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 were studied, the OprB-like protein BCAS0764 and two OmpA-like proteins BCAL2645 and BCAL2958, previously identified and predicted as immunoreactive. 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, and therefore 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.

Introduction

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 [1–3]. 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 their resistance to most of the clinically relevant antibiotics, make Bcc infections very difficult to eradicate and the most feared by CF patients [4]. Nevertheless, no standard strategy to eradicate Bcc bacteria is currently available [5], 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 into the category of subunit vaccines, with numerous promising antigens being proposed as vaccine candidates [6]. 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 [7]. 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 [8]. 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 [9] . 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 [10]. Antibodies against OmpA and several OmpA family proteins can be bactericidal, opsonic, or protective [11]. *In* silico 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 (BCAL2958, BCAL2645 and BCAL3204) were conserved in the Bcc and potentially immunogenic [7], 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* [12]. 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 [13]. In *Xanthomonas citri*, the OprB was also studied and

found to be required for biofilm production, as well as for virulence [14]. 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 [15]. It was also demonstrated in a different *B. pseudomallei* strain that OprB play an important role in virulence and quorum sensing [16]. 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-GeI 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 located 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 quantification of their IgG titers.

Materials and Methods

Bacterial strains plasmids and growth 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}).

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 [20]. 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.

Table 1. 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 (rK– mK+), λ–	Invitrogen
E. coli BL21 (DE3)	F– *ompT hsd*SB (rB–mB–) dcm gal λ(DE3)	Stratagene
Burkholderia cenocepacia J2315	Cystic fibrosis clinical isolate (Edinburgh, UK); ET12 lineage reference strain; LMG16656	[17]
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	[18]
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	[19]
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

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).

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 [21].

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).

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 comercial 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).

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 Na2PO4, 2.4 mM KH2PO4). 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.

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).

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).

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.

BCAL2958 insertion mutant construction

The mutant construction strategy was based on the strategy of Flanagan et al [22], 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.

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.

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 [23]. The alignment of amino acid sequences was performed using the bioinformatics tool Clustal Omega [24].

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.

Results

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

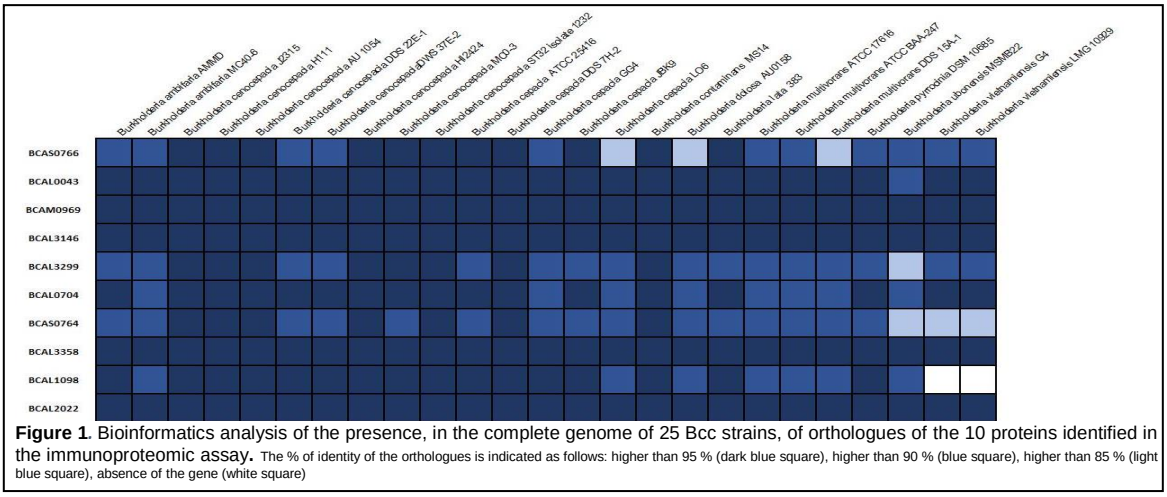

Figure 1. Bioinformatics analysis of the presence, in the complete genome of 25 Bcc strains, of orthologues of the 10 proteins identified in the immunoproteomic assay**.** The % of identity of the orthologues is indicated as follows: higher than 95 % (dark blue square), higher than 90 % (blue square), higher than 85 % (light blue square), absence of the gene (white square)

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 spectometry. These 10 proteins were then bioinformatically analyzed to access their conservation within the Bcc (figure 1).

These analyses showed that 9 of the 10 proteins were conserved in this group of bacteria. 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 [23], 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 an homology of 95% and an OprB from *B. pseudomallei* ATCC 23343 (AAQ94111.1) with an homology of 82%. An alignment of the amino acids 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 [25]. The *BCAS0764* gene is located on the third chromosome of *B. cenocepacia* J2315, more specifically in the RND 2 operon. RND is a family of 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 [26]. The RND operons are composed of three proteins, the outer membrane channel, the periplasmic membrane fusion protein and the RND permease [26]. 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 [26]. The OprM gene from *B. cenocepacia* J2315, used in the alignment in figure 7, is the outer membrane protein 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 [15].

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, including the use of the antibiotic carbenicillin instead of ampicillin due to its higher stability [27]. The overproduced 6× His-tagged protein was analyzed by SDS-PAGE (figure 2a), but no obvious overexpression was visible. Therefore, the presence of the 6× His-tagged protein expression was accessed by immunoblot experiments using the comercial monoclonal anti-polyhistidine peroxidase conjugate clone HIS-1 antibody (figures 2b). The analysis revealed one form of the recombinant

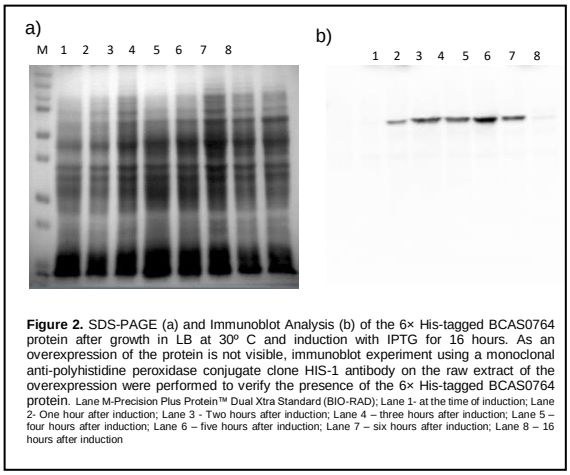

Figure 2. SDS-PAGE (a) and Immunoblot Analysis (b) of the 6× His-tagged BCAS0764 protein after growth in LB at 30º C and induction with IPTG for 16 hours. As an overexpression of the protein is not visible, immunoblot experiment using a monoclonal anti-polyhistidine peroxidase conjugate clone HIS-1 antibody 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

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 (figure 2).

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 3a), the fraction with fewer contaminants was collected for immunoreactive studies and to understand if the purification optimization will be required. Immunoblot experiments using the comercial monoclonal anti-polyhistidine peroxidase conjugate clone HIS-1 antibody were also performed to confirm that the protein purified was the BCAS0764 protein (figure 3b).

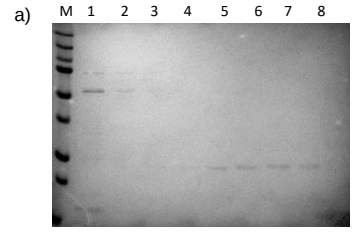

Figure 3. Purification analysis by SDS-PAGE and Western Blot. a) SDS-PAGE analysis of 6× His-tagged BCAS0764 protein purification on nickel affinity chromatography. The protein is yet to be purified without any contaminants b) Immunoblot Analysis using a monoclonal anti-polyhistidine peroxidase conjugate 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

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

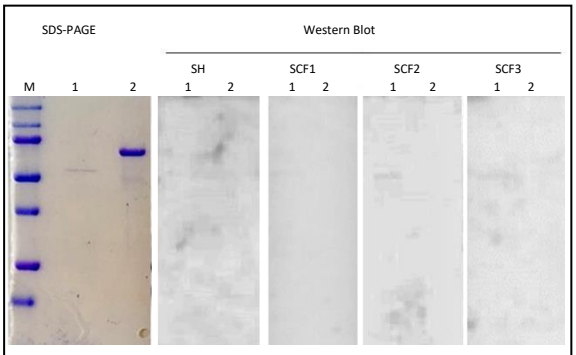

Figure 4. Western blotting of the recombinant protein BCAS0764 from *B. cenocepacia* J2315 probed with the Human serum samples SCF1, SCF2, and SCF3 from CF patients infected with Bcc bacteria, or a pool of human serum sample SH from healthy donors. The BSA protein was used in the assay as a negative control. *Lanes M*—Precision Plus Protein™ Dual Xtra Standard (BIO-RAD); *1*—recombinant BCAS2958 protein; *2*—albumin bovine fraction V (BSA, Nzytech).

(Figure 4), 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 4).

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 5a), which is in agreement with previously reported observations for other OmpA-like proteins. 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 [29]. 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 5b).

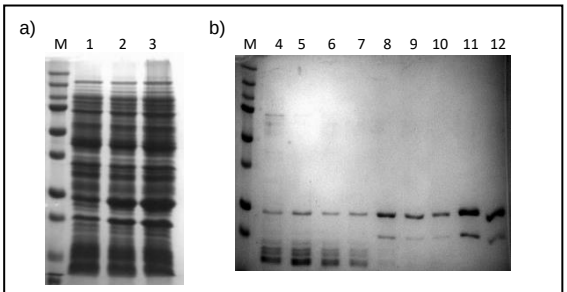

Figure 5. Analysis of 6× His-tagged BCAL2958 protein overexpression (a) and purification (b) on nickel affinity chromatography analysis by SDS-PAGE. Lane M-Precision Plus Protein™ Dual Xtra Standard (BIO-RAD); Lane 1- *At the time of induction*; Lane 2- 1 hour after induction with IPTG; Lane 3- 2 hours 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.

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 6). This suggests that during infection the protein is exposed to the immunological system of the CF patients and is immunogenic. No reactivity of

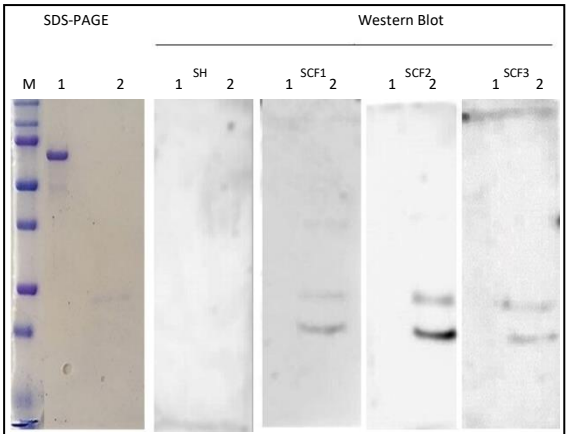

Figure 6. Western blotting of the purified recombinant protein BCAL2645 from *B. cenocepacia* J2315 probed with the Human serum samples SCF1, SCF2 and SCF3 from CF patients infected with Bcc bacteria, or a pool of human serum sample SH from healthy donors. The BSA protein was used as a negative control. Lanes M—Precision Plus Protein™ Dual Xtra Standard (BIO-RAD); 1— albumin bovine fraction V (BSA, Nzytech); 2— purified recombinant BCAL2645 protein

this protein was observed when using a sample of a pool of sera from healthy individuals (Figure 6)

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 7)

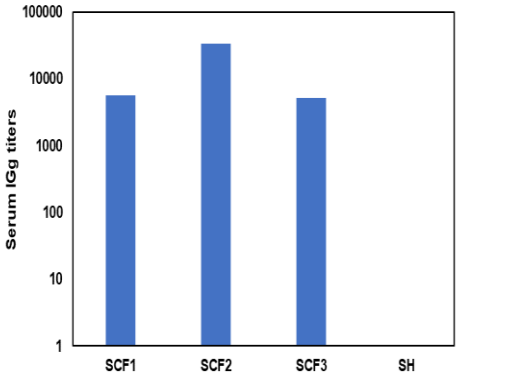

Figure 7. IgG antibody levels against BCAL2645 protein present in sera from CF patients infected with Bcc (SCF1, 2, and 3) and in healthy individuals (SH). Serum antibody concentrations were defined as endpoint titers and were calculated as the reciprocal of the highest serum dilution producing an OD_{450} nm above cutoff value. The cutoff value was determined as the mean OD_{450} nm of the blank control plus 3 standard deviations.

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 [7]. 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. 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. 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 cassete interrupting it, stays in the genome. 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 resistant. This indicates that all the tested cells recombined back to wildtype genotype.

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 8), 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

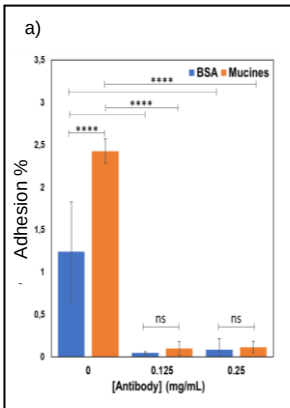

Figure 8. Effect of pre-incubation of *B. cenocepacia* J2315 with the anti-BCAL2958 antibody on the adhesion to mucins. a) The adherence to mucins by the *B. cenocepacia* J2315, was expressed as a ratio of *B. cenocepacia* J2315 total number of cells. BSA was used as the negative control. The error bars 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

(P<0.0001), which suggests that the antibody does not affect specifically the adherence to mucins but the adherence to proteins in general.

Discussion

Life expectancy, as well as life quality of CF patients, has improved in recent years [30, 31], 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 [32]. However, these new eradication strategies are mainly directed to the leading pathogen, *Pseudomonas aeruginosa*, with no strategies for the eradication of *Burkholderia* being available [5]. 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 [5, 32, 33]. 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 [34, 35]. Vaccines also have the benefit of not inducing selection pressures on the environment, and therefore do not contribute to antimicrobial resistance [36]. 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 [37]. 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. 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* [7], 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 [6]. 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 [25, 38]. 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 [15]. 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 [26]. The immunoreactivity found for this family of proteins in other species [39], 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 left 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 [40]. However bigger tags may cause perturbation of protein function and structure [40], 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* [9, 41, 42]. 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 [43], dendritic cells [44] and neutrophils [7, 45] . 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 [46], and activated neutrophils regulate the immune response assisting in the activation and maturation of dendritic cells and macrophages [7, 47, 48]. 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. 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 [49].

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. 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 [50]. 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 these 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 [10]. 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 [51, 52]. 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 [51, 52]. 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 [53] and to have a mucin-sulphatase activity that allows the degradation of mucins [54]. 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 [55]. 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.

Conclusion

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 the 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 [56], 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 an 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.

Acknowledgments

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