The *Burkholderia cenocepacia* membrane proteins BCAL2958, BCAL2645 and BCAS0764: identification, characterization, and detection of antibodies in sera from *B. cepacia* complex-infected Cystic Fibrosis patients

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Abstract

The *Burkholderia cepacia* complex (Bcc) comprises opportunistic pathogens capable of causing life-threatening infections among cystic fibrosis (CF) patients. Infections by Bcc have a severe prognostic and are particularly difficult to eradicate due to their high level of intrinsic resistance and ability to acquire resistance to antimicrobials. This resistance, together with high morbidity and mortality associated with the infection, highlight the need for novel therapeutic strategies against Bcc infections. The discovery of proteins or other bacterial components capable of eliciting an immune response is the leading research for this development. In line with this objective, three *B. cenocepacia* J2315 proteins, the OprB-like protein BCAS0764 and two OmpA-like proteins BCAL2645 and BCAL2958, previously identified and predicted as immunoreactive, were studied. The OprB-like protein revealed a low immunoactivity against sera samples from Bcc-infected CF patients and no further studies were performed. The OmpA-like protein BCAL2645 was shown to be immunoreactive against sera from CF patients infected with Bcc. BCAL2958 protein was previously characterized as immunoreactive, the study focused on the construction of a mutant on the encoding gene, and on the assessment of the interference of the anti-BCAL2958 antibody on the *B. cenocepacia* adherence to mucins. Although, the creation of a BCAL2958 mutant strain was not possible, the antibody anti-BCAL2958 was found to interfere with the adherence of *B. cenocepacia* to mucins. Despite the promising results obtained, further studies on these proteins are required to evaluate their potential use as vaccines to combat Bcc infections.

Keywords: *Burkholderia cepacia* complex, Vaccines, Antigen, OmpA, OprB, Adhesion

Resumo

O complexo *Burkholderia cepacia* (Bcc) compreende bactérias patogénicas oportunistas capazes de causar infeções fatais entre pacientes com fibrose cística (CF). As infeções por Bcc têm um prognóstico severo e são particularmente difíceis de erradicar devido ao seu alto nível de resistência intrínseca e capacidade de adquirir resistência a antimicrobianos. Esta resistência, juntamente com a alta morbilidade e mortalidade associadas à infeção, destacam a necessidade de novas estratégias terapêuticas contra infeções por Bcc. A descoberta de proteínas ou outros componentes bacterianos capazes de desencadear uma resposta imune é critica para este desenvolvimento. De acordo com este objetivo, três proteínas de *B. cenocepacia* J2315 foram estudadas, a proteína BCAS0764 semelhante a OprB e duas proteínas semelhantes a OmpA, BCAL2645 e BCAL2958, previamente identificadas e previstas como imunorreativas. A proteína semelhante a OprB revelou uma baixa imunoreatividade contra soros de pacientes CF infetados com Bcc e mais estudos não foram realizados. A proteína do tipo OmpA BCAL2645 mostrou ser imunorreativa contra soros de pacientes CF infetados com Bcc. A proteina BCAL2958 foi previamente caracterizada como imunorreativa, logo o estudo focou-se na construção de um mutante no gene codificante e na avaliação da interferência do anticorpo anti-BCAL2958 na adesão de *B. cenocepacia* a mucinas. Embora a estirpe mutante no gene *BCAL2958* não tenha sido obtida, o anticorpo anti-BCAL2958 mostrou interferir com a adesão de *B. cenocepacia* a mucinas. Apesar dos resultados promissores obtidos, estudos adicionais sobre essas proteínas são necessários para avaliar o seu uso potencial em vacinas para combater infeções por Bcc.

Palavras Chave: *Burkholderia cepacia* complex, Vacinas, Antigénios, OmpA, OprB, Adesão

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List of abbreviations

AP^r – Ampicillin resistance

ASM – Artificial sputum medium

ATP – Adenosine triphosphate

Bcc – *Burkholderia cepacia* complex

BSA – Bovine serum albumin

CF – Cystic fibrosis

CFTR – Cystic fibrosis transmembrane conductance regulator

CGD – Chronic granulomatous disease

CK13 – Cytokeratin 13

Cm^r – Chloramphenicol resistance

DsRed – Discosoma red fluorescent protein

DTPA – Diethylene triamine pentaacetic acid

DTT – Dithiothreitol

ELISA – Enzyme-linked immunosorbent assay

EPS – Exopolysaccharide

ET – Edinburgh-Toronto

HRP – Horseradish peroxidase

IgG – Immunoglobulin G

IPTG – Isopropyl β-D-1-thiogalactopyranoside

Kan^r –Kanamycin resistance

KDa – KiloDalton

LAV – Live attenuated vaccine

LB – Lennox Broth

LPS – Lipopolysaccharide

MPO – Myeloperoxidase

NADPH – Adenine dinucleotide phosphate

NC – Nitrocellulose

NO – Nitric oxide

OD – Optical density

OEP – Outer membrane efflux protein

OMP – Outer membrane protein

ORF – Open reading frame

PBS – Phosphate-buffered saline

PCR – Polymerase chain reaction

PG – Peptidoglycan

PIA – *Pseudomonas* isolation agar

RND – Resistance nodulation cell division

ROS – Reactive oxygen species

SB – Super Broth

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

T5SS – Type V Secretion System

TBST – Tris Buffered Saline with Tween 20

Tet^r – Tetracycline resistance

TNFα – Tumor necrosis factor

1. Introduction

1.1. *Burkholderia cepacia* complex – An overview

The *Burkholderia cepacia* complex (Bcc) is a group of at least 23 closely related bacterial species [1–3] that emerged in the 1980s as important pathogens to patients suffering from cystic fibrosis. Bcc bacteria can be found in a wide range of environments like water, soil, and associated with the rhizosphere of plants, causing plant diseases like 'sour skin' onion rot [4] but also having a role on the prevention of other diseases like the 'damping-off ' disease of seeds caused by fungi species like *Pythium spp*. [5] and *Rhizoctonia solani* [6], and root rot by *Aphanomyces euteiches* [7]. These bacteria are also able to inhibit the growth of gray mold and blue mold on fruit[8], to produce several antimicrobial compounds and be effective as a fungicide [9]. Some strains like *Burkholderia vietnamiensis* can increase plant growth and crop production owing to their ability to colonize plant roots and fix nitrogen [10]. Some studies have also been performed showing that Bcc have potential as a bioremediation agent, since these bacteria are able to utilize many different carbon compounds as energy sources [11], can degrade common contaminants of groundwater [12] and some synthetic herbicides [13, 14].

However, Bcc bacteria are also opportunistic pathogens capable of causing life-threatening infections in the respiratory tract of immunocompromised patients, patients with chronic granulomatous disease (CGD), and especially in patients suffering from cystic fibrosis (CF), the most common lethal inherited genetic disease among Caucasians [15]. The majority of Bcc infected CF patients experience an increased decline of pulmonary function, which is associated with chronic infection and exacerbation episodes, while a few patients experience an asymptomatic carriage. However, a significant percentage of around 20% of the patients infected with Bcc bacteria can develop a rapid and fatal necrotizing pneumonia known as the cepacia syndrome, and there is also the possibility of bacteremia as Bcc strains have the ability to cross the epithelial barrier and gain access to the blood stream, something that is not seen in other pathogens that usually infect CF patients [15]. Furthermore, several strains can be easily transmitted from patient-to-patient, which led to the adoption by CF centers worldwide of segregation measures to CF patients infected with Bcc, which is considered one of the most successful prevention strategies [16]. These measures consist on various infection-prevention interventions, ranging from emphasis on hand hygiene, family and clinician education, to single-patient rooms and showers, segregation of Bcc infected patients and elimination of close contact and socialization between all patients infected with Bcc from those not infected [17]. On top of all of this there is the intrinsic resistance of Bcc bacteria to the clinically available antimicrobials, which renders chronic infections untreatable [15]. Although only around 3.5% of the bacterial infections on CF patients worldwide are caused by Bcc bacteria, these infections are of major concern since the clinical outcome is highly variable and hitherto unpredictable [18]. All Bcc species are potential pathogens, but their prevalence varies geographically, with *Burkholderia cenocepacia* and *Burkholderia multivorans* as the predominant species worldwide [19]. In the decades of 1980s and 1990s several outbreaks were reported in Europe and North America due to specific virulent strains that disseminated within CF centers [20, 21]. One example is the Edinburgh-Toronto lineage also referred as ET12, with *B. cenocepacia* J2315 as the representative strain [22]. *B. cenocepacia* J2315 was the first Bcc strain with its complete genome available and one of the best studied Bcc strains [22]. Despite the reduced cases of outbreaks mainly

due to the segregation measures adopted by CF centers which lead to a reduction of the prevalence of infections [23], prevalence of chronic infections by Bcc in 2013 still ranged from 5-10% in Portugal, Russian Federation, Slovak Republican, Czech Republic, Luxembourg and Denmark, and reaching values of 11% in Serbia and 15% in Lithuania [24]. Even though *Burkholderia cenocepacia* and *Burkholderia multivorans* remain the predominant species worldwide, evidence show a changing epidemiology with *B. multivorans* surpassing *B. cenocepacia* in several countries, and *B. contaminans* emerging as a new problematic species [25,26].

1.2. Bcc as opportunistic pathogens in humans

Studies of Bcc strains have been carried out by several research groups worldwide, to understand the pathogenic mechanisms and traits used by these bacteria to cause infection in humans. Nonetheless the processes associated to virulence and the establishment of life-threatening infections are not still fully understood. The importance of this research is largely due to the ability of Bcc strains to cause chronic infections among CF patients. Cystic Fibrosis is the most common lethal genetic disease in Caucasian populations and is a recessive human genetic disorder caused by a loss-of-function mutation in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene, located on the long arm of Chromosome 7 [27]. The CFTR is expressed in many epithelial cells and blood cells [28], as well as neutrophils [29] and macrophages [30]. Although this regulator functions manly as a chloride channel, it has other regulatory functions like the inhibition of sodium transport through the epithelial sodium channel, the regulation of the outwardly rectifying chloride channel, regulation of ATP channels, regulation of intracellular vesicle transport, inhibition of endogenous calcium-activated chloride channels [28] along with acidification of intracellular organelles [31]. There are over 1900 mutations described for the CFTR gene that can cause CF [32]. However, only a small fraction of these have their functional importance known [32]. The most common mutation is ΔF508del, a deletion of the phenylalanine at position 508 in the CFTR, accounting for approximately two thirds of all CFTR alleles in patients with CF. The last third of the alleles are heterogeneous, and less than 20 mutations have a worldwide occurrence higher than 0.1% [32]. The F508del-CFTR defective protein is retained in the endoplasmic reticulum due to a misfolding, and is consequently degraded [33], causing a defective trafficking or a total absence of the protein at the membrane [33]. The alterations in function of CFTR have a main effect on the respiratory system, causing dehydration, acidification, and a weakened innate immunity of the airways [28, 34]. This happens due to the increase in sodium levels, which in turn leads to water reabsorption and dehydration, and cannot be corrected because of the non-functional CFTR, leading to a reduction in the lubricating layer between the epithelium and the mucus, compressing cilia and inhibiting the normal clearance of mucus [28]. A cycle of defective clearance, obstruction, infection and inflammation turns the patients more vulnerable to chronic respiratory infection and acute exacerbations, and progressively deteriorates pulmonary function leading to a considerable morbidity and mortality [35]. The microflora of airways in CF patients is characterized by their diversity, being often polymicrobial and constantly evolving [35], with the microorganism being usually confined to the airway luminal mucus,

rather than invading tissues [36, 37]. Infection of the CF lung occur in infancy or early childhood, with no dominant species and with a high diversity. A study using 16S RNA sequencing to describe the early lower airway microbiota in children with CF showed that, the most prevalent phyla was *Firmicutes* (63.7%), followed by *Proteobacteria* (21.9%), *Actinobacteria* (8.1%), *Bacteroidetes* (4.8%) and *Fusobacteria* (1.3%). In terms of genera, the *Staphylococcus* was the most prevalent (36.6%), followed by *Streptococcus* (13.7%), *Pseudomonas* (6.1%), *Neisseria* (5.4%), *Haemophilus* (4.9%), *Gemella* (4.9%), *Graniculatella* (3.9%), *Prevotella* (2.8%), *Veillonella* (2.6%) and *Streptomyces* (2.1%) [38]. However, with the increase in infections and the consequent increase to antibiotic exposure, a decrease in lung microbiota diversity is observed, as well as the growing abundance of species regarded as pathogens, such as *Haemophilus influenzae*, *Streptococcus aureus*, *P. aeruginosa,* and Bcc bacteria [39–41]. When the end stage of lung disease is reached, usually an almost complete breakdown in community diversity is observed, with the lung microbiome becoming completely dominated by specific bacteria, like *P. aeruginosa* or *Burkholderia spp*. [35, 39, 42].

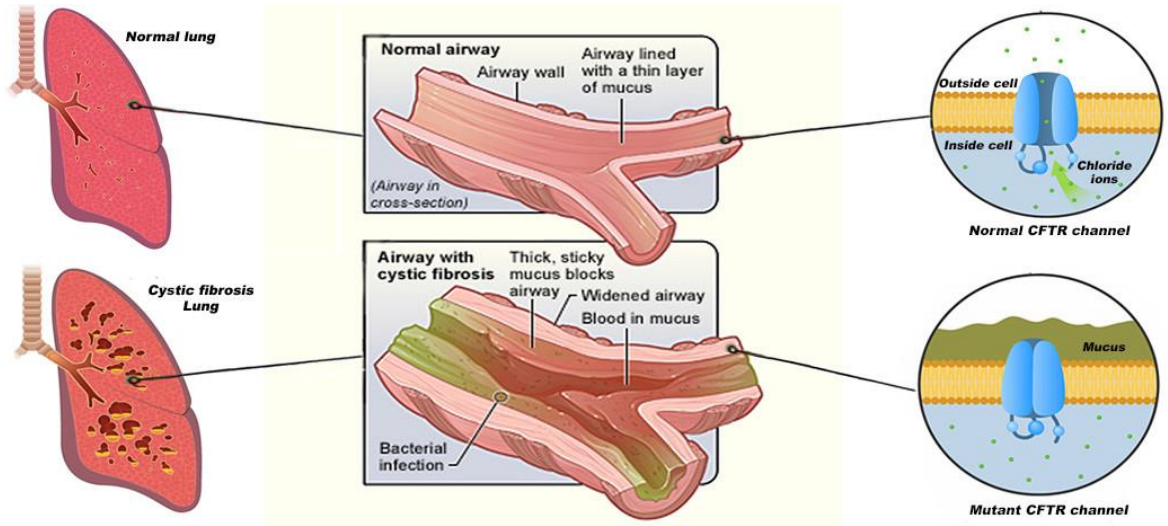

Figure 1. Comparison of normal and CF lung, with cross-sections of the airways showing the effects of CFTR mutation on the lungs and airways

Due to the prevalence of CF, many researchers focus mainly on the pathogens of Bcc in association with the disease. However, Bcc bacteria also emerged as important pathogens in other compromised patients, as is the case of patients suffering from chronic granulomatous disease (CGD). CGD is a relatively rare disease affecting about 1:250 000 individuals worldwide. It encompasses a heterogeneous group of disorders characterized by defective generation of a respiratory burst in human phagocytes, such as neutrophils, mononuclear cells, macrophages and eosinophils, and is due to a rare primary immunodeficiency [43]. CGD is caused by a defect in any of the four components of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex, resulting in the inability to generate ROS and therefore in an inability to combat infectious pathogens like *Aspergillus* species, Bcc

and *Staphylococcus aureus* [44–46]. The disease manifests as repeated, severe bacterial and fungal infections, resulting in the formation of inflammatory granulomas. Opposite to healthy neutrophils, neutrophils originated from CGD patients are incapable of killing Bcc bacteria [47]. Invasive infections and pneumonia are the main causes of death of CGD patients by Bcc [48]. Infections by Bcc have also been reported in immunocompromised patients, such as HIV and cancer [49] patients, and also amongst immunocompetent individual [50]. More recently, Bcc also emerged as nosocomial pathogen among non-CF patients, with an accumulation of nosocomial outbreaks caused by these bacteria being reported [49, 51, 52].

Respiratory infections by Bcc species have a low prevalence on CF patients when compared to *P. aeruginosa*, however they are still one of the most feared pathogens, since they generally lead to a faster decline of pulmonary function, as well as to a decreased life expectancy [19]. Acute infection of the respiratory tract by Bcc bacteria can occur, however colonization by these bacteria usually results in chronic infection [53]. Three distinct clinical patterns are usually observed : chronic asymptomatic carriage; progressive deterioration over a large period of time with episodes of recurrent fever, progressive weight loss, and repeated hospital admissions; and a rapid, typically fatal, high fever, necrotizing pneumonia and bacteremia that is known as the cepacia syndrome [54, 55]. The cepacia syndrome occurs in around 20% of CF patients infected with Bcc and in 2015 a non-CF case was reported [56, 57]. Generally, the chronic infections are caused by a single strain. Nevertheless, extended coinfection with more than one strain, as well as the replacement of the initial strain by another during the course of infection have also been reported [53, 58, 59].

Throughout the chronic infection, the airways of the CF patient represent an highly stressful and fluctuating environment due to the host immune system, antimicrobial therapy, and the reduced availability of oxygen and nutrients [60, 61]. This causes a selective pressure and increases the genetic and phenotypic diversification of the original strain/clone, which will exhibit different antimicrobial susceptibility patterns, size of biofilms, as well as other important phenotypes in the context of bacterial pathogenesis [62]. Several adaptations to this environment were studied by Coutinho *et al* [62]. that performed a proteomic and transcriptomic analysis to compare the first isolate recovered from a chronically infected patient against an isolate recovered 3 years later, after a period of exacerbated infection and intravenous antimicrobial therapy [62]. This study identified several gene/proteins up-regulated in the later isolate, which were associated with carbohydrate and amino acids metabolism, translation, iron uptake, nucleotide synthesis, and protein folding. These findings are consistent with a genetic adaptation and other phenotypic characteristics observed. For example, the up-regulation of genes related with protein synthesis, translation, and protein folding could explain the higher antimicrobial resistance exhibited by the later isolate [63]. The up-regulation of genes involved in iron-uptake was also consistent to the observed higher efficiency exhibited by the last isolate to recover iron, which is an adaptive trait that contributes to increase bacterial persistence in the CF lung [64]. Additionally, several proteins were found to be down-regulated, like those involved in the lipid A and O-antigen formation, consistent with increased ability to evade the host immune system due to the fact that lipid A has a potent pro-inflammatory effect, leading to a response from the host immune system [65]. While O-antigen deficiency is thought to contribute to the enhanced *B. cenocepacia* adhesion to

bronchial epithelial cells [66], other proteins were found in lower number in the later isolates, such as several outer membrane proteins. This decrease content of membrane proteins may explain the increase resistance to antimicrobials because it has been described that impermeability of the outer membrane can improve antibiotic resistance [67]. Another important alteration observed in later isolates was the reduction of the saturation degree of fatty acid due to the growth under severe low oxygen that accompanies the deterioration of the lung [68]. Considering this genetic adaptation occurring during the course of the chronic infection, later isolates were found to have a higher virulence potential based on their higher ability to invade epithelial cells, and to compromise the epithelial monolayer integrity, when compared to the first isolate [69]. However, studies in the non-mammalian infection models *Caenorhabditis elegans* and *Galleria mellonella* showed no consistent progressive pattern of decrease or increase of the virulence potential for a large number of *B. cenocepacia* clonal isolates obtained from chronically infected patients [60].

Considering all the above, a strategy to eradicate Bcc bacteria from CF patients is imperative, however none is currently available [70]. Despite this lack of a universal eradication strategy, several attempts have been described. For instance, a study involving CF centers in UK reported that among the 17 CF adult centers analyzed, 12 centers always attempt eradication of newly isolated Bcc, and of these, 8 estimated a success rate lower than 50% [71]. This same study followed the progression of 19 cases of CF patients infected with Bcc and registered that only 7 achieved eradication. However, 4 out of this 7 were only single isolates [71]. Eradication therapy consisted of a combination of 2–5 antibiotics for a median of 28 days and a maximum of 84 days. The antibiotics were prescribed in 14 completed cases, and only four of these patients successfully cleared the infection, demonstrating no significant association between eradication and receiving the therapy [71] and showing that eradication is unsuccessful in the majority of the cases.

Since eradication is unpredictable and mainly unsuccessful, new strategies to deal with Bcc infections are of major interest, and the strategies with the best chance of success are those that tackle the early stage of Bcc lung infections. Vaccines fit in to this concept and can also confer protection to particularly vulnerable patients, such as the CF patients, which makes them attractive strategies.

1.3. *Burkholderia cepacia* complex - Virulence factors

The factors that contribute to Bcc virulence have been the focus of many studies and a great progress has been achieved in the understanding of their contribution to lung deterioration. Nonetheless, the contribution of virulence factors to the success of the infection by Bcc remains to be fully understood. Moreover, Bcc virulence is multifactorial and does not rely on a single virulence factor [72]. These factors includ bacterial structures like the flagella [73], cable pili and the 22 Kda adhesin [74] that play roles in initial steps of infection by facilitating the adherence to the lung surface and the invasion of lung epithelial cells [73, 74]. Other factors have been described and include mechanisms that are usually involved in the evasion from clearance by the primary cellular defense mechanisms, involving invasion and intracellular survival, exopolysaccharide biosynthesis, quorum-sensing systems, resistance to antibiotics, biofilm formation and oxidative stress resistance [22, 75]. Some of these virulence factors

that have been associated with the external membrane and antimicrobial resistance will be discussed below.

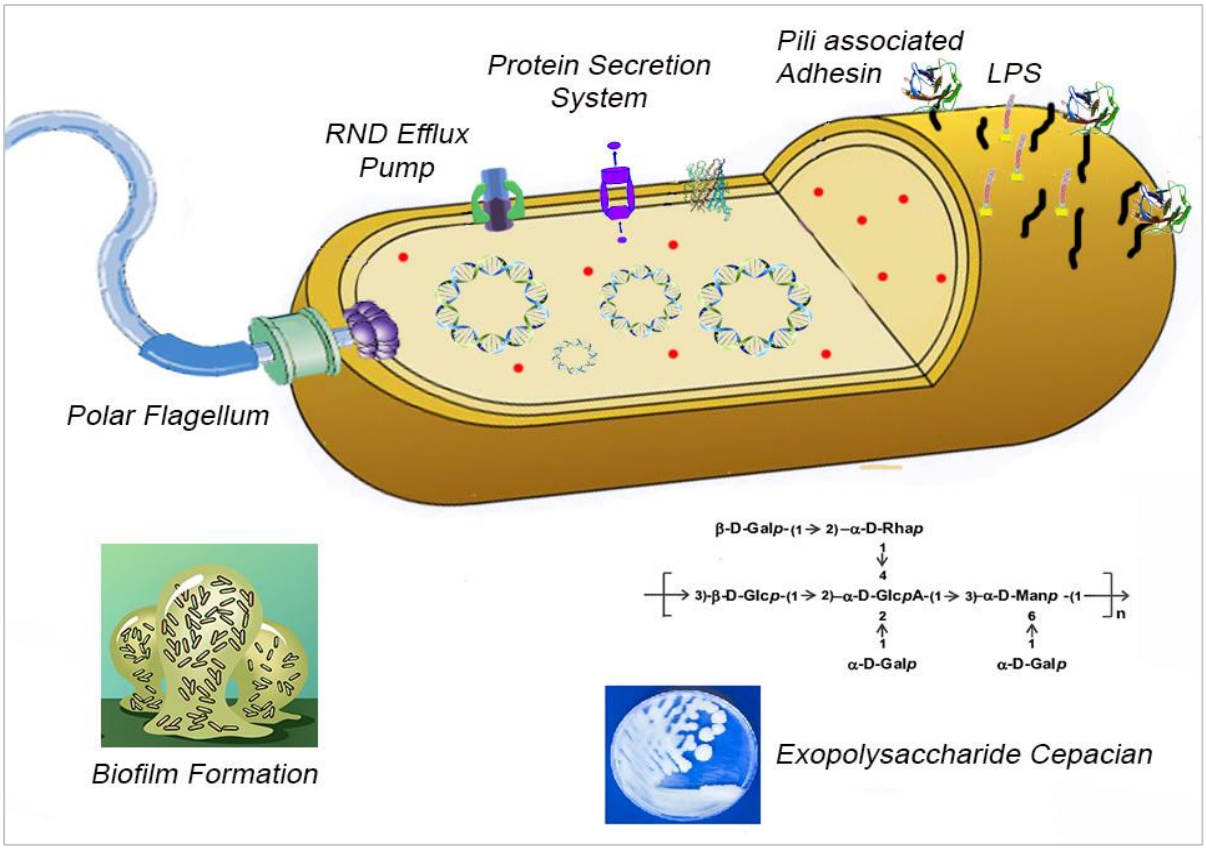

Figure 2. Schematic representation of some of the virulence factors described in the present work.

1.3.1. Polysaccharides

Polysaccharides play important roles in bacterial virulence, and in the case of Bcc bacteria this is not an exception. The lipopolysaccharide (LPS), which is a central component of gram-negative bacteria outer membrane is constituted by the Lipid A, the core oligosaccharide, and the O-antigen moieties. The LPS of Bcc bacteria greatly differs from other gram-negative bacteria by the lack of negatively charged residues, the presence of the heterodimeric disaccharide D-glycero-D-talo-oct-2-ulosonic acid-(2–4)-3-deoxy-D-manno-oct-2-ulosonic acid (Ko-(2–4)-Kdo) in the core region, the presence of an Ara4N residue, either in the core or in the lipid A moiety and the structure of O-antigen [72, 76, 77]. This composition alters the charge of the bacterial surface, inhibiting the binding and the successful action of antibiotics and antimicrobial peptides, contributing to the persistence of bacterial infection [77]. In the specific case of *B. cenocepacia* J2315, the LPS lacks the O-antigen, which was shown to increase phagocytosis by macrophages without interfering with the intracellular survival of bacteria [66]. The exopolysaccharides are also important virulence factors of Bcc, being described for several species. The most common exopolysaccharide produced by Bcc is named Cepacian and can be produced by Bcc and non-Bcc isolates from both clinical and environmental sources [78, 79]. This EPS has been shown to interfere with phagocytosis by human neutrophils, facilitating bacterial persistence in a mouse

model of infection, inhibiting the production of ROS by neutrophils, and to scavenge reactive oxygen species [72, 80–82]. Cepacian is therefore regarded as playing and important role in chronic infections [78]. Interestingly, Cepacian is not produced by *B. cenocepacia* J231, due to mutations, more specifically a frameshift mutation in the BCAM0856 gene that encodes a putative glycosyltransferase [72, 83].

1.3.2. 22 Kda Adhesin

The adherence to host cells is a very important step in bacterial infection and determines host specificity. It has been showed that Bcc bacteria are able to bind to a variety of epithelial cells, including those from nasal polyps, human buccal, airway epithelia and also to alveolar type II pneumocytes [84]. The 22 KDa adhesin that is distributed along the cable pili is used by Bcc bacteria, especially those from the ET12 lineage, to bind to human respiratory mucins [74]. This adhesin binds specifically to cytokeratin 13 (CK13), which expression is increased in CF airway epithelial cells, specifically in bronchiolar and respiratory epithelium [85, 86]. CK13 is mainly a cytoplasmic protein. However, after repeated injury and consequent repair, it is expressed on the apical surface of airway epithelium undergoing squamous metaplasia [84]. The increased expression of CK13 is due to the repeated injury of the airway epithelium that occurs in the lungs of CF patients, and is not directly linked with the CFTR mutation [87]. Studies have shown that *B. cenocepacia* strains expressing the 22 kDa adhesin accompanied by the cable pili bind more efficiently to lung sections from CF patients when compared to equivalents from normal individuals, as well as to lung sections of mice with the CFTR knocked-out when compared to wildtype mice [86].

1.3.3. Biofilms

Biofilms are microbial communities aggregated by extracellular matrices produced by cells that attach to one another or to surfaces, forming 3D-structures. These structures along with their intrinsic resistance to antimicrobials have been described in some studies as the basis for chronic infections caused by bacteria [88]. In Bcc bacteria, the formation and maturation of biofilms depends of several aspects like EPS production [89], iron availability [90], motility [91], as well as multiple gene regulatory systems like alternative sigma factors and quorum sensing[92, 93]. The relevance of these structures relies on the fact that Bcc bacteria growing in biofilms usually exhibit a higher tolerance to antimicrobials [94]. In addition, these structures contain the persister cells that are able to withstand the presence of high antibiotic concentrations by avoiding the production of ROS [95]. Biofilms act a barrier against neutrophils and masking the bacteria from recognition by professional phagocites [96]. However, recent studies suggest that Bcc bacteria are not usually present in biofilm-like structures when in the lung of CF patients, being instead present as single cells or small clusters within phagocytes and in the mucus layer [36, 97].

1.3.4. Protein Secretion Systems

Protein secretion systems are used by both gram-negative and gram-positive bacteria to secrete proteins or toxins out of the cell, either to the environment or into host cells [72]. *Burkholderia vietnamiensis* and Bcc strains from the ET12 lineage have type I and II secretion systems, used for the secretion of hemolytic proteins [98, 99]. The type II secretion system secretes two zinc metalloproteases, ZmpA and ZmpB that were shown to play a role in virulence [72, 100, 101]. Type III secretion systems were shown, in both the mouse agar bead infection model and the nematode *Caenorhabditis elegans,* to be necessary for the pathogenicity of different Bcc species [102, 103]. Two type IV secretion systems are encoded in the genome of *B. cenocepacia*, one in a plasmid and another in chromosome 2 [104]. The one in the plasmid was found to be important for intracellular survival in phagocytes [105]. In the genome of *B. cenocepacia* J2315 four type V secretions systems (T5SS) are encoded. Two T5SS contain hemagglutinin repeat domains and the other two contain pertactin domains. These T5SS were predicted to affect bacterial adhesion [106]. In addition, Bcc bacteria also contain a type VI secretion system, that plays an essential role in pathogenesis and bacterial competition [107]. In *B. cenocepacia* K56-2, it was demonstrated that this secretion system affects the actin cytoskeleton of macrophages [108] and the assembly of the NADPH oxidase complex in *Burkholderia cepacia*-containing vacuoles [109].

1.3.5. Motility

Bacterial motility is described and characterized based on the spreading of the bacteria in agar plates. Four main methods of translocation are used by bacteria: swimming, swarming, twitching and gliding, with the first two being flagella dependent [110]. However, the flagella-dependent modes of motility are the more commonly used by Bcc. Despite the role on the motility played by pili , in most cases bacterial motility is achieved through the flagellum, consisting an important virulence factor that is essential for numerous microbial processes like colonization, bacterial dispersion, biofilm formation and tactic responses [111]. In a transcriptomic analysis it was demonstrated that the growth of *B. cenocepacia* in conditions mimicking the sputum of CF patients leads to a upregulation of several genes related to the flagellar structure and regulation [112]. Another study in an agar bead model of pulmonary infection showed that the flagellum is essential to the virulence of *B cenocepacia* K56-2, since the mutant showed 0% lethality in mice compared to 40% in the wildtype [113]. A more recent work showed an increase in motility and flagellin expression, due to a rise in the number of flagella per cell in a medium mimicking CF sputum [114]. The comparison of transcriptomes of the bacteria from the blood stream at the time of the cepacia syndrome with the transcriptome of bacteria recovered from the sputum prior to the syndrome, revealed a downregulation in flagellar genes in the blood stream bacteria, suggesting that the development of the cepacia syndrome was linked with the loss of bacterial motility [56].

1.3.6. Antimicrobial Resistance

Infections caused by Bcc bacteria represent one of the more difficult to eradicate, and this is a direct consequence of its intrinsic resistance to a variety of antibiotics such as aminoglycosides, polymyxins and a great variety of β-lactams [22]. In addition these bacteria have the ability to develop resistance *in vivo* to effectively all classes of antibiotics [115, 116]. The mechanisms that lead to this multiple resistance vary from enzymes inactivation [22], drug target alteration [22, 117], integrons [118], cell wall impermeability [67, 119], and active efflux pumps [115, 116, 120]. Despite all these mechanisms having a part in antimicrobial resistance, efflux pumps of the resistance nodulation cell division (RND) family seem to be the major contributor either to intrinsic or acquired resistance [121].The genome of *B. cenocepacia* J2315 encodes 16 efflux systems of the RND family [121], and at least 6 of them are associated with antimicrobial resistance [72].

1.4. Vaccines

Vaccines work by enhancing the immunity to a particular disease or pathogen, and usually fall into 4 categories: live attenuated vaccines, toxoid vaccines, killed whole cell vaccines and subunit vaccines [122] (Figure 3). The body immune system recognizes an agent as foreign, destroys it, and then keeps a record of it, in a way that in later encounters the immune system can recognize and destroy the agent and the microorganism that produce it [123]. Vaccines can be used in a therapeutic way (usually related to cancer vaccines) [124] or more commonly as prophylactics, with reports showing that vaccines have prevented more than 100 million cases of disease in the United States, and 2.5 million deaths worldwide per year [125–127]. The enhancing of the immunity can be achieved by active or passive immunity. Active immunity refers to the exposure of the immune system to an antigen, generating an adaptive immune response. This response may take days or weeks to develop but has a long-lasting effect. Passive immunity is the process of providing IgG antibodies to achieve protection against infection, the protection it gives is immediate, but short-lived, with the effect lasting several weeks to 3 or 4 months [128] (Figure 4). This kind of immunization is particularly important in populations where the time or the function of the immune system necessary to develop an effective response to an active vaccination may not be possible. A few immunotherapies using this principle of passive vaccination for *P. aeruginosa* are in clinical trials, like the anti-PcrV, which is a surface-expressed, needle-tip-like protein component of the type III secretion apparatus of *P. aeruginosa*, with early studies with polyclonal rabbit antisera to PcrV showing protection in various acute and chronic mouse models of infection and to have the ability to block the translocation of type III secreted effectors into mammalian cells [129, 130]. A different approach is the Anti-*P. aeruginosa* IgY, with clinical trials underway using a daily mouthwash containing antibodies to *P. aeruginosa* produced from eggs. Hens are vaccinated and produce specific IgY antibodies that are transferred to the egg yolk in large quantities. The anti-*P. aeruginosa* IgY antibodies appear to inhibit the attachment of *P. aeruginosa* to epithelial cells [129, 131].

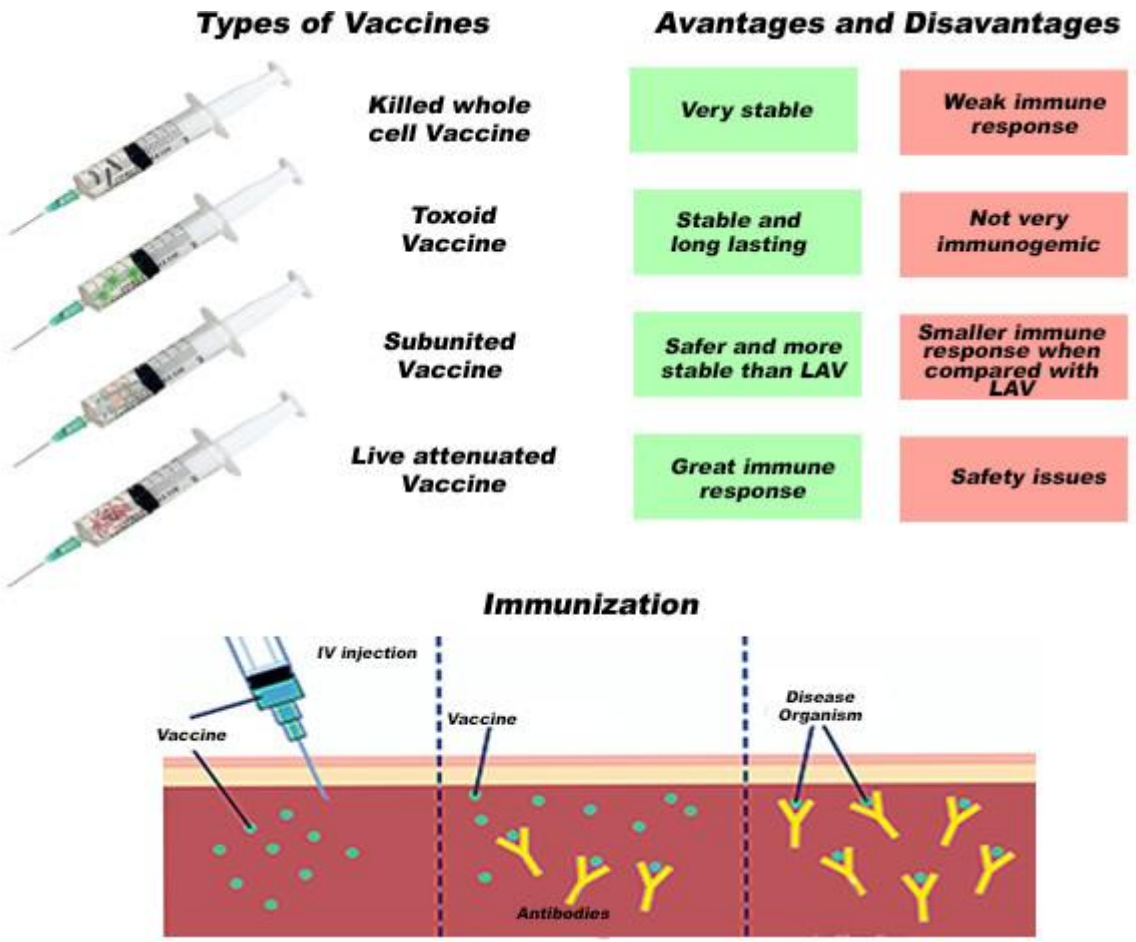

Figure 3. The four main categories of vaccines along with the active immunization process and their advantages and disadvantages

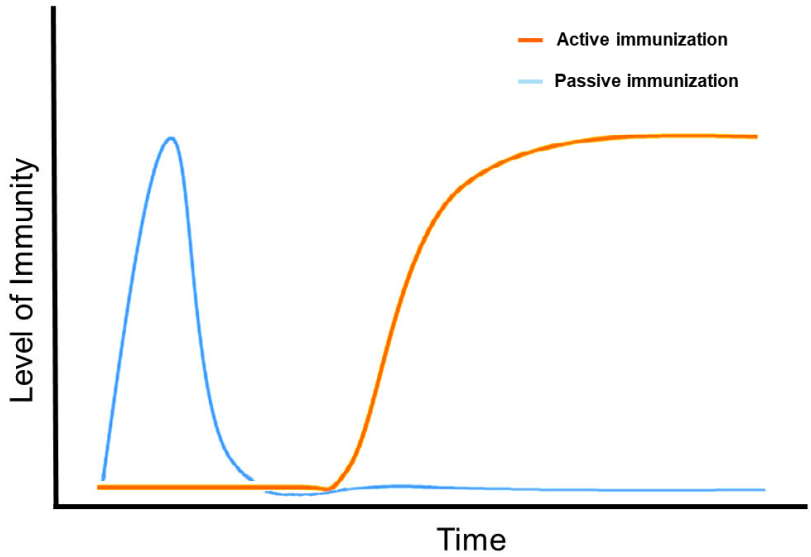

Figure 4. Schematic representation of immunity after active immunization, in which there is a delay between immunization and achieving a protective immunity and passive immunization, where an immediate but relatively short-lived protective immunity is observed**.**

1.4.1. Current strategies and development of Bcc vaccines

Currently no vaccines to protect against Bcc infections are available, nonetheless numerous studies on the immune responses caused by Bcc infection in CF patients have been performed [122, 132]. The design of vaccines against bacterial pathogens must consider the balance between Th1 and Th2 responses for an efficient pathogen clearance, as a Th1 bias will provoke a cell-mediated response and a Th2 bias a humoral immune response [133]. In the specific case of CF, the immune response seems to have a bias towards the Th2 response [134] , and the optimal host response for an efficient clearance of Bcc bacteria is still unknow [72]. Killed whole cells vaccines often induce narrow immune responses, and no reports of this kind of vaccines in Bcc species are available. However, killed whole cells vaccines of Bcc-related *B. pseudomallei* and *B. mallei* in mice were studied and found to be ineffective [135]. On the other hand, live attenuated vaccines are able to stimulate both humoral and cellular immune response, because they are able to replicate in the host but are incapable of causing disease. An example of this was described by Pradenas *et al* using a *B. cenocepacia ton*B mutant live attenuated vaccine that was able to protect mice against acute respiratory infection [136]. Subunit vaccines are usually composed of one or a small number of microbial components that are purified and usually delivered with an immunostimulating adjuvant [137]. A wide variety of virulence factors and proteins are being proposed as potential vaccine candidates [122, 138]. These proteins usually are surface exposed and involved in the first contacts with the host immune system. However, it has been shown that cytoplasmic proteins can also be immunoreactive, due to a variety of factors [122]. Subunit vaccines are thought to have a great potential, and several studies are being performed [122, 135]. The discovery of these proteins and virulence factors often relies on proteomics, more specifically immunoproteomics, which is a rapidly expanding field and includes an increasingly variety of techniques that aim at the identification of immune related proteins and peptides, derived from invading pathogens, host cells, or immune signaling molecules [139]; bioinformatics tools that predict immune properties of individual gene products [122, 140]; or a combination of the two. Examples of proteins identified by these methods are the Linocin and OmpW that were identified using a proteomic approach, and later used for an intraperitoneal immunization of BALB/c mice, where a significant reduction of the *B. cenocepacia* and *B. multivorans* cells in the lung as well as a reduction of the dissemination to the spleen was observed [138]. Linocin led to a Th1 response and OmpW to a mixed Th1/Th2 response, which in its turn was reflected in a greater reduction of the bacterial load in the lung [138]. Differently, the OmpA-like protein encoded by the BCAL2958 gene studied in this work was identified through bioinformatic tools, and despite it has not been tested in animal infection models, it has the capacity to elicit IgG antibodies in CF patients and increase the TNFα, elastase, NO, and MPO levels in neutrophils [141]. Table 1 presents the antigens tested for the use on a Bcc vaccine.

Table 1. Antigens studied for the production of vaccines against the Burkholderia cepacia complex

Antigen/Target	Type of vaccination / Immunization	Immune Response	Effect of vaccination	Animal models	Ref.
Proteins					
OmpA (BCAL2958)	Active vaccination	Ability to elicit IgG antibodies in CF patients and increase the TNFα, elastase, NO, and MPO levels in neutrophils	Not tested	No	[141]
OmpW	Active vaccination	Mixed Th1/Th2 response	Significant reduction of *B. cenocepacia* and *B. multivorans* cells in the lung	BALB/c mice	[138]
Linocin	Active vaccination	Th1 biased response	Significant reduction of *B. cenocepacia* and *B. multivorans* cells in the lung	BALB/c mice	[138]
17 kDa Omp	Active vaccination	Robust IgG and mucosal secretory IgA immune response	Protection against pulmonary colonization by *B. cenocepacia* and cross-protection against *Burkholderia multivorans*	CD-1 mice	[142, 143]
TonB	Live-attenuated vaccine	Mid- to high-total *B. cenocepacia*-specific IgG and Th1-driven immune response	*B. cenocepacia tonB* mutant was able to protect mice against acute respiratory infection	BALB/c mice	[136]
Flagella	Active vaccination	Capable of induction of the host immune responses through interaction with TLR5	Not tested	C57/BL6 mice	[113]
Zinc metalloprotease	Active vaccination	Not accessed	Decrease of about 50% in the severity of *B. cepacia* infection and lung damage	Agar bead model of chronic lung infection	[100, 135, 144]
Polysaccharides					
LPS	Active vaccination	The LPS is immunoreactive, inducing IgA antibodies	Not protective for patients with CF.	No	[145, 146]
PNAG	Passive vaccination	Antibodies are opsonic and protective	Mediates protective immunity against Bcc and also against coinfection with a MRSA strain	FVB/N mice	[147]

1.5. Thesis theme

The *Burkholderia cepacia* complex emerged in the 1980s as important pathogens for patients suffering from CF, and consist of opportunistic pathogens capable of causing life-threatening respiratory infections in the tract of immunocompromised patients, specifically those with cystic fibrosis or chronic granulomatous disease [54, 148, 149]. Several research groups worldwide have been focusing their research on Bcc bacteria, and new perceptions into the virulence of Bcc are being attained, nonetheless the process through which they establish life-threatening infections it is not yet fully understood. The unpredictability of the clinical outcome, the easy patient-to-patient spread, and the resistance to most of the clinically relevant antibiotics, make Bcc infections very difficult to eradicate and the most feared by CF patients [150]. Nevertheless, no standard strategy to eradicate Bcc bacteria is currently available [70], highlighting the need of novel strategies to successfully eradicate these bacterial infections. Strategies that tackle the early stage of Bcc infections are regarded as those having the best chances of success. Vaccines are one of the most attractive strategies, as they fit in this concept and can confer protection to particularly vulnerable patients, such as CF patients. No vaccines against the Bcc exist currently, but the most promising usually fall onto the category of subunit vaccines, with numerous promising antigens being proposed as vaccine candidates [122]. In this perspective, the identification of new potential antigens for the production of subunits vaccines is the aim of this thesis. For this purpose, 3 *B. cenocepacia* J2315 proteins previously identified and predicted to be immunoreactive by our research group were studied. Two of these proteins are OmpA-like proteins and were identified in an *in silico* approach to find immunoreactive outer membrane protein A (OmpA)-like proteins in the *B. cenocepacia* J2315 genome, performed by Sousa *et al* [141]. This reverse vaccinology approach was performed since outer membrane proteins A (OmpA) are among the most immunodominant antigens on the outer membrane of gram-negative bacteria and possess many of the characteristics desirable for a vaccine candidate. They are being studied as potential vaccine antigens for several bacterial infections and have been used successfully in the development of vaccines for Lyme disease [151]. OmpA is an abundant protein in the bacterial outer membrane and is highly conserved. OmpA protein is important in maintaining the integrity of the outer membrane and stimulates a strong antibody response. It is involved in bacterial conjugation, in bacterial attachment, as receptor for certain bacteriophages, and in porin activity [152] . OmpA is also known to be associated with pathogenesis and playing a key role during the initial process of bacterial adhesion and invasion of the host [153]. Antibodies against OmpA and several OmpA family proteins can be bactericidal, opsonic, or protective [154]. *In silico* studies revealed 10 ORF's encoding putative OmpA-like proteins. The *in silico* analysis of the conservation and immunogenicity of the 10 proteins revealed that 3 of them (BCAL2958, BCAL2645 and BCAL3204) were conserved in the Bcc and potentially immunogenic [141], and the proteins BCAL2958 and BCAL2645 were chosen for further studies. The third protein under study, BCAS0764, belongs to the OprB family which are Carbohydrate-selective porins, that play a central role of carbohydrate uptake in *Pseudomonas aeruginosa* [155]. OprB expression in *P. aeruginosa* is linked with virulence and its expression increases when exposed to human respiratory epithelial cells and was found to be strongly downregulated in 96-h biofilms when compared to planktonic cells [156]. In

Xanthomonas citri, the OprB was also studied and found to be required for biofilm production, as well as for virulence [157]. In *B. cenocepacia* no studies of the OprB were performed, however in a strain of *Burkholderia pseudomallei* the *oprB* was studied, and shown to have a role as a multidrug efflux system which extrudes macrolides, fluoroquinolones, tetracyclines, acriflavine, and, to a lesser extent, chloramphenicol [158]. It was also demonstrated in a different *B. pseudomallei* strain that OprB plays an important role in virulence and quorum sensing [159]. This protein was identified by our group in an immunoproteomic assay performed, with the intent of identifying immunogenic proteins from *B. cenocepacia*. For this purpose, the *B. cenocepacia* J2315 was grown on Artificial Sputum Medium (ASM), for 20h at 37ºC in aerobiosis and microaerofilic conditions to mimic the conditions found in the lungs of CF patients, and a 2D-Gel was performed with the extracytoplasmatic protein fraction extracted. This gel was Blotted using a mix of sera from 3 patients with Cystic fibrosis infected with Bcc and BCAS0764 was identified as one of the immunoreactive proteins and one of the few to be putatively located in the outer membrane. This protein's importance towards virulence, together with the fact that it locates in the outer membrane, makes it also a possible good candidate for vaccine development.

The immunoreactivity of the OmpA-like BCAL2958 protein was previously studied in a work by our group, and as such in this work studies to access the relevance of this protein in the virulence of *Burkholderia cenocepacia* began. For this purpose, several attempts for the construction of a mutant on the gene encoding for this protein were attempted. The effect of the anti-BCAL2958 antibody on the adhesion of *B. cenocepacia* J2315 was studied by the performance of adhesion to mucins assays. The OmpA-like protein BCAL2645 and the OprB-like protein were also studied to evaluate their potential as vaccine candidates, based on their immunoreactivity with sera from cystic fibrosis patients infected with Bcc bacteria and the quantification of their IgG titers.

2. Materials and Methods

2.1. Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this work are listed in Table 1. When in use, Bcc strains were maintained in PIA (Pseudomonas Isolation Agar, BD) plates. *Escherichia coli* strains were maintained in Lennox broth (containing 10 g/L tryptone, 5g/L yeast extract and 5g/L NaCl) agar plates, supplemented with 150 µg ampicillin mL^{-1}. Super Broth (SB) medium contained 32g/L tryptone, 20g/L yeast extract, 5g/L NaCl. Unless otherwise mentioned, liquid cultures were carried out at 37 °C in LB liquid medium supplemented with the appropriate antibiotics, with orbital agitation (250 rev min^{-1}). Bacterial growth was followed by measuring the cultures optical density at 640 nm (OD_{640}).

Table 2. Bacterial strains and plasmids used in this work.

Strain or plasmid	Genotype or description	References or source
Strains		
Escherichia coli DH5α	F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ (*lacZYA*, *argF*) U169, hsdR17 (r_{K-} m_{K+}), λ–	Invitrogen
E. coli BL21 (DE3)	F- *ompT hsd*S_B (r_{B-} m_{B-}) dcm gal λ(DE3)	Stratagene
Burkholderia cenocepacia J2315	Cystic fibrosis clinical isolate (Edinburgh, UK); ET12 lineage reference strain; LMG16656	[21]
Plasmids		
pET23a+	Cloning/expression vector, T7 promoter, C-terminal 6× His-Tag, Ap^r	Novagen
pET29a+	Cloning/expression vector, T7 promoter, thrombin recognition site, C-terminal 6× His-Tag, Kan^r	Novagen
pDrive	Cloning vector, Cm^r, Ap^r	Qiagen
pCat1	pUCBM20 with the CAT cassette cloned	[73]
pAV182	pGPI-SceI (accession number EU372690) derivative with the sacB gene and encoding a DsRed fluorescent protein; Cm^r	Kindly provided by Dr. Annette Vergunst
pDAI-SceI-SacB	pDA17 carrying the I-SceI gene and the counterselectable marker SacB, Tet^r	[160]
pSAS36	pET23a+ with *BCAL2645* gene cloned downstream of T7 promoter	This study
pSAS38	pET23a+ with pET29a+ thrombin recognition site cloned downstream of T7 promoter and upstream of C-terminal 6× His-Tag	This study
pAMS1	pSAS38 with the *BCAS0764* gene cloned downstream of T7 promoter	This study
pAMS2	pDrive with the *BCAL2958* gene and the upstream and downstream regions cloned using the XhoI and XbaI restriction enzymes	This study
pAMS3	pAMS2 with the chloramphenicol cassette cloned with be BglII restriction enzyme in the opposite orientation of the gene	This study
pAMS5	pAV182 with the *BCAL2958* gene and the upstream and downstream regions cloned from pAMS3 using the SalI and XbaI restriction enzymes	This study

2.2. Molecular Biology Techniques

Total DNA was extracted from cells harvested from exponentially-growing liquid cultures of *B. cenocepacia* strain J2315 using the High Pure PCR Template Preparation Kit (Roche). Plasmid isolation and purification (NZYTech), DNA amplification (Thermo Fisher Scientific), restriction and T4 DNA ligation (Fermentas), agarose gel electrophoresis, SDS-PAGE and *E. coli* transformation were carried out using standard procedures [161]. The primers used for amplification of BCAL2645 were UP-BCAL2645 (5′- TGACATATGAACATGAAAATCGC -3′) and LW-BCAL2645 (5′- AACTCGAGC TGATGCTGTTGC -3′), containing the NdeI and XhoI restriction sites (underlined), respectively, at their 5′end. The primers used for amplification of BCAS0764 were UP-BCAS0764 (5′- AACATATGCAAAAACATGCTTTG -3′) and LW-BCAL2645 (5′- AAGGTACCTCCGTTCGTCGC -3′), containing the NdeI and KpnI restriction sites (underlined), respectively, at their 5′end. The primers used for amplification of BCAL2958 and upstream and downstream regions were UP - BCAL2958Mut (5′- AAATCTAGAGGAAGGGAACGATATG -3′) and LW-BCAL2958Mut (5′ - AAACTCGAG GACGTCGTAGGTAC -3′), containing the XbaI and XhoI restriction sites (underlined), respectively, at their 5′end. Primers were designed based on the genome sequence of *B. cenocepacia* J2315 (available at the Sanger Institute Homepage; http://www.sanger.ac.uk/Projects/B_cenocepacia).

2.3. Cloning and overexpression of *B. cenocepacia* J2315 BCAS0764 protein

The gene *BCAS0764* was cloned using plasmid pSAS38 and the 1503 bp PCR product obtained using the primers UP-BCAS0764 and LW-BCAS0764. The plasmid pSAS38 was previously created using pET23a+ and the thrombin recognition site of pET29a+. The restriction enzymes BglII and EcoRV were used to remove the thrombin recognition site of pET29a+ followed by a fill-in using the klenow fragment. The HincII restriction enzyme was used to linearize pET23a+, and the thrombin recognition site was ligated. The *BCAS0764* fragment was ligated into the NdeI/KpnI digested pSAS38, providing pAMS1. The nucleotide sequence of this construction was confirmed by sequencing (Eurofins). pAMS1 allows the controlled expression of BCAS0764 by the T7 promoter, upon isopropyl β-D-thiogalactoside (IPTG) induction, producing a BCAS0764 derivative with a 6× His-tag at the protein C-terminus. This plasmid was transformed into *E. coli* BL21 (DE3) and the 6× His tagged protein was overexpressed by growing transformed *E. coli* BL21 (DE3) with pAMS1 in 500 mL of LB liquid medium supplemented with 150 μg/mL ampicillin at 30 °C and with orbital agitation (250 rpm). When the culture reached an OD_{640} of 0.6, IPTG was added to a final concentration of 0.4 mM and the culture was further incubated for 5 h at 30 °C, 250 rpm. Bacteria were then harvested by centrifugation for 5 min at 7000×g and 4 °C and the resulting pellet was resuspended in 20 mL sonication buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM Imidazole, pH 7.4). This cell suspension was aliquoted and stored at −80 °C until further processing. 6× His-tagged BCAL2645 overproduction was assessed by SDS-PAGE analysis and immunoblot experiments using a monoclonal anti-polyhistidine peroxidase conjugate clone HIS-1 antibody (diluted 1:2000, SIGMA) as described in Sousa *et al* [162].

2.4. Cloning and overexpression of *B. cenocepacia* J2315 BCAL2645 protein

The gene *BCAL2645* was cloned using the plasmid pET23a+ and the 659 bp PCR product obtained using the primers UP-BCAL2645 and LW-BCAL2645, digested with the restriction enzymes NdeI and XhoI. The *BCAL2645* fragment was ligated into the NdeI/XhoI digested pET23a+, yielding pSAS36. The nucleotide sequence of the cloned fragment was confirmed by sequencing (Eurofins). pSAS36 allows the controlled expression of the protein BCAL2645 by the T7 promoter upon isopropyl β-D-thiogalactoside (IPTG) induction, producing a BCAL2645 derivative with a 6× His-tag at the C-terminus. This plasmid was transformed into *E. coli* BL21 (DE3) and the 6× His tagged protein was overexpressed by growing transformed *E. coli* BL21 (DE3) in 100 mL of LB liquid medium supplemented with 150 µg/mL ampicillin at 37 °C and with orbital agitation (250 rpm). When the culture reached an OD_{640} of 0.6, IPTG was added to a final concentration of 0.4 mM and the culture was further incubated for 2 h at 37 °C, 250 rpm. Bacteria were then harvested by centrifugation for 5 min at 7000×g and 4 °C, and the resulting pellet was resuspended in 10 mL sonication buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM Imidazole, pH 7.4). This cell suspension was aliquoted and stored at −80 °C until further processing. 6× His-tagged BCAL2645 overproduction was assessed by SDS-PAGE analysis, followed by immunoblot experiments using a monoclonal anti-polyhistidine peroxidase conjugate clone HIS-1 antibody (diluted 1:2000, SIGMA).

2.5. Western Blot analyses

To confirm the expression of the 6× His-tag proteins, western blot using a commercial monoclonal anti-polyhistidine antibody was performed. For this purpose, a volume of the total cell extracts corresponding to 1 mL aliquot of a culture with an OD_{640} of 0.6 was suspended in 40 µL of sample buffer [100 mM Tris base pH 6.8, 4 % (W/V) SDS, 20 % (V/V) glycerol, 0.2 % (W/V) bromophenol blue, 200 mM DTT], incubated for 5 min at 100 °C, and separated by 12.5 % SDS-PAGE. After electrophoresis for 1h at 150 V using standard procedures, the gels were incubated in transfer buffer (48 mM Tris, 39 mM glycin, 20 % (V/V) methanol, 0.04 % (W/V) SDS, pH 9.2) for 15 min and the proteins were electrotransferred to nitrocellulose (NC) membranes (PALL corporation) using a Trans-Blot® SD (BIORAD) device apparatus at 15 mA for 1 h. After protein transfer, NC membranes were blocked at room temperature for 50 minutes with 5 % (W/V) skim milk (DIFCO) in PBS 1×. The membranes were then probed with the commercial monoclonal anti-polyhistidine peroxidase conjugate clone HIS-1 antibody (diluted 1:2000, SIGMA) for 2 h at room temperature. After three washes with PBS 1×, the membranes were treated with the peroxidase substrate ECL (Sigma). The chemiluminescence signals were detected using the FUSION Solo device (Vilber Lourmat).

2.6. Purification of *B. cenocepacia* J2315 6× His-tagged BCAL2645 and 6× His-tagged BCAS0764

Bacterial cell suspensions were lysed by ultrasonic vibration with a Branson sonifier 250 (Branson), using 6 sonication cycles of 30 s each at 40 % duty cycle. When processing cell suspensions to obtain the 6× His-tagged proteins, 2 % (V/V) Triton X-100 were added prior to the last two sonication cycles. After sonication, non-soluble cell debris were removed by centrifugation at 12,000×*g* for 30 min at 4 °C. The cleared supernatants were collected to new tubes and kept at 4 °C.

Both the 6× His-tagged proteins BCAL2645 and BCAS0764 were purified by affinity chromatography using a HisTrap FF column (GE Healthcare), with differences in the imidazole concentrations used to elute each protein. In the case of the 6× His-tagged BCAL2645 protein, the initial equilibration of the column was achieved with 10 mL of Start buffer [sodium phosphate buffer 1×, pH 7.4 (20 mM sodium phosphate, 500 mM NaCl); 20 mM Imidazole; 10 % Glycerol; 0.25 M NaCl], followed by elution with 5 mL of Start buffer containing increasing imidazole concentrations of 60, 100, 150, 200, 250, 300, 400 and 500 mM. Aliquots (1 mL) of the collected fractions of each protein were analyzed by SDS-PAGE. In the case of the 6× His-tagged BCAS0764 protein, no glycerol was used, being substituted by 1 % (V/V) Triton X-100. The increasing imidazole concentrations were 60, 100, 150, 200, 300, 500 mM, 1 mL aliquots were collected and analyzed by SDS-PAGE. Immunoblot experiments were carried out using the commercial monoclonal anti-polyhistidine peroxidase conjugate clone HIS-1 antibody (Sigma).

To produce polyclonal antibodies against the 6× His-tagged BCAL2645, endotoxin contaminations were removed from the protein purified samples using the Detoxi-Gel ™ endotoxin removing gel (Thermo Scientific), following the supplier´s instructions and eluting protein samples with 1× Phosphate Buffered Saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, 2.4 mM KH_2PO_4). Production and purification of a polyclonal goat antibody anti-6× His-tagged BCAL2645 were performed by the commercial company SICGEN (Portugal) after receiving the protein purified as described above.

2.7. Human serum samples

The serum samples S1 and S2 were collected from 2 CF patients infected with Bcc bacteria who attend the Hospital Santa Maria (Lisbon, Portugal), while serum samples S3 were obtained from a CF patient infected with Bcc who attended the Hospital de D. Estefânia (Lisbon, Portugal). Upon blood processing and serum recovery, serum samples were stored at −80 °C until further use. A pool of human blood serum from healthy persons, used as control, was obtained commercially (Sigma).

2.8. CF patients blood sera immunoreactivity against *Burkholderia cenocepacia* J2315 whole cell proteins cultivated in conditions mimicking the CF human lung

B. cenocepacia J2315 was cultivated on LB agar plates, for 24 hours at 37ºC. The cells were then harvested with 0.9% (W/V) NaCl, and the suspension was normalized to an OD_{600} of 0.5. On the surface of each of 4 Artificial Sputum Medium (ASM) agar plates, 100 µL of the suspension was spread. ASM contained 5.0 g/L porcine stomach mucin (Sigma), 4.0 g/L low molecular-weight salmon sperm DNA (Fluka), 5.9 mg/L diethylene triamine pentaacetic acid (DTPA) (Sigma) as iron-chelating agent, 5.0 g/L NaCl (Sigma), 2.2 g/L KCl (Sigma), 5.0 ml/L egg yolk emulsion (phosphatidylcholine as source of lecithin) (Sigma), 5.0 g/L casamino acids (Difco), 1.81 g/L Tris Base (Sigma), and 20 g/L agar (pH 7.0). After incubation for 20 hours at 37ºC under microaerophilic or aerobic conditions, bacterial cells were once again harvested with 0.9% (W/V) NaCl, and aliquots were boiled. Microaerophilic conditions were obtained using the CampyGenTM Compact Sachet in OxoidTM Compact Plastic Pouch (Oxoid). The samples were then loaded into 12.5 % SDS-PAGE gels and electrophoresed for 1 h at 150 V using standard procedures. The gels were then incubated in transfer buffer (48 mM Tris, 39 mM glycin, 20 % (V/V) methanol, 0.04 % (W/V) SDS, pH 9.2) for 15 min and the proteins were electrotransferred to nitrocellulose (NC) membranes (PALL corporation) using a Trans-Blot® SD (BIORAD) device apparatus at 15 mA for 1 h. After protein transfer, NC membranes were blocked overnight at 4 °C with 5 % (W/V) skim milk (DIFCO) in TBST 1×. The membranes were then washed 3 times, 10 minutes each, with TBST 1x, and probed with a pool of 3 serum samples from CF patients (1:2000 dilution in TBST) or with a pool of human sera from healthy donors (1: 2000 dilution, SIGMA) for 2 hours at room temperature. Membranes were washed 2 times with TBST 1× containing 5% (W/V) skim milk (DIFCO) and once with TBST1x, 10 minutes each, and subsequently incubated with a secondary antibody horseradish peroxidase (HRP)-conjugated Rabbit anti-Human IgG (1:5000 dilution, SANTA CRUZ biotechnology) for 1 h at room temperature. After removal of the secondary antibody and 4 washes with TBST 1x, membranes were treated with the peroxidase substrate ECL (Sigma) and signals were detected using the FUSION Solo apparatus (Vilber Lourmat).

2.9. CF patients blood sera immunoreactivity against the BCAL2645, and BCAS0764 proteins

The purified 6× His-tagged BCAS0764, 6× His-tagged BCAL2645 and BSA (used as a negative control, Nzytech) were loaded into 12.5 % SDS-PAGE gels and electrophoresed for 1 h at 150 V using standard procedures [161]. The gels were then incubated in transfer buffer (48 mM Tris, 39 mM glycin, 20 % (V/V) methanol, 0.04 % (W/V) SDS, pH 9.2) for 15 min and the proteins were electrotransferred to nitrocellulose (NC) membranes (PALL corporation) using a Trans-Blot® SD (BIORAD) device apparatus at 15 mA for 1 h. After protein transfer, NC membranes were blocked overnight at 4 °C with 5 % (W/V) skim milk (DIFCO) in PBS 1×. Membranes were then probed with serum samples from CF patients

(1:1000 dilution) or with a pool of human sera from healthy donors (1: 1000 dilution, SIGMA), for 3 h at room temperature. Membranes were washed with PBS 1× containing Tween 20 0.05 % (V/V), and subsequently incubated with a secondary antibody horseradish peroxidase (HRP)-conjugated Rabbit anti-Human IgG (1:5000 dilution, SANTA CRUZ biotechnology) for 1 h at room temperature. After removal of the secondary antibody and wash with PBS 1×Tween 20 0.05 % (V/V), membranes were treated with the peroxidase substrate ECL (Sigma) and signals were detected using the FUSION Solo apparatus (Vilber Lourmat).

2.10. Enzyme-linked immunosorbent assay (ELISA)

IgG levels against purified 6× His-tagged BCAL2645 in sera from CF patients with clinical history of Bcc were determined by enzyme-linked immunosorbent assay (ELISA). The protein solution was prepared at 2 µg/mL in 100 mM sodium carbonate buffer (pH 9.6), and 100 µL was applied per well to 96-wells ELISA plates (Greiner Microlon 600, Greiner Bio-One) and incubated overnight at 4 °C. The plates were blocked with 250 µL of 3 % BSA in PBS 1× overnight at 4 °C. Serum samples were serially diluted (1:100 to 1:10000) in PBS 1 × supplemented with 3 % BSA and 0.05 % Tween 20. The diluted serum was added to the plates and then incubated for 2 h at 25 °C. Then, the plates were washed with PBS 1× containing 0.05 % Tween 20 and were incubated with 100 µL of HRP-conjugated rabbit anti-Human IgG (SANTA CRUZ Biotechnology) antibody at 1:3000 in PBS supplemented with 3 % BSA and 0.05 % Tween 20. The plates were incubated 1 h at 25 °C. After washing the plates with PBS 1× containing 0.05 % Tween 20, 100 µL of the peroxidase substrate 3,3′,5,5′-tetramethylbenzidine (TMB, SIGMA) was added. After 20 min at 25 °C, the reaction was stopped by addition of 100 µL of 0.5 M H_2SO_4. The plates were read at 450 nm in a SPECTROstar Nano microplate reader (BMG LABTECH). A pool of sera from healthy humans (Sigma) was used as control. Internal positive and negative controls were included in each plate. All serum samples were analyzed in triplicate in three independent experiments, and the mean values were calculated. Serum antibody concentrations were defined as endpoint titers and were calculated as the reciprocal of the highest serum dilution producing an OD_{450} above the cutoff value. The cutoff value was determined as the mean OD_{450} nm of the blank control plus 3 standard deviations. A titer above the cutoff value was considered positive for the ELISA.

2.11. BCAL2958 insertion mutant construction

The mutant construction strategy was based on the strategy of Flanagan *et al* [163], using electrotransformation instead of triparental mating. First a plasmid was constructed and for this the *BCAL2958* gene and its respective upstream and downstream regions were cloned using the plasmid pDrive. The 1349 bp PCR product obtained using the primers UP-BCAL2958Mut and LW-BCAL2958Mut digested with the restriction enzymes XbaI and XhoI. The fragment was ligated into the XbaI/XhoI digested pDrive, providing pAMS2. The nucleotide sequence of the cloned fragment was confirmed by

sequencing (Eurofins). Next the chloramphenicol cassette was removed from pCAT1 using HincII restriction enzyme and cloned on to the pAMS2 previously digested with BglII followed by a fill-in using the Klenow fragment, giving rise to pAMS3. Afterwards the pAMS3 was digested with XbaI and XhoI, and the resulting fragment was ligated to the XbaI, SalI digested pAV182, providing pAMS5. The nucleotide sequence of the cloned fragment was confirmed by sequencing (Eurofins). The plasmid pAMS5 is a suicide vector containing the I-SceI restriction site and a gene encoding the Discosoma red fluorescent protein (DsRed). With the plasmid constructed the strategy consisted on the transformation of the pAMS5, onto *B. cenocepacia* J2315, and plating on selective LB with chloramphenicol 400 µg/ml. As the plasmid cannot replicate within the bacteria, it has to integrate itself in the genome. When chloramphenicol resistance colonies start to appear, the ones that had fluorescence, due to the DsRed, where picked and confirmed by PCR. The second transformation was performed using the pDAI-SceI-SacB, followed by plating on selective LB with tetracycline 350 µg/mL. This second plasmid encodes the SceI restriction enzyme, that has a recognition site on the first plasmid, forcing the bacteria to recombine and remove the plasmid from its genome. Tetracycline resistant bacteria were tested for fluorescence, and the ones that didn't exhibit fluorescence, due to the loss of the plasmid in the genome and the DsRed gene, where picked and grown on LB chloramphenicol 400 ng/ µL to confirm that the chloramphenicol cassete was inserted in to the genome. The cells that grow are the insertion mutant. To remove the replicative vector pDAI-SceI-SacB from the cells, the mutant colony is grown in LB without antibiotics. The next day, serial dilutions for up to 10^{-5} are performed. 50 µL aliquots of 10^{-3}, 10^{-4}, and 10^{-5} dilutions are plated onto LB agar plates supplemented with 5 % sucrose lacking salt and incubated at 37 °C overnight. The resulting isolated colonies are plated onto LB agar and LB agar plus 150 µg/mL tetracycline. Tetracycline sensitivity is indicative of loss of pDAI-SceI-SacB.

2.12. Mucins adhesion assays

Inhibition of bacterial adherence to mucins from porcine stomach (type II) by the antibody anti-BCAL2958, was tested as follows: to 96-wells ELISA plates (Greiner Microlon 600, Greiner Bio-One), 200 µL of a solution of Porcine gastric mucin (Sigma-Aldrich) with a concentration of 10 mg/ml in sterile PBS 1x (pH 7.4) was added and incubated overnight at 4ºC. A solution of 3% BSA was used as control. The wells were washed twice with sterile PBS 1x and incubated with 200 µL bovine serum albumin (BSA 3%) for 1h at room temperature. *Burkholderia cenocepacia* J2315 was inoculated at an OD_{640} of 0.1. When an OD_{640} of 0.6 was achieved, the cells were washed and suspended in PBS 1x to a final OD_{640} of 0.05 (5×10^7 cells/mL). The cells were then incubated for 1h at room temperature with the antibody anti-BCAL2958 at different concentrations (0; 0.125 and 0.25 mg/mL). 200 µL of each bacterial suspension was added to each coated well and incubate for 1h at 37ºC. To determine the initial number of bacteria, serial dilutions of the bacterial suspensions before and after the incubation with the antibody, were plated on LB. After incubation, the wells were washed twice with PBS 1x to remove unbound bacteria, and 200 µL of 0.5% Triton X-100 was added and shacked for 2h at room temperature to isolate attached bacteria. The bound bacteria were enumerated by plating serial dilutions on LB agar.

2.13. Bioinformatics analyses

Nucleotide and predicted amino acid sequences were analyzed using bioinformatics tools resident at the National Center for Biotechnology Information (NCBI) and the ExPASy-Prosite websites. Searches for homologous sequences within the genomes of *B. cenocepacia* J2315 and other *Burkholderia* strains were carried out using the Burkholderia Genome Database [164]. The alignment of amino acid sequences was performed using the bioinformatics tool Clustal Omega [165].

2.14. Statistical analysis

Statistical analysis was performed using GraphPad Prism software 6.0. Two-way and One-way analysis of variance (ANOVA) were performed to determine statistically significant differences. The data obtained were represented as mean ± S.D. Results with a P value <0.05 were considered statistically significant.

3. Results

3.1. Study of *B. cenocepacia* BCAS0764 protein immunoreactivity

3.1.1. Identification of *B. cenocepacia* J2315 BCAS0764 as an immunoreactive protein

Previously in our research group, immunoproteomic assays were performed, allowing the identification of the BCAS0764 protein as immunoreactive against sera samples from CF patients infected with Bcc (submitted for publication). For that, the *B. cenocepacia* J2315 was grown on Artificial Sputum Medium (ASM) for 20 h at 37ºC under aerobic or microaerophilic conditions, and 2D-gels were performed using the extracytoplasmatic protein fraction extracted from these cultures. The gels were blotted using a mix of 3 sera from 3 patients with Cystic Fibrosis infected with Bcc. A total of 31 proteins were found to be immunoreactive, and 10 of these were identified by mass spectrometry.

To show that the proteins were only immunoreactive against sera from Bcc infected CF patients and not against sera from normal individuals, a control was performed using an immunoblot assay. Using the same conditions of growth, the whole proteins from *B. cenocepacia* were separated by SDS-PAGE, transferred to a nylon membrane and incubated against the sera from healthy individuals or the mix of 3 sera from CF patients infected with Bcc (figure 5).

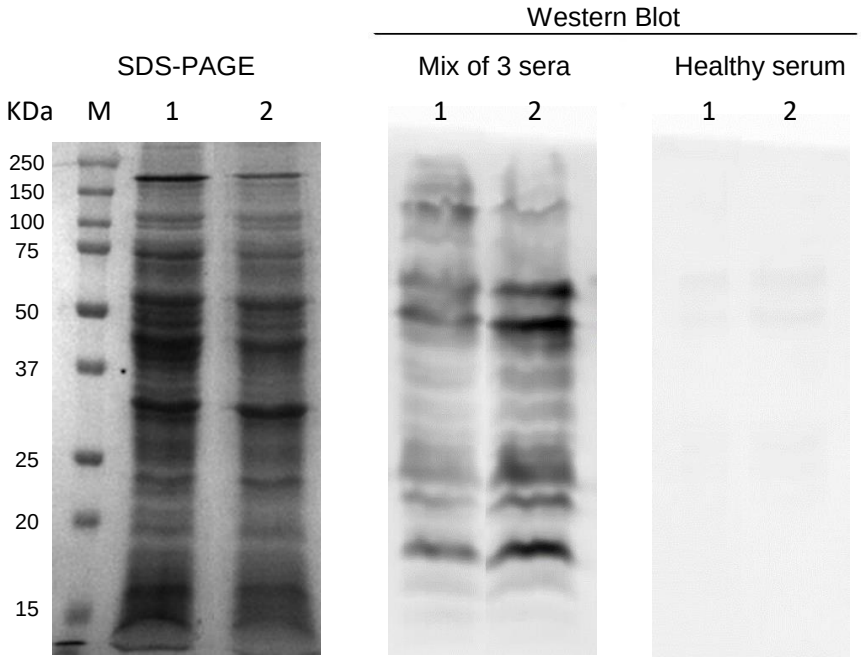

Figure 5. SDS-PAGE of *B. cenocepacia* J2315 whole proteins and the results of the immunoblot assays against the mix of the 3 sera from CF patients infected with Bcc and sera from healthy individuals. Lane M-Precision Plus Protein™ Dual Xtra Standard (BIO-RAD); Lane 1- Aerophilic growth consitions; Lane 2- Microaerophilic growth conditions;

Since the whole proteins from *B. cenocepacia* J2315 were proven not to be immunoreactive against sera from healthy individuals, these results confirm that the 10 proteins identified in the immunoproteomic assay were only identified due to their reactivity with the sera from CF patients infected with Bcc. As such, these 10 proteins were then bioinformatically analyzed to access their conservation within the Bcc (figure 6). These analyses showed that 9 of the 10 proteins were conserved in this group of bacteria.

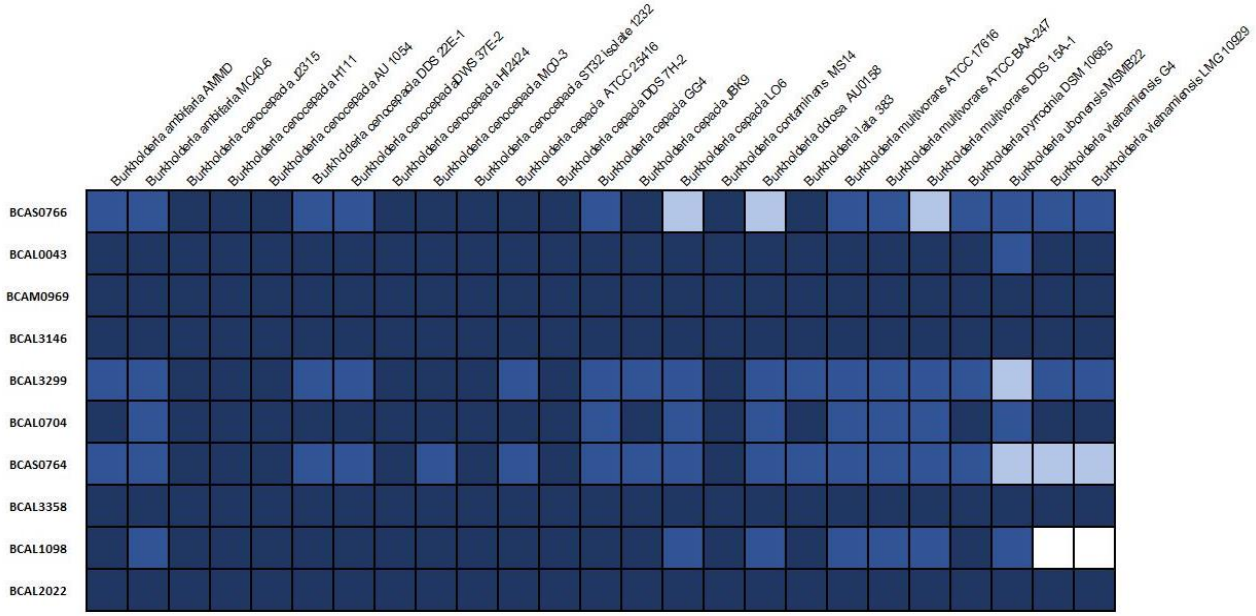

Figure 6. Bioinformatics analysis of the presence, in the complete genome of 25 Bcc strains, of orthologues of the 10 proteins identified in the immunoproteomic assay**.** The % of identity of the orthologues is indicated as follows: higher than 95 % (dark blue square), higher than 90 % (blue square), higher than 85 % (light blue square), absence of the gene (white square)

With the 9 proteins being potential antigen candidates, the BCAS0764 was chosen for further studies in this work. Since it was one of the few identified proteins to be putatively located in the outer membrane. The protein BCAS0764 is annotated as an OprB-like protein in the *Burkholderia* Genome Database [164], nonetheless a blast analysis to find similar proteins was performed. Two similar proteins were found, the OprM protein from *B. cenocepacia* J2315 (SOT40395.1) with a homology of 95% and an OprB from *B. pseudomallei* ATCC 23343 (AAQ94111.1) with a homology of 82%. An alignment of the amino acid sequence of the three proteins was performed (figure 7). This alignment showed a high conservation between the BCAS0764 protein and the other two proteins tested, all presenting the OEP motif typical of the protein family. Members of the OEP family (Outer membrane efflux protein) form trimeric channels that allow the export of a variety of substrates in Gram negative bacteria, with each member of this family being composed of two repeats [166]. The *BCAS0764* gene is located on the third chromosome of *B. cenocepacia* J2315, more specifically in the RND 2 operon. RND are drug antiporters located in the inner membrane that play several roles, including bacterial virulence, quorum sensing, plant–bacteria interactions, and detoxification of metabolic intermediates and toxic compounds, such as heavy metals, solvents, or antimicrobials [167]. The RND operons are composed of three proteins, the outer membrane channel, the periplasmic membrane fusion protein and the RND permease [167]. The

BCAS0764 is the outer membrane channel of this operon. The RND 2 is believed to be a duplication of RND 4, with the two operons sharing an identical gene organization and a high degree of similarity between the two operons in terms of nucleotide and amino acid sequences [167]. The *OprM* gene from *B. cenocepacia* J2315, used in the alignment in figure 7, is the outer membrane channel from the RND4 operon. The OprB from *B. pseudomallei* used in the alignment is also present in an RND operon and as such it also plays a putative role in extrusion of antimicrobials [158].

```
OprB B.pseudomallei         MKRKHALTA----LAVALLAAGCTLAPRYERPAAPVSGAFPADGVYAAPGAAPGARSANG  56
BCAS0764 B. cenocepacia     -MQKHALTAAAVALAAALFATGCTMAPHYKRPDAPVAQAYPAGGVYATQPGAAGARSANG  59
OprM B.cenocepacia          MMQKHALTA----IAVALFATGCTMAPHYKRPDAPVAQAYPASGVYATQPGAAGARSANG  56
                              :******    :*.**:*:***:**:*:** ***: *:**.****:  .* *******

OprB B.pseudomallei         QAAVDIGWREFFVDPRLQRLIEIALKNNRDLRVSVLNVEASRAQYQITRAGLFPTLSGTG  116
BCAS0764 B. cenocepacia     QAATAIGWREFFVDPRLQRLIEIALKNNRDLRVSVLNIEAARAQYQITRAGLFPTLDGTG  119
OprM B.cenocepacia          QAATAIGWREFFVDPRLQRLIEIALKNNRDLRVSVLNIEAARAQYQITRAGLFPTLDGTG  116
                            ***. ********************************:**:***************.***

OprB B.pseudomallei         TGTIQRTPAGVSITGQPLISRTYNVGVSASWELDLFGRVQSLKDQALAQYFATAQARKAA  176
BCAS0764 B. cenocepacia     TGNRQRLPNSLTAVPGRNITTTYNVGLSASWELDLFGRVQSLKDQALAQYLSTSYARQAS  179
OprM B.cenocepacia          TGNIQRLPAGVSTTRAPLISRTYNVGLSASWELDLFGRVQSLKDQALAQYLSTSYARQAS  176
                            **. ** * .:: .    *: *****:************************::*: **:*:

OprB B.pseudomallei         EISLVASVADQYLTLLSTDDLLQVTQNTLKSARASYDLTKLQFDNGTGSELDLRQAQTVV  236
BCAS0764 B. cenocepacia     EISLVSQVADQYLTLLSTDDLLKVTEDTLKTAQASYDLTKLQFDNGTGSELELRQAQTVV  239
OprM B.cenocepacia          EISLVSQVADQYLTLLSTDDLLKVTEDTLKTAQASYDLTKLQFDNGTGSELELRQAQTVV  236
                            *****:.***************:**::***:*:******************:********

OprB B.pseudomallei         ETALASQQAQARARAQALNALVLLIGEPLPDDLPAGLPLNAQNLLTDIPAGLPSDLLTRR  296
BCAS0764 B. cenocepacia     ETALANQQAQARARAQALNALVLLIGEPLPDDLPAGMPLDAQNLLTDVPAGLPSDLLTRR  299
OprM B.cenocepacia          ETALANQQAQARARAQALNALVLLIGEPLPDDLPAGMPLDAQNLLTDVPAGLPSDLLTRR  296
                            *****.*****************************:**:*******:************

OprB B.pseudomallei         PDIMQAEETLRAANANIGAARAAFFPKISLTSAFGTASPTLGGLFKAGTAAWSFAPNIAL  356
BCAS0764 B. cenocepacia     PDVMQAEQTLLAANANIGAARAAFFPKISLTGAFGTASPTLGGLFKAGTAAWSFAPSIAL  359
OprM B.cenocepacia          PDVMQAEQTLLAANANIGAARAAFFPKISLTGAFGTASPTLGGLFKAGTAAWSFAPSIAL  356
                            **:****:** ********************.************************.***

OprB B.pseudomallei         PIFEGGQNIANLDLAHVQKRIEIANYEKAIQSAFREVSDGLAARGTYDQQIAALERNEHA  416
BCAS0764 B. cenocepacia     PIFEGGQNIANLDLANVQKRIEIANYEKAIQSAFREVSDGLAARGTYDQQIAALERNEHA  419
OprM B.cenocepacia          PIFEGGQNIANLDLANVQKRIEIANYEKAIQSAFREVSDGLAARGTYDQQIAALERNEHA  416
                            ***************:*********************************************

OprB B.pseudomallei         QQRRYDLSDLRYRNGVDSYLSVLTAQTDLYSAQHQLISARLARWTNLVDLYRALGGGWLE  476
BCAS0764 B. cenocepacia     QQRRFDLSDLRYKNGVDSYLSVLTAQTDLYAAQQSLISARLARWTNLVDLYRALGGGWIQ  479
OprM B.cenocepacia          QQRRFDLSDLRYKNGVDSYLSVLTAQTDLYAAQQSLISARLARWTNLVDLYRALGGGWIQ  476
                            ****:*******:*****************:**:.************************::

OprB B.pseudomallei         HCAHKGPATYFHLHTEDGVIENAAWSYEEPSGIANAIRQYVAFDAACVDRIDVTS    531
BCAS0764 B. cenocepacia     RAGETPRAPDAPVDYQTATN-G---------------------------------    500
OprM B.cenocepacia          RAGETPRAPDAPVDYDKAAA-PAPASATATNG----------------------    507
                            :....  *    :. : ..
```

Figure 7. Alignment of the amino acid sequences of the protein BCAS0764 from *B. cenocepacia* J2315, the OprB from *Burkholderia pseudomallei (*AAQ94111.1*)* and the OprM protein from *B. cenocepacia* J2315 (SOT40395.1). The 2 motifs correspondent to the OEP family (Outer membrane efflux protein) (PF02321) are boxed in grey. Asterisks indicate the amino acid residues that are identical in the 3 proteins; one or two dots indicate semi-conserved or conserved substitutions, respectively

3.1.2. **Cloning, expression and purification of BCAS0764**

To further study the protein BCAS0764 and access its immunoreactive potential, the 1503 bp PCR fragment corresponding to the *BCAS0764* gene of *B. cenocepacia* J2315 was amplified using the

primers UP-BCAS0764 and LW-BCAS0764 and cloned into the expression vector pSAS38 under the control of the T7 promoter, creating pAMS1 (Table 2). The overexpression of the protein as a 6× His-tagged derivative was accomplished by transforming plasmid pAMS1 into *E. coli* BL21 (DE3) and inducing its expression by the addition of 0.4 mM IPTG. Various conditions were used to improve the amount of protein expressed (table 3), including the use of the antibiotic carbenicillin instead of ampicillin due to its higher stability [168]. The overproduced 6× His-tagged protein was analyzed by SDS-PAGE (figure 8a), but no obvious overexpression was visible. Therefore, the presence of the 6× His-tagged protein expression was accessed by immunoblot experiments using the commercial monoclonal anti-polyhistidine peroxidase conjugate clone HIS-1 antibody (figures 8b). The analysis revealed one form of the recombinant protein with an estimated molecular mass of approximately 56.3 kDa, in agreement with its predicted molecular mass. Although a high overexpression of BCAS0764 protein was not achievable, the best condition for the protein expression in *E. coli* BL21(de3) was at 30º C with 200 mL of LB with 150 µg/L of ampicillin in 500 mL flasks, with an induction time of 5 hours (table 3, figure 8).

Table 3. Conditions tested to improve the expression of the BCAS0764 protein.

Medium and Temperature	Induction time	Volume in flask	Result
LB with 150 µg/mL of ampicillin			
37ºC	2 h	100 mL in 250 mL flask	No visible overexpression
30º C	2 h	200 mL in 500 mL flask	Better expression than 100 mL
30º C	2 h	100 mL in 500 mL flask	Smaller expression than 200 mL
30º C	3 h	200 mL in 500 mL flask	Increase in expression
30º C	6 h	200 mL in 500 mL flask	Best result after 5 hours of induction with IPTG (figure 8)
30º C	16 h	200 mL in 500 mL flask	Smaller overexpression with the increase in time
16ºC	16 h	200 mL in 500 mL flask	Worst expression than 30º
LB with 150 µg/mL of carbenicillin			
30º C	16 h	200 mL in 500 mL flask	No improvement when compared to growth on ampicillin
SB with 150 µg/mL of ampicillin			
37ºC	2 h	100 mL in 250 mL flask	No improvement when compared to LB

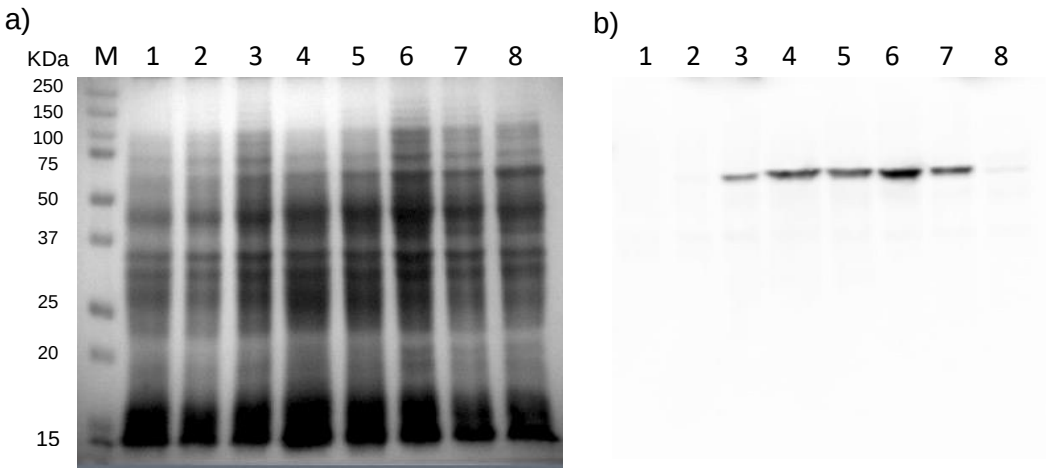

Figure 8. SDS-PAGE (a) and Immunoblot Analysis (b) of the 6× His-tagged BCAS0764 protein after growth in LB at 30º C and induction with IPTG for 16 hours. As an overexpression of the protein is not visible, immunoblot experiment using a monoclonal anti-polyhistidine peroxidase conjugate clone HIS-1 antibody on the raw extract of the overexpression were performed to verify the presence of the 6× His-tagged BCAS0764 protein. Lane M-Precision Plus Protein™ Dual Xtra Standard (BIO-RAD); Lane 1- at the time of induction; Lane 2- One hour after induction; Lane 3 - Two hours after induction; Lane 4 – three hours after induction; Lane 5 – four hours after induction; Lane 6 – five hours after induction; Lane 7 – six hours after induction; Lane 8 – 16 hours after induction

Despite the small amount of BCAS0764 protein expressed, the purification of the recombinant protein was performed using nickel affinity chromatography. A great variety of difficulties were encountered, ranging from the small amounts of protein expressed to the low binding ability of the BCAS0764 protein to the Ni-NtA column. No fraction containing the protein purified was obtained, and from the purification attempt with the best results (figure 9a), the fraction with fewer contaminants was collected for immunoreactive studies and to understand if the purification optimization will be required. Immunoblot experiments using the commercial monoclonal anti-polyhistidine peroxidase conjugate clone HIS-1 antibody were also performed to confirm that the protein purified was the BCAS0764 protein (figure 9b).

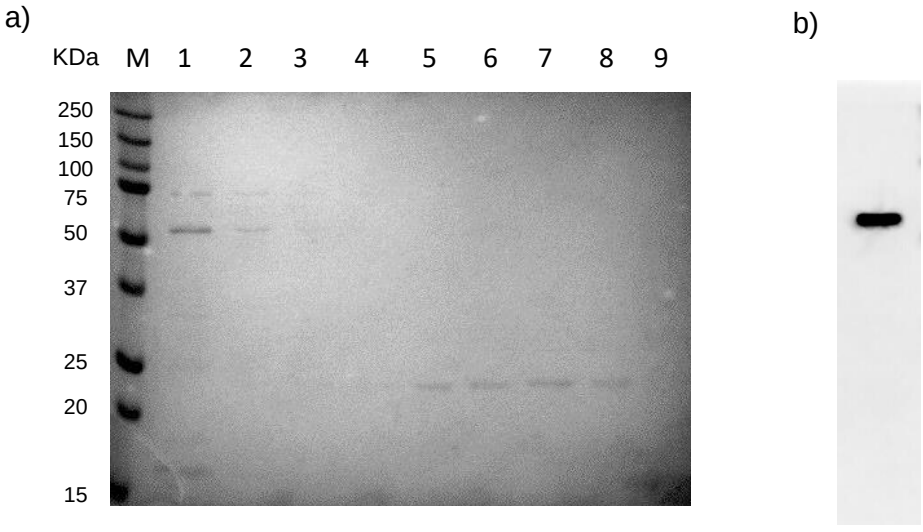

Figure 9. Purification analysis by SDS-PAGE and Western Blot. a) SDS-PAGE analysis of 6× His-tagged BCAS0764 protein purification on nickel affinity chromatography. The protein is yet to be purified without any contaminants. b) Immunoblot Analysis using a monoclonal anti-polyhistidine peroxidase conjugate clone HIS-1 antibody on the 50(3) mM imidazole fraction. Lane M-Precision Plus Protein™ Dual Xtra Standard (BIO-RAD); Lane 1- 50(3) mM imidazole; Lane 2- 70(3) mM imidazole; Lane 3- 90(2) mM imidazole; Lane 4- 90(3) mM imidazole; Lane 5- 150(2) mM imidazole; Lane 6- 150(3) mM imidazole; Lane 7- 200(2) mM imidazole; Lane 8- 200(3) mM imidazole; Lane 9- 300(3) mM imidazole

3.1.3. **The BCAS0764 protein has a low immunoreactivity against the sera from CF patients infected with Bcc**

To examine if the BCAS0764 protein has the potential to induce an immune response in CF patients during infection with Bcc, a western blot assay was performed against 3 serum samples collected from CF patients with culture-confirmed Bcc infections. The 6× His-tagged BCAS0764 protein presented a low reaction with all the serum samples (Figure 10), suggesting that the protein elicits a low IgG titer in CF patients. Bovine serum albumin fraction V was used as negative control. No reactivity of the 6× His-tagged BCAL0764 protein was observed when using a sample of a pool of serum from healthy individuals (Figure 10).

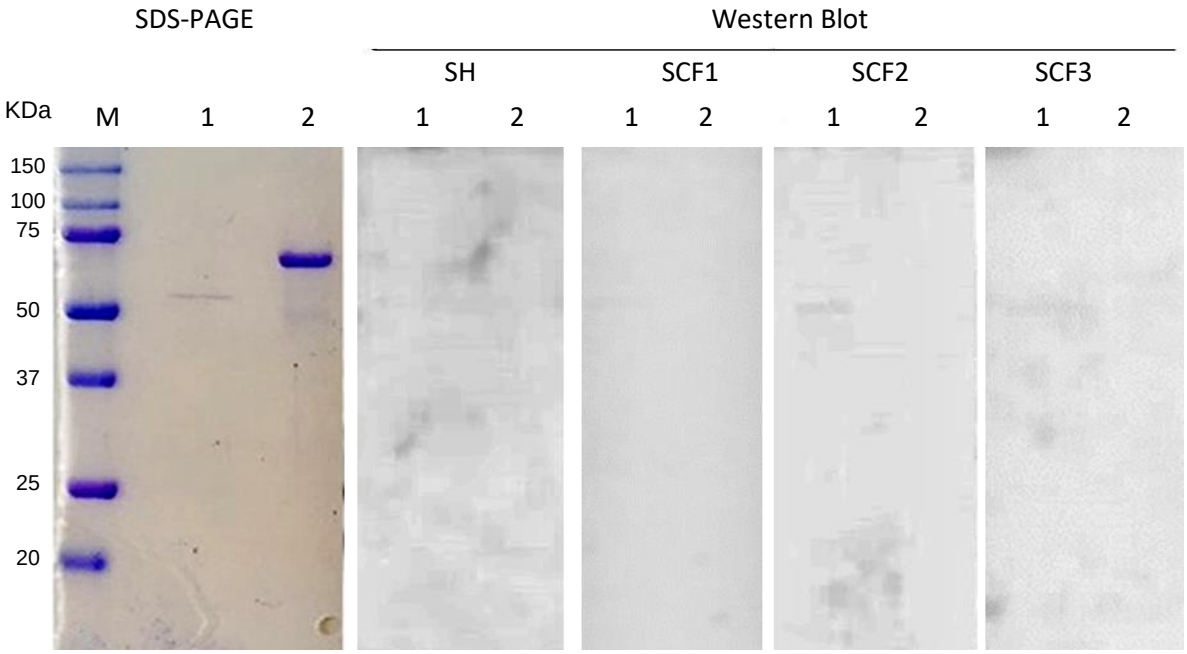

Figure 10. Western blotting of the recombinant protein BCAS0764 from *B. cenocepacia* J2315 probed with the Human serum samples SCF1, SCF2, and SCF3 from CF patients infected with Bcc bacteria, or a pool of human serum sample SH from healthy donors. The BSA protein was used in the assay as a negative control. *Lanes M*—Precision Plus Protein™ Dual Xtra Standard (BIO-RAD); *1*—recombinant BCAS2958 protein; *2*—albumin bovine fraction V (BSA, Nzytech).

3.2. Study of *B. cenocepacia* J2315 BCAL2645 protein immunoreactive

3.2.1. Bioinformatic analysis of the BCAL2645 protein

The BCAL2645 protein was identified in a previous work by our group, in an effort to characterize OmpA-like proteins in the genome of *B. cenocepacia* J2315. This study consisted of a *in silico* approach to find immunoreactive outer membrane protein A (OmpA)-like proteins in the *B. cenocepacia* J2315 genome. The study revealed 10 ORF's encoding putative OmpA-like proteins. The *in silico* analysis of the conservation and immunogenicity of the 10 proteins revealed that 3 of them were conserved in the Bcc and potentially immunogenic, with the BCAL2645 protein being one of them [141].

```
BCAL2645      ------------------------------------------------------------  0
BCAL2958      ------------------------------------------------------------  0
OmpA_E.coli   MKKTAIAIAVALAGFATVAQAAPKDNTWYTGAK-LGWSQYHDTGFINNNGPTHENQLGAG  59
OprF_P.aeru   MKLK-NTLGVVIGSLV----AASAMNAFAQGQNSVEIEAFGKRYFTDSVRNMKNADLYGG  55

BCAL2645      ------------------------------------MNM-------KIATRLSVFALAG  16
BCAL2958      ----------------------------------------------------MNK--   3
OmpA_E.coli   AFGGYQVNPYVGFEMGYDWLGRMPYKGSVENGAYKAQGVQLTAKLGYPITDDLDIYTR--  117
OprF_P.aeru   SI-GYFLTDDVELALSYGE--YHDVRGTYETGNKKVHGNL-------TSLDAIYHFGTPG  105

BCAL2645      A-----LLAGCATQ---------QGNN----TAVGTGTGA----------ALG--AGIGA  46
BCAL2958      -------LSKLAFIAA----------------------TAVMAASA----SA---QSVPA  27
OmpA_E.coli   -------LGGMVWRAD--TKSNVYGKN------HDTGVSPVFAGGV----EY---AITPE  155
OprF_P.aeru   VGLRPYVSAGLAHQNITNINSDSQGRQQMTMANIGAGLKYYFTENFFAKASLDGQYGLEK  165
                     .  .

BCAL2645      LAGGGKG-AAIGAGVGALVGGVTGYNWQAIKNKLAPSAAQ--------T--GTQVTEQPD  95
BCAL2958      SRQAVNDNWVNGTGEWVWMNGTNELCWRDAFWTPATA--NAKC-----D--GALVAQAPQ  78
OmpA_E.coli   IATRLEYQWTNNIGDAHTIGTRPDN----GMLSLGVS--YRFG-----Q--G--EAAPVV  200
OprF_P.aeru   RDNGHQGEWMAGLGV--------GFNFGGSKAAPAPEPVADVCSDSDNDGVCDNVDKCPD  217
                   :     . *                    .

BCAL2645      GSL--------------KLNVPSSVTFATNQYAITPAFTPLLNDLATTLNQNPQ--VTAS  139
BCAL2958      PPV----APVAPAITSQKITYQADALFDFDKATLKPLGKQKLDELASKIEGMNT--EVVV  132
OmpA_E.coli   APA----PAPAPEVQTKHFTLKSDVLFNFNKATLKPEGQAALDQLYSQLSNLDPKDGSVV  256
OprF_P.aeru   TPANVTVDANGCPAVAEVVRVQLDVKFDFDKSKVKENSYADIKNLADFMKQYPS--TSTT  275
                          .    .. *  ::  :.      :.:*   :.        .
                              Peptidoglycan
                              binding motif
BCAL2645      IVGYTDSTGSAQLNQTLSQNRAQSVVNALV-QRGVNGGRLSAQGMGASNPIADNATE---  195
BCAL2958      ATGYTDRIGSDKYNDRLSLRRAQAVKSYLV-SKGVPANKIYTEGKGKRNPVTTGC--NQK  189
OmpA_E.coli   VLGYTDRIGSDAYNQGLSERRAQSVVDYLI-SKGIPADKISARGMGESNPVTGNTCDNVK  315
OprF_P.aeru   VEGHTDSVGTDAYNQKLSERRANAVRDVLVNEYGVEGGRVNAVGYGESRPVADNATA---  332
                *:**  *:   *: ** .**::* . *: . *: ..:: : * *  .*:: .

BCAL2645      ------AGRAQNRRVEIYLRAPQQHQ-------    215
BCAL2958      NRKQLIACLAPDRRVEVEVVGTQQVQKTTVPAN    222
OmpA_E.coli   QRAALIDCLAPDRRVEIEVKGIKDVVTQPQA--    346
OprF_P.aeru   ------EGRAINRRVEAEVEAEAK---------    350
                     * :****  : .  .
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Figure 11. Alignment of the amino acid sequence of the protein BCAL2645 from *B. cenocepacia* J2315, and the BCAL2958 protein from *B. cenocepacia* J2315 (CAR53262), the OmpA from *E. coli* (P0A910) and the OprF from *P. aeruginosa* (AAD11568.1). The corresponding sequence of the peptidoglycan binding motif is boxed in grey. Asterisks indicate the amino acid residues that are identical in all the proteins; one or two dots indicate semi-conserved or conserved substitutions, respectively.

To continue the work on the protein, an alignment of the amino acid sequence was performed against the OmpA protein from *Escherichia coli,* the OprF from *P. aeruginosa,* and the BCAL2958 OmpA-like protein from *B. cenocepacia* J2315 (figure 11). The alignment allowed the identification of the peptidoglycan (PG) biding motif at the C-terminal region on BCAL2645. This motif consists on the following sequence, $NX_2LSX_2RAX_2VX_3L$ [169, 170] and is typical of OmpA-like proteins, being located in the periplasm to interact non-covalently with the PG layer [171]. The N-terminal region of BCAL2645, has a lower identity when compared to the other proteins (figure11). The N-terminal domain of OmpA-like proteins normally, is highly variable and is where is usually located the transmembrane domain [156, 172, 173].

3.2.2. Cloning, expression and purification of *B. cenocepacia* J2315 BCAL2645 protein

To study the potential of this protein for immunotherapies, the 659 bp PCR fragment corresponding to the *BCAL2645* gene of *B. cenocepacia* J2315 was amplified using the primers UP-BCAL2645 and LW-BCAL2645 and cloned into the expression vector pET23a+ under the control of the T7 promoter, creating pSAS36 (Table 2). The overexpression of the protein as a 6× His-tagged derivative, was accomplished by transforming plasmid pSAS36 into *E. coli* BL21 (DE3) and inducing expression by addition of 0.4 mM IPTG. The overproduced 6× His-tagged BCAL2645 protein was analyzed by SDS-PAGE. Two forms of the protein, with different molecular weights were detected (figure 12a), which is in agreement with previously reported observations for other OmpA-like proteins [174]. The sizes of these two bands correspond to the predicted molecular weight of 21.6 and 18.9 kDa for the pre-protein with the signal peptide and the mature forms of the native BCAL2645 with the addition of the 6x His-tag protein, respectively. The predicted molecular weights were obtained with the ExPASy ProtParam tool [175]. The recombinant protein was purified by nickel affinity chromatography and the fractions containing the purified his-tagged protein were dialyzed overnight against appropriate storage buffers and further studied (figure 12b).

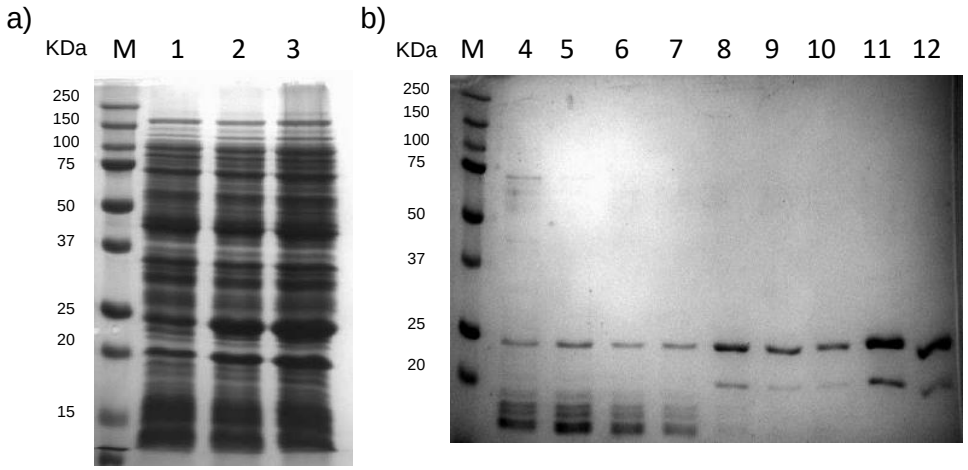


Figure 12. Analysis of 6× His-tagged BCAL2958 protein overexpression (a) and purification (b) on nickel affinity chromatography analysis by SDS-PAGE. Lane M-Precision Plus Protein™ Dual Xtra Standard (BIO-RAD); Lane 1- At the time of induction; Lane 2- 1 hour after induction with IPTG; Lane 3- 2 hours after induction with IPTG; Lane 4- 60(2) mM imidazole Lane 5- 100(3) mM imidazole Lane 6- 150(2) mM imidazole Lane 7- 150(3) mM imidazole Lane 8- 200(2) mM imidazole; Lane 9- 200(5) mM imidazole; Lane 10- 200(8) mM imidazole; Lane 11- 250(2) mM imidazole; Lane 12- 300(2) mM imidazole

After the purification process was optimized, the protein was also purified with the objective of producing a goat polyclonal antibody anti-BCAL2645. For this purpose, endotoxin contaminations were removed from the protein purified samples using the Detoxi-Gel ™ endotoxin removing gel. Two batches of the protein were produced one for the injection on the goat and the other for the purification of the antibody.

3.2.3. **BCAL2645 protein is immunoreactive against sera of CF patients infected with Bcc**

To access the potential of the BCAL2645 protein to induce an immune response in CF patients infected with Bcc bacteria, immunoblot assays, consisting of western blot and ELISA, were performed using 3 serum samples collected from CF patients with culture-confirmed Bcc infections. The purified 6× His-tagged BCAL2645 protein reacted with all the serum samples tested, contrasting with bovine serum albumin fraction V that was used as negative control (Figure 13). This suggests that during infection the protein is exposed to the immunological system of the CF patients and is immunogenic. No reactivity of this protein was observed when using a sample of a pool of sera from healthy individuals (Figure 13).

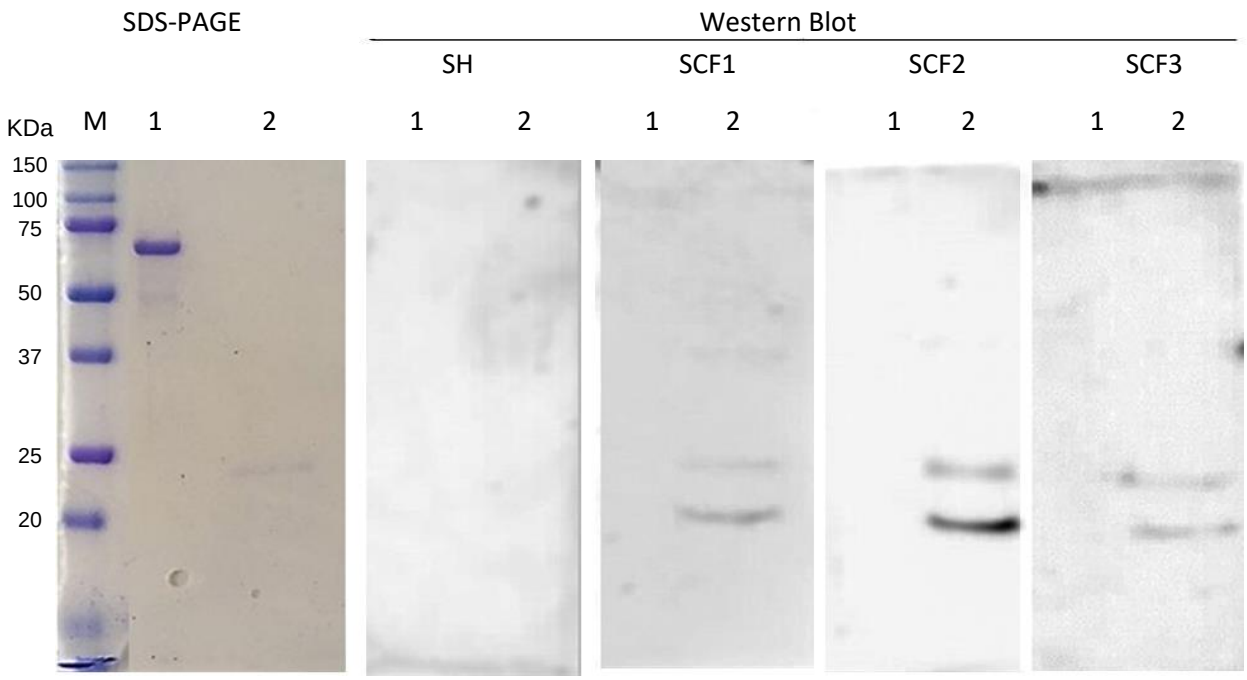


Figure 13. Western blotting of the purified recombinant protein BCAL2645 from *B. cenocepacia* J2315 probed with the Human serum samples SCF1, SCF2 and SCF3 from CF patients infected with Bcc bacteria, or a pool of human serum sample SH from healthy donors. The BSA protein was used as a negative control. Lanes M—Precision Plus Protein™ Dual Xtra Standard (BIO-RAD); 1— albumin bovine fraction V (BSA, Nzytech); 2— purified recombinant BCAL2645 protein

The IgG antibody titers of each serum sample was determined by ELISA and revealed that the samples from CF patients infected with Bcc had IgG titers higher than 5500, while the sample of a pool of serum from healthy individuals presented null IgG titers (figure 14).

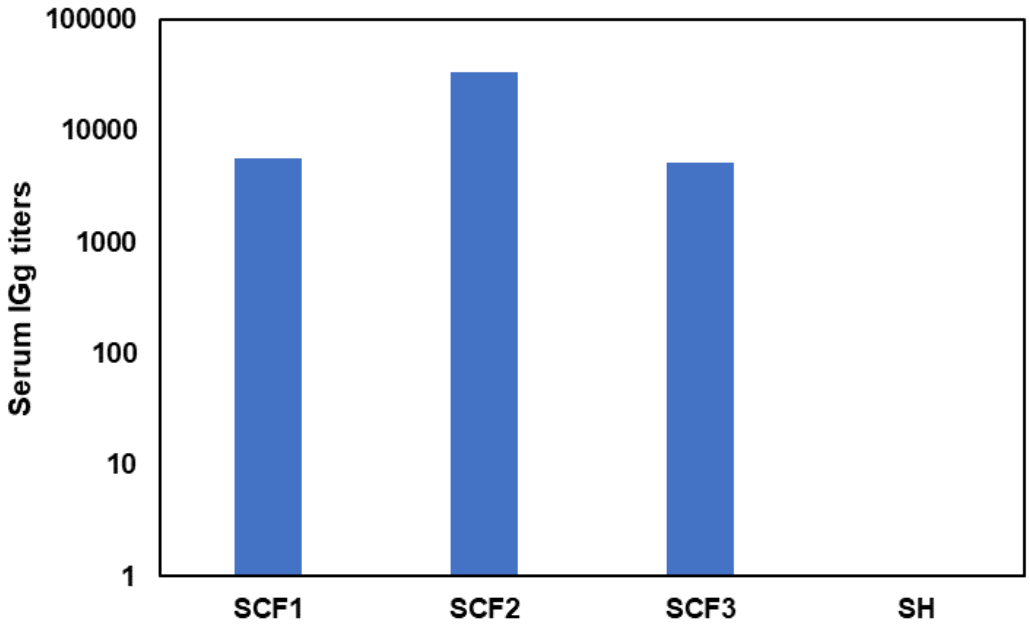


Figure 14. IgG antibody levels against BCAL2645 protein present in sera from CF patients infected with Bcc (SCF1, 2, and 3) and in healthy individuals (SH). Serum antibody concentrations were defined as endpoint titers and were calculated as the reciprocal of the highest serum dilution producing an OD_{450} nm above cutoff value. The cutoff value was determined as the mean OD_{450} nm of the blank control plus 3 standard deviations.

3.3. Study of the role of BCAL2958 protein on the virulence of *B. cenocepacia* J2315

3.3.1. *BCAL2958* gene mutant construction

The BCAL2958 protein from *B. cenocepacia* J2315 was already studied by our research group and found to elicit IgG antibodies against the sera of CF patients infected with Bcc and also the increase of TNFα, elastase, NO, and MPO levels in neutrophils [141]. To further these studies, the construction of a mutant for the gene encoding for this protein was attempted. This had the intent of discovering the role played by this protein on virulence and adhesion to host cells, to understand the potential of strategies focusing on the inhibition of this protein, like passive immunization.

For this purpose, plasmid pAMS5 was constructed, and the first step of the mutant creation consisted on the electrotransformation of this plasmid into *B. cenocepacia* J2315 cells. As this plasmid does not have a replication origin recognized by *Burkholderia*, the cells are obliged to integrate the plasmid in the

genome, so they can survive (figure 16). The chloramphenicol resistant colonies obtained where then screened for fluorescence, and the one that had the fluorescence was the one with the plasmid inserted in the genome and that was confirmed by PCR (figure 15). Two bands resulting from the PCR can be observed in a positive colony, one with 1350 nucleotides and the other with 2100 nucleotides, while in the negative control, only the band with 1350 nucleotides, consisting of the gene plus the upstream and downstream regions is obtained.

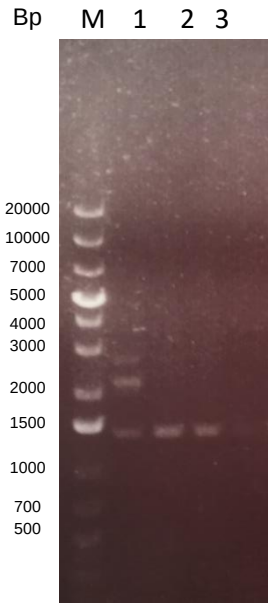


Figure 15. Confirmation by PCR of the colony screened for fluorescence
Lanes M - 1 Kb + Dna ladder; 1 - Colony with fluorescence; 2 - Colony without fluorescence; 3 – *B. cenocepacia* J2315 wildtype

The second step of this procedure consists on the occurrence of a second recombination, in a way that the wildtype gene is removed and the one present in the plasmid containing the chloramphenicol cassette interrupting it, stays in the genome (figure 16). This was attempted by performing a new electrotransformation using the pDAI-SceI-SacB. This plasmid encodes the SceI restriction enzyme, that has a recognition site on the first plasmid. The plasmid enters in the cells and encodes the enzyme that cuts the genome, forcing the bacteria to recombine and remove the plasmid from its genome. Bacteria with the selection marker, tetracycline resistance, where tested for fluorescence, and those that didn't had any, where tested for the selection marker in the gene, chloramphenicol resistance, to confirm that the gene was interrupted.

Various colonies were obtained that were tetracycline resistant, and that lacked fluorescence, however when these colonies were tested for chloramphenicol resistance none was found as resistance. This indicates that all the tested cells recombined back to wildtype genotype.

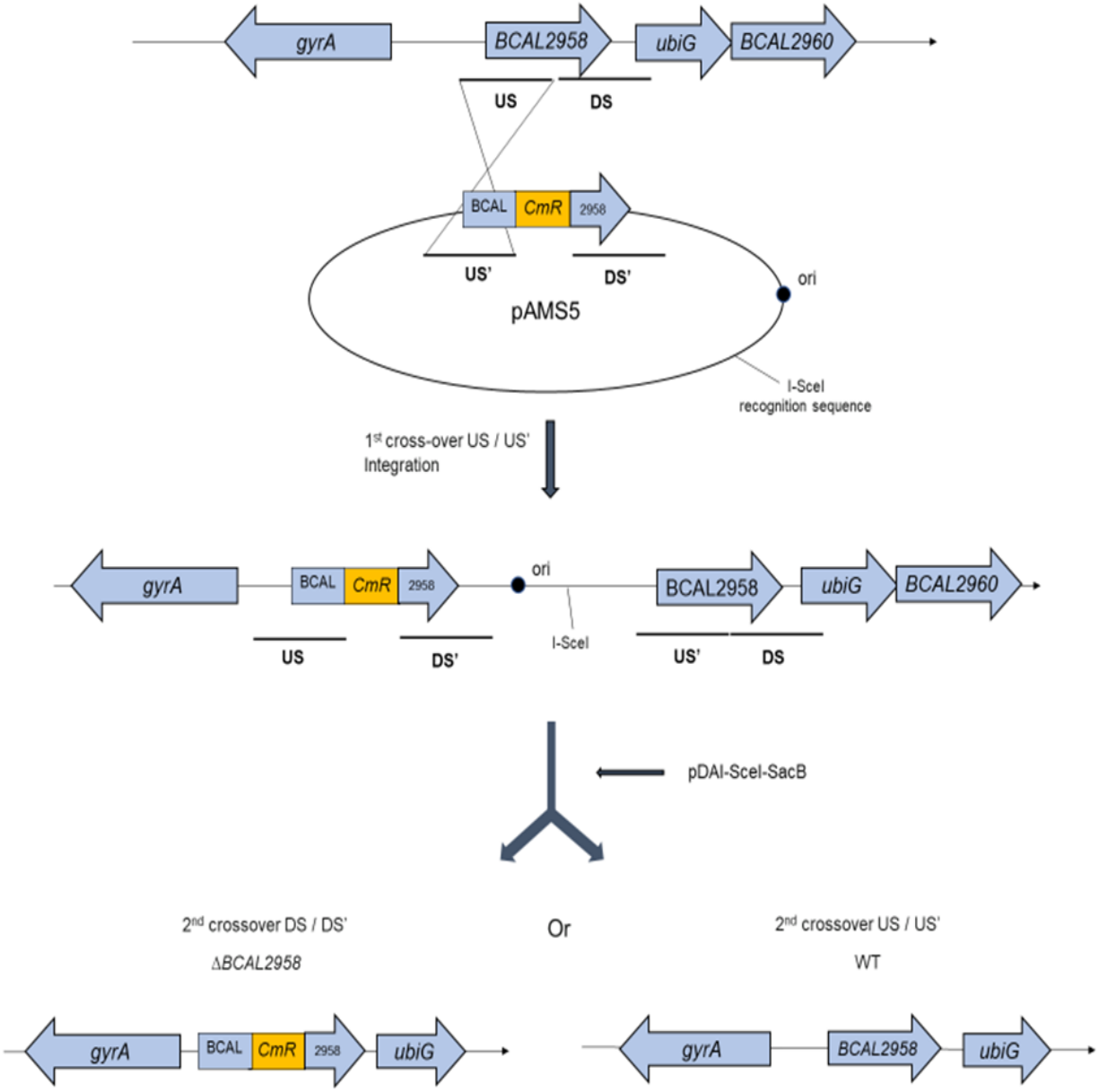
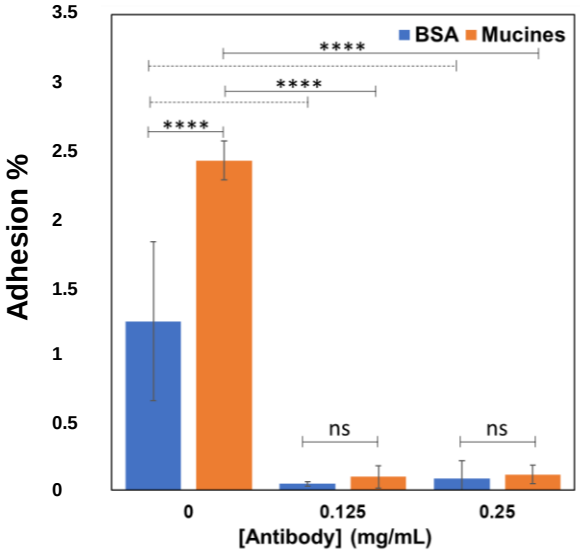


Figure 16. Strategy for the creation of the insertion mutant on the gene *BCAL2958* using the system described by Flannagan et al (2008). Genes are indicated with an arrow, the upstream (US) and downstream (DS) homology regions are shown. The suicide plasmid pAMS5 is introduced by electrotransformation into *B. cenocepacia* J2315 and integrates into the genome by homologous recombination (1st crossover) using either the US' or DS' fragments. Plasmid pDAI-SceI- SacB, which expresses the I-SceI endonuclease, is then introduced. I-SceI expression results in a double strand cut in the chromosomal DNA at the I-SceI site, which favors the 2nd crossover event by stimulating the host DNA repair machinery. A 2nd crossover occurring between DS and DS' will insert the gene with the chloramphenicol cassette and remove the one without. Reversion to wildtype occurs if recombination involves the same regions that have been involved in the 1st crossover step.

3.3.2. Anti-BCAL2958 antibody affects *Burkholderia cenocepacia* J2315 adhesion to mucins

The capacity of the anti-BCAL2958 antibody to affect the adhesion of *B. cenocepacia* J2315 to mucins was analyzed by performing adhesion assays. The preincubation of *B. cenocepacia* J2315 with anti-BCAL2958 antibody caused a significant decrease in the adherence (figure 17), with decreases in adhesion of 95% and 96% for the concentrations of 0.125 and 0.250 mg/mL of antibody, respectively. The different concentrations of the antibody tested showed no significant decrease in the relative adhesion observed when the antibody concentration is increased. Nonetheless, the incubation with the antibody showed statistically significant decreases in the adherence either to mucins or to BSA (P<0.0001), which suggests that the antibody does not affect specifically the adherence to mucins but the adherence to proteins in general.

a)



b)

[Antibody] (mg/mL)	Adherent bacteria (CFU/mL) BSA	Adherent bacteria (CFU/mL) Mucin
0	2.14E+05	3.76E+05
0.125	7.60E+03	1.78E+04
0.250	2.00E+04	1.65E+04

Figure 17. Effect of pre-incubation of *B. cenocepacia* J2315 with the anti-BCAL2958 antibody on the adhesion to mucins. a) The adherence to mucins by the *B. cenocepacia* J2315, was expressed as a ratio of *B. cenocepacia* J2315 total number of cells. BSA was used as the negative control. The error bars represent the standard deviation observed. The P-value was determined with the 2way ANOVA and are represented by **** when P<0.0001 and ns when there's no significance. b) Mean value of CFU/mL obtained for BSA and mucin after incubation with different concentration of the anti-BCAL2958 antibody

Discussion and Perspectives

4. Discussion

Life expectancy, as well as life quality of CF patients, has improved in recent years [176, 177], and many factors can be pointed out. One of them is the appearance of novel therapies for the eradication of bacterial infections from the lungs of CF patients [178]. However, these new eradication strategies are mainly directed to the leading pathogen, *Pseudomonas aeruginosa,* with no strategies for the eradication of *Burkholderia* being available [70]. Patients chronically infected with *P. aeruginosa,* are still very hard to treat, with the tackle of pathogens at the early stage of infection being the preferred course of action [70, 178, 179]. This is one of the reasons why the development of strategies for an efficient immune protection and eradication is so important, being vaccines one of the most efficient, since they have an astounding impact on public health, with the reduction of mortality and morbidity of infectious diseases [125, 126]. Vaccines also have the benefit of not inducing selection pressures on the environment, and therefore do not contribute to antimicrobial resistance [180]. Main immunogenic components of pathogenic bacteria can be studied as potential candidates for the development of strategies for efficient immune protection and eradication, like the development of subunit vaccines. A great number of strategies to identify new vaccine antigens are now available, with reverse vaccinology being one of the most used and many actual vaccines are developed due to this approach [181]. A great variety of antigens can be potential vaccine candidates however, the majority of the efficient antigens used in vaccines are involved in host cell attachment. This is the case of *Bordetella pertussis* vaccine that is composed of 5 antigens, all involved in bacterial attachment [182]. This highlights the potential that adhesins and bacterial proteins involved in interactions with host cells have as vaccine components [183]. The necessity to uncover new possible antigens capable of causing an immune response, and therefore possible candidates for vaccines against Bcc, prompt this work.

After the identification of the proteins to study, the conservation of these proteins among Bcc strains is an imperative step to access their potential as antigens for vaccine development. Since antibodies against antigens missing in some strains are incapable of providing protection against these same strains, a bioinformatics analysis of the conservation of the proteins in study was performed. Since the conservation of the two OmpA-like proteins was already accessed in the study performed by Sousa *et al* [141], only the conservation analysis of the proteins identified in the immunoproteomic assay was now performed. All the proteins, with the exception of BCAL1098, were present in the completed and publicly available genomes of the Bcc members. As not all proteins identified could be used, to restrict the number of proteins to be used for further studies, the BCAS0764 was chosen since it is one of the few that is putatively located on the outer membrane. Since surface and extracytoplasmatic proteins of pathogens are usually the first exposed to the host immune system, it is expected that they can trigger the host immune response [122]. This protein BCAS0764 belongs to the *OprB* family that includes the carbohydrate selective porins, described as playing a central role in carbohydrate uptake in *Pseudomonas aeruginosa* [155]. No studies are available for this protein in *B. cenocepacia*. However, the bioinformatics study identified two additional very similar proteins, both with a role in antimicrobial efflux, the OprB from *Burkholderia pseudomallei* and the OprM from *B. cenocepacia*. The three proteins containing a motif belonging to the OEP family of outer membrane proteins and, as such, probably

playing a role in the extrusion of a variety of substrates [166, 184]. The OprB from *B. pseudomallei* was shown to play a role as a multidrug efflux system which extrudes macrolides, fluoroquinolones, tetracyclines, acriflavine, and, to a lesser extent, chloramphenicol [158]. In *B. cenocepacia*, the OprM is present in an RND complex. RND are drug antiporters located in the inner membrane, playing several roles including bacterial virulence, quorum sensing, plant–bacteria interactions, and detoxification of metabolic intermediates and toxic compounds such as heavy metals, solvents, or antimicrobials [167]. Although this protein seems not to play a role in the interaction with the host cell, it is a porin present in the bacterial membrane and with a role in virulence in other species. The immunoreactivity found for this family of proteins in other species [185], together with the immunoproteomic assay that led to its identification, prompt the choice of this protein for further studies.

To clone the gene encoding the BCAS0764, a plasmid containing a thrombin recognition site downstream of the gene and upstream of the C-terminal 6× His-Tag, was constructed. This construction was done in order that after protein purification, the tag could be removed to produce the protein without structural changes. The tag had to be inserted in the C-terminal because the protein contains a signal peptide at the N-terminal. A variety of conditions were tested to try to produce and improve the amount of protein obtained by overexpression. However, all these attempts showed very few improvements. In addition to the minimal amounts of protein obtained, the purification process was an even bigger obstacle. The protein exit the affinity chromatography column at low concentrations of imidazole, indicating a low affinity to the column, maybe this could be addressed with a longer affinity tag, that sometimes results in an increased purity, due to the ability to use more stringent washing steps [186]. However bigger tags may cause perturbation of protein function and structure [186], which in its turn maybe could have been addressed with the removal of such tag after the purification. Various conditions of buffer composition were utilized like different concentrations of imidazole, glycerol 10%, NaCl 0.25 M and Triton-X 100 1%, all leading to few to none improvement. Purification of the inclusion bodies in denaturing conditions using urea, was also attempted with unsuccessful results. Since the contaminants exhibit a high affinity to the column, we have tested if are the result of protein degradation. With this intent, the empty plasmid, not containing the gene, was introduced in *E. coli*, and the production and purification procedures were repeated in the same conditions. Despite these efforts, the contaminants were still present.

To understand if the efforts of the purification were worth it, immunoblot assays using the not yet fully purified protein were performed. These assays showed a very low immunoreactivity of this protein BCAS0764 against sera of CF patients infected with Bcc. This new information prompted us to cease all the efforts to purify the protein BCAS0764, based on the protein's low reactivity against these sera, as it indicates that the protein cannot elicit a humoral immune response in CF patients.

The remaining two proteins are both OmpA-like proteins, that generally present significant immunogenicity and are present on the outer membrane of gram-negative bacteria. These OmpA-like proteins are frequently promising candidates for vaccine development. Examples are the OmpA from *Shigella flexneri 2a,* OmpA from *E. coli* and the OmpF from *P. aeruginosa* [152, 187, 188]. The immunogenic potential of OmpA-like proteins from other organisms prompted us to further our studies

on the two proteins BCAL2958 and BCAL2645. A bioinformatics analysis of the protein BCAL2645 of *B. cenocepacia* J2315 confirmed the protein as an OmpA-like protein. The encoding gene was cloned, overexpressed, purified, and confirmed to be immunoreactive against sera of CF patients infected with Bcc by Western Blot and the quantification of IgG titers in CF patients infected with Bcc sera. With the results obtained, we determined that the protein is highly immunogenic and the production of antibodies by a commercial company is in process. Nonetheless, the potential demonstrated by this protein as a vaccine component still requires further studies, like the assessment of its interference with neutrophil activity, since different purified forms of OmpA have been shown to activate macrophages [189], dendritic cells [190] and neutrophils [141, 191] . The importance of these assays resides on the fact that neutrophils are the first line of the innate immune defense against diseases of infectious origins [192], and activated neutrophils regulate the immune response assisting in the activation and maturation of dendritic cells and macrophages [141, 193, 194]. Experiments involving the immunization of mice with the purified recombinant protein BCAL2645, can also give important information of this protein as a potential candidate for the development of novel strategies for immunoprotection against Bcc infections. An example of this kind of study was performed by Makidon *et al* [143], who used a OMP from *B. cenocepacia* together with an adjuvant to immunize mice, showing both an elicited robust Bcc-specific serum and mucosal immune response, as well as a balanced Th1/Th2-type cellular immunity [143]. This vaccination produced antibodies capable of neutralizing *B. cenocepacia* and cross-neutralizing *B. multivorans* species. An efficient clearance of *B. cenocepacia* after experimental pulmonary infection in immunized animals was also reported [143]. Another important study necessary to demonstrate the role played by the protein is the construction of a mutant in this gene. This will allow to infer the importance of the gene for the bacterial virulence, antimicrobial resistance, biofilm formation, and other traits of *B. cenocepacia* biology [195].

The BCAL2958 OmpA-like protein was previously shown by our research group as highly immunoreactive. This work was continued with the construction of an insertion mutant in the gene encoding for the protein, to perform its functional characterization. However, this objective was not achieved despite of the two strategies used. The first consisted on the creation of a plasmid containing a 1349 bp fragment including the upstream and downstream regions of the gene, with a chloramphenicol cassette interrupting the gene. The plasmid does not contain an origin of replication recognized by *Burkholderia* and as so, when the plasmid was inserted in the cell and put in a medium containing chloramphenicol, the bacteria was forced to integrate the plasmid in the genome, using the regions of homology cloned in the plasmid, to survive. This phenomenon is known as homologous recombination [196]. A great number of colonies containing the plasmid integrated in the genome were obtained, however in all this colonies the second recombination never occurred, meaning the plasmid was integrated in the genome as well as the copy of the gene uninterrupted by the cassette. Since in this strategy there was no way of forcing the second recombination to occur and no bacteria containing only the interrupted gene were obtained, a different strategy was attempted. This new strategy is based on the work of Flannagan *et al* [163] and follows the same steps as the first, using instead, a different plasmid that contains the SceI recognition sequence. This plasmid after its integration, allows a second plasmid containing a origin of replication recognized by *Burkholderia,* a selective marker and encoding

the restriction enzyme SceI, to be inserted forcing the occurrence of the second recombination. This forcing is due to the fact that no bacteria is able to survive with a cut in its genome, and to survive the removal of the plasmid from the genome must occur. This occurrence is supposed to have a 50/50 chance of returning to the wildtype or to have the gene interrupted. Nonetheless, this gene interruption was not observed, with numerous colonies that always had return to the wildtype phenotype obtained, and none with the gene interrupted. This led to the hypothesis that the gene might be essential for the bacteria survival, with a bibliography search founding two vital genes right upstream of this gene [197]. Another possibility for the absence of the mutant bacteria might be that, although the homologous regions coincide with these essential genes and their sequence were not altered, the insertion of the cassette might cause structural alterations that affect the expression of those vital genes. Further work should focus on the creation of a conditional knockout mutant for this gene, that would allow to confirm if the gene *BCAL2958* is in fact essential to the bacteria or if there is another factor affecting the mutant creation.

BCAL2958 is an OmpA-like protein thought to be located in the outer membrane of bacteria, and possibly playing a role on the adhesion of bacteria [153]. As such, to test if the antibody against this protein had any effect on the adherence of the bacteria to host cells, adherence experiments were performed, with bacteria incubated with the anti-BCAL2958 antibody prior to their contact with mucins. Mucins comprise the major protein component of airway mucus, that covers the luminal surface of the respiratory tract, existing as secreted and cell-associated glycoproteins [198, 199]. Secreted mucins are largely responsible for the viscoelastic property of mucus, a crucial factor for an effective mucociliary clearance. Cell-associated mucins shield the epithelial surface from pathogens [198, 199]. This makes mucins one of the first points of contact with the host cells when bacteria enter the body through the respiratory track. Despite the function in epithelial cells as pathogen removal, *B. cenocepacia* has been shown to adhere to mucins in CF patients [200] and to have a mucin-sulphatase activity that allows the degradation of mucins [201]. The assay performed, allowed to conclude that the use of an anti-BCAL2958 antibody strongly inhibits the ability of the *B. cenocepacia* J2315 to adhere *in vitro* to both mucins and BSA. Unfortunately, since the mutant on this gene was not available, the effect of the antibody on cells without the protein was not possible to ascertain. Nonetheless, these findings suggest that the anti-BCAL2958 antibody has potential as a passive immunization agent. This is particularly important with the emergence of adhesin-based vaccines as attractive approaches to deal with bacterial infection [202]. Regardless of how promising the results might seem, studies on the use of the antibody as a passive immunization agent in animal model of infections are necessary.

5. Concluding Remarks

The development of novel strategies for immunoprotection against Bcc infections or of novel diagnostic methods for the early detection of Bcc infections is a particularly significant topic in the treatment of CF patients. The discovery of proteins or other components of bacteria able of eliciting strong immune responses is the leading research for this development. This work focused on 3 proteins, BCAS0764, BCAL2645 and BCAL2958, previously identified and predicted to be immunoreactive. The BCAS0764, in spite of having some favorable characteristics for this development like its conservation amongst Bcc members and in other species, was shown to lack reactivity against sera from CF patients infected with Bcc, indicating that the protein is not a good candidate for immunotherapies. In contrast, the BCAL2645 protein tested for immunoreactive, exhibited strong immunoreactivity and no reactivity against sera from healthy individuals. These results suggest a high potential of this protein for the development of this kind of therapies, however further studies are required to properly access its potential. For the BCAL2958 protein, unfortunately, a mutant in the encoding gene was not obtained. The difficulty to obtain bacteria with the gene inactivated, led us to hypothesize that the gene might be necessary for bacterial survival. Further studies are required to confirm this hypothesis. However, if the gene is found as vital, its potential as a candidate for immunoprotection or novel diagnostic method for early detection rises, because the bacteria is not able to remove this gene from its genome as a way of evading the immune system. The remaining assays involving the anti-BCAL2958 antibody, showed that this antibody has the ability to drastically reduce the adherence of the bacteria to mucins. This is of outmost importance, as bacterial adhesion to the host is a crucial step in the infection process [203], and if it is avoided, infection cannot proceed. Although promising, more studies are required to access if the antibody is capable of affecting adhesion to other components besides mucins, like bronchial epithelial cells.

Although encouraging results were obtained in this work, the identification of antigens is only the first step in a extensive development process to obtain a vaccine, with the safety, stability, formulation and an adequate adjuvant for its administration to humans, the remaining important stages in this development. The advances in this field of antigen identification have the ability to improve and accelerate the development of vaccines, but at the end, each vaccine, regardless of the potential it shows in preclinical models or other tests, must be evaluated in human trials. This step is the bottleneck in vaccine development, especially due to the high costs involved, not available to the majority of academic researchers involved in the identification of vaccine antigens.

6. References

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