Identification of an immunodominant drug efflux pump in
*Burkholderia cepacia*


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*Burkholderia cepacia*, a major pathogen amongst individuals with cystic fibrosis (CF), is intrinsically resistant to most clinically available antibiotics. We report the identification of an immunodominant antigen in CF patients infected with *B. cepacia*, a multidrug-resistance efflux pump called BcrA. The *bcrA* gene encodes a 46 kDa peptide with 14 potential α-helices that belongs to the major facilitator superfamily of drug transporters. A recombinant *Escherichia coli* strain was constructed containing the *bcrA* gene, which resulted in a four-fold increase in resistance to tetracycline and an eight-fold increase in resistance to nalidixic acid. These results demonstrate that the *bcrA* gene is part of a drug efflux system that is potentially a major contributor to the high-level antibiotic resistance observed in *B. cepacia* and thus a potential target for novel therapeutics.

**Introduction**

*Burkholderia cepacia*, originally thought to be only a phytopathogen, is now recognized as a major opportunistic pathogen more commonly associated with pulmonary infections among individuals with cystic fibrosis (CF) and chronic granulomatous disease.1

Clinically, *B. cepacia* colonization of CF patients can be asymptomatic, or it can be associated with a slow decline in lung function. Approximately 20% of colonized individuals suffer from rapid and fatal deterioration in pulmonary function accompanied by pneumonia, fever and, in some cases, bacteraemia.2 The latter is not observed with other CF pathogens, which makes infection with *B. cepacia* more difficult to treat.2,3

Treatment of *B. cepacia* is difficult because of its high resistance to most clinically available antibiotics.4 This problem is complicated by the development of resistance during therapy and the development of cross-resistance to other antimicrobials.5,6 Resistance mechanisms of *B. cepacia* include selective permeability of the cell wall, alteration of the intracellular targets of drugs so they are no longer rendered susceptible to the drug, enzymic degradation or inactivation of drugs, and the active efflux of antibiotics from the cell via drug exclusion pumps.7

In this study, we focused on the immunodominant antigens of *B. cepacia* and found that one of them is part of a multidrug efflux pump that is responsible for resistance to both nalidixic acid and tetracycline.

**Materials and methods**

**Bacterial strain and serum samples**

*B. cepacia* J2315 Edinburgh was obtained from a patient with CF. Serum samples were kindly provided from CF patients infected with *B. cepacia* attending the Bradbury Cystic Fibrosis Unit, after approval by an ethics committee. Microbiological culture and biochemical identification was carried out to confirm positive identification of *B. cepacia* isolates.

**DNA isolation and Lambda ZAP Express library preparation**

DNA was isolated and restricted according to Golbang et al.8 to produce a partial digest with the enzyme Sau3A (Promega, Southampton, UK). A Lambda ZAP Express library was prepared with an insert range of 3–5 kb according to protocols from Clontech Laboratories, Cambridge, UK.

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**Antibody screening**

Sera were taken from a patient with lower respiratory tract infection due to *B. cepacia* and used for antibody screening. *Escherichia coli* XL1–Blue cells were infected with the Lambda ZAP Express phage on L broth agar at c. 3000 pfu per 85 mm plate. This was incubated at 42°C for 3 h. Plaques were then transferred to nitrocellulose filters (0.45 μm pore size; Sartorius AG, Goettingen, Germany), impregnated with 10 mM isopropyl β-D-thio-galactopyranoside, at 37°C for 2 h. These filters were blocked overnight at 4°C with 3% (w/v) bovine serum albumin (BSA; Sigma, Poole, UK) in buffered saline (150 mM NaCl, 10 mM Tris). Serum diluted 100-fold in 3% (w/v) BSA was added to the filters and incubated at room temperature for 2 h; the filters were washed for 30 min in washing solution (150 mM NaCl, 0.05% Tween 20), before the second antibody, anti-human IgG conjugated to alkaline phosphatase (Sigma) diluted 1000-fold in 3% (w/v) BSA, was added. After 1 h at room temperature, the filters were again washed and stained with equal volumes of naphthol AS-MX phosphate (0.4 mg/mL in distilled water; Sigma) and Fast Red TR salt (6 mg/mL in 0.2 M Tris pH 8.2; Sigma). Positive plaques were stored in 200 μL SM buffer [100 mM NaCl, 50 mM Tris–HCl pH 7.5, 10 mM MgSO₄, 0.0001% (v/v) gelatin]. Plaque purification was carried out by repeating the above.

**ABI DNA sequencing**

Before sequencing, excised pBK–CMV plasmid DNA, containing the clone of interest, was purified using the Wizard Plus SV Midiprep DNA Purification Kit (Promega) according to the manufacturer’s instructions. Purified pBK–CMV plasmid DNA (50 ng/mL), containing the clone of interest, was sequenced using the chain termination method with M13 forward (−20) (5′-GTAAAGCAGCGCCGATG) and M13 reverse (5′-TTCACGAGGAAACAG) primers and d-rodhamine bid-dye terminator mix (Applied Biosystems, Warrington, UK). The DNA was sequenced on an ABI 377 Prism DNA sequencer in the Department of Biochemistry, University of Manchester.

**Immunoblotting patient sera and positive clone from antibody screening**

The positive clone obtained from antibody screening was expressed in phage lambda using lacK in Lambda ZAP Express, according to protocols from Clontech Laboratories. *B. cepacia* J2315 Edinburgh was grown at 30°C overnight on nutrient agar plates. The bacteria were harvested and washed in sterile water. The cells were crushed in an ‘X press’ (LKB, Bromma, Sweden) at a pressure of 200 MPG at −20°C. The disintegrated cells were removed from the press and centrifuged at 12 000 g for 20 min at 4°C. After heating in cracking buffer [2.6% (w/v) sodium dodecyl sulphate, 1.3% (v/v) 2-mercaptoethanol, 6% (v/v) glycerol, 0.2% (w/v) bromophenol blue, 0.05 M Tris–HCl pH 6.8] at 100°C for 5 min, 50 μg of *B. cepacia* pressate and the positive clone were loaded into separate wells in a 10% (v/v) polyacrylamide gel. Following electrophoresis and transblotting, the proteins were analysed by western blotting against the sera from a CF patient infected with *B. cepacia*. Positive proteins were detected by enhanced chemiluminescence according to the manufacturer’s instructions (ECL; Amersham Pharmacia Biotech, Little Chalfont, UK) using rabbit anti-human IgG.

**Further screening of the *B. cepacia* genomic library**

The remaining sequence of the *bcrA* gene was obtained using a 500 bp polymerase chain reaction (PCR)-derived digoxigenin-11-uridine-5′-phosphate (DIG)-labelled probe (Roche Diagnostics, Lewes, UK) derived from the 5′ end of the clone pMKC. This DIG-labelled probe was then used to screen the *B. cepacia* genomic library according to protocols from Clontech Laboratories.

**Single clone excision of the Lambda ZAP Express vector**

Positive pBK–CMV clones obtained from lambda library screening were excised from the *E. coli* XL1–Blue cells into *E. coli* XLO LR cells using ExAssist helper phage (≥1 × 10⁶ pfu/mL; Stratagene, Amsterdam, Groningen, The Netherlands), according to protocols from Clontech Laboratories. This clone was sequenced on an ABI 377 Prism DNA sequencer in the Department of Biochemistry, University of Manchester.

**Expression of the complete BcrA transporter protein**

The complete protein was expressed in *E. coli* TOP10 (Invitrogen, Groningen, The Netherlands) by amplifying the gene from a clone obtained from library screening by PCR using forward (5′-ATGTCCGCAACCGAG) and reverse (5′-ATGGCCTCATGCGCGG) primers in a final reaction volume of 25 μL in 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl₂ containing 200 μM of each deoxynucleotide triphosphate, and 5 U Taq polymerase (Roche Diagnostic Systems). Thermal cycling conditions were an initial denaturation of 5 min at 94°C followed by 30 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min. Following amplification, the samples were incubated at 72°C for 7 min and then held at 4°C. Amplified products were resolved by gel electrophoresis using a 1.0% (w/v) low-melting-point agarose gel (Promega) in Tris-acetate buffer containing 0.5 mg/L ethidium bromide and visualized under UV illumination. The band was cut out from the gel and melted by heating at 65°C for 10 min. PCR product (2 μL) was cloned into the pBAD vector by means of a pBAD–TOPO cloning kit (Invitrogen). The recombinant *E. coli* was grown at 37°C, and protein induction was

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initiated by addition of 0.2% (w/v) arabinose at mid-log phase. The cells were harvested after 4 h, and the presence of the tagged recombinant protein was confirmed by probing with a monoclonal antibody to the V5 epitope (1:2000, v/v; Invitrogen).

**MIC determination**

MICs were determined using microtitre broth dilution methodology. *E. coli* TOP10 ± bcrA gene was grown at 37°C, and protein induction was initiated by addition of 0.2% (w/v) arabinose at mid-log phase. The cells were harvested after 4 h and diluted in RPMI (Sigma), supplemented with 0.2% (w/v) arabinose, to an inoculum of 2 × 10^8 cfu/mL. To serial doubling dilutions of antibiotics, dyes, detergents, salts, acids and amino acids were added 10^4 cfu/mL *E. coli* TOP10 ± bcrA gene in 96-well round-bottom microtitre plates (Sigma). The plates were incubated at 37°C for 18–24 h. The MIC was taken as the lowest concentration at which no growth was visible.

**Results**

Identification of a positive clone after screening the *B. cepacia* library

The *B. cepacia* genomic library was cloned into the Lambda ZAP Express vector, which provided the necessary functions for expression of any inserts. Screening the *B. cepacia* genomic library led to the identification of a positive clone (pMKC). The potential immunogenicity of this clone was analysed by comparison with the immunodominant proteins in *B. cepacia* by immunoblotting (Figure). A dominant band appeared at 28 kDa in the positive clone (pMKC) (lane 2) which was not present in the Lambda ZAP control (containing no insert) but was present in an epidemic *B. cepacia* strain (Edinburgh J2315) obtained from an infected CF patient (lane 3).

This positive clone (pMKC) was sequenced and produced an open reading frame (ORF). Analysis of this ORF using the Basic Logic Alignment Search Tool (BLAST) showed this to be an incomplete sequence with the N-terminal region clearly absent. The ORF started at amino acid 46 and continued to the carboxy end of the protein. Further screening of the *B. cepacia* genomic library yielded the remaining sequence.

Sequence analysis

Further cloning using a PCR-derived DIG-labelled probe constructed from the 5’ end of clone pMKC led to the identification of the full-length sequence (EMBL new accession number AJ347753). The ORF encompasses 1554 nucleotides and encodes a 518 amino acid polypeptide with a calculated molecular weight of 46 kDa. This 46 kDa protein is referred to as the BcrA protein. The expected molecular weight of the BcrA protein, as calculated by ExPasy was 55 kDa. The difference in the observed (46 kDa) and expected (55 kDa) molecular weight was possibly due to the hydrophobicity of the BcrA protein. Hydrophobic proteins, such as BcrA, bind less SDS-PAGE sample buffer. As a result, the BcrA protein has an increased negative charge and so migrates faster when proteins are separated, therefore giving a lower molecular weight than expected.

**MIC determination**

To explore some functionality of the BcrA protein, a range of antibiotics, dyes, detergents, salts, acids and amino acids were tested against the BcrA protein. There was no difference in the MIC values between the BcrA protein and the control (*E. coli* TOP10 with no bcrA gene) against detergents (Tween 20), amino acids (arginine or glycine), salts (MgCl_2 and CaCl_2) or dyes (ethidium bromide). There was a small increase in resistance shown against potassium acetate but not to sodium acetate. The antibiotics tested against the BcrA protein are those that are used to treat infection with Gram-negative bacteria. There was no resistance shown by the BcrA protein against gentamicin, meropenem, chlorhexidine or ciprofloxacin. However, there was a four-fold increase in resistance to tetracycline (8 mg/L versus 2 mg/L) and an eight-fold increase in resistance to nalidixic acid (16 mg/L versus 2 mg/L) in the presence of the BcrA protein. Thus, the BcrA pump may confer resistance to these structurally unrelated classes of antibiotics.

**Discussion**

This study reports the identification of a novel immunodominant antigen in *B. cepacia* that encodes a 46 kDa
multidrug efflux protein, called BcrA, which was specific for both tetracycline and nalidixic acid.

We focused on identifying immunodominant antigens and identified BcrA with an apparent molecular weight of 46 kDa. Previous studies on *B. cepacia* have identified porins C and D, a 39–47 kDa outer membrane protein and a 50 kDa porin called OpcM. Described here is the isolation of a new porin protein that belongs to the 14 transmembrane domain subfamily (14-TMS) of the major facilitator superfamily (MFS), distinct from any of the previously described membrane pumps from *B. cepacia*.

Immunoblot analysis between the positive clone isolated in this study and *B. cepacia* proteins showed the presence of two bands, one at 28 kDa and another at c. 46 kDa. The immunodominant band at c. 46 kDa is likely to be the BcrA protein, and the smaller band is potentially either a breakdown product of this protein or there are two immunodominant regions of the BcrA protein.

Two potential transcriptional regulators of the *bcrA* gene were isolated in the ORF. One of these transcriptional regulators was identified downstream of the *bcrA* gene and encodes a 31.5 kDa peptide that belongs to the LysR family of transcriptional factors. Upstream of the *bcrA* gene, there is a putative transcriptional regulator that belongs to the multiple antibiotic resistance (*mar*) family of transcriptional factors. One or the other (or possibly both) of these regulatory genes may play a role in *bcrA* regulation. Construction of knockout mutants would confirm the role of these putative regulators in the control of transcription of the *bcrA* gene.

Analysis of the BcrA protein using PSORT showed that it is a classical membrane protein likely to be located within the bacterial inner membrane. The grand average of hydrophobicity (GRAVY) as determined by the method of Kyte & Doolittle showed that the BcrA protein is a hydrophobic protein giving a value of 0.612, a score similar to other known membrane proteins. Membrane topology analysis using TopPred showed that the BcrA protein contains 14-TMS with the N-terminus located on the outermost side of the membrane.

A BLAST search of all amino acid sequences similar to BcrA was carried out using BLAST version 2 at the National Centre for Biotechnology Information (Bethesda, MA, USA). All sequences that produced high-scoring segment pairs with a P(N) < 10^-10 were considered to be closely related. The BcrA protein showed significant identity to EmrB from *E. coli* and *Neisseria gonorhoeae* (L. Sabbagh, G. Cardinal & R. C. Levesque, unpublished data), PmrB from *Pseudomonas aeruginosa* and VceB from *Vibrio cholerae*. The BcrA protein showed the highest identity (84%) to an efflux protein, described by DeShazer et al., which is known to secrete exoproducts, such as lipases, proteases and phospholipase C, from *Burkholderia pseudomallei*. These proteins are all part of a complex that functions as multidrug extrusion pumps belonging to the MFS. Analysis of the BcrA protein sequence shows that it contains motifs specific to the 14-TMS of the MFS group of transporters (A, B, C, D1, E, F and H) as identified by Rouch et al. and Paulsen & Skurray.

Gram-negative organisms require accessory proteins for substrates to cross both membranes. These accessory proteins are the membrane fusion protein (MFP) and an outer membrane protein (OMP). Multidrug transporters of the MFS often require the functions of an MFP, which spans the periplasmic space, and an OMP to transport molecules across the double membrane. For example, Lomovskaya & Lewis provided indirect evidence that the EmrB protein functions cooperatively with the MFP EmrA and for the transport of molecules across the double membrane of *E. coli*. In the ORF encoding the *bcrA* gene, no sequence encoding a putative MFP was isolated. Therefore, it can be speculated that either the BcrA protein functions without an MFP or the MFP associated with the BcrA protein has yet to be identified and is instead located elsewhere in the *B. cepacia* genome.

Once characterized and cloned into a suitable expression vector, the BcrA protein increased resistance to nalidixic acid and tetracyclines. There was a four-fold increase in resistance to tetracycline and an eight-fold increase in resistance to nalidixic acid. Resistance to the quinolones, such as nalidixic acid, has previously been linked with protein-mediated outer membrane permeability changes, which provides further evidence that one of the contributing factors could be the BcrA drug efflux pump. The construction of a *bcrA* knockout would assess the impact of resistance in *B. cepacia* that the BcrA protein has to tetracycline and nalidixic acid.

That the immune system has targeted a drug efflux pump as a major antigen might indicate that the BcrA pump has a metabolic role in *B. cepacia* infection. By targeting the antibiotic resistance mechanisms, in this case the BcrA drug efflux pump, one could potentially render *B. cepacia* more susceptible to antimicrobials. Inhibiting this pump could hinder the efflux of nalidixic acid and tetracycline and could potentially allow these antibiotics to hinder the growth of the organism. A potential inhibitor may be a protein or synthetic chemical compound that may covalently bind to the efflux pump and prevent the passage of drugs, or it may act to prevent or down-regulate the synthesis of the pump at the transcriptional, translational or post-translational level. Alternatively, an inhibitor could be a novel agent such as a human antibody. Recent advances in antibody engineering have made the use of genetically engineered antibodies an achievable objective; such antibodies can be produced economically without the risk of contamination by human retroviruses or serum sickness. Human recombinant antibodies have now been produced by several groups against bacterial antigens, including antibodies against the *Staphylococcus* enterotoxin A, the *Pseudomonas* endotoxin and, more
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recently, the heat shock protein 90 antigen of *Candida albicans*; one of the latter is now in clinical trials.38

Dual therapy with an inhibitor of the BcrA pump, such as a human recombinant antibody, in combination with nalidixic acid and/or tetracycline might be of benefit in the treatment of *B. cepacia* infection. Research is continuing in our laboratories to pursue this possibility.

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**References**


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