Plant host and sugar alcohol induced exopolysaccharide biosynthesis in the *Burkholderia cepacia* complex

S. Josefin Bartholdson,1,2 Alan R. Brown,2 Ben R. Mewburn,3 David J. Clarke,1 Stephen C. Fry,3 Dominic J. Campopiano1 and John R. W. Govan2

1School of Chemistry, University of Edinburgh, Edinburgh EH9 3JJ, UK
2Centre for Infectious Diseases, University of Edinburgh, Edinburgh EH16 4SB, UK
3Institute of Molecular Plant Sciences, University of Edinburgh, Edinburgh EH9 3JH, UK

The species that presently constitute the *Burkholderia cepacia* complex (Bcc) have multiple roles; they include soil and water saprophytes, bioremediators, and plant, animal and human pathogens. Since the first description of pathogenicity in the Bcc was based on sour skin rot of onion bulbs, this study returned to this plant host to investigate the onion-associated phenotype of the Bcc. Many Bcc isolates, which were previously considered to be non-mucoid, produced copious amounts of exopolysaccharide (EPS) when onion tissue was provided as the sole nutrient. EPS production was not species-specific, was observed in isolates from both clinical and environmental sources, and did not correlate with the ability to cause maceration of onion tissue. Chemical analysis suggested that the onion components responsible for EPS induction were primarily the carbohydrates sucrose, fructose and fructans. Additional sugars were investigated, and all alcohol sugars tested were able to induce EPS production, in particular mannitol and glucitol. To investigate the molecular basis for EPS biosynthesis, we focused on the highly conserved *bce* gene cluster thought to be involved in cepacian biosynthesis. We demonstrated induction of the *bce* gene cluster by mannitol, and found a clear correlation between the inability of representatives of the *Burkholderia cenocepacia* ET12 lineage to produce EPS and the presence of an 11 bp deletion within the *bceB* gene, which encodes a glycosyltransferase. Insertional inactivation of *bceB* in *Burkholderia ambifaria* AMMD results in loss of EPS production on sugar alcohol media. These novel and surprising insights into EPS biosynthesis highlight the metabolic potential of the Bcc and show that a potential virulence factor may not be detected by routine laboratory culture. Our results also highlight a potential hazard in the use of inhaled mannitol as an osmolyte to improve mucociliary clearance in individuals with cystic fibrosis.

INTRODUCTION

The genus *Burkholderia* includes three closely related microbial species that highlight diverse evolutionary adaptation to different niches and hosts. *Burkholderia mallei* is a soliped-specific pathogen that only occasionally infects humans. *Burkholderia pseudomallei* is a free-living soil microbe and the causative agent of the subtropical human disease melioidosis. The species that presently constitute the *Burkholderia cepacia* complex (Bcc) have multiple roles; they include soil and water saprophytes, rhizosphere parasites, bioremediators, plant growth promoters and plant, animal and human pathogens. Members of the Bcc are particularly associated with life-threatening respiratory infections in patients with chronic granulomatous disease (CGD), and are the most potently virulent, transmissible and inherently resistant microbes to have emerged as cystic fibrosis (CF) pathogens in recent decades (Govan, 2006; Mahenthiralingam et al., 2005).

Although most species within the Bcc produce a variety of putative virulence factors, the role of these factors in the pathogenesis of human infection is unclear (Mahenthiralingam et al., 2005). Evidence from various model systems (mouse, rat, plant and nematode) suggests...
that the importance of individual virulence factors, or combinations of factors, depends on the infection model used (Bernier et al., 2003). In addition, studies of Bcc infections in CF patients also suggest a key role of host–pathogen interactions, since clinical outcome in individual patients cannot be predicted even during epidemic outbreaks when multiple patients are infected by the same strain (Govan et al., 1993). The first description of pathogenicity in the Bcc was based on sour skin rot of onion bulbs (Burkholder, 1950). In this study, we returned to this plant host to investigate the onion-associated phenotype of the Bcc, and reveal a link between growth conditions and exopolysaccharide (EPS) production.

EPS is a putative Bcc virulence factor that is involved in persistence of the bacteria in CF lungs (Conway et al., 2004), interactions with antimicrobial peptides (Herasimenka et al., 2005) and the formation of biofilms (Cunha et al., 2004). The EPSs of Burkholderia species have recently been comprehensively reviewed (Goldberg, 2007). Recent studies (Zlosnik et al., 2008) have also challenged the previous belief that mucoid, EPS-producing colonial morphotypes of Bcc are rare in both environmental and clinical isolates (Govan & Deretic, 1996). Other studies have shown that mucoid Bcc isolates mostly synthesize one type of EPS, with a highly branched heptasaccharide repeating unit, which was named cepacian (Moreira et al., 2003; Sist et al., 2003). EPS production has been shown to increase when the Bcc are grown in mannitol-rich yeast extract medium (MYEM) (Sage et al., 1990; Zlosnik et al., 2008).

Here we report the novel observation that many Bcc isolates, found to be non-mucoid on typical culture media, produce copious amounts of EPS when onion tissue is provided as the sole nutrient. Chemical and molecular analyses suggest that EPS biosynthesis is strain-specific and that the plant compounds responsible are primarily sugars and sugar alcohols. We show that the EPS phenotype on onion media is associated with the previously described bce cluster (Moreira et al., 2003), thought to be involved in cepacian biosynthesis.

**METHODS**

**Bacterial strains and culture conditions.** Bcc isolates used in this study are described in Table 1 and include 16 isolates from the two published Bcc strain panels (Coenye et al., 2000; Mahenthiralingam et al., 2000). Additional Bcc strains investigated included Burkholderia pyrrocinia BTS7, Burkholderia cepacia BTS2, as well as 19 Burkholderia multivorans, 14 Burkholderia cenocepacia IIIA and 11 Burkholderia cenocepacia IIIB isolates from our collection. Isolates were recovered from storage at −80 °C by subculture on nutrient agar (NA; Columbia base agar, Oxoid) and subsequently grown on media composed of 1.5% (w/v) bacteriological agar (NA; Columbia base agar, Oxoid) and subsequently grown on nutrient agar (NA; Columbia base agar, Oxoid) and subsequently grown on a Whatman no. 1 paper alongside markers (ferulic acid, rhamnose, xylose, sucrose, myo-inositol, ribitol (adonitol) and D-glucitol (sorbitol). The fructan polysaccharide inulin was also tested. As a control, isolates were grown on bacteriological agar containing 0.2% (w/v) yeast extract alone.

**Paper electrophoresis.** To fractionate the extract based on the presence or absence of functional groups, the freeze-dried aqueous phase of the onion extract was weighed and resuspended in distilled H₂O to a final concentration of 50 mg ml⁻¹ and was hydrolysed in 2 M TFA (trifluoroacetic acid; Sigma) at 60 °C or 120 °C for 1 h.

**Acid hydrolysis.** The aqueous phase residue of the ethyl acetate partitioned onion extract was redissolved in distilled H₂O, filtered, and hydrolysed in 2 M TFA (trifluoroacetic acid; Sigma) at 60 °C or 120 °C for 1 h.
Table 1. EPS biosynthesis of *B. cepacia* complex species when grown on agar supplemented with various substrates

All strains tested are from the two published Bcc panels (Coenye *et al.*, 2003; Mahenthiralingam *et al.*, 2000), except for BTS2 and BTS7, which were donated by Paola Cescutti. EPS production was scored on a scale from – (no EPS) to +++ (very mucoid). Mucoid growth described as +++ is shown in Fig. 1(b). YE, yeast extract agar.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Source</th>
<th>YE</th>
<th>Onion</th>
<th>Glucose</th>
<th>Sucrose</th>
<th>Fructose</th>
<th>Inulin</th>
<th>Glycerol</th>
<th>Mannitol</th>
<th>Glucitol</th>
<th>Ribitol</th>
<th>Inositol</th>
<th>Mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. cepacia</em></td>
<td>ATCC 25416</td>
<td>Onion</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>++</td>
<td>+++</td>
<td>–</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><em>B. cepacia</em></td>
<td>CEP509</td>
<td>CF</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><em>B. multivorans</em></td>
<td>C1576</td>
<td>CF</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td><em>B. multivorans</em></td>
<td>ATCC 17616</td>
<td>Soil</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td><em>B. cenocepacia</em></td>
<td>J2315*</td>
<td>CF</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>B. cenocepacia</em></td>
<td>K56-2*</td>
<td>CF</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>B. cenocepacia</em></td>
<td>BC7*</td>
<td>CF</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>B. cenocepacia</em></td>
<td>PC184</td>
<td>CF</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><em>B. cenocepacia</em></td>
<td>BTS2</td>
<td>CF</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><em>B. stabilis</em></td>
<td>LMG14294</td>
<td>CF</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>B. vietnamiensis</em></td>
<td>LMG10929</td>
<td>Rice</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>–</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td><em>B. vietnamiensis</em></td>
<td>PC259</td>
<td>CF</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>B. dolosa</em></td>
<td>E12</td>
<td>CF</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>B. ambifaria</em></td>
<td>AMMD</td>
<td>Soil</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>B. anthina</em></td>
<td>W92†</td>
<td>Soil</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>B. anthina</em></td>
<td>C1765</td>
<td>CF</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>B. pyrrocinia</em></td>
<td>BTS7</td>
<td>CF</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>B. pyrrocinia</em></td>
<td>C1469</td>
<td>CF</td>
<td>–</td>
<td>++</td>
<td>–</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*ET12 isolates.*
Staining and elution methods. Electrophoretograms and chromatograms were stained with silver nitrate (Fry, 2000) to reveal monosaccharides, oligosaccharides, alditols, saccharic acids and phenols, and with aniline hydrogen phthalate to reveal monosaccharides and reducing disaccharides. The paper strips of interest from both methods were eluted by a syringe method with distilled H2O (Eshdat & Mirelman, 1972). The eluted material was incorporated into 1.5% (w/v) bacteriological agar with 0.2% (w/v) yeast extract.

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD). Twenty microlitres each of the aequous phases of the onion extract and the TFA-hydrolysed samples (0.1 mg ml⁻¹) were analysed by HPAE–PAD (Dionex). The system consisted of an AS5000 autosampler, GP40 gradient pump, ED40 electrochemical detector, and PC10 pneumatic controller. The amperometry detector cell contained a gold electrode and a pH-Ag/AgCl combination reference electrode. CarboPac MA-1, PA-1 and PA-100 columns and guard columns were used for the separation of alditols, monosaccharides and oligosaccharides, respectively. Eluents, degassed by bubbling with helium, were as follows: MA-1, 600 mM NaOH at 0.4 ml min⁻¹ (isocratic); PA-1, 20 mM NaOH for 3 min, then H2O for 32 min, then a 0–200 mM NaOAc gradient over 10 min (all at 1.0 ml min⁻¹ with post-column addition of base); PA-100, 100 mM NaOH throughout, supplemented with a 0–200 mM NaOAc gradient over 30 min, then 200–800 mM NaOAc over 10 min (all at 1.0 ml min⁻¹). Analyses were identified by comparison of retention times to those of standards and quantified by integration of peak area with Chromeleon software (Dionex).

Investigation of conserved EPS gene clusters in Bcc species. Genome sequences representing five Bcc species were examined to investigate of conserved EPS gene clusters in Bcc species. Primer sequences are available upon request.

RT-PCR analysis of BCESM and chbA. Genomic DNA was prepared as described above. PCRs were performed as described previously (Mahenthiralingam et al., 1997; Sajjan et al., 1995).

Insertional inactivation of bceB in B. ambifaria AMMD. Insertional inactivation of bceB was performed using the pGP5TIp suicide vector, essentially as described previously (Flanagan et al., 2007). In brief, a 300 bp fragment internal to the bceB ORF of B. ambifaria AMMD and flanked by XbaI and EcoRI sites was PCR-amplified and ligated into the corresponding sites in pPG5TIP following appropriate restriction. Resulting plasmids were transformed into Escherichia coli GT115 competent cells (InvivoGen) and subsequently introduced into B. ambifaria AMMD by triparental mating. Resulting exconjugants were selected using gentamicin (50 mg l⁻¹) and trimethoprim (100 mg l⁻¹), and mutants identified by PCR using a chromosomal-specific primer in conjunction with the vector-specific primer RSF1300 (Flanagan et al., 2007).

RESULTS

EPS production on onion agar

When a lyophilized onion extract was incorporated into agar at 2% (w/v) as the sole nutrient, members of the Bcc were not only able to grow, but a majority of isolates also produced copious amounts of EPS, as typified by B. ambifaria AMMD (Fig. 1a, b). This phenotype is not observed when the Bcc are cultured on B. cepacia media (Mast Diagnostics), nutrient agar and other common laboratory media. Induction of EPS biosynthesis on onion agar was observed in all Bcc species investigated, but was not observed in all strains within a species (Table 1 and Supplementary Tables S1, S2 and S3, available with the online version of this paper). The exception was the single strain of Burkholderia stabilis available for testing. Of particular interest was the failure of the well-characterized B. cepacia ET12 representatives J2315, K56-2 and BC7 to produce EPS on onion agar. In addition, there was no correlation between induction of Bce EPS on onion agar and the ability of individual Bcc strains to cause maceration of onion bulbs (data not shown).
The onion factor

Attempts were made to identify the ‘onion factor’ responsible for inducing EPS biosynthesis by use of standard biochemical methods for extracting and fractionating phytochemicals. The causative factor was retained on drying in vacuo, and remained in the aqueous phase after partition with ethyl acetate at pH 7.0 and pH 2.0. When further physicochemical analyses discounted the role of proteins and lipids, attention was turned to the carbohydrate content of onion extract. After preparative paper electrophoresis in buffers at pH 2.0 and 6.5, the only biological activity recovered from paper strips co-migrated with the standard glucose, indicating the absence of ionizable functional groups such as phosphate, acid or amine (data not shown). Analytical paper chromatography and HPAE-PAD identified the major carbohydrate components as sucrose, glucose, fructose and fructans (Fig. 2). The HPAE-PAD chromatograms in Fig. 2 show characteristic peaks of glucose, fructose and sucrose. Sucrose breaks down to fructose and glucose upon mild hydrolysis, as do the fructans to fructose. Fructose breaks down under complete hydrolysis as expected for a ketose sugar, whilst the aldose sugar glucose remains stable. The ability of these and related compounds to stimulate EPS biosynthesis in Bcc was then investigated (Table 1). Glycerol and mannitol were included as these sugar alcohols have previously been noted to enhance EPS biosynthesis in *Pseudomonas aeruginosa* (Whitchurch *et al.*, 1996) and the Bcc (Sage *et al.*, 1990; Zlosnik *et al.*, 2008). Glucitol was included because of its close degradative relationship with fructose and mannitol (Allenza *et al.*, 1982). These experiments showed that within a particular Bcc species, EPS biosynthesis was strain-specific and that the most potent inducers of EPS were fructose and all alditols tested, most significantly mannitol and glucitol, as well as the cyclitol myo-inositol. Importantly, the profile of EPS biosynthesis production with these sugars was similar to that observed with onion extract (Table 1). EPS biosynthesis was not observed on agar containing yeast extract alone, nor in the presence of glucose (Table 1), galactose, lactose or maltose (data not shown) with any Bcc strains tested.

Investigation of the molecular basis for EPS biosynthesis

With the exception of the single *B. stabilis* strain tested, all Bcc species were shown to be capable of producing EPS when grown on onion agar (Table 1 and Supplementary Tables S1–S3), suggesting the presence of a conserved EPS biosynthetic gene cluster. Therefore genome sequences representing five Bcc species (*B. ambifaria*, *B. multivorans*, *B. vietnamiensis*, *B. dolosa* and *Burkholderia* sp. 383) were examined to determine if two putative EPS gene clusters within *B. cenocepacia* J2315, the bce gene cluster (Moreira *et al.*, 2003) and the wcb gene cluster (Parsons *et al.*, 2003), are conserved across the Bcc. The wcb gene cluster was found to be poorly conserved, with between one-third and one-half of J2315 ORFs having no direct homologues within the species examined (data not shown). Notably, within the EPS-producing *B. ambifaria* AMMD strain, over half of the J2315 wcb-associated ORFs have no homologues, and the remaining homologous ORFs are not organized within a gene cluster. In contrast, the bce gene cluster was conserved across all species examined, in terms of both sequence homology and organization of ORFs (Supplementary Fig. S1). In the course of these genome comparisons, a third putative polysaccharide biosynthetic gene cluster was observed on chromosome 2 of *B. cenocepacia* J2315. This cluster encodes two putative EPS transporter proteins (BCAM1330 and BCAM1331), an acyltransferase (BCAM1333), several glycosyltransferases (BCAM1335, BCAM1337, BCAM1338), a polysaccharide biosynthesis protein (BCAM1336) and a mannose-6-phosphate isomerase (BCAM1340). This cluster is conserved amongst several Bcc species, albeit to a lesser extent than the bce gene cluster (data not shown). In the EPS-producing *B. ambifaria* AMMD, this gene cluster maps to ORFs Bamb_3621 through to Bamb_3629.

---

**Fig. 1.** Growth of *B. ambifaria* AMMD on (a) nutrient agar (non-mucoid); (b) onion agar (mucoid), and (c) comparison of AMMD bceB mutant (left) and AMMD wild-type (right) on mannitol agar.
To investigate which polysaccharide gene cluster is induced by growth on mannitol, the expression of representative genes from two distinct EPS gene clusters was assessed in _B. ambifaria_ AMMD grown in the presence and absence of mannitol. The genes studied each encode homologues of the Wza EPS export protein: Bamb_5549 of the _bce_ gene cluster (equivalent to _bceE_, BCAM0858 of _B. cenocepacia_ J2315), and Bamb_3621 of the novel putative polysaccharide gene cluster described above (BCAM1330 of _B. cenocepacia_ J2315). As shown in Fig. 3, expression of Bamb_3621 was not observed under either growth condition. In contrast, expression of the _bceE_ homologue (Bamb_5549) was clearly induced by the presence of mannitol. We therefore focused on the _bce_ gene cluster to investigate why some Bcc isolates, most notably those of the _B. cenocepacia_ ET12 lineage, failed to produce EPS under any growth conditions.

A PCR assay was designed to screen isolates for an 11 bp deletion in the _bceB_ gene that has been suggested to be responsible for loss of EPS production in the CF isolate _B. cenocepacia_ J2315 (Moreira _et al._, 2003). Of the panel of strains shown in Table 1, only the _B. cenocepacia_ ET12 isolates J2315, K56-2 and BC7 harbour the 11 bp deletion (data not shown). This result prompted us to test a panel of _B. cenocepacia_ IIIA strains containing both ET12 and non-ET12 isolates. There was a clear correlation between the 11 bp deletion and the presence of both _cblA_ (cable pilus) and BCESM (_B. cepacia_ epidemic strain marker), indicating that this deletion is a conserved feature within the ET12 lineage (Fig. 4). In our study, with the exception of strain E3051, the presence of the deletion correlated with the lack of EPS production in all _B. cenocepacia_ IIIA isolates examined (Fig. 4; Supplementary Table S1). Furthermore, the 11 bp deletion in the _bceB_ gene was not observed in any _B. cenocepacia_ IIIB or _B. multivorans_ isolates studied (see Supplementary Tables S2 and S3).

Using proven methods for the complementation of gene function in _B. cenocepacia_ (Ortega _et al._, 2005), attempts were made to complement BceB function within _B. cenocepacia_ K56-2 by introducing a wild-type _bceB_ ORF.

---

**Fig. 2.** HPAE-PAD chromatograms: Carbopac PA-100 column separation of sugars in onion extract. (a) Crude onion extract; (b) onion extract hydrolysed by 2 M TFA 60 °C 1 h; (c) onion extract hydrolysed by 2 M TFA 120 °C 1 h. Glucose, fructose and sucrose peaks were clearly identified based on standards (not shown), and hydrolysis pattern. Peaks with retention times between 12 and 36 min appear to be oligosaccharides of fructose based on their degradation to fructose under mild hydrolysis.
Expression of Bamb_3621 was not detected in YE or YE+Man. Bamb_3621 is located within a distinct putative EPS biosynthetic gene cluster. Expression was assessed in yeast extract (YE) and yeast extract supplemented with 2% (w/v) mannitol (YE+Man). Expression of Bamb_3621 was not detected in YE or YE+Man. In contrast, bceE expression was strongly induced by growth in YE+Man. For each sample, RT and non-RT reactions (+/-) are shown alongside each other. Genomic DNA positive controls are shown for each gene (bceE +ve, Bamb_3621 +ve).

**DISCUSSION**

In this study, we returned to the original Bcc host and report that Bcc isolates previously considered non-mucoid produce copious amounts of EPS when onion tissue is provided as the sole nutrient, highlighting the metabolic potential of this group of organisms. This novel and surprising observation is not species-specific, and is exhibited by isolates of both clinical and environmental origin. The onion components responsible for EPS induction are primarily the carbohydrates sucrose, fructose and fructans. Additionally, all alcohol sugars tested are able to induce EPS production, in particular mannitol and glucitol.

The reasons why EPS biosynthesis is readily induced by onion extracts and, in particular, by fructose and the hexitol sugars mannitol and glucitol, require further investigation. An ability to respond to inulin was confined to certain strains, all of which responded well to fructose. This suggests that the fructan polysaccharide was only active in bacteria that can hydrolyse it to fructose. The ability of one strain (BTS7) to respond particularly strongly to inulin could possibly be due to its enhanced ability to hydrolyse inulin to fructose. Interestingly, the similar profile of EPS induction shown by fructose and alcohol sugars agrees well with a previous study which showed that the initial steps of utilization of these sugars in the Bcc differ from those in most other pseudomonads. Growth of Bcc on fructose involves active transport followed by fructokinase conversion to fructose 6-phosphate then degradation via the Entner–Doudoroff pathway (Allenza et al., 1982). Growth on mannitol and glucitol also requires this pathway following active uptake and intracellular oxidation to fructose.

The role of Bcc EPS as a putative virulence factor is unclear. The influence of mannitol and other alcohol sugars on EPS production suggests that the prevalence of EPS in the Bcc may have been underestimated. In addition, given the variety of human, animal, plant and other models studied, the role of EPS may be influenced by the host involved and the route of administration. EPS has been associated with altered Bcc clearance in a mouse model of infection (Conway et al., 2004), and EPS-deficient Bcc mutants displayed reduced mortality within a CGD mouse model (Sousa et al., 2007a). Similarly, a role for EPS in persistence amplified from B. cenocepacia PC184. Whilst expression of 6His-tagged BceB was detected in E. coli C41(DE3) cells, previously shown to support expression of membrane-bound proteins (Miroux & Walker, 1996), we were unable to detect expression within B. cenocepacia K56-2 (data not shown). Consequently, we chose to disrupt the bceB ORF of an EPS-producer. Insertional inactivation of the bceB gene resulted in loss of EPS production in B. ambifaria AMMD when grown on mannitol agar (Fig. 1c) and on onion agar (data not shown).
in human airways is suggested by its capacity to scavenge reactive oxygen species and inhibit neutrophil chemotaxis (Bylund et al., 2006). In our study, EPS biosynthesis did not correlate with the ability to cause onion rot, which is perhaps to be expected since pectinases rather than EPS are likely to play the major role in the maceration of plant tissue. Cunha et al. (2004) did not observe a clear correlation between EPS biosynthesis in vitro and the ability of Bcc strains to establish chronic infections within the CF lung. Recent evidence, however, suggests a subtle role for Bcc EPS in CF lung infection. Consistent with our findings, Zlosnik et al. (2008) reported that isolates of B. cenocepacia, the most virulent Bcc species, are most frequently non-mucoid. They also observed a mucoid to non-mucoid conversion in sequential isolates of Bcc from chronically infected CF patients. This apparent loss of mucoidity in vivo, and its absence in the virulent B. cenocepacia ET12 lineage, provides an intriguing contrast with the characteristic non-mucoid to mucoid conversion observed with alginate-producing P. aeruginosa. Zlosnik and colleagues suggest that Bcc EPS could be responsible for the persistence of Bcc in CF airways whilst loss of EPS leads to increased disease severity.

In our investigation of EPS biosynthesis determinants, we focused on the highly conserved bce gene cluster. Previously described by Moreira et al. (2003), the bce gene cluster has had several of its encoded proteins characterized (Ferreira et al., 2007; Sousa et al., 2007b; Videira et al., 2005) and is thought to be involved in cepacian biosynthesis. In the present study, we demonstrated induction of the bce gene cluster by mannitol, and found a clear correlation between the inability of representatives of the B. cenocepacia ET12 lineage to produce EPS and the presence of an 11 bp deletion within the bceB gene, originally described within the genome sequence of B. cenocepacia J2315 (Moreira et al., 2003). Consistent with this correlation, insertional inactivation of bceB, which encodes a glycosyltransferase, resulted in the loss of EPS production by B. ambifaria AMMD when grown on onion media. Combined, these observations highlight the pivotal role of the bce gene cluster in onion-induced EPS biosynthesis, and suggest that the observed EPS is cepacian. However, mutations elsewhere within the bce gene cluster, or in other EPS-related gene clusters, must be responsible for the lack of EPS biosynthesis in B. cenocepacia strain E3051 (Supplementary Table S1), and in other EPS-negative Bcc strains in our study which lack the 11 bp deletion in bceB (Supplementary Tables S2 and S3).

The ability of hexoses and hexitols, in particular mannitol, to enhance EPS biosynthesis in the B. cepacia complex has disturbing implications for therapeutic intervention in CF. Recent attempts to improve airway clearance with hypertonic saline 5% (w/v) have been handicapped by the problem of salty taste and the salt-sensitive nature of many antimicrobial peptides. Thus, attention has turned to the use of non-ionic osmolytes, including inhaled mannitol (Daviskas et al., 2008; Robinson et al., 1999; Wills, 2007), which is marketed as Bronchitol (Pharmaxis). Robinson and colleagues acknowledged that the majority of P. aeruginosa and Bcc isolates are able to utilize mannitol as a carbon and energy source. However, they felt that the nutritional influence of mannitol as a therapeutic osmolyte would be minimal given the abundance of other nutrients already present in CF respiratory secretions. On a cautionary note, they state that this potential problem would need to be confirmed by quantitative microbiology. The potential induction of virulence determinants during osmolyte therapy has to our knowledge not been considered. Our results also provide justification for the continued exclusion of CF individuals known to be infected with Bcc from ongoing trials of inhaled mannitol (http://clinicaltrials.gov; identifier NCT00117208 and NCT00251056).

ACKNOWLEDGEMENTS

We thank Catherine Doherty (University of Edinburgh, UK) for the strain panels, Rosanna Hennessy (University of Edinburgh, UK) for the RT-PCR data, Paola Cescutti (Università di Trieste, Italy) for Burkholderia pyrocinia BTS7 and B. cenocepacia BTS2, and Miguel Valvano (University of Western Ontario, Canada) for the suicide vector pGP90T and complementation vector pSCRahB3. S. J. B., A. R. B., J. R. W. G. and D. J. C. thank the CF Trust and the Big Lottery Fund, D. J. C. and D. J. C. also thank the Royal Society of Edinburgh, and S. C. F. and B. R. M. thank the Biotechnology and Biological Sciences Research Council for financial support.

REFERENCES


Exopolysaccharide from the Burkholderia cepacia complex


Edited by: P. Cornelis

http://mic.sgmjournals.org