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# The *Burkholderia cepacia bceA* gene encodes a protein with phosphomannose isomerase and GDP-D-mannose pyrophosphorylase activities

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**Abstract**

The *bceA* gene is part of the *Burkholderia cepacia* IST408 exopolysaccharide (EPS) biosynthetic cluster. It encodes a 55.3-kDa bifunctional protein (type II PMI family) with phosphomannose isomerase (PMI) and GDP-mannose pyrophosphorylase (GMP) activities. GMP activity is strongly dependent on the presence of $Ca^{2+}$ or $Mn^{2+}$, while PMI activity can use a broader variety of divalent cations ($Ca^{2+} > Mn^{2+} > Mg^{2+} > Co^{2+} > Ni^{2+}$). The lack of a functional *bceA* gene does not affect EPS production yield in a non-polar insertion *bceA* mutant. The in silico search for putative *bceA* homologues revealed the presence of 2–5 *bceA* orthologues in the *Burkholderia* genomes available. This suggests that in *B. cepacia* IST408 putative *bceA* functional homologues may compensate the *bceA* mutation. However, the viscosity of aqueous solutions prepared with the EPS produced by the *bceA* mutant was significantly reduced compared with wild-type biopolymer and the mutant forms biofilms with a size reduced by 6-fold.


*Keywords:* *Burkholderia cepacia* complex; Exopolysaccharide; Cepacian; Lipopolysaccharide; Phosphomannose isomerase; GDP-mannose pyrophosphorylase; *bceA* gene

A large percentage (around 80%) of *Burkholderia cepacia* complex (*Bcc*) isolates from cystic fibrosis (CF) patients produce the extracellular polysaccharide (EPS) cepacian [1,2]. Although the role of cepacian as a persistence and virulence factor of *Bcc* in CF patients is still unclear [1], the production of this EPS was correlated with enhanced persistence and virulence in animal models [3]. Furthermore, cepacian inhibits neutrophil chemotaxis and scavenges reactive oxygen species in vitro [4], similarly to the alginate produced by *Pseudomonas aeruginosa* in the CF lung [5], suggesting that it may protect the producing bacteria from host defenses.

Cepacian is composed of a branched acetylated heptasaccharide repeating unit with D-glucose, D-rhamnose, D-mannose, D-galactose, and D-glucuronic acid, in the ratio 1:1:1:3:1 [2]. The biochemical pathway for the sugar nucleotides necessary for cepacian biosynthesis was postulated [6]. The *bce* cluster of genes required for cepacian biosynthesis was identified (Fig. 1A), following the isolation of EPS-defective mutants by random plasposon mutagenesis and comparison of the nucleotide sequence of the interrupted genes with *B. cenocepacia* J2315 genome sequence [7]. Based on sequence analysis, *bceA* was postulated to encode a bifunctional type II phosphomannose isomerase (PMI) [7], also exhibiting GDP-mannose pyrophosphorylase (GMP) activity. PMI catalyses the reversible conversion of fructose-6-phosphate into mannose-6-phosphate, while GMP catalyses the reversible conversion of

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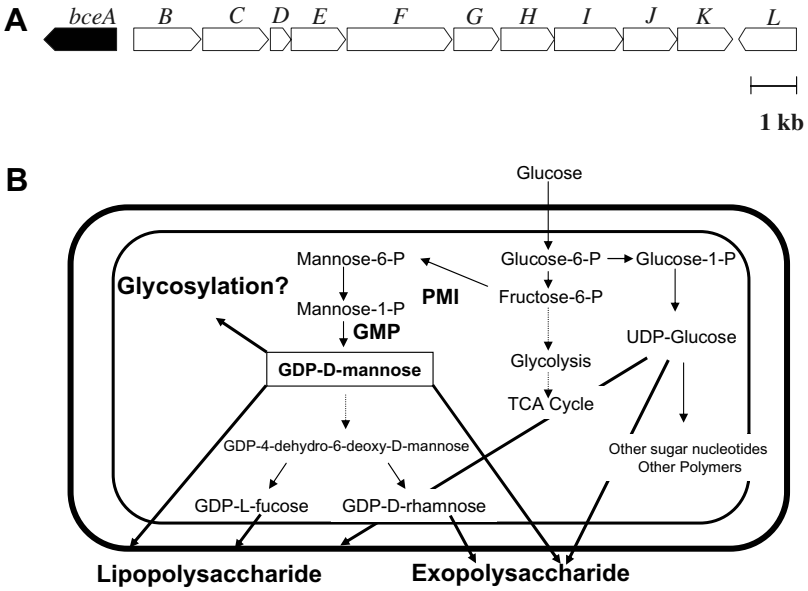


Fig. 1. (A) Physical organization of the *bce* exopolysaccharide biosynthetic locus of *B. cepacia* IST408, evidencing the gene *bceA* (in black). (B) Hypothesized metabolic pathways for mannose in *Bcc* bacteria. The enzyme reactions catalysed by PMI and GMP are indicated.

mannose-1-phosphate into GDP-D-mannose. Biochemically characterized PMI proteins are grouped in three families, types I, II, and III [8]. The characterization of type II PMIs is scarce [8].

In prokaryotes, type II PMIs are involved in several pathways including polysaccharide biosynthesis and D-mannose metabolism [9]. In *B. cepacia*, GDP-mannose is the activated sugar precursor for mannose incorporation in the EPS cepacian and the precursor for the synthesis of GDP-D-rhamnose, the activated sugar precursor of the cepacian rhamnose moiety (Fig. 1B) [6].

This work describes the cloning and functional analysis of *B. cepacia* IST408 *bceA*. The BceA protein was biochemically characterized and confirmed to exhibit both PMI and GMP activities. Based on a non-polar *bceA* mutant prepared during this study, we examined the involvement of BceA in cepacian production yield and rheological properties and in the size of biofilms formed.

## Materials and methods

*Bacterial strains, plasmids, and culture conditions.* The cepacian producer *B. cepacia* IST408 [1,2,6] and the mutant *B. cepacia* IST408::*bceA* were used and maintained in PIA (Difco) plates, containing 100 μg/ml trimethoprim for *B. cepacia* IST408::*bceA*. *Escherichia coli* αDH5 was used to host plasmids. *E. coli* BL21(DE3) was used for overproduction of His-tagged BceA. *E. coli* recombinant strains were grown in LB supplemented with ampicillin (150 μg/ml), kanamycin (50 μg/ml), chloramphenicol (25 μg/ml), or trimethoprim (100 μg/ml).

*Molecular biology techniques.* Molecular biology techniques were performed using standard procedures [10]. "Gene Images Random Prime Labelling" and "Gene images CDP-Star Detection Module" kits (Amersham) were used for DNA labelling and detection of hybridization bands, respectively.

*Cloning, overproduction, and purification of His-tagged BceA.* The gene *bceA* was amplified from *B. cepacia* IST408 genomic DNA using primers bccANheI (5′-AAAGCTAGCATGAATGCTCCGGCAG-3′) and bccA-HindIII (5′-AAAAGCTTCTGCCGGCCGTAGGTA-3′), designed based on the *bceA* gene sequence of *B. cenocepacia* J2315. After amplification and agarose gel electrophoresis, the 1541 bp PCR product was purified from the gel, digested with *Nhe*I and *Hin*dIII, and ligated directionally into pET23a+ to create pSASbceA8.

*Escherichia coli* BL21(DE3) cells harbouring pSASbceA8 were grown in SB [14] liquid medium containing 150 μg/ml ampicillin, at 30 °C, to an $OD_{640}$ of 0.6. Induction was started by the addition of 0.4 mM IPTG, followed by additional 3 h of incubation. The His-tagged BceA was purified from *E. coli* lysates in phosphate buffer (20 mM sodium phosphate, 0.5 mM NaCl, pH 7.4) containing 10 mM imidazole. After removal of cell debris by centrifugation at 20,000*g* for 1 h at 4 °C, the His-tagged BceA was purified by affinity chromatography using a Hi-Trap column (Amersham) according to the manufacturer's instructions. After loading the column with *E. coli* lysates containing the overproduced His-tagged BceA, the matrix was washed with phosphate buffer. Bound proteins were eluted with 5 ml of sodium phosphate buffer containing increasing concentrations of imidazole (60–500 mM). Proteins were visualized after SDS–PAGE and staining with Coomassie brilliant blue R-250.

*PMI and GMP enzyme activity assays.* The PMI and GMP activities were assayed in the reverse direction as previously described [6]. Enzyme activities were calculated from the initial linear rates of cofactor reduction.

The reaction mixture for PMI assay contained, in 1 ml, 50 mM Tris buffer, pH 7.6, 5 mM $MgCl_2$, 1 mM NADP, 1 U each of glucose-6-phosphate dehydrogenase and phosphoglucose isomerase, and mannose-6-phosphate concentrations ranging from 0.5 to 25 mM. The reaction was initiated by the addition of 1 μg of purified His-tagged BceA.

The reaction mixture for GMP assay contained, in 1 ml, 50 mM Tris buffer, pH 7.6, 4 mM glucose, 1 mM ADP, 1 mM NADP, 10 mM $MnCl_2$, 1 U each of hexokinase, nucleoside-5′-diphospho-kinase, and glucose-6-phosphate dehydrogenase, and GDP-mannose concentrations ranging from 0.05 to 5 mM. The reaction was initiated by the addition of 1 μg of His-tagged BceA and 2 mM sodium pyrophosphate.

PMI and GMP activities were also determined in cell-free crude extracts from *B. cepacia* IST408 or *B. cepacia* IST408 *bceA*::Tp.

Protein concentration was estimated with bovine serum albumin fraction *V* (Merck) as standard [11].

One unit of enzyme activity was defined as the amount of enzyme that catalysed the reduction of 1 μmol of NADP per minute under the assay conditions. Specific activity was expressed as units of enzyme activity per

mg of purified BceA or per mg of total protein in crude extracts. Results are the mean values of at least three enzyme assays using two independently prepared fractions of purified His-tagged BceA.

*Construction of a bceA insertion mutant from B. cepacia IST408.* The 986 bp *Xba*I fragment from pUC-Tp [12] containing the trimethoprim (Tp) cassette was inserted in the single *Cla*I restriction site of the *bceA* gene, creating pSASbceA8T. A 2.51-kb *Xba*I–*Hin*dIII fragment from pSASbceA8T containing the inactivated *bceA* gene was inserted into pDrive to form pSASbceA8TD. This plasmid was introduced into *B. cepacia* IST408 by electroporation [10]. Insertional inactivation of the *bceA* gene was confirmed by PCR and Southern hybridization.

*EPS production and properties.* EPS production by *B. cepacia* IST408 and the mutant IST408 *bceA*::Tp was quantified as described before [6]. For viscosity determinations and acetyl content estimation EPSs from 72 h cultures were recovered and purified as follows: after ethanol precipitation and drying, EPSs were dissolved in water, centrifuged for 1 h at 20,000*g*, extensively dialysed (cut-off 12 kDa), and lyophilised. The viscosity of 5 g/L aqueous solutions of purified EPSs was measured at 30 °C using a cone and plate Brookfield Viscometer, Model LVIIT. The acetyl content of the exopolysaccharides produced was determined using glucose pentaacetate as standard [13]. Glucose and galactose content of the polysaccharide was quantified by enzyme assays after hydrolysis of the EPS with HCl and subsequent neutralization [14,15].

Results are mean values of at least three independent experiments.

*Biofilm formation assay.* Biofilm formation assays were performed based on previously described methods [1]. Results are mean values of at least five repeats from three independent experiments.

*Nucleotide and amino acid sequence analysis.* DNA sequences were analysed using ORFinder (NCBI). The algorithm BLAST [16] was used to compare deduced amino acid sequences with sequences available in databases. Amino acid sequence alignments and analysis were performed with programs CLUSTALW [17], Protparam, and MotifScan.

The amino acid or nucleotide sequences of *bceA* homologues were obtained from the DOE Joint Genome Institute (*B.* strain 383, *B. cenocepacia* strains AU1054 and HI2424, *B. vietnamiensis* G4, *B. ambifaria* AMMD, and *B. xenovorans* LB400), the Wellcome Trust Sanger Institute (*B. cenocepacia* J2315 and *B. pseudomallei* K96243), the Broad Institute (*B. dolosa* AU0158), and the Institute for Genomic Research (*B. mallei* ATCC 23344 and *B. thailandensis* E264) websites. The phylogenetic tree of BceA homologues was constructed with CLUSTALX 1.81 using the neighbour-joining method with a minimum of 100 bootstraps.

*Nucleotide sequence accession number.* The *bceA* gene nucleotide sequence was deposited in GenBank under the Accession No. DQ463418.

## Results and discussion

### *Cloning and sequence analysis of B. cepacia IST408 bceA*

The translated nucleotide sequence of the 1541 bp DNA fragment obtained from *B. cepacia* IST408 total DNA with primers bccANheI and bccAHindIII, exhibitied a similarity higher than 70% with putative type II PMIs from several strains of β- and γ-proteobacteria with their genome sequences deposited in GenBank (data not shown). The *B. cepacia* IST408 BceA was predicted to be a 55.3-kDa protein with a p*I* of 6.03. This is consistent with the 55-kDa molecular mass estimated by SDS–PAGE analysis. Analysis

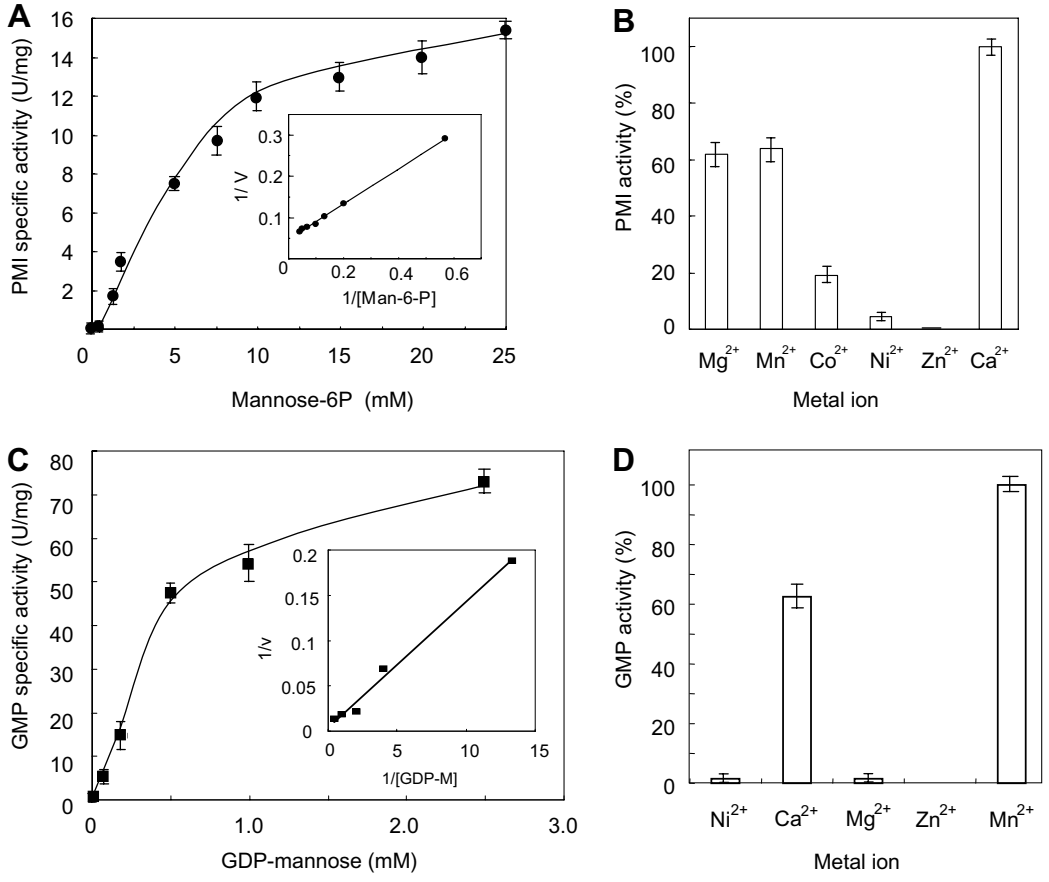


Fig. 2. Kinetic analysis of the (A) PMI and (C) GMP activities of the $His_6$-BceA, and effect of the different divalent cations on PMI (B) and GMP (D) activities. (B,D) Values are given as the percentage of maximal activity of PMI or GMP, registered with $Ca^{2+}$ or $Mn^{2+}$ (set as 100%), respectively.

of BceA sequence allowed the identification of (i) the nucleotidyl transferase domain, found in enzymes which transfer nucleotides onto phosphosugars [8] spanning amino acid residues 27–326, (ii) the mannose-6-phosphate isomerase domain spanning amino acid residues 337–503, typical of PMIs [8], (iii) a cupin signature domain, characterized by six β-strands within a conserved β-barrel structure [9], spanning amino acid residues 425–476. The alignment of BceA with *P. aeruginosa* AlgA [18] and *X. campestris* XanB [19] (both with experimentally demonstrated PMI and GMP activities) indicated that BceA amino acid sequence is 50% identical and 67% similar to AlgA and 53% identical and 69% similar to XanB. This analysis also evidenced the conservation of: (i) the N-terminal highly conserved pyrophosphorylase signature sequence $^{34}$GXGXRLXPLX$_5$PK$^{49}$ [8], (ii) the active site $^{205}$FVEKP$^{209}$ [18], and (iii) the zinc binding motif $^{431}$QXH$^{433}$ [8] (X represents any amino acid).

### *BceA exhibits PMI and GMP activities*

The PMI and GMP activities of His-tagged BceA (purified by affinity chromatography) were both determined in the reverse direction. For the PMI activity, a $V_{max}$ of 21 U/mg, a $K_m$ of 9.01 mM, and a $K_{cat}$ of 19.4 $s^{-1}$ were calculated (Fig. 2A), using $Mg^{2+}$ as co-factor at a final concentration of 5 mM. Divalent ions were found necessary for PMI maximal activity, being the order of activation $Ca^{2+} > Mn^{2+} > Mg^{2+} > Co^{2+} > Ni^{2+}$ (Fig. 2B). No PMI activity was detected when using $Zn^{2+}$ as co-factor. No PMI activity was detected when 2 mM EDTA was added to the dialysis buffer prior to enzyme activity assay, indicating that PMI is a metalloenzyme.

The PMI-catalysed reaction was not affected when mannose-1-phosphate, GTP, GDP-mannose or pyrophosphate was added to the reaction mixture, at a final concentration of 1 mM.

For the GMP activity, a $V_{max}$ of 212 U/mg, a $K_m$ of 2.9 mM, and a $K_{cat}$ of 195.9 $s^{-1}$ were calculated, using 10 mM of $Mn^{2+}$ and 2 mM pyrophosphate (final concentrations) (Fig. 2C). GMP activity was specifically dependent on the presence of either $Mn^{2+}$ or $Ca^{2+}$ as co-factors (Fig. 2D). The eventual effect of $Co^{2+}$ on GMP activity could not be determined, since $Co^{2+}$ addition to the reaction mixture led to a visible precipitate. Requirement for divalent cations is characteristic of other microbial type II PMIs [8,18,19]. The kinetic parameters $K_m$ and $V_{max}$ obtained for the BceA PMI reaction are comparable to those indicated for the PMI reaction of *P. aeruginosa* AlgA (3.03 mM for $K_m$ and 0.87 U/mg for $V_{max}$, respectively) [18]. However, for the GMP reaction of BceA the $K_m$ and $V_{max}$ values significantly differ from those reported for *P. aeruginosa* AlgA ($K_m$ 0.014 mM and $V_{max}$ 5.17 U/mg [18]).

### *Construction and characterization of a bceA mutant*

A mutant with the *bceA* gene inactivated was constructed and confirmed by Southern blot and PCR (results not shown). The growth curves of the mutant and the wild-type strains in S liquid medium were identical, as well as the amount of EPS produced (Fig. 3A). Apparently, the EPSs

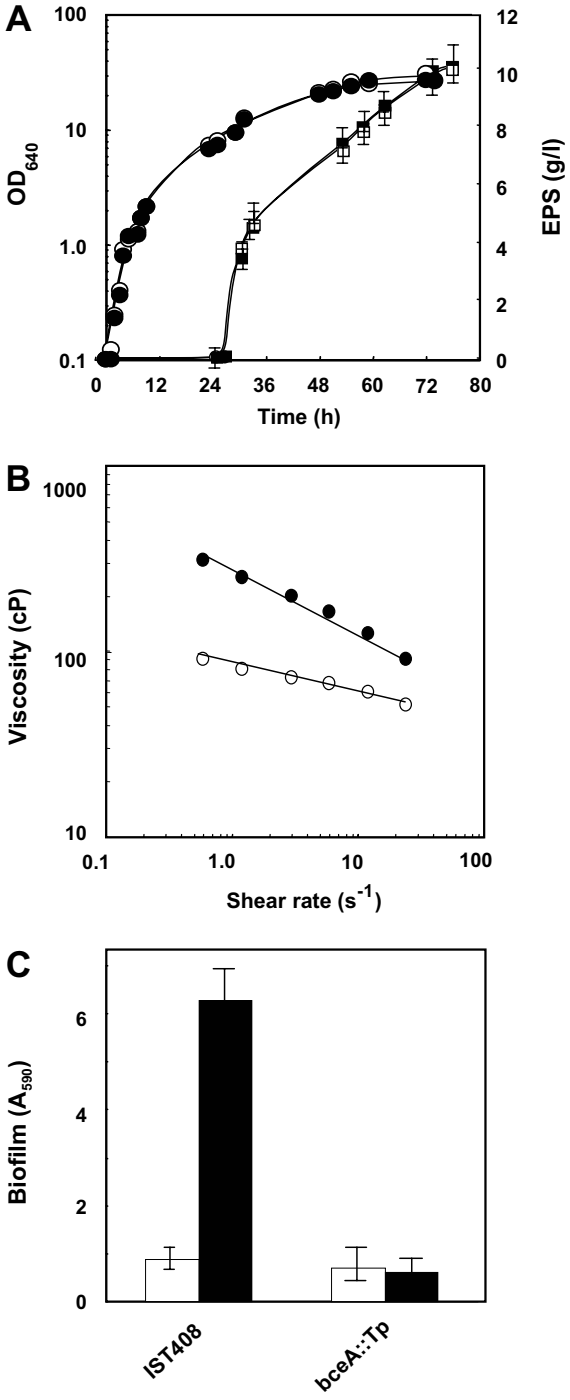


Fig. 3. (A) Comparison of *B. cepacia* IST408 (closed symbols) and the *bceA* mutant (open symbols) growth curves (circles) and amount of cepacian produced in S liquid medium (squares), at 30 °C with orbital agitation; (B) viscosity of 5 g/L aqueous solutions prepared with purified EPSs recovered from cultures after 72 h of incubation; (C) size of the biofilm formed by *B. cepacia* IST408 and the *bceA* mutant strain in polystyrene microtitre plates after 24 (white bars) or 48 h (black bars). $A_{590}$ quantifies the amount of crystal violet associated with the biofilm after staining.

produced are identical with respect to neutral sugar composition, with glucose and galactose in a ratio 1:3. The average number of acetyl groups per repeating unit is also similar ($3.3 \pm 0.2$ and $3.0 \pm 0.1$ for the wild-type and the mutant polymers, respectively). Contrasting with our results, mutations in *X. campestris xanB* and in *P. aeruginosa algA*, both encoding type II PMIs, led to the abolishment of xanthan and alginate production, respectively [18,19].

These results suggest that PMI and GMP global cell activities are encoded by other genes besides *bceA*. This hypothesis is consistent with the observation that cell-free crude extracts of the *bceA* mutant still exhibit 50% and 80% of the PMI and GMP activities detected in extracts from the wild-type strain (results not shown). Furthermore, Southern blot experiments using *Eco*RI-digested total DNA from *B. cepacia* IST408 and the *bceA* gene as probe originated a strong hybridization band and an additional hybridization band of weaker intensity, indicating the presence of DNA regions with significant homology to BceA.

The search for *bceA* homologues within the complete and partial genome sequences of 11 strains from 9 *Burkholderia* species revealed the presence of 2–5 *bceA* homologues in the genomes examined (Table 1). In *P. aeruginosa*, 3 type II PMI-encoding genes were reported [20].

However, the viscosity of aqueous solutions prepared with the same concentration of the purified EPSs recovered from cell-free supernatants of 72 h cultures of the mutant was remarkably below the wild-type EPS solution viscosity (Fig. 3B), suggesting that the mutant produces, with an identical yield, a similar EPS of reduced molecular mass.

Recently, it was demonstrated that cellular concentration of nucleotide sugar precursors is critical in determining capsular polysaccharide chain length [21]. It is likely that the intracellular pool of GDP-mannose, together with

Table 1
Identity and similarity of BceA and the putative type II PMIs identified in the indicated *Burkholderia* strains

| Species | Chromosome/contig | Identity/similarity (%) | Accession No. or Gene No./localization |
|---|---|---|---|
| *B.* strain 383 | Contig 233 | 49/65 | Bcep18194A3856 |
| | | 42/57 | Bcep18194A3979 |
| | Contig 234 | 61/74 | Bcep18194B1812 |
| | | 96/98 | Bcep18194B2277 |
| *B. cenocepacia* J2315 | Chromosome I | 49/66 | nt 3,554,927–3,556,411 |
| | Chromosome II | 92/94 | nt 942,079–940,541 |
| | | 60/74 | nt 1,487,810–1,486,365 |
| *B. cenocepacia* AU1054 | Contig 94 | 60/74 | 4160 |
| | Contig 94 | 91/94 | 4544 |
| | Contig 95 | 42/60 | 0397 |
| *B. cenocepacia* HI2424 | Contig 356 | 60/74 | 2891 |
| | Contig 363 | 91/94 | 3414 |
| | Contig 384 | 49/65 | 6044 |
| *B. vietnamiensis* G4 | Contig 161 | 61/74 | Gene 2428 |
| | Contig 164 | 64/76 | Gene 2694 |
| | Contig 174 | 89/93 | Gene 3606 |
| | Contig 176 | 42/57 | Gene 3860 |
| | Contig 192 | 60/71 | Gene 6676 |
| *B. dolosa* AUO158 | Supercontig 1.1 | 45/59 | nt 2,859,918–2,858,464 |
| | Supercontig 1.2 | 89/93 | nt 24,594–23,102 |
| | | 60/73 | nt 539,661–538,216 |
| *B. ambifaria* AMMD | Contig 38 | 46/59 | Gene 3456 |
| | Contig 39 | 60/74 | Gene 3834 |
| | Contig 41 | 91/94 | Gene 4884 |
| | Contig 42 | 60/72 | Gene 6151 |
| *B. pseudomallei* K96243 | Chromosome I | 63/74 | CAH34598 |
| | | 48/64 | CAH36820 |
| | Chromosome II | 78/88 | CAH39311 |
| *B. mallei* ATCC23344 | Chromosome I | 63/74 | AAU48935 |
| | | 48/64 | AAU49814 |
| *B. xenovorans* LB400 | Chromosome I | 68/79 | ABE30721 |
| | | 61/72 | ABE29131 |
| | | 58/70 | ABE30459 |
| | Chromosome II | 62/75 | ABE34236 |
| | Chromosome III | 61/75 | ABE36957 |
| *B. thailandensis* E264 | Chromosome I | 63/74 | I0522 |
| | | 50/65 | I1324 |
| | Chromosome II | 77/87 | II0542 |

Putative proteins are identified by their Accession No., gene code used by the respective sequencing project, or nucleotide (nt) localization. BceA orthologues with a percentage of identity/similarity higher than 65/75 are underlined.

GDP-rhamnose, may limit the rate of formation of the repeating unit for polymerization in the *bceA* mutant.

The ability of both the wild-type strain and the *bceA* mutant to form biofilms in vitro was assessed using experimental conditions previously shown to lead to maximal EPS production and biofilm formation [1]. During the first 24 h of incubation, when the amounts of EPS produced by both wild-type and *bceA* mutant cultures are negligible (Fig. 3A), the amounts of biofilm formed are similar (Fig. 3C). After 48 h of incubation following the production of a significant concentration of EPS, the amount of biofilm formed by the wild-type strain was 6-fold higher compared to the mutant strain (Fig. 3C). This observation indicates that a functional *bceA* is required for the formation of biofilms of maximal size, while no involvement in cepacian production yield, under the experimental conditions used, could be demonstrated. It is possible that the modified rheological properties of the EPS produced by the *bceA* mutant affect the development of biofilms of maximal size. In *Stenotrophomonas maltophilia* WR-C, the disruption of the *bceA* gene homologue *xanB* also led to a drastic reduction of the bacteria ability to form biofilms, together with a reduced ability to produce EPS and alterations in LPS structure [22]. However, we were unable to detect any differences when comparing the wild-type and mutant LPS profiles by SDS–PAGE analysis (unpublished).

### *Phylogenetic analysis of BceA homologues from Burkholderia strains*

The alignment of the BceA orthologues indicated in Table 1 revealed that the encoding genes with highest degree of identity/similarity with *bceA* all belong to clusters similar to the *bce* gene cluster involved in polysaccharide biosynthesis (cluster A in Fig. 4 and underlined in Table 1). No *bce* gene cluster could be identified in the genome of *B. mallei*. The BceA homologues in cluster B Fig. 4 belong to the capsular polysaccharide genomic island [23]. Although I1324 was included in cluster B, only part of this genomic island was identified in *B. thailandensis* E264 genome. Other BceA orthologues are located mainly within clusters presumably involved in LPS biosynthesis.

In conclusion, this work demonstrates that *bceA* encodes a type II PMI with both PMI and GMP activities. Although apparently not required for the production of maximal levels of cepacian, inactivation of the *bceA* gene leads to the production of cepacian with rheological properties distinct from wild-type and to the impairment of the mutant to form biofilms with maximal size. Data here

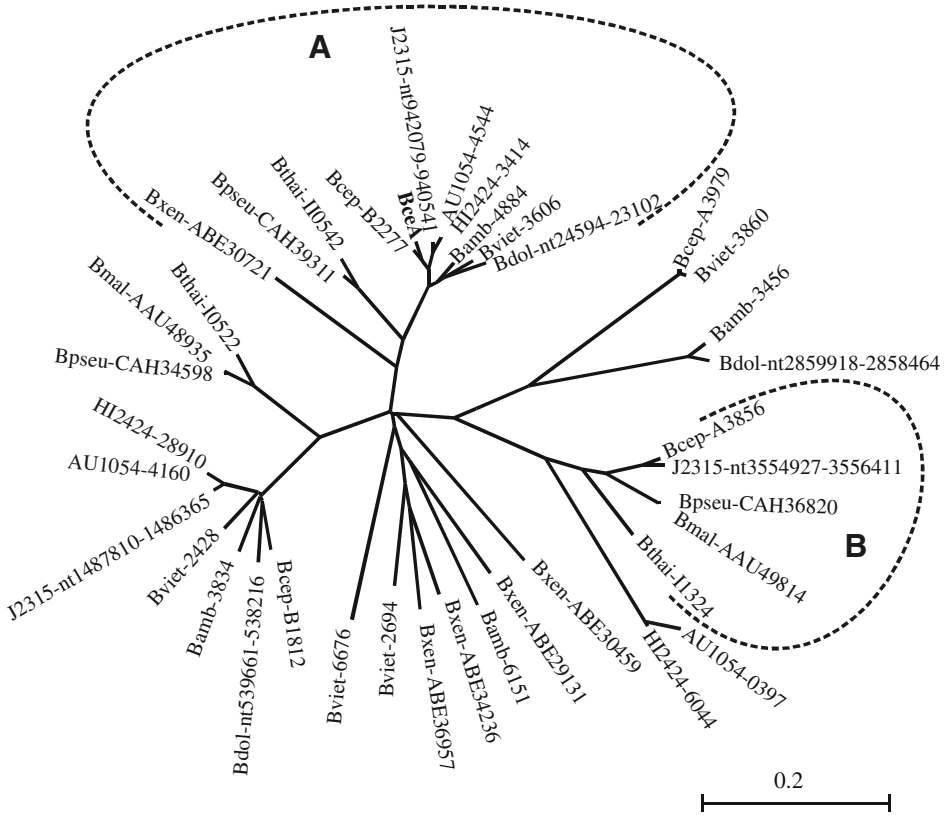


Fig. 4. Unrooted phylogenetic tree for *B. cepacia* IST408 BceA and the putative BceA orthologues (identified as in Table 1) from *Burkholderia* strain 383, *B. cenocepacia* strains J2315, AU1054 and HI2424, *B. dolosa* AUO158, *B. ambifaria* AMMD, *B. vietnaminensis* G4, *B. xenovorans* LB400, *B. thailandensis* E264, *B. mallei* ATCC 23344, and *B. pseudomallei* K96243. (Cluster A) Putative type II PMIs located in gene clusters similar to the *bce* gene cluster involved in cepacian biosynthesis [7]. (Cluster B) Putative type II PMIs located in gene clusters similar to the capsular polysaccharide genomic island [23].

presented strongly support the hypothesis that the phenotypes observed for the *bceA* mutant most probably result from additional functional BceA homologues encoded within *B. cepacia* IST408 genome.

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## References

[1] M.V. Cunha, S.A. Sousa, J.H. Leitão, L.M. Moreira, P.A. Videira, I. Sá-Correia, Studies on the involvement of the exopolysaccharide produced by cystic fibrosis-associated isolates of the *Burkholderia cepacia* complex in biofilm formation and in persistence of respiratory infections, J. Clin. Microbiol. 42 (2004) 3052–3058.

[2] P. Cescutti, M. Bosco, F. Picotti, G. Impallomeni, J.H. Leitão, J.A. Richau, I. Sá-Correia, Structural study of the exopolysaccharide produced by a clinical isolate of *Burkholderia cepacia*, Biochem. Biophys. Res. Commun. 273 (2000) 1088–1094.

[3] B.A. Conway, K.K. Chu, J. Bylund, E. Altman, D.P. Speert, Production of exopolysaccharide by *Burkholderia cenocepacia* results in altered cell-surface interactions and altered bacterial clearance in mice, J. Infect. Dis. 190 (2004) 957–966.

[4] J. Bylund, L.A. Burgess, P. Cescutti, R.K. Ernst, D.P. Speert, Exopolysaccharides from *Burkholderia cenocepacia* inhibit neutrophil chemotaxis and scavenge reactive oxygen species, J. Biol. Chem. 281 (2006) 2526–2532.

[5] S.S. Pedersen, A. Kharazmi, F. Espersen, N. Høiby, *Pseudomonas aeruginosa* alginate in cystic fibrosis sputum and the inflammatory response, Infect. Immun. 58 (1990) 3363–3368.

[6] J.A. Richau, J.H. Leitão, I. Sá-Correia, Enzymes leading to the nucleotide sugar precursors for exopolysaccharide synthesis in *Burkholderia cepacia*, Biochem. Biophys. Res. Commun. 276 (2000) 71–76.

[7] L.M. Moreira, P.A. Videira, S.A. Sousa, J.H. Leitão, M.V. Cunha, I. Sá-Correia, Identification and physical organization of the gene cluster involved in the biosynthesis of *Burkholderia cepacia* complex exopolysaccharide, Biochem. Biophys. Res. Commun. 312 (2003) 323–333.

[8] B. Wu, Y. Zhang, R. Zheng, C. Guo, P.G. Wang, Bifunctional phosphomannose isomerase/GDP-mannose pyrophosphorylase is the point of control for GDP-D-mannose biosynthesis in *Helicobacter pylori*, FEBS Lett. 519 (2002) 87–92.

[9] J.M. Dunwell, S. Khur, P.J. Gane, Microbial relatives of the seed storage proteins of higher plants: conservation of structure and diversification of function during evolution of the cupin superfamily, Microbiol. Mol. Biol. Rev. 64 (2000) 153–179.

[10] J. Sambrook, E.F Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, second ed., Cold Spring Harbour Laboratory Press, New York, 1989.

[11] M.M. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.

[12] P.A. Sokol, P. Darling, D.E. Woods, E. Mahenthiralingam, C. Kooi, Role of ornibactin biosynthesis in the virulence of *Burkholderia cepacia*: characterization of *pvdA*, the gene encoding L-ornithine N(5)-oxygenase, Infect. Immun. 67 (1999) 4443–4455.

[13] E.A. McComb, R.M. McCready, Determination of acetyl in pectin and acetylated carbohydrate polymers, Anal. Chem. 29 (1957) 819–821.

[14] H.O. Beutler, Lactose and D-galactose: UV method, in: H.U. Bergemeyer (Ed.), Methods of Enzymatic Analysis, Verlag Chemie, Weinheim, 1984, pp. 104–112.

[15] A. Kunst, B. Draeger, J. Zoegenhorn, UV methods with hexokinase and glucose-6-phosphate dehydrogenase, in: H.U. Bergemeyer (Ed.), Methods of Enzymatic Analysis, Verlag Chemie, Weinheim, 1984, pp. 163–172.

[16] S.F. Altschul, T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search Programs, Nucleic Acids Res. 25 (1997) 3389–3402.

[17] J.D. Thompson, D.G. Higgins, T.J. Gibson, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Res. 22 (1994) 4673–4680.

[18] D. Shinabarger, A. Berry, T.B. May, R. Rothmel, A.M. Fialho, A.M. Chakrabarty, Purification and characterization of phosphomannose isomerase-guanosine diphospho-D-mannose pyrophosphorylase: a bifunctional enzyme in the alginate biosynthetic pathway of *Pseudomonas aeruginosa*, J. Biol. Chem. 266 (1991) 2080–2088.

[19] R. Köplin, W. Arnold, B. Hötte, R. Simon, G. Wang, A. Pühler, Genetics of xanthan production in *Xanthomonas campestris*: the *xanA* and *xanB* genes are involved in UDP-glucose and GDP-mannose biosynthesis, J. Bacteriol. 174 (1992) 191–199.

[20] H.L. Rochetta, J.C. Pacan, J.S. Lam, Synthesis of the A-band polysaccharide sugar D-rhamnose requires RmD and WbpW: identification of multiple AlgA homologues, WbpW and ORF488, in *Pseudomonas aeruginosa*, Mol. Microbiol. 29 (1998) 1419–1434.

[21] C.L. Ventura, R.T. Cartee, W.T. Forsee, J. Yother, Control of capsular polysaccharide chain length by UDP-sugar substrate concentrations in *Streptococcus pneumoniae*, Mol. Microbiol. 61 (2006) 723–733.

[22] T.-P. Huang, E.B. Somers, A.C.L. Wong, Differential biofilm formation and motility associated with lipopolysaccharide/exopolysaccharide-coupled biosynthetic genes in *Stenotrophomonas maltophilia*, J. Bacteriol. 188 (2006) 3116–3120.

[23] Y.N. Parsons, R. Banasko, M.G. Detsika, K. Duangsonk, L. Rainbow, C.A. Hart, C. Winstanley, Suppression-subtractive hybridization reveals variations in gene distribution amongst the *Burkholderia cepacia* complex, including the presence in some strains of a genomic island containing putative polysaccharide production genes, Arch. Microbiol. 179 (2003) 214–223.