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Phosphomannose isomerase and phosphomannomutase gene disruptions in *Streptomyces nodosus*: Impact on amphotericin biosynthesis and implications for glycosylation engineering

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ABSTRACT

Streptomycetes synthesise several bioactive natural products that are modified with sugar residues derived from GDP-mannose. These include the antifungal polyenes, the antibacterial antibiotics hygromycin A and mannopeptimycins, and the anticancer agent bleomycin. Three enzymes function in biosynthesis of GDP-mannose from the glycolytic intermediate fructose 6-phosphate: phosphomannose isomerase (PMI), phosphomannomutase (PMM) and GDP-mannose pyrophosphorylase (GMPP). Synthesis of GDP-mannose from exogenous mannose requires hexokinase or phosphotransferase enzymes together with PMM and GMPP. In this study, a region containing genes for PMI, PMM and GMPP was cloned from *Streptomyces nodosus*, producer of the polyenes amphotericins A and B. Inactivation of the *manA* gene for PMI resulted in production of amphotericins and their aglycones, 8-deoxyamphoteronolides. A double mutant lacking the PMI and PMM genes produced 8-deoxyamphoteronolides in good yields along with trace levels of glycosylated amphotericins. With further genetic engineering these mutants may activate alternative hexoses as GDP-sugars for transfer to aglycones *in vivo*.

1. Introduction

Amphotericins A and B (Fig. 1) are antifungal antibiotics that are produced by *Streptomyces nodosus* (Gold et al., 1956). The mycosamine sugar residues of amphotericins and related polyenes are synthesised from GDP-mannose (Nedal et al., 2007). However, polyene biosynthetic gene clusters do not include genes for GDP-mannose formation (Aparicio et al., 2003). Identification of these genes will extend knowledge of polyene biosynthesis and assist production of analogues by glycosylation engineering.

GDP-mannose has various metabolic functions in prokaryotic and eukaryotic cells. It is essential for biosynthesis of glycoproteins and glycolipids that contain D-mannose and derived sugars (Freeze and Aebi, 1999; Jensen and Reeves, 2001). GDP-mannose is also a biosynthetic precursor of ascorbic acid in plants (Conklin et al., 1999) and the osmotic stabiliser mannosyl-glycerate in thermophilic bacteria (Empadinhas et al., 2001). While most of the deoxyhexoses found in natural products are synthesised from dTDP-glucose, several compounds contain sugar residues derived from GDP-mannose. This group is not limited to polyenes and includes the anticancer agents bleomycin (Du et al., 2000) and neocarzinosatin (Liu et al., 2005), as well as the antibacterial antibiotics hygromycin A (Palaniappan et al., 2006) and mannopeptimycins (Magarvey et al., 2006).

GDP-mannose is synthesised from the glycolytic intermediate fructose-6-phosphate through the actions of three enzymes: phosphomannose isomerase (PMI), phosphomannomutase (PMM) and GDP-mannose pyrophosphorylase (GMPP) (Fig. 2). PMI catalyses the interconversion of fructose 6-phosphate and mannose 6-phosphate. Mannose-6-phosphate is converted to mannose-1-phosphate in a reaction catalysed by PMM. Finally, GMPP catalyses the formation of GDP-mannose from GTP and mannose-1-phosphate. Cells can also use exogenous mannose which is phosphorylated at C-6 by hexokinase (HK) or by a phosphotransferase system component to provide a substrate for PMM.

The genes for PMI, PMM and GMPP are usually named *manA*, *manB* and *manC*, respectively. These genes have been intensively studied in bacterial, fungal and protozoan pathogens (Patterson et al., 2003; Wills et al., 2001; Garami and Ilg, 2001). In general, inactivating these genes reduces virulence by interfering with

Abbreviations: ESMS, electrospray mass spectrometry; GMPP, GDP-mannose pyrophosphorylase; GPDH, glucose-6-phosphate dehydrogenase; HK, hexokinase; ORF, open reading frame; PGI, phosphoglucose isomerase; PMI, phosphomannose isomerase; PMM, phosphomannomutase; RMM, relative molecular mass; SIS, sugar isomerase; SACE, *Saccharopolyspora erythraea* gene; SAV, *S. avermitilis* gene; SCAB, *S. scabies* gene; SCO, *S. coelicolor* gene; SGR, *S. griseus* gene

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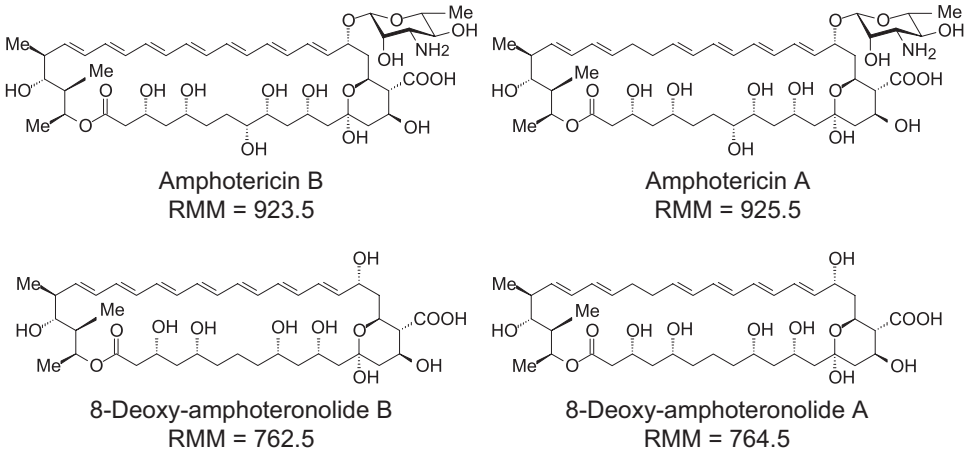

Fig. 1. Structures and masses of amphotericins A and B and 8-deoxyamphoteronolides A and B.

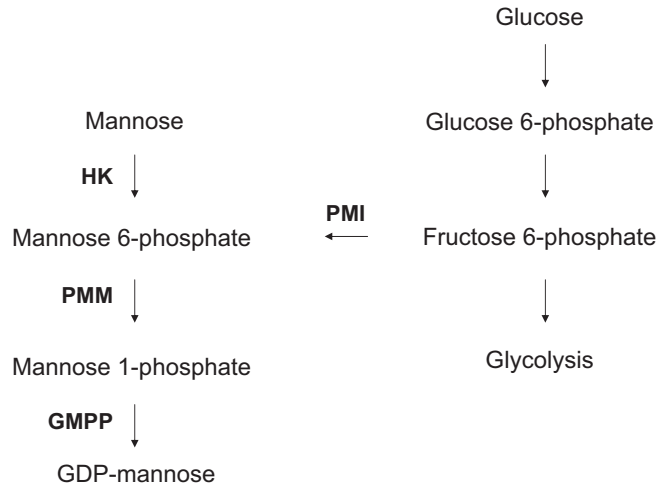

Fig. 2. Overview of GDP-mannose biosynthesis.

biosynthesis of cell surface glycoproteins and glycolipids. There have been no reports on disruption of these genes in a micro-organism that requires GDP-mannose for glycosylation of a bioactive secondary metabolite.

Modification of sugar residues of natural products can have profound effects on biological activities. Sugars have been manipulated by genetic engineering of producer micro-organisms, or by exploiting *in vitro* glycosylation systems (Thibodeaux et al., 2007; Salas and Méndez, 2007). With *in vitro* glycorandomisation, diverse arrays of synthetic sugars are C-1 phosphorylated and attached to nucleotides by engineered sugar-flexible enzymes (Yang et al., 2004; Moretti and Thorson, 2007). The resulting NDP-sugars are used for *in vitro* enzymatic glycosylation of aglycones to give libraries of new compounds (Williams et al., 2008).

Valuable compounds might be obtained in high yields by feeding unnatural sugars to aglycone-producing bacteria transformed with genes for appropriate anomeric kinases, nucleotidyl transferases and glycosyl transferases. A promiscuous sugar-1-kinase has already been shown to phosphorylate a range of sugars fed to *Escherichia coli* (Hui et al., 2007). Subsequent formation of unnatural NDP-sugars might be impaired by competing hexose-1-phosphates that are normally present in host cells. In polyene producers, the process might be facilitated by reducing intracellular levels of mannose-1-phosphate. This study aimed to investigate the effects of disrupting PMI and PMM genes on amphotericin biosynthesis in *S. nodosus*. This should lead to production of polyene aglycones. At a later stage the mutants might serve as host strains for efficient activation of alternative hexoses as GDP-sugars for transfer to aglycones.

2. Materials and methods

2.1. Bacterial strains, plasmids and phages

E. coli XL1-Blue was used as a host for general cloning. *E. coli* ET12567 was used to obtain non-methylated DNA. *Streptomyces lividans* 1326 was used for propagation of recombinant phages. *S. nodosus* was used for inactivation of *manA* and *manB* genes. *Saccharomyces cerevisiae* was used as an indicator organism to assess antifungal activity.

Cosmid B3 containing GDP-mannose biosynthetic genes was obtained from the previously described cosmid library of *S. nodosus* genomic DNA (Caffrey et al., 2001). Plasmid B3-1 is a pUC118 subclone containing nucleotides 7763–19,669 of the sequenced region. This plasmid was used to fill in the internal NotI site in the *manA* gene. The KC-UCD1 vector was used to construct recombinant phages.

2.2. DNA methods

DNA sequencing was carried out by Biotica Technology Ltd. and MWG Biotech. General molecular cloning, construction of recombinant phages, PCR and Southern hybridisation were carried out as described previously (Kieser et al., 2000; Carmody et al., 2004). Oligonucleotide primers are listed in Table 1.

The *Streptomyces* annotation server StrepDB, available at http://Streptomyces.org.uk, was used to search the genome sequences of *Streptomyces coelicolor*, *Streptomyces avermitilis*, *Streptomyces griseus* and *Streptomyces scabies*. The *Saccharopolyspora erythraea* genome was accessed through http://131.111.43.95:65400/gnmweb/index.html.

2.3. Enzyme assays

The quantities of mannose in growth media were determined with an assay kit from Megazyme, Ireland. In the assay procedure, glucose, fructose and mannose in a test sample are phosphorylated by the action of HK to glucose-6-phosphate,