Enhancement of avermectin and ivermectin production by overexpression of the maltose ATP-binding cassette transporter in *Streptomyces avermitilis*

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ARTICLE INFO

ABSTRACT

We investigated the function of maltose ABC transporter system encoded by *malEFG-a* and the effect of its overexpression on antibiotic production in *Streptomyces avermitilis*. A *malEFG-a* deletion mutant was unable to grow in a minimal medium with maltose as sole carbon source and produce avermectin. Maltose utilization and avermectin production were restored by introduction of a single copy of *malEFG-a*. RT-PCR analysis showed that the expression of *malE-a* was induced by maltose, and was strongly repressed by glucose. When multi-copy, integrative *malEFG-a* gene expression vectors were introduced into wild-type strain ATCC31267 and ivermectin-producer OI-31, antibiotic production increased by 2.6- to 3.3-fold and the time required for fermentation decreased by about 10%. The overexpression of *malEFG-a* improved the utilization rate of starch, and thereby enhanced avermectin production. Such an approach would be useful for the improvement of commercial antibiotic production using starch as the main carbon source in the fermentation process.

1. Introduction

Starches are used widely in the antibiotic production and fermentation industries as carbon and energy sources (Neway, 1989). They are large polysaccharide molecules, and are generally unable to pass through cell membranes. Starch utilization generally requires the presence of amylases secreted into the medium (Boos and Shuman, 1998) and the transport of the degradation products, mostly maltose and maltodextrins, into the cytoplasm by members of the ATP-binding cassette (ABC) transport system superfamily (Young and Holland, 1999; Holland and Blight, 1999). ABC transporters are ubiquitous membrane protein complexes that transport solutes across the membrane using energy generated by ATP hydrolysis (Higgins, 1992, 1995, 2001; Rees et al., 2009). They do not phosphorylate or otherwise modify their substrates during the transport process (Higgins, 1992; Davidson and Chen, 2004; Davidson et al., 2008). Substrate-binding proteins of ABC transporters are soluble in the periplasm of Gram-negative bacteria, but are anchored to the membrane by lipid modifications in Gram-positive bacteria (Linton and Higgins, 1998). Many reports show that ABC uptake systems specific for maltose and maltodextrin are widespread among bacteria (Davidson et al., 2008). Sequence alignment analysis shows that maltose ABC transporter belongs to the CUT1 subfamily, which comprises transporters specific for di- and oligosaccharides, glycerol phosphate, and polyols (Schneider, 2001). The

maltose ABC transporter in *Streptomyces coelicolor* A3 (2) is composed of the periplasmic maltose-binding protein (MBP) MalE, a membrane-bound complex comprising hydrophobic subunits MalF and MalG, and two copies of the ATPase subunit, MsiK, which is involved in cellobiose and maltose uptake in whole cells (van Wezel et al., 1997). In the process of maltose transport, MalE and membrane-bound complex MalF and MalG cooperate with each other. The maltose ABC transporters in other *Streptomyces*, such as *S. lividins* (Schlösser et al., 2001) and *S. retuculi* (Schlösser et al., 1999), were also characterized and showed a high degree of similarity.

Avermectins, a group of anthelmintic and insecticidal agents (A1a, A1b, A2a, A2b, B1a, B1b, B2a, B2b) produced by *Streptomyces avermitilis*, are widely used in the agricultural, veterinary, and medical fields (Miller et al., 1979; Burg et al., 1979). Among these compounds, the B1 types have the strongest antiparasitic activity. Ivermectins (22,23-dihydroavermectin B1), which are derived from avermectin B1, show less toxic side effects than avermectin B1, and are used in livestock production and in health care of animals and humans (Campbell et al., 1983; Fernandez-de-Mera et al., 2004). We previously reported construction of genetically engineered strain *S. avermitilis* OI-31, which can produce ivermectins directly, by domain swaps of avermectin polyketide synthase; however, the yield of ivermectins needs to be improved (Zhang et al., 2006; Li et al., 2008).

Starch is the most important carbon source in the process of avermectin or ivermectin fermentation (Chen et al., 2007). The amount of starch in fermentation medium is a crucial factor in avermectin production (Ikeda et al., 1988). Since starch is utilized

by *S. avermitilis* for formation of maltose and maltodextrin, the efficiency of maltose/maltodextrin transporters is probably crucial for *S. avermitilis* growth and antibiotic production. *S. avermitilis* contains a homologue of *malEFG* (designated as *malEFG-a*) which is involved in maltose/maltodextrin transport in *S. coelicolor* and other *Streptomyces* (Ikeda et al., 2003). However, the exact function of *malEFG-a* remains to be determined.

We describe here characterization of *malEFG-a* encoding maltose ABC transporter in *S. avermitilis* and potential strain improvement based on the overexpression of maltose ABC transporter for increasing the yield of avermectin or ivermectin.

2. Methods

2.1. Strains and plasmids

Strains used were: *S. avermitilis* ATCC31267 (wild-type; avermectin-producer), *S. avermitilis* OI-31 (ivermectin-producer) (Li et al., 2008), *Escherichia coli* DH5α (used for routine DNA manipulation), *E. coli* ET12567 (*dam dcm hsdS*; used to propagate non-methylated DNA for transformation into *S. avermitilis*) (MacNeil and Klapko, 1987).

E. coli–Streptomyces high copy number vector pKC1139 (Bierman et al., 1992), which contains a *Streptomyces* temperature-sensitive origin of replication, was used for gene disruption by homologous recombination and for introducing multi-copies of *malEFG-a* into *S. avermitilis*. pSET152 (Bierman et al., 1992), which can integrate into the *Streptomyces* chromosome by site-specific recombination at the bacteriophage ΦC31 attachment site (*attB*), was used to introduce a single copy of *malEFG-a* into *S. avermitilis*. pIJ963 (Kieser et al., 2000) was used to provide hygromycin resistance gene (*hyg*) for constructing gene replacement vector. T-vector pMD18-T (TaKaRa, Dalian, China) was used for cloning PCR products.

2.2. Media and culture conditions

E. coli DH5α was grown at 37 °C in Luria–Bertani (LB) medium (Sambrook et al., 1989) and ampicillin (100 μg/ml) or apramycin (100 μg/ml) was added when necessary for plasmid propagation. *S. avermitilis* was grown at 28 °C on solid YMS (yeast extract–malt extract–starch) medium (Ikeda et al., 1988) or on SFM (soya flour–mannitol) medium (Hobbs et al., 1989) for sporulation. RM14 medium (MacNeil and Klapko, 1987) was used for regeneration of protoplasts. YEME (yeast extract–malt extract) medium (Kieser et al., 2000) containing 25% sucrose was used to grow mycelia for the purpose of extracting DNA and preparing protoplasts. When necessary, apramycin was added at a concentration of 10 μg/ml for YMS, 20 μg/ml for RM14, or 5 μg/ml for YEME; hygromycin was added at 50 μg/ml for YMS. Seed medium for avermectin or ivermectin fermentation was composed of 30 g soluble starch, 4 g yeast extract, 2 g soya peptone, and 10 mg $CoCl_2 \cdot 6H_2O$ per liter H_2O. Fermentation medium was composed of 70 g soluble starch, 16 g yeast power, 0.5 g $K_2HPO_4 \cdot 3H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 4 g KCl, 10 mg $CoCl_2 \cdot 6H_2O$, and 2 g $CaCO_3$ per liter H_2O (Chen et al., 2007). SMM (supplemented liquid minimal medium), used for analyzing utilization of carbon sources, was composed of 81.9 ml PEG 6000 (6.1% w/v in distilled water), 5 mM $MgSO_4 \cdot 7H_2O$, 25 mM TES (Tris (hydroxymethyl)-methyl-2-aminoethanesuphonic acid) buffer (pH 7.2), 1 mM NaH_2PO_4, 1 mM K_2HPO_4, 0.1 ml trace element solution (0.1 g/l $ZnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $CaCl_2 \cdot 6H_2O$ and NaCl), and carbon sources (1% w/v) per 100 ml H_2O (Strauch et al., 1991). SYFT (starch–yeast–fermentation medium) was composed of 50 g soluble starch, 12 g yeast extract, 0.5 g $MgSO_4 \cdot 7H_2O$, 4 g KCl, 5 mg $CoCl_2 \cdot 6H_2O$, 0.5 g $K_2HPO_4 \cdot 3H_2O$ for the purposes of extracting RNA.

2.3. DNA manipulation and sequencing

Plasmid and chromosomal DNA were isolated by standard techniques from *Streptomyces* (Kieser et al., 2000) or *E. coli* (Sambrook et al., 1989). DNA cloning and transformation of competent *E. coli* cells were performed as described by Sambrook et al. (1989). *S. avermitilis* protoplast formation, transformation, and regeneration were performed as described previously (Li et al., 2008). PCR was carried out using *PfuUltra* polymerase (Stratagene) as recommended by the manufacturer. DNA sequencing was performed by TaKaRa Biotechnology Co., Ltd. (Dalian, China).

2.4. Construction of malEFG-a deletion mutant

The *malEFG-a* gene replacement vector was constructed as follows: Using genomic DNA of *S. avermitilis* ATCC31267 as template, a 0.87-kb fragment upstream of *malE-a* start codon was amplified by PCR with primers mal1 (5′-ATA TCTAGA GCCAGCAGGAA-GCCG AAG-3′: the engineered *Xba*I site is underlined) and mal2 (5′-AGA AGATCT AGCGGTGCGTAGGGTGT-3′: *Bgl*II), and a 0.83-kb fragment downstream of *malG-a* stop codon was amplified with primers mal3 (5′-CTC AGATCT CCCAACCACTCCTCCGAC-3′, *Bgl*II) and mal4 (5′-ATA GAATTC CCCTGGTAGAGGTAGGCGG-3′, *Eco*RI). The two PCR fragments were then digested with *Xba*I/*Bgl*II and *Bgl*II/*Eco*RI, respectively. The 1.7-kb hygromycin resistance gene (*hyg*) cassette was excised from pIJ963 by *Bgl*II digestion. The above three fragments were simultaneously ligated into *Xba*I/*Eco*RI digested pKC1139 to generate plasmid pMAL4 (Fig. 1A).

Transformation of pMAL4 into ATCC31267 and selection of double-crossover recombination strains were performed as described previously (Li et al., 2008). The putative *malEFG-a* deletion mutant was termed MA18d, and confirmed by PCR analysis using primers mal5 (5′-CGGATGATTCCCGCAACGAAA-3′), hhz (5′-CCATCCCAGC TCGGCAAG-3′), hqf (5′-CGGGATCGCCAATCTCTAC-3′), and mal6 (5′-GAAGAACACGGAGACGGGTA-3′). Primers mal5 and mal6 flank the exchange regions, while primers hhz and hqf are specific for *hyg*. When primer pairs mal5/hhz and hqf/mal6, which are specific for replacement of *malEFG-a* with *hyg*, were used for PCR analysis of MA18d, a 0.75-kb band and a 0.55-kb band appeared, respectively, whereas such bands were not detected when genomic DNA of wild-type strain ATCC31267 was used as the template. These results confirmed that MA18d was a *malEFG-a*-deleted mutant in which the *malEFG-a* gene was completely replaced with *hyg* by double-crossover recombination (Fig. 1A). The deletion in MA18d was also confirmed by Southern analysis as described by Kieser et al. (2000). DNA probe was labeled with digoxigenin-11-dUTP according to the protocols of the manufacturer. Southern blotting were performed by using the digoxigenin-labeled 2-kb fragment of *malEFG-a* as a probe, which was amplified with primers malS1 (5′-GGCTGTTCTTCCTGTCCCTC-3′) and malS2 (5′-CGTC GTGGTTGGACAGCA-3′). The chromosomal DNAs of MA18d and ATCC31267 were isolated and digested with *Pvu*II. There are two *Pvu*II sites in the 3.7-kb fragment of *malEFG-a* and in the probe, whereas only one *Pvu*II site in *hyg* fragment (Fig. 1A). One 1.9-kb hybridizing signal was therefore supposed to be present in the *malEFG-a* deletion mutant, while three hybridizing bands (3.7, 1.5 and 0.6-kb) would be present in the wild-type strain.

2.5. Complementation of the malEFG-a deletion mutant

The *malEFG-a* promoter region with a putative transcriptional start 143-bp upstream of the initiation codon (ATG) of *malE* was predicted using software NNPP 2.2 (http://www.fruitfly.org/seq_-tools/promoter.html). A 3.7-kb fragment of *malEFG-a* carrying the putative promoter and coding region of *malEFG-a* was amplified by PCR from the wild-type strain with primers mal7