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Enzymes Leading to the Nucleotide Sugar Precursors for Exopolysaccharide Synthesis in *Burkholderia cepacia*

João A. Richau, Jorge H. Leitão, and Isabel Sá-Correia[1]
Centro de Engenharia Biológica e Quımica, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

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Based on the chemical composition of the exopolysaccharide produced by the cystic fibrosis bacterial isolate *Burkholderia cepacia* IST408, we postulated and confirmed, based on the specificity of enzymes detected in crude cell-free extracts, the pathway leading to the presumptive activated sugar precursors: UDP-D-glucose, UDP-D-galactose, UDP-D-glucuronic acid, GDP-D-mannose, and GDP-D-rhamnose. Results also suggest that regulation of the expression of the mucoid phenotype in *B. cepacia* does not occur at the level of synthesis of any of these enzymes. © 2000 Academic Press

***Key Words: Burkholderia cepacia;* exopolysaccharide; exopolysaccharide biosynthetic enzymes; cystic fibrosis.**

Burkholderia cepacia, originally described as a saprophyte that causes the soft rot in onions (1), has become an important multidrug resistant pathogen in patients with cystic fibrosis (CF) and a cause of nosocomial infection (2). Contrasting with the extensive information on the role of alginate in the colonization and persistence, in the CF lung, of *Pseudomonas aeruginosa*, the most frequent pathogen in respiratory infections of CF patients (3), there is no clear data about the possible contribution of the polysaccharide produced extracellularly by some *B. cepacia* isolates (4) in the development of pulmonary infections in these patients (2). Indeed, only very recently (4), evidences were obtained indicating that exopolysaccharide (EPS) production by *B. cepacia* CF isolates is not as rare as thought before (2). During this systematic study, carried out among the Portuguese CF population, we found that, under optimal conditions, approximately 70% of the *B. cepacia* isolates examined produce EPS (4), suggesting that *B. cepacia* EPS may play a role similar to alginate in CF infection by *P. aeruginosa*. The structural analysis of the EPS produced by one of the high mucoid CF isolates identified in that study, strain IST408, indicated that this is an acetylated acidic polysaccharide with a branched heptasaccharide repeating unit (Fig. 1) (5). This is composed of D-glucose (D-glu*p*), D-rhamnose (D-rha*p*), D-mannose (D-man*p*), D-galactose (D-gal*p*), and D-glucuronic acid (D-Glc*p*A), in the molar ratio 1.0:1.0:1.0:3.0:1.0 (5).

This structure is very similar, if not identical, to the structure of the EPS produced by a French clinical isolate, recently revealed by Cérantola *et al.* (6). Moreover, the sugar composition of the *B. cepacia* IST408 EPS is similar to the composition of the EPSs produced by other *B. cepacia* isolates from patients with CF in Portugal (5), in the United States (7), and in the United Kingdom (8), suggesting that this polysaccharide may be characteristic of *B. cepacia* species.

Based on the chemical composition of the EPS produced by *B. cepacia* IST408, we postulated the pathway leading to the presumptive activated sugar precursors that are the donors of monomers for the synthesis of the referred heptasaccharide unit: UDP-D-glucose, UDP-D-galactose, UDP-D-glucuronic acid, GDP-D-mannose, and GDP-D-rhamnose (Fig. 2). Consistent with the postulated pathway (Fig. 2), the specific activities of all the enzymes presumed to be involved in the biosynthesis of these activated precursors were detected in crude cell-free extracts prepared from cells of *B. cepacia* IST408. The role of these enzymes in EPS production during batch growth and in the expression of the mucoid phenotype was also examined.

METHODS

B. cepacia strains and media culture. The high EPS producing strain *Burkholderia cepacia* IST408, isolated from a Portuguese cystic fibrosis (CF) patient (4), and the non-mucoid variant, IST408N, spontaneously obtained during cultivation of IST408 on A medium plates, were used in this work. This variant was isolated based on the typical non-mucoid phenotype of the colonies formed on the surface of A medium agar plates [containing, in g l^{-1}, yeast extract (Difco) 2.0, glucose 10.0, agar 20.0] after 5 days of incubation. The strains were maintained at −70°C in 40% (w/v) glycerol and, when in use, they were cultivated on PIA (Pseudomonas Isolation Agar, Difco) plates.

Abbreviations used: CF, cystic fibrosis; EPS, exopolysaccharide.

[1] To whom correspondence should be addressed. Fax: 351-218480072. E-mail: pcisc@alfa.ist.utl.pt.

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Ribotyping of mucoid and non-mucoid B. cepacia variants. Isolation of total DNA, restriction with *Eco*RI (Gibco BRL), DNA blotting, and hybridization with the acetylaminofluorene-labeled 16S + 23S rRNA probe from *E. coli* (Eurogentec) were carried out according to standard procedures (9) and as described by Grimont *et al.* (10) and Richau *et al.* (4).

Growth and EPS production in liquid medium. Batch culture of *B. cepacia* variants was carried out in 100 ml of S liquid medium [containing, in g l^{-1}, Na_2HPO_4 12.5, KH_2PO_4 3.0, K_2SO_4 1.0, NaCl 1.0, $MgSO_4 \cdot 7H_2O$ 0.2, yeast extract (Difco) 1.0, casamino acids (Difco) 1.0, $CaCl_2 \cdot 2H_2O$ 0.01, $FeSO_4 \cdot 7H_2O$ 0.001, and glucose 20.0], contained in 250 ml shake flasks and incubated at 30°C with orbital agitation (250 rev min^{-1}). Growth was monitored by following the cultures OD at 640 nanometers (OD_{640}). The EPS was precipitated from cell-free supernatants of liquid cultures by the addition of 2.5 volumes of cold ethanol (96% v/v). EPS production was estimated by weighing the ethanol precipitates, dried overnight at 80°C. Results are means ± standard deviation of at least three independent determinations of the dry weight of EPS samples obtained from three independent cultures. The increase of broth viscosity, following EPS production, was measured at 30°C with a cone and plate Brookfield viscometer, model LVIIT at shear rate 24 s^{-1}. Results are means of three viscosity determinations, from at least three independent batch growth experiments.

Preparation of crude cell-free extracts. Cells from batch cultures of mucoid and non-mucoid *B. cepacia* variants were harvested by centrifugation at 10,000*g* for 10 min at 4°C. The cell pellets obtained were washed once with NaCl 0.9% (w/v), resuspended in sonication buffer (Tris 100 mM, DTT 1 mM, and PMFS 0.5 mM, pH 7.6), and immediately disrupted by four cycles of sonication at 4°C (70 Watt for 30 s each followed by 1 min holding the sample at 4°C) (Vibracell Sonics Material) or kept at −70°C until used. Cell free crude cell-free extracts were the supernatants obtained after centrifugation (20,000*g* for 1 h at 4°C) of the cell suspensions, in sonication buffer, disrupted by ultrasonic vibration.

Enzyme assays. The activities of *B. cepacia* EPS biosynthetic enzymes were assayed in crude cell-free extracts based on NAD or NADP reduction, or NADPH oxidation, at 30°C using coupled reaction systems and recording the increase or decrease in optical density at 340 nm in a Hitachi U-2000 double-beam spectrophotometer.

Enzyme activities were calculated from the initial linear rates of cofactor reduction or oxidation, after subtraction of endogenous activity (measured in enzyme assays lacking the substrate). Specific activities are median values of at least three enzyme assays in crude cell-free extracts prepared from three or four independent growth experiments. Protein concentration in crude cell-free extracts ranged from 5 to 7.5 mg l^{-1}, as estimated by the method of Bradford (11), with BSA fraction V (Merck) as the standard. All enzymes used in coupled reaction, obtained from Sigma, were added in excess relatively to the enzymatic activity under study. One unit of enzyme activity was defined as the amount of enzyme that reduced 1 μmol of NAD or NADP or oxidized 1 μmol of NADPH per minute under the assay conditions.

Enzymes used in coupled reactions, phosphorylated sugars, sugar nucleotides, NAD, NADP, NADPH, DTT, and PMFS were from Sigma.

Phosphoglucose isomerase (PGI) (EC 5.3.1.9). The assay for PGI was based on the method described by Slein (12). The reaction mixture contained, in a final volume of 1 ml, 30 mM Tris–HCl buffer pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 5 mM fructose-6-phosphate, 1 U of glucose-6-phosphate dehydrogenase, and 1 mM NADP. Enzyme reaction was initiated by the addition of 50 μl of crude extract.

Phosphoglucomutase (PGM) (EC 5.4.2.5). The PGM assay was based on the method described by Slein (12). The reaction mixture contained, in a final volume of 1 ml, 30 mM Tris buffer, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM glucose-1,6-diphosphate, 1 mM glucose-1-phosphate, 1 U glucose-6-phosphate dehydrogenase, and 1 mM NADP. Enzyme reaction was started by the addition of 50 μl of crude extract.

UDP-glucose pyrophosphorylase (UGP) (EC 2.7.7.9). This assay was based on the method described by Bernstein and Robbins (13). The reaction mixture contained in a final volume of 1 ml, 100 mM Tris–Cl buffer, pH 7.8, 10 mM $MgCl_2$, 2 mM of UDP-glucose, 1 U each of phosphoglucomutase (PMM) and glucose-6-phosphate dehydrogenase, and 1 mM NADP. Enzyme reaction was started by the addition of 100 μl of crude extract and 2 mM sodium pyrophosphate.

UDP-glucose dehydrogenase (UGD) (EC 1.1.1.22). This assay was based on the method described by Strominger *et al.* (14). The reaction mixture contained, in a final volume of 1 ml, 100 mM glycine-NaOH buffer, pH 8.7, 10 mM $MgCl_2$, 0.5 mM UDP-glucose, and 1 mM NAD. Enzyme reaction was initiated by the addition of 100 μl of crude extract.

GDP-rhamnose synthetase (GRS). This assay was based on the method described by Markowitz (15) for the overall reaction catalyzed by the GRS system. At least two enzyme activities are involved: the first, catalyzed by GDP-mannose dehydrogenase converting GDP-mannose to GDP-4-keto-D-rhamnose and the second catalyzed by GDP-4-keto-D-rhamnose reductase, converting GDP-4-keto-D-rhamnose in to GDP-D-rhamnose. The reaction mixture contained, in a final volume of 1 ml, 50 mM phosphate buffer pH 8.0, 2 mM $MgCl_2$, 0.25 mM cystein, 0.1 mM GDP-D-mannose, 0.1 mM NAD, and 0.12 mM NADPH. Enzyme reaction was initiated by the addition of 100 μl of crude extract.

UDP-glucose epimerase (UGE) (EC 5.1.3.2). This assay was based on the method described by Fukasawa *et al.* (16). The reaction mixture contained, in a final volume of 1 ml, 100 mM glycine-NaOH buffer, pH 8.7, 0.3 mM UDP-galactose, 0.8 mM NAD, and 0.1 U of glucose-6-phosphate dehydrogenase. Enzyme reaction was initiated by the addition of 100 μl of crude extract.

Phosphomannose isomerase (PMI) (EC 5.3.1.8). This assay was based on the method used by Sá-Correia *et al.* (17). The reaction mixture contained, in a total volume of 1 ml, 25 mM Tris–Cl buffer pH 7.6, 10 mM $MgCl_2$, 1 mM NADP, 3 mM mannose-6-phosphate and 1 U each of phosphoglucose isomerase and glucose-6-phosphate dehydrogenase. Enzyme reaction was started by the addition of 50 μl of crude extract.

Phosphomannomutase (PMM) (EC 5.4.2.8). This assay was based on the method used by Sá-Correia *et al.* (17). The reaction mixture contained, in a total of 1 ml, 20 mM Tris–Cl buffer pH 7.6, 10 mM $MgCl_2$, 1 mM NADP, 0.1 mM glucose-1,6-diphosphate, 1 mM mannose-1-phosphate, and 1 U each of phosphoglucose isomerase, glucose-6-phosphate dehydrogenase, and phosphomannose isomerase. Enzyme reaction was initiated by the addition of 100 μl of crude extract.

GDP-D-mannose pyrophosphorylase (GMP) (EC 2.7.7.13). This assay was based on the method used by Sá-Correia *et al.* (17). The reaction mixture contained, in a total volume of 1 ml, 20 mM Tris–Cl buffer pH 7.6, 1 mM NADP, 0.1 mM ADP, 4 mM glucose, 0.4 mM GDP-D-mannose, 10 mM $MgCl_2$, and 1 U each of hexokinase, nucleoside-5′-diphospho-kinase, and glucose-6-phosphate dehydrogenase. Enzyme reaction was initiated by the addition of 50 μl of crude extract and 2 mM sodium pyrophosphate.

RESULTS AND DISCUSSION

Enzymes Involved in the Formation of Sugar Nucleotides in Mucoid B. cepacia IST408

Based on the sugar composition of *B. cepacia* IST408 EPS and on the data available in the literature for other EPS producing microbial systems, we postulated

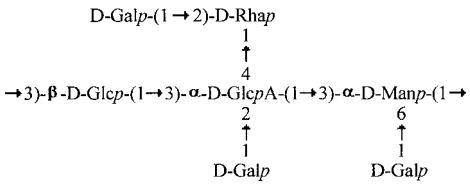

FIG. 1. Heptasaccharide repeating unit of *B. cepacia* IST408 EPS (5).

the pathway, indicated in Fig. 2, for the synthesis of the nucleotide sugar precursors UDP-D-glucose, UDP-D-galactose, UDP-D-glucuronic acid, GDP-D-mannose, and GDP-D-rhamnose. These activated precursors were presumed to be the donors of monomers for the biosynthesis of the heptasaccharide repetitive unit of *B. cepacia* IST408 EPS (Fig. 1). The postulated pathway was confirmed by detecting, in crude cell-free extracts prepared from cells of this mucoid isolate, all the enzyme activities thought to be involved in the precursor's synthesis (Figs. 2 and 3), by using slightly modified well-established techniques. The specific activities were measured in crude cell-free extracts prepared from cells harvested, at intervals, from mid-exponential phase to late stationary phase of *B. cepacia* IST408 batch growth curve (Figs. 3 and 4a). In general, maximal specific activities of these biosynthetic enzymes were observed at the late exponential phase (PGM, UGE, and UGD) or at the early stationary phase (PMM, UGP, and PMI) (Figs. 3 and 4a). However, PGI and GRS specific activities increased during stationary phase and GMP specific activity was independent of the phase of growth. Most of the EPS production, following the increase of broth viscosity during cultivation, was observed at the end of exponential phase of growth (Fig. 4a). Significantly, the producing cells exhibit, at those growth phases, high levels of all the enzymes responsible for the precursors' formation (Figs. 3 and 4a). An identical pattern of growth-phase dependent EPS production was observed with all the mucoid CF isolates identified before [(4), and J. A. Richau, J. H. Leitão, and I. Sá-Correia, unpublished results].

Activity of Enzymes Responsible for Sugar Nucleotide Formation in the Non-Mucoid Variant B. cepacia IST408N

During successive cultivations in S liquid medium or on A, S, and PIA agar plates, all the high-EPS producing CF isolates of *B. cepacia* examined before (4) showed no detectable colonial variation concerning

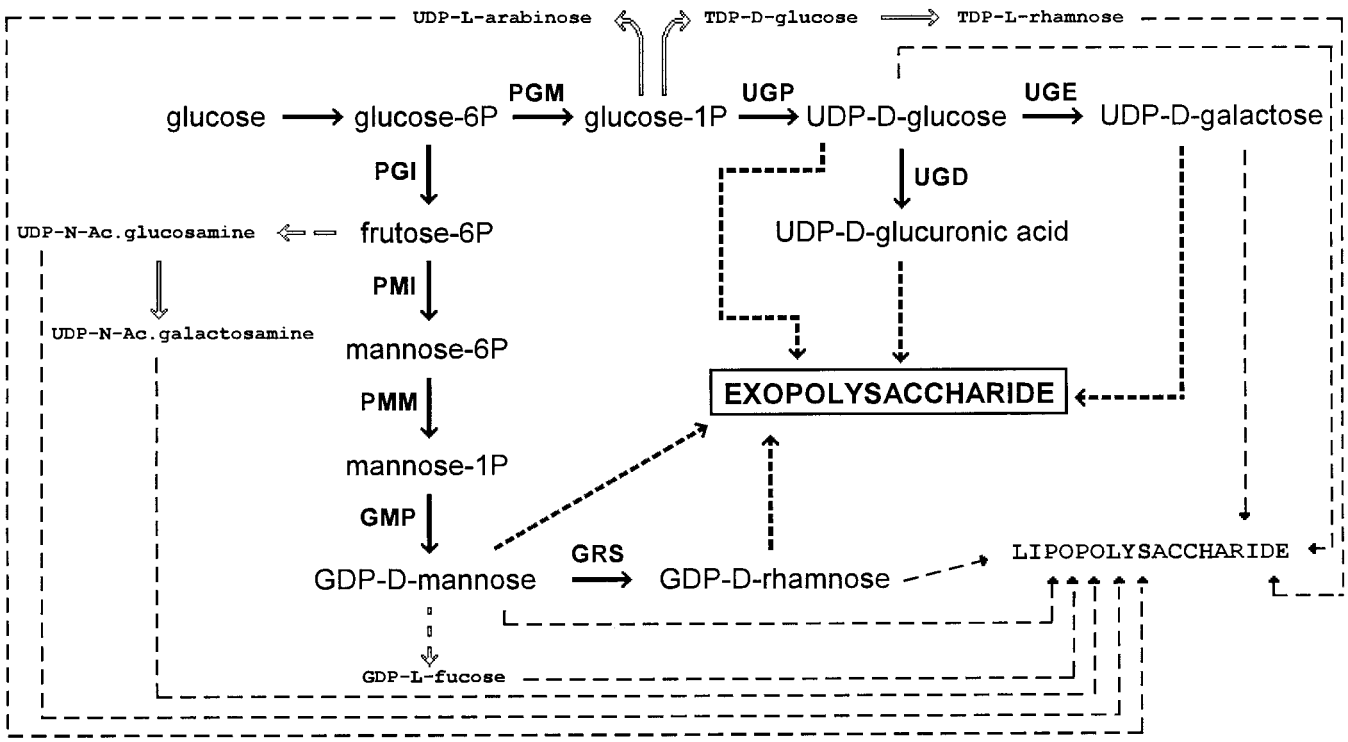

FIG. 2. Postulated pathway leading to the nucleotide sugar precursors presumed to be involved in the biosynthesis of *B. cepacia* IST408 exopolysaccharide. Also shown are the major sugar precursors presumably involved in lipopolysaccharide(s) synthesis (24, 25, 26). Abbreviations: GDP, guanosine-5′-diphosphate; UDP, uridine-5′-diphosphate; PGM, phosphoglucomutase; UGE, UDP-glucose epimerase; PMM, phosphomannomutase; UGP, UDP-glucose pyrophosphorylase; PGI, phosphoglucose isomerase; GMP, GDP-D-mannose pyrophosphorylase; UGD, UDP-glucose dehydrogenase; PMI, phosphomannose isomerase; GRS, GDP-rhamnose synthetase.

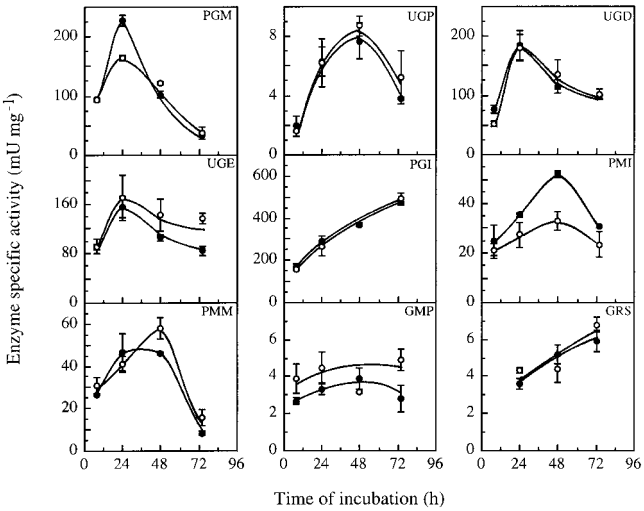

FIG. 3. Specific activities of PGM, UGE, PMM, UGP, PGI, GMP, UGD, PMI, and GRS in crude cell-free extracts prepared from cells of mucoid *B. cepacia* IST408 (●) and the spontaneous non-mucoid variant IST408N (○) during batch growth at 30°C and 250 rev min^{-1}. The abbreviations for the biosynthetic enzymes are as in Fig. 1 legend. Error bars represent standard deviation between triplicates in a single experiment.

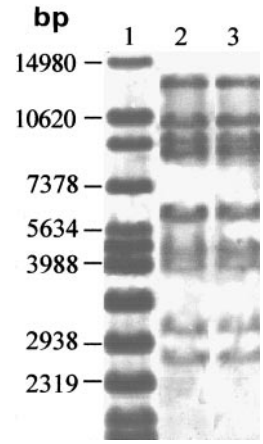

FIG. 5. Ribopatterns obtained with *B. cepacia* variants, after digestion of total DNA with *Eco*RI and hybridization with the acetylaminofluorene-labeled 16S + 23S rRNA from *E. coli*. Lane 1, molecular size standard *Raoul*I; lane 2, *B. cepacia* isolate IST408; lane 3, *B. cepacia* IST408N. Molecular masses are given in base pairs (bp).

mucoidy. This contrasts with the high frequent conversion of mucoid *P. aeruginosa* to the non-mucoid phenotype when cultivated in the laboratory (2). After several attempts, we finally succeeded in isolating the non-mucoid spontaneous variant *B. cepacia* IST408N on A agar plates, based on differences in colony morphology. The ribopatterns, with *Eco*RI, generated by the original mucoid isolate *B. cepacia* IST408 and by this non-producing spontaneous variant were undistinguishable, indicating a very close relationship between the two forms (Fig. 5). They also indicate that ribotyping is inadequate to monitor the genomic modifications accompanying the spontaneous variation of the mucoid phenotype. This was also observed with mucoid and non-mucoid *P. aeruginosa* variants either using ribotyping or RFLP-PFGE (18).

In liquid culture, *B. cepacia* IST408N confirmed its inability to produce EPS or to lead to the increase of broth viscosity during cultivation (Fig. 4b). The specific activity of all the biosynthetic enzymes examined before in crude cell-free extracts prepared from producing cells were also determined under the same conditions, in crude cell-free extracts prepared from IST408N cells, harvested at identical phases of the growth curve. Results indicate that these activities are, in general, similar to those determined in the mucoid cells and that the growth-dependent pattern of enzyme activity also reproduces the pattern observed for the mucoid strain. Only phosphomannose isomerase and possibly phosphoglucomutase consistently exhibited in the mucoid strain levels that were slightly above those detected in the non-mucoid form (Fig. 3). In contrast, the specific activity of GDP-mannose pyrophosphorylase and UDP-glucose epimerase appeared to be slightly higher in the non-mucoid variant (Fig. 3).

Mucoid strains of *P. aeruginosa* that chronically colonize the lungs of CF patients are thought to evolve, in the CF respiratory tract, from initial non-mucoid colonizing environmental strains (2). This also contrasts with the fact that there are environmental strains of *B.*

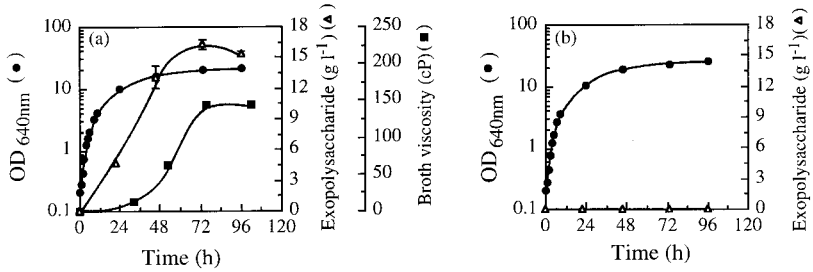

FIG. 4. Culture $OD_{640\ nm}$ (●), EPS production (△), and broth viscosity (□) (in centipoise, cP, measured at shear rate 24 s^{-1}) during batch growth, at 30°C and 250 rev min^{-1}, of (a) mucoid *B. cepacia* IST408 and (b) spontaneous non-mucoid variant *B. cepacia* IST408N. Error bars represent standard deviation between at least three independent determinations of the dry weight of EPS samples obtained from three independent cultures.

cepacia that overproduce EPS (4). Several lines of evidence clearly indicate that the step catalyzed by GDP-mannose dehydrogenase (GMD) is a key step in the control of the alginate pathway in *P. aeruginosa* (2, 19); this irreversible step channels GDP-D-mannose into the alginate pathway via the activated sugar precursor GDP-mannuronic acid, which is specific of this biosynthetic process. Indeed, consistent with the undetectable levels of *algD* mRNA in non-mucoid forms (20), GMD activity was undetectable or showed negligible values in non-mucoid variants, compared with the mucoid forms (21). However, the three other enzyme activities necessary to the synthesis of GDP-D-mannose, specifically, phosphomannose isomerase, phosphomannomutase, and GDP-D-mannose pyrophosphorylase, exhibit activities in the non-mucoid forms only slightly (40–70%) below the values in the mucoid forms (21). This is consistent with the requirement of these enzyme activities for the formation of GDP-D-mannose, indispensable to lipopolysaccharide synthesis (22). Results of the present work indicate that the spontaneous regulation of the expression of the mucoid phenotype in *B. cepacia* does not occur at the level of the synthesis of any of the enzymes leading to the formation of the activated sugar precursors for EPS synthesis. In contrast to GDP-mannuronic acid which is specifically used for alginate biosynthesis in *P. aeruginosa* (2, 19), the sugar nucleotides which are the activated precursors for EPS biosynthesis in *B. cepacia* are also involved in other essential biosynthetic pathways, in particular in the formation of cell wall polysaccharides (23) and lipopolysaccharide(s) (24, 25, 26). The control of EPS synthesis in *B. cepacia* may be exerted at the level of glycosyl transferases, specific for each sugar linkage in the repetitive sub-units, or proteins involved in the polymerization and export of the polysaccharide (19).

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