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Distribution of Cepacian Biosynthesis Genes among Environmental and Clinical *Burkholderia* Strains and Role of Cepacian Exopolysaccharide in Resistance to Stress Conditions[▽]

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The genus *Burkholderia* includes strains pathogenic to animals and plants, bioremediators, or plant growth promoters. Genome sequence analyses of representative *Burkholderia cepacia* complex (*Bcc*) and non-*Bcc* strains for the presence of the *bce-I* gene cluster, directing the biosynthesis of the exopolysaccharide (EPS) cepacian, further extended this previously described cluster by another 9 genes. The genes in the *bce-II* cluster were named *bceM* to *bceU* and encode products putatively involved in nucleotide sugar precursor biosynthesis and repeat unit assembly, modification, and translocation across the cytoplasmic membrane. Disruption of the *B. cepacia* IST408 *bceQ* and *bceR* genes, encoding a putative repeat unit flippase and a glycosyltransferase, respectively, resulted in the abolishment of cepacian biosynthesis. A mutation in the *bceS* gene, encoding a putative acyltransferase, did not affect EPS production yield significantly but decreased its acetylation content by approximately 20%. Quantitative real-time reverse transcription-PCR experiments confirmed the induction of genes in the *bce-I* and *bce-II* clusters in a *Burkholderia multivorans* EPS producer clinical isolate in comparison to the level for its isogenic EPS-defective strain. Fourier Transform infrared spectroscopy analysis confirmed that the exopolysaccharide produced by 10 *Burkholderia* isolates tested was cepacian. The ability of *Burkholderia* strains to withstand desiccation and metal ion stress was higher when bacteria were incubated in the presence of 2.5 g/liter of cepacian, suggesting that this EPS plays a role in the survival of these bacteria by contributing to their ability to thrive in different environments.

Many bacteria produce exopolysaccharides (EPSs), which play a wide range of roles in their biology. Besides their contribution to the fitness of the producing microorganism to their ecological niche (14, 36), EPSs are often important virulence determinants produced by pathogens of plants, animals, and humans. Cepacian is the major EPS produced by a large percentage of clinical isolates of the *Burkholderia cepacia* complex (*Bcc*) (11, 20, 37, 52). The *Bcc* comprises at least 17 distinct bacterial species, including soil and water saprophytes, rhizosphere parasites, bioremediators, plant growth promoters, and plant and animal pathogens (49). *Bcc* members are receiving particular attention due to their increasingly recognized importance as opportunistic pathogens in immunocompromised patients and in patients suffering from cystic fibrosis (CF) or chronic granulomatous disease (CGD) (27).

Several studies have pointed out cepacian as a virulence factor contributing to the overall pathogenicity of *Bcc* members and thus to their success as pathogens. For instance, Conway et al. (10) have shown that the EPS produced by a mucoid *Burkholderia cenocepacia* clinical isolate interfered with phagocytosis of bacteria by human neutrophils and facilitated bacterial persistence in the BALB/c mice model of infection. In a study performed using the gp91$^{phox-/-}$ CGD mouse model of infection, Sousa et al. (43) have shown that mutants defective in cepacian production were less virulent than the wild-type cepacian-producing strain or completely avirulent. Cepacian was also found to inhibit neutrophil chemotaxis and the production of reactive oxygen species, both essential components of the innate host defenses (5). The persistence of infections has been correlated with the ability of bacterial pathogens to form biofilms. Several studies have demonstrated the ability of the *Bcc* to form biofilms alone or together with other bacteria (11, 23). Studies performed with cepacian-defective mutants have demonstrated that, although not required for the initiation of biofilm formation, cepacian is required to the formation of thick and mature biofilms (11).

Cepacian is composed of a branched acetylated heptasaccharide repeat unit with D-glucose, D-rhamnose, D-mannose, D-galactose, and D-glucuronic acid in the ratio 1:1:1:3:1 (6). The biochemical pathway leading to the activated sugar nucleotides necessary for repeat unit formation was postulated, and the predicted enzyme activities were detected in crude extracts prepared from a cepacian-producing *Bcc* clinical isolate (38). A strategy based on random plasposon mutagenesis of the cepacian producer clinical isolate *B. cepacia* IST408 allowed the identification of the 16.2-kb *bce* cluster of genes involved in cepacian biosynthesis (31).

Although the *bce* clustered genes encode several proteins and enzymes required for the biosynthesis of the EPS (31), not

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristic(s)[a]	Source or reference
Strains		
B. cepacia IST408	Cystic fibrosis clinical isolate; cepacian producer	37
B. multivorans ATCC 17616	Soil isolate	48
B. cenocepacia J2315	Cystic fibrosis clinical isolate	19
B. vietnamiensis G4	Isolated from a water treatment facility	32
B. dolosa AUO158	Cystic fibrosis clinical isolate	8
B. ambifaria AMMD	Root-colonizing bacterium	9
B. lata 383	Soil isolate	44
B. phytofirmans PsJN	Soil isolate; plant growth-promoting bacterium	40
B. phymatum STM815	Soil isolate; nitrogen fixation	47
B. xenovorans LB400	Soil isolate; degradation of polychlorinated biphenyl compounds	18
B. cepacia IST408 *bceQ*::pIS58-1	pIS58-1 integrated into the *bceQ* gene region	This work
B. cepacia IST408 *bceR*::pIS58-2	pIS58-2 integrated into the *bceR* gene region	This work
B. multivorans ATCC 17616 *bceS*::pSF71-8	pSF71-8 integrated into the *bceS* gene region	This work
B. multivorans D2095	Mucoid cystic fibrosis clinical isolate	D. P. Speert
B. multivorans D2214	Nonmucoid cystic fibrosis clinical isolate	D. P. Speert
Escherichia coli XL1-Blue	*recA1 lac* (F′*proAB lacI*^{q} ZαM15 Tn*10* [Tc^r]) *thi*	4
Plasmids		
pDrive	3.85-kb vector; *lacZ* α-peptide; Ap^r Km^r	Qiagen
pBCKS	3.4-kb phagemid derived from pUC19; *lac* promoter; Cm^r	Stratagene
pMLBAD	pBBR1 *ori*; *araC*-P_{BAD}; Tp^r; *mob*$^+$	25
pSF71-8	pBCKS derivative carrying a 579-bp EcoRI/XbaI fragment with an internal fragment from the *bceS* gene	This work
pIS58-1	pDrive derivative carrying a 1,133-bp HindIII fragment with an internal fragment from the *bceQ* gene	This work
pIS58-2	pDrive derivative carrying a 1,028-bp HindIII/XbaI fragment with an internal fragment from the *bceR* gene	This work
pIS94-1	pMLBAD derivative carrying a 2,509-bp KpnI/HindIII fragment with the coding region of the *bceR* gene	This work

[a] Ap, ampicillin; Km, kanamycin; Tp, trimethoprim; Cm, chloramphenicol.

all the proteins required for cepacian biosynthesis are encoded within this cluster. In the present work, we report the identification and partial functional analysis of a second cluster of genes, here named *bce-II*. The *bce-II* cluster contains genes encoding enzymes putatively involved in the synthesis of the D-rhamnose and D-glucose moieties of cepacian, a glycosyltransferase, a repeat unit flippase, and acyltransferases presumably required for the acetylation of cepacian. We also report results showing that the *bce-I* and *bce-II* clusters are widespread within all the sequenced *Burkholderia* strains, with the exception of *Burkholderia mallei* strains. In agreement with the results of these *in silico* studies, the abilities of several *Bcc* and non-*Bcc* strains to produce cepacian were confirmed. The ability of cepacian to confer resistance against desiccation and metal ion stress is also reported.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The strains and plasmids used in this study are listed in Table 1. *Burkholderia* strains were maintained in pseudomonas isolation agar (Difco) plates at 30 or 37°C. Mannitol medium (MM) (7) or S medium (37) supplemented with mannitol instead of glucose (SM) were used to quantify EPS production by *Burkholderia* strains at 30°C. *Escherichia coli* strains were grown in Lennox broth (LB) at 37°C. Growth media were supplemented with antibiotics when required, to maintain selective pressure, at the following final concentrations: for *Burkholderia* strains, 300 μg/ml chloramphenicol, 600 μg/ml kanamycin, and 100 μg/ml trimethoprim, and for *E. coli*, 100 μg/ml ampicillin, 50 μg/ml kanamycin, 25 μg/ml chloramphenicol, and 50 μg/ml trimethoprim.

DNA manipulation techniques. Total DNA and plasmid DNA isolation, DNA restriction, agarose gel electrophoresis, Southern blot experiments, and *E. coli* transformation were carried out using standard procedures (39). *Burkholderia multivorans* ATCC 17616 or *B. cepacia* IST408 electrocompetent cells, prepared as described previously (15), were transformed by electroporation using a Bio-Rad Gene Pulser II system (200 Ω, 25 μF, 2.5 kV) and grown overnight before being plated in selective medium. Triparental conjugation was performed as described previously (15).

Construction of insertion mutants and phenotype complementation. The primers *bceQ*-Fw/*bceQ*-Rev (Table 2) were used to amplify the 1,133-bp region encoding the putative flippase BceQ of *B. cepacia* IST408. The fragment obtained was digested with HindIII and ligated into the pDrive vector, originating plasmid pIS58-1. To amplify the glycosyltransferase-encoding gene *bceR*, primers *bceR*-Fw/*bceR*-Rev were used (Table 2). The 1,028-bp fragment amplified from *B. cepacia* IST408 was cloned into the XbaI/HindIII sites of the previously digested pDrive vector. The resulting plasmid was named pIS58-2. An internal 579-bp fragment from the *B. multivorans* ATCC 17616 *bceS* gene was amplified by PCR with primers *bceS*-Fw/*bceS*-Rev, and the amplified product was cloned into the XbaI/EcoRI sites of pBCKS, generating pSF71-8. The nucleotide sequences of the cloned internal gene regions of pIS58-1, pIS58-2, and pSF71-8 were confirmed by sequencing. Insertion mutations in each of the *bceQ*, *bceR*, and *bceS* genes were prepared from *B. cepacia* IST408 or *B. multivorans* ATCC 17616 by electroporation. Candidate mutants were further characterized by PCR amplification or Southern hybridization.

Plasmid pIS94-1 was constructed to complement the EPS-deficient phenotype of the *B. cepacia bceR* mutant as follows. The *bceR* coding region was amplified by PCR using primers C*bceR*-Fw/C*bceR*-Rev and *B. cepacia* IST408 genomic DNA as a template. The amplified fragment was restricted with KpnI/HindIII and inserted into the same restriction sites of the pMLBAD vector. The nucleotide sequence of the cloned gene was confirmed by sequencing.

Quantitative real-time RT-PCR experiments. Cells of the *B. multivorans* strains D2095 and D2214 and the *B. cepacia* strains IST408 and *bceQ*::pIS58-1 were harvested at the late exponential phase of growth, and total RNA was extracted using an RNeasy minikit (Qiagen) with DNase treatment by following the recommendation of the manufacturer. RNA integrity was checked with an Agilent 2100 Bioanalyzer coupled with an RNA Nano-Assay (Agilent Technol-

TABLE 2. Primers used in this study

Primer	Sequence (5′–3′)[a]
bceQ-Fw	CCT*AAGCTT*GGACGCTGATCGGCTAT
bceQ-Rev	CCT*AAGCTT*CGGATCGCCGACAGG
bceR-Fw	GCT*TCTAGA*GATCGTCGCGTGCT
bceR-Rev	CGA*AAGCTT*GCCGCCGGAACGTG
bceS-Fw	CAT*TCTAGA*CGGTCGTTCGAACA
bceS-Rev	CTC*GAATTC*GAAGTGCAGTTCTA
CbceR-Fw	CAT*GGTACC*CGATCTGGCGGAAC
CbceR-Rev	GCA*AAGCTT*CCGATGCGAAAGGC
*bceB*_RT_Fw	TTCGTGAACATCCGCTTCATT
*bceB*_RT_Rev	CCGAGCACCTCGACCACTT
*bceE*_RT_Fw	CCGAGACCTATCCGGTTCATT
*bceE*_RT_Rev	CTTTCTGCAGCTGGTCCATCA
*bceG*_RT_Fw	ACGCTGTCCGGCAAGATC
*bceG*_RT_Rev	TAGCTCATGTTCGCGCCTTT
*bceM*_RT_Fw	GGCGAAGCGCATGAAGTC
*bceM*_RT_Rev	CAGTGTGCCGGTCGTATACG
*bceR*_RT_Fw	TTCGGCGAGGACGACTATG
*bceR*_RT_Rev	TGGAACCCGAGGAAATGC
*bceS*_RT_Fw	AACGGCCTCGTCCATCAC
*bceS*_RT_Rev	GCGTCCAGAACCAGACGAAA
*gyrB*_RT-Fw	GCGGACTGCCAGGAGAAAG
*gyrB*_RT-Rev	GACCCACCTGCCGAGTCA

[a] Restriction sites are in italic.

ogies). For the reverse transcription step, 1 μg of total RNA from *Burkholderia* strains, derived from three independent samples, was used. cDNA was synthesized using TaqMan^R reverse transcription reagents (Applied Biosystems) in accordance with the manufacturer's instructions. The primers used to amplify the *bce* genes (Table 2) were designed using Primer Express 3.0 software (Applied Biosystems). Reverse transcription (RT) mixtures were properly diluted to use 400 ng of template cDNA, 2× SYBR green PCR master mix, and 0.4 mM reverse and forward primers for each gene in a total volume of 25 μl. Reaction mixtures containing nuclease-free water (Eppendorf) instead of the reverse transcriptase were included as a negative control. Reactions were performed with a model 7500 instrument from Applied Biosystems. The expression ratio of the target genes relative to the reference gene *gyrB*, which showed no variation in transcription abundance under the conditions tested, was determined. Relative quantification of gene expression by real time RT-PCR was determined using the $\Delta\Delta C_T$ method (34).

Production and characterization of bacterial exopolysaccharides. EPS production was assessed based on the dry weight of the ethanol-precipitated polysaccharide recovered from 100-ml culture samples of the different strains grown in liquid MM over 3 or 6 days at 30°C with orbital agitation, based on methods described before (37). For Fourier transform infrared (FTIR) spectroscopy analysis, the ethanol-precipitated EPSs were air dried and redissolved in distilled water prior to dialysis (molecular mass cutoff, 12 kDa) against water for 3 days at 4°C, followed by centrifugation at 10,000 × *g* for 30 min and freeze drying. The acetyl content of the EPSs produced was determined as described by McComb and McCready (30), using glucose penta-acetate as standard. The results shown are the mean values for at least 3 independent determinations.

***In vivo* complementation of the EPS-deficient phenotype of the IST408 *bceR* mutant.** Plasmid pIS94-1, carrying the parental *bceR* gene, was mobilized into the *B. cepacia* IST408 *bceR* mutant strain by triparental conjugation. The complemented IST408 *bceR*::pIS58-2/pIS94-1 strain was grown in solid MM supplemented with 1% (wt/vol) of L-arabinose, in order to induce the expression of the cloned gene from the P_{BAD} promoter of *E. coli* present in this vector, at 30°C for 5 days, and the mucoidy of the corresponding colonies was assessed.

FTIR analysis. The EPSs produced by the *Burkholderia* strains under study were analyzed by condensed-phase infrared spectroscopy in the wave number range of 400 to 4,000 cm^{-1} at a spectral resolution of 2 cm^{-1}. The data were obtained with an FTIR spectrometer (Jasco FTIR 4100) in transmission mode, with at least 16 scans per sample. At least two independently prepared samples of each EPS with the same massic concentration (0.5 mg/50 mg), prepared with spectrometric-grade potassium bromide (Merck), were analyzed.

Metal ion stress assays and desiccation sensitivity. Overnight-grown cultures of *Burkholderia* strains in SM were incubated at 30°C in the presence or absence of 2.5 g/liter of purified cepacian and of 50 mM $FeSO_4$ or 50 mM $ZnCl_2$. Viable cell counts (CFU) in LB solid medium were determined. For the quantification of bacterial survival to desiccation, 10 μl of overnight stationary-phase *Burkholderia* cultures was mixed with or without cepacian (final concentration, 2.5 g/liter) and aliquoted into the wells of a microtiter plate. After drying, the plate was incubated at 30°C for several days. Each viable count was performed by adding 100 μl of saline buffer to rehydrate, followed by serial dilutions and plating on solid LB medium. Experiments were performed at least three times.

Computational analysis of nucleotide and protein sequences. Nucleotide and amino acid sequences were retrieved from Integrated Microbial Genomes (28) for the following strains: *B. multivorans* ATCC 17616; *B. cenocepacia* strains J2315, AU10546, HI2424, MC0-3, and PC184; *Burkholderia ambifaria* strains MC40-6 and AMMD; *Burkholderia dolosa* AUO158; *Burkholderia vietnamiensis* G4; *B. mallei* strains 2002721280, ATCC 23344, FMH, GB8 horse 4, JHU, NCTC 10229, NCTC 10247, SAVP1, and PRL-20; *B. pseudomallei* strains 1106a, 1106b, 1655, 1710a, 1710b, 305, 406e, 668, K96243, Pasteur, S13, 112, 14, 7894, 9, 91, B7210, BCC215, DM98, and NCTC 13177; *Burkholderia lata* sp. 383; *Burkholderia thailandensis* E264, BT4, MSMB43, ATCC 700388, and TXDOH; *Burkholderia phymatum* STM815; *Burkholderia phytofirmans* PsJN; *Burkholderia oklahomensis* strains EO147 and C6786; *Burkholderia ubonensis* Bu; *Burkholderia graminis* C4D1M; and *Burkholderia xenovorans* LB400. The BLAST algorithm (1) was used to compare the deduced amino acid sequences to database sequences available at the NCBI. Alignments were performed using the program CLUSTAL W (46). Transmembrane regions were predicted by TMHMM server v2.0. Signal sequences and subcellular localization were predicted by PSORTb v2.0. The *B. vietnamiensis* G4 genome sequence was used as a reference in the bioinformatic analysis.

RESULTS

Identification of a second gene cluster involved in cepacian biosynthesis. In order to identify the cepacian biosynthesis genes missing from the *bce* cluster, the genome sequences of 7 representative *Bcc* strains and 8 non-*Bcc* strains were examined by bioinformatic tools. A genomic region containing 11 genes homologous to the *bce* cluster (*bceA* to *bceK*) previously identified by Moreira et al. (31), followed by 8 genes encoding proteins putatively involved in polysaccharide biosynthesis were identified in the genome sequences of the non-*Bcc* strains *B. xenovorans* LB400, *B. phymatum* STM815, *B. phytofirmans* PsJN, and *B. graminis* C4D1M (Fig. 1a). The search for homologues to these newly identified genes within the genomes of *Bcc* strains (*B. cenocepacia* J2315, *B. ambifaria* AMMD, *B. dolosa* AU0158, *B. vietnamiensis* G4, *B. lata* sp. 383, *B. ubonensis* Bu, and *B. multivorans* ATCC 17616) and other non-*Bcc* strains (*B. pseudomallei* 1106a, *B. oklahomensis* C6786, and *B. thailandensis* Bt4) indicated that they were present in all these genomes, approximately 155 to 314 kb downstream of the *bceA-K* genes, depending on the strain. The minimal distance between the two chromosomal regions was observed for *B. cenocepacia* J2315 (155 kb), with the maximum distance being observed for *B. multivorans* ATCC 17616 (314 kb). These size distances depend on the number/size of genes encoding phage-related proteins and insertion sequences present between them. *B. mallei* strains have this second region, but the previously identified *bce* genes are absent from their genomes. The second region was named the *bce-II* cluster, and the genes therein were named *bceM* through *bceU* (Fig. 1a and b).

A thorough analysis revealed that strains with the two clusters together have the *bceV* gene encoding a putative acylesterase/lipolytic protein (Fig. 1a). Strains with the *bce-II* cluster separated possess the *bceM* gene encoding a putative NAD-dependent epimerase/dehydratase (Fig. 1b). Since the putative acyltransferase-encoding gene *bceU* was found in the genome sequences of all the *Bcc* strains as well as in *B. thailandensis*, *B.*

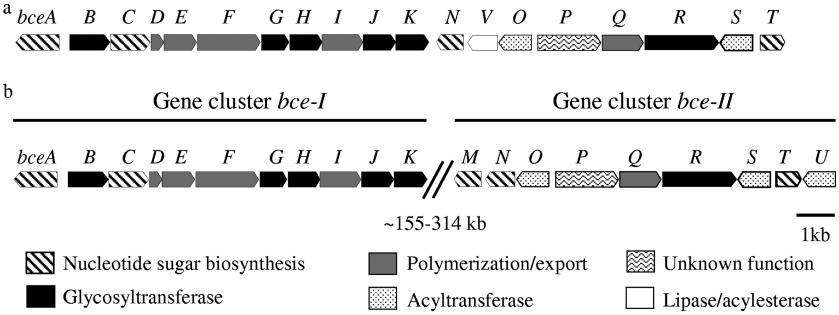

FIG. 1. Genetic organization of the *bce* gene cluster directing the biosynthesis of cepacian by *Burkholderia* bacteria. In representative strains of the species *B. xenovorans*, *B. phymatum*, *B. phytofirmans*, and *B. graminis*, the *bce* genes are clustered together in the same genomic region (a), while, in representative strains of the *Burkholderia cepacia* complex, comprising *B. pseudomallei*, *B. oklahomensis*, and *B. thailandensis*, the *bce* genes are split into two regions 155 to 314 kb apart (b). Strains from *B. mallei* have the *bce-II* cluster only. The locus tags for each gene in the *B. vietnamiensis* G4 genome are as follows: for *bceA*, Bcep1808_4200; for *bceB*, Bcep1808_4201; for *bceC*, Bcep1808_4202; for *bceD*, Bcep1808_4203; for *bceE*, Bcep1808_4204; for *bceF*, Bcep1808_4205; for *bceG*, Bcep1808_4206; for *bceH*, Bcep1808_4207; for *bceI*, Bcep1808_4208; for *bceJ*, Bcep1808_4209; for *bceK*, Bcep1808_4210; for *bceM*, Bcep1808_4471; for *bceN*, Bcep1808_4472; for *bceO*, Bcep1808_4473; for *bceP*, Bcep1808_4474; for *bceQ*, Bcep1808_4475; for *bceR*, Bcep1808_4476; for *bceS*, Bcep1808_4477; for *bceT*, Bcep1808_4479; and for *bceU*, Bcep1808_4480.

oklahomensis, and *B. pseudomallei* strains, it was considered to belong to this genetic cluster (Fig. 1b).

Assignment of putative functions to the *bce-II* gene products. The deduced amino acid sequences of the *bce-II* genes of *B. vietnamiensis* G4 were compared with protein sequences deposited in GenBank. BceT showed identity with putative or confirmed bacterial UDP-glucose pyrophosphorylases (Table 3) catalyzing the reversible formation of UDP-glucose from UTP and glucose-1-phosphate. The *bceM* and *bceN* genes were predicted to encode a GDP-6-deoxy-D-lyxo-4-hexulose reductase (RMD) and a GDP-D-mannose 6,4-dehydratase (GMD), respectively (Table 3). These two enzyme activities use the precursor GDP-D-mannose to synthesize GDP-D-rhamnose, the donor of the D-rhamnose moiety of cepacian. The *B. vietnamiensis* G4 *bceR* gene encodes a putative 817-amino-acid protein that, based on sequence similarity, shows two domains of the glycosyltransferase family GT4, which comprises retaining glycosyltransferases with a wide variety of donor and acceptor specificities (Table 3). With the exception of *Burkholderia* strains, no other bifunctional glycosyltransferase homologous to BceR was identified. The *B. vietnamiensis* G4 *bceO*, *bceS*, and *bceU* genes encode proteins homologous to functionally uncharacterized putative proteins (Table 3). In spite of the weak conservation, BceO, -S, and -U are also homologous to proteins that define a family of integral membrane proteins involved in the acylation of carbohydrate moieties of extracytoplasmic molecules. These proteins include OafA from *Salmonella enterica* serovar Typhimurium, which acetylates O antigen (41); NodX from *Rhizobium leguminosarum* biovar viciae, responsible for acetylation of the Nod factor and for conferring host range specificity (16); and ExoZ from *Sinorhizobium meliloti*, responsible for succinoglycan acetylation (3). The *B. vietnamiensis* G4 *bceP* gene encodes a predicted protein homologous to uncharacterized putative proteins from other bacteria (Table 3). Gene *bceQ* encodes a putative protein homologous to flippase or translocase proteins of the Wzx family, postulated to be involved in the transport of oligosaccharide repeat units of the lipopolysaccharide (LPS) O antigen, capsular polysaccharides, and EPSs across the inner membrane (29). The closest characterized homologues of *B. vietnamiensis* G4 BceQ were AceE from *Gluconacetobacter xylinus*, Wzx from *E. coli*, and RfbE from *Shigella flexneri* (Table 3). In a previous work (31), the putative Bce flippase was incorrectly assigned to the *bceL* gene in the *bce-I* cluster. However, the analysis of other *Burkholderia* genomes, such as those of *B. phytofirmans* or *B. xenovorans*, indicated that this protein is absent from the *bce* cluster.

Cepacian production is widespread among *Burkholderia* strains. To determine whether *Bcc* and non-*Bcc* strains were able to produce EPS, 10 strains were grown in liquid MM for 3 to 6 days at 30°C. The results indicate that after 3 days of growth, the non-*Bcc* soil isolates *B. xenovorans* LB400, *B. phytofirmans* PsJN, and *B. phymatum* STM815 and the *Bcc* strains *B. cepacia* IST408, *B. multivorans* ATCC 17616, and *B. ambifaria* AMMD produced more than 2.6 g/liter of EPS (Fig. 2a). *B. vietnamiensis* G4, *B. dolosa* AU0158, and *B. lata* sp. 383 produced less than 0.9 g/liter of EPS after 3 days of incubation (Fig. 2a), but after 6 days of growth, 1.1, 0.6, and 0.3 g/liter of EPS were obtained, respectively. The *B. cenocepacia* J2315 isolate from the ET12 lineage was unable to produce EPS. Although the genome of *B. cepacia* IST408 is not sequenced, cepacian is the sole EPS produced by this strain (6), being used as a reference strain in this work.

The EPSs obtained were analyzed by Fourier transform infrared (FTIR) spectroscopy using, as a reference, *B. cepacia* IST408 cepacian. All the EPSs originated spectra indistinguishable from the spectra obtained for *B. cepacia* IST408 cepacian, indicating that all strains analyzed produced cepacian. As an example, the FTIR spectra obtained for the EPSs of *B. cepacia* IST408 and *B. phytofirmans* PsJN are shown in Fig. 3.

Altogether, our results suggest that cepacian production may be a common feature within the genus *Burkholderia*, independently of whether the source of the strain is environmental (*B. xenovorans* LB400, *B. phytofirmans* PsJN, *B. phymatum* STM815, *B. multivorans* ATCC 17616, *B. vietnamiensis* G4, *B. lata* sp. 383, and *B. ambifaria* AMMD) or clinical (*B. cepacia* IST408 and *B. dolosa* AU0158).

TABLE 3. Features of the Bce proteins from *Burkholderia*

Protein	Predicted function	Homologue	% Identity/ % similarity[a]	Organism	Conserved domains[b]	GenBank accession no.
BceM	GDP-6-deoxy-D-lyxo-4-hexulose reductase (RMD)		54/67	*Pseudomonas syringae*	NADP(H) binding Wierenga motif $G(X)_2G(X)_2G$; catalytic domain $Y(X)_3K$ and S/T	YP_273224
		Rmd	53/68	*Xanthomonas campestris*		CAP53110
		Rmd	32/48	*Pseudomonas aeruginosa*		ABJ14840
BceN	GDP-D-mannose 4,6-dehydratase (GMD)	Gmd	58/72	*Pseudomonas aeruginosa*	NADP(H) binding Wierenga motif $G(X)_2G(X)_2G$; catalytic domain $Y(X)_3K$ and S/T	NP_254140
		Gmd	52/66	*Klebsiella pneumoniae*		YP_002920358
		Gmd	51/67	*Escherichia coli*		NP_288559
BceO	Acyltransferase		46/65	*Acidobacteria bacterium*	9 transmembrane domains	ABF40784
			32/61	*Rhizobium etli*		ZP_03501439
			32/45	*Solibacter usitatus*		ABJ83241
BceP	Unknown		38/55	*Nodularia spumigena*	Six-bladed beta propeller TolB-like domain	EAW43145
			38/56	*Nostoc punctiforme*		ACC81483
			38/54	*Anabaena variabilis*		ABA20764
BceQ	Repeat unit flippase	Wzx	26/48	*Escherichia coli*	12 transmembrane domains	AAT85651
		RfbE	26/49	*Shigella flexneri*		CAA50771
		AceE	35/52	*Gluconacetobacter xylinus*		CAA64437
BceR-I	Glycosyltransferase	WbaZ	66/81	*Bacillus cereus*	Amino acids 46 to 424; two β-α-β Rossman-like domains characteristic of GT-B fold proteins	ZP_04230623
		RfaG	64/81	*Desulfotomaculum reducens*		YP_001114454
		WbaZ-like	62/78	*Salmonella enterica*		EDZ22349
BceR-II	Glycosyltransferase	YqgM_like	42/60	*Nostoc punctiforme*	Amino acids 428 to 817; two β-α-β Rossman-like domains characteristic of GT-B fold proteins	ACC81485
		YqgM_like	44/60	*Nodularia spumigena*		EAW42619
		RfaG	44/60	*Rhodothermus marinus*		YP_003290427
BceS	Acyltransferase		27/43	*Cytophaga hutchinsonii*	8 transmembrane domains	ABG58185
			29/44	*Methylobacterium* sp.		ACA19306
			26/43	*Lentisphaera araneosa*		EDM29042
BceT	UDP-glucose pyrophosphorylase (UGP)	GalU	53/68	*Xanthomonas campestris*	Nucleotide binding domain: ^{12}GXGTRXLPXTK $(X)_3$KEXLP$(X)_4P^{35}$; glucose-1-P binding domain: ^{191}EKP$(X)_4$APSXL $(X)_3$GRY^{208}	Q8P8Q1
		GalU	51/65	*Escherichia coli*		AP_001862
		ExoN	46/62	*Sinorhizobium meliloti*		AAA16043
BceU	Acyltransferase		35/50	*Bradyrhizobium japonicum*	9 transmembrane domains	BAC47625AC
			31/45	*Agrobacterium radiobacter*		ACM30377
			32/46	*Sinorhizobium meliloti*		CAC49254

[a] All the hits with proteins from *Burkholderia* strains were excluded from this table due to the high identity scores obtained by BLAST analysis. Instead, shown is the level of identity between Bce proteins and experimentally characterized ones or, in the absence of biochemical data, the best hits with uncharacterized proteins.
[b] Domains refer to *B. vietnamiensis* G4 protein sequences.

Characterization of the EPS phenotype of *Burkholderia bceQ*, *bceR*, and *bceS* mutants. To clearly demonstrate the involvement of *bce-II* cluster genes in cepacian biosynthesis, the insertional inactivation of the genes *bceQ*, *bceR*, and *bceS*, encoding putative flippase, glycosyltransferase, and acyltransferase, respectively, was performed. The mutagenized strains were *B. cepacia* IST408 for the *bceQ* and *bceR* genes and *B. multivorans* ATCC 17616 for the *bceS* gene. The resulting mutants were named *B. cepacia* IST408 *bceQ*::pIS58-1, *B. cepacia* IST408 *bceR*::pIS58-2, and *B. multivorans* ATCC 17616 *bceS*::pSF71-8 (Table 1). The *bceQ* and *bceR* mutants were grown in liquid MM to assess EPS production. Under these conditions, no EPS production was detected over the 72 h of cultivation of the *bceQ* and *bceR* mutants, while the parental strain produced EPS (Fig. 2b). This result allowed for the conclusion that the *bceQ* and *bceR* genes are required for cepacian biosynthesis. In *trans* complementation using the *bceR* gene cloned in the replicative plasmid pMLBAD was performed. For this purpose, solid MM supplemented with 1% of arabinose was used. Under these conditions, the introduction of pIS94-1 into the *bceR* mutant led to the recovery of cepacian biosynthesis, associated with the mucoid phenotype of the colonies in solid medium (Fig. 2d).

The disruption of the *B. multivorans* ATCC 17616 *bceS* gene had no significant effect on cepacian biosynthesis, since the mutant and the parental strain produced similar quantities of EPS (Fig. 2b). In order to evaluate whether the mutation of *bceS* could have an impact in cepacian acetylation, the acetyl

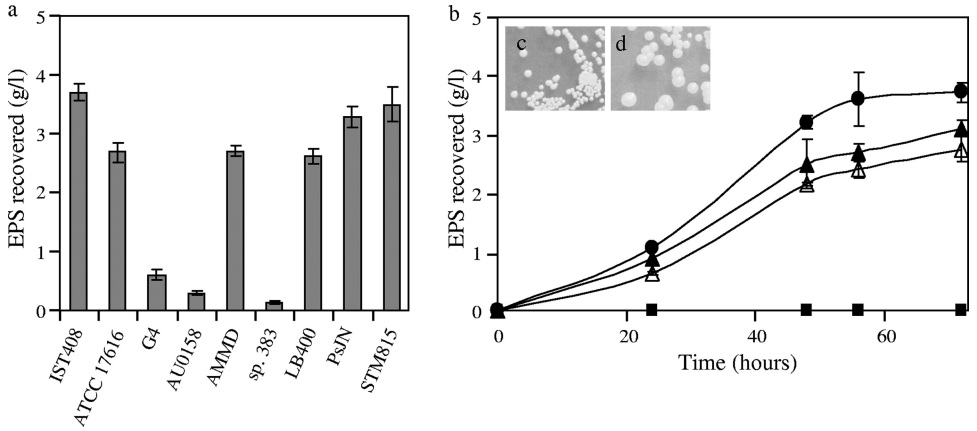

FIG. 2. EPS production by *Burkholderia* strains. Cells from different *Burkholderia* species (a) and from *B. cepacia* IST408 (●), *B. cepacia* IST408 *bceR*::pIS58-2 (■), *B. multivorans* ATCC 17616 (▲), and *B. multivorans* ATCC 17616 *bceS*::pSF71-8 (△) (b) were grown in MM for 3 days at 30°C and EPSs quantified by dry weight after ethanol precipitation. Data represent the means of results from at least three independent experiments. Error bars show standard deviations. Phenotype of *B. cepacia* IST408 *bceR*::pIS58-2 harboring pMLBAD (c) or pIS94-1 containing the *bceR* gene (d) grown in solid MM supplemented with 1% arabinose.

content of the EPSs produced by *B. multivorans* ATCC 17616 and the *bceS* mutant was quantified. The results obtained indicated 3.9 ± 0.1 and 3.1 ± 0.1 acetyl groups per repeat unit for the EPSs produced by the parental and the mutant strains, respectively. This reduction of approximately 20% in the acetyl content of the EPS produced by the *bceS* mutant is consistent with the predicted acetyltransferase activity of BceS.

Expression of *bce* genes in mucoid and nonmucoid strains. To assess whether the genes from clusters *bce-I* and *bce-II* have similar expression patterns, quantitative real-time RT-PCR was performed with the two *Bcc* isogenic *B. multivorans* strains D2095 and D2214, isolated from a chronically infected cystic fibrosis patient (52), for the *bceB*, *bceE*, and *bceG* transcripts from the *bce-I* cluster and for the *bceM*, *bceR*, and *bceS* transcripts from the *bce-II* cluster. The expression values obtained indicate a 2-fold induction of the transcription of all the genes under study in the mucoid D2095 strain, compared to the level for the D2214 nonmucoid strain (Fig. 4a).

The expression of *bceB*, *bceE*, *bceM*, and *bceS* genes in the *B. cepacia* IST408 parental strain and in the *bceQ*::pIS58-1 mutant strain was also determined after 24 h of growth in SM. As is shown in Fig. 4b, the disruption of the *bceQ* gene had no significant influence on the expression levels of the *bce-I* region genes *bceB* and *bceE*. Concerning the two genes from *bce-II* region, the expression value of *bceS* was not found to change significantly when the parental and the mutant strain were compared, although a 2.5-fold induction was observed for the *bceM* gene in the parental strain *B. cepacia* IST408 (Fig. 4b).

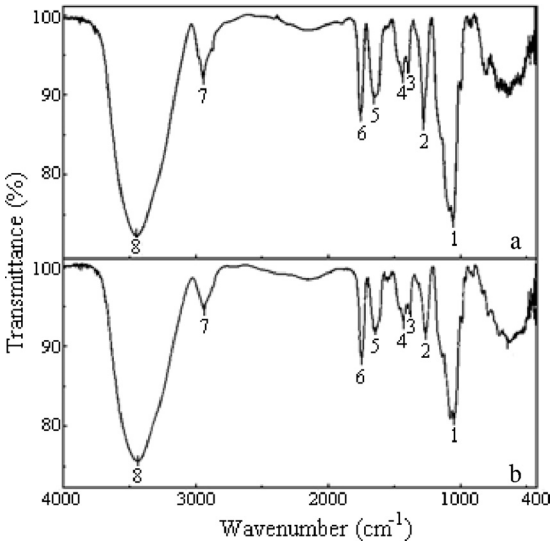

FIG. 3. FTIR analysis of the purified EPS produced by *Burkholderia cepacia* IST408 (a) and *B. phytofirmans* PsJN (b), showing similar spectra. Peaks: 1 and 2, carboxylic and/or hydroxyl C-O; 3 and 4, C-H; 5, water; 6, carbonyl; 7, C-C bonds; 8, water and hydroxyl groups of the EPS.

Effect of the polysaccharide on the desiccation sensitivity of *Burkholderia* strains. To determine whether the EPS produced by *Burkholderia* could contribute to their resistance to desiccation, the soil isolates *B. xenovorans* LB400 and *B. multivorans* ATCC 17616, the clinical isolate *B. cepacia* IST408, and the *bceR*::pIS58-2 mutant derivative were incubated in the presence or absence of 2.5 g/liter of EPS and kept dry for 7 days, and the numbers of CFU were determined (Fig. 5a). The results showed drastic reductions of CFU for *B. xenovorans* and *B. multivorans* incubated in the absence of the EPS in the first 24 h, and after 3 days, no viable cells were recovered (Fig. 5a). Contrastingly, when cells were dried in the presence of cepacian, viable cells could be recovered even after 7 days of incubation (Fig. 5a). *B. cepacia* IST408 and its mutant derivative *bceR*::pIS58-2 were highly susceptible to desiccation conditions, since no viable cells could be obtained after 24 h of exposure to desiccation either in the presence or in the absence of cepacian.

Effect of the exopolysaccharide on protection from metal ion stress. The protective effects of cepacian against toxic levels of Fe^{2+} and Zn^{2+} were investigated by challenging *B. xenovorans* LB400, *B. multivorans* ATCC 17616, and *B. cepacia* IST408 with iron or zinc ions in the presence or absence of EPS. When

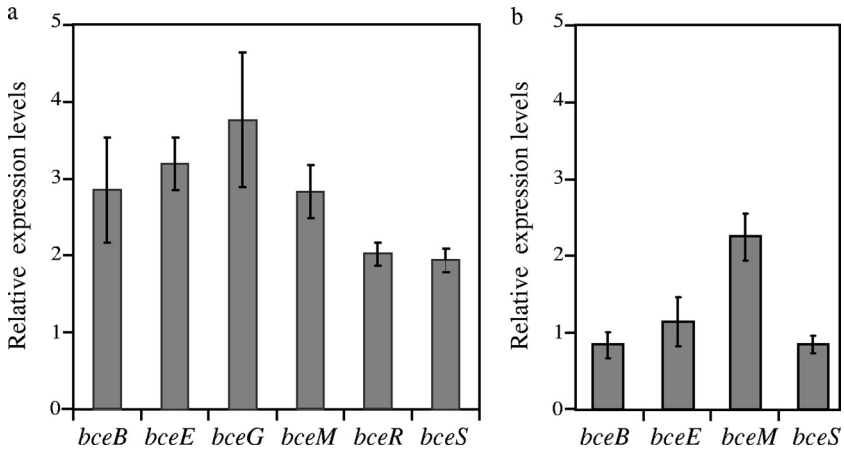

FIG. 4. Quantitative real-time RT-PCR analysis of the relative transcript abundances in *B. multivorans* D2095 with respect to *B. multivorans* D2214 after 17 h of growth (a) and *B. cepacia* IST408 with respect to *bceQ*::pIS58-1 after 24 h of growth (b). For each gene, the data were standardized to values obtained for the internal control gene *gyrB*. The results were obtained from three independent experiments. Error bars represent standard deviations.

these strains were challenged with 50 mM ferrous sulfate, the numbers of CFU of the strains incubated with 2.5 g/liter of EPS were 1 to 2 logs higher than those observed with the cells incubated with Fe^{2+} in the absence of EPS after 1 h of incubation (Fig. 5b). In the presence of EPS, only a slight increase in the survival rates was observed after treatment with zinc (data not shown). *B. xenovorans* and *B. multivorans* strains incubated with EPS seem to be more resistant to iron stress than *B. cepacia* under the same tested conditions. Indeed, we observed in 1 h a 1.5-log reduction when IST408 was incubated with EPS, while the two other strains exhibited no significant reduction in the number of viable cells. The *B. cepacia* IST408 *bceR*::IS58-2 mutant exhibited behavior similar to that observed for the parental strain (data not shown).

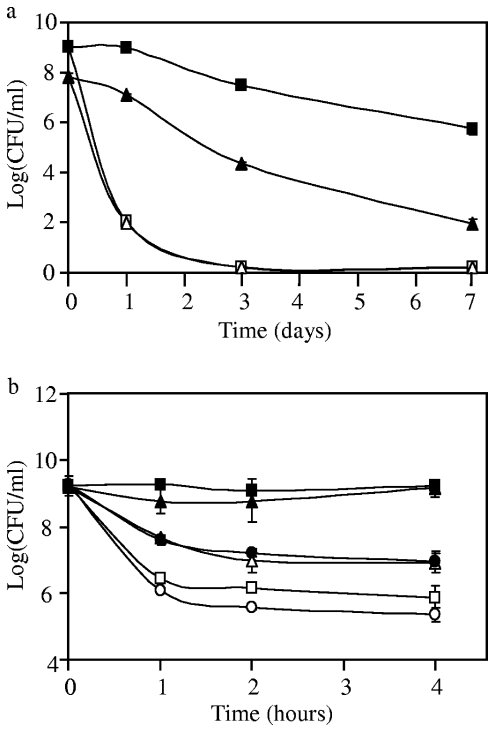

FIG. 5. Protective role of EPS against desiccation and iron ion stress. Cells from overnight grown cultures of *B. xenovorans* LB400 (■), *B. multivorans* ATCC 17616 (▲), and *B. cepacia* IST408 (●) were harvested by centrifugation and exposed to desiccation (a) or 50 mM ferrous sulfate (b) at 30°C in the presence (closed symbols) or absence (open symbols) of 2.5 g/liter of cepacian. The remaining viable bacterial counts were determined at different time points by determining the numbers of CFU. The data represents the means of results from three independent experiments. Error bars show standard deviations.

DISCUSSION

In this study, we report the identification of a second cluster of genes required for cepacian biosynthesis and compared the abilities of environmental and clinical *Burkholderia* strains to produce this EPS. In a previous work, Moreira et al. (31) identified 11 *bce* genes from *B. cepacia* IST408 as being involved in cepacian biosynthesis. Since the heptasaccharide repeat unit of cepacian is composed of 5 different sugars (6), it was evident that some protein activities were missing. A thorough analysis of the available *Burkholderia* genome sequences revealed that the genome sequences of four rhizosphere strains (*B. graminis* C4D1M, *B. xenovorans* LB400, *B. phymatum* STM815, and *B. phytofirmans* C6786) possess 8 additional genes immediately following the previously identified *bce* genes. These extra genes were also found in all other *Burkholderia* strains with a sequenced genome available, but, most probably due to genomic rearrangements caused by phages and insertion sequences, they are located several hundred kilobases apart from the initially identified *bce-I* cluster. The two clusters, *bce-I* and *bce-II*, account for most of the genes needed for cepacian biosynthesis, as depicted in Fig. 6. Most of the enzymes required for nucleotide sugar precursor synthesis are encoded by *bce* genes, with the exception of the genes encoding phosphoglucomutase (PGM), phosphoglucose isomerase (PGI), phosphomannomutase (PMM), and UDP-glucose epimerase (UGE). These enzyme activities are also involved in metabolic processes, such as the biosynthesis of lipopolysac-

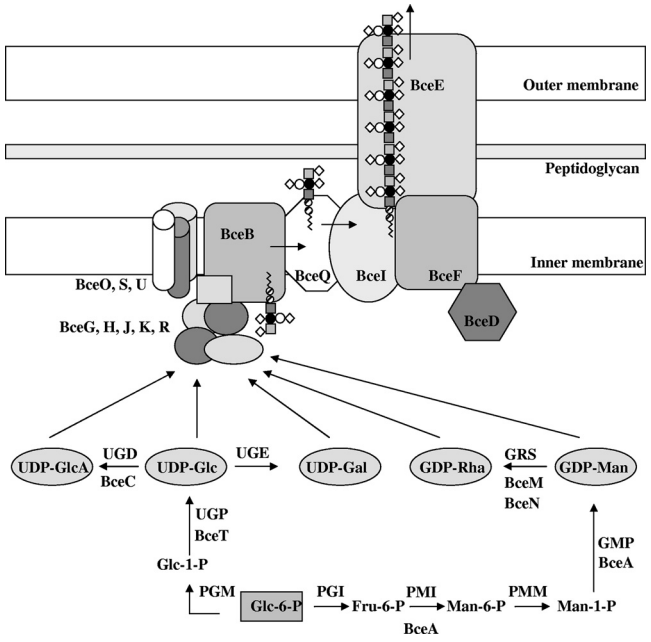

FIG. 6. Pathway leading to the nucleotide-sugar precursors for cepacian biosynthesis by *Burkholderia* and model for the assembly and export of the EPS. With the exception of BceP, all the Bce proteins have confirmed or putative roles in EPS biosynthesis, as described in the text. Abbreviations: Glc, glucose; GlcA, glucuronic acid; Gal, galactose; Rha, rhamnose; Man, mannose; GDP, guanosine-5′-diphosphate; UDP, uridine-5′-diphosphate; PGM, phosphoglucomutase; UGE, UDP-glucose epimerase; PMM, phosphomannomutase; UGP, UDP-glucose pyrophosphorylase; PGI, phosphoglucose isomerase; GMP, GDP-D-mannose pyrophosphorylase; UGD, UDP-glucose dehydrogenase; PMI, phosphomannose isomerase; GRS, GDP-rhamnose synthetase.

charide and other cell polysaccharides. The search for homologues to these proteins in *Burkholderia* genome sequences revealed that they are present in more than one copy and distributed in different locations of chromosomes 1 and 2 (data not shown). Two Bce proteins involved in nucleotide sugar precursor biosynthesis have been characterized: BceA, a bifunctional protein with phosphomannose isomerase and GDP-D-mannose pyrophosphorylase activities required for GDP-mannose biosynthesis (42), and BceC, a UDP-glucose dehydrogenase involved in UDP-glucuronic acid synthesis (26).

The assembly of the heptasaccharide repeat unit of cepacian requires the priming glycosyltransferase BceB, catalyzing the addition of the first sugar, glucose, to the lipid carrier (50). The sequential addition of the six remaining sugars must be performed by BceG, -H, -J, and -K and the presumably bifunctional protein BceR, although the protein sequences do not give clues as to their specific glycosyltransferase activity. The presumably bifunctional protein BceR seems to have a unique organization, probably due to a process of fusion of two glycosyltransferase domains. Except for the orthologues found in the genome sequences of *Burkholderia* strains, no similar proteins could be found in databases.

The EPS produced by *B. cepacia* IST408, as well as from other strains, is acetylated. The exact number and position of the acetyl groups are still unknown (6). Three putative acyltransferases were found within the *bce-II* cluster, encoded by the *bceO*, *bceS*, and *bceU* genes. We show results indicating that the disruption of *bceS* caused a decrease in the acetylation content of cepacian, suggesting that BceS is involved in the repeat unit acetylation. Hydrophobicity analysis of the BceO, BceS, and BceU amino acid sequences suggests that they are probably located in the inner membrane. This is consistent with their possible role in the acetylation of cepacian since the repeat unit is synthesized on the cytoplasmic face of the inner membrane. O-acetylated extracellular and cell surface polysaccharides are synthesized by a wide range of bacterial pathogens (24), and this structural modification appears to play an important role in host-pathogen interactions. In many cases, the O-acetyl groups constitute prominent immunogenic epitopes critical for the host immune responses against the microorganism and for the development of protective vaccines (24, 35). The role of cepacian acetylation remains unknown, although one can expect a role similar to that observed for other acetylated polysaccharides.

The last steps in extracellular polysaccharide biosynthesis are the export of the repeat units to the periplasmic side of the inner membrane, their polymerization, and export of the nascent polymer. All evidence indicates that cepacian biosynthesis proceeds via the Wzy-dependent pathway. In this model, the lipid carrier-linked heptasaccharide repeat units are exported across the inner membrane by the putative flippase BceQ, being polymerized at the periplasmic face by the putative polymerase BceI (Fig. 6). BceQ and BceI are integral membrane proteins, and their involvement in cepacian biosynthesis is demonstrated by the EPS-deficient phenotype of the respective insertion mutants (31). In many bacteria, polymerization activity is influenced by an additional protein, referred to as polysaccharide copolymerase (PCP) (reviewed in reference 12). BceF is a PCP protein, having an N-terminal domain that spans twice the inner membrane with a large periplasmic loop and a C-terminal cytoplasmic domain with tyrosine kinase activity (15). The precise role(s) of this type of protein in polysaccharide biosynthesis is still unknown, but these proteins seem to play a critical role in the translocation of the polysaccharide chains from the periplasm to the cell surface through interaction with an outer membrane protein (12), which is likely to be BceE (Fig. 6).

Although several studies report the ability of *Bcc* clinical and environmental isolates to synthesize EPS (2, 11, 37, 52), not much is known on EPS biosynthesis by non-*Bcc* strains. This study demonstrates that, with the exception of *B. mallei* strains, all the *Burkholderia* strains with their genomes sequenced do have the *bce* gene cluster (together or fragmented) and most likely produce cepacian. This is the case of the rhizosphere non-*Bcc* species *B. xenovorans*, *B. phymatum*, and *B. phytofirmans*, which produced large amounts of cepacian. Most of the *Bcc* clinical isolates are EPS producers, with the exception of isolates of the *B. cenocepacia* species (52).

Polysaccharides secreted by bacteria play different roles in their biology and are frequently essential virulence determinants in pathogens of humans, livestock, and plants. Extracellular polysaccharides are also important in symbiotic interactions such as biological nitrogen fixation symbiosis between bacteria and plants, in adhesion to soil particles or roots, and as a barrier to harmful compounds, among other functions. In

natural environments, bacterial polysaccharides may have different roles, depending on the ecological niche. One of the functions ascribed to EPSs is a role in the initial plant colonization and enhancement of survival of bacteria such as those of the genera *Agrobacterium*, *Erwinia*, and *Pseudomonas*, among others (13). The EPS produced by the alfalfa-symbiotic bacterium *Sinorhizobium meliloti* also functions as a signaling molecule, triggering a developmental response or suppressing defense responses by the plant (17). The EPS from the plant growth-promoting species *Burkholderia gladioli* was shown to elicit induced systemic resistance on cucumber (33). *Bcc* clinical and environmental strains were assessed for EPS production and onion tissue maceration ability, but no correlation could be established between EPS production and the ability to cause maceration of onion tissue (2). Other functions attributed to EPSs are of a protective nature, namely, barriers against desiccation, predation, antibiotics, or binding of toxic metal ions. In this study, we demonstrate that cepacian seems to play a protective role against desiccation. Moreover, only the soil isolates *B. xenovorans* LB400 and *B. multivorans* ATCC 17616, but not the clinical isolate *B. cepacia* IST408, were desiccation resistant. Due to the limited number of tested strains, no broader conclusions can be drawn regarding the fitness of environmental versus clinical strains in relation to desiccation. According to Vriezen et al. (51), the desiccation process comprises 3 phases. Phase I is the drying of the cells where metabolic processes slow down due to the lack of water. Phase II is the storage phase, where the decline of cell viability occurs. Phase III is the rehydration of the cells. It is possible that, due to cepacian hygroscopic properties, it retains more water, retarding the loss of cell viability during phase II, explaining the observed results. It is generally stated that the presence of EPS confers desiccation resistance to bacterial cells. Yet, only a few studies have been done correlating the presence of EPS with drying tolerance. One such study was performed by Tamaru et al. (45), showing that EPS-producing *Nostoc commune* colonies were highly stress tolerant, while EPS-depleted cells lost most of this ability. In addition, Tamaru and colleagues showed that the amount of EPS could be correlated with the degrees of both desiccation and freezing tolerance (45). In a recent work, Knowles and Castenholz (21) lend additional support to the role of EPS in stress tolerance by examining the effect of released EPS on neighboring cells within rock-inhabiting microbial communities. These authors showed that the addition of EPS significantly enhanced the desiccation tolerance of *Chlorella* sp. CCMEE 6038 and of *Chroococcidiopsis* sp. CCMEE 5056 (21). Another condition here imposed to *Burkholderia* strains was metal ion stress. Although appropriate concentrations of metals are required for bacterial growth, excessive concentrations can be lethal. In this study, the presence of high concentrations of two metal ions showed that the EPS plays an important role in survival against iron stress but provides only a slight protection from zinc stress. Overall, the results obtained in our study clearly show the importance of cepacian in desiccation and ion metal tolerance induced by *Burkholderia*, confirming a crucial function of the EPS in stress resistance.

The role of the EPS of *Burkholderia* as a virulence determinant has been demonstrated with mice (10, 43). In those studies, strains producing EPS and non-EPS derivative producers were compared, and the latter ones turned out to be less virulent. Nevertheless, there are no available data indicating whether EPS is produced *in vivo* by the *Bcc*. In addition, a limited survey of the clinical outcome in patients colonized with EPS- and non-EPS-producing *Burkholderia* strains concluded that no correlation could be established between EPS production ability and the persistence or virulence of bacteria (11). In a more recent study, Zlosnik et al. (52) carried out a larger survey of clinical isolates from 100 CF patients, showing that strains of *B. cenocepacia*, one of the most virulent *Bcc* species, are frequently nonmucoid. Additionally, they observed that isolates from chronically infected CF patients convert predominantly from mucoid to nonmucoid, opposite to the *P. aeruginosa* nonmucoid-to-mucoid conversion associated with the acute-to-chronic transition of infection (22). Whether the *Bcc* mucoid-to-nonmucoid conversion leads to a poorer clinical outcome is not yet known. Nevertheless, these authors suggested that nonmucoid isolates may be associated with increased disease severity and that the mucoid phenotype may be associated with persistence. These observations indicate that cepacian is unlikely to be a good marker for assessing pathogenicity in *Burkholderia* which depends on both bacterial and host determinants.

This study demonstrated the power of using comparative genomics in combination with genetics and microbial physiology to link EPS production by *Burkholderia* strains to the genes required for its biosynthesis. Furthermore, the widespread distribution of cepacian biosynthesis ability among clinical and environmental *Burkholderia* strains points out a multitude of possible roles of the EPS in host colonization, biofilm formation, or protection against hazardous compounds, making the elucidation of cepacian biosynthesis regulation crucial for understanding the biology of these microorganisms.

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