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Mechanisms Controlling the Expression of the Exopolysaccharide of *Burkholderia* and Role in Niche Adaptation

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1. Introduction

Bacteria from the genus *Burkholderia* are widespread in nature, with strains being isolated from rhizosphere, aquatic environments, man-made environments and in association with hosts causing disease or beneficial interactions. One common feature across the genus is the ability to produce an exopolysaccharide (EPS) termed cepacian. This ability is confirmed by the presence of the *bce* biosynthetic genes in all *Burkholderia* sequenced genomes with the exception for *Burkholderia mallei* (Ferreira *et al.*, 2010). Cepacian is composed of a branched acetylated heptasaccharide repeat-unit with D-glucose, D-rhamnose, D-mannose, D-galactose and D-glucuronic acid in the ratio of 1:1:1:3:1 (Cescutti *et al.*, 2000). Cepacian biosynthesis starts with the synthesis of the nucleotide sugar precursors, catalyzed by isomerases, mutases, epimerases, among other enzymes (Ferreira *et al.*, 2010). This step is followed by the assembly of the repeat-units by the sequential addition of sugars to an isoprenoid lipid by dedicated glycosyltransferases. The last step of cepacian biosynthesis comprises polymerization and export of the polysaccharide to the extracellular environment. A multienzyme complex including a repeat-unit translocase, a polysaccharide polymerase, an outer membrane protein, among others are involved in this process (Ferreira *et al.*, 2010; Moreira *et al.*, 2003). Insights into the biosynthetic steps will be covered in this chapter.

Within the genus *Burkholderia*, a particular group of strains belonging to the so called *Burkholderia cepacia* complex (Bcc) have been isolated from the lungs of patients suffering of cystic fibrosis (CF). The analysis of sequential isolates from the same patient revealed that during the bacterial process of lung infection and colonization, a transition between mucoid to nonmucoid phenotype occurs (Zlosnik *et al.*, 2008). This phenotypic conversion is predominantly from mucoid to nonmucoid and the relevance of it in disease progression is still under debate. It is therefore of importance not only to understand the factors triggering the mucoid phenotype conversion, but also the molecular mechanisms involved in cepacian biosynthesis regulation. Concerning EPS biosynthesis regulation, the present knowledge includes regulation at transcriptional level, namely through quorum sensing mechanisms. Indeed, it is known that exopolysaccharide production in plant-associated *Burkholderia* is regulated by N-acyl homoserine lactones (Suarez-Moreno *et al.*, 2010). Another *Burkholderia* gene recently characterized and implicated in cepacian production is *hfq* encoding a RNA chaperone involved in the riboregulation of target mRNAs by small regulatory non-coding

RNAs (Sousa *et al*., 2010). Cepacian biosynthesis is also regulated at post-translational level through a mechanism of phosphorylation and dephosphorylation of conserved tyrosine residues in specific proteins. Involved in this process are two enzymes, namely the tyrosine autokinase BceF and the phosphotyrosine phosphatase BceD (Ferreira *et al*., 2007). BceF tyrosine phosphorylation activity is entirely required for cepacian biosynthesis, while BceD activity is not. The possible role of the two enzymes in controlling EPS chain length will be discussed.

Lastly, is our aim to present and discuss data on the role of cepacian in cell adaptation to different environments such as the rhizosphere or eukaryotic host cells. For example, the role of cepacian in protecting cells against desiccation and high concentration of ion metals and some considerations on EPS involvement in biofilm formation and as a putative virulence factor both *in vitro* and *in vivo* models will be covered.

2. *Burkholderia* genus

The genus *Burkholderia* includes one of the most versatile group of gram-negative bacteria, being able to occupy a wide range of ecological niches that include soil, water, plant rhizosphere and fungal mycelia. Some *Burkholderia* species are able to cause plant disease while others bring beneficial effects to crops; some can degrade natural and man-made pollutants and others are important opportunistic pathogens. The first *Burkholderia* isolate was described by Burkholder in the 40's as being a phytophatogenic bacterium, causing rot of onion bulbs (Burkholder, 1950). This isolate was then called cepacia, meaning "onion", and was included in the *Pseudomonas* genus for many years due to broad and vague phenotypic characteristics. After molecular taxonomic analysis in 1992, several *Pseudomonas* species were transferred to *Burkholderia* genus, being *Burkholderia cepacia* the type species (Yabuuchi *et al*., 1992). There are currently more than 50 *Burkholderia* species and candidates identified.

Phylogenetic trees, based on 16S rRNA sequence analysis, separate the genus *Burkholderia* into two major clusters (Caballero-Mellado *et al*., 2007). The first cluster includes pathogens such as *B. glumae*, *B. pseudomallei*, *B. mallei* and bacteria belonging to *Burkholderia cepacia* complex. *B. glumae* has become an important plant pathogen in Japan, Korea and Taiwan, due to its ability to rot of rice grains and seedlings (Coenye & Vandamme, 2007). *B. mallei* and *B. pseudomallei* are known primary pathogens. The first species causes glanders in horses, mules and donkeys, which is characterized by the formation of nodular lesions in the lungs and ulceration of the mucous membranes in the upper respiratory tract. *B. pseudomallei* is the etiological agent of melioidosis in humans and animals. The disease can be an asymptomatic infection or cause severe lung nodulation, pneumonia or skin infections (Coenye & Vandamme, 2007). *Burkholderia cepacia* complex are a group of phenotypically similar bacteria that share a high degree of similarity at 16S rRNA gene sequence (98% to 100%) and of *recA* gene sequence (94% to 95%) as well as moderate levels of DNA-DNA hybridization (30% to 50%) (Coenye *et al*., 2001; Vandamme *et al*., 1997; Vandamme *et al*., 2000; Vandamme *et al*., 2002b; Vandamme *et al*., 2003; Vermis *et al*., 2004). There are currently seventeen species belonging to the Bcc complex where *B. cepacia*, *B. multivorans* and *B. cenocepacia* are the most studied species, due to the high percentage of patients infected by these species. Bcc bacteria have emerged as important opportunistic pathogens, mainly for patients with cystic fibrosis, chronic granulomatous disease and immunocompromised patients; are easily transmissible by social contact between patients and intrinsically resistant to several antibiotics (Nzula *et al*., 2002). Bcc infections clinical outcome may vary greatly, ranging from chronic lung infection with little or no impact

on lung function to rapid deterioration that in the worst cases can lead to septicemia and death (Isles *et al.*, 1984). The second *Burkholderia* cluster based on 16S rRNA sequence analysis comprises more than 25 environmental non-pathogenic species, the majority found in association with plants. Bacteria belonging to this second cluster have the ability to colonize the rhizosphere or the internal intercellular spaces in several plants. Some species were shown to promote plant growth, such as *B. kururiensis* and *B. phytofirmans*, and others to increase plant nutrient availability via nitrogen fixation and/or phosphate solubilization, like *B. unamae*, *B. tropica* and *B. silvatlantica* (Caballero-Mellado *et al.*, 2007; Mattos *et al.*, 2008; Perin *et al.*, 2006; Reis *et al.*, 2004). Many species have the ability to form symbiotic interactions with plants or with mosses, like *B. tuberum*, *B. phymatum*, *B. mimosarum*, *B. nodosa, B. sabiae*, *B. megapolitana* and *B. bryophila* (Chen *et al.*, 2007; Chen *et al.*, 2008; Elliott *et al.*, 2007; Vandamme *et al.*, 2002a). Even though these *Burkholderia* species seem to be very promising from the biotechnological point of view, the specific molecular mechanisms that enable such potential are poorly understood and only a few studies are available on this second *Burkholderia* cluster.
Overall, bacteria belonging to *Burkholderia* genus have large genomes, with two or more chromosomal replicons, encoding in average more than 7000 genes (Mahenthiralingam *et al.*, 2005). The enormous amount of genomic information contained in such a large genome, enables *Burkholderia* to grow on a wide range of substrates, being able to use several different carbon sources and survive to the most diverse stress conditions.

3. Exopolysaccharides produced by *Burkholderia* genus

Exopolysaccharides (EPSs) have been described in many bacteria as being important in adaptation to different stress conditions and being involved in the establishment of symbiotic and pathogenic relationships. Several EPSs have been isolated from *Burkholderia* and their chemical structure determined, but only EPS-II was shown to be produced by the majority of the species, thereby designated cepacian. This EPS was shown to be produced by both clinical and environmental Bcc isolates (Chiarini *et al.*, 2004; Cunha *et al.*, 2004; Richau *et al.*, 2000a; Zlosnik *et al.*, 2008). Even though most of the studies were conducted in species belonging to Bcc complex, recent literature shows that cepacian biosynthesis also occurs in non-Bcc species (Ferreira *et al.*, 2010; Hallack *et al.*, 2010). Ferreira and co-authors showed that the rhizosphere non-Bcc bacteria *B. xenovorans*, *B. phymatum* and *B. phytofirmans* were able to produce large amounts of an exopolysaccharide in EPS-promoting media, confirmed to be cepacian by Fourier transform infrared spectrometry (FTIR) (Ferreira *et al.*, 2010). Cepacian production was also recently confirmed in the diazotrophic *B. kururiensis* (Hallack *et al.*, 2010). Fig. 1 shows the mucoid phenotype presented by colonies of different *Burkholderia* species grown in yeast extract-mannitol (YEM) medium. Mucoidy is due to the over-production of cepacian. *B. cenocepacia* J2315 is unable to produce cepacian, thereby displaying nonmucoid colonies when growing under the same conditions (Fig. 1).
The primary structure of the exopolysaccharide cepacian produced by the clinical isolate *Burkholderia cepacia* IST408 has been determined. Cepacian is an acetylated acidic polysaccharide with a branched heptasaccharide repeat-unit composed of D-glucose, D-rhamnose, D-mannose, D-galactose and D-glucuronic acid in the molar ratio of 1:1:1:3:1 (Table 1) (Cescutti *et al.*, 2000; Richau *et al.*, 2000a; Sist *et al.*, 2003). The amount of acetyl groups per repeat-unit seems to be strain dependent, varying from 2 to 4 groups. The location of the *O*-acetyl groups was recently described in the polymeric structure produced by *B. kururiensis* using a Smith degradation analysis. The results indicated that some

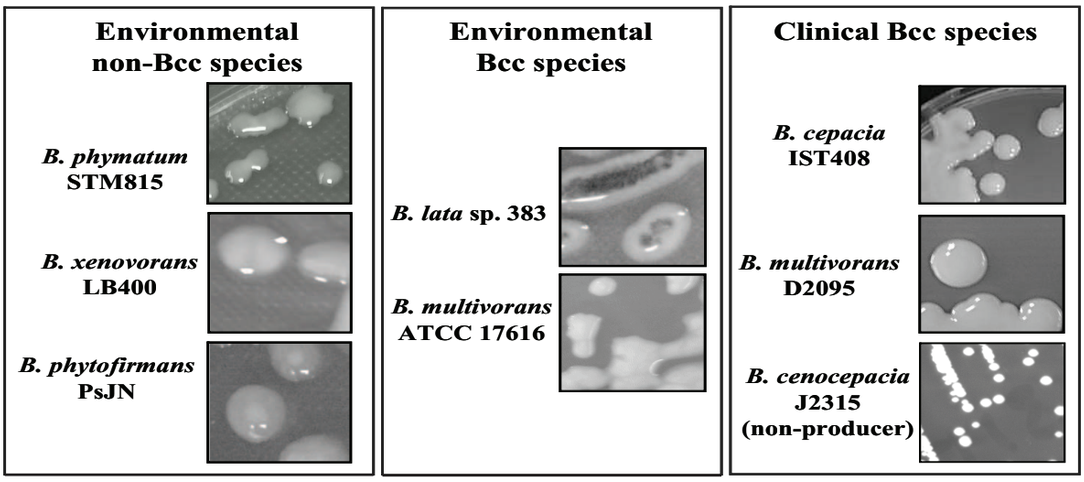

Fig. 1. EPS production by different *Burkholderia* strains gives the colonies a mucoid phenotype when growing in EPS-promoting medium. Contrastingly, the clinical isolate *B. cenocepacia* J2315 is unable to produce cepacian and is therefore nonmucoid.

galactosyl units were substituted by the acetyl group at *O*-4 and the other *O*-acetylation site might be the *O*-4 position of the rhamnose residue (Hallack *et al.*, 2010).

Besides cepacian, other exopolysaccharides have been identified and characterized from the structural point of view. For instance, several *B. pseudomallei* clinical strains were shown to produce a linear tetrasaccharide repeat-unit consisting of three galactose residues, one bearing a 2-linked *O*-acetyl group, and a 3-deoxy-D-*manno*-2-octulosonic acid residue (Table 1) (Nimtz *et al.*, 1997). This polymer was also identified in a *B. cepacia* clinical isolate, which also produce levan, an exopolysaccharide composed of D-frutose monomers (Table 1) (Nogueira *et al.*, 2005). Cérantola and co-authors identified a third EPS structure produced by a *B. cepacia* clinical isolate and named EPS-I. As presented in Table 1, such EPS repeat-unit is composed of D-galactose and D-glucose with carboxyethylidene as substituent (Cerantola *et al.*, 1996). Furthermore, *B. kururiensis*, which is an endophytic diazotrophic bacterium, was shown to produce large amounts of two acidic EPSs that were designated EPS A and EPS B being, respectively, a pentasaccharide composed of D-glucose, L-rhamnose and D-glucuronic acid in the proportion 2:2:1 (Table 1) and a mixture of two polymers composed of a hepta- and octasaccharide repeat-unit, that differ only by the presence of a terminal glycosyl residue (Table 1). The heptasaccharide repeat-unit was identified as being cepacian and the octasaccharide repeat-unit as being EPS B (Hallack *et al.*, 2010; Mattos *et al.*, 2001). Interestingly, the authors also showed that the production of EPS A and EPS B by *B. kururiensis* could be modulated under different growth conditions. This was proposed to play an important role in the endophytic-host plant association, as has been described in the case of *Sinorhizobium meliloti* (Hallack *et al.*, 2010). The variety of EPSs being produced, the ability to control their production according to nutrient availability and the production of a common EPS among the *Burkholderia* genus, seem to indicate an important function of these polymeric structures in multiple environments colonized by these bacteria. As will be discussed, several authors have hypothesized the importance of *Burkholderia* EPS in the establishment of symbiotic and pathogenic interactions. Thereby several studies have been conducted to understand EPS biosynthesis and its role in *Burkholderia* life style. Since cepacian is the most common EPS among the *Burkholderia* genus, it will be further described within this chapter.

Polymer	Repeat-unit structure	Producing strain/reference
Cepacian or EPS-II	1-α-D-Gal*p* (↓2); 1-β-D-Gal*p* (↓6) →3)-β-D-Glc*p*-(1→3)-α-D-Glc*p*A-(1→3)-α-D-Man*p*-(1→ 4 (↑) 1-α-D-Rha*p*2←1-β-D-Gal*p*	Many Bcc and non-Bcc (Ferreira *et al.*, 2010)
EPS B	1-α-D-Gal*p* (↓2); 1-β-D-Gal*p* (↓6) →3)-α-D-Glc*p*A-(1→3)-α-D-Man*p*-(1→3)-β-D-Glc*p*-(1→ 4 (↑) 1-α-D-Rha*p*2←1-β-D-Gal*p* 3 (↑) 1-β-D-Glc*p*(+/-)	*B. kururiensis* (Hallack *et al.*, 2010)
Levan	→6)-β-D-Fru*f*-(2→	*B. cepacia* (Nogueira *et al.*, 2005)
EPS-I	→3)-β-D-Glc*p*-(1→3)-[4,6-*O*-(1-carboxyethylidene)]-α-D-Gal*p*-(1→	*B. cepacia* (Cerantola *et al.*, 1996)
EPS A	→4)-α-D-Glc*p*-(1→2)-α-L-Rha*p*-(1→4)-α-D-Glc*p*A-(1→3)-β-L-Rha*p*[2*O*Ac]-(1→4)-β-D-Glc*p*-(1→	*B. kururiensis* (Mattos *et al.*, 2001)
	→5)-β-D-Kdo*p*-(2→3)-β-D-Gal*p*2Ac-(1→4)-α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→	*B. pseudomallei* (Nimtz *et al.*, 1997)

Abbreviations: Gal – galactose; Glc – glucose; GlcA – glucuronic acid; Man – mannose; Rha – rhamnose; Fru – fructose; Kdo - 3-deoxy-D-manno-2-octulosonic acid.

Table 1. Exopolysaccharides identified in *Burkholderia*. The table shows the structural formula of the different EPSs repeat-units found in the literature.

4. Cepacian biosynthesis and regulation

The first studies on cepacian biosynthesis were reported by Richau and co-authors in the year 2000 (Richau *et al*., 2000b). By that time, the chemical structure of cepacian's repeat-unit was determined and this information was used to postulate a pathway leading to the biosynthesis of the activated sugar precursors UDP-D-glucose, UDP-D-galactose, UDP-D-glucuronic acid, GDP-D-mannose and GDP-D-rhamnose. Still, at the time, nothing was

known about the genes encoding the enzymes involved in activated sugar precursor biosynthesis or the ones involved in the latter steps. This was clarified by the identification of the *bce* gene cluster (*Burkholderia cepacia* exopolysaccharide) encoding several enzymes involved in cepacian biosynthesis (Ferreira *et al.*, 2010; Moreira *et al.*, 2003).

4.1 Genes involved in cepacian biosynthesis

The first cluster of genes involved in cepacian biosynthesis was described in the year 2003. The cluster was identified by using random plasposon insertion mutagenesis to screen mutants unable to produce EPS on the clinical strain *B. cepacia* IST408 (Moreira *et al.*, 2003). Three of the obtained mutants had the transposon inserted into the same genetic location. This region located in chromosome 2, is composed of 11 genes designated *bceA* through *bceK* (Fig. 2a). Homology studies indicated that the cluster encoded two proteins, BceA and BceC, involved in the formation of activated nucleotide sugars; five glycosyltransferases, BceB, BceG, BceH, BceJ and BceK; and four proteins putatively involved in the polymerization and export of cepacian, that include BceD and BceF hypothesized to be involved in the post-translational control of exopolysaccharide biosynthesis; BceE, encoding a putative outer membrane auxiliary protein; and BceI, a putative polysaccharide polymerase protein (Fig. 2a) (Moreira *et al.*, 2003). Despite the identification of these gene products, several enzymes required for cepacian biosynthesis were still missing. Further insight came after full genome sequences of several *Burkholderia* strains from different species became available on-line. Comparative genomic studies showed that gene *bceA* through *bceK* (now named *bce-I* cluster) was present in all Bcc and non-Bcc sequenced strains with the exception of *B. mallei*. Furthermore, the bioinformatic approach used, showed that the non-Bcc species *B. xenovorans* LB400, *B. phymatum* STM815, *B. phytofirmans* PsJN and *B. graminis* C4D1M contained 8 additional genes encoding proteins putatively involved in polysaccharide biosynthesis, located immediately downstream the *bceK* gene. The search for a homologous region in the genome of other sequenced *Burkholderia* species showed that it was present in all Bcc and in the remaining non-Bcc strains, approximately 155 to 314 kb downstream of the *bce-I* cluster depending on the strain (Fig. 2a) (Ferreira *et al.*, 2010). This second genomic region was named gene cluster *bce*-II and the genes present were denominated *bceM* through *bceU*. *In silico* analysis of *bce*-II genes indicated that they encode proteins putatively involved in nucleotide sugar biosynthesis (*bceM*, *bceN* and *bceT*), repeat-unit formation and translocation through the inner membrane (*bceR* and *bceQ* respectively), acyltransferases (*bceO*, *bceS* and *bceU*) and a gene of unknown function (*bceP*) (Ferreira *et al.*, 2010). These genes are present in all Bcc and non-Bcc species, with the exception of *bceM* and *bceU* genes, that are absent from *Burkholderia* species with *bce* genes clustered together - *B. xenovorans* LB400, *B. phymatum* STM815, *B. phytofirmans* PsJN and *B. graminis* C4D1M.

Clusters *bce-I* and *bce-II* account for most of the genes needed for cepacian biosynthesis. Still, some of the genes required for the formation of the nucleotide sugar precursors are absent. These genes encode phosphoglucomutase (PGM), phosphoglucose isomerase (PGI), phosphomannomutase (PMM) and UDP-D-glucose epimerase enzymes. However, since these enzymes are also involved in other metabolic processes, their encoding genes can be located outside the genomic regions involved in cepacian biosynthesis. Indeed, search for genes encoding these four proteins in *Burkholderia cenocepacia* J2315 genome sequence revealed that they are present with more than one copy and distributed in different locations of chromosomes 1 and 2.

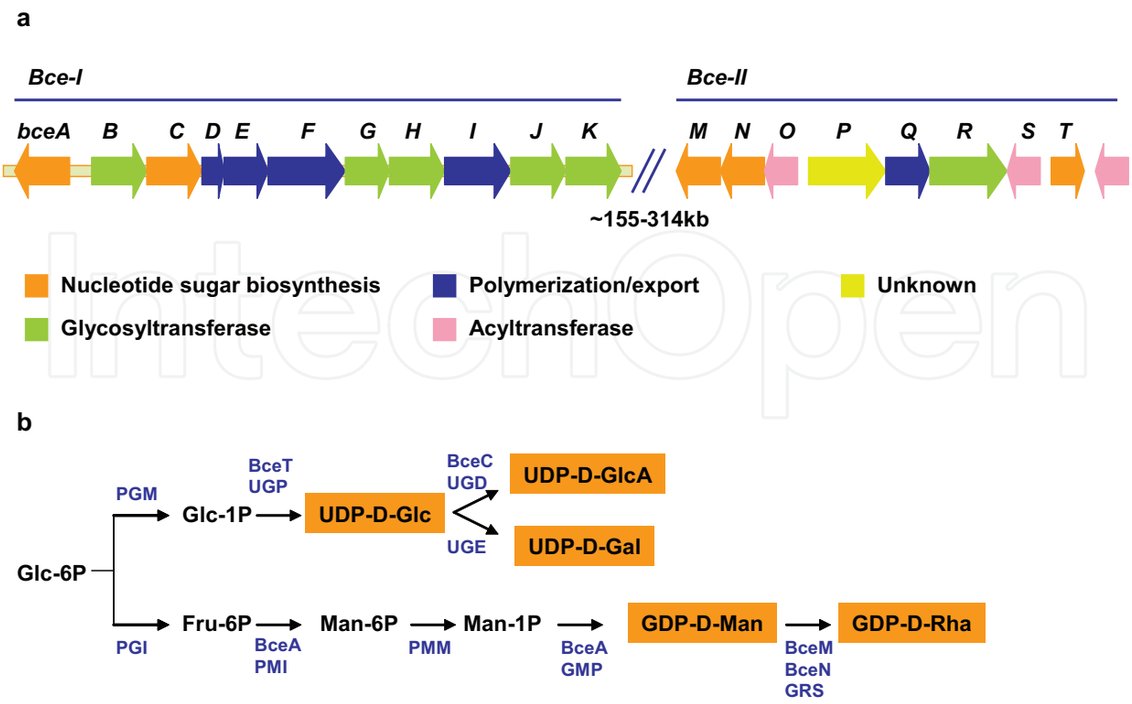

Fig. 2. (a) Genetic organization of the *bce* genes directing cepacian biosynthesis in *Burkholderia*. In representative strains of the species *B. xenovorans*, *B. phymatum*, *B. phytofirmans* and *B. graminis* the *bce* genes are clustered together (not shown), while in representative strains from Bcc, *B. pseudomallei*, *B. oklahomensis* and *B. thailandensis*, the *bce* genes are clustered into two regions (*bce-I* and *bce-II*) approximately 155-314 kb apart. (b) Pathway leading to the nucleotide-sugar precursors, required for cepacian biosynthesis. Abbreviations: Glc, glucose; GlcA, glucuronic acid; Gal, galactose; Rha, rhamnose; Man, mannose; GDP, guanosine-5´-diphosphate; UDP, uridine-5´-diphosphate; PGM, phosphoglucomutase; UGP, UDP-glucose pyrophosphorylase; UGD, UDP-glucose dehydrogenase; UGE, UDP-glucose epimerase; PGI, phosphoglucose isomerase; PMI, phosphomannose isomerase; PMM, phosphomannomutase; GMP, GDP-D-mannose pyrophosphorylase; GRS, GDP-rhamnose synthase (adapted from Ferreira *et al.*, 2010).

4.2 Biosynthetic pathway

The first step of cepacian biosynthesis is the formation of the activated nucleotide sugar precursors. Several enzymes are involved in this process. Their activities were first proposed by Richau and co-authors, that also showed the activities of the biosynthetic enzymes in cell extracts (Richau *et al.*, 2000b). Two Bce proteins involved in this step have been characterized. One is BceA, a bifunctional protein with phosphomannose isomerase and GDP-D-mannose pyrophosphorylase activities required for GDP-D-mannose biosynthesis (Fig. 2b) (Sousa *et al.*, 2007a; Sousa *et al.*, 2008). The other is BceC, a UDP-glucose dehydrogenase involved in UDP-glucuronic acid biosynthesis (Fig. 2b) (Loutet *et al.*, 2009). *Burkholderia* mutants unable to synthesize BceA or BceC have been constructed, but the strains still produce approximately the same EPS levels, suggesting that other functional homologues present in the genome may compensate for those mutations (Sousa *et al.*, 2007a). The synthesis of UDP-D-glucose is probably mediated by BceT, a putative UDP-

glucose pyrophosphorylase. The nucleotide sugar precursor, GDP-D-rhamnose, is putatively synthesized by the concerted action of BceM and BceN. BceN is predicted to be a GDP-mannose-4,6-dehydratase (GMD), catalyzing the dehydration of GDP-D-mannose to the unstable intermediate GDP-6-deoxy-D-*lyxo*-4-hexulose, which is further converted to the final GDP-D-rhamnose by GDP-6-deoxy-D-*lyxo*-4-hexulose reductase (RMD), predicted to be encoded by *bceM*. The synthesis of UDP-D-galactose from UDP-D-glucose is catalyzed by a UDP-glucose epimerase enzyme, putatively encoded by gene Bmul_2501 in *Burkholderia multivorans* ATCC 17616.

The second step of cepacian biosynthesis is the assembly of the heptasaccharide repeat-unit, which requires the presence of an undecaprenyl phosphate glycosyl-phosphate transferase (UndPGPT) catalyzing the transfer of the first sugar to the lipid carrier, and six other glycosyltransferases adding the remaining sugars in a specific order. The priming glycosyltransferase is BceB, catalyzing the addition of the first sugar, glucose, to the lipid carrier (Fig. 3) (Videira *et al.*, 2005). The sequential addition of the six remaining sugars must be performed by the deduced BceG, H, J, K and R glycosyltransferases, but none is yet characterized and the specific activity of each enzyme cannot be predicted from their amino acid sequences. An insertion mutant on *bceR* gene confirmed that this protein with two glycosyltransferase domains is essential for cepacian biosynthesis (Ferreira *et al.*, 2010).

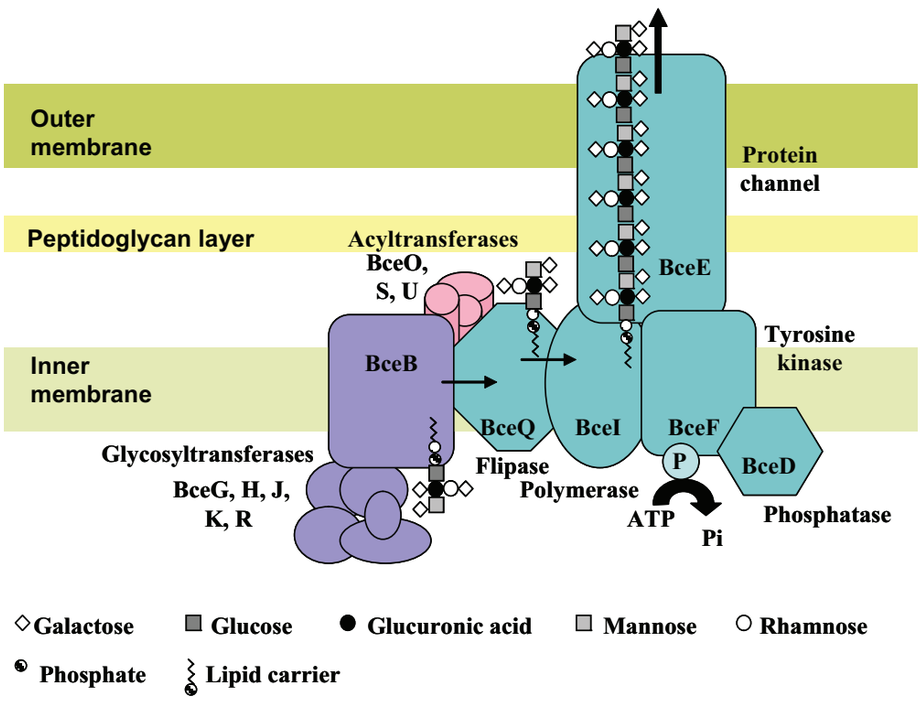

Fig. 3. Model for assembly and export of cepacian. Activated sugar nucleotides are sequentially added to a lipid carrier (undecaprenyl pyrophosphate (und-PP)) to form cepacian repeat-units by sequential activity of BceBGHJKR glycosyltransferases. These synthesized und-PP-linked repeat-units are then translocated across the inner membrane in a process requiring BceQ and polymerized by BceI. The BY-kinase BceF and the phosphotyrosine phosphatase BceD proteins are central in regulation of the EPS biosynthetic complex, possibly connecting polymerization and export, and regulating polysaccharide chain length. Cepacian is exported by BceE, which may act as a channel. (Draws are not at scale).

By using mass spectrometric analysis, Cescutti and collaborators detected ions belonging to the tri-, tetra-, penta-, and hexasaccharide biosynthetic intermediates of the cepacian repeat-unit and proposed the sequence order for sugar addition (Cescutti *et al.*, 2010). According to these authors, to the trisaccharide composed of glucose and mannose substituted with galactose, would follow the addition of glucuronic acid, then galactose as the first substituent of glucuronic acid and a rhamnose followed by galactose, as a second substituent of glucuronic acid (Cescutti *et al.*, 2010). Non-saccharide substitutions occur during the repeat-unit assembly process, such as acetylation. The number of acetyl groups per repeat-unit seems to be strain dependent (Cescutti *et al.*, 2000; Cescutti *et al.*, 2010). Three putative acyltransferases were found to be encoded within the *bce-II* cluster. These are BceS, BceT and BceU (Fig. 3). Their orthologues are membrane proteins, involved in the acylation of carbohydrate moieties of extracytoplasmic molecules. Disruption of *bceS* gene from *B. multivorans* ATCC 17616 was shown to cause a decrease in cepacian acetylation content, suggesting that it may be involved in repeat-unit acetylation (Ferreira *et al.*, 2010). Hydrophobicity analysis of the putative amino acid sequences of BceO, BceS and BceU suggests that they are probably located in the inner membrane with eight to nine transmembrane domains. The fact that they might be present in the inner membrane is consistent with their possible role in cepacian acetylation since the repeat-unit is synthesized on the cytoplasmic face of the inner membrane attached to an undecaprenol diphosphate lipid carrier. It is then possible that the acetylation reaction takes place prior to the translocation of the repeat-unit, linked to undecaprenol diphosphate, to the periplasmic face of the cytoplasmic membrane, although no evidence is yet available.

After assembly, the oligosaccharide repeat-unit coupled to the lipid carrier should be transferred across the cytoplasmic membrane to the periplasmic face of the membrane, where the polymerization is carried out by a polysaccharide polymerase. Data available points out 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 inner membrane by the putative polymerase BceI (Fig. 3). BceQ and BceI are predicted integral membrane proteins and their involvement in cepacian biosynthesis was demonstrated by the EPS deficient phenotype of the *B. cepacia* IST408 insertion mutants for *bceI* and *bceQ* genes (Ferreira *et al.*, 2010; Moreira *et al.*, 2003). BceD, BceF and BceE proteins are also involved in the polymerization and export process (Fig. 3). The putative BceE protein is a Wza homologue and therefore is predicted to be an outer membrane auxiliary protein acting as a channel. The activities of BceF as a bacterial tyrosine autokinase and BceD as phosphotyrosine phosphatase protein have been determined and the two proteins are believed to be involved in the regulation of cepacian production and perhaps in the control of the polymer molecular weight (Ferreira *et al.*, 2007).

4.3 Regulation of cepacian biosynthesis

Several studies indicate that bacteria colonizing cystic fibrosis lungs undergo through a process of adaptation which involves several phenotypic changes, including EPS production ability. For example, in *Pseudomonas aeruginosa*, it was observed that bacteria initially not producing alginate, become mucoid during the course of lung infection. That phenomenon was shown to be tightly regulated at the transcriptional level by several proteins, such as the transcriptional sigma factor AlgU, which controls the expression of alginate biosynthetic operon; MucA that is an anti-sigma factor and regulates AlgU; or AlgR, a response regulator

responsible for of expression *algC* gene encoding a protein involved in the formation of the activated sugar precursors; among many others regulators that have been described (reviewed in Ramsey & Wozniak, 2005). Recent studies indicate that phenotypic changes involving exopolysaccharide production are also frequent in *Burkholderia* clinical isolates (Zlosnik *et al.*, 2008). Furthermore, the importance of EPS biosynthesis regulation has also been suggested in the establishment of interactions between *Burkholderia* and host plants (Hallack *et al.*, 2010). Still, the genes directly involved in the regulation of cepacian biosynthesis have not been identified. Nevertheless, there are evidences of the involvement of quorum sensing at the transcriptional level and tyrosine phosphorylation/dephosphorylation at the post-translational level.

4.3.1 Regulation at transcriptional level

Quorum sensing (QS) consists of a chemical communication process that enables cells to modulate gene expression in neighboring cells, by synthesizing and detecting QS molecules. Several QS molecules have been identified, but the most studied are N-Acyl Homoserine Lactones (AHL). AHLs are synthesized by synthase proteins and are detected by transcriptional regulators, which regulate the expression of many different target genes. The presence of well conserved QS systems have been described within Bcc species, where the system is composed of the synthase CepI and the regulator CepR (Lewenza *et al.*, 1999); and in plant associated *Burkholderia*, which have a system based on the synthase BraI and the regulator BraR (Lewenza *et al.*, 1999; Suarez-Moreno *et al.*, 2008). These two conserved systems are not homologous and were found to be based on different AHL molecules: CepI/R system secretes and responds to C6-HSL and C8-HSL; and the BraI/R system to 3-oxo-C12-HSL and 3-oxo-C14-HSL depending in the strain (Lewenza *et al.*, 1999; Suarez-Moreno *et al.*, 2008). Recent studies on the plant associated bacteria *B. kururiensis*, *B. xenovorans* and *B. unamae*, indicate that BraI/R system is involved in the regulation of EPS biosynthesis. The mutants on *braI* and *braR* genes were shown to produce reduced amount of EPS, but the mucoid phenotype of colonies was re-gained by the addition of 3-oxo-C14-HSL to the *braI* mutant and by introducing the regulator gene *in trans* into the *braR* mutant. Although these results showed that EPS production is positively controlled by quorum sensing in plant associated *Burkholderia* (Suarez-Moreno *et al.*, 2010), the targets of such regulation are still unknown and the question whether quorum sensing is able to regulate EPS production in all *Burkholderia* species still needs confirmation.

Another *Burkholderia* gene that has been recently associated to cepacian production is *hfq* encoding a RNA chaperone involved in the riboregulation of target mRNAs by small regulatory non-coding RNAs (Sousa *et al.*, 2010). *B. cepacia* IST408 *hfq* mutant showed a strong decrease in cepacian production over time. The meaning of such findings is not clarified and no small RNA involved in controlling the expression of EPS-related genes is known (Sousa *et al.*, 2010).

4.3.2 Regulation at post-translational level

Protein phosphorylation is an important and widespread post-translational covalent modification in both eukaryotes and prokaryotes, being responsible for the regulation of many cellular functions. It consists in a reversible modification in which phosphoryl groups are added by protein kinases and removed by phosphoprotein phosphatases. In particular, the control of tyrosine phosphorylation and dephosphorylation mediated by bacterial-

tyrosine kinases (BY-kinases) and phosphotyrosine phosphatases (PTP) has an important role in EPS biosynthesis by bacteria. Although their precise role is not yet known, several studies indicate their involvement in the regulation of polysaccharide amount and chain length (Bugert & Geider, 1997; Minic *et al.*, 2007; Vincent *et al.*, 2000; Wugeditsch *et al.*, 2001). Genes encoding BY-kinase and PTP proteins are well conserved within polysaccharide gene clusters and are also present on *bce* gene cluster, being encoded by *bceF* and *bceD* genes, respectively (Moreira *et al.*, 2003).

Like other members of the BY-kinase family, BceF possesses two transmembrane domains, a periplasmic domain and an intracellular catalytic domain. BceF catalytic domain contains a well conserved Walker A and Walker B ATP-binding motifs, and a tyrosine-rich cluster at C-terminal end. The importance of Walker A motif for the autophosphorylation process was demonstrated by site-directed mutagenesis on a well conserved lysine residue followed by immunoblotting assays, indicating that replacement of the conserved lysine to an alanine causes loss of BceF autophosphorylation activity (Ferreira *et al.*, 2007). Phosphotyrosine phosphatases, such as BceD, promote autokinases dephosphorylation, regulating their biological function. The PTP protein encoded by *bceD* gene was demonstrated to be a phosphatase by its ability to cleave the artificial *p*-nitrophenol phosphate (PNPP) substrate. The dephosphorylation of BceF by BceD was demonstrated *in vitro* by immunoblotting, using an antibody against the presence of phosphotyrosine residues (Ferreira *et al.*, 2007). Disruption of *bceF* gene abolished cepacian production, but *bceD* mutant was still able to accomplish 75% of the production. Still, the EPS produced by *bceD* mutant showed a lower viscosity in solution than the one recovered from the parental strain, suggesting that the EPS produced might have a lower molecular weight.

The role of tyrosine phosphorylation and dephosphorylation of BY-kinase proteins in polysaccharide biosynthesis is not yet established but, some hypotheses have been raised. Structural studies on *E. coli* Wza protein, a BceE homologue, showed that the protein forms complex structures with the cognate BY-kinase protein Wzc, which are important for capsular polysaccharide translocation to the cell exterior (Collins *et al.*, 2006). According to this, it was proposed that the phosphorylated state of the BY-kinase protein could regulate the export of polysaccharides by introducing conformation changes to the Wza-Wzc complex, which could control the EPS export. Even though attractive, this hypothesis needs further investigation. Another hypothesis is that BY-kinases could also be involved in the regulation of early steps of the EPS production. It is known that BY-kinases can phosphorylate heterologous substrates, including enzymes involved in the biosynthesis of nucleotide sugar precursors. For instance the *E. coli* UDP-glucose dehydrogenase (UGD), involved in colanic acid biosynthesis, is phosphorylated by Wzc and UGD tyrosine phosphorylation state influences the amount of colanic acid formed by *E. coli*. Therefore, it was proposed that tyrosine phosphorylation and dephosphorylation can act at two different levels: the polymerization/export of the polysaccharide and on the synthesis of the repeat-unit (Lacour *et al.*, 2008). Concerning cepacian biosynthesis, no studies have been done on the role of tyrosine phosphorylation within the EPS biosynthetic complex.

5. Role of cepacian in *Burkholderia* adaptation to different environments

Extracellular polysaccharides produced by bacteria have been described as essential virulence determinants in pathogens of humans, livestock and plants; as important in symbiotic interactions between bacteria and plants, and as a barrier to harmful compounds.

Depending on the ecological niche of bacteria, exopolysaccharides play important roles on the adaptation to environmental conditions and endow bacteria with tools for the colonization of adverse environments. In Bcc bacteria, the production of EPS has been pointed out as a virulence factor, contributing to the overall pathogenicity of those bacteria.

5.1 Cepacian as a virulence factor

The importance of the EPS produced by Bcc bacteria as a virulence factor in human lung infections has been studied *in vitro* and *in vivo* models of infection. For instance, *B. cepacia* mutants unable to produce cepacian were less virulent than EPS producing wild-type strain in gp91$^{phox-/-}$mice (Sousa *et al.*, 2007b). Also, the EPS produced by a mucoid *B. cenocepacia* clinical isolate was shown to interfere with phagocytosis of bacteria by human neutrophils *in vitro* when compared to a nonmucoid clonal isolate from the same CF patient (Conway *et al.*, 2004). Additionally, the overproduction of EPS was demonstrated to facilitate the bacterial persistence in a mouse model of infection and it was suggested that the EPS masks bacterial surface structures, normally recognized by neutrophils and macrophages, and thus interfering with recognition and clearance in mice (Conway *et al.*, 2004). Cepacian was shown to inhibit chemotactic migration of human neutrophils and to scavenge reactive oxygen species (ROS) produced by activated cells (Bylund *et al.*, 2006). Since EPS can neutralize ROS and render neutrophils unable to kill Bcc bacteria by oxidative means, the antimicrobial action of those cells will be entirely dependent on the non-oxidative cationic antimicrobial peptides. However, Bcc bacteria are also resistant to non-oxidative antimicrobial peptides and this ability was attributed to the presence of EPS (Herasimenka *et al.*, 2005). In fact, due to the negative charge given by acetyl substituents, cepacian was shown to interact with positively charged antimicrobial peptides forming complexes that lower the antimicrobial peptides biological activity (Herasimenka *et al.*, 2005). If EPS produced by Bcc can both neutralize oxidative species and interact with non-oxidative antimicrobial peptides, then it might be an important virulence factor, since it would leave neutrophils without means to clear the offending bacteria. Against the importance of EPS in virulence is the study of Bartholdson and colleagues where Bcc clinical and environmental strains were assessed for EPS production and onion tissue maceration ability (Bartholdson *et al.*, 2008). According to their results no correlation could be established between EPS production and onion tissue maceration. In another study, a survey of the clinical outcome in patients colonized with EPS and non-EPS producing *Burkholderia* isolates, concluded that no correlation could be established between EPS production ability and the persistence or virulence of bacteria in the lungs (Cunha *et al.*, 2004). Despite the evidence that EPS interacts with the host immune system, the precise role of EPS production in *Burkholderia* infection process remains an open question.

5.1.1 Mucoid to nonmucoid phenotype switch

Phenotypic variation during the course of an infection is a common phenomenon in pathogenic bacteria. In *P. aeruginosa*, the conversion to a mucoid phenotype with the bacteria overproducing alginate in advanced stages of the disease is well described. The initial lung colonizer is usually a non-producer strain, which eventually converts to the mucoid phenotypic over the infection period. The overproduction of EPS is also followed by other phenotypic changes, such as decreased motility and lower toxin production, being associated to the establishment of chronic infections (reviewed in Ramsey & Wozniak, 2005).

In the case of Bcc infections, it was reported the existence of such phenotypic conversion in CF clinical isolates. Zlosnik and co-authors analyzed EPS production by *Burkholderia* sequential isolates from hundred CF patients and observed thirteen mucoid to nonmucoid conversions and only two nonmucoid to mucoid conversions (Zlosnik *et al.*, 2008). The nonmucoid to mucoid conversions occurred in *B. cenocepacia* and *B. vietnamiensis* isolates and the mucoid to nonmucoid conversions occurred in isolates from *B. multivorans*, *B. cenocepacia* and *B. vietnamiensis* species. The high frequency of mucoid phenotype transition raises the possibility that while mucoid phenotype may be associated to persistence in CF lung, nonmucoid isolates may be associated to increased disease severity. It is possible that EPS may be required for colonization and persistence in the CF lung during the early stages of infection, but once bacteria have colonized the CF lung, the EPS production is no longer required and its production can be shutdown, since it represents a great spending of energy to the cell. The nonmucoid bacteria can then present a competitive advantage in the lung. Interestingly, the Bcc species considered the most virulent and responsible for infection outbreaks, *B. cenocepacia* has many strains unable to produce cepacian. The nonmucoid phenotype was proposed to be associated with an increase of Bcc virulence, which can be consistent with the development of cepacia syndrome, characterized by the development of septicemia. Still, no conclusive studies are available and the importance of such transitions in Bcc infections is not understood. Also, the molecular mechanisms by which Bcc undergo mucoid phenotype switch still need to be investigated as well as the environmental factors triggering the mucoid phenotype conversion.

5.1.2 Biofilm formation

The ability of opportunistic pathogens to colonized lung tissues, forming microcolonies that eventually will evolve into mature biofilm structures has been suggested by many authors. Bcc ability to form microcolonies and mature biofilms has been shown on abiotic surfaces and on well-differentiated human epithelial cells (Schwab *et al.*, 2002). Studies on clinical isolates from four cystic fibrosis patients indicated that the size of the biofilms formed by the isolates increases over the infection period. This suggests an *in vivo* microevolution that selects *Burkholderia* populations with higher ability to survive in the CF lung context, which includes survival under antibiotic stress (Savoia & Zucca, 2007). The EPS produced by Bcc bacteria seems to play an important role in the establishment of thick and mature biofilms, even though *in vitro* assays indicate that is not essential for the initial steps of the biofilm formation (Cunha *et al.*, 2004). Since bacteria within biofilms can more efficiently withstand host immune responses and antibiotic action than plancktonic cells, it was hypothesized that by promoting the formation of mature biofilms, the EPS may enhance bacterial survival in the CF lung during the course of infection. The importance of cepacian in the formation of mature biofilms has also been confirmed by mutants constructed on *bce* genes. Mutants unable to produce cepacian, such as *bceF* and *bceE* insertion mutants, and mutants that were shown to produce cepacian with lower molecular weight or having a lower amount of acetyl groups per repeat-unit, such as *bceD* and *bceS* insertion mutants, respectively, exhibited a thinner biofilm when compared to the one produced by the corresponding parental strains (Ferreira *et al.*, 2007; Ferreira *et al.*, 2010).

5.2 Protection against environmental stress

Protective functions against desiccation and toxic metal ions concentration have also been attributed to EPS and these roles are of major importance concerning the adaptation of

bacteria to different environments. Given its hygroscopic properties, EPS may decrease the rate of water loss from the cells and provide bacterial cells with means to survive drying and desiccation (Potts, 1994). In *Burkholderia*, EPS was suggested to play a role in the survival of bacteria by contributing to their ability to withstand desiccation. The external addition of EPS to *B. xenovorans* LB400 and *B. multivorans* ATCC 17616 isolates prior desiccation was shown to enhance their survival (Ferreira *et al.*, 2010). Other studies in cyanobacteria *Nostoc commune* (Tamaru *et al.*, 2005) and *Chroococcidiopsis* sp. CCMEE 5056 also correlated the amount of EPS produced to the drying and freezing tolerance of bacteria (Knowles & Castenholz, 2008). Ferreira and co-authors also demonstrated a protective effect of cepacian on *Burkholderia* isolates exposed to metal ion stress (Ferreira *et al.*, 2010). The protection conferred by EPS to toxic concentrations of metal ions was also demonstrated in other bacteria as it is the case of *Klebsiella oxytoca* (Baldi *et al.*, 2009). The metal-binding properties of EPS might be due to the occurrence of carbonyl, carboxyl and hydroxyl groups within the EPS matrix that attach cations and scavenge metals (Potts, 1994). The ability of *Burkholderia* strains to withstand desiccation and metal ion stress in the presence of the exopolysaccharide cepacian suggest that this EPS plays a role in survival of these bacteria, thus representing an advantage for bacteria to thrive in adverse environments.

6. Conclusion

Cepacian biosynthesis ability is a common feature among the *Burkholderia* genus. Although some knowledge about the genes and enzymes involved in its biosynthesis already exists, the regulatory mechanisms underlying its production are almost unknown. Quorum sensing seems to be important, but the transcriptional regulators mediating *bce* gene expression are not yet identified. That knowledge would be crucial to understand mucoid to nonmucoid transitions, a phenomenon that occurs with frequency in chronically infected lungs of CF patients. Moreover, a better understanding of the environmental factors that trigger this phenotypic conversion would also be relevant in order to control this process, with expected health benefits for CF patients colonized with *Burkholderia*.

7. Acknowledgments

This work was supported by FEDER and Fundação para a Ciência e a Tecnologia, Portugal (contract PTDC/BIA-MIC/66977/2006), Ph.D. grant to I.N.S., and a Post-doc grant to A.S.F..

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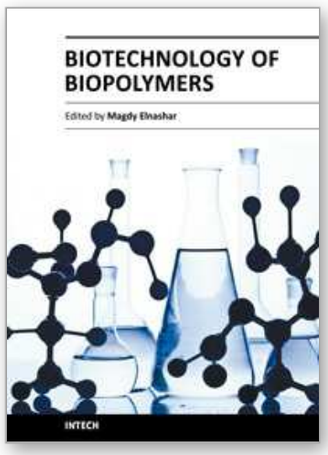

Biotechnology of Biopolymers

Edited by Prof. Magdy Elnashar

ISBN 978-953-307-179-4

Hard cover, 364 pages

Publisher InTech

Published online 24, June, 2011

Published in print edition June, 2011

The book "Biotechnology of Biopolymers" comprises 17 chapters covering occurrence, synthesis, isolation and production, properties and applications, biodegradation and modification, the relevant analysis methods to reveal the structures and properties of biopolymers and a special section on the theoretical, experimental and mathematical models of biopolymers. This book will hopefully be supportive to many scientists, physicians, pharmaceutics, engineers and other experts in a wide variety of different disciplines, in academia and in industry. It may not only support research and development but may be also suitable for teaching. Publishing of this book was achieved by choosing authors of the individual chapters for their recognized expertise and for their excellent contributions to the various fields of research.

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