 19 Distribution of minimum inhibitory concentration and minimum bactericidal concentration values determined by microdilution broth assay. Values are expressed as percentage of data obtained from 60 CF *S. maltophilia* isolates against EGCg. MIC= minimum inhibitory concentration; MBC= minimum bactericidal concentration.

The $MIC_{50/90}$ and the $MBC_{50/90}$ for EGCG was 256 mg/L in all four cases. Time-killing curve data shows that EGCg, through a 24 h incubation period, was bactericidal against reference strain ATCC 13637, Sm1 and Sm2 at 2×MIC and 4×MIC (Figure 20).

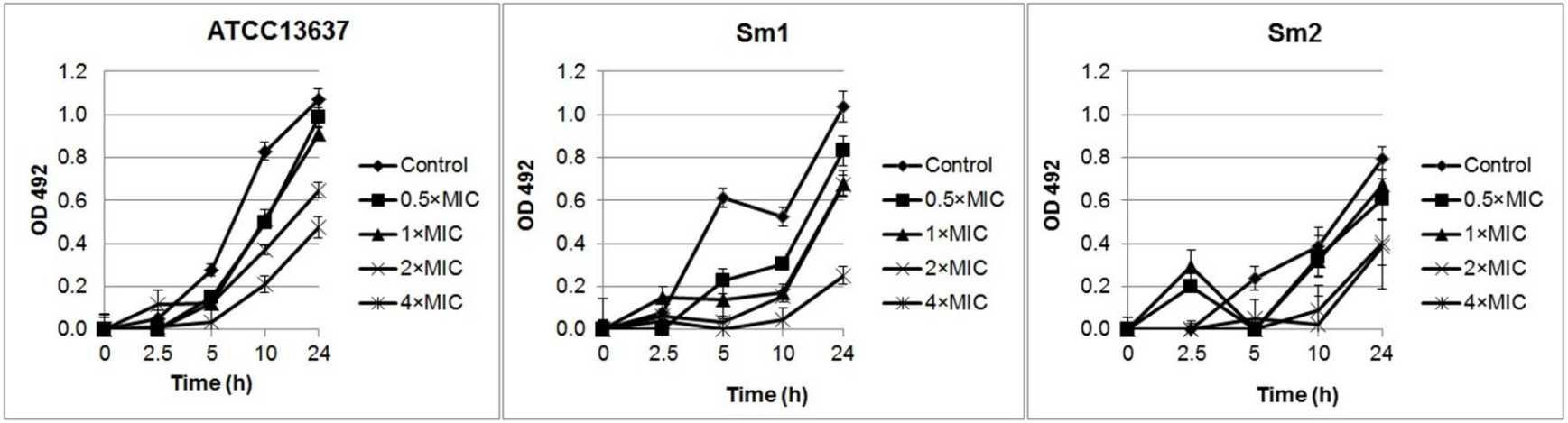

Figure 20 Kinetics of the killing effect of EGCg on *S. maltophilia* ATCC 13637 and two clinical isolates. The concentrations of EGCg ranged from 0.5×MIC to 4×MIC. Bacterial viability over a 24 h period was determined by measurement of optical density (OD) at 492 nm visualised through XTT conversion. Control samples consisted of bacterial cells grown in tryptic soy broth (TSB) in the absence of EGCg. Experiments were designed in three independent sets performed in octuplicate, and the results are expressed as means ± standard deviation (SD).

Given the positive *in vitro* antimicrobial effects of EGCg against *S. maltophilia*, an *in vivo* toxicity assay was conducted to determine if diverse doses of EGCg would be lethal against uninfected nematodes. It was observed that after 48 h exposition to 256, 512 and 1,024 mg/L of EGCg no significant lethal effects on *C. elegans* were observed (Figure 21). A complete mortality assay of *C. elegans* infected with Sm 1 was carried out over a period of five days (Figure 22). Nematodes infected with *S. maltophilia* and, subsequently, exposed to 1,024 mg/L of EGCg displayed enhanced survival up to a rate of 57.0 %, which was not considered significantly different from the control survival rate ($P>0.05$). Nematodes from the grows “only infected” and “infected/treated with EGCg (512 mg/L)” showed a survival rate to the level of 37.0% and 47.0%, respectively, which was considered statistically different from the control group ($P=0.0002$ and $P=0.0059$, respectively). Approximately 45.0% of the animals from the groups “infected/treated” with COL (64mg/L and 128mg/L) and “infected only” survived, suggesting a significant difference in the survival rate between control and these groups ($P=0.0002$ for all of them).

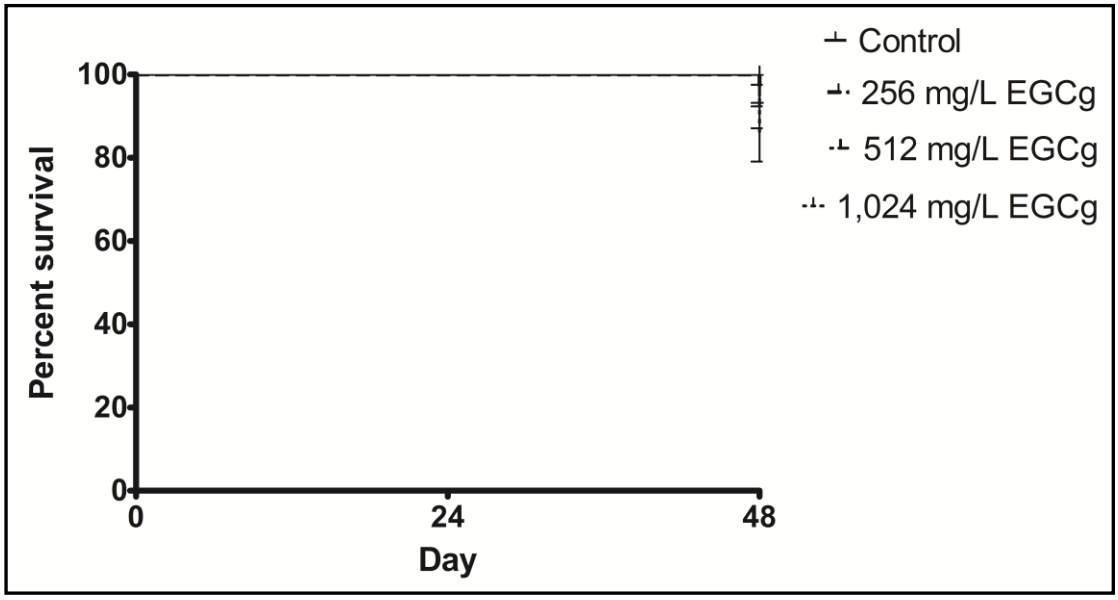

Figure 21 Percentage mortality of wild-type *C. elegans* exposed during 48 h to diverse concentrations of EGCg (256, 512 and 1,024 mg/L). Data express the mean values of two independent experiments performed in triplicated, SDs are shown.

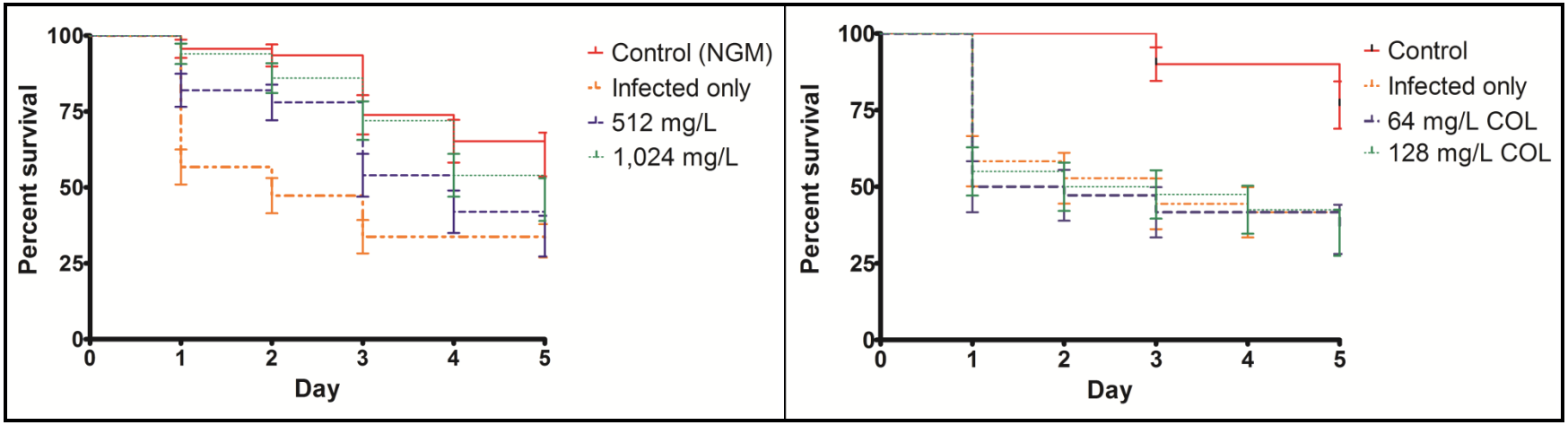

Figure 22 EGCg enhances the survival of *C. elegans* infected with *S. maltophilia* clinical isolate (Sm1). Results are shown as mean values of three independent experiments performed in triplicated, SDs are shown.

In order to study whether EGCg could be useful as a novel natural compound for the treatment (prophylactic and therapeutic) of acute *S. maltophilia* pulmonary infection in comparison to COL, three groups composed of wild type mice were treated either with EGCg, COL or placebo (1×PBS) (Figure 23A). Mice were euthanised before their overall fitness was considered severe. A treatment regimen of nebulised EGCg significantly reduced the number of CFU/mL of *S. maltophilia* (P=0.0127) when compared with the untreated, but infected, and COL-treated (P=0.0106) groups. Infected mice which were nebulised with COL did not exhibit significantly reduced numbers of bacteria in the lungs in relation to the control individuals (P=0.4964). Remarkably, the data were similar to those obtained for nematode experiments. Therefore, these results encouraged us to investigate if EGCg would also affect the bacterial loads in *Cftr* mutant mice during *S. maltophilia* acute pulmonary infection. Bacterial counts in the lungs of the *Cftr* mutant mice nebulised with EGCg was significantly lower (P=0.0039) than in those nebulised with sterile distilled water (Figure 23B).

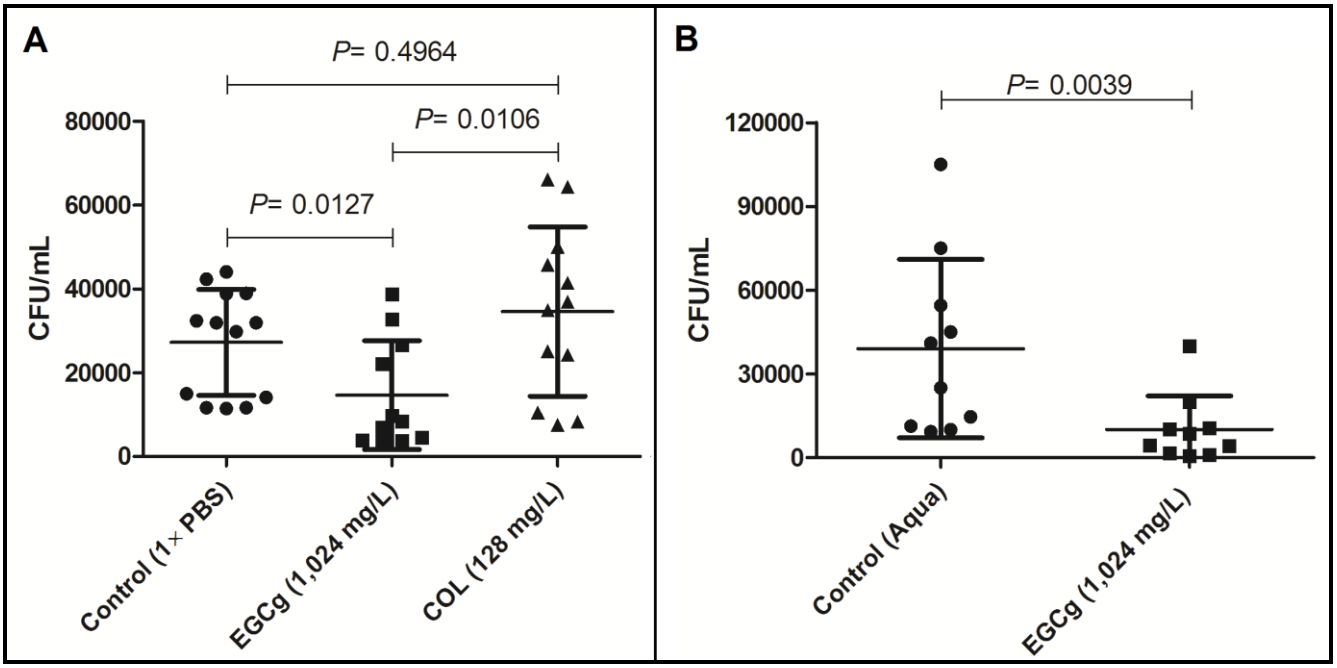

Figure 23 Bacterial load after intratracheal instillation of *S. maltophilia* in C57BL/6 and *Cftr* mutant mice. A) Bacterial count in the lungs of C57BL/6 mice after infection with Sm1 nebulised (2 h before infection and 1 h post-infection) with 1×PBS (n=13), COL (n=12) and EGCg (n=11). Mice nebulised with EGCg exhibited significantly lower bacterial count (P=0.0127) in comparison to non-treated group (1×PBS). Shown are mean ± SD and the distribution of the values. B) Bacterial count in the lungs of *Cftr* mutant mice after infection with Sm1 nebulised (2 h before infection and 1 h post-infection) with aqua (n=10) and EGCg (n=10). Mice nebulised with EGCg exhibited significantly lower bacterial count (P=0.0039) in comparison to the non-treated group (aqua). Displayed are means ±SD and individual values. Aqua= sterile distilled water.

Eradication of *S. maltophilia* colonisation/infection in CF patients is often problematic due to *S. maltophilia*'s inherent resistance to antibiotics and also due to its ability to form biofilm. Therefore, the ability of the studied isolates to form biofilm was investigated. Only 10.0% of the CF *S. maltophilia* isolates did not produce biofilms, while the majority (90.0 %) were able to adhere and to form biofilms. The isolates were classified as weak (11.7 %), moderate (15.0 %), or strong (63.3 %) biofilm producers. The reference strain (ATCC 13637) and the tested clinical isolates (Sm1 and Sm2) were considered strong biofilm producers.

Although subinhibitory concentrations (sub-MICs) of antimicrobial agents might not be able to kill bacteria, they could actually be able to modify their physical-chemical features, the architecture of the outermost surfaces and interfere with some of their functions (Davies and Spielman, 2006). Taking this into account, subinhibitory concentrations of COL and EGCg were employed to ascertain if they could also affect the biofilm development of the three tested strains in the growth medium TSB (Figure 24) in comparison to the positive controls (untreated). Biofilms (ATCC13637, Sm1 and Sm2) treated with COL and EGCg displayed a significant biofilm growth reduction. ANOVA analyses demonstrated significant reduction of biomass in all isolates, suggesting that COL and EGCg exert anti-biofilm forming effects even at subinhibitory concentrations.

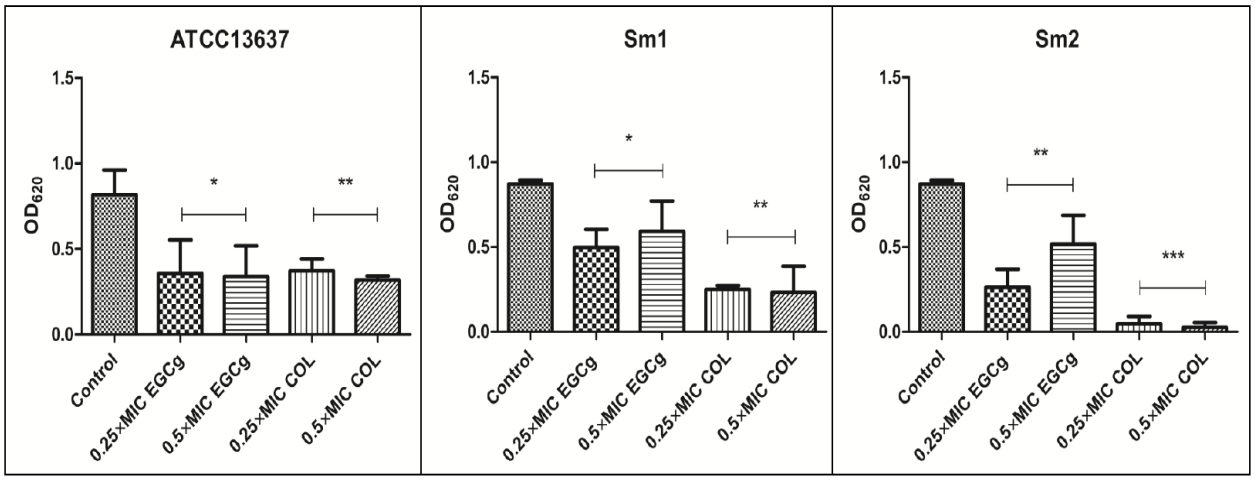

Figure 24 Effects of COL and EGCg against *S. maltophilia* biofilm formation. Reference strain ATCC13637 and two clinical isolates (Sm1, and Sm2) were used. Biofilms were stained with crystal violet and their biomasses were determined by optical density (OD) measurement at 620 nm. Compared to untreated control cells, samples exposed to EGCg and COL exhibited a significant reduction in the number of *S. maltophilia* sessile cells of ATCC13637 and Sm1. Results are expressed as average OD ± SD. Experiments were performed in triplicate. **P*<0.05; ***P*<0.01; ****P*<0.0001.

Biofilms are thought to play a key role in bacterial persistence during chronic infections in CF patients. Therefore, *in vitro* dynamics of *S. maltophilia* biofilms when treated with COL and EGCg were assessed. As illustrated in Figure 25, after exposure to COL or EGCg for 24 h, metabolic activity of young (24-h-old) and mature (7-day-old) biofilm cells produced by tested samples (ATCC13637, Sm1 and Sm2) showed a mean decrease viability in comparison to untreated biofilms. COL showed significant inhibitory effects against young biofilm cells of all tested samples, but only Sm1 mature biofilm cells were significantly inhibited. EGCg significantly reduced the metabolic activity of ATCC13637 and Sm2 young biofilm cells. On the other hand, only mature biofilm cells of ATCC13637 were significantly reduced when treated with EGCg. Remarkably, the relative effects of COL and EGCg on the viability of mature biofilms were not considered dose-dependent.

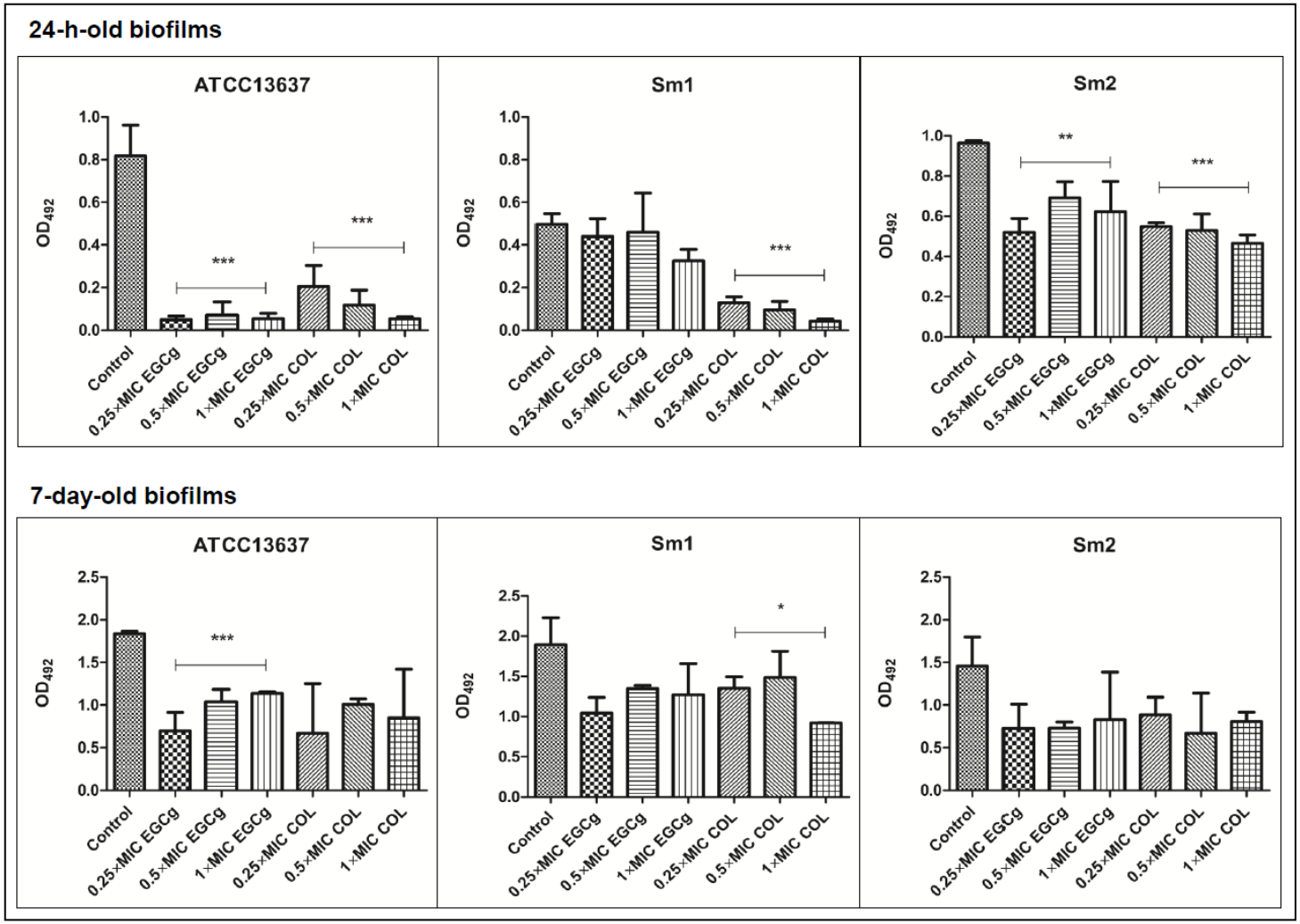

Figure 25 Effects of COL and EGCg on 24-h- and 7-day-old established biofilms of *S. maltophilia*. Reference strain ATCC13637 and clinical isolates (Sm1 and Sm2) had their biofilm metabolic activity defined by XTT viability assay. OD measurement was determined at 492 nm, and results are expressed as average OD ± SD. Experiments were performed in triplicate. *P<0.05; **P<0.01; ***P<0.0001.

To verify the effects of COL and EGCg on morphology and viability of the biofilms produced by CF strains of *S. maltophilia*, we performed CLSM on 48-h-old biofilms produced by the tested samples. Figure 26 illustrates representative biofilm sections of the acquired image stacks. Biofilms produced by isolates Sm1 and Sm2, as visible in the images, demonstrated significant modification in biofilm morphology with increasing concentrations of EGCg, in contrast to COL treated biofilms (Figure 26). Although ATCC13637 strain was not structurally altered by EGCg or by COL, the red biofilm proportion increased with higher concentrations of these compounds. On the other hand, Sm2 structure was indeed modified, but the red biovolume proportion did not change. Quantitative results indicated reduction of total biovolume in all samples at highest concentrations (1×MIC), except for COL treated ATCC 13637.

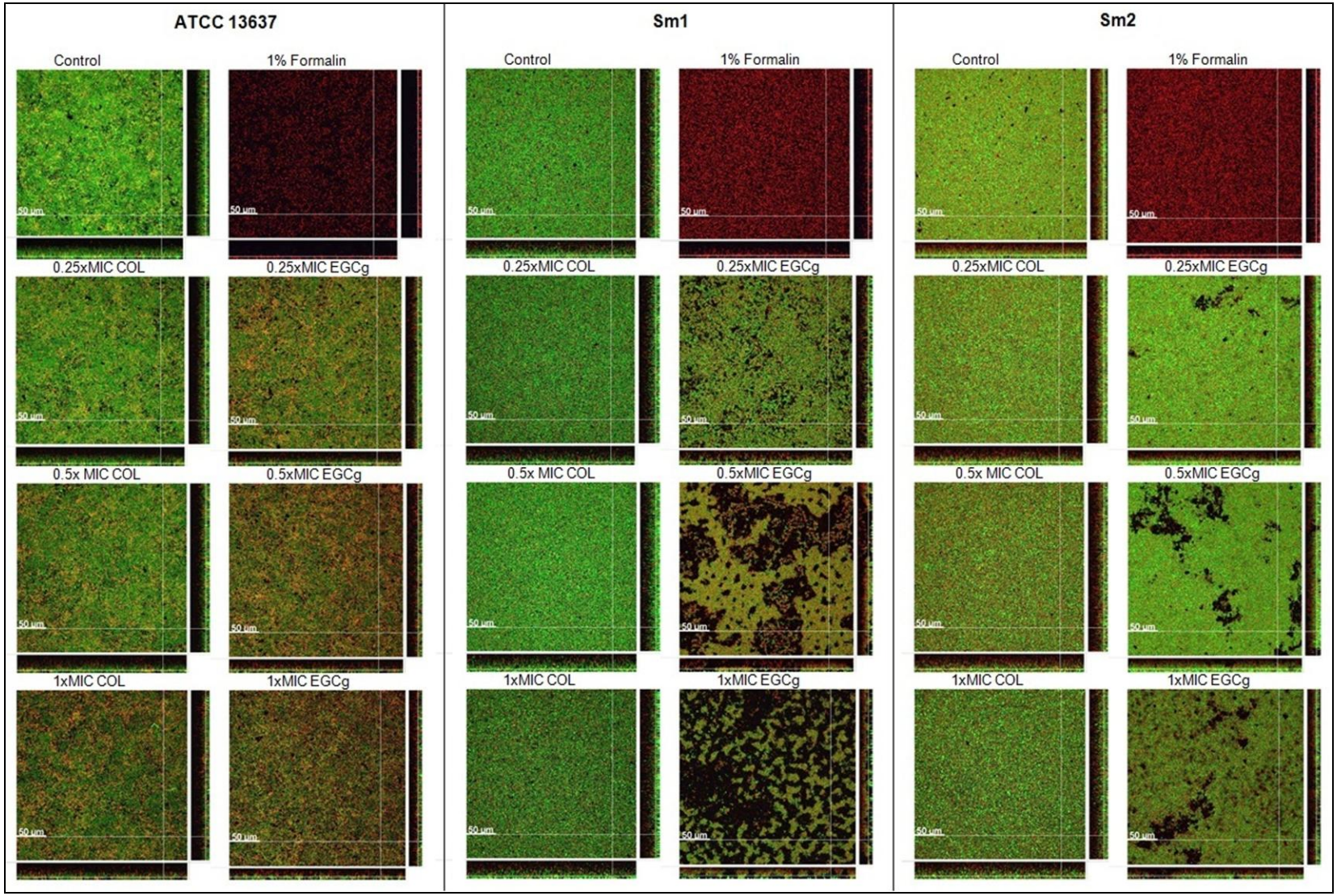

Figure 26 Optical sections of 48-h-old *S. maltophilia* biofilms treated with EGCg and COL at 0.25×MIC, 0.5×MIC, 1×MIC. Biofilms were treated with formalin as killing control. Live bacteria are stained in green (Syto9), dead bacteria in red (propidium iodide [PI]) or yellow (overlapping regions). Experiments were performed in duplicate (image data: 1024 ×1024 pixel with a pixel-size of 0.284 µm; z-step-size: 2 µm). Length of size bar: 50 µm.

4. Discussion

CF can be caused by a variety of different mutations in the CFTR gene, which usually disrupt exocrine function of multiple organ systems (Rowe *et al*., 1995). Dehydration of airway surfaces and impaired mucociliary transport are hallmarks of CF lung disease (Buzzetti *et al*., 2009; Goss and Burns, 2007). Therefore, under conditions of increased viscosity and osmolarity, adequate therapies to eliminate the presence of pathogens in these patients are problematic, normally leading to chronic lung colonisation and/or infection (Rowe *et al*., 2005). In addition, bacterial microorganisms are being increasingly described as a significant cause of morbidity and mortality amongst CF patients (de Vrankrijker *et al*., 2010).

Recently, several centres worldwide have reported increased isolation of *S. maltophilia* from CF patients. Optimal clinical management relies on precise identification and susceptibility testing of this pathogen in the microbiology laboratory. Standardised and low-cost selective media have already been designed to isolate medically important pathogens such as *P. aeruginosa* and *Burkholderia cepacia*, but there is no institutionally recognised selective medium for *S. maltophilia* according to international guidelines (UK Cystic Fibrosis Trust, 2009; Hogardt *et al*., 2006) nor is such a medium yet commercially available. According to the British and German guidelines, detection of *S. maltophilia* and other atypical respiratory microorganisms should be conducted using Columbia blood agar and MacConkey agar. In the late 1980s, a selective medium was developed for the isolation of *S. maltophilia* (XMSM) from soil and rhizosphere (Juhnke and des Jardin, 1989). Subsequently, the formulation of VIA agar, which contained the selective agents vancomycin (5 mg/L), imipenem (32 mg/L), and amphotericin B (2.5 mg/L), was published (Kerr *et al*., 1996). Since *S. maltophilia* does not produced acid from mannitol, a mannitol/bromothymol indicator system was included in the formulation of this medium. A comparison between the selective properties of VIA and XMSM agar, by using faecal samples obtained from 32 children with haematological malignancy, revealed that VIA agar was clearly superior in selection of *S. maltophilia* in comparison to XMSM, since it allowed exclusive growth of *S. maltophilia*. Further, it was shown that isolation of *S. maltophilia* from 814 sputum samples obtained from CF

patients, using modified VIA agar cultures (amphotericin B concentration increase to 4 mg/L), improved the detection of this pathogen. The authors noted that VIA agar supported the growth of 54.8 % more *S. maltophilia* isolates than bacitracin-chocolate agar.

The slightly modified SMA presented in this study has no obvious advantages over VIA agar. Nonetheless, the improved detection of *S. maltophilia* by SMA confirms previous findings (Denton *et al*., 2000). Given the increasing prevalence of this emerging pathogen in the last decade, the use of a selective agar for isolation of *S. maltophilia* in sputum samples from CF patients is strongly advised. As shown in this work, both media, in addition to others, could identify the presence of *S. maltophilia* in 7.1% of all analysed samples, whilst SMA verified the presence of this pathogen in 11.0%, supporting the growth of 64.0% more than the conventional media. Although SMA should only be used for a period of three weeks post-preparation, due to the gradual degradation of imipenem activity, the easy formulation and fast processing of sputum makes it a potentially useful medium.

There are only a few available studies regarding antimicrobial susceptibility of *S. maltophilia* samples from CF patients. The results from the current study indicated that tigecycline, trimethoprim-sulfamethoxazole, fosfomycin and levofloxacin exhibited remarkable *in vitro* activity against *S. maltophilia*, whereas ceftazidime, colistin and ticarcillin-clavulanate acid produced inferior results against this bacterium.

A Spanish study has verified the effects of 41 antimicrobial agents against 76 *S. maltophilia* isolates from CF patients admitted to the Ramón y Cajal Hospital CF Unit, Madrid (Cantón *et al*., 2003). The reported resistance rates for ceftazidime (70.0 %), ticarcillin-clavulanate acid (47.0 %) and trimethoprim-sulfamethoxazole (25.4 %) were higher than those presented in this study whilst, in comparison, moxifloxacin and levofloxacin exhibited lower resistance rates.

An antimicrobial sensitivity investigation of 673 *S. maltophilia* samples from patients attending the CF Referral Center at Columbia University (New York City, USA) was carried out through broth microdilution assay (San Gabriel *et al*., 2004). Although MIC results for ticarcillin-clavulanate were similar to ours, the resistance rate for

trimethoprim-sulfamethoxazole (84.0 %) was, surprisingly, found to be higher than the one described in this study.

On the subject of *S. maltophilia* susceptibility to levofloxacin, a Turkish group analysed genotype and susceptibility features of a small number of isolates (n=11) (Nazik *et al*., 2007). The results demonstrated that 82.0 % of these isolates were susceptible to levofloxacin, revealing its efficacy against this pathogen. In addition, the authors also observed that all isolates were susceptible to trimethoprim-sulfamethoxazole.

In Northern Ireland, *S. maltophilia* isolates from adult and paediatric CF patients (n=10) had their antibiotic susceptibility (MIC test strips) against levofloxacin characterised (McKnight *et al*., 2005). The data suggested, again, that levofloxacin showed excellent inhibitory activity against this bacterial agent.

Antimicrobial effects of five drugs were tested against Gram-negative bacteria, among them *S. maltophilia* (n=51), obtained from CF patients attending either an American CF centre or the French hospital Jean Minjoz, Besançon (King *et al*., 2010). Levofloxacin was considered the most potent drug against all tested isolates. The aforementioned results were similar to our finding that 92.0 % of CF *S. maltophilia* isolates were susceptible to levofloxacin.

Although, in the current investigation, colistin exhibited restricted activity against *S. maltophilia* isolated from CF patients, this data should be carefully interpreted. Colistin is administrated via inhalation to treat patients colonised and/or infected with Gram-negative bacteria, but interpretative *in vitro* criteria for susceptibility testing is assumed to translate into effective intravenous administration of this drug. Thus, further investigations are required to define the potential inhibitory effects of inhaled therapeutic agents. Unfortunately, breakpoints and interpretative criteria for moxifloxacin and colistin against *S. maltophilia* are yet to be established by the CLSI.

Lipopolysaccharide (LPS) is an important pathogenic factor of Gram-negative bacteria. LPS is composed of three distinct regions: O-antigen, core and lipid A. The O-antigen and the core consist of polysaccharide chains, while lipid A is primarily composed of

fatty acid and phosphate substituents bonded to a central glucosamine dimer (Raetz and Whitfield, 2002). Since lipid A is able to induce cytokine release, it is, therefore, considered the major signalling component of LPS. In addition, previous evidence suggests that lipid A produced by *P. aeruginosa* could influence the pathogenesis of CF chronic lung disease (Ernst *et al*., 2003; Hajjar *et al*., 2002; Ernst *et al*., 1999). According to these studies, *P. aeruginosa* is able to synthesize a range of lipid A structures that could potentially promote bacterial survival or colonisation, since it can exhibit different inflammation-stimulating properties, or modify its lipid A structure to confer resistance against elements of host innate immunity.

Previous studies demonstrated that LPS plays a role in the development and maintenance of various Gram-negative bacterial colonies (DeShazer *et al*., 1998; Goldberg *et al*., 1995). An early report addressing the temperature-dependent variation of *S. maltophilia* clinical isolates (n=38) revealed that their susceptibility to aminoglycoside antibiotics was significantly linked to changes in the LPS structures (Rahmati-Bahram *et al*., 1996). In the present study *S. maltophilia* samples from CF individuals produced a significantly higher amount of fatty acids in comparison to samples obtained from ICU patients and the environment. Additionally, the occurrence of distinguished groups of *S. maltophilia* categorised as “CF chronic colonist” and “CF non-chronic colonist” groups, suggests an adaptation mechanism of this microorganism to the CF airway. However, further investigations should be carried out to elucidate if antibiotic treatment against Gram-negative bacteria, during chronic infection, will influence fatty acid production. It is possible that *S. maltophilia* isolates from CF patients have a tendency to produce more fatty acids as a defense mechanism against long-term and repeated treatment with colistin (the mechanism of action relies on its ability to displace bacterial counter ions in LPS).

MALDI-TOF MS is considered a method of phenotypic classification, which has been successfully applied in the past, specifically for species identification based on the protein composition of bacterial cells (Mellmann *et al*., 2008; Degand *et al*., 2008). This method is based on the detection of molecular masses of high abundant proteins in the regarded mass range, which produce species specific mass-spectral fingerprints

(Demirev *et al*., 1999). However, MALDI-TOF MS analyses of *S. maltophilia* mass spectra, from diverse sources, in order to unravel phylogenetic relationships and support genotype data, are still scarce.

PCA is an analytical tool used to identify patterns in data and to express the results in a manner to emphasize their similarities and differences (Smith, 2002). Interestingly, the current results derived from PCA demonstrated that *S. maltophilia* CF isolates exhibited a particular MALDI-TOF spectra pattern from the other isolates (ICU and environmental), allowing them to cluster along the first principal component coordinate axis, therefore, suggesting a possible adaptation of this pathogen to the CF airway environment. In contrast, samples of this microorganism obtained from ICU individuals and the environment clustered in a mixed manner.

Recently, a research group showed that mass spectra of nine environmental *S. maltophilia* isolates exhibited a particular fragment, approximately 6080Da in size (Vasileuskaya-Schulz *et al*., 2011). However, the present study could not confirm the presence of the referred peak in the environmental isolates and neither in the clinical isolates (CF and ICU). Taking this into consideration, it is still too early to be sure that the presence of 6080Da fragment is a species-specific marker. Therefore, further investigations are advisable.

Natural genetic diversity could eventually explain most phenotypic differences amongst bacteria, e.g. geographic distribution, pathogenicity, host specificity, antibiotic resistance and virulence (Andrei and Zervos, 2006). Phylogenetic studies, at strain level, attempt to uncover convergence and divergence in evolution among samples of a species' population. Therefore, typing methods often allow a better understanding of the ecology, as well as the dynamics of bacterial populations (Li *et al*., 2009). Through the years, typing methods have evolved from phenotypic to genotypic ones, due to the fact that traditional phenotypic markers are relatively unstable and do not provide satisfactory resolution power for strain discrimination (Li *et al*., 2009; Andrei and Zervos, 2006). Genotypic methods have the advantage of not being confounded by convergent evolution. Currently, a wide variety of complementary molecular genetic techniques are

available, that when used together, allow a comprehensive analysis of the species (Singh *et al*., 2006). In this study, heterogeneity of *S. maltophilia* was investigated using a set of isolates collected from CF and ICU patients, as well as from the environment. These strains were characterised and typed using the DL system and ERIC-PCR.

Many typing methods revealed that *S. maltophilia* in CF patients and from other sources have high genetic diversity (Wu *et al*., 2011; Pompilio *et al*., 2011a; Marzuillo *et al*., 2009; Nazik *et al*., 2007; Valdezate *et al*., 2001b; Denton *et al*., 1998).

Denton *et al*. analysed 45 *S. maltophilia* isolates obtained from 41 paediatric CF patients (one isolate from each patient and fives isolates from a single patient). In this study they verified the presence of 41 ERIC-PCR types (Denton *et al*., 1998).

A Spanish research group also examined the genetic features of *S. maltophilia* obtained from 25 CF patients (children and adults). This time, using a ribotyping technique and pulsed-field gel electrophoresis (PFGE). Among 76 isolates, DNA restriction enzyme digestion using XbaI identified 47 PFGE profiles (SID value of 0.97); whereas when digested with HindIII (SID value of 0.899) and BamHI (SID value of 0.915) 21 and 20 ribotypes were detected, respectively (Valdezate *et al*., 2001b).

Further, a study conducted in a Turkish hospital analysed the genotypes of 11 samples of *S. maltophilia* recovered from eight CF patients by using random amplified polymorphic DNA (RAPD)-PCR. The authors observed the presence of nine distinct RAPD types (Nazik *et al*., 2007).

In Italy, molecular heterogenicity of *S. maltophilia* isolates collected from CF patients was also confirmed by the presence of 65 major PFGE profiles (Marzuillo *et al*., 2009). Another study of 98 *S. maltophilia* isolates demonstrated the presence of 36 out 47 and 29 out 41 different PFGE profiles, among non-CF and CF isolates, respectively (Pompilio *et al*., 2011b).

Recently, the DL rep-PCR typing method was used to study the clonal relatedness of *S. maltophilia* 47 clinical non-CF isolates. Molecular analysis through the DL system detected the presence of ten different clusters with 80.0 % similarity, while ten other samples did not cluster, suggesting unique strains (Wu *et al*., 2011).

Our results showed consistency with those presented, confirming the high degree of genetic heterogenicity among both clinical (CF and ICU) and environmental isolates supported and detected by the DL system and ERIC-PCR fingerprinting data. For the first time, a large number of *S. maltophilia* isolates, obtained from different sources, was evaluated at the molecular level by means of the DL system and ERIC-PCR. Based on the AR and W coefficient values, the current study could not establish congruence between those two typing methods. Given the fact that each technique is based on different attributes of genetic variation, it is not surprising that they do not entirely concur with one another. However, the results shown by one technique do not invalidate the data from the other.

The adaptation of bacterial populations exposed to new or challenging environments, such as the CF airway, normally tend to result in spontaneous generation of mutator phenotypes (Giraud *et al*., 2001; Tenaillon *et al*., 1999). A mutable pathogen displays an increased spontaneous rate of mutation due to occurrence of defects in DNA proofreading and repair functions (Oliver, 2010; Oliver and Mena, 2010).

Initially, to better understand the conditions required for bacterial colonisation/persistence, genetic diversity of *S. maltophilia* samples, consecutively isolated from chronically colonised CF patients, was examined. A high degree of diversity among these isolates was noted. This fits with results from previous studies (Valdezate *et al*., 2001b; Denton *et al*., 1998).

A British study collected 45 *S. maltophilia* isolates during a period of 28 months from CF patients (one single isolate from each of 40 patients and five from a single patient). Through ERIC-PCR analysis, these clinical isolates were grouped into 41 different genotypes, of which 22 patients carried a unique type, four pairs of patients shared the same type and one patient was colonised with five different strains (Denton *et al*., 1998).

Further, a Spanish prospective observational study conducted at the Hospital Ramón y Cajal (CF Unit) characterised the PFGE profiles of 76 *S. maltophilia* isolates from 25 CF patients. PFGE analysis revealed the presence of 47-well defined genomic profiles (Valdezate *et al*., 2001b).

Over the past decade, prevalence of mutator strains of *P. aeruginosa* in CF patients has been extensively investigated. For example, high prevalence of *P. aeruginosa* mutators in chronically colonised CF patients has been reported in Spain (37.0 %) and Denmark (54.0 %) as well as a wide range of other studies (Ferroni *et al*., 2009; Kenna *et al*., 2007; Ciofu *et al*., 2005; Maciá *et al*., 2005; Oliver *et al*., 2000). Unfortunately, data regarding other CF pathogens is still limited (Turrientes *et al*., 2010; Román *et al*., 2004; Prunier *et al*., 2003).

Mutation frequency of *S. maltophilia* isolates from the CF population was only partially addressed in a study conducted in Spain (Turrientes *et al*., 2010). According to the data presented, 16.7 % of the 48 clinical isolates obtained from 13 CF patients (three of them providing a single isolate) were considered strong hypermutators.

In the current work, a high proportion of mutable *S. maltophilia* isolates (approximately 31.0 %) were detected, suggesting their rapid adaptation to the CF lung environment. However, it was noted that genotypes shared by a number of patients tended, over time, to revert to their original nonmutator state. This can be justified by the fact that accumulation of deleterious mutations might decrease the overall fitness of the bacterial population (Taddei *et al*., 1997).

Currently, antibiotic resistance is recognised as an increasing phenomenon and is a cause for concern, consequently resulting in limited choice of therapeutics, due to mutation playing a role in the evolution of bacterial resistance (Chopra *et al*., 2003). Different studies have shown that *P. aeruginosa* mutators were frequently more resistant to antibiotics in comparison to non mutator isolates (Henrichfreise *et al*., 2007; Ciofu *et al*., 2005; Oliver *et al*., 2000). In contrast, the present work showed no significant correlation between *S. maltophilia* mutators and increased antibiotic resistance. The reason for this discrepancy remains unclear, but a plausible explanation could be the scarcity of concrete guidelines for eradication of *S. maltophilia* (therapy duration and antimicrobial agents are not yet established). Therefore, selective pressure that would normally be higher if the pathogen was exposed to efficient therapeutic agents, is actually reduced, giving strains more chances to adapt (Döring *et al*., 2012). This

phenomenon has been observed in *P. aeruginosa* studies (Oliver, 2010; Hogardt and Heeseman, 2010; Ciofu *et al*., 2005).

In general, chronic respiratory infection in CF patients is normally associated with progressive lung dysfunction and poor clinical status (Waters *et al*., 2013; Waters *et al*., 2011; Razvi *et al*., 2009; Lambiase *et al*., 2006). Although suitable antibiotic treatment against *S. maltophilia* is not yet well established, current evidence indicates that eradication of bacterial pathogens improves the patient's lung function (Dörig *et al*., 2012; Tacetti *et al*., 2012). However, reliable diagnosis of *S. maltophilia* airway infection poses problems due to the difficulty in identifying this pathogen in CF patients who do not expectorate sputum, particularly young children (Ratjen *et al*., 2007; Kappler *et al*., 2006). Thus, possible colonisation of the lower airway in these patients may not be properly recognised and consequently the correct immunological screening may not be carried out. This, together, could lead to incorrect patient clinical status interpretation.

The importance of *S. maltophilia* in CF patients is hotly debated, since it is still unclear whether this pathogen is a real marker of disease severity or if it is causally related to disease progression (Waters *et al*., 2013; Waters *et al*., 2011). For this reason, there is a rising interest in exploring host-pathogen interactions that are responsible for progressive CF lung disease (Turrientes *et al*., 2010; Valdezate *et al*., 2001b; Denton *et al*., 1998).

Previous studies have shown that serological tests are useful tools for monitoring therapeutic response and have also revealed that high levels of specific antibodies are associated with an increased risk for developing chronic infection (Dalbøge *et al*., 2011; Ratjen *et al*., 2007; Kappler et al., 2006). Since chronic exposure to a pathogen usually leads to a specific immune response, a simple and practical serological quantitative IFA to detect specific *S. maltophilia* antibody levels in CF patients was developed.

S. maltophilia antibody titres correlated well with colonisation status. The data revealed that CF patients with "chronic *S. maltophilia*" colonisation status exhibited a significantly higher antibody titre in comparison to all other characterised groups in this study (healthy subjects ($P < 0.0001$), "CF never *S. maltophilia*/*P. aeruginosa*" ($P = 0.0002$), "CF intermittent *S. maltophilia*" ($P = 0.0315$)).

Similar findings were also observed in Danish and Canadian cohorts. The amount of precipitating *S. maltophilia* antibodies was measured by crossed immunoelectrophoresis. The results revealed that chronically colonised patients (n=21) attending the Copenhagen CF centre had antibody titres, that increased during a period of two years (Dalbøge *et al*., 2011). Likewise, research conducted at the Hospital for Sick Children and St. Michael's Hospital (Toronto, Canada), using ELISA, showed that *S. maltophilia* antibody titres against whole cell (P=0.0004) and flagellin (P<0.0001) were higher in CF "chronic" than CF "intermittent" or "never *S. maltophilia*" groups (Waters *et al*., 2011)

Identification and differentiation of serological responses to *P. aeruginosa* and *S. maltophilia*, expressed by the majority of CF "*S. maltophilia* chronic" patients, particularly, ruling out cross-reactivity, was one of the biggest technical obstacles in developing an IFA. In order to reduce the presence of any residual antibodies in the sera from the CF patient groups, non-specific antibody absorption using *P. aeruginosa* whole cells was conducted with all samples. *S. maltophilia* whole-cells were used as the target antigen for the IFA. The reason for choosing *S. maltophilia* whole-cells was based on the fact that some epitopes are unsuitable because they are buried within the outer membrane and consequently may not be available on the bacterial surface (Bakri *et al*., 2002). Thus, antibodies capable of binding to intact bacteria are more likely to provide a protective immune response (Bakri *et al*., 2002).

Interestingly, ROC data allowed us to establish a cut-off value of >1:120 titre with a high degree of confidence based on satisfactory specificity (81.8 %) and sensitivity (78.5 %). Since specificity and sensitivity are not determined by the prevalence of *S. maltophilia* colonisation in the evaluated CF population, the NPV value was measured. A NPV value of 84.7 % indicated that only 15.3 % of the CF patients produced false positive results when tested for *S. maltophilia* antibodies. Therefore, from a clinical standpoint, assessment of both culture and serology results might represent a unique window for intervention to eradicate the organism before the onset of chronic infection, and also to monitor CF patients' *S. maltophilia* colonisation status.

CF patients are generally susceptible to bacterial respiratory infections due to impaired mucociliary clearance competence (O'Sullivan and Freedman, 2009). Furthermore, treatment of these bacterial infections play a major role in assuring lung function by reducing the impact of infection, inflammation, and reduces the incidence of subsequent lung injury (Döring *et al*., 2012). Notably, *in vitro* evidence showed that epigallocatechin-3-gallate (EGCg), an abundant polyphenol found in green tea (*Camellia sinesis*), is an effective antimicrobial agent against a range of Gram-positive and Gram-negative bacterial and fungal pathogens (Steinmann *et al*., 2013; Xu *et al*., 2011; Gordon and Wareham, 2010; Osterburg *et al*., 2009; Hirasawa and Takada, 2004).

EGCg *in vitro* antimicrobial activity was evaluated using 60 *S. maltophilia* isolates obtained from different CF patients. Interestingly, the MIC ($MIC_{50/90}$= 256 mg/L) and time-killing data were similar to those described by Gordon and Wareham with a cohort of 40 clinical isolates from non-CF patients (Gordon and Wareham, 2010). In addition, *in vivo* antibacterial efficacy of EGCg was tested by performing intratracheal instillation infection in wild type and CF mice. The results revealed that bacterial counts in the lungs of mice treated with EGCg were significantly reduced, indicating a bactericidal effect and possible suppression of bacterial dissemination. On the other hand COL, solely administrated, was not as effective as EGCg, a fact that has been previously observed in a different *in vivo* model when infected with *P. aeruginosa* (Herrmann *et al*., 2010).

MIC and MBC *per se* do not provide suitable evidence regarding the efficacy of antimicrobial drugs against bacteria that live in biofilm mode because these results are obtained from assays using planktonic cells. It is important to remember that slow growth rate, low metabolic activity and production of a protective matrix of extracellular polymeric substances are biofilm-specific features that will contribute to pathogen resistance and poor host response (Costerton *et al*., 1999). Thus, biofilm formation is a survival strategy for bacteria.

In the current study, a high percentage of *S. maltophilia* isolates from CF patients (90.0 %) were able to produce biofilm on polystyrene surfaces. An Italian research group has described similar data and they also observed that *S. maltophilia*'s biofilm

formation ability was influenced by different environmental conditions, such as temperature, oxygen availability and pH (Di Bonaventura *et al*., 2007).

Since the biofilm mode of growth is an effective defence mechanism developed by the bacteria to survive in the challenging environment of the CF airway, the impact of COL and EGCg on biofilm formation and on various maturation stages of the biofilm was assessed. It was verified that COL and EGCg at subinhibitory concentrations considerably reduced biofilm formation as well as cell viability of *S. maltophilia* samples tested, with the strongest effect observed at the 0.5×MIC concentration for both compounds. Previous reports have demonstrated that quinolones also display anti-biofilm properties against *S. maltophilia* at sub-MIC concentrations (Pompilio *et al*., 2010a; Di Bonaventura *et al*., 2004). For example, ciprofloxacin, grepafloxacin, and norfloxacin significantly reduced not only biomass, but also the viability of *S. maltophilia* biofilm at concentration of 0.25×MIC (Di Bonaventura *et al*., 2004). Furthermore, the same research group also noticed that subinhibitory concentration of moxifloxacin induced a significant reduction in adhesion and biofilm formation of two *S. maltophilia* isolates from CF patients (Pompilio *et al*., 2010a).

The present study verified, for the first time, whether bactericidal and/or subinhibitory concentrations of COL and EGCg were able to damage young and mature biofilms. At various concentrations, *in vitro* data showed that COL and EGCg reduced 24-h-old and 7-day-old biofilm biovolume. In addition, when biofilm susceptibility testing was assessed by CLSM, strong effects were noticed after EGCg treatment both with regard to morphology and viability. Conversely, COL only decreased the total biovolume of the clinical isolates, as previously evidenced by crystal violet staining experiments.

Although anti-biofilm therapies against medically relevant bacterial pathogens have accrued a significant level of interest, currently there are, as yet, no clearly established treatments to specifically target biofilms. Thus, novel therapeutic solutions for preventing and/or controlling biofilm-associated bacterial infections are urgently needed (Høiby, 2002).

Green tea is not only attractive as a foodstuff, but is also recognised as a millenary substance used in traditional medicine in most Asian countries; and interest in its

medicinal properties are rising in the Western world (Benelli *et al*., 2002). Diverse epidemiological investigations have shown that green tea positively affects the health of patients suffering from cancer, cardiovascular and neurological diseases (Zaveri, 2006; Benelli *et al*., 2002). It also exhibits antimicrobial activity. Several epidemiological studies verified that sub-MIC concentrations of EGCg were effective against biofilms produced by other microbial agents, such as *Streptococcus mutans*, *Staphylococcus aureus*, and *Candida albicans* (Steinmann *et al*., 2013; Evensen and Braun, 2009; Sudano Roccaro *et al*., 2004). Further analyses demonstrated that EGCg in normal human lung cells, even at high concentrations, shows a low toxicity, suggesting it as a potential candidate for development of novel therapies to treat respiratory infections in CF patients (Wu *et al*., 2012; Isbrucker *et al*., 2006a; Isbrucker et al., 2006b). One pharmacokinetic challenge to the therapeutic utilisation of EGCg is the instability of the EGCg molecule (1.8 and 4.9 h for free EGCG and between 1.9 and 4.6 h for total EGCG (Ulmann *et al*., 2003)); but nebulisation emerges as a promising alternative strategy for improving mucociliary impairment and respiratory function among CF patients (O'Connell *et al*., 2011). For example, a clinical study performed with elderly disabled patients demonstrated that inhalation of a green tea extract was able to eradicate Methicillin-resistant *S. aureus* from the upper respiratory tract (Yamada *et al*., 2006).

The mechanism of action involved in the activity of EGCg against bacterial growth remains unclear. It has been proposed that EGCg is an efficient inhibitor of the *S. maltophilia* dihydrofolate reductase enzyme, which consequently leads to disruption of DNA synthesis (Navarro-Martínez *et al*., 2005). Contrarily, other studies suggested that catechins might play a crucial role in damaging bacterial membranes or cell membranes of various pathogens (Arakawa *et al*., 2004; Hirasawa and Takada, 2004). Recently, atomic force microscopy showed evidence that important morphological changes to Gram-negative bacterial cell surfaces induced by EGCg were highly depended on the release of hydrogen peroxide (H_2O_2) (Cui *et al*., 2012). One molecule of EGCg, in phosphate buffer at neutral pH, can produce up to two molecules of H_2O_2 (Arakawa *et al*., 2004). Hydroxyl radicals derived from H_2O_2 are recognised for their ability to damage polyunsaturated fatty acids in membranes and initiate lipid peroxidation, altering the membrane properties, such as fluidity (Cabiscol *et al*., 2002).

Based on these facts, it was demonstrated that treatment of *E. coli* 0157:H7 with sub-MIC of EGCg led to temporary pore-like lesions in the cell wall and degradation of the outer membrane (Cui *et al*., 2012).

Biofilms usually have a high level of cellular activity at the surface, whereas regions towards the core are considered to have low activity and slow growth or no detectable growth (Yamada *et al*., 2006). The hypothesis is that EGCg's anti-biofilm effects are not related to metabolic activity, but instead are related to its ability to bind and damage bacterial membranes (Cui *et al*., 2012).

5. Conclusions and future research

This dissertation has given an account of, and the reason for, the emergence of *S. maltophilia* as a progressive cause of morbidity and mortality in CF patients. The purpose of the current study was to better understand the role of this pathogen in the CF-lung environment.

The following conclusions can be drawn from the present study:

- Implementation of a selective medium for *S. maltophilia* improved the rate of identification for this pathogen in comparison to conventional media.
- Findings of characterisation experiments showed that *S. maltophilia* isolates had a high degree of genetic heterogeneity among clinical (CF and ICU) and environmental isolates, and also revealed that CF *S. maltophilia* samples produced a significantly higher amount of fatty acids in comparison to others cited above.
- The presence of *S. maltophilia* mutator strains recovered from chronically colonised CF patients and their lack of increasing resistance in comparison to non-mutator isolates serve as reliable markers of this pathogen's biological fitness for long-term persistence in the CF-lung.
- Patients chronically colonised by *S. maltophilia* exhibited a specific immune response and displayed significantly higher antibody titres in comparison to other individual groups.
- EGCg, the main component of green tea, exhibited antimicrobial properties against *S. maltophilia* acute pulmonary infection and *in vitro* biofilm.

The evidence from this study suggests that *S. maltophilia* is a versatile bacterium. Its multi-drug resistance coupled with its biofilm-producing characteristic, raises strong concerns in the medical community. Consequently, development of measures to control infection in the CF population caused by this pathogen is urgently required.

However, a number of limitations should be considered. Although it is considered representative, the current number of ICU and environmental samples included was relatively small. An issue that was not addressed was whether naturally hypermutable

strains have an associated biological benefit or cost when colonising a host. Finally, the study did not evaluate the use of EGCg as either a prophylactic or therapeutic treatment.

Further experimental investigation should determine the relevance of increased *S. maltophilia* antibody titres over time; the significance of seroconversion in childhood; and whether antibody titre is reduced after specific therapy against *S. maltophilia*. In addition, the experiments described in this work demonstrated that EGCg is detrimental to *S. maltophilia* biofilm integrity *in vitro*. It would be interesting to assess the role of EGCg in *in vivo* CF models during *S. maltophilia* chronic infection.

6 Summary

Cystic fibrosis (CF) is an autosomal recessive disorder that affects mainly the Caucasian population. CF patients frequently suffer from chronic bacterial airway infections, which are progressively described as an important cause of high morbidity and mortality rates among these individuals. In this scenario, *Stenotrophomonas maltophilia*, an environmental Gram-negative rod, has been lately reported as a global emerging bacterium by CF centres worldwide. At the moment, it is still unclear whether *S. maltophilia* is causally related to disease progression or if this pathogen is real a marker of the CF disease's severity. In the present study, the relevance and importance of *S. maltophilia* was investigated. The major findings were:

- The use of selective medium for *S. maltophilia* supported the growth of the S*. maltophilia* 64.0 % more than the conventional media. It was also verified that tigecycline, trimethoprim-sulfamethoxazole, fosfomycin and levofloxacin exhibited good *in vitro* activity against *S. maltophilia*, while ceftazidime, colistin and ticarcillin-clavulanate acid had low activity against this bacterium.
- Different fingerprinting methods, Enterobacterial Repetitive Intergenic Consensus - polymerase chain reaction (ERIC-PCR) and semi-automated Repetitive Sequence-Based-polymerase chain reaction (rep-PCR) DiversiLab® system, demonstrated high degree of genetic heterogeneity among both clinical (CF and ICU) and environmental *S. maltophilia* isolates. Further, gas chromatography data revealed that *S. maltophilia* samples from CF individuals produced a significant higher amount of fatty acids in comparison to samples obtained from ICU patients and environment. Interestingly, principal component analysis based on Matrix-Assisted Laser Desorption Ionisation-Time of Flight Mass Spectrometry (MALDI-TOF MS) spectra revealed that *S. maltophilia* isolates from CF patients are clustered together in a separated group, whereas ICU and environmental samples grouped in a mixed manner indicating adaptation or strains that show different disease specific characteristics.
- Development of a quantitative immunofluorescence assay (IFA) showed that CF patients with "chronic *S. maltophilia*" had a specific immune response and had a significantly higher *S. maltophilia* antibody levels compared with healthy

individuals, CF patients with “intermittent” or “never *S. maltophilia*/*Pseudomonas aeruginosa*”. A discriminant cut-off value of >1:120 titre was established to differentiate “CF chronic *S. maltophilia*” from the other groups. This assay could be useful to screen *S. maltophilia* colonisation status of CF patients at an early stage of disease and it may also assist on the decision of whether initiation of targeted therapy should be made or not.

- *S. maltophilia* bacterial population recovered from chronically colonised CF patients was highly diverse and revealed the presence of mutator strains. However, these mutator isolates did not show increased resistance to antibiotics in comparison to non-mutator isolates. This kind of adaptation mechanism cost fitness. These results suggest that *S. maltophilia* attempts to sustain its biological fitness as mechanism for long-term persistence in the CF lung.

- EGCg, a natural compound from green tea, displayed *in vivo* antimicrobial effects against *S. maltophilia* acute pulmonary infection and *in vitro* anti-biofilms properties against *S. maltophilia* isolates recovered from CF individuals. This compound might be a promising novel therapeutic inhaling agent against *S. maltophilia* colonisation and infection in patients with CF.

7 References

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Award

Scientific Competition in the context of Annual Meeting 2012 - EXCELLENCE AWARD for excellent PhD thesis, PhD Graduate Course 1045 (Deutsche Forschungsgemeinschaft) - Universität Duisburg-Essen.

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1. *Goncalves Vidigal P,* Grosse-Onnebrink J, Mellies U, Buer J, Rath PM, Steinmann J. *Stenotrophomonas maltophilia* in cystic fibrosis: improved detection by the use of selective agar and evaluation of antimicrobial resistance. **J Cyst Fibros** 2011;10:422-427.

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3. *Gonçalves Vidigal P*, Müsken M, Becker KA, Häuβler S, Wingender J *et al*. Effects of green tea compound epigallocatechin-3-gallate against *Stenotrophomonas maltophilia* infection and biofilm. **Plos One** 2014;9:e92876.

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Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, g der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „***Stenotrophomonas maltophilia* in cystic fibrosis**“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Pedrina Gonçalves Vidigal befürworte.

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