Increased Sensitivity to Chloramphenicol by Inactivation of manB in Streptomyces coelicolor

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Phosphomannomutase (ManB) is involved in the biosynthesis of GDP-mannose, which is vital for numerous processes such as synthesis of carbohydrates, production of alginites and ascorbic acid, and post-translational modification of proteins. Here, we discovered that a deletion mutant of manB (BG101) in Streptomyces coelicolor (S. coelicolor) showed higher sensitivity to bacteriostatic chloramphenicol (CM) than the wild-type strain (M145), along with decreased production of CM metabolites. Deletion of manB also decreased the mRNA expression level of drug efflux pumps (i.e., cmtr1 and cmtr2) in S. coelicolor, resulting in increased sensitivity to CM. This is the first report on changes in antibiotic sensitivity to CM by deletion of one glycolysis-related enzyme in S. coelicolor, and the results suggest different approaches for studying the antibiotic-resistant mechanism and its regulation.

Keywords: Streptomyces coelicolor, phosphomannomutase, manB, antibiotic resistance, chloramphenicol, mannosylation

Phosphomannomutase (ManB) is involved in the synthesis of GDP-mannose that is used for the production of carbohydrates [2, 5], ascorbic acid [4], alginate polymer for cell encapsulation [26], lipo- and exopolysaccharides in many pathogenic bacteria [15, 24, 27], and branched glycosyl and glycoconjugates [16] in prokaryotes and eukaryotes. The function of ManB (also known as PMM) is the reversible conversion of mannose-6-phosphate to mannose-1-phosphate, which is subsequently modified to GDP-mannose [19]. In the report, we showed ManB from S. coelicolor to be bifunctional, having activity for both glucose-6-phosphate and mannose-6-phosphate, as well as being different from ManB from E. coli, which is specific for mannose-6-phosphate only [25]. In addition, we also showed that deletion of manB from S. coelicolor greatly increased actinorhodin (ACT) production in Difco nutrient media. Moreover, blocking of PMM-regulated conversion of mannose-6-phosphate to mannose-1-phosphate has a considerable effect on ACT production, suggesting that ManB plays an important role in the direct and/or indirect control of antibiotic production.

As a sequential work on ManB, we examined the sensitivities of the wild-type strain and manB deletion mutant (BG101) to different antibiotics, as there have been several reports of decreased susceptibility of deletion mutants related to GDP-mannose and inositol biosynthesis, including enzymes such as GDP-mannose pyrophosphorylase, phosphoglcomutase, phosphoisomerase, and inositol monophosphate phosphatase [3, 7, 8, 11, 18]. The loss of these enzymes results in attenuated or complete loss of virulence: changes in cell wall rigidity, capsule thickness, permeability, intracellular survival rate, and cell size [3, 7, 8, 11, 18]; reduced bacterial resistance to oxidative stress; and higher susceptibility to the antimicrobial peptide cecropin P due to the inability to produce wild-type LPS [6, 14]. In this paper, we discuss the properties of a deletion mutant of manB in S. coelicolor showing variable sensitivity to chloramphenicol (CM) in comparison with the wild-type strain as well as its effects on major
facilitator superfamily drug efflux pumps such as cmlR1 and cmlR2 [22]. To our knowledge, this is the first report on changes in sensitivity to CM in S. coelicolor by deletion of a glycolysis-related enzyme.

MATERIALS AND METHODS

Bacterial Strains and Media

All Streptomyces strains used in this work are listed in Table 1. The culture method for strain M145 followed standard procedures [13]. Briefly, fresh spores of wild-type strain (M145) (7.92 x 10^6 colonies/μl) and manB deletion mutant (BG101) (5.32 x 10^5 colonies/μl) were collected on R55 composed of 103 g of sucrose, 0.25 g of K2SO4, 10.12 g of MgCl2·6H2O, 10 g of glucose, 0.1 g of Difco casein amino acids, 2 ml of a trace element solution (composed of 40 mg of ZnCl2, 200 mg of FeCl3·6H2O, 10 mg of CuCl2·2H2O, 10 mg of MnCl2·4H2O, 10 mg of Na2B4O7·10H2O, and 10 mg of (NH4)2MoO4·4H2O in 1 L of distilled water), 5 g of yeast extract, 5.73 g of TES buffer, and 7 ml of 1 N NaOH in 1 L of distilled water.

RT-PCR (Reverse Transcription-PCR)

RNA extraction from late-treated cells was carried out using an RNeasy mini kit (Qiagen, USA) according to the manufacturer's instructions. The concentration of RNA was determined by measuring the absorbance at 260 nm (A260) using a NanoDrop ND-1000 spectrophotometer. The purity of the RNA was estimated from the absorbance ratio at 260 nm and 280 nm (A280/A260). RNA was reverse-transcribed into first-strand cDNA in the presence of 1 μg of total RNA, 5x First-Strand Buffer, 0.5 mM dNTP mix, 200 units of SuperScript III Reverse Transcriptase (Invitrogen, USA), 40 units of RNaseOUT recombinant RNase inhibitor, 3 μg of random hexamer, and 5 mM DTT in a final reaction volume of 20 μl. Reactions were carried out in a water bath at 25°C for 5 min and 50°C for 30 min, followed by heating to 70°C for 15 min and then cooling to 4°C. The synthesized cDNA was amplified by PCR in a total volume of 50 μl containing 10 μM of each primer, 2 μl of cDNA, 4 units of LA Taq DNA polymerase (TAKARA, Japan), 2x GC II buffer, and 200 μM dNTP mix. HsdB was used as a control to normalize the expression level of each mRNA as well as sample-to-sample variations. Gel band intensities of hsdB, SOC1234, SOD526 (cmlR1), and SOC7662 (cmlR2) were compared after 25 cycles of PCR to ensure that DNA amplification was in the linear range for each template in kinetic analysis. The PCR products were visualized by 2% (w/v) agarose gel electrophoresis with ethidium bromide staining.

Cell Viability Test

For the preparation of S. coelicolor cells, 0.5 μl of sterile stock solution (5.4 x 10^6 colonies/μl) was cultured in 20 ml of R55 medium in a 250 ml baffled flask. One microliter of each sample was taken after 24 h, followed by centrifugation in a microcentrifuge for 5 min, after which cells were washed twice. Then, 1 ml of sterilized water was added, and 100 μl of the cells was spread by bead. After fully

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drying, filter paper was set on the cell. Then, 1 μl of each antibacterial reagent was added, and the plates were incubated for 24 h at 30°C.

HPLC Analysis of CM and Its Metabolites
To compare the changed amount of CM metabolites between M145 and BG101, cells were inoculated and grown in two 100 ml flasks containing 15 ml of R5 medium each for 48 h. Cells were then harvested by centrifugation at 13,000 ×g and sequentially washed with sterilized water two times before inoculation. Both cell types were added to a 50 ml falcon tube at a wet weight of 300 mg and then mixed with 30 ml of CM solution (250 μg/ml of CM in distilled water). This was followed by incubation for 12 h at 30°C, after which 100 μl of sample was used for HPLC analysis. The amount of CM and its metabolites were analyzed using a HPLC (Autochrom-3000; Young Lin, South Korea) equipped with a UV/Vis detector and Waters symmetry C18 column (4.6 mm × 150 mm, 5.0 μm particle size; Waters, Milford, MA, USA). The flow rate was 1 ml/min and the injection volume was 10 μl. The mobile phase solvent was acetonitrile:water [3:7 (v/v)].

RESULTS

Increased Sensitivity to CM by Deletion of manB
Wild-type M145 and the manB deletion mutant of S. coelicolor (BG101) were exposed to various chemicals and peptide antibacterial reagents as described in Materials and Methods. The used antibiotics included cyclic peptides (bacitracin and nisin), glycopeptide (vancomycin), aminoglycosides (kanamycin and apramycin), β-lactams (ampicillin and carbenicillin), and CM. These antibacterial agents target cell wall synthesis (bacitracin, nisin, vancomycin, ampicillin, and carbenicillin) and protein synthesis (kanamycin, apramycin, and CM) [20]. Among these examined antibacterial reagents, wild-type S. coelicolor M145 was found to be sensitive to apramycin (5 μg/μl), kanamycin (5 μg/μl), vancomycin (2.5 μg/μl), and bacitracin (5 μg/μl); however, it survived when exposed to carbenicillin (2.5 μg/μl), CM (5 μg/μl), nisin (5 μg/μl), and ampicillin (10 μg/μl) (Fig. 1A). BG101 exhibited a similar sensitivity pattern to each antibacterial reagent at the same concentration, except for CM (Fig. 1B). Compared with M145, BG101 clearly showed increased sensitivity to CM at 5 μg/μl (Fig. 1). At increased concentration of CM (50 μg/μl), both strains did not survive (data not shown); however, at 5 μg/μl, only BG101 did not survive.

Complementation Studies with pgm, pnm from E. coli, and manB from S. coelicolor
Based on the increased sensitivity of BG101 to CM, different complementation strains were examined to determine

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Fig. 1. Antibacterial sensitivity tests of antibacterial reagents with M145 (A) and BG101 (B). 1, 1/10, 1/100, and 1/1,000 stand for the serial dilutions of antibacterial reagents.
which function of ManB, either its phosphoglucomutase or phosphomannomutase activity, alters sensitivity to CM. M145 and BG101 cells were treated with 5 μg or 2.5 μg CM (data not shown). It was found that only M145 cells could survive whereas BG101 died eventually, as explained in the previous section. The same amount of CM was treated to BG103 cells containing phosphoglucomutase from S. coelicolor, BG104 containing manB from E. coli, which has phosphomannomutase activity only in BG101, and BG105 containing manB (which has both phosphomannomutase and phosphoglucomutase activities) from S. coelicolor (Fig. 2). BG103 cells did not come through the CM treatment (Fig. 2A), whereas BG104 and BG105 did survive (Fig. 2B and 2C), suggesting that phosphomannomutase activity is critical for the sensitivity of S. coelicolor to CM.

**Changes in CM Metabolites**

To monitor the direct effect of manB deletion on CM level, we measured the decrease in CM as well as the production of CM metabolites after incubating cells with CM (Fig. 3). At 0 h, there was no substrate except CM in the sample (Fig. 3A). However, after 12 h, 79% of CM was converted to CM metabolites in M145 (Fig. 3B). At this point, the relative level of CM metabolites in BG101 cells was found to be 60% of that in M145 (Fig. 3D). Both strains demonstrated changes in metabolite production (Fig. 3B and 3C), which explains the antibiotic resistance. Initially it was expected to be phosphorylated CM like *Streptomyces venezuelae* [17]; however, consecutive analysis of CM metabolites by ESI-MS proved it was not a CM metabolite but CM derivatives based on MS/MS, although its structure was not fully understood (data not shown). The decreased amount of CM was responsible for the increased sensitivity of BG101 to CM as compared with M145 [17]. The reason why it showed less amount of CM metabolites in BG101 was not clear yet; however, considering that deletion of inositol monophosphate phosphatase in *Mycobacterium* results in alteration of the cell surface, thereby decreasing the permeability to lipophilic molecules, the increased sensitivity of BG101 could be explained by simple alteration.

![HPLC analysis of CM and metabolites.](image)

**Fig. 3.** HPLC analysis of CM and metabolites. HPLC peaks at 0 h (A), at 12 h in M145 (B), and at 12 h in BG101 (C) are shown. The relative amounts of CM metabolites were compared with the amount of CM with M145 (100%) (D). Peak areas of HPLC were divided by wet weight.
of the cell surface [18] or other reasons such as drug efflux pump or resistance genes like acetylation or phosphorylation [17, 22].

**DISCUSSION**

Phosphomannomutase is known to be involved in the reversible conversion of mannose-6-phosphate to mannose-1-phosphate, which is subsequently modified to GDP-mannose [19]. ManB is thought to supply the building blocks of various polymers, such as phosphatidylinositol mannoside, exopolysaccharide, and alginate [15, 16, 26], and function in metabolic pathways. However, its sensitivity to antibiotics has never been reported, although decreased virulence factor was reported [3, 7]. When the amount of CM metabolites was compared, we found that deletion of manB resulted in less production of CM metabolite. When the mRNA expression level of CM drug efflux pump genes such as cmlR1 and cmlR2 were compared, decreased expression of both cmlR1 and cmlR2 was observed, which resulted in increased sensitivity to CM in BG101 cells. There are other possible explanations for the increased sensitivity of BG101 cells to CM, like changes in hydrophobicity of the cell wall [18], deglycosylation of key regulators resulting in loss of activity [23], and changes in the phosphorylation of CM [17]. Among them, we found that increased sensitivity to CM by BG101 cells and direct changes in the gene expression of both transporters in *S. coelicolor* resulted in altered phosphorylation of CM. Although one reason for the antibiotic resistance of BG101 to CM was elucidated here, the whole process seems to be more complex, considering the varying roles of mannose and glycosylation in prokaryotes and eukaryotes [1] and the unknown regulatory links from ManB to CM resistance. Nevertheless, these results provide a new direction for drug development and a different approach to avoiding antibiotic-resistant strains involving both a synthetic inhibitor such as manB and an antibiotic reagent such as CM.

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