Contributions of two UDP-glucose dehydrogenases to viability and polymyxin B resistance of *Burkholderia cenocepacia*

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Burkholderia cenocepacia is highly resistant to antimicrobial peptides and we hypothesized that the conversion of UDP-glucose to UDP-glucuronic acid, a reaction catalysed by the enzyme UDP-glucose dehydrogenase (Ugd) would be important for this resistance. The genome of *B. cenocepacia* contains three predicted *ugd* genes: *ugd*_{BCAL2946}, *ugd*_{BCAM0855} and *ugd*_{BCAM2034}, all of which were individually inactivated. Only inactivation of *ugd*_{BCAL2946} resulted in increased sensitivity to polymyxin B and this sensitivity could be overcome when either *ugd*_{BCAL2946} or *ugd*_{BCAM0855} but not *ugd*_{BCAM2034} was expressed from plasmids. The growth of a conditional *ugd*_{BCAL2946} mutant, created in the Δ *ugd*_{BCAM0855} background, was significantly impaired under non-permissive conditions. Growth could be rescued by either *ugd*_{BCAL2946} or *ugd*_{BCAM0855} expressed *in trans*, but not by *ugd*_{BCAM2034}. Biochemical analysis of the purified, recombinant forms of Ugd_{BCAL2946} and Ugd_{BCAM0855} revealed that they are soluble homodimers with similar *in vitro* Ugd activity and comparable kinetic constants for their substrates UDP-glucose and NAD⁺. Purified Ugd_{BCAM2034} showed no *in vitro* Ugd activity. Real-time PCR analysis showed that the expression of *ugd*_{BCAL2946} was 5.4- and 135-fold greater than that of *ugd*_{BCAM0855} and *ugd*_{BCAM2034}, respectively. Together, these data indicate that the combined activity of Ugd_{BCAL2946} and Ugd_{BCAM0855} is essential for the survival of *B. cenocepacia* but only the most highly expressed *ugd* gene, *ugd*_{BCAL2946}, is required for polymyxin B resistance.

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INTRODUCTION

Burkholderia cenocepacia belongs to the *Burkholderia cepacia* complex (Bcc), a group of ten phenotypically similar environmental Gram-negative bacteria (Balandreau *et al.*, 2001), which are also opportunistic human pathogens causing chronic, sometimes fatal, pulmonary infections in cystic fibrosis (CF) patients (Isles *et al.*, 1984; Mahenthiralingam *et al.*, 2005). Treatment of these infections is difficult as Bcc bacteria are inherently resistant to most clinically relevant antimicrobial agents (Aaron *et al.*, 2000; Burns *et al.*, 1996; Gold *et al.*, 1983), including

antimicrobial peptides (APs) (Loutet *et al.*, 2006; Turner *et al.*, 1998).

APs are short, amphipathic, positively charged peptides produced by organisms from bacteria to mammals (Brogden, 2005). They are part of the innate immune response (Ganz, 2003; Zanetti, 2004) and have been shown to kill bacteria through the disruption of the inner membrane (Brogden, 2005) and also through inhibition of intracellular processes (Patrzykat *et al.*, 2002). APs have been proposed as agents for treatment of infections by other CF lung pathogens, such as *Pseudomonas aeruginosa* (Zhang *et al.*, 2005). Unfortunately, due to the extraordinary resistance of *B. cenocepacia* to the killing effects of APs, they are unlikely to be useful agents for treatment of lung infections by *B. cenocepacia*. Understanding the determinants of AP resistance in *B. cenocepacia* is important because they could provide targets for the

Abbreviations: APs, antimicrobial peptides; Ara4N, 4-amino-4-deoxy-L-arabinose; CF, cystic fibrosis; DDM, dodecylmaltoside; pmB, polymyxin B; Ugd, UDP-glucose dehydrogenase.

Supplementary methods and tables of strains and primers are available with the online version of this paper.

development of novel antimicrobial agents that could be co-administered with APs. For example, in *B. cenocepacia* L-glycero-D-manno-heptose sugars of the lipopolysaccharide (LPS) core oligosaccharide are required for AP resistance and *in vivo* survival (Loutet *et al.*, 2006) and small molecule inhibitors of the synthesis of these sugars have been identified (De Leon *et al.*, 2006).

A widely recognized mechanism of AP resistance in Gram-negative bacteria is the decoration of lipid A phosphate residues with the positively charged sugar 4-amino-4-deoxy-L-arabinose (Ara4N), which requires the synthesis of a UDP-Ara4N precursor (Ernst *et al.*, 1999; Helander *et al.*, 1994; Nummala *et al.*, 1995; Vaara *et al.*, 1981). Ara4N substitution reduces the net negative charge of the lipid A molecule and hampers the ability of APs to bind to the outer membrane (Vaara *et al.*, 1981). In *P. aeruginosa* and *Salmonella enterica*, these substitutions are induced upon treatment with APs (Bader *et al.*, 2005; McPhee *et al.*, 2003) and are dispensable for growth under normal laboratory conditions. In *B. cenocepacia*, a species closely related to *B. cenocepacia*, Ara4N is constitutively incorporated into both lipid A and the LPS core oligosaccharide (Silipo *et al.*, 2005). Preliminary evidence suggests that this is also true in *B. cenocepacia* (X. P. Ortega & M. A. Valvano, unpublished data) and UDP-Ara4N synthesis was recently shown to be essential for *B. cenocepacia* viability (Ortega *et al.*, 2007). These results highlight the importance of Ara4N in this organism and its abundance in LPS likely contributes significantly to the organism's unusually high AP resistance.

The initial step in the synthesis of UDP-Ara4N is the conversion of UDP-glucose to UDP-glucuronic acid, which is catalysed by the enzyme UDP-glucose dehydrogenase (Ugd) (Breazeale *et al.*, 2002; Raetz & Whitfield, 2002; Strominger *et al.*, 1957). A recent study by Hung *et al.* (2007) described two Ugd-encoding genes in *P. aeruginosa*. Both Ugd enzymes were purified as homodimers, and biochemical analysis confirmed Ugd activity, but revealed distinct expression and enzymic properties, indicating different roles for the two Ugd proteins *in vivo*.

The *B. cenocepacia* genome (Holden *et al.*, 2009) contains three predicted *ugd* genes, designated *ugd_{BCAL2946}*, *ugd_{BCAM0855}* and *ugd_{BCAM2034}* (gene designations correspond to the Sanger Centre annotation of the strain J2315 genome, http://www.sanger.ac.uk/Projects/B_cenocepacia/, where BCAL denotes genes in the large chromosome and BCAM genes in the mid-size chromosome). In this study, we examined in detail the function of these genes and show that only *ugd_{BCAL2946}* is required for resistance to polymyxin B (pmB), while the combination of *ugd_{BCAL2946}* and *ugd_{BCAM0855}* is required for bacterial survival. We purified Ugd_{BCAL2946}, Ugd_{BCAM0855} and Ugd_{BCAM2034} but only the recombinant forms of Ugd_{BCAL2946} and Ugd_{BCAM0855} possess Ugd activity with similar substrate reaction kinetics. Finally, we show that in cells the number of RNA transcripts of *ugd_{BCAL2946}* is substantially higher than either *ugd_{BCAM0855}* or *ugd_{BCAM2034}*.

METHODS

Reagents. Unless otherwise noted all chemicals and antibiotics, restriction endonucleases and DNA polymerases were purchased from Sigma-Aldrich, Roche Diagnostics and Qiagen, respectively.

Bacterial strains and culture conditions. Strains and plasmids used in this study are described in Supplementary Table S1, available with the online version of this paper. *B. cenocepacia* and *Escherichia coli* strains were routinely cultured at 37 °C either in Luria Broth (LB) supplemented with 1.6% (w/v) Bacto Agar (Becton Dickinson) or in liquid LB. When required, trimethoprim or tetracycline was added at a final concentration of 100 µg ml⁻¹ for selection of *B. cenocepacia* strains, and at 50 µg ml⁻¹ and 20 µg ml⁻¹, respectively, for selection of *E. coli* strains. Kanamycin and gentamicin were used at 40 µg ml⁻¹ and 50 µg ml⁻¹, respectively.

Mutagenesis and cloning. A complete description of the mutagenesis and cloning experiments can be found in Supplementary Methods and the primers used are listed in Supplementary Table S2. To inactivate the putative *ugd* genes, suicide plasmids containing internal fragments of each *ugd* gene were created. The fragments were PCR amplified, restriction enzyme digested, ligated into the plasmid pGPlox (Table S1), used to transform *E. coli* SY327 (Miller & Mekalanos, 1988), and confirmed by colony PCR and restriction digest. The resulting plasmids – pSL23 (*ugd_{BCAL2946}*), pSL29 (*ugd_{BCAM2034}*) and pSL31 (*ugd_{BCAM0855}*) – were transferred into *B. cenocepacia* strain K56-2 (Mahenthalingam *et al.*, 2000) by triparental mating with the pRK2013 helper plasmid (Figurski & Helinski, 1979). Exconjugants were screened for plasmid insertion into the correct location by PCR and Southern blot hybridization. The resulting mutants were named SAL8 (*ugd_{BCAL2946}::pSL23*), SAL12 (*ugd_{BCAM2034}::pSL29*) and SAL15 (*ugd_{BCAM0855}::pSL31*).

To construct an unmarked *ugd_{BCAM0855}* deletion mutant, two fragments of this gene were PCR amplified, restriction enzyme digested, ligated as a triple ligation into pGPI-SceI (Flannagan *et al.*, 2008), screened as described above, and the resulting plasmid, pSL43, was transferred to K56-2. Putative mutants (SAL22) were screened by PCR for recombination of the plasmid into the chromosome. To delete the backbone of pSL43 from the chromosome, pDAI-SceI (Flannagan *et al.*, 2008) was introduced into SAL22, and this resulted in the isolation of strain SAL23, which carries a 986 bp deletion removing most of *ugd_{BCAM0855}*. pDAI-SceI was cured from SAL23 by serial passage in the absence of tetracycline for 1 week. The final mutant construct was confirmed by Southern blot hybridization.

Plasmids for the conditional mutagenesis of *hldA* and *ugd_{BCAL2946}* were constructed as described above with fragments corresponding to the 5' ends of the two genes cloned into the plasmid pSC201 (Ortega *et al.*, 2007), resulting in plasmids pSL26 (*hldA*) and pSL27 (*ugd_{BCAL2946}*). These plasmids were transferred into SAL23, resulting in strains SAL25 (conditional *hldA*) and SAL26 (conditional *ugd_{BCAL2946}*). A conditional *ugd_{BCAL2946}* mutant in the K56-2 background (SAL10), also constructed with pSL27, was previously described (Ortega *et al.*, 2007).

For complementation experiments each of the putative *ugd* genes was cloned into pDA17 (Aubert *et al.*, 2008). Plasmids were confirmed as described above, inserts were verified by sequencing (Robarts Research Institute DNA Sequencing Facility, London, ON, Canada), and the resulting plasmids were named pSL37 (*ugd_{BCAL2946}*), pSL38 (*ugd_{BCAM2034}*) and pSL39 (*ugd_{BCAM0855}*).

For expression and purification of the Ugd proteins, the *B. cenocepacia* J2315 *ugd_{BCAL2946}*, *ugd_{BCAM0855}* and *ugd_{BCAM2034}* genes were PCR amplified, cloned using the pGEM-T Easy vector system (Promega), and sequenced. Genes were liberated by restriction enzyme digest, ligated into pET28a (Novagen), and used to transform

E. coli Top10 cells. The resulting plasmids were confirmed by restriction digests and named pJB1 (pET28a/*ugd*_{BCAL2946}), pJB2 (pET28a/*ugd*_{BCAM0855}) and pJB3 (pET28a/*ugd*_{BCAM2034}), with each *ugd* expressed with an N-terminal His₆ fusion tag.

Growth curves. Cells were cultured overnight to stationary phase and then diluted to an OD₆₀₀ of 0.05 in LB. Cultures were aliquoted to 100-well honeycomb plates in 300 µl volumes. Plates were incubated in a Bioscreen C automated growth curve reader at 37 °C with constant, low shaking for 24 h with OD₆₀₀ readings taken every hour. For growth curves with pmB, cells were diluted to approximately 2×10^5 c.f.u. ml⁻¹ in LB. Then 10 × stock solutions of pmB dissolved in 0.2 % (w/v) BSA + 0.02 % (v/v) acetic acid were added to cells to give final concentrations of pmB ranging from 16 to 1024 µg ml⁻¹ in twofold increments. Cells were also treated with the pmB diluent indicated above as a vehicle control. Triplicate 300 µl aliquots of the cells with either vehicle control or the various concentrations of pmB were incubated in the Bioscreen C automated growth curve reader, as described above, for 18 h. MIC₅₀ values reported are the mode of three independent experiments. An assay for growth of conditional mutants was performed as previously described (Ortega *et al.*, 2007). Expression of putative essential genes was repressed with 1.0 % (w/v) glucose and induced with 1.0 % (w/v) rhamnose.

LPS extraction and analysis. LPS samples were prepared and analysed as previously described (Hitchcock & Brown, 1983; Loutet *et al.*, 2006).

Western blotting. Cells were grown overnight in liquid LB with appropriate antibiotics. A volume of culture equivalent to 1 ml at an OD₆₀₀ of 0.2 was centrifuged for 1 min at 8000 g; cells were resuspended in 20 µl H₂O and 10 µl of 3 × loading dye, and boiled for 10 min. Aliquots (15 µl) of boiled samples were separated by gel electrophoresis using 14 % (w/v) SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was washed briefly with H₂O and stained with a solution of 0.1 % (w/v) Ponceau S in 5 % (v/v) acetic acid to confirm the transfer of equal amounts of protein to the membrane in each lane. The membrane was rinsed with Tris-buffered saline, pH 7.5 (TBS) to remove Ponceau S stain and then blocked for 2 h with 10 % (v/v) Western blocking reagent (Roche) in TBS at room temperature and incubated with murine anti-FLAG M2 monoclonal antibody diluted 1:10 000 overnight at 4 °C. The membrane was incubated at room temperature for 2 h with goat anti-mouse IgG antibody conjugated to Alexa Fluor 680 (Molecular Probes) diluted 1:20 000. Proteins were visualized using a Licor Infrared Imaging System with Odyssey software version 2.1.

Purification of recombinant Ugd proteins. To prepare recombinant Ugd_{BCAL2946} and Ugd_{BCAM0855} proteins for biochemical analysis, a large-scale culture (4 l LB with kanamycin) was performed with *E. coli* HMS 174 (DE3) transformed with pJB1 or pJB2. Each batch was grown to an OD₆₀₀ of 0.6, and induced with 0.5 mM IPTG for 3 h at 37 °C with shaking. Both Ugd_{BCAL2946} and Ugd_{BCAM0855} were highly overexpressed in soluble forms and cell-free extracts were prepared by sonic disruption of the *E. coli* cell paste. The enzymes were isolated from ultrafiltered cell-free extracts by FPLC (ÄKTA Basic instrument) using a 5 ml HisTrap column (GE Healthcare). The binding buffer consisted of 20 mM Tris, 500 mM NaCl, 5 mM imidazole, pH 7.5, and the elution buffer was identical except that it contained 500 mM imidazole. The eluted His-tagged Ugd proteins were judged >90 % pure after this step. The enzymes were dialysed against 150 mM NaCl and 20 mM Tris, pH 7.5, and further purified by size exclusion chromatography using a HiPrep 26/60 Sephacryl S-200 column (GE Healthcare). The calibrated column was equilibrated with 150 mM NaCl and 20 mM Tris, pH 7.5. The molecular masses of the Ugd proteins, estimated from their column elution volumes and by non-

denaturing gel electrophoresis, were consistent with dimers (data not shown). The subunit molecular masses were confirmed by electrospray-mass spectrometry (ESI-MS) and were within 0.1 % of the theoretical values (data not shown). The protein concentration was determined by a BCA assay (Pierce) and a typical yield was ~3 mg Ugd per litre of *E. coli* culture.

To prepare recombinant Ugd_{BCAM2034}, a large-scale culture (4 l LB with kanamycin) was performed with *E. coli* BL21 (DE3) transformed with pJB3. Each batch was grown to an OD₆₀₀ of 0.6, and induced by addition of 0.2 mM IPTG for 16 h at 15 °C with shaking. To solubilize Ugd_{BCAM2034} for purification, the pellet was resuspended and homogenized in 5 ml binding buffer containing 25 % (w/v) sucrose, 1 mM EDTA and a protease inhibitor tablet per gram of cell pellet. Thirty mg lysozyme, 5 µl DNase and 5 mM MgCl₂ were added and the cell paste was left to stir for 1 h at 4 °C. A cell-free extract was prepared by sonic disruption of the *E. coli* cell paste, which was incubated with 1 % (w/v) dodecylmaltoside (DDM) and 20 % (v/v) glycerol for 1 h at 4 °C with stirring before centrifugation at 25 000 g for 45 min. The supernatant was incubated with equilibrated nickel NTA agar beads (Qiagen) for 3 h. The beads were then washed in elution buffer [20 mM Tris, pH 7.5, 500 mM NaCl, 0.1 % (w/v) DDM and 10 % glycerol] with 10 mM imidazole. Protein was eluted in elution buffer containing 20, 50, 100 or 200 mM imidazole. The molecular mass of Ugd_{BCAM2034} was estimated from size-exclusion chromatography (as above), ESI-MS and non-denaturing gel electrophoresis (data not shown).

In vitro Ugd activity assay. Ugd oxidizes UDP-glucose to UDP-glucuronic acid, and in the process also reduces 2 mol NAD⁺ to 2 mol NADH. The absorption coefficient of NADH is 6.220 mM⁻¹ cm⁻¹ at 340 nm. A standard Ugd activity assay was set up using 200 µl H₂O, 100 µl purified Ugd (1 mg ml⁻¹), 200 µl buffer (100 mM Tris, pH 7.5), 250 µl NAD⁺ (10 mM; Roche) at 25 °C. The reaction was initiated by the addition of 250 µl UDP-glucose (1 mM; Calbiochem) and the production of NADH was monitored at 340 nm on a Cary 50 UV visible spectrophotometer (Varian). Initial velocities were determined for UDP-glucose using concentrations ranging from 0 to 0.25 mM, and for NAD⁺ using concentrations ranging from 0 to 2.5 mM. To determine kinetic constants, one substrate was held constant at the maximum concentration, while the other was varied. The kinetic parameters (k_{cat} , K_m , V_{max}) were determined by non-linear regression from $V = V_{max} [S]^h / ([S]^h + K_m^h)$ using Origin 6.1 software. Substrate specificity was investigated by replacing the substrate UDP-glucose with either UDP-galactose, UDP-acetylglucosamine or GDP-mannose (Calbiochem), and performing the standard assay.

Real-time PCR analysis. *B. cenocepacia* strain K56-2 was grown overnight in liquid LB, diluted to an OD₆₀₀ of 0.1 and grown to a final OD₆₀₀ between 0.5 and 0.7. RNA was prepared from approximately 5×10^8 c.f.u. with the Ribo-Pure Bacteria kit (Ambion) according to the manufacturer's instructions. RNA was treated with DNase I (Ambion) and the concentration of RNA was determined with an ND-1000 spectrophotometer (NanoDrop Technologies). One microgram of RNA was converted to cDNA using Transcriptor Reverse Transcriptase (Roche) in a reaction volume of 30 µl with reverse primers 3136 (*hisD*), 3246 (*ugd*_{BCAM0855}), 3247 (*ugd*_{BCAL2946}) and 3364 (*ugd*_{BCAM2034}) according to the manufacturer's instructions and supplemented with Protector RNase Inhibitor (Roche), 0.5 × Q solution (Qiagen) and 10 % (v/v) DMSO. A second reaction containing nuclease-free water (Qiagen) instead of Transcriptor Reverse Transcriptase was included as a negative control.

The real-time PCRs were conducted using the Rotor-Gene 6000 (Corbett Life Science) in final reaction volumes of 20 µl. Reactions of 18 µl, containing FastStart SYBR Green (Roche), primer pairs 3247/3255 (*ugd*_{BCAL2946}), 3361/3364 (*ugd*_{BCAM2034}), 3246/3248

(*ugd_{BCAM0855}*) or 3136/3143 (*hisD*), and nuclease-free water were prepared and to these 2 µl of the reverse transcription reactions was added in triplicate. Negative controls were 2 µl of the reverse transcription reaction done without Transcriptor Reverse Transcriptase and 2 µl of nuclease-free water. The segment of each gene analysed was also amplified from chromosomal DNA and used in standard curve reactions containing 10⁶, 10⁵, 10⁴, 10³ and 10² copies of the PCR products per reaction. The thermal cycling conditions were 95 °C for 10 min, 35 cycles at 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 30 s. Data were acquired on the green channel at the end of each cycle. After the PCR a melt curve was obtained by increasing the temperature from 72 °C to 95 °C in 1 °C increments, with data acquired on the green channel at each degree. Data were analysed using the Rotor-Gene 6000 software provided by the manufacturer. For each analysed gene, the number of copies per reaction was quantified by comparison to the standard curve and adjusted based on the PCR efficiency calculated from the standard curve. A mean value from three replicates was calculated for the internal control *hisD*. Each replicate value of each *ugd* gene was divided by the mean of the *hisD* results in order to obtain a ratio of each value to *hisD*.

Statistical analyses. Statistical analyses were done with GraphPad Prism 4.

RESULTS

Identification of three putative *ugd* genes in *B. cenocepacia*

A TBLASTN analysis of the genome of *B. cenocepacia* strain J2315 (Holden *et al.*, 2009) using the *P. aeruginosa* strain PAO1 Ugd (PA2022; accession no. NP_250712) gave three high scoring hits: *ugd_{BCAL2946}*, *ugd_{BCAM0855}* and *ugd_{BCAM2034}*. The presence of these genes was also confirmed in strain K56-2, which is clonally related to J2315 (Mahenthiralingam *et al.*, 2000), but much more amenable to genetic manipulation. The chromosomal arrangements of these genes are shown in Fig. 1(a). *ugd_{BCAL2946}* is directly upstream of *hldA* and *hldD* within a six-gene cluster. *hldA* and *hldD* encode proteins required for the synthesis of L-glycero-D-manno-heptose, a critical residue of the LPS inner-core oligosaccharide (Loutet *et al.*, 2006). *ugd_{BCAM0855}*, annotated as *bceC* by Moreira *et al.* (2003), is in a ten-gene cluster (the final five genes of which are not depicted in Fig. 1a) that plays a role in exopolysaccharide synthesis in *B. cepacia* (Moreira *et al.*, 2003). This exopolysaccharide is not produced in *B. cenocepacia* strains J2315 or K56-2 due to the presence of transposable insertion elements within other genes of this cluster (M. S. Saldías & M. A. Valvano, unpublished data). *ugd_{BCAM2034}* is in a four-gene cluster that contains two open reading frames similar to *galE* (UDP-galactose 4-epimerase) as well as a predicted glycosyltransferase. The sequences of the three predicted *ugd* genes in strain K56-2 are identical to those in the sequenced J2315 strain.

The three loci *ugd_{BCAL2946}*, *ugd_{BCAM0855}* and *ugd_{BCAM2034}* are predicted to encode proteins sharing, respectively, 53 %, 52 % and 39 % amino acid sequence identity with *P. aeruginosa* PA2022. The predicted proteins Ugd_{BCAL2946}

and Ugd_{BCAM0855} share 74 % amino acid sequence identity. The predicted Ugd_{BCAM2034} shares 44 % and 45 % amino acid sequence identity with Ugd_{BCAL2946} and Ugd_{BCAM0855}, respectively. Fig. 1(b) shows a CLUSTAL W alignment of the three predicted Ugd proteins of *B. cenocepacia*, *P. aeruginosa* Ugd PA2022 and *Streptococcus pyogenes* Ugd (accession no. AAA26899). Despite low pair-wise sequence identity (~21 %) with the *S. pyogenes* Ugd, for which high-resolution crystal structures are available (Protein Data Bank, accession nos 1DLI and 1DLJ) (Campbell *et al.*, 2000), each of the three predicted Ugd proteins contains a predicted Rossmann fold for NAD⁺ binding (Rossmann, 1981) at the N terminus, and residues involved in the catalytic mechanism (Campbell *et al.*, 2000), such as Cys260 involved in thioester formation and Arg244 involved in UDP-sugar specificity, are conserved (equivalent residues Cys274 and Arg258 in Ugd_{BCAL2946}, Cys270 and Arg254 in Ugd_{BCAM0855}, and Cys266 and Arg250 in Ugd_{BCAM2034}).

A mutation in *ugd_{BCAL2946}* confers sensitivity to polymyxin B

To assess the function of the candidate *ugd* genes, three mutant strains were constructed, each with a different putative *ugd* gene inactivated by insertional mutagenesis: SAL8 (*ugd_{BCAL2946}::pSL23*), SAL12 (*ugd_{BCAM2034}::pSL29*) and SAL15 (*ugd_{BCAM0855}::pSL31*). SAL12 and SAL15 grew similarly to the parental strain, K56-2 (Fig. 2). In contrast, SAL8 exhibited a slightly slower growth rate in exponential phase, but eventually reached the same cell density as K56-2, SAL12 and SAL15 (Fig. 2). There were no detectable differences in the LPS profiles of mutant strains compared to that of the parental K56-2 when LPS was prepared from cells, separated by gel electrophoresis, and stained with silver (data not shown); however, detection of subtle changes to LPS structure may require a more sensitive method of analysis. The *ugd* mutants were tested for their ability to grow in LB medium containing pmB at a concentration of 1024 µg ml⁻¹. Fig. 3(a) shows that the growth of SAL8 was significantly impaired at this concentration of pmB, in comparison to K56-2 and the other two mutants. In vehicle control incubations, K56-2, SAL12 and SAL15 all grew equivalently and the growth of SAL8 was slightly slower in the exponential phase of growth (Fig. 3a inset), similar to the results shown above with LB alone. The MIC₅₀ values for pmB were also calculated for all the strains. For K56-2, SAL12 and SAL15 the MIC₅₀ values for pmB were all greater than 1024 µg ml⁻¹ (the highest concentration tested). For SAL8, the MIC₅₀ value for pmB was 128 µg ml⁻¹.

Next, SAL8 was transformed with a series of plasmids, each containing a different putative *ugd* gene fused to the FLAG epitope at the 3' end of the gene. Growth of SAL8(pDA17) containing a vector control or SAL8(pSL38) encoding *ugd_{BCAM2034}* was poor in 128 µg ml⁻¹ pmB (Fig. 3b). In fact, the plasmids actually appeared to exacerbate the

Fig. 1. Identification and chromosomal arrangement of putative *ugd* genes. (a) Each *ugd* gene is schematically depicted with neighbouring genes. The nomenclature below each gene is that used in the annotation of the sequenced genome of *B. cenocepacia* strain J2315. Designations above the genes are based on either TBLASTX analysis or previous publications (Iwanicka-Nowicka *et al.*, 2007; Loutet *et al.*, 2006; Moreira *et al.*, 2003). The grey bars within the *ugd* genes represent the internal fragments that were cloned into the *ugd* mutagenesis plasmids (pSL23, pSL29 and pSL31 for *ugd*_{BCAL2946}, *ugd*_{BCAM2034} and *ugd*_{BCAM0855}, respectively). The dashed lines below *hldA* and *ugd*_{BCAL2946} represent the PCR fragments used to generate plasmids for conditional mutagenesis (pSL26 and pSL27 for *hldA* and *ugd*_{BCAL2946}, respectively). The dotted lines below *ugd*_{BCAM0855} and its neighbouring genes represent the two fragments that were cloned into the plasmid (pSL43) used for unmarked mutagenesis of *ugd*_{BCAM0855}. The solid lines below each *ugd* gene represent the regions analysed by real-time PCR. (b) CLUSTAL W alignment of the three putative Ugd proteins of *B. cenocepacia*, *P. aeruginosa* PA2022 and Ugd from *S. pyogenes*. Residues conserved in all five proteins, four of the five, or three of the five are highlighted in black, dark grey and light grey, respectively. The line below the alignment indicates the conserved GXGXXG motif of the Rossmann fold required for NAD binding. Asterisks denote residues Arg-244 and Cys-260 of *S. pyogenes* Ugd shown to be important for catalysis.

Fig. 2. *B. cenocepacia* K56-2 and the panel of *ugd* mutants grow similarly. Growth of K56-2 (■), SAL8 (▲), SAL12 (▼) and SAL15 (◆) was monitored in a Bioscreen C automated growth curve reader for 24 h. Shown are the means of data from three independent experiments done in triplicate; error bars denote the standard error of the mean.

sensitivity to pmB in SAL8 since growth was impaired by >95 % at this concentration of pmB. The growth of SAL8 transformed with pSL37 (*ugd*_{BCAL2946}) after 18 h was not statistically different in the presence or absence of 128 µg pmB ml⁻¹, similar to the growth of K56-2 under these conditions. SAL8 transformed with pSL39 (*ugd*_{BCAM0855}) regained in part the ability to grow in the presence of 128 µg pmB ml⁻¹. Western blot analysis showed that all three putative Ugd proteins were expressed in SAL8 (Fig. 4), although not to the same levels. The predicted masses for Ugd-FLAG_{BCAL2946}, Ugd-FLAG_{BCAM2034}, and Ugd-FLAG_{BCAM0855} are 51.7 kDa, 50.9 kDa and 52.1 kDa, respectively. Bands indicated with arrows correspond approximately to the predicted sizes. In addition, we observed numerous faster-migrating bands for the Ugd-FLAG constructs that were consistent between experiments. These bands were interpreted as degradation products due to proteolytic cleavage either in the cell or during the sample preparation. Ponceau S staining of the membrane before Western blotting indicated that equal amounts of protein were transferred to the membrane in each lane (data not shown). Similar complementation results were obtained with a series of vectors expressing Ugd proteins not fused to the FLAG epitope (data not shown). Together, these experiments indicate that of the three putative *ugd* genes, only *ugd*_{BCAL2946} is required for the full resistance of *B. cenocepacia* strain K56-2 to pmB and also that the protein encoded by *ugd*_{BCAM0855} can compensate for the function of the protein encoded by *ugd*_{BCAL2946}.

The combined activity of Ugd_{BCAL2946} and Ugd_{BCAM0855} is essential for viability

Due to the role of Ugd in the synthesis of UDP-Ara4N, we hypothesized that the combined Ugd activity of *B. cenocepacia* is essential for viability of the organism. Based on the results described above, we predicted that the combination of *ugd*_{BCAL2946} and *ugd*_{BCAM0855} would be

Fig. 3. SAL8 is the only *ugd* mutant that is sensitive to pmB. (a) Growth of K56-2 (■, □), SAL8 (▲, △), SAL12 (▼, ▽) and SAL15 (◆, ◇) in either a vehicle control (filled symbols, shown in inset) or 1024 µg pmB ml⁻¹ (empty symbols) was monitored for 18 h. (b) End point OD₆₀₀ of K56-2 and SAL8 with either pDA17 (plasmid control), pSL37 (*ugd*_{BCAL2946}), pSL38 (*ugd*_{BCAM2034}) or pSL39 (*ugd*_{BCAM0855}) grown for 18 h in either a vehicle control (horizontally striped bars) or 128 µg pmB ml⁻¹ (vertically striped bars). Shown are the mean and standard error of the mean for data from three independent experiments done in triplicate. *, Statistically significant difference ($P < 0.05$) between vehicle control and pmB treatments by unpaired *t*-test.

essential. To test this, a strain containing an unmarked *ugd*_{BCAM0855} deletion was created (SAL23). Like SAL15, SAL23 did not show impaired resistance to pmB (data not shown). SAL23 was then used for the creation of conditional mutants in *ugd*_{BCAL2946} (SAL26) and the downstream gene, *hldA* (SAL25).

These strains were tested for their ability to grow under inducing (rhamnose) and repressing (glucose) conditions. The wild-type strain K56-2 transformed with pSCrhaB2 (to allow for growth in medium supplemented with trimethoprim) grew equally well on plates with either glucose or rhamnose (Fig. 5, first lane). XOA11, a positive control for a conditional mutant of an essential gene required for Ara4N transfer to lipid A, grew well in the presence of rhamnose but growth was substantially impaired in the

Fig. 4. All three Ugd-FLAG constructs are expressed in SAL8 and SAL26. Western blot analysis using anti-FLAG antibodies was done for both SAL8 and SAL26 containing either pDA17 (plasmid control), pSL37 (*ugd_{BCAL2946}*), pSL38 (*ugd_{BCAM2034}*) or pSL39 (*ugd_{BCAM0855}*). Arrows indicate bands that correspond to the full-length Ugd-FLAG constructs. This is a representative image of three independent experiments.

presence of glucose (Fig. 5, second lane), as previously demonstrated (Ortega *et al.*, 2007). Two negative control strains, SAL10 (a *ugd_{BCAL2946}* conditional mutant with an intact *ugd_{BCAM0855}* gene) and SAL25 (the conditional *hldA* mutant lacking *ugd_{BCAM0855}* but with *ugd_{BCAL2946}* intact) both grew equally well in the presence of either glucose or rhamnose (Fig. 5, third and fourth lanes, respectively). Finally, SAL26, the conditional *ugd_{BCAL2946}* mutant lacking an intact *ugd_{BCAM0855}* gene, grew well in the presence of rhamnose but like XOA11 its growth was very poor in the presence of glucose (Fig. 5, fifth lane).

Next, experiments were carried out to determine which of the three putative *ugd* genes could complement the conditional growth defect of SAL26. In the presence of rhamnose, the growth of SAL26 transformed with any of the plasmids was similar to that of untransformed SAL26 cells (Fig. 5, top right panel). However, growth of SAL26 in the presence of glucose improved significantly in cells transformed with either pSL37 or pSL39 but not with pSL38 or the plasmid control, pDA17 (Fig. 5, bottom right panel). Western blot analysis showed that all three Ugd-FLAG constructs were expressed in SAL26, with protein profiles similar to those in SAL8 (Fig. 4); Ponceau S staining of the membrane showed that there was equal transfer of proteins in each of the lanes (data not shown). Together, these experiments indicate that the combined activity of the proteins encoded by *ugd_{BCAL2946}* and *ugd_{BCAM0855}* is required for the viability of *B. cenocepacia*.

Ugd_{BCAL2946} and Ugd_{BCAM0855} have similar reaction kinetics

To further characterize the proteins encoded by *ugd_{BCAL2946}*, *ugd_{BCAM0855}* and *ugd_{BCAM2034}*, the genes were

Fig. 5. The combined activity of Ugd_{BCAL2946} and Ugd_{BCAM0855} is essential for viability of *B. cenocepacia* strain K56-2. Left panels: K56-2 with pSCrhaB (for growth of wild-type on agar plates with trimethoprim), XOA11 (positive control for a strain with a conditional mutant in an essential gene), SAL10 (negative control, *ugd_{BCAL2946}* conditional mutant in the presence of a functional copy of *ugd_{BCAM0855}*), SAL25 (negative control, *Δugd_{BCAM0855}* strain with a conditional mutation in *hldA*, the gene downstream of *ugd_{BCAL2946}*) and SAL26 (*Δugd_{BCAM0855}* strain with a conditional mutation in *ugd_{BCAL2946}*) were grown overnight, serially diluted, and 10 μ l drops were plated on media containing either rhamnose (inducing conditions, top panel) or glucose (repressing conditions, bottom panel). Mutants with a conditional lethal phenotype grow well on agar plates with rhamnose but grow much more poorly on glucose. Right panels: growth of SAL26 in the presence of either pDA17 (plasmid control), pSL37 (*ugd_{BCAL2946}*), pSL38 (*ugd_{BCAM2034}*) or pSL39 (*ugd_{BCAM0855}*) under inducing or repressing conditions of chromosomally encoded *ugd_{BCAL2946}*. Shown is a representative image of three independent experiments.

cloned into a pET28 vector to add an N-terminal His₆ tag. Ugd_{BCAL2946} and Ugd_{BCAM0855} were overexpressed in *E. coli* and purified using nickel affinity and size exclusion chromatography. The Ugd_{BCAL2946} and Ugd_{BCAM0855} proteins were soluble and behaved as homodimers (~106 kDa) with similar elution profiles (data not shown). Ugd_{BCAM2034} was insoluble and required low-temperature induction and an additional solubilization step prior to purification as a mixture of monomer (~52 kDa) and homodimer (~104 kDa) (data not shown). The enzyme assay was based on the Ugd mechanism proposed by

Campbell *et al.* (2000) and Ge *et al.* (2004): UDP-glucose is converted to UDP-glucuronic acid in a two-step oxidation via a covalently bound thioester intermediate. Conversion of this to the final free UDP-glucuronic acid product requires a second mole of NAD^+ so overall the process reduces two NAD^+ to two NADH. To determine the kinetic parameters, the initial velocity was calculated by measuring the increase in absorbance at 340 nm, due to the production of NADH, divided by two. The data were fitted by non-linear regression with the Hill equation ($V = V_{\text{max}} [S]^h / ([S]^h + K_m^h)$). The enzyme turnover (k_{cat}) and catalytic efficiency (k_{cat}/K_m) were calculated from the V_{max} and K_m values obtained. Both Ugd_{BCAL2946} and Ugd_{BCAM0855} showed Ugd activity with very similar kinetic constants (Table 1), while Ugd_{BCAM2034} showed no *in vitro* activity with UDP-glucose as a substrate. To investigate any substrate specificity variation between the Ugd proteins, the spectrophotometric assay was repeated with three additional UDP-sugars (UDP-galactose, UDP-acetylglucosamine and GDP-mannose) that replaced UDP-glucose. None of these molecules were substrates for any of the enzymes (data not shown). From these experiments we conclude that Ugd_{BCAL2946} and Ugd_{BCAM0855} have very similar enzymic activities and confirm biochemically the functional assignment of these proteins as UDP-glucose dehydrogenases.

Expression of *ugd*_{BCAL2946} is higher than that of *ugd*_{BCAM0855} or *ugd*_{BCAM2034}

Since both Ugd_{BCAL2946} and Ugd_{BCAM0855} demonstrated very similar kinetic parameters, we investigated whether these proteins are differentially expressed *in vivo*. RNA was prepared from *B. cenocepacia* cells in exponential growth and the number of RNA transcripts of each *ugd* gene was quantified and compared to the number of transcripts of the constitutively active gene *hisD*. The number of *ugd*_{BCAL2946} transcripts was on average 5.4 times and 135 times higher than the number of *ugd*_{BCAM0855} or *ugd*_{BCAM2034} transcripts, respectively (Fig. 6). Melting curves for each portion of each gene analysed by real-time PCR were similar to the positive controls and there were no detectable differences in band sizes by agarose gel

electrophoresis (data not shown), indicating that the products amplified after the reverse transcription reaction were the same as those amplified in the positive controls. These results indicate that *ugd*_{BCAL2946} is highly expressed relative to the expression of *ugd*_{BCAM0855}, while the expression of *ugd*_{BCAM2034} under the condition tested (exponential growth) is negligible. These results help to explain why only the mutant defective in *ugd*_{BCAL2946} has a phenotype in terms of pmB sensitivity, and also why the double mutant (Δ *ugd*_{BCAM0855}, *ugd*_{BCAL2946} conditional) fails to grow under non-permissive conditions.

DISCUSSION

We demonstrate here that the proteins encoded by two *B. cenocepacia* genes, *ugd*_{BCAL2946} and *ugd*_{BCAM0855}, possess the predicted dehydrogenase activity, which results in the conversion of UDP-glucose into UDP-glucuronic acid. Ugd_{BCAL2946} and Ugd_{BCAM0855} are efficient catalysts with low micromolar K_m values for UDP-glucose and NAD^+ and relatively fast turnover. The similar catalytic profile for these enzymes is not surprising since they show 74% amino acid sequence identity. The two *P. aeruginosa* Ugd proteins described by Hung *et al.* (2007) utilized UDP-galactose and UDP-*N*-acetylglucosamine as substrates, but with much lower activity compared with UDP-glucose. However, apart from UDP-glucose, the two *B. cenocepacia* Ugd proteins did not exhibit any activity when any of the other three UDP-sugar substrates were used. This substrate specificity correlates with the high catalytic efficiency (k_{cat}/K_m) of the enzymes, and the similar biochemical profiles of Ugd_{BCAL2946} and Ugd_{BCAM0855} explain why they can compensate each other's function *in vivo* during complementation assays.

B. cenocepacia is highly resistant to APs and understanding the molecular basis of this resistance is a longstanding goal of our groups. Here, we show that only *ugd*_{BCAL2946} is necessary for resistance to the AP pmB, as the insertional inactivation of this gene led to an eightfold increase in sensitivity to pmB. This phenotype does not appear to be due to a polar effect on *hldA* and *hldD*, the two genes downstream of *ugd*_{BCAL2946}, based on two experimental

Table 1. Kinetic parameters of Ugd_{BCAL2946} and Ugd_{BCAM0855}

Parameters were determined by non-linear regression from $V = V_{\text{max}} [S]^h / ([S]^h + K_m^h)$ using Origin6.1 software.

Protein	V_{max} (nmol min ⁻¹)	K_m (mM)	h	k_{cat} (min ⁻¹)	k_{cat}/K_m (mM ⁻¹ min ⁻¹)
UDP-glucose					
Ugd _{BCAL2946}	14.38 ± 0.73	0.02 ± 0.01	1.25 ± 0.16	7.99 ± 0.13	392.36 ± 0.37
Ugd _{BCAM0855}	13.73 ± 0.90	0.02 ± 0.01	1.41 ± 0.12	7.63 ± 0.15	340.35 ± 0.10
NAD⁺					
Ugd _{BCAL2946}	16.61 ± 1.11	0.17 ± 0.03	1.51 ± 0.31	9.23 ± 0.15	53.95 ± 0.19
Ugd _{BCAM0855}	16.88 ± 1.22	0.21 ± 0.04	1.50 ± 0.31	9.38 ± 0.16	44.50 ± 0.19

Fig. 6. *ugd_{BCAL2946}* is more highly expressed than either *ugd_{BCAM2034}* or *ugd_{BCAM0855}*. Shown are real-time PCR results from three experiments completed in triplicate; error bars represent the standard error of the mean. For each *ugd* gene the data are standardized to values obtained for the internal control gene, *hisD*. *, Statistically significant difference ($P < 0.01$) compared to results for *ugd_{BCAL2946}* by unpaired *t*-test.

observations: (i) the sensitivity can be restored *in trans* when *ugd_{BCAL2946}* is expressed from a plasmid, and (ii) disruption of *hldA* and *hldD* has a profound effect on the structure of the LPS molecule (Loutet *et al.*, 2006), which we did not observe in the *ugd_{BCAL2946}* mutant. In fact, no differences were found in the LPS profiles of any of the *ugd* mutants compared to the LPS profile of the parental strain, although we cannot rule out subtle modifications in the LPS molecule that would require a more detailed analysis, currently under way in our laboratories. The pmB-sensitive phenotype of the *ugd_{BCAL2946}* mutant was also complemented by a plasmid-encoded *ugd_{BCAM0855}* but not by *ugd_{BCAM2034}*. The lack of complementation by *ugd_{BCAM2034}* was not due to lack of protein expression since all three *ugd* genes revealed a polypeptide of the expected molecular mass when cloned in a plasmid and expressed as protein-FLAG fusions. Also, lack of complementation was not due to the presence of the C-terminal FLAG epitope, since the same results were obtained in experiments using untagged proteins. These data suggest that Ugd_{BCAL2946} accounts for the cellular Ugd activity required for the resistance of *B. cenocepacia* to pmB.

We have previously demonstrated that the synthesis of UDP-Ara4N is essential for the viability of *B. cenocepacia* (Ortega *et al.*, 2007). Since the conversion of UDP-glucose into UDP-glucuronic acid is an obligatory step for the synthesis of UDP-Ara4N, we reasoned that a mutant lacking both *ugd_{BCAL2946}* and *ugd_{BCAM0855}* would not be viable. Indeed, a double mutant in which *ugd_{BCAM0855}* is deleted and *ugd_{BCAL2946}* is conditionally expressed was non-viable under non-permissive conditions. This result suggests that Ugd_{BCAL2946} and Ugd_{BCAM0855} account for the majority of the cellular Ugd activity required for UDP-Ara4N synthesis.

Studies in other Gram-negative bacterial species have shown that the expression of *ugd* genes can be complex. In

Salmonella, a variety of regulatory systems control the expression of the *ugd* gene. In the presence of high Fe^{3+} , *ugd* expression is upregulated by PmrA, the response regulator of the two-component regulatory system PmrA-PmrB (Wösten *et al.*, 2000). Under low Mg^{2+} conditions, activation of the two-component regulatory system PhoP-PhoQ occurs, activated PhoP upregulates expression of another protein, PmrD, which in turn activates the PmrA-PmrB system, and *ugd* expression is upregulated by PmrA (Kox *et al.*, 2000). Finally, the RcsC-YojN-RcsB phosphorelay system can also control the expression of the *ugd* gene in *Salmonella* from a second promoter that is independent of PmrA (Mouslim & Groisman, 2003). A recent study in *P. aeruginosa* demonstrated that this common CF lung pathogen also possesses two *ugd* genes and that the two genes are differentially expressed (Hung *et al.*, 2007). Hung *et al.* (2007) showed that in *P. aeruginosa* strain PAO1 the expression of PA3559 was induced under low Mg^{2+} conditions and that this gene was required for pmB resistance, while PA2022 was more constitutively expressed and was not required for pmB resistance. Similar results were obtained for PA3559 by McPhee *et al.* (2006), who also showed that the promoter of PA3559 has two binding sites each for the PhoP and PmrA proteins of *P. aeruginosa*. With three genes predicted to encode UDP-glucose dehydrogenase, the regulation of Ugd activity may be even more complicated in *B. cenocepacia*. We have shown that in cells in the exponential growth phase *ugd_{BCAL2946}* is more highly expressed than either *ugd_{BCAM0855}* or *ugd_{BCAM2034}*. Little else is known about the regulatory control of expression of *ugd_{BCAL2946}*, *ugd_{BCAM0855}* and *ugd_{BCAM2034}* and it is something we are currently investigating. None of these *ugd* genes is directly linked to the *arn* locus in *B. cenocepacia*, which itself has an unusual gene order (Ortega *et al.*, 2007), and this also suggests that there are differences in operon regulation between *Burkholderia* and other Gram-negative organisms. It is interesting to note that the response regulators of *B. cenocepacia* most similar to the PhoP and PmrA proteins of *P. aeruginosa* and *Salmonella* are not required for pmB resistance (Flannagan *et al.*, 2007), which suggests that they are probably not required for expression of at least *ugd_{BCAL2946}*. *B. cenocepacia* does, however, contain numerous other two-component regulatory systems that could participate in the response to pmB. The finding that *ugd_{BCAL2946}* is more highly expressed than the other two *ugd* genes suggests to us that this difference may account for the fact that sensitivity to pmB is only seen in the *ugd_{BCAL2946}* mutant strain.

We have shown that the protein encoded by *ugd_{BCAM2034}* does not appear to be a player in the total cellular Ugd activity: mutation of the gene neither affects pmB resistance nor is required for the essential synthesis of UDP-Ara4N, the gene cannot rescue the pmB sensitivity of SAL8 or the inability of SAL26 to grow under non-permissive conditions, the gene is poorly expressed in rapidly dividing cells, and the purified form of the protein

does not have *in vitro* Ugd activity under the conditions we tested. Further analysis of the protein encoded by *BCAM2034* is required before an enzymic function can be assigned to it.

In summary, we have demonstrated that the most highly expressed *ugd* gene of *B. cenocepacia* is required for resistance to pmB and that the two most highly expressed genes are required for viability. However, we cannot conclusively state why disruption of *ugd*_{BCAL2946} results in increased sensitivity to pmB. We hypothesize that it is due to small decreases in UDP-Ara4N in the LPS molecule of SAL8 at some point in growth; however, it is also possible that UDP-glucuronic acid is required for some other pathway required for pmB resistance in *B. cenocepacia*. Studies are ongoing in our laboratories to determine why UDP-Ara4N is essential for the survival of *B. cenocepacia*, where Ara4N is located in the LPS molecule and whether there are changes in the Ara4N content of the LPS molecules of our conditional mutants as well as the LPS molecules of the individual *ugd* mutants described in this study.

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