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RESUMO

A diversidade e multifuncionalidade dos exopolissacáridos (EPSs) produzidos pelos organismos procariotas são consideráveis, supondo-se que estes têm um papel fundamental na protecção dos microrganismos que os produzem. Mais de 70% das estirpes pertencentes ao complexo *Burkholderia cepacia* (Bcc), consideradas importantes patogénios oportunistas em doentes com fibrose quística (FQ) ou com doença granulomatosa crónica, são produtoras do EPS Cepaciano. O Bcc é um grupo de bactérias da subdivisão β-proteobacteria, que são genotipicamente distintas, mas fenotipicamente semelhantes, estando estas divididas em pelo menos 17 espécies diferentes.

Recentemente, vários estudos têm evidenciado o papel do Cepaciano como um factor de virulência que contribui para a patogenicidade global dos membros do Bcc e portanto para o seu sucesso como patogénios. Observou-se que a produção de EPS por um isolado clínico mucóide de *B. cenocepacia* interfere com a fagocitose das bactérias pelos neutrófilos humanos e facilita a persistência bacteriana nos ratinhos BALB/c, usados como modelo de infecção. Por outro lado, usando-se os ratinhos gp91$^{phox-/-}$ como modelo de infecção, verificou-se que os mutantes relativamente à produção de EPS eram menos virulentos ou completamente avirulentos quando comparados com a estirpe selvagem produtora de EPS. Estes resultados sugerem que a produção de EPS por bactérias do Bcc pode contribuir para a persistência das infecções pulmonares. Foi também descrito que o Cepaciano produzido por um isolado clínico de *B. cenocepacia* interfere com a função dos neutrófilos humanos *in vitro*, inibe a quimiotaxia e a produção de espécies reactivas de oxigénio, que são componentes essenciais da resposta imune inata do hospedeiro. Vários estudos têm demonstrado a capacidade das bactérias do Bcc para formarem biofilmes sozinhas ou em associação com outras bactérias. Estudos realizados com mutantes defectivos na produção de EPS demonstraram que apesar de não ser requerido para a formação inicial dos biofilmes, o EPS é importante para a formação de biofilmes espessos e maduros.

O Cepaciano é composto por uma unidade repetitiva heptassacarídica ramificada e acetilada apresentando na sua constituição D-ramnose, D-manose, D-glucose, D-galactose e ácido D-glucurónico, numa proporção de 1:1:1:3:1. Curiosamente este polímero apresenta duas características incomuns, a presença de um D-isómero da ramnose que é mais comum no antigénio O dos lipopolissacáridos e a presença de um resíduo de ácido glucorónico tri-substituído, que confere ao polímero uma estrutura secundária rígida e alongada.

Só após ter sido proposta a via biossintética dos precursores dos nucleótidos de açúcar do Cepaciano, é que se tentaram identificar os genes codificantes das enzimas intervenientes nesta via, estando estes localizados nos agrupamentos de genes *bce-I* e *bce-II* do cromossoma 2 do Bcc. O agrupamento *bce-I* foi identificado após mutagénese aleatória através da inserção de plasposões e seleccionando os mutantes defectivos na produção de EPS. O agrupamento *bce-II* foi identificado posteriormente recorrendo a análises bioinformáticas e a genómica comparativa.

Ainda que tenham sido identificados cinco dos genes que codificam proteínas provavelmente envolvidas na síntese dos precursores dos nucleótidos de açúcar (BceA, BceC, BceM, BceN e BceT), apenas as proteínas BceA e BceC foram funcionalmente caracterizadas. A via biossintética da D-

ramnose não se encontra ainda caracterizada nas bactérias do Bcc. No entanto, análises bioinformáticas sugerem que o gene *bceN*, que se encontra no agrupamento genético *bce-II*, codifica para uma proteína com actividade de GDP-D-manose 4,6-desidratase (GMD), envolvida na síntese do precursor GDP-D-ramnose a partir de GDP-D-manose. Desta forma, as actividades descritas no presente trabalho visam a análise funcional do gene *bceN* e o estudo do seu papel na síntese do EPS Cepaciano, bem como o seu envolvimento na formação de biofilmes.

As sequências aminoacídicas das proteínas BceN de *B. cepacia* IST408 e *B. cenocepacia* J2315 foram analisadas bioinformaticamente, verificando-se a presença de motivos conservados da família das proteínas Desidrogenases/reductases de cadeia curta (SDR), nomeadamente o motivo de Wierenga, importante para a ligação do co-factor; a tríade catalítica, essencial para a catálise; e o domínio de Rossman, igualmente associado à ligação do co-factor à região N-terminal da proteína. Os motivos e os resíduos típicos das enzimas com actividade GMD, nomeadamente os resíduos conservados glutamato (posição 133) e arginina (posição 190), e a região do "RR loop", também estão presentes na proteína BceN.

Com vista a confirmar os resultados bioinformáticos obtidos o gene *bceN* foi clonado num vector de expressão, e a proteína BceN foi sobrexpressa com cauda de histidinas e purificada por cromatografia de afinidade. Através da análise dos produtos obtidos por espectroscopia de ressonância magnética nuclear (NMR), demonstrou-se *in vitro* que a proteína BceN catalisa a conversão de GDP-D-manose em GDP-4-ceto-6-desoxi-D-manose, tendo portanto actividade GDP-D-manose 4,6-desidratase. Ainda que os resultados obtidos sugerissem que esta proteína apresenta um modelo cinético de inibição pelo substrato, os ensaios realizados não possibilitaram a determinação dos parâmetros cinéticos da proteína BceN. No entanto foi possível constatar que a actividade GMD desta proteína é bastante dependente da presença de iões bivalentes Mg^{2+}, que os co-factores oxidados NAD e NADP não são essenciais para a actividade da mesma e que esta é inibida pelo substrato GDP-D-manose. Ainda que algumas proteínas com actividade GMD tenham a capacidade de catalisar a reacção de desidratação e redução que leva à formação de GDP-D-ramnose a partir de GDP-D-manose, os ensaios realizados sugerem que a proteína BceN não tem dupla actividade.

Um outro objectivo deste trabalho consistiu no estudo do papel da proteína BceN na produção do EPS Cepaciano na estirpe clínica mucóide *B. cepacia* IST408, construindo-se para tal um mutante de inserção do gene *bceN*. Uma vez que este gene está envolvido na síntese da ramnose, um dos açúcares que constituem o Cepaciano, seria de esperar que a capacidade de produzir EPS fosse afectada na estirpe mutante. Todavia, a capacidade de produzir EPS é mantida pela estirpe mutada, sendo a quantidade de EPS produzida apenas ligeiramente inferior à da estirpe selvagem. Ainda que não se tenha determinado a composição do EPS produzido pelo mutante relativamente à estirpe selvagem, os resultados obtidos sugerem que a proteína BceN não é essencial para a síntese do EPS Cepaciano. A capacidade da estirpe mutante para produzir EPS sugere assim que outros genes funcionais homólogos ao gene *bceN* possam existir no genoma de *B. cepacia* IST408. A pesquisa destes genes nas sequências genómicas disponíveis de 14 estirpes do género *Burkholderia* revelou a presença de um, dois ou três genes que possivelmente codificam proteínas com actividade GMD.

Esta constatação sugere assim que a mutação do gene *bceN* em *B. cepacia* IST408 pode ser compensada por homólogos funcionais deste gene.

Uma vez que a produção de EPS está envolvida na formação de biofilmes e esta por sua vez está relacionada com a motilidade por swarming, estes dois últimos comportamentos multicelulares foram comparados entre a estirpe selvagem e o mutante no gene *bceN* de *B. cepacia* IST408. A quantidade de biofilmes produzida pelo mutante foi inferior à produzida pela estirpe selvagem, o que é consistente com a ligeira redução da capacidade do mutante para produzir o Cepaciano, e com o requerimento deste para a formação de biofilmes de dimensões máximas. Por outro lado a mutação do gene *bceN* também afectou a motilidade por swarming, apresentando o mutante uma diminuição desta capacidade.

Com base nos estudos realizados e com vista a melhorar a percepção do papel da actividade GDP-D-manose 4,6-desidratase na virulência das bactérias do Bcc seria necessária a construção de uma estirpe mutante em que todos os genes homólogos ao gene *bceN* estivessem inactivados.

Palavras-chave:

Complexo *Burkholderia cepacia*;
Exopolissacárido Cepaciano;
Agrupamento de genes *bce-II*;
Gene *bceN*;
GDP-D-manose 4,6-desidratase.

ABSTRACT

Burkholderia cepacia complex (Bcc) bacteria have gained notoriety as pathogens in cystic fibrosis (CF) because they are easily transmitted among CF individuals, difficult to identify and almost impossible to eradicate.

Isolates of the Bcc may produce large amounts of the exopolysaccharide (EPS) Cepacian, a virulence factor contributing to the overall pathogenicity of Bcc members and thus to their success as pathogens. The genes required for Cepacian biosynthesis by Bcc strains are located in clusters *bce-I* and *bce-II* of chromosome 2.

One of the clustered genes, *bceN*, encodes a 39 kDa protein with GDP-D-mannose 4,6-dehydratase (GMD) activity. This dehydratase activity of BceN protein is strongly dependent on the presence of Mg^{2+} ions, is unaffected by the addition of exogenous NAD(P) and is susceptible to inhibition by the substrate GDP-D-mannose. This protein appears to have the substrate inhibition kinetic model, however additional assays are required to confirm this hypothesis and to determine kinetic parameters.

The disruption of the *B. cepacia* IST408 *bceN* gene resulted in a slightly reduced EPS production yield compared to the wild type strain. The search for putative *bceN* homologues revealed the presence of one, two or three *bceN* orthologues in the *Burkholderia* genomes available. This suggests that in *B. cepacia* IST408 putative *bceN* functional homologues may compensate the *bceN* mutation. However, the size of the biofilm produced *in vitro* by the *bceN* mutant strain is smaller than the size of the biofilm formed by the parental strain and the mutant showed a decreased swarming ability.

Keywords:

Burkholderia cepacia complex;
Exopolysaccharide Cepacian;
bce-II gene cluster;
bceN gene;
GDP-D-mannose 4,6-dehydratase

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CONTENTS

1. INTRODUCTION

1.1. The *Burkholderia cepacia* complex (Bcc) – an overview

Burkholderia cepacia, previously known as *Pseudomonas cepacia*, was described originally by Walter Burkholder in 1950 as a phytopathogen associated with the soft rot of onion bulbs (sour skin disease) (Burkholder, 1950). In 1992, *Pseudomonas cepacia* and six other species were moved from the *Pseudomonas* genus to the new genus *Burkholderia* based on 16S rRNA sequences, DNA-DNA homology values, cellular lipid and fatty acid composition, and phenotypic characteristics (Yabuuchi *et al.*, 1992). From the mid 1990s on, researchers had noticed that there was a marked heterogeneity among strains identified as *B. cepacia* using traditional and molecular identification approaches, making their correct identification problematic (Stead, 1992; Larsen *et al.*, 1993; Simpson *et al.*, 1994; Kiska *et al.*, 1996). The diversity among these presumed *B. cepacia* strains and the lack of reliable identification schemes led Vandamme and colleagues to perform a polyphasic taxonomic study demonstrating that *B. cepacia* strains belonged to at least five distinct genomovars (I-V). These genomovars represented groups of phenotypically similar isolates with sufficient genetically differences to be designated as new species (Vandamme *et al.*, 1997). Presently, *Burkholderia cepacia* complex (Bcc) comprises at least 17 distinct species (Table 1.1), sharing a high degree of 16S rRNA gene (98 to 100%) and *recA* gene (94 to 95%) sequence similarity, and moderate levels of DNA-DNA hybridization (30 to 50%) (Coenye *et al.*, 2001; Vanlaere *et al.*, 2009).

Table 1.1 – *Burkholderia cepacia* complex species and strains with their genome completely sequenced.

Bcc Species	Sources and Relevant characteristics	Strains sequenced	References
B. cepacia	Infections in Humans (CF and non-CF); bioremediation and biocontrol agent		(Vandamme *et al.*, 1997)
B. multivorans	Infections in Humans (CF and non-CF)	ATCC 17616	(Vandamme *et al.*, 1997)
B. cenocepacia	Infections in Humans (CF and non-CF); biocontrol agent	J2315, AU1054, HI2424, MCO-3, PC184	(Vandamme *et al.*, 2003)
B. stabilis	Infections in Humans (CF and non-CF)		(Vandamme *et al.*, 2000)
B. vietnamiensis	Infections in Humans (CF and non-CF); bioremediation and biocontrol agent	G4	(Vandamme *et al.*, 1997)
B. dolosa	Infections in CF patients	AU0158	(Vermis *et al.*, 2004)
B. ambifaria	Infections in Humans (CF and non-CF); biocontrol agent	AMMD, MC40-6, MEX-5, IOP40-10	(Coenye *et al.*, 2001)
B. pyrrocinia	Infections in CF patients; biocontrol agent		(Vandamme *et al.*, 2002)
B. anthinia	Infections in Humans (CF and non-CF)		(Vandamme *et al.*, 2002)
B. ubonensis	Nosocomial infection	Bu	(Vanlaere *et al.*, 2008)

B. lateens	Infections in CF patients		(Vanlaere *et al.*, 2008)
B. diffusa	Infections in Humans (CF and non-CF), isolated from water and soil		(Vanlaere *et al.*, 2008)
B. arboris	Infections in Humans (CF and non-CF), environmental sources		(Vanlaere *et al.*, 2008)
B. seminalis	Infections in Humans (CF and non-CF), environmental sources		(Vanlaere *et al.*, 2008)
B. metallica	Infections in CF patients		(Vanlaere *et al.*, 2008)
B. contaminans	Infections in CF patients and in animals		(Vanlaere *et al.*, 2009)
B. lata	Isolates from forest soil	383	(Vanlaere *et al.*, 2009)

As a member of the *Burkholderia* genus, the Bcc comprises Gram-negative bacteria of the β-proteobacteria subdivision. These microorganisms are aerobic, motile and non-spore-forming rods that are nutritionally quite versatile, being able to utilize a variety of simple and complex carbohydrates, alcohols, and amino acids as carbon sources (Palleroni, 1984; Vermis *et al.*, 2003). Most of them are mesophilic, with optimal growth temperatures between 30ºC and 37ºC (Palleroni, 1984). The genome of these bacteria consists of three chromosomes and many of them contain also plasmids (Holden *et al.*, 2009).

Bcc bacteria exhibit an extraordinary metabolic capacity, allowing their adaptation to a wide range of environments. Among these bacteria, several strains of potential environmental application have been identified due to their ability to degrade important pollutants in water and soils (e.g., crude oils, herbicides, recalcitrant aromatic compounds such as trichloroethylene, and xenobiotics). Although Bcc was originally recognized as a pathogen of onions in their natural environment, most Bcc species are considered highly beneficial (Mahenthiralingam *et al.*, 2005). In fact, many Bcc isolates are able to produce antifungal compounds, to fix atmospheric nitrogen, and to promote plant growth. These properties have attracted significant commercial interest on the use of some strains as biocontrol, bioremediation, and plant-growth promoting agents, mainly due to their ability to colonize the rhizosphere of several crops of economical interest, like corn, maize, rice, pea, and sunflower (Chiarini *et al.*, 2006). However, these ecologically and biotechnologically useful features of Bcc bacteria highly contrast with severe and often fatal infections that they can cause as human opportunistic pathogens. For this, there is a general consensus that the large-scale use of organisms of the *Burkholderia* genus is imprudent until more is known about the fate of biocontrol strains after their release in the environment (Mahenthiralingam *et al.*, 2005; Sousa *et al.*, 2011).

1.2. Bcc as opportunistic pathogens in Humans

In the last 30 years, Bcc bacteria have emerged as highly problematic opportunistic human pathogens, mainly in Cystic Fibrosis (CF) and Chronic Granulomatous Disease (CGD) patients (Isles

et al., 1984; Goldman and Klinger, 1986; Johnston, 2001). Lately, increasing number of infections caused by Bcc in immunocompromised individuals was observed (Mann *et al*., 2010). This fact could be attributed to the increasing numbers of immunocompromised patients, increased population age of these patients, a more accurate identification of Bcc organisms, the social and behavioral changes that have allowed closer contact between patients, and the increased use of antibiotics to which Bcc bacteria are resistant (LiPuma, 1998; Banerjee and Stabieforth, 2000; Moore and Elborn, 2001; Chernish and Aaron, 2003; Mahenthiralingam *et al*., 2005).

CF patients are particularly susceptible to lung infections with Bcc bacteria, which are not commensal organisms. Although *Pseudomonas aeruginosa* remains the most prevalent CF pathogen, increased morbidity and mortality rates are registered in Bcc-infected patients (Courtney *et al*., 2004). Bcc infections are particularly feared by CF patients and their caregivers, because the clinical outcome is highly variable and unpredictable, ranging from asymptomatic carriage to the cepacia syndrome (Mahenthiralingam *et al*., 2005; Sousa *et al*., 2011). Moreover, in the vast majority of CF patients, pulmonary colonization with Bcc is associated with a worst prognosis, including an accelerated decline of the patients' clinical status and an increased risk of death (Lyczak *et al*., 2002).

Patients who suffer from CGD are also at risk of invasive Bcc infections due to a primary immune disorder of polymorphonuclear leukocytes, which are defective in oxidative killing and therefore cannot kill these bacteria. Infection with Bcc bacteria leading to sepsis and pneumonia is the second most common cause of death of CGD patients (Johnston, 2001).

Furthermore, there are some reports of Bcc infections in immunocompromised patients such as cancer and HIV patients, and also among immunocompetent individuals (Marioni *et al*., 2006; Mann *et al*., 2010). In immunocompetent patients, Bcc strains have been isolated in cases of chronic suppurative otitis media complicated by cerebellar and cerebello-pontine abscesses, pharyngeal infection, and paediatric neck infections (Marioni *et al*., 2006). In recent years, an increasing number of bacteraemia cases caused by Bcc among non-CF hospitalized patients have been reported. Most of these patients have comorbidities such as chronic hemodialysis, diabetes mellitus, congestive heart failure, and malignancy. Among these hospitalized non-CF patients, hemodialysis, the permanence in intensive care units, the use of central venous catheters, indwelling urinary catheters, and endotracheal tubes are now recognized as risk factors contributing for Bcc acquisition. The accumulating reports of nosocomial outbreaks caused by Bcc led to the recognition of these bacteria as emergent nosocomial pathogens among non-CF patients, in particular among oncology patients (Mann *et al*., 2010; Sousa *et al*., 2011).

1.2.1. Bcc infections in Cystic Fibrosis patients

CF is the most common autosomal recessive disorder among Caucasians, with a frequency of about 1 to 2500 livebirths. The disease results from mutations in a single gene of chromosome 7, which encodes a 1480 amino acid polypeptide, the so-called cystic fibrosis transmembrane regulator (CFTR) (Collins, 1992). CFTR functions as a cAMP-dependent chloride channel of low conductance, mainly expressed in the apical membrane of epithelial cells (Collins, 1992). It also regulates other

conductance pathways, such as the ORCC chloride channels and the ENaC sodium channels (Vankeerberghen *et al*., 2002). Point mutations at the level of the *CFTR* gene produce abnormal forms of the protein, which results in defects in epithelial ion and water transport, mainly affecting cells in the respiratory, gastrointestinal, hepatobiliary and reproductive tracts (Welsh and Smith, 1995). As a consequence, mucociliary clearance of bacteria from the lungs is impaired by the highly viscous nature of airway secretions (Govan and Deretic, 1996). This situation favours chronic pulmonary infections by opportunistic bacterial pathogens such as *P. aeruginosa*, Bcc, *Staphylococcus aureus*, *Haemophilus influenza* and *Stenotrophomonas maltophilia*, which remain the leading cause of death among these patients (Ratjen and Döring, 2003).

Although Bcc is reported to cause infections in only 3.5% of CF patients worldwide, bacteria of this complex have emerged as a serious CF pathogen, mainly due to the reasons described below (Courtney *et al*., 2004).

Bcc infection can cause the cepacia syndrome, a condition occurring in approximately 20% of infected CF patients, characterized by high fever, necrotizing pneumonia and bacteraemia that results in a rapid and uncontrollable clinical deterioration and, in most cases early death (Isles *et al*., 1984). This fatal decline of the patient clinical condition has not been observed with any other CF pathogen and the key determinants associated with this syndrome are not clear. Clonal isolates can be isolated from patients with or without cepacia syndrome, suggesting that both bacterial and host factors play important roles in determining the clinical prognosis (Govan *et al*., 1993; Jones *et al*., 2004).

Furthermore, Bcc is inherently resistant to most of the clinically available antimicrobials and increased resistance is observed on formation of Bcc biofilms *in vitro* (Caraher *et al*., 2007). Therefore, although current treatment strategies use double or triple antibiotic combinations to achieve bactericidal activity, they rarely result in the eradication of the pathogen, particularly in the case of chronic infections (George *et al*., 2009).

Some Bcc strains can also be transmitted from patient-to-patient. There are strains of *B. multivorans* and *B. cenocepacia* which are highly transmissible, though other species, such as *B. dolosa* and *B. cepacia*, also show this ability (Holmes *et al*., 1999; Mahenthiralingam *et al*., 2002; Biddick *et al*., 2003). Due to the easy transmission (through aerosol droplets, direct physical contact with infected individuals or contaminated surfaces) of highly virulent strains among these patients, segregation measures of Bcc-infected patients have been successfully implemented to reduce the transmission of Bcc strains (LiPuma *et al*., 1990; Festini *et al*., 2006).

1.3. Virulence Factors

Bcc bacteria can produce a wide variety of potential virulence factors that are crucial for its pathogenicity. Some isolates have been demonstrated to be capable of surviving within eukaryotic cells, such as respiratory epithelial cells, macrophages and amoebae (Burns *et al*., 1996; Saini *et al*., 1999; Martin and Mohr, 2000).

Moreover, these bacteria express specific fimbriae, the cable pili, which, in association with adhesins, have shown to play an essential role in the invasion of the respiratory epithelium. These

bacteria also possess flagella that not only make bacteria motile, but also serve as adhesins enabling the pathogens to invade host cells (Sajjan *et al*., 2000; Tomich *et al*., 2002). In addition to the processes of attachment and invasion, Bcc strains also produce virulence factors such as lipases, exotoxins, metalloproteases and serine proteases, which enhance its pathogenicity to epithelial cells (McClean and Callaghan, 2009). Other virulence factors that have been identified by the use of different infection models include type III and IV secretion systems (Tomich *et al*., 2003; Engledow *et al*., 2004; Glendinning *et al*., 2004), components of the quorum sensing system (Huber *et al*., 2001; Sokol *et al*., 2003; Malott *et al*., 2005; Wopperer *et al*., 2006), lipopolysaccharide (LPS) (Shaw *et al*., 1995; Vinion-Dubiel and Goldberg, 2003), exopolysaccharide (EPS) (Conway *et al*., 2004; Cunha *et al*., 2004; Bylund *at al*., 2006), biofilm formation (Huber *et al*., 2001), iron acquisition (Visser *et al*., 2004), iron-chelating siderophores (Darling *et al*., 1998), an intrinsic antimicrobial resistance to multiple antibiotics (Chernish and Aaron, 2003), among other factors (Hutchison *et al*., 1998; Baldwin *et al*., 2004; Mahenthiralingam *et al*., 2005; Valvano *et al*., 2005). These virulence factors are thought to play critical roles for the success of the pathogen, however the relative contribution of each of these factors to the overall pathogenicity of Bcc in CF lung disease is still not sufficiently defined (Leitão *et al*., 2010).

EPS production, that can be a potent virulence factor responsible for avoidance of the host immune response, elicitation of an inflammatory response, or attachment to host cells, is discussed in detail below.

1.3.1. Exopolysaccharides (EPSs) of Bcc

Microorganisms synthesize a wide spectrum of multifunctional polysaccharides, some of which are excreted, retaining only a limited association with the cell surface, and these are often referred to as extracellular polysaccharides or exopolysaccharides (Sutherland, 2001; Cuthbertson *et al*., 2009).

EPSs play many different roles in the biology of microorganisms and are often important virulence determinants in bacteria that infect plants, animals and humans, promoting the evasion from the host defense mechanisms, as well as the bacterial adhesion to surfaces and resistance to antibacterial agents (Costerton *et al*., 1987; Roberts, 1996).

Some strains of Bcc produce an acidic EPS, exhibiting a mucoid phenotype when grown on solid media (Cérantola *et al*., 2000; Richau *et al*., 2000a). This phenotype has been found among both clinical and environmental isolates (Ferreira *et al*., 2010). A study by Richau *et al*. (2000a) and subsequent studies (Cunha *et al*., 2004) demonstrated that more than 80% of Bcc isolates involved in respiratory infections in CF patients were EPS producers. The EPS isolated from a clinical *B. cenocepacia* isolate has been shown to interfere with the function of human neutrophils *in vitro* by inhibiting chemotaxis and the generation of reactive oxygen species, both essential components of the host innate defenses (Bylund *et al*., 2005).

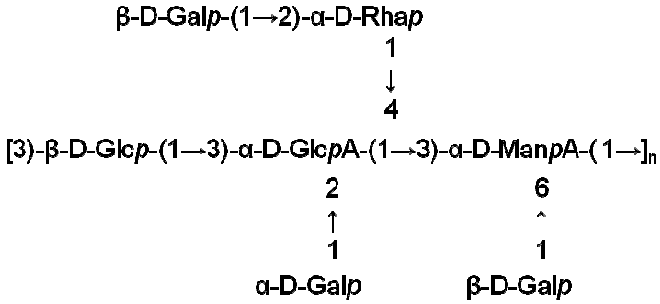
Several studies using mouse infection models revealed the importance of the EPS production. For instance, Conway *et al*. have shown that the EPS produced by a mucoid *B. cenocepacia* clinical isolate interfered with the bacterial phagocytosis by human neutrophils and facilitated the bacterial persistence in the BALB/c mice model of infection (Conway *et al*., 2004). In a study performed using

the gp91$^{phox-/-}$ CGD mice model of infection, Sousa *et al*. have shown that mutants defective in EPS production were less virulent or completely avirulent when compared to the wild type EPS-producing strain (Sousa *et al*., 2007b). These results suggest that the production of EPS plays a role in the persistence of Bcc pulmonary infections.

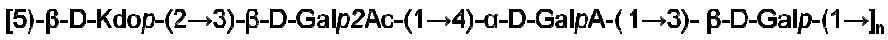
The ability of Bcc to form biofilms alone or in association with other bacteria has been demonstrated (Cunha *et al*., 2004; Komlos *et al*., 2005). Studies performed with EPS-defective mutants have demonstrated that, although not required for the initiation of biofilm formation, EPS is important to the formation of thick and mature biofilms (Cunha *et al*., 2004).

There are at least five EPSs that have been described in strains from Bcc, whose production, apparently, depends on the culture conditions used for growth (Figure 1.1; Chiarini *et al*., 2004; Conway *et al*., 2004). Several studies indicated that Cepacian, an heptasaccharide, is the major EPS produced by a large percentage of clinical isolates of the Bcc (Cérantola *et al*., 2000; Cescutti *et al*., 2000; Linker *et al*., 2001; Sist *et al*., 2003; Chiarini *et al*., 2004).

Cepacian



Galactan



Levan

PS-I

Figure 1.1. - Structure of four polysaccharides produced by Bcc strains. Abbreviations: Glu – Glucose, Gal – Galactose, Fru – Fructose, Man – Mannose, Glc – Glucuronic acid, Rha – rhamnose (adapted from Chiarini *et al*., 2004).

1.3.1.1. Exopolysaccharide Cepacian

Cepacian is composed of a branched acetylated heptasaccharide repeating unit with D-glucose, D-rhamnose, D-mannose, D-galactose, and D-glucuronic acid, in the ratio 1:1:1:3:1, conferring this latter sugar residue the acidic character to the polymer (Cèrantola *et al*., 1999; Cescutti *et al*., 2000). Curiously two unusual characteristics of this polymer were observed: the presence of a D-isomer of rhamnose that is more common in the O-antigenic polysaccharides of LPS, and the presence of a trisubstituted glucuronic acid residue, that confer a rigid and elongated secondary

structure to the polymer (Sist *et al*., 2003). Cepacian is also partially O-acetylated and can also have pyruvate as an additional substituent, however the position and number of the acetyl groups in Cepacian are still unknown (Cescutti *et al*., 2000). This feature could be relevant since it was described that acetylated polysaccharides confer a higher resistance to opsonic phagocytosis killing (Pier *et al*., 2001).

After the pathway leading to the nucleotide sugar precursors necessary for Cepacian biosynthesis have been proposed, genes encoding the enzymes involved in this pathway were searched. For this, a strategy based on random plasposon insertion mutagenesis was performed on the highly mucoid *B. cepacia* strain IST408 (Moreira *et al*., 2003). Based on comparison of the nucleotide sequence of the interrupted genes with the available genome of the ET12 lineage *B. cenocepacia* strain J2315, a polysaccharide biosynthetic gene cluster, the *bce-I* cluster, was identified (Moreira *et al*., 2003). This cluster was localized in chromosome 2, is about 16.2 kb, and includes eleven genes (*bceA* to *bceK*) predicted to encode enzymes required for the synthesis of nucleotide sugar precursors, and those involved in glycosyltransferase, EPS polymerization, and export (Moreira *et al*., 2003). Interestingly, a sequence comparison revealed that the *bceB* gene, encoding a putative glycosyltransferase, was interrupted with a frameshift in the J2315 strain, which does not produce detectable amounts of EPS (Moreira *et al*., 2003).

Although the *bce-I* clustered genes encode several proteins and enzymes, not all the proteins required for Cepacian biosynthesis are encoded within this cluster. In order to identify the missing genes, the available genome sequences of several Bcc strains were examined by bioinformatics tools and a second gene cluster, the *bce-II* cluster, was identified (Figure 1.2; Ferreira *et al*., 2010).

The *bce-II* cluster contains nine genes (*bceM* to *bceU*) encoding enzymes putatively involved in the D-rhamnose and D-glucose precursor's biosynthesis, a glycosyltransferase, a repeat-unit flippase, and acyltransferases presumably required for the acetylation of Cepacian (Figure 1.2). With the exception of *B. mallei* strains, the *bce-I* and *bce-II* clusters are widespread within all the sequenced strains of the *Burkholderia* genus (Ferreira *et al*., 2010).

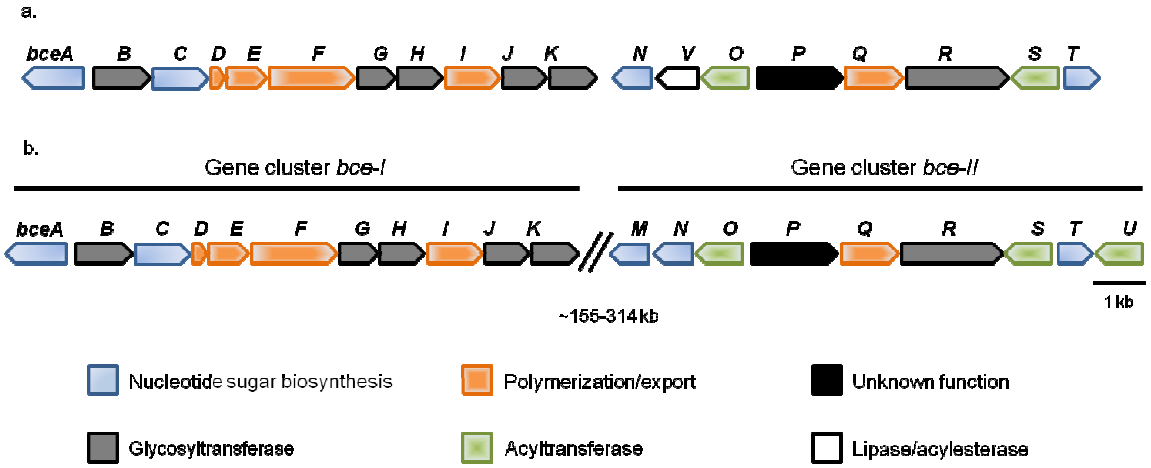


Figure 1.2. - Genetic organization of the *bce* gene cluster directing the biosynthesis of Cepacian by *Burkholderia* bacteria. (a) In representative strains of the species *B. xenovorans*, *B. phymatum*, *B. phytofirmans*, and *B. graminis*, the bce genes are clustered together in the same genomic region; (b) in representative strains of the *Burkholderia cepacia* complex, *B. pseudomallei*, *B. oklahomensis*, and *B. thailandensis*, the *bce* genes are split into two regions 155 to 314 kb apart (adapted from Ferreira *et al*., 2010).

1.3.1.2. Biosynthetic Pathway of the Exopolysaccharide Cepacian

The biosynthetic pathway that leads to the formation of the activated sugar precursors necessary for the synthesis of Cepacian was first postulated based on its chemical composition (Richau *et al*., 2000b). However, new data and hypothesis were acquired with the identification of gene clusters *bce-I* and *bce-II* (Moreira *et al*., 2003; Ferreira *et al*., 2010).

Cepacian biosynthetic pathway was hypothesized to start in the bacterial cytoplasm by the conversion of glucose-6-P into the five activated nucleotide sugar precursors (UDP-D-glucose, UDP-D-galactose, UDP-D-glucuronic acid, GDP-D-mannose, and GDP-D-rhamnose) that are the donors of monomers to synthesize the heptasaccharide unit (Figure 1.3; Richau *et al*., 2000b). The cluster *bce-I* only includes two of the genes (*bceA* and *bceC*) coding for these biosynthetic enzymes. These two proteins have been characterized: BceA is a bifunctional protein with phosphomannose isomerase (PMI) and GDP-D-mannose pyrophosphorylase (GMP) activities required for GDP-D-mannose biosynthesis (Sousa *et al*., 2007a), while BceC is a UDP-glucose dehydrogenase (UGD) involved in UDP-glucuronic acid synthesis (Loutet *et al*., 2009). The cluster *bce-II* includes three additional genes coding for enzymes involved in the nucleotide sugar precursor biosynthetic pathway. The gene *bceT* encodes a putative UDP-glucose pyrophosphorylase (UGP) catalyzing the reversible formation of UDP-glucose from UTP and glucose-1-phosphate. The *bceM* and *bceN* genes were predicted to encode a GDP-4-keto-6-deoxy-D-mannose reductase (RMD) and a GDP-D-mannose 4,6-dehydratase (GMD), respectively, two enzymes that use the precursor GDP-D-mannose to synthesize GDP-D-rhamnose, the donor of the D-rhamnose moiety of Cepacian (Figure 1.3; Ferreira *et al*., 2010). However, genes encoding the remaining enzymes necessary for the formation of nucleotide sugar precursors (phosphoglucomutase, PGM; phosphoglucose isomerase, PGI; phosphomannomutase, PMM; and UDP-glucose epimerase, UGE) are absent from the *bce* clusters, as found in other Gram-negative bacterial species. The scattered location of genes involved in the synthesis of these sugar nucleotides may reflect the fact that they are not specific for Cepacian synthesis, but are also involved in other metabolic processes, such as the biosynthesis of lipopolysaccharide and other cell polysaccharides (Ferreira *et al*., 2010).

In several Gram-negative bacteria, the synthesis of EPS involves undecaprenyl intermediates. The translocation of the repeating units through the membrane occurs in an undecaprenyl-dependent manner (White, 2000). Therefore, the assembly of the heptasaccharide repeat unit of Cepacian requires the priming glycosyltransferase BceB, catalyzing the addition of the first sugar residue, glucose, to the lipid carrier, which is located on the cytoplasmic surface of the cell membrane (Videira *et al*., 2005). The sequential addition of the six remaining sugars must be performed by BceG, H, J, K, and presumably by the bifunctional BceR, although the protein sequences do not give clues on their specific glycosyltransferase activity (Moreira *et al*., 2003; Ferreira *et al*., 2010).

The modification of the polymer with non-saccharidic substitute, as O-acetyl groups, can occur during this phase of the biosynthetic pathway, catalyzed by enzymes required for EPS acetylation and/or other modifications. Three putative acyltransferases were found within the *bce-II* cluster, encoded by the *bceO*, *bceS* and *bceU* genes. Some analysis of BceO, BceS and BceU amino acid

sequences suggest that they probably locate in the inner membrane and that BceS is involved in the repeat unit acetylation (Ferreira *et al.*, 2010).

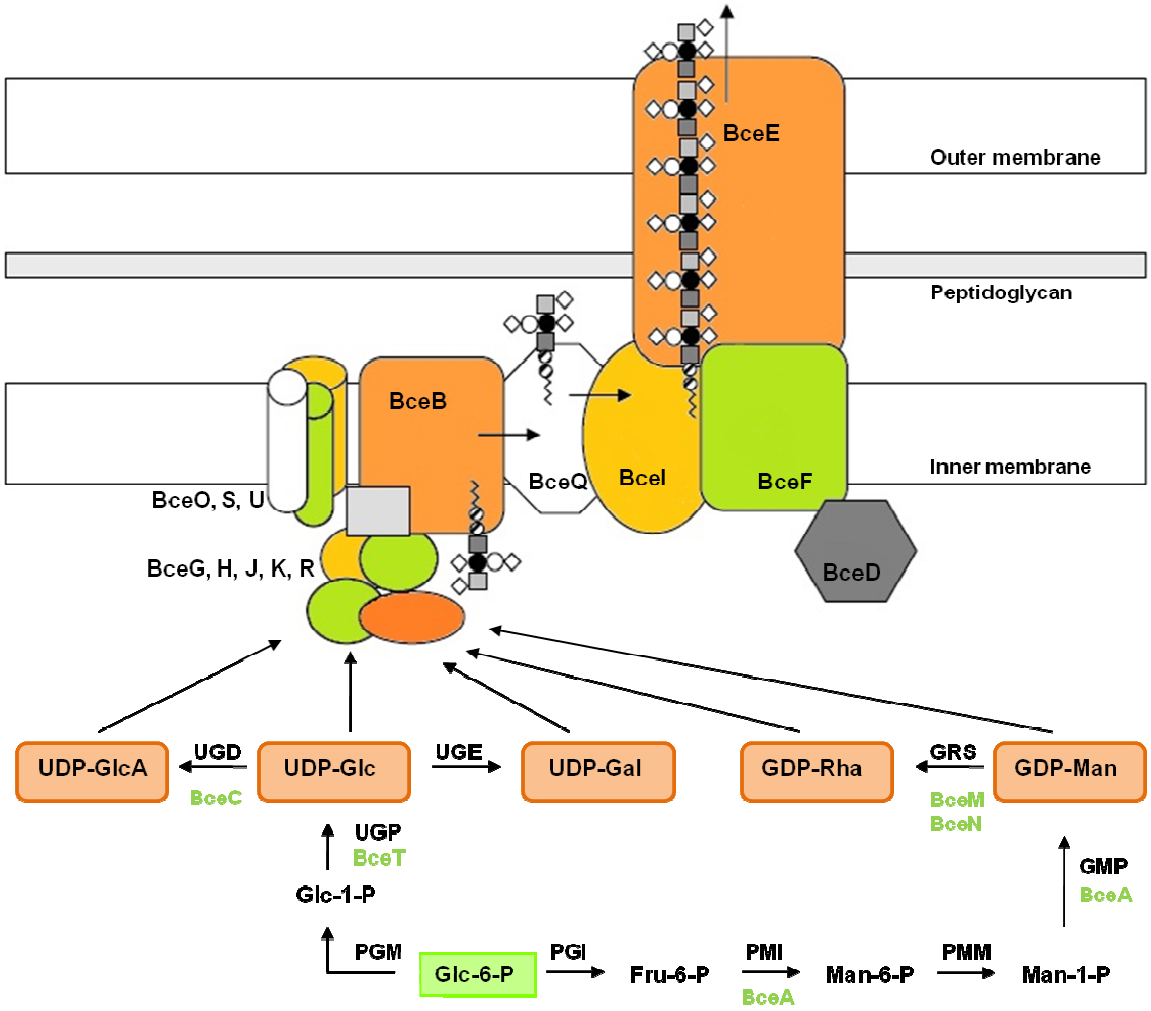


Figure 1.3. - Postulated pathway leading to the formation of the nucleotide-sugar precursors required for the Cepacian biosynthesis by *Burkholderia* and model for EPS assembly and export. The enzyme activities postulated to be involved in the formation of these precursors are: phosphoglucomutase (PGM), converting glucose-6-P into glucose-1-P; UDP-glucose pyrophosphorylase (UGP), converting glucose-1-P into UDP-D-glucose; UDP-glucose dehydrogenase (UGD), converting UDP-D-glucose into UDP-D-glucuronic acid; UDP-galactose epimerase (UGE), converting UDP-D-glucose into UDP-D-galactose; phosphoglucose isomerase (PGI), converting glucose-6-P into fructose-6-P; phosphomannose isomerase (PMI), converting fructose-6-P into mannose-6-P; phosphomannomutase (PMM), converting mannose-6-P into mannose-1-P; GDP-mannose pyrophosphorylase (GMP), converting mannose-1-P into GDP-D-mannose; and GDP-rhamnose synthase (GRS), converting GDP-D-mannose into GDP-D-rhamnose (adapted from Ferreira *et al.*, 2010).

After assembly, the polysaccharide repeat units coupled to the lipid carrier have to be exported to the periplasmic side of the inner membrane, polymerized, and the nascent polymer has to be exported. All evidence indicates that Cepacian biosynthesis proceeds via the Wzy-dependent pathway. In this pathway, the lipid carrier-linked heptasaccharide repeat units are exported across the inner membrane by the putative flippase BceQ, being polymerized at the periplasmic face of the plasma membrane by the putative polymerase BceI (Figure 1.3; Ferreira *et al.*, 2010). 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 (Moreira *et al*., 2003; Ferreira *et al*., 2010).

In *Escherichia coli*, and in many other bacteria, polymerization activity is also influenced by an additional protein, referred to as the polysaccharide copolymerase (PCP) (Cuthbertson *et al*., 2009). Wzc protein, an *E. coli* PCP, plays a critical role in the translocation of polysaccharides from the periplasm to the cell surface and is crucial for the interactions between the final component of the EPS apparatus and the Wza protein (a member of outer membrane polysaccharide export family). Together, Wza and Wzc comprise a molecular scaffold that is required for the final stage of polymer export (Cuthbertson *et al*., 2009). BceF, as a Wzc protein, is a PCP protein, whose precise role(s) in Cepacian biosynthesis is still unknown. Nevertheless, these proteins seem to play a critical role in the translocation of the polysaccharide chains from the periplasm to the cell surface through the interaction with an outer membrane protein, which is likely to be BceE in the case of Cepacian. BceE protein is a homologue of the *E. coli* Wza protein, suggesting that it plays a role in the translocation of the EPS (Figure 1.3; Cuthbertson *et al*., 2009, Ferreira *et al*., 2010).

1.4. Introduction to the thesis theme

Cepacian, the main exopolysaccharide produced by Bcc isolates, has been pointed out as a virulence factor that is involved in persistence of these bacteria in CF lungs, interactions with antimicrobial peptides and formation of biofilms, contributing to the overall pathogenicity of Bcc members and, thus to their success as pathogens (Richau *et al*., 2000a; Cunha *et al*., 2004; Conway *et al*., 2004; Sousa *et al*., 2007b). Therefore, the elucidation of the Cepacian biosynthesis is of crucial importance for the understanding of the biology of these microorganisms.

Recent bioinformatics analysis revealed that the genes involved in Cepacian biosynthesis are clustered into two *loci* on the chromosome 2 of sequenced Bcc bacteria (Ferreira *et al*., 2010). Although some of the *bce* genes have already been functionally characterized, little is known about the genes involved in the synthesis of GDP-D-rhamnose, the activated sugar precursor of the Cepacian D-rhamnose moiety (Richau *et al*., 2000b). The rare sugar D-rhamnose has been identified only in bacteria, including pathogens of animals (Kocharova *et al*., 2000; Senchenkova *et al*., 1996) and plants (Molicaro *et al*., 2003), where it is a component of cell surface polysaccharides.

The biosynthetic pathway for GDP-D-rhamnose has been elucidated in the Gram-positive thermophile *Aneurinibacillus thermoaerophilus* L420-91^T, in which D-rhamnose is a component of the S-layer proteoglycan and, more recently, in *Pseudomonas aeruginosa*, whose A-band O polysaccharide includes a D-rhamnose homopolymer (Kneidinger *et al*., 2001; King *et al*., 2009). Two reaction steps are required to convert GDP-D-mannose into GDP-D-rhamnose. In the first step, the enzyme GDP-D-mannose 4,6-dehydratase (GMD; EC 4.2.1.47) catalyzes the dehydration of GDP-D-mannose to produce the unstable intermediate GDP-4-keto-6-deoxy-D-mannose. The mechanism of this reaction has been proposed to involve a protein-bound pyridine dinucleotide (NAD^+ or $NADP^+$) as the responsible for the transfer of an hydrogen from the C-4 to the C-6 position of the deoxy-monosaccharide (Sturla *et al*., 1997). The 4-keto moiety of the intermediate is then reduced to GDP-D-rhamnose by the enzyme activity GDP-4-keto-6-deoxy-D-mannose reductase (RMD; EC 1.1.1.281).

The two proteins involved in GDP-D-rhamnose biosynthesis are members of the nucleotide diphosphate (NDP)-sugar modifying subfamily of the short-chain dehydrogenase/reductase (SDR) superfamily (Kavanagh *et al*., 2008). Although the members of this family share low sequence identity, the three-dimensional structures are quite conserved. The most conserved feature of these proteins is the Rossman-fold motif, involved in dinucleotide binding, consisting of an α/β folding pattern, with seven parallel β-sheets flanked by six or seven α-helices on each side (Lesk, 1995). In the N-terminal region the glycine-rich Wierenga motif, GXXGXXG, is also present, which is the specific region for the binding of the cofactor NADP(H). These proteins also share the conserved catalytic triad Tyr-XXX-Lys and Ser/Thr in their catalytic centers (Webb *et al*., 2004; Kavanagh *et al*., 2008).

The GMD enzyme activity is widespread in nature, and serves as a branching point for the biosynthesis of sugars including L-fucose, D-talose, and D-perosamine (Webb *et al*., 2004) (Figure 1.4). The involvement of GMD as the initiator enzyme in all these pathways makes it a particularly interesting target of research. Bioinformatics analysis suggests that the closest paralog of RMD is GMD. This conclusion is also supported by the existence of GMD proteins with bifunctional activity,

which catalyzes the dehydration of GDP-D-mannose and the reduction of the 4-keto sugar nucleotide to a 6-deoxysugar nucleotide (King *et al.*, 2009).

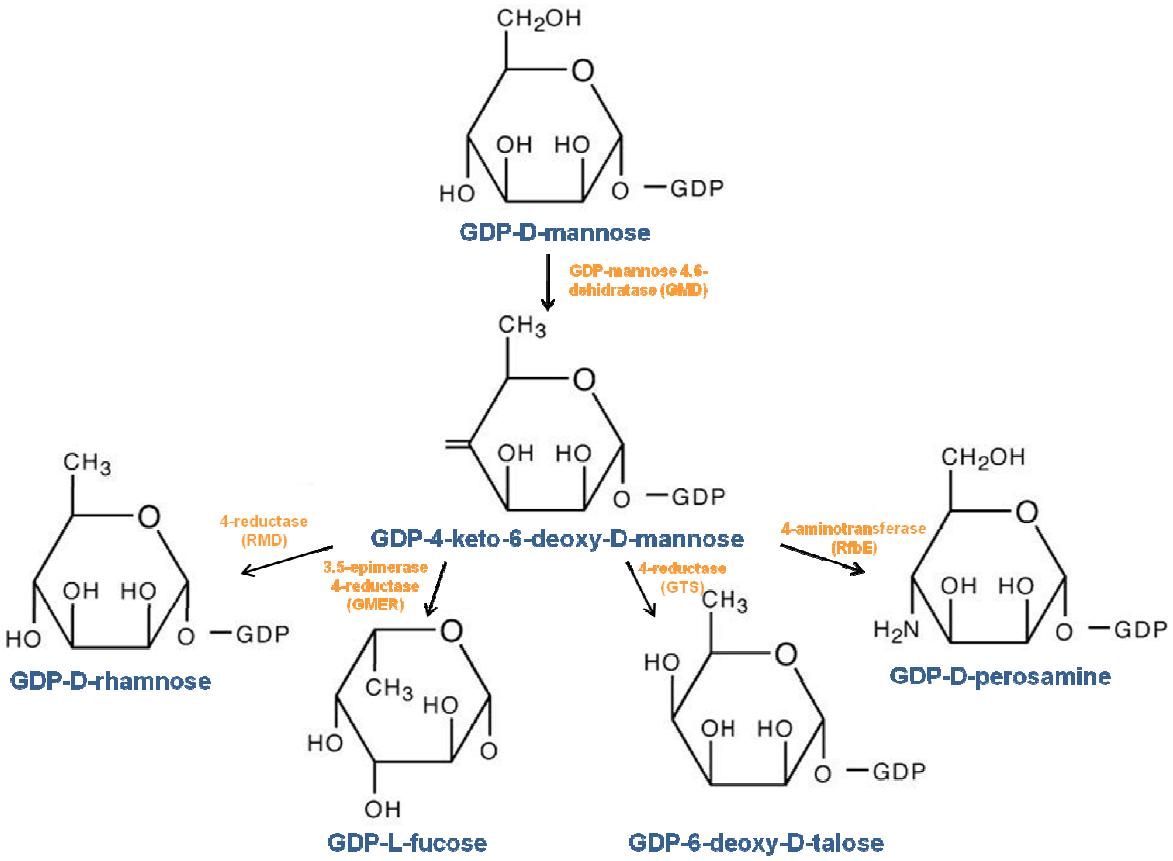


Figure 1.4. - The biosynthetic pathway of several different deoxyhexoses originating from the same 4-keto-6-deoxy intermediate. GMD converts GDP-D-mannose to the common intermediate, which undergoes reduction, epimerization, or amination, resulting in a variety of deoxy sugars (adapted from Webb *et al.*, 2004).

Until now the biosynthetic pathway for GDP-D-rhamnose has not been elucidated in bacteria of the Bcc. However, recently Ferreira and colleagues predicted that *bceM* and *bceN* genes, of the *bce*-II cluster, encode a RMD and a GMD, respectively (Ferreira *et al.*, 2010). The present study aims at the cloning and functional analysis of *bceN* gene, which encodes a protein exhibiting homology to GMD proteins, and also the study of its role on the synthesis of the Cepacian EPS.

2. MATERIALS AND METHODS

2.1. Bacterial strains, plasmids and culture conditions

Strains and plasmids used in this study are listed in Table 2.1. To preserve cultures for longer periods of time, bacterial strains were kept at -80ºC in 40% (v/v) glycerol. When in use, *Burkholderia* and *Escherichia coli* strains were maintained, respectively, in Pseudomonas Isolation Agar (PIA, Difco) plates and Lennox broth (LB, Pronadisa) agar plates (2% agar). Cultivation of *E. coli* and *Burkholderia* strains on solid media was carried out by incubation at 37ºC for 1 day and 30ºC for 2 days, respectively. When required, growth media were supplemented with antibiotics to maintain selective pressure, at the following final concentrations (µg/ml): for *Burkholderia*, kanamycin 600, trimethoprim 150; for *E. coli*, ampicillin 150, kanamycin 50, trimethoprim 100.

Overnight liquid cultures were prepared by transferring one isolated bacterial colony of Bcc or *E. coli*, previously grown in appropriate solid media, into 25 ml of LB liquid media and incubating ±16 hours at 30ºC or 37ºC, respectively, with orbital agitation (250 rpm).

Table 2.1 – Strains and plasmids used in this study.

Strain/ Plasmid	Description	Source
Bcc strains		
B. cenocepacia LMG 16656	J2315; Cystic fibrosis clinical isolate (UK); genome sequenced; ET12 lineage reference strain.	Gingues *et al.*, 2005
B. cepacia IST408	Cystic fibrosis clinical isolate (Hospital Santa Maria, Portugal), Cepacian producer.	Richau *et al.*, 2000a.
B. multivorans ATCC 17588	ATCC 17616, Soil isolate (Berkeley California, USA).	Vandamme *et al.*, 1997.
***E. coli* strains**		
DH5α	*sup*E44 (ϕ80 *lac*ZΔM15) *hsd*R17(r_K^- m_K^+) *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1 *deo*R Δ(l*ac*ZYA-*arg*F)U169.	Invitrogen
BL21 (DE3)	F^- *ompT hsd*S_B ($r_B^-m_B^-$) dcm gal λ(DE3).	Stratagene
SCS110	*dam dcm endA1 supE44 hsdR17 thi leu rpsL1 lacY galK galT ara tonA thr* Δ(*lac-proAB*)/F' [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15].	Stratagene
HB101	*thi-1 hsd*S20(r_B^-, m_B^-) *sup*E44 *rec*A13 *ara*-14 *leu*B6 *pro*A2 *lac*Y1 *gal*K 2*rps*L20 (Str^r) *xyl*-5 *mti*-1.	Promega
Plasmids		
pET23a+	Cloning vector, Ap^r.	Novagen
pDrive	Cloning vector, *lacZ* α-peptide, Ap^r Km^r.	Qiagen
pRK2013	Mobilizing vector, ColE1 tra$(RK2)^+$; Km^r.	Figurski and Helenski, 1979.

pMLBAD	pBBR1 ori, araC-P_{BAD}, Tp^r, mob^+.	Lefebre and Valvano, 2002.
pACM4	pDrive containing the *bceN* gene disrupted by the trimethoprim cassette, Ap^r, Km^r, Tp^r.	This work
pACM2	pDrive with a 2,272bp KpnI-HindIII fragment containing the *bceN* gene and flanking regions, Ap^r, Km^r.	This work

2.2. Production of Exopolysaccharide (EPS) in liquid medium

Overnight grown liquid cultures of Bcc isolates were used to inoculate 25 ml of S or S_{ARA} liquid medium contained in 100 ml Erlenmeyer flasks, at an initial optical density at 640 nm (OD_{640nm}) of 0.1. After 3 days of incubation at 30ºC or 37ºC with orbital agitation (250 rpm), cells were removed by centrifugation (7000xg, 15 minutes). The EPS produced was precipitated from the cell-free supernatant by the addition of 2.5 volumes of 95% (v/v) cold ethanol and dried during some days at room temperature. EPS production was estimated by weighing the dried precipitates. The results are the means of at least three independent determinations.

S medium composition – 12.5 g/l $Na_2HPO_4.2H_2O$, 3 g/l KH_2PO_4, 1 g/l K_2SO_4, 1 g/l NaCl, 1 g/l yeast extract, 1 g/l casamino acids, 20 g/l glucose, 0,2 g/l $MgSO_4.7H_2O$, 0.01 g/l $CaCl.2H_2O$, 0.001 g/l $FeSO_4.7H_2O$.

S_{ARA} medium composition – 12.5 g/l $Na_2HPO_4.2H_2O$, 3 g/l KH_2PO_4, 1 g/l K_2SO_4, 1 g/l NaCl, 1 g/l yeast extract, 1 g/l casamino acids, 20 g/l arabinose, 0,2 g/l $MgSO_4.7H_2O$, 0.01 g/l $CaCl.2H_2O$, 0.001 g/l $FeSO_4.7H_2O$.

2.3. Biofilm formation assay

Biofilm formation assays were based on the methodology described by O'Toole and Kolter (1998). Overnight liquid cultures of Bcc strains were transferred to S liquid medium and grown at 30ºC with orbital agitation until the mid-exponential phase was reached. The cultures were then diluted to a standardized culture OD_{640nm} of 0.5, and 20 µl of this cell suspension were used to inoculate the wells of a 96-well polystyrene microtiter plate (Greiner Bio-One) containing 180 µl of S liquid medium. Plates were incubated at 30ºC for 24 or 48 hours without agitation. Wells containing sterile growth medium were used as negative controls.

For biofilm quantification, the culture medium and unattached bacterial cells were removed by rinsing the wells three times with 200 µl of distilled water. Adherent bacteria were stained with 200 µl of a 1% (wt/v) crystal violet solution for 15 minutes at room temperature. After three gentle rinses with 200 µl of distilled water, the dye associated with the attached cells was solubilized in 200 µl of 95% ethanol. The biofilm formed was quantified by measuring the absorbance of the solution at 590 nm in a VERSAmax tunable microplate reader (Molecular Devices). Results are mean values of at least five repeats from three independent experiments.

Crystal violet solution 1% – 50 ml of the solution was prepared by dissolving 0.5 g crystal violet in 10 ml of 95% (v/v) ethanol and 40 ml of water containing 0.4 g of ammonium oxalate was added.

2.4. Motility Assays

2.4.1. Twitching motility assay

Twitching motility was assayed by the subsurface agar method (Déziel *et al.*, 2001). Bcc cells were stab inoculated with a toothpick through a thin (approximately 3-mm) LB agar layer (1% agar) to the bottom of the petri dish. After incubation for 24 to 48 hours at 30 and 37ºC, a hazy zone of growth at the interface between the agar and the polystyrene surface was observed.

2.4.2. Swimming assay

Swimming assays were performed using tryptone swim plates (Déziel *et al.*, 2001). Plates were spot-inoculated with 1 µl of an overnight Bcc culture, diluted to a standardized culture OD_{640nm} of 2, and the swimming zone was measured after incubation for 24 and 48 hours at 30 and 37ºC.

Tryptone swim plates – 1% (wt/v) tryptone, 0.5% (wt/v) NaCl, 0.3% (wt/v) agar.

2.4.3. Swarming assay

For the examination of swarming motility ABC minimal medium (Clark and Maaløe, 1967) supplemented with 0.1% (wt/v) Casamino acids and 1% (wt/v) glucose was solidified with 0.4% (wt/v) agar. Plates were spot-inoculated with 1 µl of an overnight Bcc culture, diluted to a standardized culture OD_{640nm} of 2, and incubated at 30 and 37ºC for 24, 48 and 72 hours.

ABC minimal medium – Component A: 20 g/l $(NH_4)_2SO_4$, 60 g/l Na_2HPO_4, 30 g/l KH_2PO_4, 30 g/l NaCl; Component B: 2 mM $MgCl_2.6H_2O$, 100 µM $CaCl_2.2H_2O$, 3 µM $FeCl_2.6H_2O$; Component C: 1 M Sodium Citrate. Sterilize the three components by autoclaving. Add 900 ml of Component B, 100 ml of Component A and 10 ml of component C.

2.5. Molecular biology methods

2.5.1. DNA manipulation techniques

2.5.1.1. Extraction and purification of genomic and plasmid DNA

Plasmid DNA was extracted from overnight cultures of *E. coli* host strains grown in LB medium supplemented with appropriate antibiotics, by using the Easy Spin Plasmid DNA Minipreps kit (Citomed), following the manufacturer's instructions. DNA fragments used in cloning procedures were purified from agarose gels with a NucleoSpin Extract II kit (Macherey-Nagel), according to the manufacturer's instructions. Total genomic DNA from Bcc isolates was extracted from bacterial cells harvested from overnight grown liquid cultures at 30ºC, using the High Pure PCR Template Preparation kit (Roche), following the manufacturer's instructions. The concentration of genomic and

plasmid DNA solutions was estimated by measuring the absorbance at 260 nm in a Spectrophotometer ND-1000 (NanoDrop).

2.5.1.2. Polymerase chain reaction (PCR) conditions

Genomic DNA from *B. cepacia* IST408 strain and plasmid DNA from pACM2 were used as templates for PCR amplification of different products (Table 2.2) in a C1000 Thermal cycler (Bio-Rad). The general conditions used were: 150 ng of template DNA, 0.4 µM of each primer, 200 µM of each deoxynucleotide (Fermentas) and 0.02 U/µl of Phusion® High-Fidelity DNA Polymerase (Finnzymes). The samples were subjected to an initial denaturation at 98ºC for 2 minutes, followed by 30 cycles of: denaturation (98ºC for 10 seconds), annealing (55ºC for 30 seconds) and elongation (72ºC for 30 seconds/kb of product expected). After a final elongation at 72ºC for 7 minutes, the samples were stored at 4ºC until used.

Table 2.2 – PCR amplification conditions.

Amplification product	Template	Primers	Annealing temperature	Product size (bp)
bceNJ	pACM2	bccNNheI/ bccNHindIII	55ºC	1061
bceN8	*B. cepacia* IST408	bccNNheI/ bccNHindIII	55ºC	1061
Insertionally inactivated *bceN8*	*B. cepacia* IST408::*bceN*	bccNNheI/ bccNHindIII	55ºC	1972

The oligonucleotide primers were design based on the genome sequence of *B. cenocepacia* J2315 (http://www.sanger.ac.uk/Projects/B_cenocepacia), using the OLIGO 4.0 (IDT) program, and were synthesized by Operon MWG Eurofins (Germany) (Table 2.3).

Table 2.3 – Oligonucleotide primers used for PCR amplification. The start codon (ATG) is in bold and the recognition sites for restriction enzymes are underlined.

Primer	DNA sequence
BccNheI	5'-TTG CTA GC**A TG**A GCC AAA CTC-3'
BccNHindIII	5´-AAA AAG CTT GAA CGT GTC ATG-3'

2.5.1.3. Agarose gel electrophoresis

Agarose gel electrophoresis was carried out as described by Sambrook and Russel (2001). Concentrations of agarose (Seakem LE, FMC bioproducts) ranging from 0.7% to 1% (wt/v) in TAE 1X buffer were used to separate the DNA fragments. Prior to electrophoresis the gel loading dye was added to DNA sample solution. The molecular weight marker GeneRuler™ 1 kb Plus DNA Ladder (Fermentas) was also used. DNA fragments were separated by gel agarose electrophoresis in TAE 1X

buffer at 4V/cm. The gel was stained with GelRed™ Nucleic Acid Gel Stain (Biotium) and the DNA was visualized under short wave UV light in a transilluminator (Bio-Rad).

TAE 50X – 242 g/l Tris.base, 57.1 ml/l acetic acid, 37.2 g/l $Na_2EDTA.2H_2O$. The pH is adjusted to 8 with acetic acid. Dilution of 1:50 of this buffer was performed to obtain TAE 1X.

Gel loading dye 6X – 0.25% (wt/v) Bromophenol blue (Merck), 0.25% (wt/v) Xylene cyanol FF (Sigma), 30% (v/v) glycerol.

2.5.1.4. Construction of plasmids and mutagenesis strategies

2.5.1.4.1. Construction of plasmids

For sequencing the *bceN* gene of *B. cepacia* IST408, the plasmid pJRF2 was constructed. With this purpose, the *bceN* coding region was amplified by PCR using *B. cepacia* IST408 genomic DNA as template. The blunt end amplification product was inserted into the *HincII* site of pDrive vector. The resulting plasmid was introduced into *E. coli* DH5α by electroporation.

In order to overexpress the protein encoded by the *bceN* gene as an His_6-tagged protein, the amplified product *bceNJ* was digested with *NheI/HindIII* (Takara) and cloned into the same sites of the linearized pET23a+ expression vector. The plasmid obtained, 4669 bp, was named pJRF1 and was used for the overproduction of this protein in *E. coli* BL21(DE3).

2.5.1.4.2. Construction of a *bceN* insertion mutant

For the construction of a *bceN* insertion mutant of *B. cepacia* IST408, a 911 bp *EcoRI/SphI* fragment from pUC-Tp (Sokol *et al.*, 1999), containing the trimethoprim resistance cassette, was inserted into the same sites of the pACM2 plasmid, creating pACM4. This plasmid was introduced into *B. cepacia* IST408 by electroporation, after extraction and purification from *E. coli* SCS110 (*dam*, *dcm*). Insertional inactivation of *bceN* was confirmed by PCR (see 2.4.1.2.).

2.5.1.4.3. Construction of a plasmid expressing a *bceN* antisense RNA

In order to analyse the *bceN* gene function in *B. multivorans* ATCC 17616, a DNA fragment of this gene was cloned downstream of an inducible promoter whose expression would lead to the transcription of a *bceN* antisense RNA. For the construction of antisense plasmid, the 1061 bp *XbaI/HindIII* fragment from plasmid pJRF1, containing the *bceN* gene coding region was cloned into the same sites of pDrive. The plasmid obtained, pJRF5, was digested with *KpnI/XbaI* (Takara) and, the fragment obtained (1125 bp) was cloned in the same sites of pMLBAD, resulting in antisense plasmid pJRF6. This plasmid was introduced into *B. multivorans* ATCC 17616 by triparental conjugative plasmid transfer.

2.5.1.4.4. DNA and protein sequence analysis

DNA sequencing reactions were performed by Operon MWG Eurofins (Germany).

The nucleotide sequences were analyzed using the Open Reading Frame (ORF) Finder tool, resident at the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). The algorithm BLAST (Altschul *et al*., 1997) was used to compare sequences of the deduced amino acids to sequences available at the NCBI database. Alignments were performed using the program CLUSTAL W2 (Thompson *et al*., 1994). The phylogenetic tree was constructed based on results of the amino acid sequences with CLUSTAL X2 using the neighbour-joining method with a minimum of 100 bootstraps.

2.5.2. Methods for insertion of foreign DNA in bacterial cells

2.5.2.1. Transformation by electroporation

The DNA solutions to be used for electroporation must have a very low ionic strength and a high resistance, so the DNA solutions were previously precipitated, with 3 M sodium acetate and 2.5 volumes of ethanol, incubated at -70ºC as described by Sambrook and Russel (2001).

B. cepacia and *E. coli* electrocompetent cells were prepared as described by Sambrook and Russel (2001). Overnight liquid cultures of the appropriate host strains were used to inoculate 100 ml of LB liquid medium at a final OD_{640nm} of 0.1 and incubated at 37ºC, with orbital agitation (250 rev./min), until the bacterial culture reached the mid-log phase (OD_{640nm}≈0.8). The cells were harvested by centrifugation and the cell pellets were washed three times with sterile ice-cold distilled water to eliminate salts. The pellets were then resuspended in 2 ml of 10% (v/v) glycerol and aliquots of 110 µl were frozen at -70ºC, until used.

Electrocompetent cells were transformed with foreign DNA by electroporation using a Gene Pulser™ apparatus (Bio-Rad) set at 2.5 kV, 25 µF capitance, and 4 kΩ (*E. coli*) or 2 kΩ (Bcc strains) resistance was used. After the electric pulse, cells were incubated in rich medium (e.g. LB liquid medium) for 1 hour or overnight, when using *E. coli* or Bcc host strains, respectively. After incubation, the bacterial cultures were plated on appropriate selective medium.

2.5.2.2. Triparental conjugative plasmid transfer

Triparental conjugation was used to transfer plasmids of interest from *E. coli* to Bcc strains. The *E. coli* donor strain and the helper strain *E. coli* HB101 (pRK2013) were grown overnight at 37ºC in LB supplemented with the appropriate antibiotics. The recipient strain of Bcc was grown overnight at 30ºC in LB. Cells from 0.4 ml of culture of the recipient strain and from 0.2 ml of liquid cultures of the donor and the helper strains were harvested by centrifugation, washed with sterile 0.9% (wt/v) NaCl and resuspended in the same solution. The three bacterial cultures were mixed together and harvested by low speed centrifugation for 5 minutes. The pellet was resuspended in 80 µl of 0.9% (wt/v) NaCl and spot-inoculated on the surface of a 13 mm diameter filter disk (Supor® -200, 0.2 µm

pore size, Pall Corporation), previously placed onto the surface of an LB agar plate. After 12 hours of incubation at 30ºC, the bacterial layer on the surface of the filter was resuspended in 1 ml of 0.9% (wt/v) NaCl. Serial dilutions were prepared and 100 µl aliquots were plated on appropriate selective medium, thus enabling the positive selection of transconjugants.

2.5.3. Protein manipulation techniques

2.5.3.1. Overproduction of His_6-tagged proteins and preparation of cell extracts

After transformation of *E. coli* BL21(DE3) cells with the pJRF1 plasmid, 100 ml of LB liquid medium containing 150 µg/ml ampicillin were inoculated at an initial OD_{640nm} 0.1 and incubated at 37ºC with orbital agitation (250 rpm). When the culture reached an OD_{640nm} of 0.65, induction was started by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the growth medium, followed by incubation for an additional 2 hours, under the same conditions. To confirm that the protein of interest was overproduced, aliquots of culture were recovered before and after IPTG induction. These samples were centrifugated and the resulting pellet was resuspended in 40 µl of gel loading buffer solution. Proteins were visualized after polyacrylamide gel electrophoresis and staining with Comassie Blue R-250.

Cells from cultures induced with IPTG for 2 hours were harvested by centrifugation at 7000xg for 5 minutes at 4ºC and resuspended in sonication buffer. Cells were disrupted by ultrasonic vibration (six cycles for 30 seconds each, 70 Watts) with a Branson sonifier 250 (Branson). Cell debris and intact bacteria were removed by centrifugation at 4ºC for 1 hour at 12500xg. The clear supernatant was carefully transferred to a clean container and kept at 4ºC, until further use.

Gel loading buffer – 20% (v/v) glycerol, 4% (wt/v) SDS, 100 mM Tris.Cl (pH 6.8), 0,2% (wt/v) bromophenol blue, 200 mM DTT.

Sonication buffer – Phosphate buffer 1X (pH 7,4), 10 mM Imidazole.

2.5.3.2. Purification of His_6-tagged proteins by affinity chromatography

The recombinant His_6-tagged BceN protein was purified in a one-step procedure using the Hi-Trap™ Chelating HP system (GE Healthcare). After regeneration of the column with 0.5 ml of 0.1 M nickel sulphate salt solution, the column was washed with distilled water and equilibrated with Start buffer. Then the sample was applied into the column and washed with Start buffer. Elution of His_6-tagged protein was carried out using 5 ml of Elution buffer containing increased concentrations of imidazole (60, 80, 100, 200 and 500 mM). Samples from the collected fractions were analyzed using SDS-PAGE and those containing the purified His_6-tagged protein were pooled and dialyzed overnight at 4ºC in a Slide-A-Lyzer® Dialysis Cassette (PIERCE) against Dialysis buffer. The protein concentration of the purified His-tagged protein solution was estimated by the method of Bradford (1976), using bovine serum albumin as standard.

Phosphate buffer 8X – 80 mM $Na_2HPO_4.2H_2O$, 80 mM $NaH_2PO_4.H_2O$, 4 mM NaCl. Adjust pH to 7.4 with NaOH 3M. Keep at dark.

Start buffer – Phosphate buffer 1X (pH 7.4), 10 mM Imidazole.

Elution buffer – Phosphate buffer 1X (pH 7.4), 60 mM to 500 mM Imidazole.

Dialysis buffer - 25 mM NaPO4 (pH 7.4), 20% (v/v) glycerol, 50 mM NaCl.

2.5.3.3. Analysis of proteins by electrophoresis in polyacrylamide gels

2.5.3.3.1. Denaturating polyacrylamide gel electrophoresis (SDS-PAGE)

The discontinuous gel system of Laemmli (1970) was used to separate proteins on polyacrylamide gels. The system is composed of two different gels: the running gel (12.5% or 15% polyacrylamide) that allows the proteins to separate according to their relative molecular mass, and the stacking gel (4% polyacrylamide), were proteins are applied and concentrated. The composition of each gel is detailed in Table 2.4.

Table 2.4 – Composition of 5 ml of 12.5 or 15% running gels and of 2 ml of stacking gel.

Stock solution	Running gel (12.5%)	Running gel (15%)	Stacking gel (4%)
Separating buffer	1.25 ml	1.25 ml	—
Stacking buffer	—	—	0.5 ml
Acrylamide stock	2.08 ml	2.5 ml	0.27 ml
H_20	1.64 ml	1.22 ml	1.22 ml
10% (p/v) APS	50 µl	50 µl	20 µl
TEMED	2.5 µl	2.5 µl	2 µl

The ammonium persulphate (APS) solution was freshly prepared and the N,N,N',N'-tetramethylethylenediamine (TEMED) was added just before pouring the gel to prevent premature polymerization of acrylamide. After polymerization, the gel was immersed in the running buffer 1X and 10-20 µl of protein sample (previously resuspended in gel loading buffer and incubated for 5 minutes at 100ºC) were applied in each well of the gel. Separation of proteins was achieved by applying 160 V, until the dye contained in the loading buffer reached the lower end of the gel.

The acrylamide gels were stained with coomassie blue staining solution for 20 minutes, after boiling the solution. The gel was then placed into a previously boiled distaining solution for further 20 minutes.

Separating buffer – 1.5 M Tris.base (pH 8.8), 0.4% (v/v) SDS.

Stacking buffer – 0.5 M Tris.base (pH 6.8), 0.4% (v/v) SDS.

Acrylamide stock solution – 30% (wt/v) acrylamide, 0.8% (wt/v) bis-acrylamide. Stored at 4ºC.

Running buffer 10X – 0.25 M Tris.base, 1.92 M glycin, 1% (wt/v) SDS. This buffer was diluted 1:10 before use.

Coomassie blue staining solution - 0.2% (wt/v) coomassie blue R-250, 10% (v/v) acetic acid, 47.5% (v/v) ethanol, mix thoroughly and filter with Whatman nº1 filter paper.

Distaining solution - 10% (v/v) acetic acid, 26.25% (v/v) ethanol.

2.5.3.4. Western Blot analysis of His_6-tagged proteins

Western blotting allows the determination of the relative amounts of the target protein present in the different samples using a specific antibody. After electrophoresis the gel was immersed in 100 ml Towbin buffer for at least 15 minutes. The nitrocellulose (NC) membrane (Biotrace® NT, Pall Corporation) was cut to the appropriate size and incubated in Towbin buffer for 5 minutes. Six 3MM Whatmann filter papers with the same size of the NC membrane were rapidly immersed in the Towbin buffer. The system was assembled (Figure 2.1) and the electrotransfer of proteins to the NC membrane was performed at 15V, 120 mA for 50 minutes in a semi-dry electrophoretic transfer unit (Trans-Blot® SD, Bio-Rad).

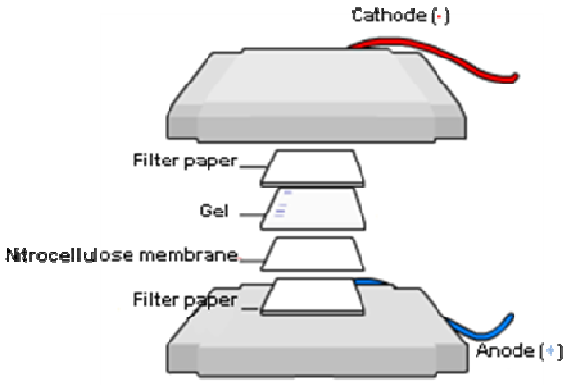


Figure 2.1. – Schematic representation of a western-blot apparatus assembled for a semi-dry electrophoretic transfer unit.

After the transfer of proteins, the membrane was blocked by incubation with 5% (wt/v) skim milk in PBS 1X with gentle agitation for 1 hour at room temperature. The membrane was washed three times in PBS 1X containing 0.05% Tween 20. The membrane was then incubated with an Anti-polyHistidine, Peroxidase conjugate antibody (Sigma-Aldrich) for 2 hours, according to the manufacturer's instructions. The membrane was further incubated with the peroxidase substrate 3,3',5,5'Tetramethylbenzidine (TMB) (Sigma), following the manufacturer's instructions.

Towbin buffer – 48 mM Tris, 39 mM Glycine, 20% (v/v) methanol, 0.04% (wt/v) SDS.

2.6. Enzymatic assays

2.6.1. GDP-mannose 4,6-dehydratase (GMD) activity (EC 4.2.1.47)

The ability of the His_6-tagged BceN protein to convert GDP-D-mannose to GDP-4-keto-6-deoxy-D-mannose was assayed using GDP-D-mannose as substrate and analyzing the reaction products by Nuclear Magnetic Resonance (NMR) spectroscopy.

NMR experiments were performed on a Bruker AVANCE II 400 MHz (^1H) spectrometer, with a BBO 5 mm probe, in 10% D_2O/90% H_2O. The spectra were treated with the software Topsin version 2.1. 500 µl of the reaction buffer (25 mM $NaPO_4$, 50 mM NaCl pH 7.4, 90% H_2O/10%D_2O) were placed in the NMR tube (Norrel 100MHz 5 mm) with 2.5, 3 or 5 mM GDP-D-mannose (Sigma), and $MgCl_2$ (of a ratio of 1 mol $MgCl_2$: 1 mol protein). As internal reference, the signal correspondent to the resonance of the methyl group of acetone was fixed at δ_H = 2.225 ppm. The reaction was started at 37ºC by the addition of 90 µg BceN protein to the reaction buffer, and the proton spectrum was recorded at fixed periods of time. The relative concentrations of each sugar nucleotide (in terms of their anomeric proton signal integrals) were plotted versus time. Different concentration of $MgCl_2$ (0 mM, 25 mM and 1mol:1mol protein) and NADP (0 mM, 2.5 mM and 5 mM) (Sigma) were tested.

2.6.2. GDP-4-keto-6-deoxy-D-mannose reductase activity (EC 1.1.1.281)

GMD reductase activity of BceN protein was carried out in the same conditions of GMD activity assays, but using NADPH and GDP-4-keto-6-deoxy-D-mannose as substrate. 5 mM of GDP-D-mannose were converted to GDP-4-keto-6-deoxy-D-mannose using 90 µg of His_6-BceN in 25 mM $NaPO_4$ pH 7.4, 50 mM NaCl, at 37ºC for 1 hour.

After the first reaction step additional BceN enzyme and 5 mM NADPH (Sigma) were added to the reaction tube. Reaction products were analyzed by NMR spectroscopy as described before (see 2.6.1).

3. RESULTS AND DISCUSSION

3.1. Sequence analysis of the *bceN* gene product

The genes required for the exopolysaccharide Cepacian biosynthesis by Bcc strains were previously identified in clusters *bce-I* and *bce-II* of chromosome 2 (Moreira *et al*., 2003; Ferreira *et al*., 2010). Five of these genes encode proteins putatively involved in nucleotide sugar precursor biosynthesis (BceA, BceC, BceM, BceN, and BceT) and only two of them were already functionally characterized (BceA and BceC) (Sousa *et al*., 2007a; Loutet *et al*., 2009; Ferreira *et al*., 2010). To functionally characterize the *bceN* gene the sequence analysis of the BceN protein from *B. cenocepacia* J2315 and *B. cepacia* IST08 was performed in this study.

The nucleotide sequence of the *bceN* gene from the *bce-II* cluster of *B. cenocepacia* J2315 was retrieved and appropriate primers were designed to amplify by PCR the 1061 bp fragment bearing the *bceN* gene from *B. cepacia* IST408. The DNA fragment obtained was cloned in plasmid pDrive, resulting in plasmid pJRF2, which was used to determine the *bceN* gene nucleotide sequence of *B. cepacia* IST408 (Table 3.1).

Table 3.1 - Plasmid constructions required for the functional analysis of the *bceN* gene product.

Plasmid	Description
PJRF1	pET23a+ with 1061 bp *NheI-HindIII* fragment containing *bceN* gene of *B. cenocepacia* J2315, Ap^r.
pJRF2	pDrive with a blunt-end fragment from 1061 bp containing *bceN* gene of *B. cepacia* IST408 in *Hinc-II* site, Ap^r, Km^r.

Comparison of the *bceN* translated nucleotide sequences of *B. cenocepacia* J2315 and *B. cepacia* IST408 with protein sequences deposited in GenBank revealed a high level of similarity to proteins with GDP-D-mannose 4,6-dehydratase (GMD) activity. These proteins are members of the (NDP)-sugar modifying subfamily of the short-chain dehydrogenases/reductases (SDR) family and catalyze the irreversible conversion of GDP-D-mannose to the intermediate GDP-4-keto-6-deoxy-D-mannose (Yamamoto *et al*., 1993; Sturla *et al*., 1997; Somoza *et al*., 2000).

The *B. cenocepacia* J2315 and *B. cepacia* IST408 BceN proteins are both composed of 348 amino acid residues with a predicted molecular mass of approximately 38.96 kDa and 39.27 kDa, respectively, and a pI of 6.35. The alignment of the two BceN proteins of Bcc revealed that they are 97% identical and 99% similar. The amino acid sequences of these two proteins were aligned with 38 GMD sequences obtained from data bases, including GMD proteins from Bacteria as well as from Eukarya. The alignment showed a high level of overall identity in all sequences used. The highest homology to the BceN sequence of *B. cepacia* IST408 was obtained with GMD proteins from other members of the *Burkholderia* genus (data not shown).

Since the enzyme activity of GMD proteins from *P. aeruginosa* (AAG08838), *Aneurinibacillus thermoaerophilus* L420-91^T (AAS55711) and *E. coli* (AAC77842) were experimentally demonstrated,

we have performed their alignment with the BceN protein of *B. cenocepacia* J2315 and *B. cepacia* IST408 using the Clustal W2 software (Figure 3.1). Results show that both Bcc BceN amino acids sequences are 58% identical and 73% similar to *P. aeruginosa* GMD, 55% identical and 68% similar to the *A. thermoaerophilus* GMD, and 51% identical and 66% similar to the *E. coli* GMD. The crystal structures of GMD proteins from *E. coli* and *P. aeruginosa* were reported, and several conserved amino acid residues were proposed to play a role in substrate and cofactor binding, and in catalysis (Somoza *et al*., 2000; Webb *et al*., 2004).

The analysis of the amino acid sequences evidenced the presence of the following features in BceN proteins:

1) The glycine-rich *Wierenga motif*, G-XX-G-XX-G, present in the N-terminal region of the proteins (amino acid positions 12 to 18). This domain corresponds to the specific region for the binding of the cofactor NADP(H), a characteristic feature of the SDR protein family (Jörnvall *et al*., 1995);
2) The G-XX-RR motif (amino acid positions 34 to 38), which is conserved among all GMDs sequenced so far, except for the *P. aeruginosa* GMD that exhibits the sequence G-XXX-RR. The two positively charged arginine residues mark the beginning of the RR loop (Webb *et al*., 2004).
3) The conserved RR loop (Arg37-Arg46) that in the *P. aeruginosa* GMD protein closes over the adenosyl phosphate end of the cofactor, thus playing an important role in cofactor binding, as well as in the tetramer interface (Webb *et al*., 2004).
4) A conserved catalytic triad of S/T and Y-XXX-K (amino acid positions 131, 155 and 159, respectively), which is crucial for catalysis (Kavanagh *et al*., 2008). In the GMD protein from *E. coli* the strictly conserved residue Tyr155 has been described as a general base facilitating the deprotonation of the C-4 hydroxyl group, formation of the 4-keto group, and hydride transfer to NADP, whereas the Thr 131 might act as a proton shuttle in this reaction (Somoza *et al*., 2000). On the other hand, the highly conserved residue Lys159 lowers the pK_a of Tyr155, making the catalysis of the oxidation reaction possible (Jörnvall *et al*., 1999).
5) The glutamic acid residue (Glu133) that is characteristic of dehydratases (Somoza *et al*., 2000; Allard *et al*., 2002). It has been proposed that the Glu133 plays a role in the deprotonation/reprotonation of C5 during catalysis, acting as an additional active-site base that abstracts the C5 proton in the dehydration reaction (Somoza *et al*., 2000).
6) A conserved amino acid side chain, Arg190, which may be critical for the correct orientation of the GDP-D-mannose substrate and the $NADP^+$ cofactor in the active site for the dehydratase reaction (King *et al*., 2009).

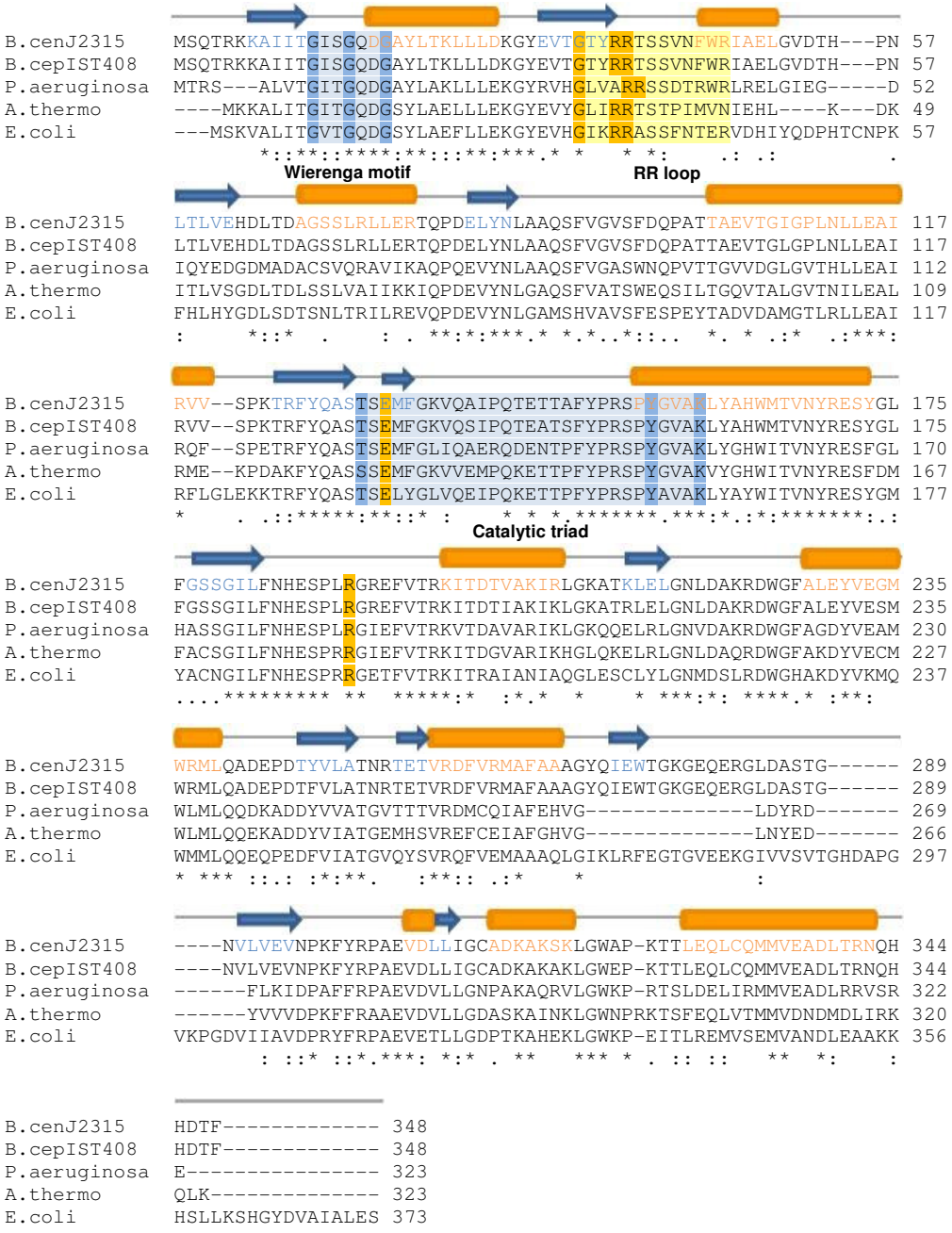


Figure 3.1. – Alignment of the amino acid sequences of the BceN proteins from *B. cenocepacia* J2315 (CAR54861) and *B. cepacia* IST408, the GMD from *P. aeruginosa* (AAG08838), the GMD from *A. thermoaerophilus* (AAS55711) and the GMD from *E. coli* (AAC77842). The Wierenga motif and the catalytic triad typical of the SDR family of proteins are highlighted in blue, and the specific GMD motifs in orange. Asterisks indicate the amino acid residues that are identical in all proteins. One or two dots indicate semi-conserved or conserved substitutions, respectively. The secondary structure elements of Bcc strains are shown above the alignment segments, where cylinders represent α-helices and arrows represent β-sheets. Alignments were performed with CLUSTAL W2 and the secondary structure predictions with PSIPRED and PredictProtein softwares.

The secondary structures of *B. cenocepacia* J2315 and *B. cepacia* IST408 BceN proteins were predicted by PSIPRED (Jones, 1999) and PredictProtein (Rost *et al.*, 2004) servers. Based on the

structures obtained it is possible to identify the N-terminal Rossman fold domain, typical of the sugar nucleotide-modifying proteins of the SDR family. This motif is composed of an α/β folding pattern, with seven β-strands flanked by six or seven α-helices on each side and is involved in cofactor binding (Kavanagh *et al*., 2008).

The secondary structural elements of *B. cepacia* IST408 and *B. cenocepacia* J2315 BceN proteins superimpose totally, which was expected since the two proteins differ by only 11 amino acid residues, whose position does not coincide with the proposed regions of critical importance for the GMD activity. Furthermore, only one of the eleven substitutions is not conserved or semi-conserved, the Ala322 of *B. cenocepacia* J2315 BceN, which was replaced by Glu322 in *B. cepacia* IST408 BceN.

The presence of GMDs conserved motifs and the homology of Bcc BceN proteins with other microbial GMDs strongly suggest that the *bceN* gene encodes a protein with GDP-D-mannose 4,6-dehydratase activity.

3.2. BceN exhibits GMD activity

In order to prove that BceN possess GDP-D-mannose 4,6-dehydratase enzyme activity, the protein was purified and the hypothesized enzymatic activity was assessed. For that, the *bceN* gene from the *B. cenocepacia* J2315 strain lacking the stop codon TGA was PCR amplified from pACM2 plasmid and cloned in the expression vector pET23a+. The resulting plasmid, pJRF1, was used to transform *E. coli* BL21 (DE3) cells, and after IPTG induction, the *bceN* gene was overproduced as a histidine-tagged derivative (with a C-terminal addition of 12 amino acid residues, including 6 histidines) and confirmed by western-blotting (data not shown).

The His_6-tagged BceN protein of *B. cenocepacia* J2315 was purified to homogeneity by affinity chromatography followed by dialysis overnight at 4ºC against dialysis buffer (25 mM sodium phosphate buffer, pH 7.4; 20% (v/v) glycerol; 50 mM NaCl). This glycerol concentration is crucial to improve the protein stability and to avoid its precipitation.

Fractions of cell extracts and of the purified fusion protein were analysed by SDS-PAGE, revealing that the apparent molecular mass of the fusion protein BceN-His_6 was around 40 kDa (Figure 3.2), which is consistent with the predicted molecular mass of 38.96 kDa.

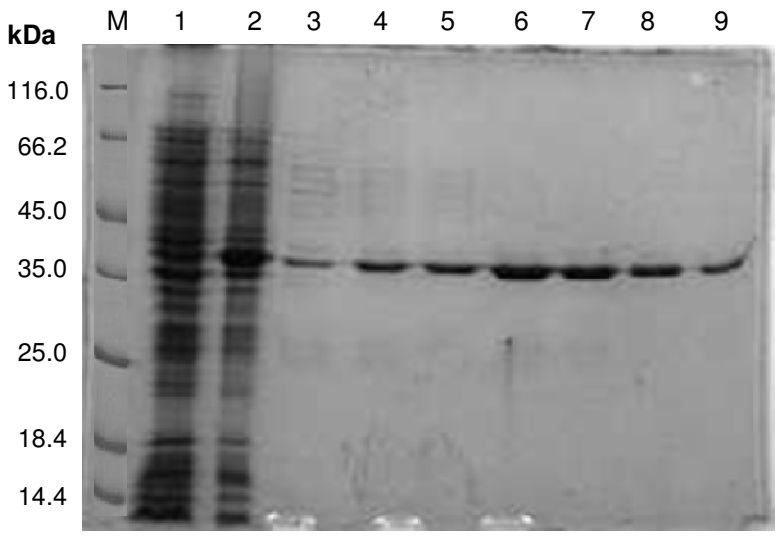


Figure 3.2. – SDS-PAGE analysis of the fractions obtained during overproduction and purification of the His_6-BceN protein of *B. cenocepacia* J2315 from *E. coli* BL21 (DE3). Lanes: M – Molecular mass standard; 1 – total soluble proteins from *E. coli* BL21 (DE3) with plasmid pJRF1, before IPTG induction; 2 - total soluble proteins from *E. coli* BL21 (DE3) with plasmid pJRF1 after 2 hours of IPTG induction; 3 - BceN protein eluted from the Ni^{2+}-NTA affinity chromatography column with 60 mM imidazole; 4, 5 – 100 mM imidazole; 6, 7, 8 - 200 mM imidazole; 9 - 500 mM imidazole.

Based on recent determinations of GMD activity by NMR spectroscopy analysis (King *et al.*, 2009), the purified His_6-tagged BceN protein from *B. cenocepacia* J2315 was incubated with GPD-D-mannose in the NMR spectrometer, being the reaction monitored directly in the tube over the time and the products identified based on the ^1H spectra. The spectra obtained indicate that the BceN protein catalyzed the dehydration of GDP-D-mannose to GDP-4-keto-6-deoxy-D-mannose, thus having the

GDP-D-mannose 4,6-dehydratase enzyme activity. As observed in Figure 3.3, over the course of incubation with BceN, the monitorization of the anomeric region of the one-dimensional ^{1}H- spectrum shows a progressive depletion of signals from GDP-D-mannose (A) and an increase in the intensity of the peaks corresponding to the anomeric resonances of the 4-keto (B) and gem-diol (C) forms of GDP-4-keto-6-deoxy-D-mannose.

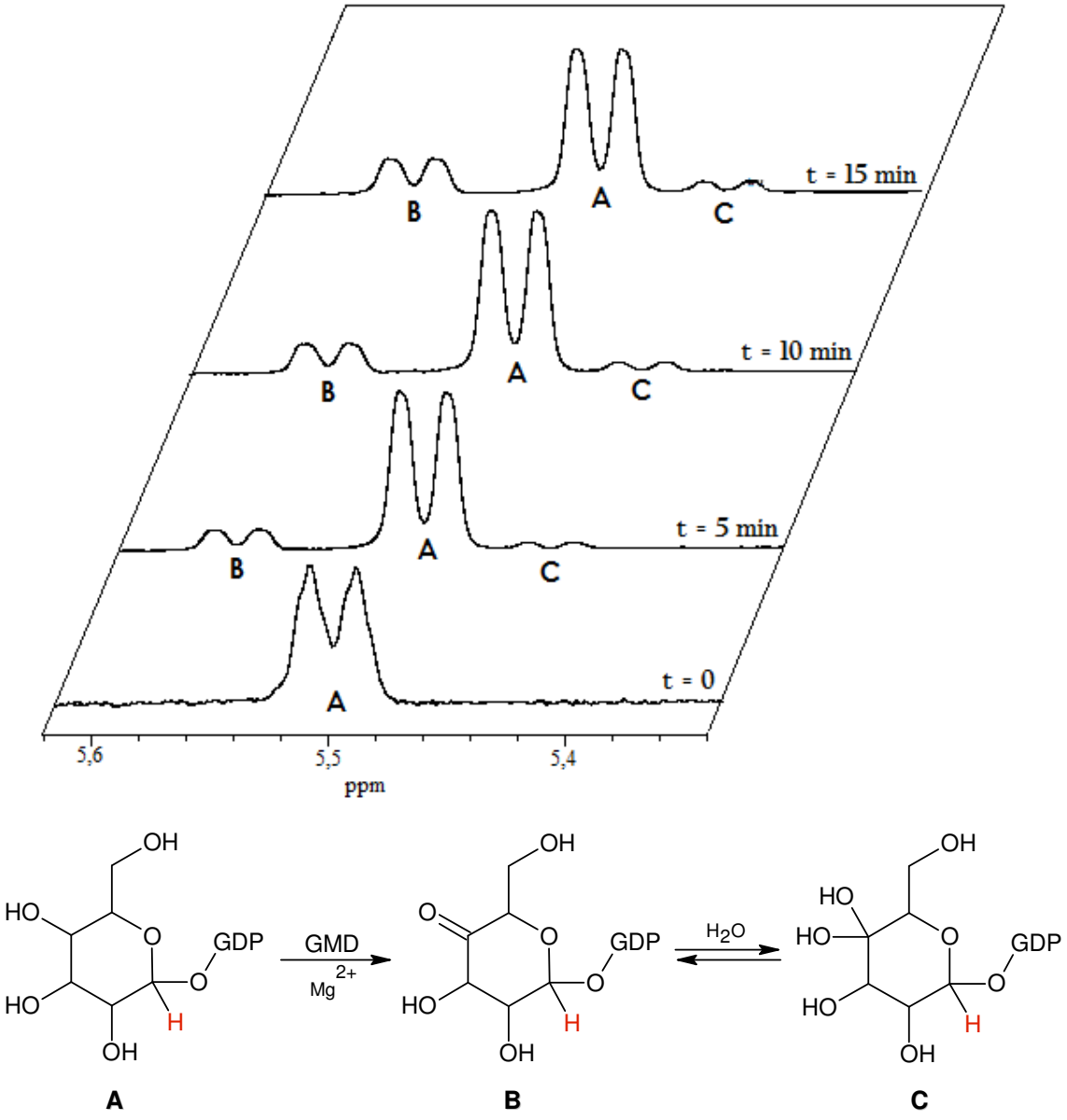


Figure 3.3. – NMR spectroscopy of the active His_6-BceN reaction directly in aqueous reaction buffer. ^{1}H-NMR spectra of the sample with 5 mM of GDP-D-mannose, incubated at 37ºC in 25 mM sodium phosphate buffer (10% D_2O) with Mg^{2+} (1 mol $MgCl_2$:1mol protein) and 90 µg of protein, taken each 5 minutes. Results indicate the conversion of GDP-D-mannose (A) into GDP-4-keto-6-deoxy-D-mannose (B) and the gem-diol form of compound B (C). The reaction catalyzed by *B. cenocepacia* J2315 GMD is also showed.

Although GMD enzymes have been reported to require a dinucleotide cofactor for an internal redox reaction between the C-4 and the C-6 of the GDP-D-mannose (Oths *et al.*, 1990), the GMD activity of the BceN protein was unaffected by the addition of exogenous NAD^+ or $NADP^+$ (results not

shown). The no requirement of exogenous NAD(P) was described to other GMD proteins, such as the *E. coli* GMD and the *P. aeruginosa* GMD (Sturla *et al*., 1997; King *et al*., 2009). We also noticed that the His_6-tagged BceN activity is dependent on the presence of magnesium ions, being the enzyme inactive in its absence (results not shown). The presence of $MgCl_2$ was reported as leading to a slight inhibitory effect on the activity of some GMD proteins (King *et al*., 2009). However, this divalent ion seems to be an effective BceN protein cofactor.

P. aeruginosa, Klebsiella pneumonia and *A. thermoaerophilus* GMDs are bifunctional, being able to catalyze the dehydratase and reductase reactions leading to the formation of GDP-D-rhamnose (King *et al*., 2009; Yamamoto *et al*., 1993; Kneidinger *et al*., 2001). Therefore, the GDP-4-keto-6-deoxy-D-mannose reductase (RMD) activity of BceN protein from *B. cenocepacia* J2315 was evaluated. This study, based on the method described by Kneidinger and colleagues (2001), revealed that the BceN protein had no ability to convert the GDP-4-keto-6-deoxy-D-mannose into GDP-D-rhamnose, even after incubation with NADPH over 24 hours, suggesting that the *B. cepacia* protein is not bifunctional. However, it is important to note that the reductase activity of the already characterized GMD proteins is much slower than their dehydratase activity or than RMD proteins acting only as reductases. Therefore, it is also possible that the NMR experiments could not detect a weak reduction. Recently, King *et al*. (2009) demonstrated the bifunctionality of *P. aeruginosa* GMD in a non-sequential way. After the first reaction step, these authors removed the dehydratase by ultrafiltration and then added fresh GMD enzyme and NADPH to the reaction mixture from which GMD was removed. This methodology suggests that a sequential procedure could interfere in GMD activity. To exclude this hypothesis the ability of BceN protein to convert the GDP-4-keto-6-deoxy-D-mannose into GDP-D-rhamnose should be tested again.

To determine the kinetic parameters of the BceN protein, the initial rate of GDP-4-keto-6-deoxy-D-mannose production, for different substrate concentrations, was monitored by NMR spectroscopy. The results obtained suggest that this enzyme is substrate-inhibited, since its reaction rate decreases with the increase of substrate concentrations tested (Figure 3.4). However, the kinetic behaviour of BceN enzyme at lower substrate concentrations is unknown, limiting the determination of the BceN kinetic. This fact could be due to the sensitivity limits of the NMR instrument at lower substrate concentrations. Other GMD proteins, as *P. aeruginosa* His_6-GMD (King *et al*., 2009), exhibit non-Michaelis-Menten kinetics producing typical curves corresponding to the substrate inhibition model, in which the enzyme reaction rate increases with increasing substrate concentration until it reaches a maximum, after which increases in substrate concentration will decrease the reaction velocity.

To confirm the substrate inhibition kinetic hypothesis, further assays are required using lower concentrations of substrate to determine the kinetic parameters of the BceN protein. Given the limitations of the method currently used, methods such as capillary electrophoresis analysis may be required to experimentally determine BceN kinetic parameters.

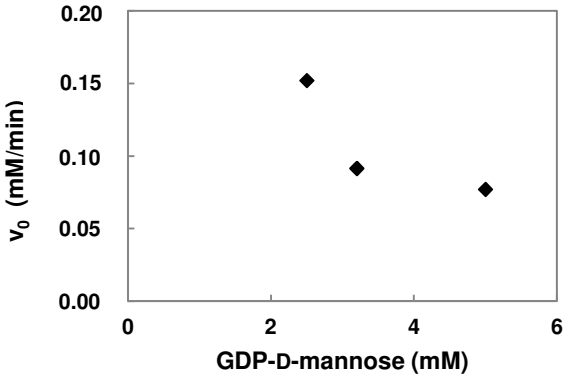


Figure 3.4. – Initial rate of GDP-mannose 4,6-dehydratase enzymatic reaction of *B. cenocepacia* J2315 BceN using Mg^{2+} as cofactor in function of substrate concentration.

3.3. Construction and characterization of a *bceN* insertion mutant

To investigate the role of BceN in Cepacian biosynthesis, a mutant derived from the highly mucoid *B. cepacia* IST408 strain bearing an insertionally inactivated *bceN* gene was constructed. For this purpose, the previously created pACM4 plasmid, resulting from the insertional inactivation of the *bceN* gene present in pACM2 plasmid with a 911pb *EcoRI/SphI* fragment containing the trimethoprim (Tp) resistance cassette from pUC-TP, was demethylated by passage through *E. coli* SCS110 cells. The demethylated plasmid was introduced by electrotransformation into *B. cepacia* IST408 cells and, after recombination, the insertional inactivation of the *bceN* gene was confirmed by PCR (results not shown). The demethylation step, carried out by the extraction of the transforming DNA from a *E. coli dam dcm* host strain prior to electrotransformation of some *Burkholderia* strains, has been shown to be 10 to 20 times more efficient than electrotransformation with methylated DNA, due to the evasion to the *Burkholderia* restriction systems specific for methylated sites (Dubarry *et al*., 2009).

The ability of the wild-type strain *B. cepacia* IST408 and the mutant strain *B. cepacia* IST408::*bceN* to produce EPS was compared in S liquid medium at 30ºC with orbital agitation. Both strains showed similar growth curves and, the *B. cepacia* IST408::*bceN* mutant is also able to produce EPS, although with a slightly reduced yield compared to the IST408 wild-type strain (Figure 3.5). Since GMD activity is required for biosynthesis of the activated sugar precursor of the Cepacian rhamnose moiety, it was expected that the loss of this function would influence EPS production.

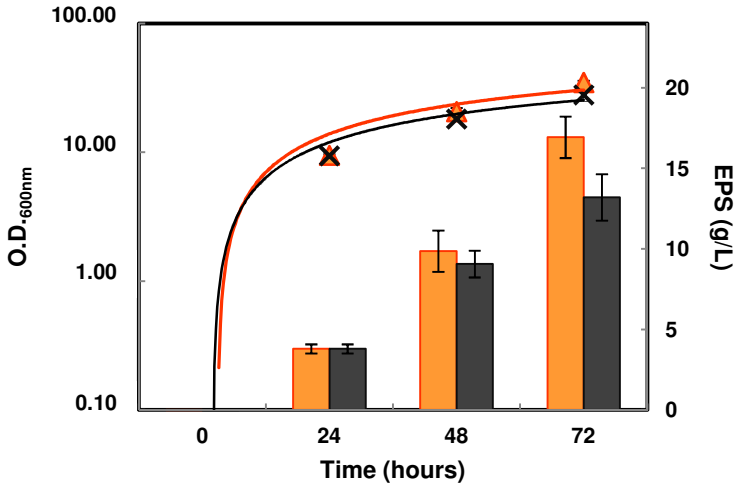


Figure 3.5. – Phenotypical characterization of EPS production by *B. cepacia* IST408 (orange) and the mutant *B. cepacia* IST408::*bceN* (black). Growth curve and amount of Cepacian produced in S liquid medium at 30ºC with orbital agitation. Error bars indicate Standard Deviation (SD).

Based on this, although no experiments have been carried out to determine the EPS composition of the mutant relative to the wild-type strain, the results obtained suggest the presence of additional GMD activity encoding genes in the *B. cepacia* IST408 genome that compensate the lack of

BceN activity. However, this hypothesis cannot be confirmed by bioinformatics analysis, because the genome sequence of *B. cepacia* IST408 is not yet available.

Homology searches for putative GMD encoding genes within publicly available genome sequences of strains of Bcc allowed the identification of strains in which one, two or three putative GMD encoding genes were present (further described in 3.4). Some Bcc strains had only one *bceN* homologous gene. One of these strains, *B. multivorans* ATCC 17616, is especially interesting for the present study because besides not having additional GMD encoded genes, it also produces EPS. Despite several attempts, the construction of *bceN* mutant in *B. multivorans* was not successful, which may simply result from the difficulty in constructing a mutant in bacteria of *Burkholderia* genus, or may highlight the importance of this gene.

In order to examine the *bceN* gene functional importance in *B. multivorans* ATCC 17616, an antisense RNA strategy, based on interference of mRNA expression with antisense RNA, was employed. This approach has been used in *Staphylococcus aureus* and Mycobacteria to examine specific gene essentiality and more recently in *B. cenocepacia* J2315 (Parish and Stoker, 1997; Ji *et al*., 1999; Wang and Kuramitsu, 2003; Ramos *et al*., 2011). With this purpose, the pMLBAD vector harbouring the DNA fragment of *bceN* gene in the antisense orientation downstream of the pBAD promoter was constructed, resulting in plasmid pJRF6. This plasmid was introduced into *B. multivorans* ATCC 17616 cells by triparental conjugation, yielding *B. multivorans* ATCC 17616 (pJRF6). Upon induction with arabinose, the antisense transcript of *bceN* is expressed, blocking the translation of target mRNAs. Since the expression of the antisense transcript cloned in pMLBAD is dependent on induction by arabinose and is repressed by glucose, the standard medium for EPS production (S medium) could not be used for these experiments. As an alternative, S medium without glucose and supplemented with 2% arabinose (S_{ARA} medium) was used.

To assess Cepacian production, the wild-type and mutant strains of *B. multivorans* ATCC 17616 were grown in S_{ARA} liquid medium at 30ºC and 37ºC, since the assay conditions in S_{ARA} medium have not been optimized to the highest Cepacian production yield. A comparation of results obtained suggest that in S_{ARA} medium the highest EPS production yield was obtained at 37ºC (data not shown). Under these conditions, although growth curves were similar, no ethanol-precipitated polysaccharide was obtained with the mutant strain *B. multivorans* ATCC 17616 (pJRF6), in contrast to wild-type strain (Figure 3.6). Although the observations suggest an influence of *bceN* gene in EPS production, results obtained for mutant strain expressing the antisense *bceN* gene cannot be inferred due to inability of control strain, *B. multivorans* ATCC 17616 transformed with pMLBAD plasmid, to produce EPS. These control strain also showed a lower growth rate compared to wild-type strain (results not shown).

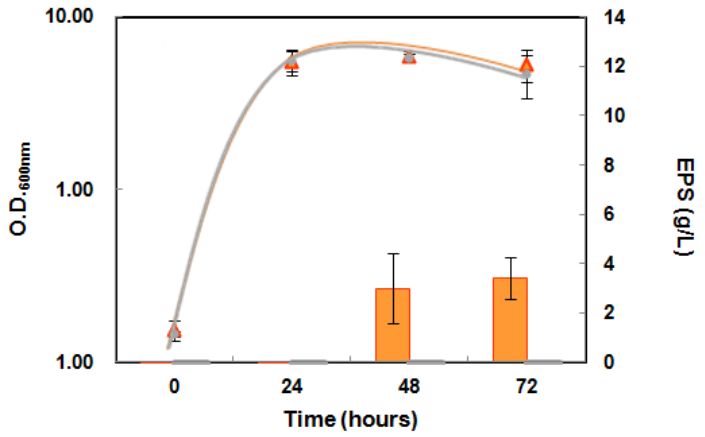


Figure 3.6. - Characterization of EPS production by *B. multivorans* wild type (orange) and *B. multivorans* (pJRF6) (grey). Growth curves and amounts of Cepacian produced were assessed in S_{ARA} liquid medium at 37ºC with orbital agitation. Error bars indicate SD.

Since the EPS has been reported to be involved in biofilms formation (Wai *et al.*, 1998; Cunha *et al.*, 2004; Jackson *et al.*, 2004), the ability of *B. cepacia* IST408 wild-type and *bceN* mutant strains to form biofilms *in vitro* was compared after 24 hours of incubation in S medium, at 30ºC. After incubation under these conditions the amount of biofilm formed by *B. cepacia* IST408 wild-type strain was two fold higher than the amount of biofilm formed by *B. cepacia* IST408::*bceN* mutant strain (Figure 3.7). This result is consistent with the requirement of Cepacian for the formation of thick and mature biofilms of maximal size and with the slightly reduced ability of the mutant to produce Cepacian.

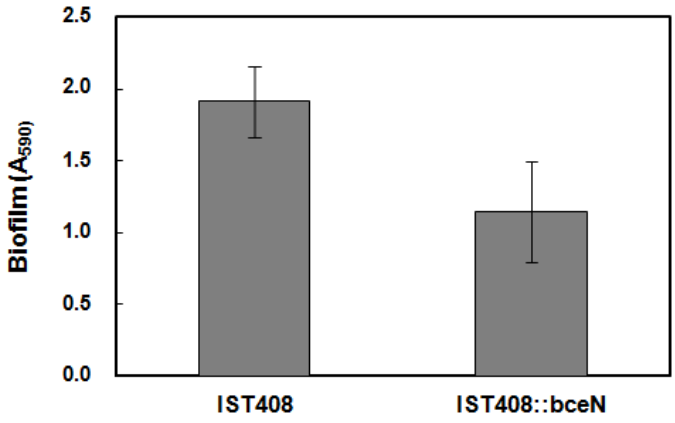


Figure 3.7. – Biofilm formed by *B. cepacia* IST408 and the mutant *B. cepacia* IST408::*bceN* in polystyrene microtiter plates. Biofilm formation was quantified after 24 hours of incubation at 30ºC in S medium without agitation. Error bars indicate SD.

Previous studies have shown that biofilm formation is closely related to the swarming ability in many bacterial species, including *Salmonella enterica*, *P. aeruginosa* and *B. cepacia* (Mireles *et al.*,

2001; Déziel *et al*., 2001; Huber *et al*., 2001). Since the *bceN* gene is required for the formation of biofilms of maximal size, experiments were performed to determine whether the swarming motility was also affected. To investigate this feature, the *B. cepacia* IST408 wild-type and the *bceN*-defective mutant were inoculated on ABC-swarming agar plates with casamino acids and glucose. While at 30ºC similar results were obtained, after 48 or 72 hours of incubation at 37ºC, *B. cepacia* IST408::*bceN* mutant exhibited a decreased swarming capacity when compared to the wild-type strain (Figure 3.8). Although the effects of *bceN* deletion in two other types of motility have been tested, the flagellar-mediated swimming motility and the type IV pili-mediated twitching motility, no differences were observed between the wild-type and the deletion mutant (data not shown). These results suggest that the alterations in swarming motility are most probably due to defects other than the lack of functional flagella and type IV pili.

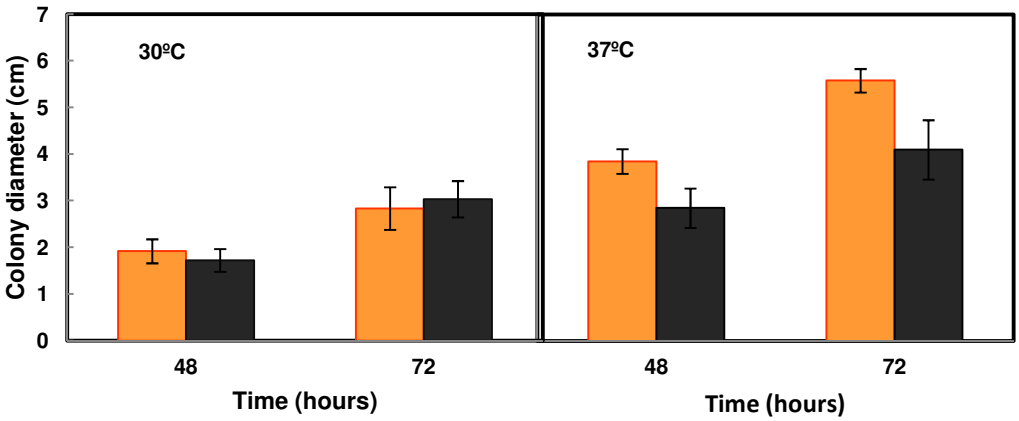


Figure 3.8. - Effects of *bceN* gene mutation on swarming motility of *B. cepacia* IST408. Bars showing the spread of *B. cepacia* IST408 wild type (orange) and the mutant *B. cepacia* IST408::*bceN* (black) at 30 or 37ºC, after inoculation onto swarming agar plates. Error bars indicate SD.

3.4. Phylogenetic analysis of *bceN* homologues from strains of the *Burkholderia* genus

Given the importance of GMD activity, the results described above suggest that more than one gene coding for GMD could be present within the *B. cepacia* IST408 genome sequence. This is a rather common feature in Bcc genomes, as illustrated for example by the presence of two to five *bceA* homologues in the genome sequences of 11 *Burkholderia* strains analysed (Sousa *et al*., 2007a). This possibility prompted us to search for *bceN* homologues within publicly available genome sequences of other strains of the *Burkholderia* genus. 14 strains from nine *Burkholderia* species were included in this analysis (Table 3.2).

Table 3.2 – Putative GMDs identified in the genome sequences of strains belonging to the *Burkholderia* genus. Putative proteins are identified by GenBank accession number and the identity of *B. cepacia* IST408 BceN protein and the identified putative GMDs is shown.

Organism	Chromosome/ Contig	Identity (%)	No. of amino acids	GenBank accession No.
***B. lata* 383**	Chromosome II	344/348	348	ABB12226
***B. cenocepacia* AU1054**	Chromosome I	260/341	372	ABF75312
	Chromosome II	338/348	348	ABF79279
***B. cenocepacia* HI2424**	Chromosome I	260/341	372	ABK07633
	Chromosome II	338/348	348	ABK10707
***B. cenocepacia* MC0-3**	Chromosome II	338/348	348	ACA92711
***B. cenocepacia* J2315**	Chromosome II	337/348	348	CAR54861
***B. cenocepacia* PC184**	Supercontig 1.1	263/345	351	EAY62633
	Supercontig 1.1	263/341	370	EAY62635
	Supercontig 1.2	337/348	348	EAY65184
***B. dolosa* AU0158**	Supercontig 1.1	265/343	346	EAY69559
	Supercontig 1.2	331/348	348	EAY70152
***B. ambifaria* MC40-6**	Chromosome I	265/345	346	ACB63266
	Chromosome II	325/348	348	ACB66330
***B. ambifaria* AMMD**	Chromosome I	268/345	346	ABI86318
	Chromosome II	325/348	348	ABI88913
***B. vietnamiensis* G4**	Chromosome II	322/348	348	ABO57437
	Chromosome III	185/349	372	ABO59420
***B. multivorans* ATCC 17616**	Chromosome II	325/348	347	ABX18290
***B. thailandensis* E264**	Chromosome I	259/341	345	ABC38232
	Chromosome II	302/348	347	ABC34776
***B. pseudomallei* K96243**	Chromosome II	304/348	347	CAH39161
***B. mallei* ATCC 23344**	Chromosome II	304/348	347	AAU45732

As shown in Table 3.2, one, two or three putative GMD homologues were found to be present in the genomes sequences examined. No association could be established between the number of genes putatively encoding GMDs and the virulence of strains or their ability to produce EPS.

The deduced amino acid sequences of the 23 identified putative GMD homologues present in databases were obtained and aligned, together with the *B. cepacia* IST408 BceN, using the CLUSTAL X2 software. In order to obtain additional clues on the biological functions of the various GMDs, the sequence of the surrounding genes was also examined (Figure 3.9).

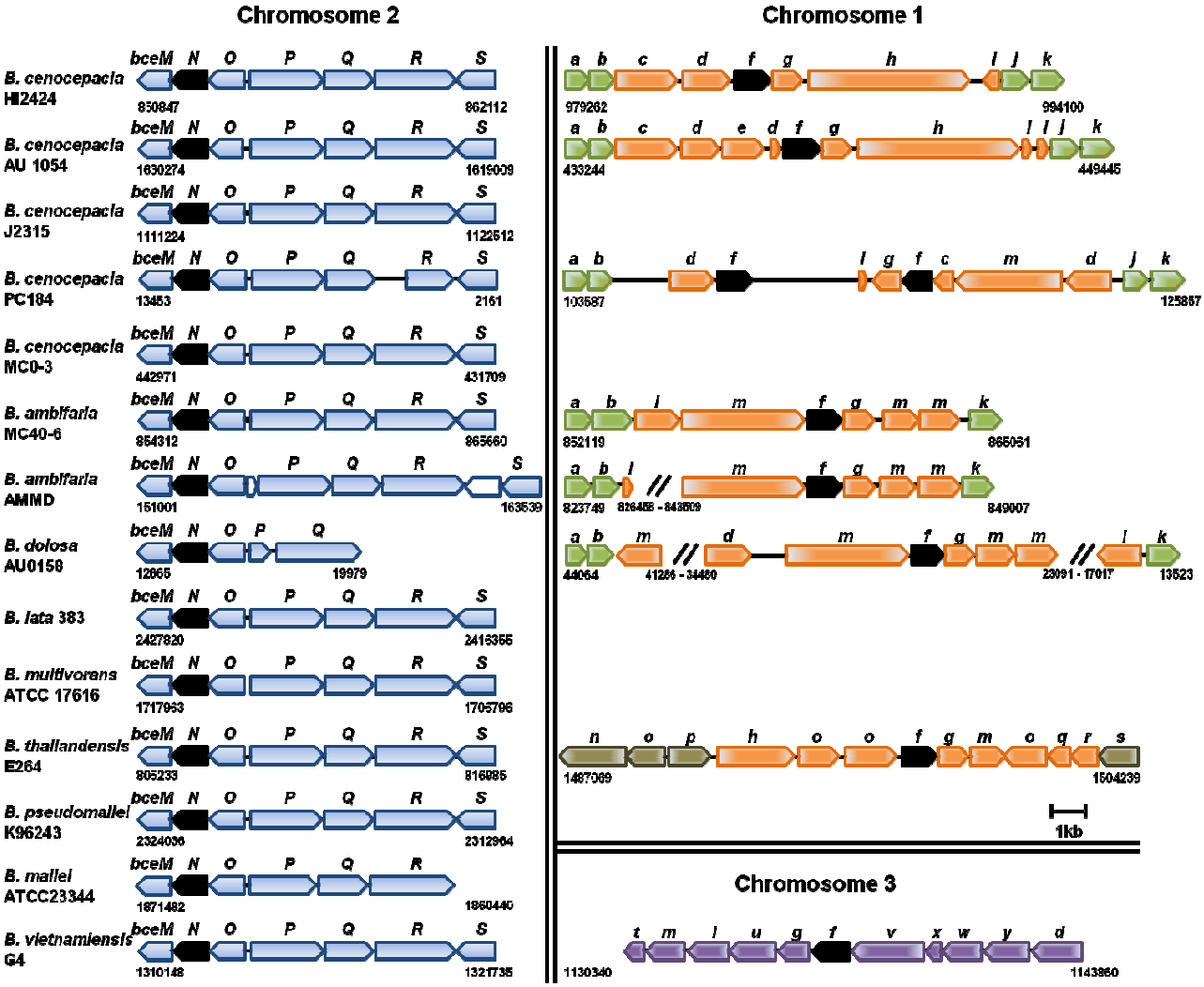


Figure 3.9. – Genetic organization of the chromosomal region in the vicinity of *bceN* homologous genes, from 14 strains of the *Burkholderia* genus with genome sequences available. Genes are represented as box-like arrows with the transcription orientation indicated by the arrow head, the numbers refer to the nucleotide position of the genes in bacteria genomes sequence. In the figure are represented genes homologous to *bceN* gene (black boxes); genes of the *bce-II* cluster (blue boxes); genes involved in the biosynthesis of the O-antigen (green boxes), genes involved in the capsular polysaccharide biosynthesis (brown boxes), genes putatively involved in polysaccharides biosynthesis and transport (purple boxes), and genes present in insertional sequences (orange boxes). Genes name or putative function: **N** – *bceN*; **O** – *bceO*; **P** – *bceP*; **Q** – *bceQ*; **R** – *bceR*; **S** – *bceS*; **a, b** – ABC transporters; **c, j, m, o** – glycosiltransferases; **d** – type 2 mannose-6-phosphate isomerases; **e** – transposase; **f** – GDP-D-mannose 4,6-desydratases; **g, k**– NAD-dependent epimerases/dehydratases; **h** – methyltransferases FkbM; **i** – hypothetical proteins; **l** – methyltransferase type 11; **n** – *wcbA*; **p** – *wcbC*; **q** – *bexA*; **r** – *bexB*; **s** – *wcbD*; **t** – transferase hexapeptide repeat containing protein; **u** – polysaccharide biosynthesis protein; **v** – exopolysacharide transport protein; **x** – tyrosine phosphatase; **w** – polysaccharide export protein; **y** – sugar transferase.

The *bceN* orthologues with the highest degree of identity at the amino acid level were located, in all the genome sequences analysed, on chromosome 2, more specifically in the *bce-II* gene cluster that encode proteins and enzymes putatively involved in polysaccharide biosynthesis (Figure 3.9). This cluster is widespread within all *Burkholderia* strains analyzed, being the *bceN* gene and neighbour sequences extremely conserved between bacteria of the *Burkholderia* genus (Figure 3.9).

The proteins encoded by these genes are represented as clade A in Figure 3.10 where it is possible to see that putative GMDs present on chromosome 2 share a higher level of homology with BceN protein and among themselves, especially the proteins encoded by Bcc strains.

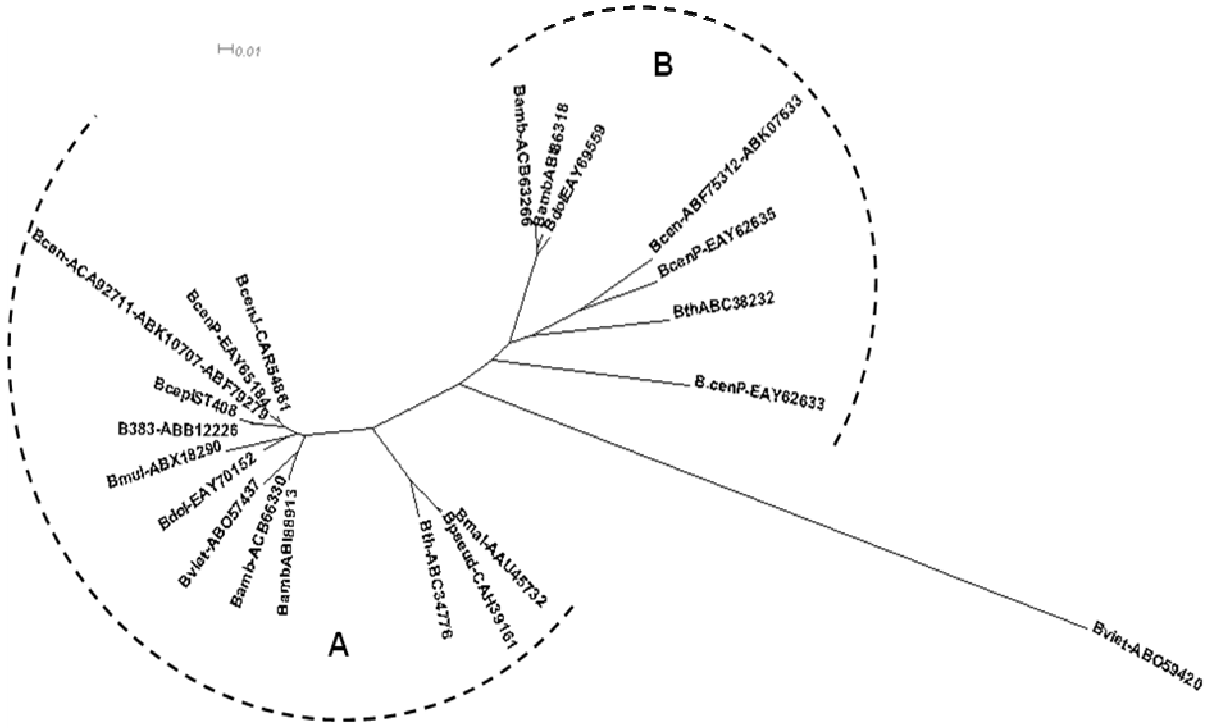


Figure 3.10. – Unrooted phylogenetic tree for the *B. cepacia* IST408 BceN (BcepIST408) and the putative BceN orthologues from *B. lata* sp. 383 (B383), *B. cenocepacia* J2315 (BcenJ), *B. cenocepacia* AU1054, *B. cenocepacia* HI2424 and *B. cenocepacia* MC0-3 (Bcen), *B. cenocepacia* PC184 (BcenP), *B. ambifaria* MC40-6 and *B. ambifaria* AMMD (Bamb), *B. dolosa* AUO158 (Bdol), *B. vietnamiensis G4* (Bviet), *B. thailandensis E264* (Bthai), *B. multivorans ATCC* 17616 (Bmul), *B. mallei* ATCC23344 (Bmal) and *B. pseudomallei* K96243 (Bpseud). The phylogenetic tree was constructed based on the alignment of the amino acid sequences with CLUSTAL X2 using the neighbour-joining method with a minimum of 100 bootstraps. Clade A, including *B. cepacia* IST408 BceN, includes the putative GMDs located in a gene cluster similar to the *bce-II* gene cluster involved in Cepacian biosynthesis. Clade B includes the *Burkholderia* putative GMDs located on chromosome 1, surrounded by genes located in clusters most probably involved in LPS biosynthesis. The BceN ortologue of *B. vietnamiensis* is not included in clades A or B and is the only one encoded by a gene located in chromosome 3.

The second copy of *bceN* homologues genes is located in a distinct chromosome, being the genetic organization in the vicinity of its genes less conserved. The BceN homologues ABF75312, ABK07633, EAY62635, EAY69559, ACB63266, ABI86318 and ABC38232 identified in the genomes of *B. cenocepacia* AU1054, *B. cenocepacia* HI2424, *B. cenocepacia* PC184, *B. dolosa* AU0158, *B.*

ambifaria MC40-6, *B. ambifaria* AMMD and *B. thailandensis* E264, respectively, are located on chromosome 1. While BceN homologues ABF75312, ABK07633, EAY62635, EAY69559, ACB63266 and ABI86318 are encoded by genes present in the insertion sequence element IS402 of the O-antigen lipopolysaccharide synthesis cluster (previously described by Ortega *et al*., 2005), the BceN homologue ABC38232 of *B. thailandensis* E264 is encoded by a gene homologue to those present in the capsular polysaccharide biosynthetic insertion sequence reported by Parsons *et al*. (2003) (Figure 3.9). Although insertion sequence elements show a low level of conservation, these BceN homologue proteins share a high homology to each other, forming the separate clade B (Figure 3.10).

The identified orthologue exhibiting the lowest identity to the *B. cepacia* IST408 *bceN* is the *B. vietnamiensis* G4 BceN-homologue protein ABO59420, which is the only ortologue located on chromosome 3 (Figure 3.9 and 3.10). In this case, the encoding gene is flanked by proteins putatively involved in polysaccharide and lipopolysaccharide biosynthesis and exopolysaccharide transport.

The search for genes encoding proteins with GMD activity in other bacterial genomes showed that the presence of one or multiple copies is variable among strains of the same species or genus, which is consistent with the results obtained by analysis of *Burkholderia* strains sequences. For example, *E. coli* str. K12 and *P. aeruginosa* PAO1 have only one gene encoding a protein with GMD activity. However *E. coli* O157:H7 str. Sakai and *P. syringae* pv. syringae 642 have two genes encoding GMDs that map in two different chromosomal regions. Therefore, these data suggest that an additional gene encoding GMD, besides *bceN*, could be present in the genome of *B. cepacia* IST408.

4. CONCLUSIONS AND PERSPECTIVES

In the last years, Bcc infections have instigated an increased research interest, mainly due to the ability of some virulent strains to easily transmit between CF patients, through social contact and in the hospital environment (Chen *et al*., 2001); to their intrinsic resistance to antimicrobials of clinical significance (Chernish and Aaron, 2003) and to the possibility of some strains cause a severe necrotizing infection, named cepacia syndrome (Govan *et al*., 1996; Mahenthiralingam and Vandamme, 2005). Although some advances in Bcc identification have been achieved, the knowledge on the molecular mechanisms of Bcc pathogenicity and progress on the development of new therapeutic agents are still limited (Leitão *et al*., 2010).

Bcc virulence is multifactorial and differs widely between species and even within species however, the relative contribution of the potential virulence factors in the pathogenesis of human disease is still not sufficiently defined. Taking in consideration the importance of EPS production in the persistence of infections caused by other bacterial species, several studies have been performed pointing out Cepacian as a virulence factor contributing to the overall pathogenicity of Bcc members and thus to their success as pathogens (Conway *et al*., 2004; Sousa *et al*., 2007b). The genes required for Cepacian biosynthesis are located in the clusters *bce-I* and *bce-II* of chromosome 2 (Moreira *et al*., 2003; Ferreira *et al*., 2010). In these clusters, only two of the five genes encoding proteins putatively involved in nucleotide sugar precursors biosynthesis were already functionally characterized, the *bceA* and *bceC* gene products (Sousa *et al*., 2007a; Loutet *et al*., 2009; Ferreira *et al*., 2010).

In this study we report results on the functional analysis of the BceN protein of the *bce-II* cluster from the opportunistic human pathogen *Burkholderia cenocepacia* and investigated the role of BceN protein of *B. cepacia* IST408 on Cepacian biosynthesis and on the size of the biofilms formed. Analysis of the amino acid sequences of BceN protein evidenced the presence of the typical domains of the short-chain dehydrogenases/reductases enzymes family: the Wierenga motif, the conserved catalytic triad and the Rossman fold (Jörnvall *et al*., 1995; Kavanagh *et al*., 2008). In addition, the BceN protein also has the typical motifs and residues of GDP-D-mannose 4,6-dehydratase enzymes, namely the conserved glutamic acid (Glu133) and arginine (Arg190) residues and the RR loop region (Somoza *et al*., 2000; King *et al*., 2009; Webb *et al*., 2004). To prove this putative activity, functional studies were performed and demonstrated that the BceN protein has GDP-D-mannose 4,6-dehydratase activity, catalyzing the conversion of GDP-D-mannose to GDP-4-keto-6-deoxy-D-mannose. Although the GMD protein from *A. thermoaerophilus* and *Helicobacter pylori* have been reported to require $NADP^+$ as cofactor (Broschat *et al*., 1985; Kneidinger *et al*., 2001; Wu *et al*., 2001), the catalytic activity of the BceN enzyme was unaffected by the addition of exogenous NAD(P), similarly to the GMDs purified from *P. aeruginosa* and *E. coli*, suggesting that the cofactor had copurified with the protein (Sturla *et al*., 1997; King *et al*., 2009). The GMD activity of BceN protein is also strongly dependent on the presence of Mg^{2+} ions, being the effect of these divalent ions variable between GMDs. While Mg^{2+} ions have a slight inhibitory effect on activity of *P. aeruginosa* GMD, these cations activated the GMD of *E. coli* and had little effect on the *H. pylori* GMD (King *et al*., 2009; Sturla *et al*., 1997; Wu *et al*., 2001). On the other hand, the BceN protein is susceptible to inhibition by the

substrate GDP-D-mannose. We therefore anticipate that this protein exhibits a substrate inhibition kinetics model, as described for *P. aeruginosa* GMD (King *et al*., 2009). Despite these conclusions are taken from only three experimental determinations of the reaction rate *versus* substrate concentration, results clearly show a decrease of the BceN activity with the increase of the substrate concentration. Some GMD enzymes are also able to catalyze the reductase reaction leading to GDP-D-rhamnose, but this ability was not detected for the BceN protein. Effectively, BceN is most certainly unable to catalyze reduction reaction; however, several problems of the reaction cascade, such as the instability of GDP-4-keto-6-deoxy-D-mannose may have limited the putative synthesis of GDP-D-rhamnose for NMR analysis.

Although some differences between GMD proteins have been highlighted, it is important to note that these enzymes are highly conserved throughout the domains Archaea, Bacteria, in higher eukaryotic organism, such as plants and animals and even in virus (Kneidinger *et al*., 2001). This fact may reflect the importance of the enzyme throughout evolution. It is also noteworthy that the intermediate resulting from the dehydration of GDP-D-mannose by GMD serves as a branching point for the synthesis of several different deoxyhexoses, including GDP-D-rhamnose, GDP-L-fucose; GDP-6-deoxy-D-talose, and the GDP-dideoxy amino sugar GDP-D-perosamine. The involvement of GMD as the initial enzyme in all the pathways leading to the above mentioned sugar nucleotides makes it a particularly interesting target of research.

Mutant strains are required to study the effect of GMD proteins in the cell. Only two descriptions of bacterial mutants in genes encoding for enzymes with GMD activity were found in literature (*P. fluorescens* and the plant root-colonizing Gram-negative bacteria *Azospirillum brasiliense* Sp7) and at least in one of the mutated strains an additional gene encoding for a second GMD protein was identified (Pimenta *et al*., 2003; Lerner *et al*., 2009). The *bceN* gene from the highly mucoid *B. cepacia* strain was inactivated. Based on role of this gene in the synthesis of the Cepacian rhamnose moiety, the mutant was expected to be affected in its EPS production ability. However, the *bceN* mutant strain was able to produce EPS with a slightly reduced yield compared to the wild type, indicating that the BceN protein is not essential for EPS biosynthesis. Similarly, no differences in EPS concentration were found between *A. brasiliense* Sp7 wild type and mutant strains (Lerner *et al*., 2009). Nevertheless, whether or not the Cepacian produced by the mutant strain has a sugar composition similar to that of the wild-type remains unknown.

The ability of *bceN* mutant to produce EPS suggests that other functional *bceN* homologues may exist in *B. cepacia* IST408 genome sequence. One, two or three putative GMD encoding genes were identified within the available genome sequences of 14 strains of the *Burkholderia* genus with their genome sequences available. This observation supports the hypothesis of the existence of multiple functional copies of *bceN* within the *B. cepacia* IST408 genome. There are other examples in the literature of the presence of multiple copies of genes encoding GMD proteins in bacterial genomes. In *A. brasiliensis* for example, in addition to *noeL*, another gene encodes a protein with predicted GMD activity (Lerner *et al*., 2009).

In accordance with results obtained for the *gmd* mutant of *P. fluorescens* (Pimenta *et al*., 2003), defects in swarming motility and in biofilms formation were detected in *bceN* mutant strain

when compared with the wild type *B. cepacia* IST408 strain. In *P. fluorescens,* the *gmd* gene seems also to be involved in LPS synthesis and mutants in this gene are affected in adherence to human fibronectin, being also avirulent in an *in vivo Drosophila* virulence model (Pimenta *et al*., 2003).

Based on this and in order to further our understanding on the role of the GMD activity in the virulence of Bcc bacteria it would be necessary to construct and study mutants in which all the genes homologous to the *bceN* gene were knocked out.

5. BIBLIOGRAPHY

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