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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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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, *Sacc. erythraea* gene; SAV, *S. avermitilis* gene; SCAB, *S. scabies* gene; SCO, *S. coelicolor* gene; SGR, *S. griseus* gene.

Abstract

Streptomyces 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*.

Keywords: GDP-mannose biosynthesis, polyenes, natural products

1. Introduction

Amphotericins A and B (Figure 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 neocarzinostatin (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) (Figure 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 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 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 Mendez, 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 to 19669 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., 2001; 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 *Sacc. 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 hexokinase to glucose-6-phosphate, fructose-6-phosphate and mannose-6-phosphate. The enzyme glucose-6-phosphate dehydrogenase (GPDH) catalyses oxidation of the glucose-6-phosphate to 6-phosphogluconolactone, with the reduction of NADP^+ to NADPH. The amount of NADPH formed is proportional to the amount of glucose in the original sample, and is quantified by measuring the increase in absorbance at 340 nm. When the oxidation of glucose-6-phosphate is complete, phosphoglucose isomerase (PGI) is added. This catalyses conversion of fructose-6-phosphate to glucose-6-phosphate, which is then oxidised by GPDH. This gives a further increase in the level of NADPH that is a measure of the amount of fructose. Finally, PMI is added to catalyse conversion of mannose-6-phosphate to fructose-6-phosphate. This is isomerised to glucose-6-phosphate and further oxidation gives another increment in the A_{340} from which the amount of mannose can be calculated. Fructose was omitted from samples of production media used for mannose determination.

For detection of PMI and PMM activity in *S. nodosus* and mutant strains, cells were grown at 30°C with shaking for 40 hours in 100ml volumes of

Streptomyces medium (10g glucose, 1g yeast extract, 1g Lab Lemco powder, 2g tryptone, 10 mg FeSO₄ per litre). Mycelial cells were harvested by centrifugation, washed twice with 10mM MOPS (pH 7. 2) 2mM dithiothreitol and resuspended in 10ml volumes of the same buffer. Cells were disrupted by means of a French Pressure cell. Lysates were centrifuged at 40,000 x g for 30 min at 4°C. The supernatant fractions were tested for enzyme activity. PMI and PMM assays were carried out as described by (Sa-Correia et al., 1987). Protein concentrations were determined by the method of Lowry and co-workers (1951).

2. 4 Isolation and analysis of polyenes

S. nodosus and the *manA* and *manAB* mutants were grown in production medium (60g dextrin, 20g fructose, 30g soya flour 10g CaCO₃ per litre) containing 5% (w/v) Amberlite XAD16 and 100mM glycerol (Power et al., 2008; Recio et al., 2006). Flasks were shaken at 30°C for 60-72 hours. Cultures were centrifuged at 10,000 x g for 10 min at 4°C. Methanol was used to extract polyenes from sedimented mycelium and resin. Polyenes were purified by gel filtration on a Sephadex LH20 column (50 x 2 cm) equilibrated with methanol. UV-visible absorption spectra were scanned in the wavelength range 250 to 450 nm. Amphotericin A has the absorption spectrum characteristic of a tetraene with maxima at 280, 292, 305 and 320nm. The extinction co-efficient at 320nm is 0.78×10^5 litres mole⁻¹ cm⁻¹. The amphotericin B heptaene absorbs at 346, 364, 382 and 405nm. The extinction co-efficient at 405nm is 1.7×10^5 litres mole⁻¹ cm⁻¹. ESMS was carried out as described previously (Power et al., 2008). Antifungal activity was assessed by agar diffusion assay (Caffrey et al., 2001), or as detailed in Section 3. 6.

3. Results

3. 1 *Streptomyces* genes for phosphomannomutase, phosphomannose isomerase and GDP-mannose pyrophosphorylase

Some of the mutations that confer resistance to actinophage phiC31 map in mannosyl transferase genes (Cowlshaw and Smith, 2002). These studies showed that the phage receptor is a mannoprotein. Otherwise, little is known about the role of GDP-mannose in general metabolism in streptomycetes. The genome sequences of *S. coelicolor*, *S. avermitilis*, *S. griseus*, *S. scabies* and *Sacc. erythraea* (Bentley et

al., 2001; Ikeda et al., 2003; Ohnishi et al., 2008; Oliynyk et al., 2007) were examined for genes involved in GDP-mannose formation. Several genes encode homologues of known PMI, PMM and GMPP enzymes. Some of these seemed unlikely to be involved in mannose metabolism because their context suggested an alternative role, or because they are not present in all these organisms. The SCO0064, SAV1412, and SACE6227 genes encode hypothetical proteins that are weakly homologous to phosphohexose isomerases. Conserved phosphosugar mutase genes (SCO4916, SAV13343, SGR2627, SCAB34691 and SACE6548) are homologous to PMM genes but are adjacent to genes for purine nucleoside phosphorylase (SCO4917, SAV13342, SGR2626, SCAB34681 and SACE6547). This enzyme converts purine nucleosides to free purine bases and ribose- or deoxyribose-1-phosphates. This suggests that the associated phosphosugar mutases are phosphopentomutases that catalyse conversion of pentose-1-phosphates to pentose-5-phosphates. The SCO1388, SAV6977, SGR6141 and SCAB76231 genes encode bifunctional enzymes with N-terminal nucleotidyl transferase and C-terminal phosphohexomutase domains. Another GMPP homologue is encoded by SCO4238, SAV3964, SGR4022 and SCAB76231. *S. avermitilis* has genes for two further GMPP homologues SAV358 and SAV1013. These are both located in similar clusters that include homologues of genes for UDP-glucose-4-epimerase, NDP-hexose-4-ketoreductase and glycosyl transferases.

All of the annotated genome sequences contain linked PMI and PMM genes (SCO3025 and SCO3028; SAV5051 and SAV5048; SGR4511 and SGR4508; SCAB55161 and SCAB55131; SACE6457 and SACE6460) located near GMPP genes (SCO3039, SAV5037, SGR4497, SCAB55031 and SACE6470). The genome of *Mycobacterium tuberculosis* contains a similar region with Rv3255, Rv3257 and Rv3264 genes for PMI, PMM and GMPP. There is experimental evidence that these *M. tuberculosis* genes are essential for mannose catabolism and biosynthesis of mannose-containing cell surface glycoconjugates (Patterson et al., 2003; McCarthy et al., 2005). This indicates that these genes encode the major GDP-mannose biosynthetic enzymes in actinomycetes. This region was targeted in *S. nodosus* to examine the effects on polyene glycosylation (see below).

3. 2 Analysis of *S. nodosus* genes involved in GDP-mannose biosynthesis

The *S. nodosus* genome has not yet been sequenced. A cosmid library of *S. nodosus* genomic DNA was previously used to clone amphotericin polyketide synthase genes (Caffrey et al., 2001). This work also yielded a cosmid clone B3 of an unlinked region containing the putative GDP-mannose biosynthetic genes. This study reports the sequence of 19669 bp of chromosomal DNA containing GMPP, PMI and PMM genes (GenBank accession number EU834702).

Genes were identified by BLAST searches and by comparison with the corresponding regions of the annotated *Streptomyces* and *Sacc. erythraea* genome sequences. The gene organisation is similar in all six organisms. The *S. nodosus* genes are listed in Table 2. ORF1 is homologous to *manC* genes encoding GMPP enzymes that have been characterised biochemically (McCarthy et al., 2005). ORF2 encodes an endonuclease involved in excision repair of DNA. ORFs 3 and 4 encode homologues of enzymes involved in the late stages of biosynthesis of the redox cofactor F₄₂₀. ORF5 is homologous to a gene for a cysteine dioxygenase (Dominy et al., 2006). This enzyme oxidises cysteine to cysteine sulphinic acid which may function as a developmental signal compound in *S. coelicolor* (Gehring et al., 2000). ORF6 is homologous to *whiB* that encodes a sporulation regulatory protein (Davis and Chater, 1992). ORF7 encodes a putative membrane-bound glycosyl transferase. The ORF7 stop codon overlaps the start codon for the downstream ORF8 which encodes a hypothetical secreted protein. ORF9 and ORF10 encode hypothetical proteins of unknown function.

ORF11 (*manB*) encodes PMM. ORF12 encodes a protein with a sugar isomerase (SIS) domain. SIS domains appear in phosphosugar isomerases that catalyse aldose-ketose interconversions (Bateman, 1999). The SIS domain also appears in non-catalytic transcriptional regulators that control expression of genes involved in phosphosugar metabolism. Binding of the phosphosugar to the SIS domain enables the protein to bind to its DNA target site and to influence transcription initiation. ORF13 (*manA*) encodes PMI. ORF14 encodes a homologue of a cation efflux protein that exports zinc and cadmium from bacteria. Interestingly, both zinc and cadmium are potent inhibitors of PMI enzymes (Wells et al., 1993). The end of the sequenced region contains a fragment of a gene for S-adenosyl homocysteinase. This enzyme is essential for viability of most cells because it

degrades S-adenosylhomocysteine, an inhibitory by-product of S-adenosylmethionine-dependent methylations.

3.3 Insertional inactivation of the *manA* gene

For insertional inactivation of the *S. nodosus manA* gene, an internal fragment was amplified by PCR with oligonucleotides ManA-F and ManA-R as primers. The 1086 bp product was phosphorylated with T4 kinase, digested at one end with BamHI, and cloned between the BamHI and ScaI sites of the KC-UCD1 phage vector. The recombinant phage was named KC-*manA1*. This was plated on *S. nodosus* and a lysogen was obtained by selecting for the thiostrepton resistance gene in the phage vector. Genomic DNA was extracted from the lysogen and analysed by Southern hybridisation (Figure 3). The *manA* gene is located within a 3640 bp PvuII fragment of the chromosome. Integration of the KC-*manA1* phage should result in loss of this fragment and formation of PvuII and EcoRI-PvuII junction fragments of 2027 and 3758 bp respectively (Figure 3A). Analysis of EcoRI-PvuII digests of genomic DNA from the lysogen revealed that the phage had integrated correctly (Figure 3B). The mutant strain was named *S. nodosus manA*.

It was not known whether insertional inactivation of *manA* would have polar effects on downstream genes. Charaniya and co-workers (2007) have developed algorithms for predicting how *S. coelicolor* genes are grouped into operons. In *S. coelicolor*, the *manB* gene is predicted to be the sole gene in a transcription unit. The SIS protein gene and the downstream *manA* are predicted to form an operon that does not include the cation efflux protein gene which lies 241 bp further downstream. The same predictions apply to *S. nodosus* because the genes and intergenic sequences are conserved. Since *manA* and *manB* appear to be the last genes in their transcription units, insertional inactivation should not have polar effects on downstream genes.

3.4 Generation of a *manA-manB* double mutant

A *manA-manB* double mutant was obtained during attempts to replace the chromosomal *manA* gene with a mutated version. A frameshift mutation was introduced into a plasmid-borne copy of the gene by cutting an internal NotI site, filling in the cohesive ends and religation. Resequencing showed that the NotI site (nucleotides 16899-16906 of EU834702) had been removed correctly (not shown).

A 5100 bp BclI-ScaI fragment (nucleotides 14246 – 19346 of EU834702) was cloned between the ScaI and BamHI sites of the KC-UCD1 vector to give recombinant phage KC-*manA2*. Propagation on *S. nodosus* gave a single thiostrepton-resistant lysogen. The expected integration event is shown in Figure 4. Southern hybridisation analysis of genomic DNA revealed that the lysogen contained a deletion in the *manB* region (not shown). The extent of the deletion was determined by PCR analysis (Figure 5). Primers were designed to cover the start and stop codons of ORF12, *manB*, ORF10 and ORF9. All primer pairs amplified the expected products from wild-type genomic DNA. Analysis of genomic DNA from the KC-*manA2* lysogen revealed that the *manB* and ORF10 genes had been deleted. ORF9 and the upstream genes remained intact. The *manA* sequence amplified from the lysogen was resistant to digestion with NotI. These results show that the deletion is as shown in Figure 4. The KC-*manA2* lysogen was deficient in both the *manA* and *manB* genes and was named *S. nodosus* Δ *manAB*. This mutant is also deficient in ORF10 which encodes a hypothetical protein. BLAST searches revealed that homologues of ORF10 are located just upstream from the PMM genes of over forty actinomycete bacteria but not other organisms. This suggests that the ORF10 product may have an auxiliary role in GDP-mannose biosynthesis in actinomycetes, possibly by acting as a chaperone for PMM.

3. 5 Characterisation of *manA* and *manAB* mutants

The *manA* and the *manAB* mutant strains grew normally on enriched media. All three strains were grown on *Streptomyces* medium and cell lysates were assayed for PMI and PMM. Wild type *S. nodosus* contained PMI with a specific activity of 1.032 units / mg protein. PMI activity was undetectable in the *manA* and *manAB* mutants. These results indicated that the *manA* gene encodes the major PMI activity in cells grown on this medium. PMM activity in lysates was below the detection limit of the standard assay, even with wild type *S. nodosus*. It was not possible to assess directly the effect of the *manB* deletion on PMM activity.

S. nodosus and the *manA* and *manAB* mutants showed identical growth when streaked on mannose-free minimal medium agar containing glucose as sole carbon source. Growth of the two mutants was barely discernible with mannose as sole carbon source. Growth on mannose would require PMI-catalysed conversion of

mannose-6-phosphate to fructose-6-phosphate, which would enter the central pathways of carbohydrate catabolism.

Inclusion of mannose in enriched solid media was found to enhance sporulation in *S. nodosus* and the *manA* mutant. The *manAB* double mutant sporulated poorly. Some of the genes in the *manA-manB* region have been implicated in differentiation and development of streptomycetes. The cysteine dioxygenase gene is important for aerial mycelium formation and the *whiB* regulatory gene of *S. coelicolor* is essential for sporulation. These observations suggest that GDP-mannose may be essential for synthesis of a glycoconjugate found in spore walls.

3. 6 Effects of *manA* and *manAB* mutations on polyene glycosylation

Amphotericins A and B are potent antifungal agents whereas their aglycones have no activity (Byrne et al., 2003). A bioassay method was devised for sensitive qualitative detection of active glycosylated polyenes in crude samples. Spores of *S. nodosus* and the two mutants were grown as dense cross-streaks on glucose minimal agar (Kieser et al., 2001) and on glucose minimal agar supplemented with 1% (w/v) (55.6 mM) mannose. 0.25ml sections of agar adjacent to the bacterial growth were excised and extracted with 1 ml volumes of butanol. Scanning of UV-visible absorption spectra revealed that these extracts contained approximately 1 µg polyene per ml, along with contaminating material. The agar plates were overlaid with molten cooled yeast agar seeded with *S. cerevisiae* cells. After overnight incubation, lawns of yeast cells formed with clear zones of inhibition around streaks of strains producing active amphotericins. The *manA* mutant produced antifungal activity even when grown in the absence of mannose. This suggests that at least some polyene glycosylation occurs despite inactivation of the *manA* gene. The *manAB* mutant produced no antifungal activity in the absence of mannose but gave a slight inhibition zone when supplemented with mannose.

Attempts were made to grow *S. nodosus* on defined liquid minimal media in which the mannose content could be controlled. However, very low yields of biomass and polyenes were obtained. The three strains were grown on standard production medium to obtain material for analysis by mass spectrometry. The concentration of free mannose in this medium was found to be 12.19 µM. Inclusion

of 100mM glycerol in the medium maximised yields but increased the tetraene to heptaene ratio so that tetraenes were predominant. All three strains yielded about 50mg total polyene per litre. Polyenes were purified by gel filtration in methanol and the major tetraene peaks were analysed by electrospray mass spectrometry (ESMS). Typical results are shown in Figure 6. The *S. nodosus* polyene consisted of amphotericin A ($[M - H]^- = 924.5$) (Figure 6A). The polyene from the *manA* mutant contained amphotericin A ($[M - H]^- = 924.5$) and the aglycone 8-deoxyamphoteronolide A ($[M - H]^- = 763.5$). The two species were equally prominent in the mass spectrum (Figure 6B). Previous work has shown that C-8 hydroxylation of the macrolactone ring occurs after mycosamine is added (Byrne et al., 2003). The free mannose content of the growth medium (12.19 μ moles/litre) would account for partial glycosylation of the total polyene (62.5 μ moles/litre). Additional mannose may be available from polysaccharides in the growth medium. Further partial glycosylation might result from weak complementation of the PMI deficiency by another activity (Seeholzer, 1993). The bioassay data (see above) suggest that this is the case. However, the fact that glycosylation was incomplete shows that *manA* is important for amphotericin biosynthesis.

The *manAB* mutant gave the aglycone as the major product (Figure 6C). A minor component with antifungal activity was found to elute just ahead of the main inactive aglycone peak. ESMS analysis of the active fraction revealed mostly aglycone with a trace of amphotericin A (not shown). This shows that complementation of the *manB* deletion by other genes occurs, but only at a low level. *S. coelicolor* has a phosphoglucomutase encoded by SCO7443 that catalyses conversion of glucose-6-phosphate to glucose-1-phosphate for glycogen biosynthesis (Ryu et al., 2006). Some bacterial phosphoglucomutases catalyse slow conversion of mannose-6-phosphate to mannose-1-phosphate (Regni et al., 2004). The detection of traces of amphotericin A suggests that the *manAB* mutant can still carry out limited synthesis of other mannose-containing glyconjugates.

Polyene aglycones have also been produced by inactivating genes responsible for later stages of the mycosamine pathway. Inactivation of *manB* results in efficient aglycone formation and depletion of mannose-1-phosphate. Thus *manB* mutants should be useful for *in vivo* glycorandomisation.

4. Discussion

This study has shown that the *manA* and *manB* genes are important for amphotericin glycosylation. Disruption of both genes did not impair growth or production of amphotericin aglycones. A previous study reported inactivation of the *amphDIII* gene, which encodes a GDP-mannose 4, 6 dehydratase that catalyses the first step in the mycosamine-specific pathway (Byrne et al., 2003). The *amphDIII* mutant produced mostly 8-deoxyamphoteronolide A along with some mannosyl-amphoteronolide A. This showed that in the absence of GDP-mycosamine, the AmphDI glycosyl transferase can catalyse slow transfer of mannose from GDP to 8-deoxyamphoteronolides. The *manAB* mutant did not produce high levels of abnormally glycosylated amphotericins. This indicates that *S. nodosus* does not normally contain alternative hexose-1-phosphates that can be efficiently activated as GDP-sugars and transferred to amphotericin aglycones. *In vitro* studies have shown that typical GMPP enzymes can only activate a narrow range of alternative sugar-1-phosphates in the absence of mannose-1-phosphate (Watt et al., 2000; Yang et al., 2005). *In vivo* glycorandomisation of polyene aglycones will require co-expression of engineered anomeric kinases, guanosine nucleotidyl-transferases and glycosyl transferase genes. Inactivation of *manA* and *manB* genes could also facilitate engineering glycosylation of other bioactive natural products that contain GDP-mannose-derived sugars.

In future work, *manAB* mutants could be used to evaluate a different strategy for introducing unnatural hexose-1-phosphates into streptomycete cells. In humans, mutations in PMM genes cause the most common of the congenital deficiencies in glycosylation, severe inherited diseases that result from defective mannosylation of glycoproteins. Different membrane-permeable derivatives of mannose-1-phosphate have been synthesised as prodrugs that bypass the block in the pathway to GDP-mannose (Eklund et al., 2005; Muus et al., 2004). The negative charges on the phosphate group were neutralised with acetoxymethyl or cyclosaligenyl groups whereas the sugar hydroxyls were esterified with acetyl or ethylcarbonate groups to increase hydrophobicity. On feeding to cultured fibroblast cells, these neutral derivatives traversed the cytoplasmic membrane and were converted to mannose-1-phosphate in the cytosol. They were incorporated into GDP-mannose more efficiently than mannose and restored normal glycosylation in PMM-deficient cultured fibroblast cell lines. The *S. nodosus manAB* mutant can now be used to

assess whether similarly protected hexose-1-phosphates can enter streptomycete cells.

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Figure legends

1. Structures and masses of amphotericins A and B and 8-deoxyamphoteronolides A and B.
2. Overview of GDP-mannose biosynthesis.
3. Insertional inactivation of *manA* gene. Integration of phage KC-*manA1* results in loss of a 3.6 kb PvuII fragment and formation of new 3.7 kb PvuII-EcoRI and 2.0 kb PvuII fragments (panel A). Panel B shows Southern hybridisation analysis of EcoRI-PvuII digests of genomic DNA from *S. nodosus* (lane 1) and the lysogen (lane 2). A digoxigenin-labelled internal sequence from *manA* was used as probe.
4. Formation of a strain with inactivated *manA* and deleted *manB* genes. The KC-*manA2* phage integrated as shown but the right hand junction region underwent a deletion that extended as far as ORF10. PCR evidence is shown in Figure 5.
5. Analysis of *manAB* mutant by PCR. Genomic DNA was isolated from *S. nodosus* and from the *manAB* mutant. PCR was used to detect the *manA*, ORF12, *manB*, ORF10 and ORF9 sequences. Odd and even-numbered lanes contain DNA amplified from wild type and the *manAB* mutant, respectively. Reaction products were analysed by agarose gel electrophoresis as follows: lanes 1 and 2, *manA*; 3 and 4, ORF12; 5 and 6, *manB*; 7 and 8, ORF10; 9 and 10, ORF9. The *manB* and ORF10 sequences were not detected in the *manAB* mutant (lanes 6 and 8). The *manA* region

amplified from *S. nodosus* was digested with NotI (lane 11) whereas this region amplified from the *manAB* mutant was resistant to NotI digestion (lane 12). In control digests, the *manA* region amplified from both strains was cut by BclI (lanes 13 and 14). These results show that the lysogen has an inactive *manA* gene and a deletion of *manB* and ORF10, as shown in Figure 4.

6. Analysis of polyenes by ESMS. Polyenes were analysed in negative ion mode. Panels A, B and C show analyses of polyenes from *S. nodosus*, the *manA* mutant and the *manAB* mutant. The major peaks at 924.6 (panels A and B) are consistent with amphotericin A ($[M - H]^- = 924.5$). The peaks of 763.5 (panels B and C) are consistent with the aglycone 8-deoxyamphoteronolide A ($[M - H]^- = 763.5$). The peak at 908.6 in panel A corresponds to 8-deoxyamphotericin A ($[M - H]^- = 908.5$). The peak at 922.6 results from amphotericin B ($[M - H]^- = 922.5$). The peak at 779.5 in panel C is amphoteronolide B ($[M - H]^- = 779.5$). Other peaks result from the natural abundance of carbon-13. Peaks at 925.6 and 926.6 represent amphotericin A molecules containing one or two ^{13}C atoms, respectively. Peaks at 764.5 and 765.5 result from 8-deoxyamphoteronolide A molecules containing one or two ^{13}C atoms respectively.

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Table. 1 Oligonucleotides used in this study.

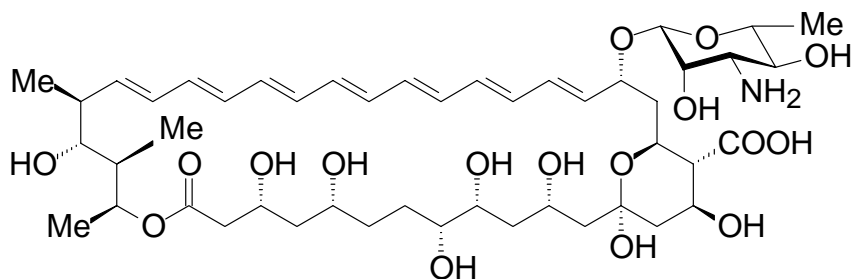
Oligonucleotide	Sequence 5' → 3'
ManA-F	GATCGGATCCGGACACCTCGACCTTCTCGC
ManA-R	GATCCTGCAGCTAGCGCTGGGGCTCCACCAC
ORF10-F	TCAGTTGTCCGGTGAGCGGAGCAC
ORF10-R	AGGGTCGTGAGCCCTGTACGTC
ORF12-F	CAGGATCACGCCCCGAAGC
ORF12-R	TGCCGACCTGCTCGACGAAAC
ManB-F	GACATCAGCCCCGGATGATCTC
ManB-R	GTTGGCCGTGGCTGCTGATCTG
ORF9-F	TAGGCTTCGACGGTGATGGACA
ORF9-R	CTGAAGAAGCGGCTGAAG AAG

Table 2. Functions of genes in sequenced region.

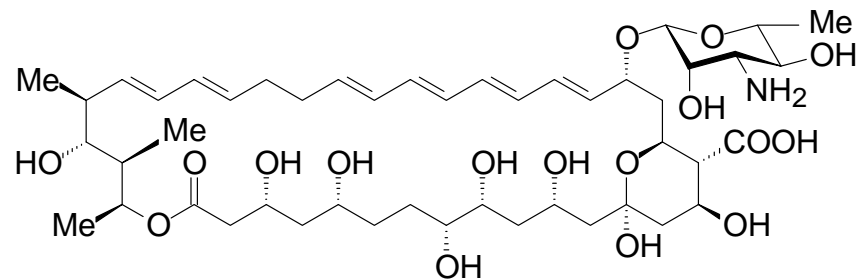
ORF	Relative Orientation	Protein	Size (AA)	% Identity ^a
1 (<i>manC</i>)	↓	GDP-mannose pyrophosphorylase	360	88
2	↑	Endonuclease	333	77
3	↑	F ₄₂₀ O-γ-glutamyl ligase	426	90
4	↑	F ₄₂₀ phospholactate transferase	319	91
5	↑	Cysteine dioxygenase	187	83
6	↓	Sporulation regulatory protein	87	100
7	↓	Glycosyl transferase	1534	76
8	↓	Hypothetical protein	176	81
9	↑	Hypothetical protein	149	81
10	↓	Hypothetical protein	125	94
11 (<i>manB</i>)	↓	Phosphomannomutase	454	86
12	↓	SIS regulatory protein	375	79
13 (<i>manA</i>)	↓	Phosphomannose isomerase	383	82
14	↓	Cation efflux protein	326	83
15 (truncated)	↓	S-adenosylhomocysteinase	(230)	90

^a % Identity refers to amino acid sequence identity with *S. coelicolor* homologue.

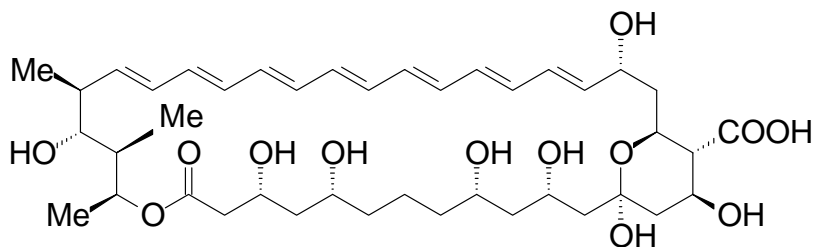
Figure 1



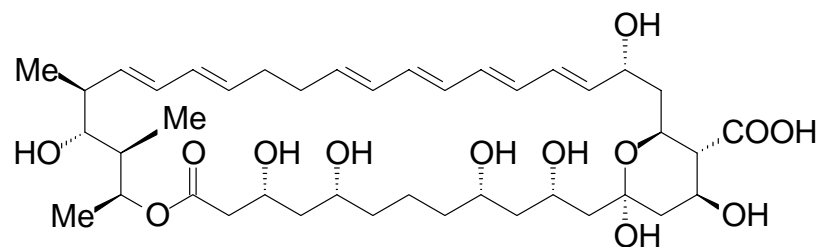
Amphotericin B
RMM = 923.5



Amphotericin A
RMM = 925.5



8-Deoxy-amphoterolide B
RMM = 762.5



8-Deoxy-amphoterolide A
RMM = 764.5

Figure 2

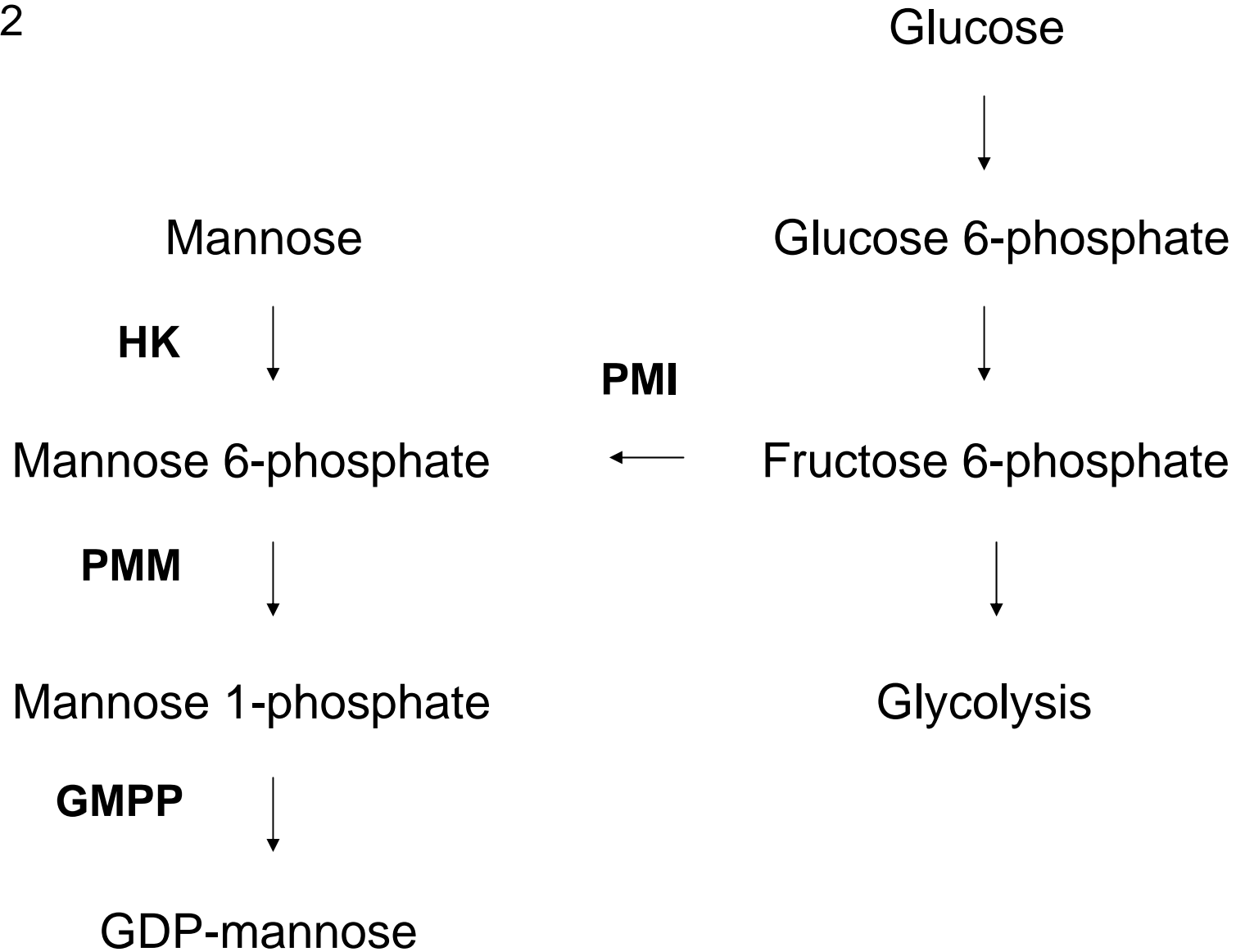


Figure 3

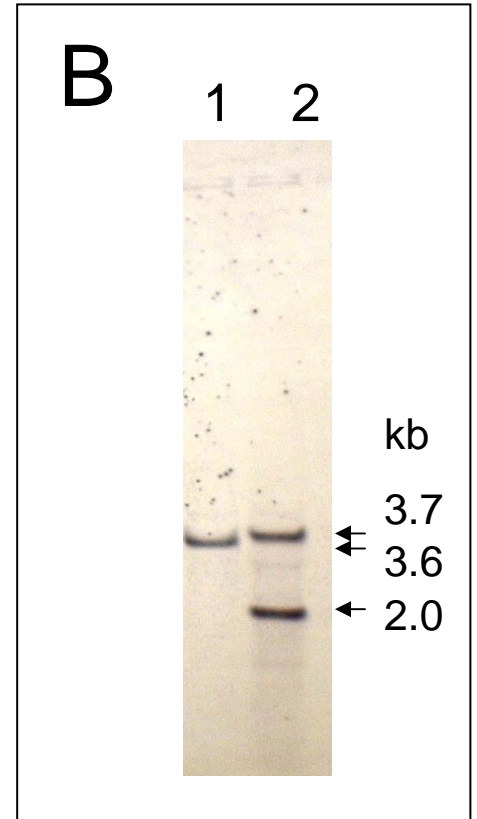
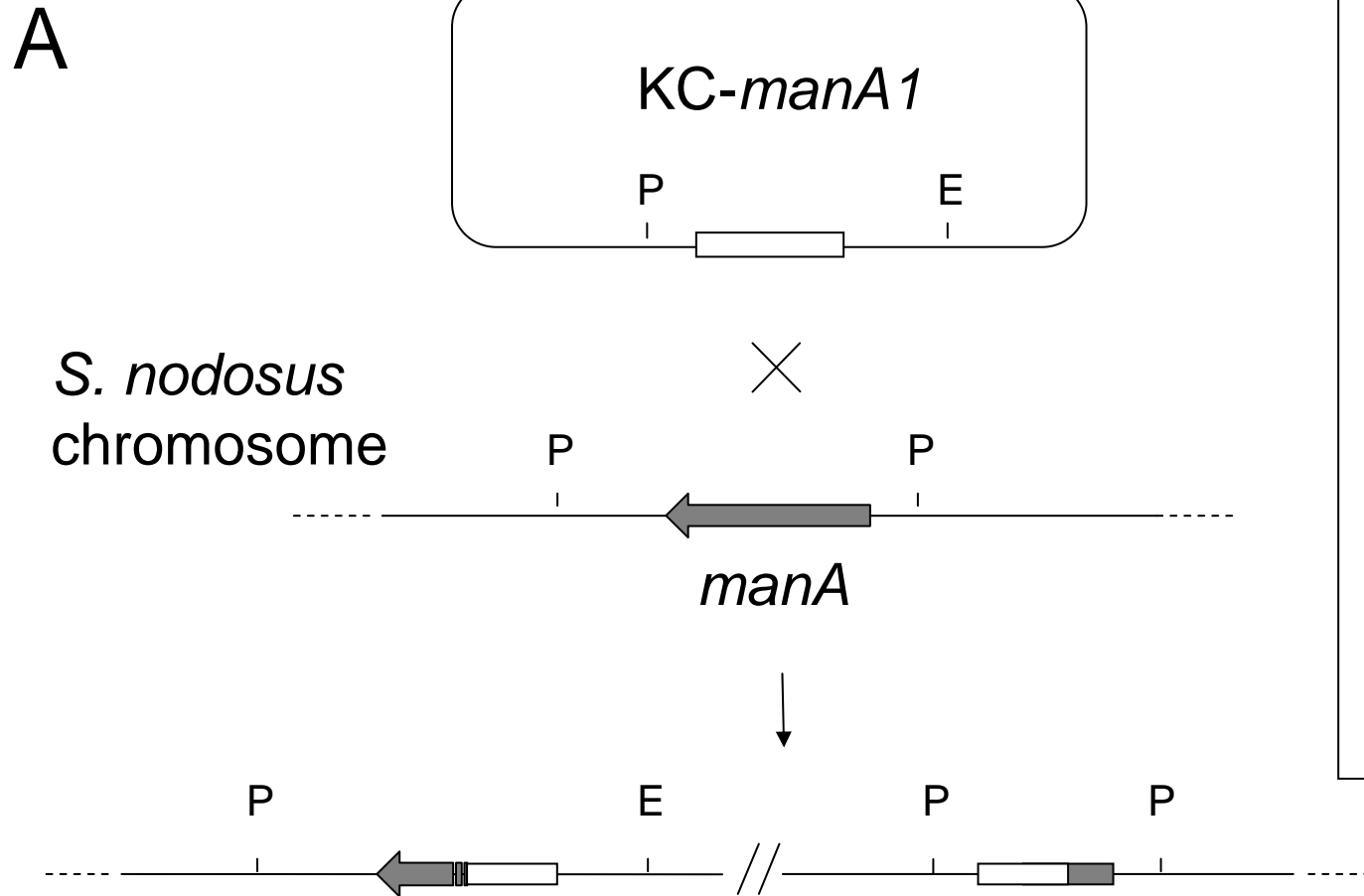


Figure 4

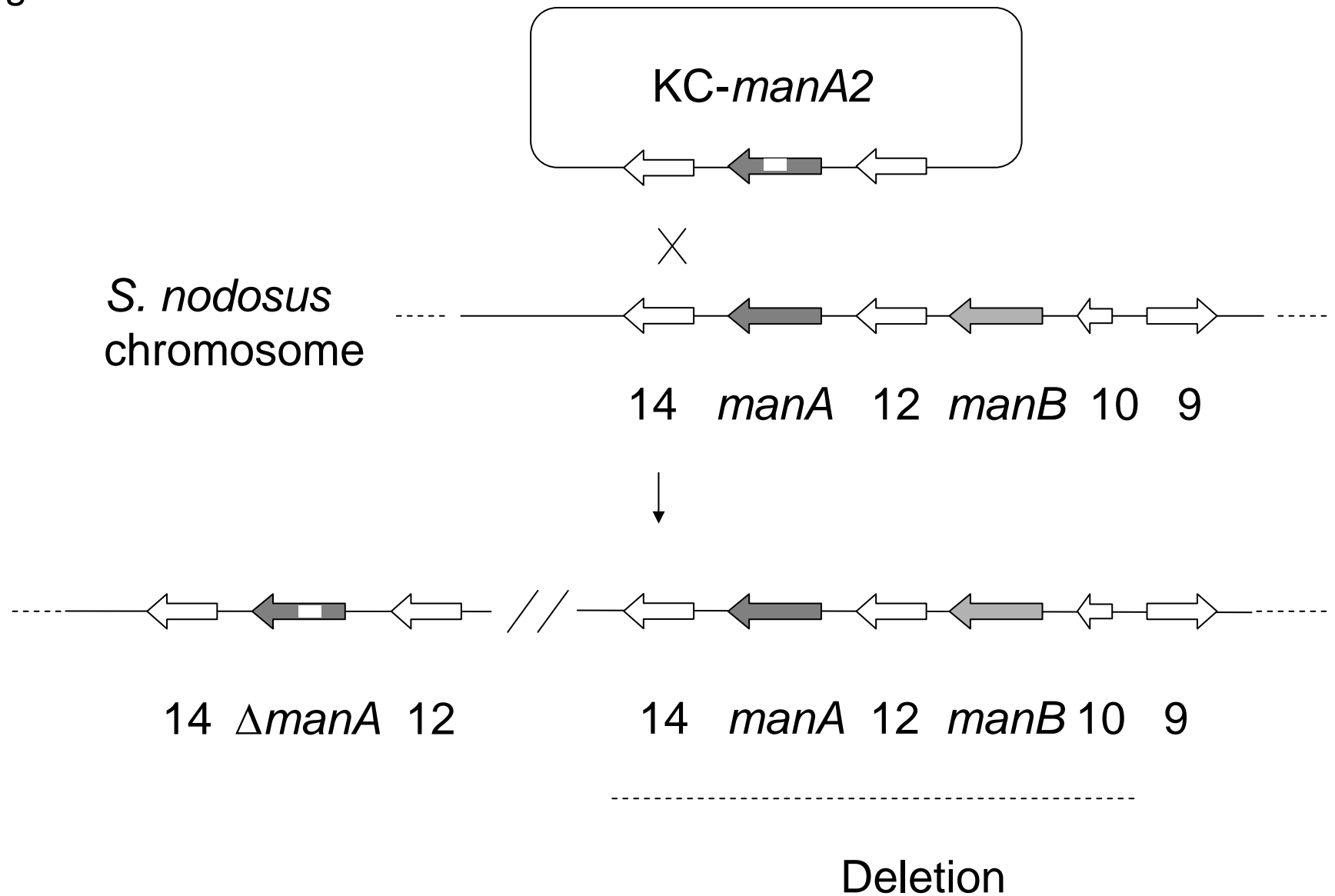


Figure 5

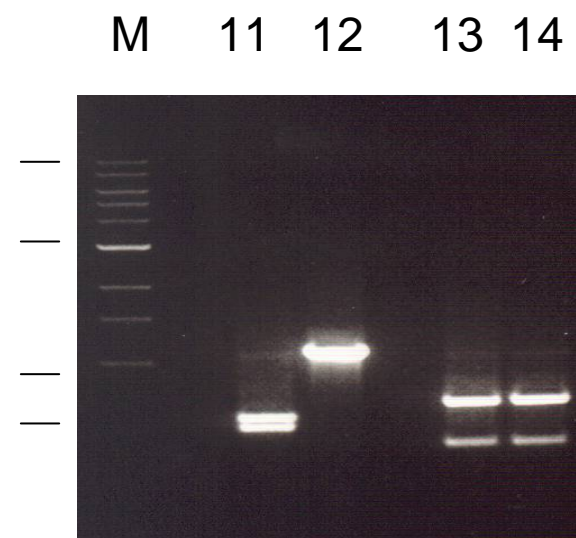
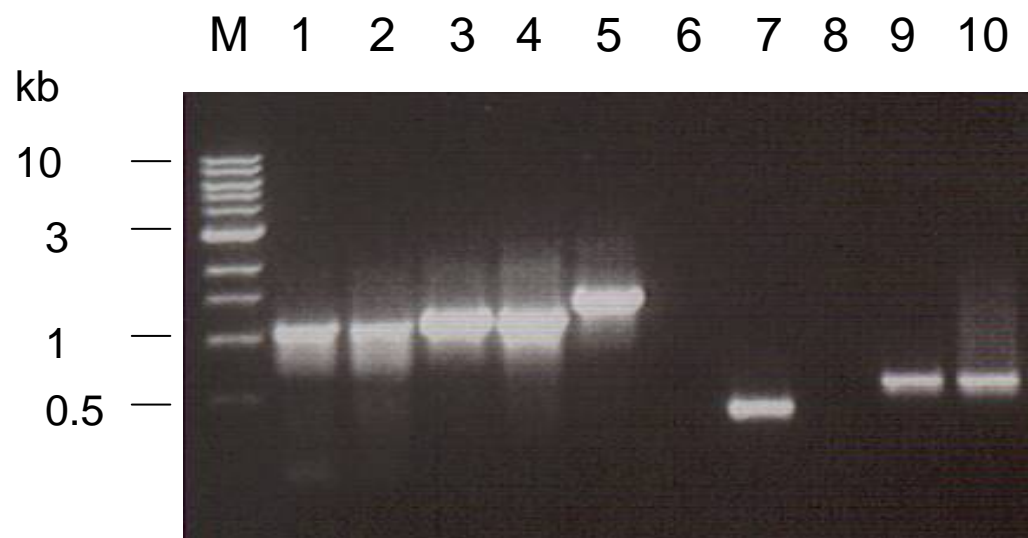


Figure 6

