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# Bacterial Type II PMIs: Exploitable Bifunctional Enzymes for Biotechnological Applications and the Rational Design of Antimicrobials

Sílvia A. Sousa, Christian G. Ramos, Joana Feliciano and Jorge H. Leitão  
 IBB – Institute for Biotechnology and Bioengineering, CEBQ, IST  
 Portugal

## 1. Introduction

Phosphomannose isomerases (PMIs, E.C. 5.3.1.8), first described by Slein (1950) and isolated from brewers' yeast by Gracy and Noltmann (1968), are metal-dependent aldose-ketose isomerases that catalyze the reversible isomerization of D-fructose-6-phosphate (F6P) into D-mannose-6-phosphate (M6P) in prokaryotic and eukaryotic cells (Fig. 1).

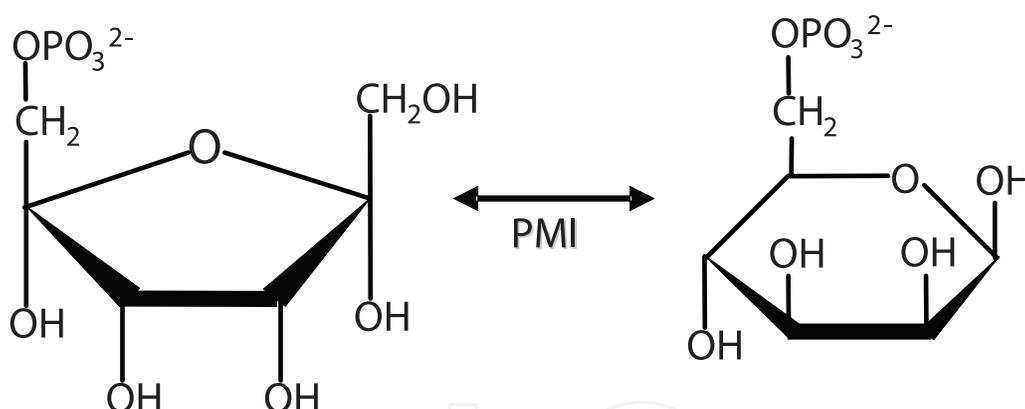


Fig. 1. The reversible conversion of fructose-6-phosphate (left) into mannose-6-phosphate (right), catalyzed by PMI enzymes.

The reaction catalyzed by PMIs is the first step of the mannose pathway leading to the generation of guanosine diphosphate (GDP)-D-mannose (Fig. 2). In this pathway, M6P is subsequently converted into mannose-1-phosphate (M1P) by the phosphomannomutase (PMM, E.C.5.4.2.8) enzymatic activity, followed by the conversion of M1P into GDP-D-mannose by GDP-D-mannose pyrophosphorylase (GMP, E.C.2.7.7.22). GDP-D-mannose is an important precursor of many mannosylated structures such as glycoproteins, nucleotide sugars, glycolipids, cell wall components found in fungi, and bacterial polysaccharides (Dunwell et al., 2000). As shown in Fig. 2, GDP-D-mannose is also the precursor of the activated sugar nucleotides GDP-L-fucose, GDP-D-rhamnose, GDP-colitose, and GDP-perosamine, that are required for the biosynthesis of several glycoconjugates, including lipopolysaccharide (LPS) O-antigens, exopolysaccharides (EPS) and glycoproteins (Richau et al., 2000b; Vinion-Dubiel and Goldberg, 2003, Fig. 2).

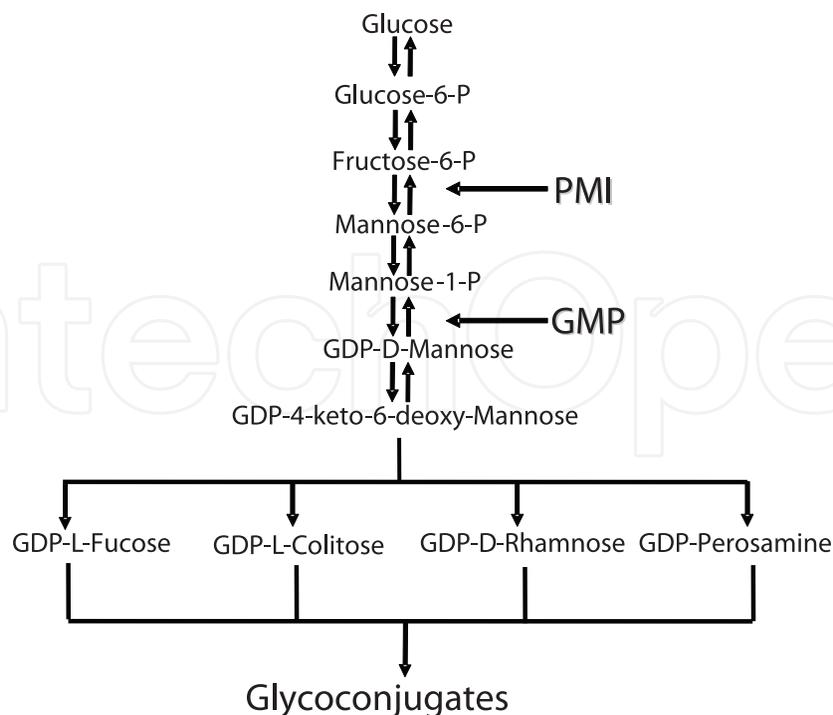


Fig. 2. Enzymatic conversions leading to GDP-D-mannose, the precursor for the synthesis of the sugar residues L-fucose, L-colitose, D-rhamnose, and D-perosamine, sugar residues commonly found in bacterial lipopolysaccharide O-antigens and other glycoconjugates.

## 2. The PMI family of proteins is divided in four classes

PMIs are a family of proteins that are members of the cupin superfamily of prokaryotic and eukaryotic proteins. Besides PMIs, the cupin superfamily includes several enzymes and proteins that bind sugars and other compounds (Dunwell et al., 2000). The term cupin derives from the latin word *cupa* (small barrel), and was given to this protein superfamily since their members possess a six-stranded  $\beta$ -barrel structural domain (the cupin domain) (Khuri et al., 2001).

The PMI protein family was divided into four distinct classes that are structurally unrelated, except for a small conserved amino acid sequence motif that belongs to the active site for the M6P to F6P isomerization reaction (Jensen & Reeves, 1998; Hansen et al., 2004). The type I class of PMIs is composed of monofunctional enzymes mainly found in eukaryotes and that only catalyze the F6P to M6P isomerization reaction. For example, the type I PMI isolated from *Saccharomyces cerevisiae* has been shown to be a zinc-dependent metalloenzyme with one metal ion per monomer (Gracy & Noltmann, 1968).

The type II class of proteins is only found in prokaryotes, and are bifunctional proteins with both PMI and GMP enzyme activities in separate catalytic domains of the protein (Jensen & Reeves, 1998). The GMP enzyme activity catalyses the reversible conversion of mannose-1-phosphate into GDP-D-mannose (Fig. 2).

The type III class of proteins comprises only a single protein from *Sinorhizobium meliloti* (Jensen & Reeves, 1998).

The type IV class includes atypical proteins from several aerobic crenarchaeota such as *Aeropyrum pernix*, *Thermoplasma acidophilum*, *Archaeoglobus fulgidus*, and *Pyrobaculum aerophilum*. This class of proteins is also bifunctional, with both phosphoglucose isomerase

(PGI, EC 5.3.1.9) and PMI enzyme activities (Hansen et al., 2004). PGI catalyses the reversible isomerization of glucose-6-phosphate (G6P) to F6P.

### 3. PMIs are critical for microbial survival and pathogenesis

PMIs have been reported as important enzymes for the survival and / or pathogenesis of several bacterial species (*Escherichia coli*, *Salmonella enterica*, *Mycobacterium smegmatis*, *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Burkholderia cepacia complex*), yeasts (*Saccharomyces cerevisiae*, *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus nidulans*) and protozoan parasites (*Leishmania mexicana*) (Patterson et al., 2003; Shinabarger et al., 1991; Wu et al., 2002; Sousa et al., 2007a; Garami and Ilg, 2001; Payton et al., 1991; Smith et al., 1995; Wills et al., 2001; Smith and Payton, 1994; Jensen and Reeves, 2001). The requirement of a functional PMI activity for the survival and pathogenesis was recently demonstrated in the case of *Leishmania*. Species of the *Leishmania* genus synthesize large amounts of mannose-containing glycoconjugates, including the unusual glycoinositolphospholipids (GIPLs), the conserved protein-linked glycosylphosphatidylinositol (GPI) membrane anchors, glycoproteins with uncommon N-linked glycans, and phosphoglycan-modified molecules such as lipophosphoglycan (LPG), and proteophosphoglycans (PPGs) (Garami and Ilg, 2001). These glycoconjugates are responsible for the remarkable resistance of *Leishmania* parasites against the hostile habitats they find within their host organisms (Garami and Ilg, 2001). To gain insights on the role played by the PMI activity in the biosynthesis of these glycoconjugates, *L. mexicana* deletion mutants on the *lmexpmi* gene (encoding the protein with PMI activity) were generated (Garami and Ilg, 2001). As a consequence of the deletion of the *lmexpmi* gene, the mutants had a lower PMI activity. However, they were still able to grow in media deficient for mannose, but were unable to synthesize the phosphoglycan repeats [-6-Gal $\beta$ 1-4Man $\alpha$ 1-PO $_4^-$ ], and the mannose-containing glycoinositolphospholipids. As a consequence, these mutants exhibited a reduced ability to express the glycosylphosphatidylinositol-anchored dominant surface glycoprotein leishmanolysin. When compared to the wild-type strain, the mutants were less virulent in mice and exhibited a lower ability to colonize macrophages *in vitro* (Garami and Ilg, 2001).

Mannose is also a key component of several cell-wall and intracellular molecules in mycobacteria, including mannanolipids (phosphatidylinositol mannoside, lipomannan, lipoarabinomannan), the cytoplasmic 3-O-methylmannose polysaccharide and O-mannosylated glycoproteins. To understand the PMI role in the mannose metabolism in mycobacteria, Patterson et al. (2003) generated a *M. smegmatis* mutant auxotrophic for mannose by deleting the *manA* gene. The mutant cells were found to be shorter in length than the wild-type, and presented a mild hyperseptation phenotype. These changes caused an exponential loss of cell viability after 10 hours of growth in mannose-free medium, revealing the essentiality of the mannose metabolism for growth and viability of *M. smegmatis* (Patterson et al., 2003).

The PMI activity is also essential for the viability of the yeast *C. albicans*. In this pathogenic yeast, cell lysis was reported to occur in the absence of the *pmi* gene (Smith et al., 1995).

In the bacterium *H. pylori*, the LPS O-antigen contains fucosyloligosaccharides similar to the human Lewis X and Lewis Y antigens (Wu et al., 2002). These molecules contribute to the mimicry of the host and to the development of an autoimmune response, leading to the pathogen increased persistence in the host (Wu et al., 2002). The major pathway for biosynthesis of GDP-L-fucose, the precursor for L-fucose, starts from GDP-D-mannose (Fig.

2). Wu et al. (2002) have shown that the type II protein HP0043 was the point control for GDP-D-mannose biosynthesis in *H. pylori*.

Due to the important roles played by these enzymes for the survival and pathogenesis, PMIs have been considered as promising targets for the development of antibacterial, antifungal, and antiparasitic agents.

#### 4. Type II phosphomannose isomerases

In contrast with the well-studied type I PMIs, type II PMI proteins remain poorly characterized. The bacterial type II PMIs that have been functionally characterized so far include the *E. coli* and *S. enterica* ManC (Jensen and Reeves, 2001), the *P. aeruginosa* AlgA (Shinabarger et al., 1991), the *Xanthomonas campestris* XanB (Köplin et al., 1992), the *Gluconacetobacter xylinum* AceF (Griffin et al., 1997), the *Sphingomonas chungbukensis* DJ77 PMI (Tran et al., 2009), the HP0043 protein from *H. pylori* (Wu et al., 2002), and the BceA and BceA<sub>J</sub> proteins from *B. cepacia* IST408 and *B. cenocepacia* J2315, respectively (Sousa et al., 2007a; Sousa et al., 2008). The sequence analysis of these type II PMIs revealed that they are composed of two domains, with four conserved motifs, as shown in Fig. 3: a GMP domain at the N-terminus and a PMI domain at the C-terminus. The GMP active site motif, that includes the conserved sequence FVEKP, is present in the N-terminus of the amino acid sequence and is essential for M1P binding, being the lysine (K) residue responsible for the binding of the phosphate moiety (May et al., 1994). The N-terminal region of the proteins also contain the highly conserved pyrophosphorylase signature sequence, GXGXR(L)-PK (where X represents any amino acid residue), similar to the activator-binding site of the bacterial XDP-sugar pyrophosphorylases (Jackson et al., 2004). In the C-terminal domain, two conserved motifs are also present, the zinc-binding motif QXH, and the putative PMI active site EN(Q/E)SX(Y/F)I (Jackson et al., 2004).

Sousa et al. (2008) have performed a prediction analysis of the secondary structure of the type II PMI BceA<sub>J</sub>. These authors have found that, while the GMP domain of BceA<sub>J</sub> was putatively composed of  $\alpha$ -helices interspaced by  $\beta$ -strands, the PMI domain of the protein was almost composed of  $\beta$ -strands. This predicted secondary structure is in good agreement with the occurrence of two distinct domains in type II PMIs (Sousa et al., 2008).

Both the PMI and GMP enzyme activities were detected in all the purified type II PMIs mentioned before. The requirement for divalent metal ions (e.g. Mg<sup>2+</sup>, Ca<sup>2+</sup>, Co<sup>2+</sup>, Mn<sup>2+</sup>, Ni<sup>2+</sup>, or Zn<sup>2+</sup>) for catalysis has also been demonstrated for those proteins. In fact, the type II PMIs extracted and purified from *X. campestris* and *B. cepacia* were totally inhibited by the metal chelator ethylenediaminetetraacetic acid (EDTA) (Sousa et al., 2007a; Papoutsopoulou & Kyriakidis, 1997).

In the type II PMI from *H. pylori*, a difference in pH dependence between the forward and reverse direction of PMI reaction was observed, the forward reaction exhibiting a pH optimum of 7, while the reverse reaction had an optimal pH range of 7-9 (Wu et al., 2002). Due to these results, it was suggested that this enzyme requires proper ionization of either M1P and/or GTP for binding and catalysis.

GDP-D-mannose was found to inhibit the PMI activity of the *P. aeruginosa* PlsB and the *H. pylori* HP0043 proteins, suggesting feedback regulation of the pathway in these bacterial species (Lee et al., 2008; Wu et al., 2002). This observation led to the suggestion that the mannose group in GDP-D-mannose might compete with M6P at the PMI active site to regulate the mannose utilization in these pathogens. However, no inhibition by GDP-D-mannose was observed for the *P. aeruginosa* AlgA protein (Shinabarger et al., 1991). In

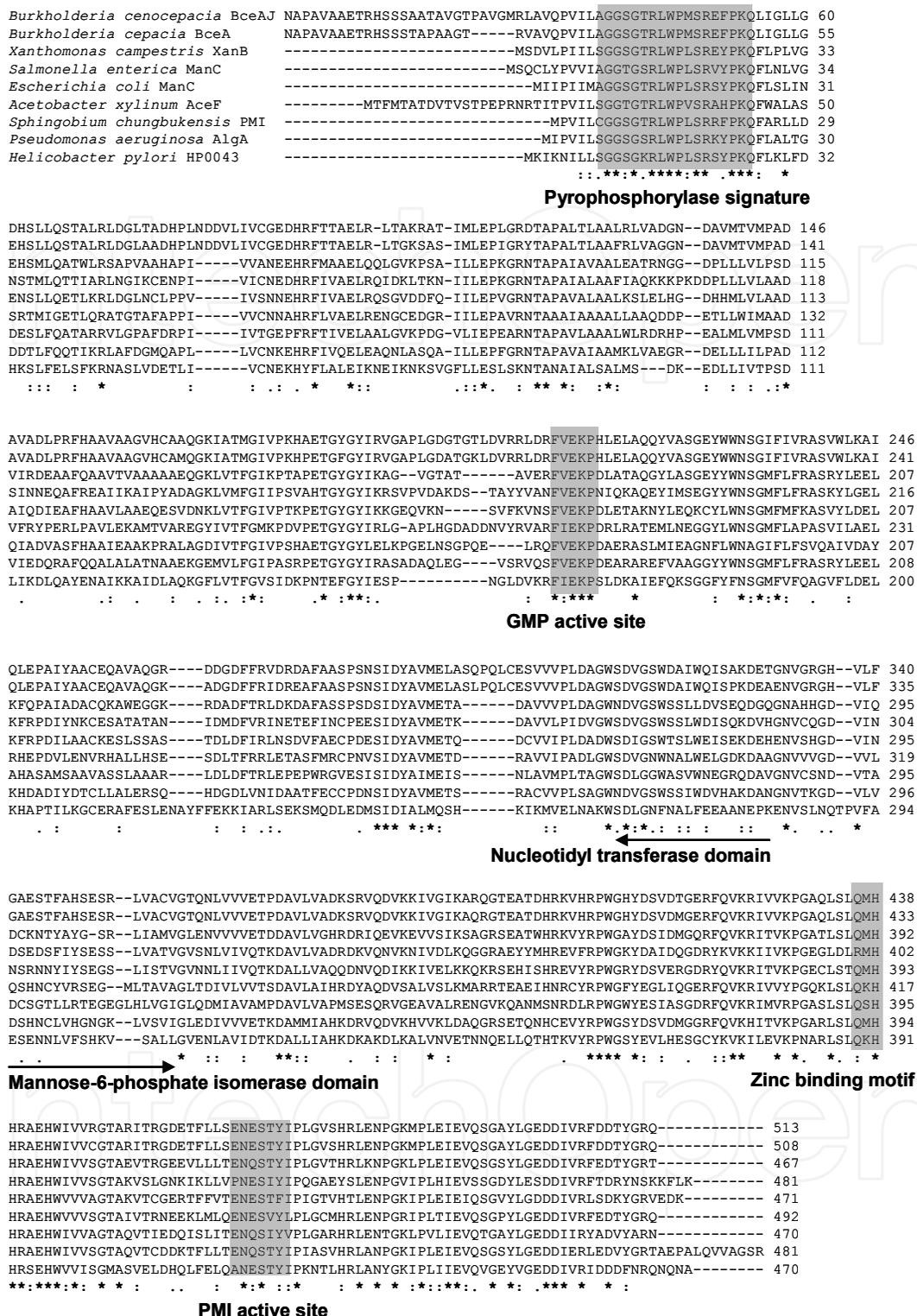


Fig. 3. Amino acid sequence alignment of experimentally characterized type II PMIs from *B. cenocepacia*, *B. cepacia*, *X. campestris*, *S. enterica*, *E. coli*, *A. xylinum*, *S. chungbukensis*, *P. aeruginosa*, and *H. pylori*. The putative GMP and PMI domains are indicated by arrows. The conserved motifs are boxed in grey. Asterisks indicate the amino acid residues that are identical in all proteins. One or two dots indicate semi-conserved or conserved substitutions, respectively. Alignments were performed with ClustalW software.

addition, some reducing agents such as DTT and  $\beta$ -mercaptoethanol, have also been shown to strongly inhibit the PMI activity, suggesting the involvement of disulfide bonds in the formation of the active site and / or the substrate binding site of the enzyme (Shinabarger et al., 1991; Papoutsopoulou & Kyriakidis, 1997). The XanB protein was also inhibited by diethyl pyrocarbonate (DEPC), being the protection from inactivation obtained after addition of the substrate M6P, revealing that the amino acid residue affected is located at or near the active site (Papoutsopoulou & Kyriakidis, 1997). DEPC modifies lysyl (K), histidyl (H), cysteinyl (C), seryl (S) and tyrosyl (Y) residues of proteins. To identify the modified amino acid residues, different compounds were tested, such as PMSF for serine residues, N-acetylimidazole for tyrosine and lysine residues, hydrogen peroxide for cysteine and methionine (M) residues, and p-mercurybenzoate for cysteine residues (Papoutsopoulou & Kyriakidis, 1997). None of these compounds were able to inhibit the PMI activity. However, the treatment of the carboxy-PMI with hydroxylamine, that reverses histidine and serine modifications, restored the activity to about 82% of the initial activity, revealing that the modified amino acid was histidine (Papoutsopoulou & Kyriakidis, 1997). The PlsB protein was inhibited by 2,3-butanedione, suggesting the presence of a catalytic arginine (R) residue (Lee et al., 2008). Site-directed mutagenesis revealed that the residue R408 of PlsB was required for the PMI catalysis, but not for the GMP activity (Lee et al., 2008). Therefore, it was concluded that this residue must participate in the interconversion of sugar moieties by providing the binding of the sugar phosphate group or by forming the hydrogen bond of sugar hydroxyl group, stabilizing the binding between substrate and enzyme (Lee et al., 2008).

A summary of the kinetic parameters of the type II PMIs AlgA and PlsB from *P. aeruginosa* PAO1, AceF from *A. xylinum*, XanB from *X. campestris* ATCC13951, BceA from *B. cepacia* IST408, BceA<sub>I</sub> from *B. cenocepacia* J2315, HP0043 from *H. pylori* 26695 and PMI from *S. chungbukensis*, is presented in Table 1.

In most of these bacterial species, the type II PMIs are involved in the biosynthetic pathway of sugar precursors that are required for the production of polysaccharide-containing polymers, such as EPSs and LPSs. In bacteria, EPSs have been shown to provide protection against dehydration, engulfment by macrophages, bacteriophages, antibiotics and other toxic compounds like heavy metal ions (Ferreira et al., 2010). Due to their physico-chemical properties, like high viscosity and pseudoplasticity, the EPSs from some bacterial species are also used in many applications ranging from food processing to pharmaceutical production (Pettitt, 1979). We will focus on the EPS xanthan as an example of an EPS with several industrial applications, and on alginate and cepacian, both known to play a role on the pathogenesis of the producing organisms to the human host. In Fig. 4, the chemical structures of these three bacterial EPSs are shown, as well as photographs of Petri plates containing colonies of the respective EPS producing bacteria.

Type II PMIs XanB, AlgA, and BceA are involved in the biosynthesis of the sugar nucleotides necessary for polymerization of the EPS xanthan, alginate and cepacian, respectively. In the case of xanthan synthesis, the GDP-D-mannose is the activated sugar precursor for the mannose moiety found in the repeating unit (Fig. 5). However, in the case of the two other EPSs, the GDP-D-mannose is either directly used to form the repeating unit (cepacian), or further converted into GDP-D-rhamnose or to GDP-D-mannuronic acid, before being incorporated, respectively, into the repeating unit of cepacian or added into the growing poly-mannuronate polymer produced by *P. aeruginosa*.

Table 1. Comparison of the kinetic parameters of different bacterial type II PMIs. The PMI and GMP reactions were performed in the presence of the best metal ion activator. Abbreviations: ND, not determined.

Protein	Metals ions for PMI activity	$V_{\max}$ PMI activity (U/mg)	$K_m$ PMI activity ( $\mu$ M)	Metals ions for GMP activity	$V_{\max}$ GMP activity (U/mg)	$K_m$ GMP activity ( $\mu$ M)	In
BceA <sub>J</sub>	Mg <sup>2+</sup> >Ca <sup>2+</sup> >Mn <sup>2+</sup> >Co <sup>2+</sup> >Ni <sup>2+</sup>	27.03	12390	Mg <sup>2+</sup> >Ca <sup>2+</sup>	4.56	24	N
BceA	Ca <sup>2+</sup> >Mn <sup>2+</sup> >Mg <sup>2+</sup> >Co <sup>2+</sup> >Ni <sup>2+</sup>	<sup>a</sup> 21.10	<sup>a</sup> 9010	Mn <sup>2+</sup> >Ca <sup>2+</sup> >Mg <sup>2+</sup> >Ni <sup>2+</sup>	212.8	2940	ED
AlgA	Co <sup>2+</sup> >Ni <sup>2+</sup> >Mn <sup>2+</sup> >Mg <sup>2+</sup> >Ca <sup>2+</sup> >Zn <sup>2+</sup>	0.83	3030	Mg <sup>2+</sup> >Mn <sup>2+</sup>	5.17	14.2	D
PlsB	Co <sup>2+</sup> >Mn <sup>2+</sup>	ND	1180	Mg <sup>2+</sup> >Co <sup>2+</sup> >Mn <sup>2+</sup>	ND	110	GM m bu
HP0043	Co <sup>2+</sup> >Mg <sup>2+</sup> >Mn <sup>2+</sup> >Zn <sup>2+</sup>	ND	55.56	Mg <sup>2+</sup> >Mn <sup>2+</sup>	ND	101	GM m
XanB	Co <sup>2+</sup> >Zn <sup>2+</sup> >Mn <sup>2+</sup> >Ni <sup>2+</sup> >Ca <sup>2+</sup>	33.5	2000	ND	ND	ND	ED m ol
AceF	ND	<sup>b</sup> 0.04	ND	ND	<sup>b</sup> 0.01	ND	N
PMI	Co <sup>2+</sup> >Mg <sup>2+</sup> >Ca <sup>2+</sup> >Ni <sup>2+</sup> >Zn <sup>2+</sup>	ND	ND	Mg <sup>2+</sup>	ND	ND	GM m

<sup>a</sup>The PMI reaction was performed in presence of MgCl<sub>2</sub>.

<sup>b</sup>The specific activity was determined in crude cell extracts of the *E. coli* CD1 (*pmi*-) and CV

Since its discovery in 1950, the xanthan EPS produced by the phytopathogen *X. campestris*, generated a great scientific and industrial interest, and was first approved as a food additive in 1969 by the Food and Drug Administration (FDA, USA) (Born et al., 2002). This high molecular weight acidic heteropolysaccharide consists of repeating units containing D-glucose, D-mannose and D-glucuronic acid in a molar ratio of 2:2:1, respectively (Fig. 4). The glucose residues are linked to form a  $\beta$ -1,4-D-glucan cellulosic backbone, with alternate glucose residues decorated with a short branch with a glucuronic acid residue sandwiched between two mannose residues (Born et al., 2002). The type II PMI XanB is one of the key enzymes in the pathway of xanthan biosynthesis, leading to the formation of the precursor GDP-D-mannose (Papoutsopoulou & Kyriakidis, 1997). Work performed by Papoutsopoulou & Kyriakidis (1997) revealed that the half life of this enzyme was 3 times higher in the presence of zinc ions, implying a structural role for this metal ion. This study also revealed that a histidyl residue at or near the PMI active site was essential for the isomerization reaction.

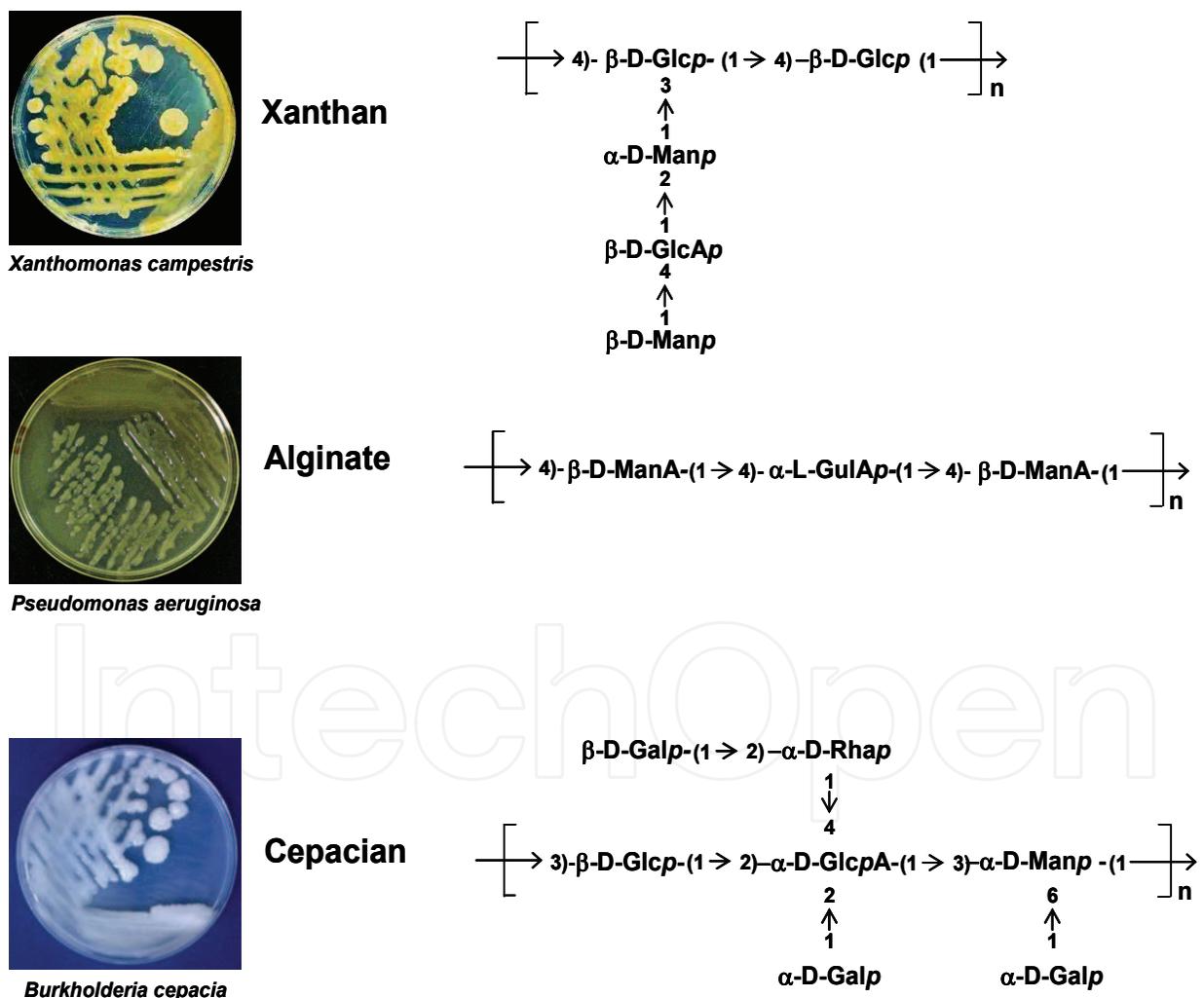


Fig. 4. Chemical structures of xanthan, alginate and cepacian (right) and photographs of Petri plates with isolated colonies of the respective producing bacterial strains, evidencing their heavy mucoid phenotype (left). Abbreviations: Glc, glucose; Man, mannose; GlcA, glucuronic acid; ManA, mannuronic acid; GulA, guluronic acid; Gal, galactose; Rha, rhamnose.

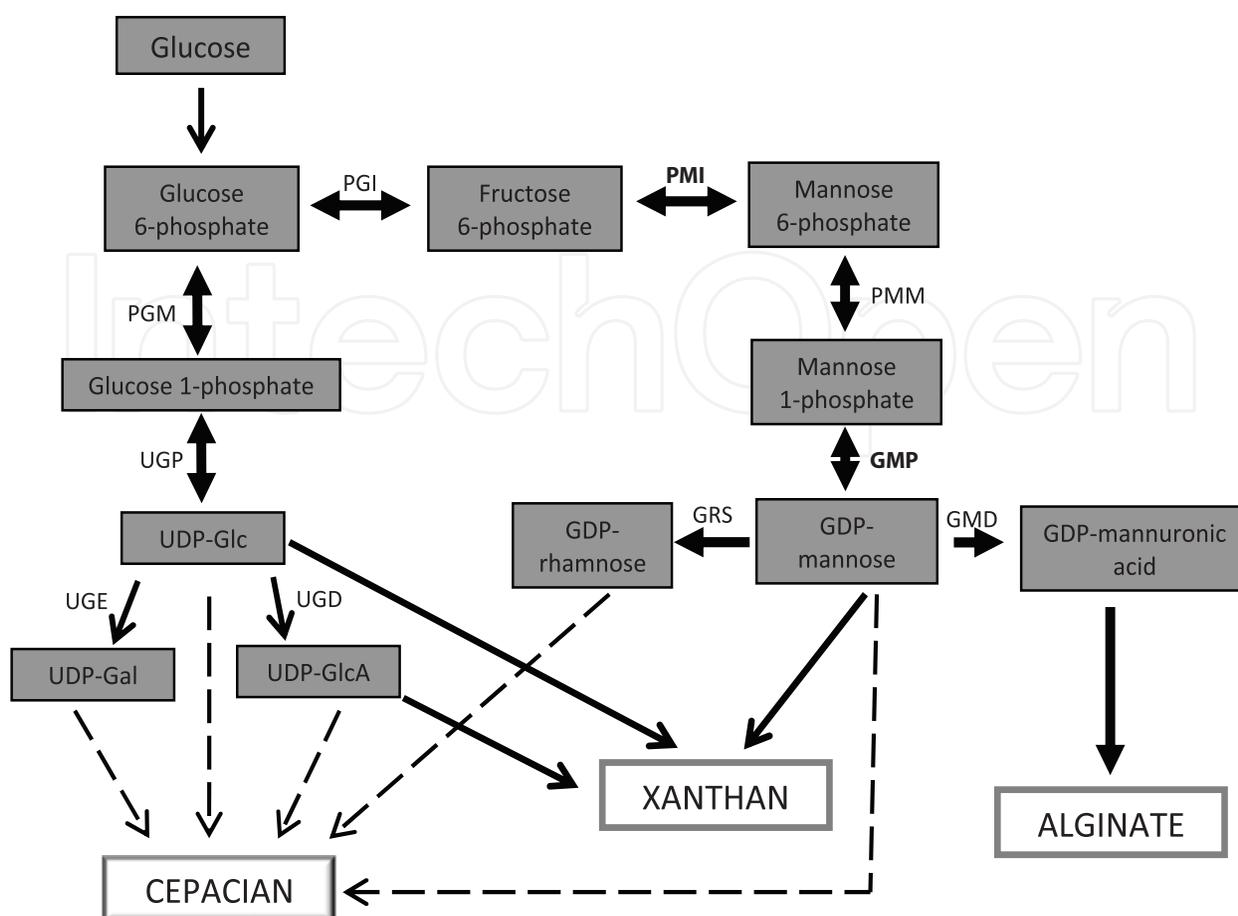


Fig. 5. Biosynthetic pathways leading to the activated sugar precursors necessary for assembly of the repeating units of cepacian, xanthan and alginate. The enzyme activity catalyzing each step is shown close to the arrow. Abbreviations: **PMI**, phosphomannose isomerase; **GMP**, GDP-D-mannose pyrophosphorylase; PGI, phosphoglucose isomerase; PGM, phosphoglucomutase; UGP, UDP-glucose pyrophosphorilase; UGE, UDP-galactose epimerase; UGD, UDP-glucose dehydrogenase; PMM, phosphomannomutase; GRS, GDP-rhamnose synthase; GMD, GDP-mannose dehydrogenase. The **PMI** and **GMP** activities of bifunctional type II PMIs are evidenced in bold.

In *P. aeruginosa* PAO1, three genes (*algA*, *wbpW*, *pslB*) encoding proteins homologous to type II PMIs have been described (Sá-Correia et al., 1987; Rocchetta et al., 1998; Jackson et al., 2004). These genes are located within three distinct polysaccharide biosynthesis gene clusters. *WbpW* participates in the production of the A-band LPS, while *PslB* is required for EPS production and biofilm formation (Rocchetta et al., 1998; Jackson et al., 2004). The occurrence in *P. aeruginosa* of multiple type II PMIs dedicated to the synthesis of distinct polysaccharide molecules is thought to enable the independent regulation of each specific pathway and thus to promote the adaptation of the bacterium to different environments. Nevertheless, *AlgA* is the best known characterized type II PMI from *P. aeruginosa*. This 56-kDa bifunctional enzyme catalyzes the first and third steps in the biosynthesis of alginate and was originally described by Sá-Correia et al. (1987). The *P. aeruginosa* alginate is a highly acetylated polymer, mostly composed of 1,4-linked  $\beta$ -D-mannuronic acid residues with few interspaced guluronic acid residues (Shinabarger et al., 1991; Fig. 4). This contrasts with the structure of the alginates produced by brown algae, which contain blocks of L-guluronic

acid, responsible for the ability of alginate to originate gels in the presence of  $\text{Ca}^{2+}$  (Sabra et al., 2001). *P. aeruginosa* alginate has medical significance due to its production during the conversion of *P. aeruginosa* strains to a mucoid phenotype, in association with chronic infections in the lungs of cystic fibrosis patients (Shankar et al., 1995). This conversion is induced by several conditions, including nutrient starvation, use of energetically poor substrates, and presence of metabolic inhibitors (Leitão and Sá-Correia, 1997). The production of alginate by *P. aeruginosa* protects the bacterium from the host immune responses and against the action of antibiotics (Govan and Deretic, 1996). Leitão and Sá-Correia (1993) have shown that the alginate pathway from *P. aeruginosa* can be manipulated by increasing the amounts of the type II PMI AlgA. This result revealed that the modification and control of critical steps in complex microbial EPS pathways at the genetic level can allow the increase of the EPS production yield and the alteration of the rheological properties of aqueous solutions prepared with the biopolymer.

In another CF pathogen, the Bcc bacteria, multiple type II PMI encoding genes were also found (2 to 5). In particular, three type II PMI encoding genes were associated with polysaccharide biosynthetic clusters, the *bceA*, of the cepacian biosynthetic cluster, the *pmi* of the capsular polysaccharide genomic island, and a third *pmi* involved in LPS biosynthesis (Sousa et al., 2007a). Cepacian is the major exopolysaccharide produced by a large percentage of clinical isolates of Bcc (Cunha et al., 2004; Herasimenka et al., 2007; Richau et al., 2000a; Zlosnik et al., 2008; Ferreira et al., 2010). The production of cepacian has been correlated with higher persistence and virulence of the producing bacterium in animal models, inhibition of neutrophil chemotaxis and scavenging of reactive oxygen species *in vitro*, suggesting a role for cepacian in the protection of bacteria against the host immune response (Sousa et al., 2007b; Bylund et al., 2006). This EPS is composed of a branched heptasaccharide repeating-unit, with D-glucose, D-rhamnose, D-mannose, D-galactose and D-glucuronic acid, in the ratio 1:1:1:3:1, respectively (Cescutti et al., 2000; Fig. 4). The GDP-D-mannose formed by the type II PMI BceA is the activated sugar precursor for the incorporation of D-mannose in cepacian and is also the precursor for the synthesis of GDP-D-rhamnose, the activated sugar precursor of the D-rhamnose moiety of cepacian (Richau et al., 2000b). The 55.3-kDa BceA protein was also shown to be required for thick biofilm formation and for the production of EPSs that lead to aqueous solutions with higher viscosity (Sousa et al., 2007a). However, the lack of a functional *bceA* gene did not affect the EPS production yield, suggesting that other *bceA* functional homologues may compensate the *bceA* mutation (Sousa et al., 2007a). The BceA protein from *B. cepacia* IST408 and the BceA<sub>I</sub> from *B. cenocepacia* J2315 exhibited no PMI and GMP activities in the presence of zinc, in spite of the existence, in their primary sequences, of a zinc-binding motif (Sousa et al., 2007a; Sousa et al., 2008; Fig. 3). Similarly, the PMI and GMP activities of PlsB could not be activated in presence of  $\text{ZnCl}_2$  (Lee et al., 2008).  $\text{Zn}^{2+}$  was also reported to be the less effective activator of the type II PMI HP0043 from *H. pylori*, suggesting that the combination between the zinc metal ion and the PMI domain is loose and less specific than in the type I PMI proteins (Wu et al., 2002).

## 5. Biotechnological potential of PMIs

L-ribose has been proposed as a potential starting material for the synthesis of many L-nucleoside-based pharmaceutical compounds (e.g. the antiviral drug for the treatment of hepatitis B, Clevudine) (Okano, 2009). However, L-ribose is not an abundant sugar in

nature, and therefore L-ribose has been produced mainly by chemical synthesis from L-arabinose, L-xylose, D-glucose, D-galactose, D-ribose, or D-mannono-1,4-lactone (Okano, 2009). Recently, the isomerization activity of the PMI enzyme from *B. subtilis* was reported to be specific for aldose substrates possessing hydroxyl groups oriented in the same direction at the C-2 and C-3 positions, such as the D and L forms of ribose, lyxose, talose, mannose, and allose (Yeom et al., 2009). This enzyme also exhibited the highest activity with L-ribulose, among all pentoses and hexoses tested (Yeom et al., 2009). Recently, experimental conditions have been reported as leading to the production of L-ribose at a final concentration of 213 g liter<sup>-1</sup> from 300 g liter<sup>-1</sup> of L-ribulose, using mannose-6-phosphate isomerase at 40°C for 3 h, with a conversion yield of 71% and a volumetric productivity of 71 g liter<sup>-1</sup> h<sup>-1</sup> (Yeom et al., 2009). This was the highest volumetric productivity and product concentration reported until now for the biological manufacture of L-ribose. In addition, the downstream purification methodology was simpler than the previously used for the preparation of L-ribose, either by chemical methods or fermentation. The production of the expensive sugar L-ribose from the rare sugar L-ribulose by PMI may prove to be a valuable industrial process, because the L-ribulose sugar can be produced from the low-cost sugar L-arabinose, using the L-arabinose isomerase from *Geobacillus thermodenitrificans* (Yeom et al., 2008).

Antibiotic and herbicide resistance genes have been used for the selection of transgenic plants (Min et al., 2007). However, the use of these genetic markers has generated a widespread public concern. For this reason, alternative methods of selection have been developed, in particular the use of mannose as the carbon source in selective media, since plants have an inefficient ability to metabolize this sugar (Lee and Matheson, 1984). The *pmi* gene isolated from *E. coli* has been used as a selectable marker for the transformation of several plant species, including sugar beet, cassava, maize, wheat, *Arabidopsis*, pepper, sweet orange, pearl millet, tomato, papaya, onion, almond, Chinese cabbage, and cucumber (Miles and Guest, 1984; Min et al., 2007). The transgenic plants expressing the *E. coli pmi* gene and growing in mannose medium, are able to convert the M6P to F6P, thus providing a carbon and energy source to survive to the high selective pressure.

The study of the biological function of oligosaccharides from human milk such as lactose, D-galactose, N-acetylglucosamine, sialic acid, and L-fucose, has received an increasing interest in the last years. This interest derives from the properties of fucosylated oligosaccharides (e.g. the Lewis blood group antigen) in protecting infants against enteric pathogens (Boehm and Stahl, 2007). The availability of large amounts of fucosylated oligosaccharides will be useful for the development of therapeutic and protective strategies to prevent infections by pathogens, to improve the immune system response, and to reduce the inflammatory process (Newburg et al., 2004). Enzymatic fucosylation of oligosaccharides requires GDP-L-fucose as the donor of L-fucose. However, the high cost of GDP-L-fucose limits its application for large-scale production of these oligosaccharides. The conversion F6P to GDP-L-fucose requires the activity of five enzyme activities (mannose-6-phosphate isomerase ManA, phosphomannomutase ManB, mannose-1-phosphate guanyltransferase ManC, GDP-D-mannose-4,6-dehydratase Gmd, and GDP-L-fucose synthase WcaG), with GDP-D-mannose as the intermediate. Recently, a recombinant *E. coli* BL21star(DE3) strain overexpressing *gmd*, *wcaG*, *manB* and *manC* genes was developed to maximize the production of GDP-L-fucose (Lee et al., 2009). This advance might lead to the future availability of affordable fucosylated oligosaccharides.

## 6. PMIs are promising targets for the development of new antimicrobials

PMIs have been considered as suitable targets for the development of antibacterial, antiparasitic, and antifungal agents (Bhandari et al., 1998; Roux et al., 2004; Roux et al., 2007). Roux et al. (2004) reported the inhibition of a yeast type I PMI and a *P. aeruginosa* type II PMI by 5-phospho-D-arabinohydroxamate (5PAH). However, phosphorylated compounds have a limited therapeutical interest, not only because of their ionic character which do not allow them to freely cross the barrier that is the hydrophobic cell membrane, but also because of their high susceptibility to hydrolysis by endogenous phosphatase enzyme activity (Foret et al., 2009). Recently, the 6-deoxy-6-(dicarboxymethyl)-D-mannopyranose, a non-hydrolysable M6P analogue in which the phosphate group was replaced by a dicarboxymethyl group, was reported to be a strong inhibitor of the enzymatic activity of PMIs from *E. coli* and *S. cerevisiae* (Foret et al., 2009).

Sequence alignment studies have revealed that the type I PMIs from pathogenic microorganisms such as fungi and protozoa, exhibit a high level of amino acid sequence identity (>40%) with the humans type I PMIs, especially in the binding site region (Jensen and Reeves, 1998). This high degree of identity hampers the development of species-specific inhibitors against fungal or bacterial type I PMIs that do not inhibit the human type I PMIs. In most human tissues, the inhibition of the PMI activity will most probably do not impair the global metabolism, because the majority of the M6P that is utilized for glycoprotein synthesis is most likely not derived from F6P, but originates from efficient uptake of D-mannose through a specific exogenous mannose transporter, followed by its phosphorylation by hexokinases (Panneerselvam et al., 1997). However, in organs such as the liver and the intestine, where this pathway is less efficient, a deficiency in human PMI activity leads to the carbohydrate deficient glycoprotein syndrome type 1b (CDGS 1b), a severe metabolic disorder with hepatic and intestinal manifestations (Niehues et al., 1998). Nevertheless, this disease has been successfully treated by oral administration of D-mannose (Niehues et al., 1998). Therefore, a therapeutical strategy combining the enzyme inhibitor and D-mannose supplementation should alleviate the side-effects of PMI inhibition on humans.

In the pathogenic yeast *C. neoformans*, the pathway for exogenous mannose uptake was reported to be much less efficient than in humans, and the type I PMI from this pathogen was considered as an excellent therapeutic target (Wills et al., 2001). However, the efficiency of the pathway for M6P formation in other microorganisms remains poorly studied. The PMI inhibition therapy will also be possible for local treatments, such as against yeast infections (e.g. candidiasis) and cutaneous leishmaniasis, without affecting human metabolism.

No significant sequence identity has been found between Type I and Type II PMI enzymes, except for a very small conserved amino acid sequence motif in the active site of the protein (Jensen and Reeves, 1998). For this reason, specific inhibition of the type II PMI activity, while leaving the human type I protein unaffected, should be achieved more easily than for the type I PMI proteins from pathogens. This opens the door for the rational design of potent and highly species-specific inhibitors against the targeted PMIs from pathogens. However, for this purpose, structural information on type I and type II PMIs is necessary. High-resolution X-ray crystal structures have been reported for the type I PMIs of *Candida albicans* (Cleasby et al., 1996; PDB code 1pmi), *Salmonella typhimurium* (Gowda et al., 2008; PDB codes 2wfp, 3h1y, 3h1w, and 3h1m), *Helicobacter pylori* (PDB code 2qh5), *Bacillus subtilis*

(PDB code 1qwr) and the type IV PMIs from *A. fulgidus* (PDB code 1zx5) and *P. aerophilum* (Swan et al., 2004; PDB codes 1x9i, 1x9h, 1tzb and 1tzc). The analysis of the crystal structure of the type I PMI from *C. albicans* allowed the identification of the active site and the zinc metal cofactor binding site of the enzyme (Cleasby et al., 1996). Although the roles of the individual amino acid residues of the active site and the catalytic mechanisms are still poorly known, Gracy and Noltman (1968) proposed that the zinc ion coordinates with the carbonyl and hydroxyl oxygens on C1 and C2, activating the  $\alpha$ -hydrogen to the carbonyl, which is abstracted by the nonprotonated nitrogen of an imidazole group to form the transient enediol intermediate. In contrast, the type II PMIs don't bind  $Zn^{2+}$  specifically. This observation reveals that there are some differences between the different classes of PMIs. Contrasting with the wealth of structural information on types I and IV PMIs, no structural information on type II and III PMIs is available, difficulting the determination of the roles of individual amino acid residues of the active site and the catalytic mechanisms of these proteins. Therefore, knowledge on the three-dimension structure of these enzymes is necessary to allow the understanding of the mechanisms of the isomerization reaction and the role played by the metal ions in catalysis. In turn, this information will certainly give crucial information for the exploitation of these important enzymes as targets for the rational design of inhibitors, thus enabling us with new antimicrobials to fight infections caused by microorganisms resistant to the clinically relevant antibiotics available nowadays.

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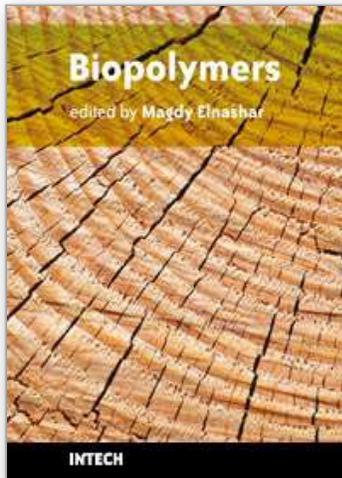
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Phone: +86-21-62489820  
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