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Improved oxytetracycline production in *Streptomyces rimosus* M4018 by metabolic engineering of the G6PDH gene in the pentose phosphate pathway

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ABSTRACT

The aromatic polyketide antibiotic, oxytetracycline (OTC), is produced by *Streptomyces rimosus* as an important secondary metabolite. High level production of antibiotics in *Streptomycetes* requires precursors and cofactors which are derived from primary metabolism; therefore it is exigent to engineer the primary metabolism. This has been demonstrated by targeting a key enzyme in the oxidative pentose phosphate pathway (PPP) and nicotinamide adenine dinucleotide phosphate (NADPH) generation, glucose-6-phosphate dehydrogenase (G6PDH), which is encoded by *zwf*1 and *zwf*2. Disruption of *zwf*1 or *zwf*2 resulted in a higher production of OTC. The disrupted strain had an increased carbon flux through glycolysis and a decreased carbon flux through PPP, as measured by the enzyme activities of G6PDH and phosphoglucose isomerase (PGI), and by the levels of ATP, which establishes G6PDH as a key player in determining carbon flux distribution. The increased production of OTC appeared to be largely due to the generation of more malonyl-CoA, one of the OTC precursors, as observed in the disrupted mutants. We have studied the effect of *zwf* modification on metabolite levels, gene expression, and secondary metabolite production to gain greater insight into flux distribution and the link between the fluxes in the primary and secondary metabolisms.

1. Introduction

The aromatic polyketide antibiotic, OTC, is produced by *Streptomyces rimosus* as an important secondary polyketide metabolite. OTC is used particularly heavily in aquaculture, where 500 kg doses may be used in one treatment [1]. In addition, OTC and related tetracycline polyketide compounds are potent inhibitors of bacterial protein synthesis displaying broad-spectrum activity against both Gram-positive and Gram-negative pathogens. Although the clinical use of the tetracyclines has declined in recent years due to the emergence of resistant strains of bacteria, OTC remains the drug of choice for the treatment of intracellular infections caused by *Rickettsia*, *Chlamydia*, and mycoplasma in penicillin-sensitive patients incapable of tolerating macrolides [2].

Current methods used to increase the productivity of industrial microorganisms range from the classical random mutagenesis performed in close association with the optimization of large-scale industrial fermentations, to the use of more rational methods. One of these is metabolic engineering, which is a powerful tool for the introduction and optimization of new cellular processes. There have been several methods and many reports of metabolic engineering to maximize product yields. Since genetic manipulation of a producing organism can be used to alter the metabolic flux distribution and improve the efficiency of production [3], primary metabolic fluxes are redirected by the introduction of genetic modifications through recombinant DNA technology, in a manner that supports high productivity of secondary metabolites [4–6]. In addition, it has been reported that engineering of the availability of coenzyme A (CoA) activated fatty acid precursors in the producer organisms can enhance production of several polyketides including erythromycin [7], oligomycin [8], monensin B [9] and actinorhodin [10], this proves the importance of the availability of precursors and cofactors which are generally formed through the catabolism of various carbon substrates such as fatty acids, monosaccharides or proteins [11,12].

Due to the fact that the supply of intermediates or precursors from the primary metabolic pathway is a prerequisite for the biosynthesis of secondary metabolites, genetic modification of the primary metabolism is likely to be one of the most effective strategies. One of the important primary metabolic pathways is PPP, which provides essential cofactors, such as NADPH, and intermediates, such as acetyl-CoA for cell growth (Fig. 1). Although still largely unclear, links between primary and secondary metabolism

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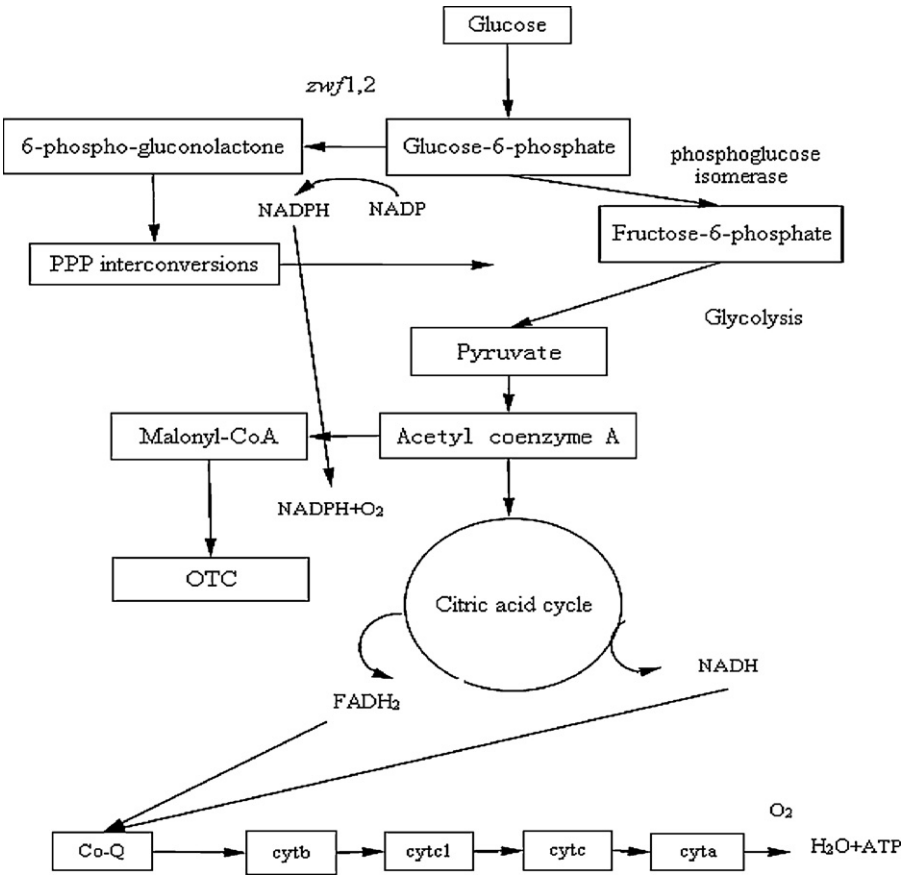

Fig. 1. Simplified carbon central pathways in *S. rimosus*.

have been reported. The physiological effect on cell growth and/or antibiotic production of *zwf*-encoding isozymes of the housekeeping enzyme G6PDH in PPP has been investigated in a variety of bacteria, such as *Escherichia coli*, *Streptomyces lividans*, and *Streptomyces coelicolor*. In one study, deletion of *zwf*1 or *zwf*2 improved actinorhodin (Act) and undecylprodigiosin (Red) production in *S. lividans* [6]. Meanwhile, a positive correlation between methylenomycin production and carbon flux into PPP was observed in *S. coelicolor* [10]; however, until now it has not been clearly shown in *S. rimosus*. Furthermore, to date there have been few reports on the effects of increased G6PDH activity on carbon metabolic flux and antibiotic production in *Streptomyces*, except in the case of *E. coli* [13]. Therefore, in this study, we will show that disruption of *zwf*1 or *zwf*2 can drastically increase OTC production in *S. rimosus* M4018, and interestingly, that a *zwf* gene knock-in strategy reduces production. To investigate this further, the effect of zwf intensification or disruption on metabolite levels, gene expression and secondary metabolite production will be studied to gain greater insight into the flux distribution and the link between the fluxes in the primary and secondary metabolisms.

2. Materials and methods

2.1. Bacterial strains, media, and plasmids

E. coli strains DH5α [14] and ET12567 [15] were used for routine subcloning and conjugation into *S. rimosus* M4018. Organisms were grown at 37 °C in Luria–Bertani medium (1% tryptone, 1% NaCl, 0.5% yeast extract), and standard procedures were used for transformation [16]. *E. coli* transformants were selected with ampicillin (100 mg mL^{-1}), apramycin (50 mg mL^{-1}), kanamycin (10 mg mL^{-1}), or chloramphenicol (25 mg mL^{-1}). The cloning vectors used were pSET152 [17], pKC1139 (Iain S Hunter, University of Strathclyde), pMD19-T (TaKaRa), pT7b3zwf(ri) and pT7b3zwf2. *S. rimosus* M4018 (type strain isolated by Pfizer Ltd. and derived from *S. rimosus* ATCC10970) was grown and manipulated as previously described [17]. For conjugation from *E. coli*, helper plasmid pUZ8002 was used [18]. *S. rimosus* M4018 exconjugants were selected on master plates of mannitol of soy flour (MS) agar [16] containing 50 mg mL^{-1} nalidixic acid and 500 μg mL^{-1} apramycin. The spores from the master plates were harvested to make dense spore plates, which provided inocula for further studies.

2.2. Construction of plasmids and mutant strains

The mutant strains made in this work are listed in Table 1.

2.3. Construction of S. rimosus M4018-1 (zwf1$^+$) and S. rimosus M4018-2 (zwf2$^+$)

To create overexpression of *zwf*1 or *zwf*2, a DNA fragment that contained *zwf*1 (length, 1.7 kb) or *zwf*2 (length, 1.4 kb) was first amplified using primers *zwf*1F and *zwf*1R or *zwf*2F and *zwf*2R (Table 2). The genomic DNA of *S. rimosus* M4018 was used as the template DNA. The *zwf*1 or *zwf*2 was digested with *Nde* I and *Hin* dIII, then cloned into pSET152, and digested with the same restriction enzymes to give pSETzwf1 or pSETzwf2. The plasmids were then transferred via *E. coli* ET12567/pUZ8002 to *S. rimosus* M4018 by conjugation. Integration occurred between the *att*P site located in the plasmid pSETzwf1 or pSETzwf2 and the *att*B site in the chromosome of *S. rimosus* M4018. The transformants were selected by apramycin (500 μg mL^{-1}) and PCR certification. The template for PCR was 1 μL of genomic DNA from the transformants. With respect to *zwf*1$^+$ confirmation, genomic DNA of the control *S. rimosus* (Pfizer) was used as the negative control template, and the SC5A7 cosmid and pT7b3zwf2 vector as the positive control template. As the pSET152 vector integrates site-specifically into the *Streptomyces* chromosome at the ΦC31 attachment site *att*B [19], PCR primers designed to amplify the *att*P and new *att*L and *att*R integration sites formed in the *S. rimosus* chromosome were used (Emma Tilley, personal communication; see Table 2).

2.4. Construction of S. rimosus M4018-3 (Δzwf1)

A single-crossover disruption was constructed in the gene to eliminate Zwf1 activity without affecting the expression of the downstream genes (*opc*, *pgi*, *gnd*, and two hypothetical genes of unknown function). A DNA fragment that contained part of *zwf*1 (length, 1.2 kb) was generated by PCR. Plasmid pT7b3zwf(ri) was used as the template DNA with part of *zwf*1F and part of *zwf*1R (Table 2) as primers, and the amplified fragment was digested with *Eco*R I and *Hin* dIII, then ligated with