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Culture temperature affects gene expression and metabolic pathways in the 2-methylisoborneol-producing cyanobacterium *Pseudanabaena galeata*

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ABSTRACT

A volatile metabolite, 2-methylisoborneol (2-MIB), causes an unpleasant taste and odor in tap water. Some filamentous cyanobacteria produce 2-MIB via a two-step biosynthetic pathway: methylation of geranyl diphosphate (GPP) by methyl transferase (GPPMT), followed by the cyclization of methyl-GPP by monoterpene cyclase (MIBS). We isolated the genes encoding GPPMT and MIBS from *Pseudanabaena galeata*, a filamentous cyanobacterium known to be a major causal organism of 2-MIB production in Japanese lakes. The predicted amino acid sequence showed high similarity with that of *Pseudanabaena limnetica* (96% identity in GPPMT and 97% identity in MIBS). *P. galeata* was cultured at different temperatures to examine the effect of growth conditions on the production of 2-MIB and major metabolites. Gas chromatograph–mass spectrometry (GC–MS) measurements showed higher accumulation of 2-MIB at 30 °C than at 4 °C or 20 °C after 24 h of culture. Real-time-RT PCR analysis showed that the expression levels of the genes encoding GPPMT and MIBS decreased at 4 °C and increased at 30 °C, compared with at 20 °C. Furthermore, metabolite analysis showed dramatic changes in primary metabolite concentrations in cyanobacteria grown at different temperatures. The data indicate that changes in carbon flow in the TCA cycle affect 2-MIB biosynthesis at higher temperatures.

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Introduction

Tastes and odors in tap water are major problems experienced by water utilities worldwide, with the number of consumer complaints being the highest during summer. Two main off-flavor compounds found in water are geosmin and 2-methylisoborneol (2-MIB). The sensitivity of humans to the odor of these compounds is very high (below 10 ng/L). Moreover, these compounds are too stable to be metabolized (Westerhoff et al., 2005). Although treatment with activated carbon removes these compounds (Lalezary-Craig et al., 1988; Cook et al., 2001), this approach is too expensive for continuous use. Furthermore, it is difficult to predict the amount of these compounds in water year to year because the intensity of the odor changes annually even though the condition of the water remains similar. Therefore, a method to predict the occurrence of geosmin and 2-MIB in tap water is needed to reduce consumer complaints and the cost of odor treatments.

Geosmin and 2-MIB are volatile terpenes (sesquiterpene and monoterpene, respectively). The geosmin biosynthesis pathway has been analyzed in actinomycetes and cyanobacteria. Sesquiterpene, farnesyl diphosphate, is converted to geosmin by the bifunctional single sesquiterpene cyclase, geosmin synthase (Cane and Watt, 2003; Gust et al., 2003; Jüttner and Watson, 2007; Agger et al., 2008; Krishnani et al., 2008). The genes encoding geosmin synthase have been identified in several organisms (*Cyc2* from *Streptomyces coelicolor*, *GeoA* from *S. avermitilis*, and *NPUNMOD* from *Nostoc punctuforme*) (Jiang et al., 2007; Ludwig et al., 2007; Giglio et al., 2008).

2-MIB is a methylated monoterpene alcohol, with the additional methyl group derived from S-adenosyl-L-methionine (SAM) (Bentley and Meganathan, 1981). Komatsu et al. (2008) identified

Abbreviations: 2-MIB, 2-methylisoborneol; GPP, geranyl diphosphate; GPPMT, GPP methyltransferase; MIBS, monoterpene cyclase; SAM, S-adenosyl-L-methionine; *P. galeata*, *Pseudanabaena galeata*; CE, capillary electrophoresis; TCA, tricarboxylic acid; *mtf*, methyl transferase; *mtc*, monoterpene cyclase.

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the monoterpene cyclase (*mtc*) gene and the SAM-dependent methyltransferase gene from actinomycetes. Functional analyses demonstrated that these genes are involved in an operon with an unknown functional nucleotide binding protein gene, and that these two genes are responsible for the reaction of geranyl diphosphate (GPP) to 2-MIB via a two-step process (Supplemental Fig. S1): GPP is methylated by methyltransferase (GPPMT), followed by the cyclization of 2-methyl-GPP by monoterpene cyclase (MIBS). MIBS has two Mg^{2+} binding motifs, usually -VDDxxxE/Dx- and -NxxxSxxxE- (where x signifies any amino acid). The conversion of 2-methyl-GPP to 2-MIB occurs in the presence of Mg^{2+}.

Many species of cyanobacteria, including *Anabaena*, *Planktothrix*, *Pseudanabaena* (Planktonic), *Phormidium*, *Oscillatoria* and *Lyngbya* have been confirmed as geosmin and 2-MIB producers (Izaguirre and Taylor, 1998, 2004; Zimba et al., 1999; Acinas et al., 2009; Zahang et al., 2009). The two 2-MIB related genes of *Pseudanabaena limnetica* were identified using Next Generation Sequencing technology (Giglio et al., 2011). These genes have high homology to those from actinomycetes.

In this study, using nucleotide sequence data from *P. limnetica* and *Pseudanabaena* sp. dqh15, the genes encoding GPPMT and MIBS, named *Pseudanabaena galeata* (*P. galeata*) methyl transferase (*pgmtf*) and *P. galeata mtc* (*pgmtc*) were isolated from the Japanese cyanobacterium strain *P. galeata. P. galeata* was originally collected from Aichi Prefecture in Japan. To date, little research has been conducted at the gene expression and metabolic level on the mechanism by which 2-MIB is biosynthesized. In order to elucidate the mechanism of 2-MIB production in *P. galeata*, the effects of growth temperature on 2-MIB production, gene expression and the concentration of fundamental metabolites were analyzed.

Materials and methods

Strain and culture conditions

Pseudanabaena galeata NIES-512, a known 2-MIB producer, was obtained from the Microbial Culture Collection at the National Institute for Environmental Studies (Japan). This strain was cultured in BG-11 medium (Rippka et al., 1979) buffered with HEPES-KOH (pH 8.0) at 20 °C under continuous light (20 μmol photons/m^2/s). Cell density was estimated by OD_{730} using an Ultraspectro3000 (Pharmacia Biotech, USA). For growth analysis and 2-MIB concentration measurements, the strain was grown to an OD_{730} = 0.05–0.1 at 20 °C. Then, the cultures were shifted to different growth temperatures (4 °C and 30 °C) or maintained at 20 °C (control) and grown for 24 h.

GC–MS analysis of 2-MIB

Cell culture (1 mL) was added to 1 mL methanol and 1 mL hexane and vortexed. The hexane extract (upper layer) was transferred to a glass tube. A portion of the extract was directly analyzed using a GC–MS (Shimadzu GCMS-QP2010, 70 eV EI, positive ion mode; 30 m × 0.25 mm Rtx-1 column; temperature program of 60–260 °C, temperature gradient of 5 °C/min, 3 min for resolution). Helium was used as the carrier gas. 2-MIB was identified by comparison with the mass spectrum and retention time of a 2-MIB standard solution (Wako, Japan).

DNA extraction

Cyanobacteria cells were collected from 1.5 mL cultures by centrifugation (15,000 rpm, 4 °C, 2 min). The pellet was shaken with glass beads to break the cell walls, and the lysate was then extracted using a DNeasy Plant Mini Kit (QIAGEN, Japan) following the manufacturer's instructions. The extracted DNA was dissolved in 30 μL ddH_2O and stored at −30 °C.

Cloning and sequencing of pgmtf and pgmtc

DNA fragments of *pgmtf* were amplified using the primers GPPMT1 (5′-CACCTATTCACCAGTAACACATTCT-3′) and GPPR2 (5′-TGGTGCGGGTTAT GTTTTGGATAATC-3′), while *pgmtc* was amplified with the primer MIBF2 (5′-CTGCCCTA ATAAAGGGATAAGAGC-3′) and MIBR2 (5′-GGTTGAACTATGTGCGCTCCTATAA-3′).

Genomic DNA (20 ng) was added to a PCR reaction mixture containing 2 μL of 10 × buffer, 2 μL of 2.5 mM dNTP, 1.6 μL of 25 mM $MgCl_2$ and 0.5 U Takara Ex *Taq* polymerase (Takara, Japan) in a total volume of 20 μL. PCR was performed using a Bio-RAD thermal cycler. The program initiated denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing for 30 s, and extension at 72 °C for 1 min. The PCR products were ligated into pGEM-T easy vector (Promega, USA). The ligated mixture was transformed into *Escherichia coli* XL1-Blue by the heat shock method. Positive clones were selected and sequenced using a CEQ8000 (Beckman Coulter, USA).

RNA extraction for gene expression analysis

Cultures in 10 mL tubes were grown to OD_{730} = 0.05–0.1 at 20 °C. The control culture was maintained at 20 °C, while the cultures for the temperature shift experiment were switched to low (4 °C) or high (30 °C) temperature incubators for 1, 3, 6, 12 and 24 h prior to being harvested for gene expression analysis.

For RNA isolation, cells were harvested from 30 mL cultures (3 × 10 mL cultures). The cultures were transferred to centrifuge tubes containing 5 mL stop solution (5% phenol in ethanol). After centrifugation (8000 rpm, 4 °C, 3 min), the supernatants were discarded and the pellets were frozen with liquid nitrogen to break the cell walls. The pellets were resuspended in 1 mL TRIzol reagent (Invitrogen, USA). Following the manufacturer's instructions, total RNA was extracted and treated with DNase at 37 °C for 30 min to remove contaminating DNA. Finally, the total RNA was dissolved in 25 μL RNase-free ddH_2O and RNA purity was evaluated by determining the A260/A280 absorption ratio (1.8–2.0).

Real-time RT-PCR

Total RNA (1 μg) was used for cDNA synthesis using a SuperScript III kit (Invitrogen, USA). Random hexamer primers (50 ng) and 1 μL of 10 mM dNTPs were added to 1 μg RNA in a total volume of 10 μL. For primer annealing, the mixture was incubated at 65 °C for 5 min and 1 min on ice. For reverse transcription, 2 μL of 10× buffer, 2 μL of 0.1 mM DTT, 4 μL of 25 mM $MgCl_2$ and 1 μL of SuperScript reverse transcriptase were added, the mixture was incubated for 10 min at room temperature, then heated for 50 min at 50 °C and 5 min at 85 °C to stop the reaction. The remaining RNA was digested using RNase (20 min incubation at 37 °C), then 25 μL of RNase-free water was added. For real-time PCR, 1 μL of cDNA solution was used.

Real-time RT-PCR using an ABI7300 system (Applied Biosystems, USA) was performed to investigate the expression levels of the target genes (*pgmtf* and *pgmtc*) following incubation of the cells at several temperatures. Primer pairs were referenced with Wang et al. (2011). GPP-RTF (5′-CGATTGGTCGGTATTAGAGGCT-3′) and GPP-RTR (5′-ATCACGCGGTCA TCAGGCTT-3′) were used to amplify *pgmtf*, and MIBS-RTF (5′-CGCTCGCTTTGTG AGTGAGATAG-3′) and MIBS-RTR (5′-GGCAGTAGAGTGGTGAGGCAGTT-3′) were used to amplify *pgmtc*. 16S rRNA was selected as the control for normalizing the expression level of the target genes. Primer sets for 16S rRNA were 16S-RTF (5′-ACGGAGTTAGCCG ATGCTTATTC-3′) and 16S-RTR (5′-CGAAAGCCTGACGGAGCAATA-3′).

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