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 acetyltransferase, 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 gp91phox−/− 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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or relevant characteristic(s)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. cepacia IST408</td>
<td>Cystic fibrosis clinical isolate; cepacian producer</td>
<td>37</td>
</tr>
<tr>
<td>B. multivorans ATCC 17616</td>
<td>Soil isolate</td>
<td>48</td>
</tr>
<tr>
<td>B. cenocepacia J2315</td>
<td>Cystic fibrosis clinical isolate</td>
<td>19</td>
</tr>
<tr>
<td>B. vietnamiensis G4</td>
<td>Isolated from a water treatment facility</td>
<td>52</td>
</tr>
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<td>B. dolosa AU0158</td>
<td>Cystic fibrosis clinical isolate</td>
<td>8</td>
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<tr>
<td>B. ambifaria AMMD</td>
<td>Root-colonizing bacterium</td>
<td>9</td>
</tr>
<tr>
<td>B. lata 383</td>
<td>Soil isolate</td>
<td>44</td>
</tr>
<tr>
<td>B. phytofirmans PsJN</td>
<td>Soil isolate; plant growth-promoting bacterium</td>
<td>40</td>
</tr>
<tr>
<td>B. phymatum STM81</td>
<td>Soil isolate; nitrogen fixation</td>
<td>47</td>
</tr>
<tr>
<td>B. xenovorans LB400</td>
<td>Soil isolate; degradation of polychlorinated biphenyl compounds</td>
<td>18</td>
</tr>
<tr>
<td>B. cenocepacia IST408 bceQ::pIS58-1</td>
<td>pIS58-1 integrated into the bceQ gene region</td>
<td>This work</td>
</tr>
<tr>
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<td>pIS58-2 integrated into the bceR gene region</td>
<td>This work</td>
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<tr>
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<td>pSF71-8 integrated into the bceS gene region</td>
<td>This work</td>
</tr>
<tr>
<td>B. multivorans D2095</td>
<td>Mucoid cystic fibrosis clinical isolate</td>
<td>D. P. Speert</td>
</tr>
<tr>
<td>B. multivorans D2214</td>
<td>Nonmucoid cystic fibrosis clinical isolate</td>
<td>D. P. Speert</td>
</tr>
<tr>
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<td>recA1 lac (F′proAB lacI&quot; ZmM15 Tn10 [Tc&quot;) thi]</td>
<td>4</td>
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<td>Plasmids</td>
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<td></td>
</tr>
<tr>
<td>pDrive</td>
<td>3.85-kb vector; lacZ α-peptide; Ap' Km'</td>
<td>Qiagen</td>
</tr>
<tr>
<td>pBCKS</td>
<td>3.4-kb phagemid derived from pUC19; lac promoter; Cm'</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pMLBAD</td>
<td>pBRR1 ori; araC-PBAD; Tp'; mob'</td>
<td>25</td>
</tr>
<tr>
<td>pSF71-8</td>
<td>pBCKS derivative carrying a 579-bp EcoRI/XbaI fragment with an internal</td>
<td>This work</td>
</tr>
<tr>
<td>pIS58-1</td>
<td>pDrive derivative carrying a 1,133-bp HindIII/XbaI fragment with an internal</td>
<td>This work</td>
</tr>
<tr>
<td>pIS58-2</td>
<td>pDrive derivative carrying a 1,028-bp HindIII/XbaI fragment with an internal</td>
<td>This work</td>
</tr>
<tr>
<td>pIS94-1</td>
<td>pMLBAD derivative carrying a 2,509-bp KpnI/HindIII fragment with the coding region of the bceR gene</td>
<td>This work</td>
</tr>
</tbody>
</table>

* 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-I. The bce-I 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. Mannit 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 V, 25 μF, 2.5 kV) and grown overnight before being plated in selective medium. Tripaternal 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 1,333-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 Cheec-Fw/Cheec-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 Techno-
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.

**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. 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 v.2.0. Signal sequences and subcellular localization were predicted by PSORTb v.2.0. The B. vietnamiensis G4 genome sequence was used as a reference in the bioinformatic analysis.

For the reverse transcription step, 1 μg of total RNA from Burkholderia strains, derived from three independent samples, was used. cDNA was synthesized using TagMan® 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 analyzed by condensed-phase infrared spectroscopy in the wave number range 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.

**TABLE 2. Primers used in this study**

<table>
<thead>
<tr>
<th>Primer Sequence (5’–3’)</th>
<th>Primer Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>bceQ_Fw.................CTTAAAGTTTGGACCGTATCGGCTAT</td>
<td>bceQ-Rev</td>
</tr>
<tr>
<td>bceR_Fw.................GCTTACTAGATGTCGTCGTGCT</td>
<td>bceR-Rev</td>
</tr>
<tr>
<td>bceR_Fw.................CGAAGCTTTGCCGCCGACCTG</td>
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</tr>
<tr>
<td>bceS_Rev.................CTTGATACGATCTGAGCTTCA</td>
<td>bceS_Rev</td>
</tr>
<tr>
<td>Cb_Rev........................CAGTTGACCTGGGAGAGAAC</td>
<td>Cb_Rev</td>
</tr>
<tr>
<td>RT_Fw.....................AACGGCCTCGTCCATCAC</td>
<td>RT_Fw</td>
</tr>
<tr>
<td>bceR_Rev..................TGGAACCCGAGGAAATGC</td>
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</tr>
<tr>
<td>bceE_Rev..................CGGACATCTGCGTCATT</td>
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<td>bceG_Rev..................TCCGTCGCGCAAGACAT</td>
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</tr>
<tr>
<td>bceM_Rev..................GGCGAAGCACTGAAATG</td>
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<td>bceR_Rev..................TGGACCCGAGAAATGC</td>
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</tr>
<tr>
<td>bceS_Rev..................CGCCTGACAGACAGCG</td>
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</tr>
<tr>
<td>gvpB_Rev..................GCCGGATGCAGAAGAGAG</td>
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<td>gvpB_Rev..................GCCGATGCAGAAGAGAG</td>
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</tr>
<tr>
<td>gvpB_Rev..................GACCCATGCCGAGG</td>
<td>gvpB_Rev</td>
</tr>
</tbody>
</table>

*Restriction sites are in italic.*

**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 (34).

**RESULTS**

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A thorough analysis revealed that strains with the two clusters together have the bceV gene encoding a putative acyltransferase/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.
predicted to encode a GDP-6-deoxy-D-lyxo-4-hexulose reduc-
saccharides, and EPSs across the inner membrane (29). The
genes of the lipopolysaccharide (LPS) O antigen, capsular poly-
lated to be involved in the transport of oligosaccharide repeat
to flippase or translocase proteins of the Wzx family, postu-
gous to uncharacterized putative proteins from other bacteria
amiensis deposited in GenBank. BceT showed identity with putative or
B. vietnamiensis G4 were compared with protein sequences
ponsible for succinoglycan acetylation (3). The
host range specificity (16); and ExoZ from
responsible for acetylation of the Nod factor and for conferring
tigen (41); NodX from
monella enterica
involved in the acylation of carbohydrate moieties of extracy-
proteins that define a family of integral membrane proteins
weak conservation, BceO, -S, and -U are also homologous to
ally uncharacterized putative proteins (Table 3). In spite of the
bceU
bceS
bceT
bceP
bce-2
bce-1

Assignment of putative functions to the bce-II gene prod-
ucts. The deduced amino acid sequences of the bce-II genes of
B. vietnamiensis G4 were compared with protein sequences
independent of whether the source of the strain is environmen-
Burkholderia strains, no other bifunctional glycosyltransferase homo-
acceptor specificities (Table 3). With the exception of
ing glycosyltransferases with a wide variety of donor and
of the glycosyltransferase family GT4, which comprises retain-
complex, comprising B. pseudomallei 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.
viethamiensis 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 bceL,
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.

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
5 to 6 days at 30°C. The results indicate that after 3 days of
growth, the non-Bcc soil isolates B. xenovorans LB400, B. phyto-
firmans PsJN, and B. phymatum STM815 and the Bcc strains
B. cepacia IST408, B. multivorans ATCC 17616, and B. ambif-
aria AMMD produced more than 2.6 g/liter of EPS (Fig. 2a). B.
viethamiensis 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
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.
viethamiensis 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 bceL,
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.

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.
viethamiensis 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 bceL,
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.

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

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 reduc-
tase (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. viethamiensis 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 retain-
ing 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. viethamiensis G4 bceO, bceS, and bceU genes encode proteins homologous to function-
ally 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 extracy-
plasmic molecules. These proteins include OafA from Salmonella enterica serovar Typhimurium, which acetylates O ant-
tigen (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 mel-
iloti, responsible for succinoglycan acetylation (3). The B. viethamiensis G4 bceP gene encodes a predicted protein homol-
ous 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, postu-
lated 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. viethamiensis G4 BceQ were AceE from Gluconacetobacter xylinus, Wzx from E. coli, and RibE 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.
TABLE 3. Features of the Bce proteins from Burkholderia

<table>
<thead>
<tr>
<th>Protein</th>
<th>Predicted function</th>
<th>Homologue</th>
<th>% Identity/% similarity</th>
<th>Organism</th>
<th>Conserved domains</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BceM</td>
<td>GDP-6-deoxy-α-l-lyo-4-α-hexulose reductase (RMD)</td>
<td>Pseudomonas syringae</td>
<td>54/67</td>
<td>NADP(H) binding Wierenga motif G(X)2,G(X)2,G; catalytic domain Y(X)2,K and S/T</td>
<td></td>
<td>YP_273224</td>
</tr>
<tr>
<td>BceN</td>
<td>GDP-α-mannose 4,6-dehydratase (GMD)</td>
<td>Pseudomonas aeruginosa</td>
<td>58/72</td>
<td>NADP(H) binding Wierenga motif G(X)2,G(X)2,G; catalytic domain Y(X)2,K and S/T</td>
<td></td>
<td>NP_254140</td>
</tr>
<tr>
<td>BceO</td>
<td>Acytflerase</td>
<td>Escherichia coli</td>
<td>46/65</td>
<td>Acidobacteria bacterium</td>
<td>9 transmembrane domains</td>
<td>ABF40784</td>
</tr>
<tr>
<td>BceP</td>
<td>Unknown</td>
<td>Nostoc punctiforme</td>
<td>38/55</td>
<td>Six-bladed beta propeller TolB-like domain</td>
<td></td>
<td>EAW43145</td>
</tr>
<tr>
<td>BceQ</td>
<td>Repeat unit flippase</td>
<td>Escherichia coli</td>
<td>26/48</td>
<td>12 transmembrane domains</td>
<td></td>
<td>AAA85651</td>
</tr>
<tr>
<td>BceR-II</td>
<td>Glycosyltransferase</td>
<td>Bacillus cereus</td>
<td>66/81</td>
<td>Amino acids 46 to 424; two β-α-β</td>
<td></td>
<td>ZP_04302632</td>
</tr>
<tr>
<td>BceR</td>
<td>Glycosyltransferase</td>
<td>Desulfitomaculum reducens</td>
<td>64/81</td>
<td>Rossman-like domains characteristic of GT-B fold proteins</td>
<td></td>
<td>YP_001114454</td>
</tr>
<tr>
<td>BceR</td>
<td>Glycosyltransferase</td>
<td>Salmonella enterica</td>
<td>62/78</td>
<td></td>
<td></td>
<td>EDZ22349</td>
</tr>
<tr>
<td>BceS</td>
<td>Acrlytransferase</td>
<td>YggM-like</td>
<td>42/60</td>
<td>Amino acids 428 to 817; two β-α-β</td>
<td></td>
<td>ACC81485</td>
</tr>
<tr>
<td>BceT</td>
<td>UDP-glucose pyrophosphorylase (UGP)</td>
<td>Xanthomonas campestris</td>
<td>53/68</td>
<td>Nucleotide binding domain:</td>
<td></td>
<td>Q8PSQ1</td>
</tr>
<tr>
<td>BceU</td>
<td>Acrlytransferase</td>
<td>Escherichia coli</td>
<td>51/65</td>
<td></td>
<td></td>
<td>AP_001862</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Syntrophobacter mobilis</td>
<td>46/62</td>
<td></td>
<td></td>
<td>AAA16043</td>
</tr>
</tbody>
</table>

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 organisms as shown.

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::pISS8-1, B. cepacia IST408 bceR::pISS8-2, 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 acetylation, the acetyl...
The 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 \( B. c\) cepacia isogenic \( B. m\) ultivorans 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).

Effect of the polysaccharide on the desiccation sensitivity of \( B. c\) cepacia strains. To determine whether the EPS produced by \( B. c\) ephthaleria could contribute to their resistance to desiccation, the soil isolates \( B. x\) enovorans LB400 and \( B. m\) ultivorans ATCC 17616, the clinical isolate \( B. c\) ephthaleria IST408, and the \( bce\)Q::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 \( B. x\) enovorans and \( B. m\) ultivorans 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). \( B. c\) ephthaleria IST408 and its mutant derivative \( bce\)R::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 \( \text{Fe}^{2+} \) and \( \text{Zn}^{2+} \) were investigated by challenging \( B. x\) enovorans LB400, \( B. m\) ultivorans ATCC 17616, and \( B. c\) ephthaleria IST408 with iron or zinc ions in the presence or absence of EPS. When
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$^{2+}$/H$_{100}$ 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).

**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), phosphoglucone isomerase (PGI), phosphomannomutase (PMM), and UDP-glucose epimerase (UGE). These enzyme activities are also involved in metabolic processes, such as the biosynthesis of lipopolysac-
transferases were found within the \textit{bce-II} cluster, encoded by the \textit{bceO}, \textit{bceS}, and \textit{bceU} genes. We show results indicating that the disruption of \textit{bceS} caused a decrease in the acetylation content of cepacian, suggesting that \textit{BceS} is involved in the repeat unit acetylation. Hydrophobicity analysis of the \textit{BceO}, \textit{BceS}, and \textit{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 \textit{BceQ}, being polymerized at the periplasmic face by the putative polymerase \textit{BceI} (Fig. 6). \textit{BceQ} and \textit{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). \textit{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 \textit{BceE} (Fig. 6).

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

FIG. 6. Pathway leading to the nucleotide-sugar precursors for cepacian biosynthesis by \textit{Burkholderia} and model for the assembly and export of the EPS. With the exception of \textit{BceP}, all the \textit{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-β-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 \textit{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 \textit{Bce} proteins involved in nucleotide sugar precursor biosynthesis have been characterized: \textit{BceA}, a bifunctional protein with phosphomannose isomerase and GDP-β-mannose pyrophosphorylase activities required for GDP-mannose biosynthesis (42), and \textit{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 \textit{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 \textit{BceG}, -H, -J, and -K and the presumably bifunctional protein \textit{BceR}, although the protein sequences do not give clues to their specific glycosyltransferase activity. The presumably bifunctional protein \textit{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 \textit{Burkholderia} strains, no similar proteins could be found in databases.

The EPS produced by \textit{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 acetyl...
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. CCME 6038 and of *Chroococcidiopsis* sp. CCME 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 iron metal ion 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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