Identification of a cytosine methyltransferase that improves transformation efficiency in *Methylomonas* sp. DH-1

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Research

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Abstract

Background: Industrial biofuels and other value-added products can be produced from metabolically engineered microorganisms. *Methylomonas* sp. DH-1 is a candidate platform for bioconversion that uses methane as a carbon source. Although several genetic engineering techniques have been developed to work with *Methylomonas* sp. DH-1, the genetic manipulation of plasmids remains difficult because of the restriction-modification (RM) system present in the bacteria. Therefore, the RM system in *Methylomonas* sp. DH-1 must be identified to improve the genetic engineering prospects of this microorganism.

Results: We identified a DNA methylation site, TGGCCA, and its corresponding cytosine methyltransferase for the first time in *Methylomonas* sp. DH-1 through whole-genome bisulfite sequencing. The methyltransferase was confirmed to methylate the fourth nucleotide of TGGC*CA. In general, methylated plasmids exhibited better transformation efficiency under the protection of the RM system than non-methylated plasmids did. As expected, when we transformed *Methylomonas* sp. DH-1 with plasmid DNA harboring the *psy* gene, the metabolic flux towards carotenoid increased. The methyltransferase-treated plasmid exhibited an increase in transformation efficiency of 3.9×10^3 CFU/μg (110 %). The introduced gene increased the production of carotenoid by 7.2 %.

Conclusions: Plasmid DNA methylated by the discovered cytosine methyltransferase from *Methylomonas* sp. DH-1 had a higher transformation efficiency than non-treated plasmid DNA. The RM system identified in this study may facilitate the plasmid-based genetic manipulation of methanotrophs.

Background

Although methane contributes to the greenhouse effect much more than carbon dioxide does, it is a useful feedstock for methanotrophs, which are bacteria that utilize methane as a carbon source [1–3]. Methane can be converted into methanol via a methanotroph bioprocess [4], and methanol is a chemical precursor for the production of value-added chemicals such as L-glutamate [5, 6], L-lysine [7, 8], cadaverine [9, 10], α-humulene [11], mesaconate, and (2S)-methyl-succinate [12] in metabolically engineered bacteria [13].

For efficient genetic engineering, genetic manipulation tools have been developed to work with methanotrophs [14–17]. Recently, the type I *Methylomonas* sp. DH-1 was isolated from brewery waste sludge, and several engineering tools have been developed [18]. This bacterium has been favored in diverse examples of metabolic engineering: the conversion of methane to methanol [18] and the production of value-added chemicals such as acetone [19, 20], succinate [21], and D-lactate [22].

The first hurdle in genetic engineering is to develop an efficient transformation method. In prokaryotes, DNA methylation and degradation by restriction-modification (RM) systems, which are rudimentary bacterial immune systems, are yet to be identified [23]. Usually, foreign DNA is not methylated and is thus destroyed by host restriction enzymes. The methylation of particular sequences in the host genome protects those sequences from cleavage by host restriction enzymes [24, 25]. A previous study showed

that 88% of bacterial genomes contain RM systems and that 44% of bacterial genomes carry four or more RM systems [26].

Although several genetic manipulation techniques have been developed to metabolically engineer *Methylomonas* sp. DH-1 [27], the low transformation efficiency due to the inherent RM system has been an obstacle. In this study, we aimed to identify the RM system in *Methylomonas* sp. DH-1 and use it for enhanced genetic manipulation with plasmid DNA. Discovering the RM system of *Methylomonas* sp. DH-1 would enable the establishment of transformation techniques for efficient genetic manipulation.

Results And Discussion

Identification of *Methylomonas* sp. DH-1 methylation site

To identify the RM system, the genome of *Methylomonas* sp. DH-1 was analyzed by whole-genome bisulfite sequencing (WGBS). Interestingly, only the TGGCCA motif (Fig. 1) was identified. In the REBASE database [28, 29], *Methylomonas* sp. DH-1 contains twelve RM systems in its genome and two in its native plasmid (Fig. 1). According to REBASE, it was predicted that the cytosine methyltransferase AYM39_01025 would recognize the GGCC sequence for methylation, which is similar to the identified methylation site TGGC*CA, in which the fourth nucleotide (C) was methylated in our results. Therefore, this cytosine methyltransferase was selected as a potential methylase for TGGCCA.

Digestion protection assay

To investigate whether the selected cytosine methyltransferase (AYM39_01025) was able to recognize the identified sequence (TGGCCA) instead of the predicted sequence (GGCC), we conducted a DNA protection assay against digestion, using several restriction enzymes. When the cytosine methyltransferase protein was over-expressed in *E. coli* BL21 (DE3), the protein formed an inclusion body even though it was co-expressed with chaperones (pGro7 and pTf16). Thus, we could not perform the *in vitro* assay requiring purified methyltransferase. Instead, we introduced a plasmid harboring the methyltransferase gene and TGGCCA sites into the JM110 strain (*dam* and *dcm* methylase genes were deleted). Since the cytosine methyltransferase was under the control of the T5 promoter with a *lac* operator, we could obtain a non-methylated or methylated plasmid by IPTG. For further analysis, the plasmid was extracted from JM110.

According to the REBASE annotations, the cytosine methyltransferase of *Methylomonas* sp. DH-1 was predicted to methylate the GGCC sequence, while the only methylation site identified in *Methylomonas* sp. DH-1 by WGBS was TGGCCA. To confirm that the cytosine methyltransferase recognized TGGCCA instead of GGCC, several restriction enzymes that contain GGCC in their restriction sites were used: *Msc*I (TGGCCA), *Apa*I (GGGCCC), and *Not*I (GCGGCCGC). We also used *EcoR*I (GAATTC) and *Xba*I (TCTAGA) restriction enzymes as negative controls. The plasmid harboring the cytosine methyltransferase gene contained all of the above-mentioned restriction sites, as well. If the methylation site was GGCC, the

restriction enzymes (*Msc*I, *Apa*I, and *Not*I) would not be able to cleave the plasmid DNA. As shown in (Fig. 2a), most restriction enzymes were able to cleave both the non-methylated and methylated plasmids, but *Msc*I failed to cleave the methylated plasmid. This result indicated that the cytosine methyltransferase recognized TGGCCA and not GGCC.

To identify the cytosine nucleotide methylated by the cytosine methyltransferase, the methylated plasmid was analyzed by bisulfite sequencing. In bisulfite sequencing, only non-methylated cytosines are converted to uracil, and during PCR, the uracil is converted to T. Methylated cytosines are not changed by bisulfite sequencing. As shown in (Fig. 2b), TGGCCA in the non-methylated plasmid was converted to TGGTTA, indicating that the cytosines were non-methylated, as expected. In the methylated plasmid, only the fifth cytosine in TGGCCA was converted to T, indicating that the fourth cytosine was methylated by the cytosine methyltransferase.

Methylation of plasmid DNA increased transformation efficiency

The plasmid harboring the *psy* (phytoene synthase) gene was constructed (Fig. 3a) and co-transformed into *E. coli* JM110 with the plasmid harboring the cytosine methyltransferase gene *psy*. The *psy* gene is involved in the biosynthetic pathway that produces carotenoids. For the methylation of the plasmid containing *psy*, the media were supplemented with 0.1 mM IPTG to induce the expression of the cytosine methyltransferase. Since *E. coli* contains two plasmids (*psy* plasmid + cytosine methyltransferase plasmid), the plasmids were separated by gel electrophoresis, and the *psy* plasmid was extracted from the gel. The non-methylated plasmid was also extracted from the cell without added IPTG to create a control sample in which the expression of the cytosine methyltransferase was not induced.

The extracted plasmids were transformed into *Methylomonas* sp. DH-1 by electroporation. The transformation efficiency of the methylated DNA of the *psy* plasmid was 3.9×10^3 CFU/μg. The efficiency was increased by 110% compared with that of the non-treated plasmid DNA (Fig. 4b). This result indicated that the methylation of plasmid DNA by the identified cytosine methyltransferase protected the plasmid from the RM system of *Methylomonas* sp. DH-1. Consequently, the methylation increased the transformation efficiency, which may facilitate the genetic manipulation of *Methylomonas* sp. DH-1.

Methylomonas sp. DH-1 carries a complete MEP pathway for carotenoid production [19]. The selected gene, *psy*, is involved in the carotenoid biosynthetic pathway. The gene was designed to be expressed by the promoter of the *mxaF* gene [30] (Fig. 4a). When the gene was introduced into the genome of *Methylomonas* sp. DH-1, *psy* increased carotenoid synthesis by 7.2% (Fig. 4c). This result indicated that the methylation of plasmid DNA by cytosine methyltransferase would be useful in the metabolic engineering of *Methylomonas* sp. DH-1.

(a) The overall biosynthetic pathway towards carotenoid. The *psy* gene is indicated. (b) Transformation efficiency of non-methylated plasmids (light gray bar) and methylated plasmid (dark gray bar) in

Methylomonas sp. DH-1. The map of the two plasmids is shown in Fig. 3a. Standard deviations were calculated from triplicates. The asterisk (*) denotes *p*-values < 0.01. (c) Carotenoid intensity in *Methylomonas* sp. DH-1 cells after transformation with the methylated plasmid. The intensity was measured by using multi-detection micro-plate reader, and the carotenoid intensity was obtained 8 h after cultivation. Standard deviations were calculated from triplicates.

Conclusions

In this study, we identified a novel cytosine methyltransferase and its methylation site for the first time in *Methylomonas* sp. DH-1. The methylase was utilized to increase transformation efficiency by protecting plasmid DNAs from the RM system of *Methylomonas* sp. DH-1. Transformation is the first barrier in the genetic manipulation of bacteria, and with the aid of the methylase, the transformation barrier was effectively overcome. The use of the methylase for methylating insertional genes may facilitate the metabolic engineering of value-added products in *Methylomonas* sp. DH-1.

Methods

Strains, antibiotics, primers, and culture conditions

The *E. coli* DH5α strain was used for gene cloning and plasmid preparation, and the *E. coli* JM110 strain was used for plasmid methylation. *E. coli* cells were cultured in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, and 1% NaCl) or on LB plates (1.5% w/v agar) at 37 °C in the presence of appropriate antibiotics (25 μg/mL of chloramphenicol and/or 50 μg/mL of kanamycin). A cytosine methyltransferase expression vector was then constructed, and its expression was regulated by IPTG. For methylation, 0.1 mM IPTG was used. Next, the *psy* plasmid was constructed.

Methylomonas sp. DH-1 was cultured in a nitrate mineral salt (NMS) medium containing 10 μM $CuCl_2.2H_2O$ as described previously [18]. *Methylomonas* sp. DH-1 cells were cultured in a 500 mL baffled flask sealed with a screw cap containing 100 mL of NMS medium at 30 °C and 250 rpm. Methane was supplied to a final concentration of 30% (v/v) by gas substitution using a gas-tight syringe, and the headspace was refreshed daily. During carotenoid intensity measurement, methanol (0.1%) was used as a carbon source instead of methane because the *Methylomonas* sp. DH-1 cells were cultured in a 96-well plate.

Whole genome bisulfite sequencing and plasmid construction

The *Methylomonas* sp. DH-1 genome was fragmented and sequenced after bisulfite treatment to identify methylation sites (Macrogen, Seoul, South Korea).

Methylomonas sp. DH-1 electroporation

Methylomonas sp. DH-1 was grown in a nitrate mineral salt (NMS) plate containing 10 μM $CuCl_2 \cdot 2H_2O$ as described previously [23]. Cells were collected from plates using a spreader, resuspended in 10 mL of distilled water (DW), and then harvested by centrifugation at 5000 rpm at 4 °C for 10 min. The pellet was washed with 10 mL of DW, transferred to a 15 mL conical tube, and centrifuged again at 5000 rpm at 4 °C for 10 min. The resulting pellet was resuspended in 100 μL of DW and placed on ice. Fifty microliters of the cell suspension was gently mixed with plasmid DNA (three to five microliters), and the mixture was transferred to an ice-cold one-millimeter micropulser electroproation cuvettes (Bio-Rad, Seoul, South Korea). Electroporation was performed using a micropulser electroporator (Gene Pulser II system,Bio-Rad, Seoul, South Koera) at 25 μF and 200 Ω. After electrical discharge, 1 mL of NMS medium was immediately added to the cells. The cells were transferred into a 250 mL serum bottle with 10 mL of NMS medium and then incubated with 0.02% methane gas. After incubation at 30 °C for 4 h, the cells were collected by centrifugation at 5000 rpm for 10 min at 25 °C. The cells were resuspended with 1 mL of NMS medium and spread onto selective NMS plates.

Carotenoid measurement

Methylomonas sp. DH-1 transformed with *psy* plasmid was cultured in NMS medium containing methanol (0.1%) at 30 °C until the stationary phase was reached. The *Methylomonas* sp. DH-1 was transferred to a 96-well plate containing 200 μL of NMS medium and grown in a shaking format at 30 °C. The OD_{450} absorbance at 8 h was measured to infer the relative carotenoid concentration by using a multi-detection microplate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA).

List Of Abbreviations

restriction modification (RM), phytoene synthase (*psy*), nitrate mineral

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Author's contributions

DN supervised this study and revised the manuscript. JR carried out the bisulfite sequencing experiments and drafted the manuscript. HL performed the transformation of *Methylomonas* sp. DH-1. TD carried out the construction of the plasmids. All authors read and approved the final manuscript.

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Figures

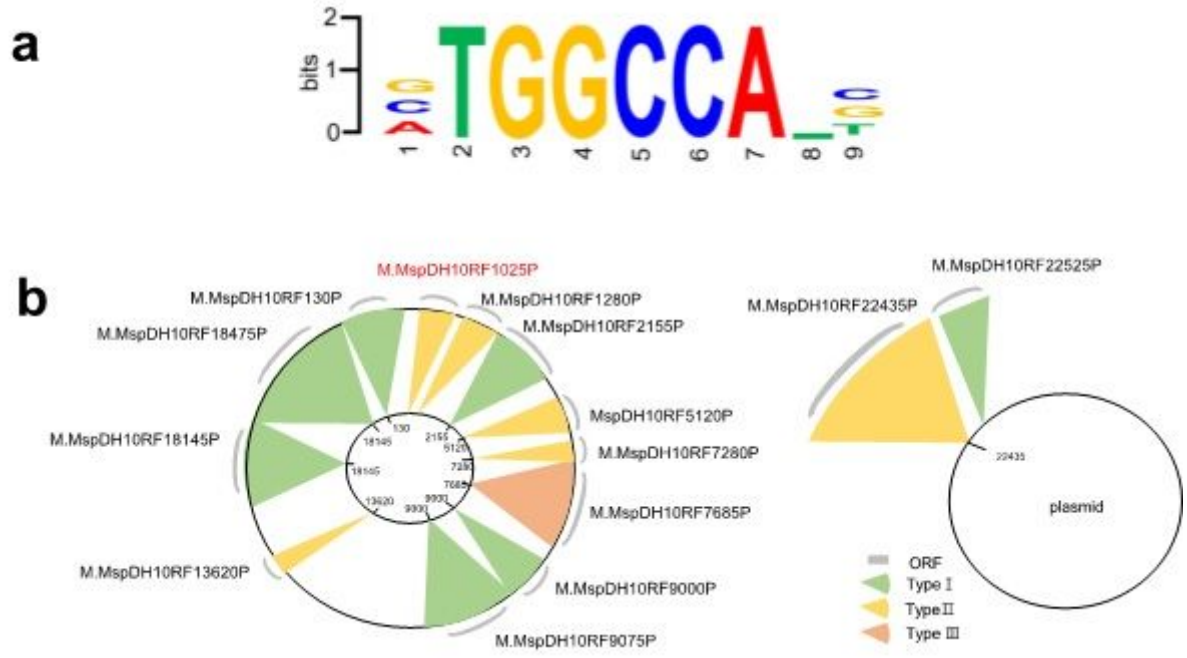

Figure 1

Identification of a methylation site and its corresponding methyltransferase in Methylomonas sp. DH-1. (a) The TGGCCA methylation site was the only site discovered by WGBS. (b) The potential

methyltransferases in Methylomonas sp. DH-1. The REBASE-predicted methyltransferases in its genome and native plasmid are shown.

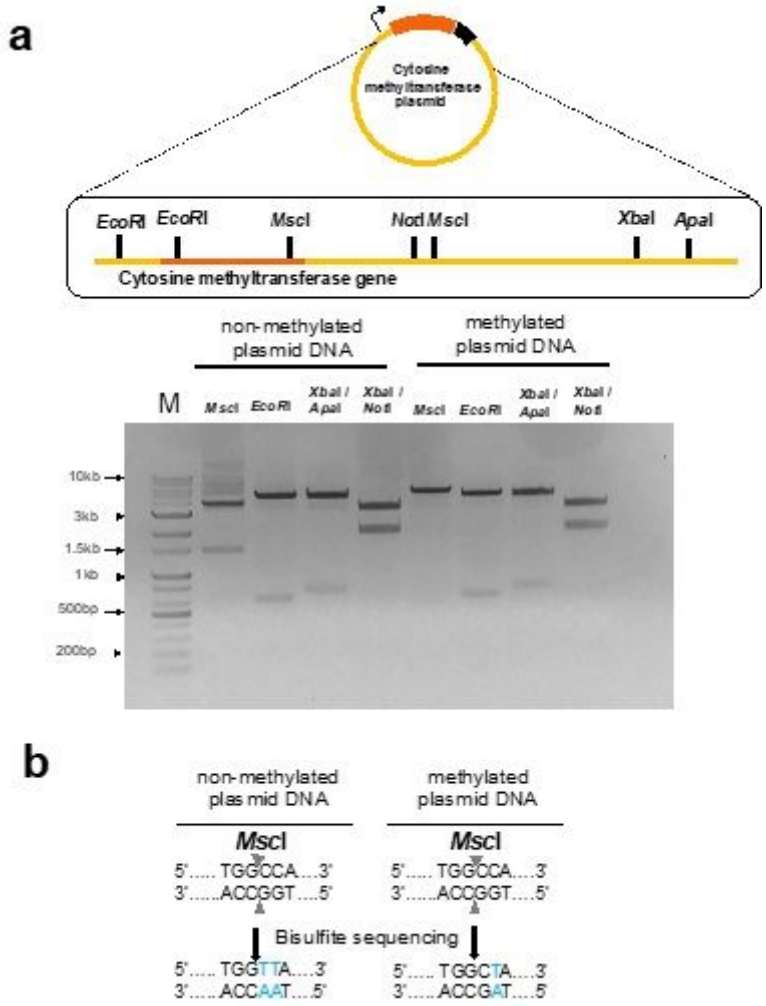

Figure 2

Agarose gel electrophoresis of the plasmid DNA cleaved by restriction enzymes. (a) Restriction patterns of non-methylated and methylated plasmid DNA. The plasmid map and restriction sites are also shown. Methylation was induced by 0.1 mM IPTG, which initiates the expression of the cytosine methyltransferase gene. M denotes a DNA marker. The treated restriction enzymes are shown in each lane. (b) The conversion of non-methylated and methylated TGGCCA sequences during bisulfite sequencing. Only non-methylated cytosines are converted to thymines during bisulfite sequencing and PCR. The left-hand figure shows that the two cytosines were converted to uracils, which means there were no methylated cytosines. Conversely, the right-hand figure shows that the fourth cytosine was not changed to uracil, indicating that it was methylated by the cytosine methyltransferase.

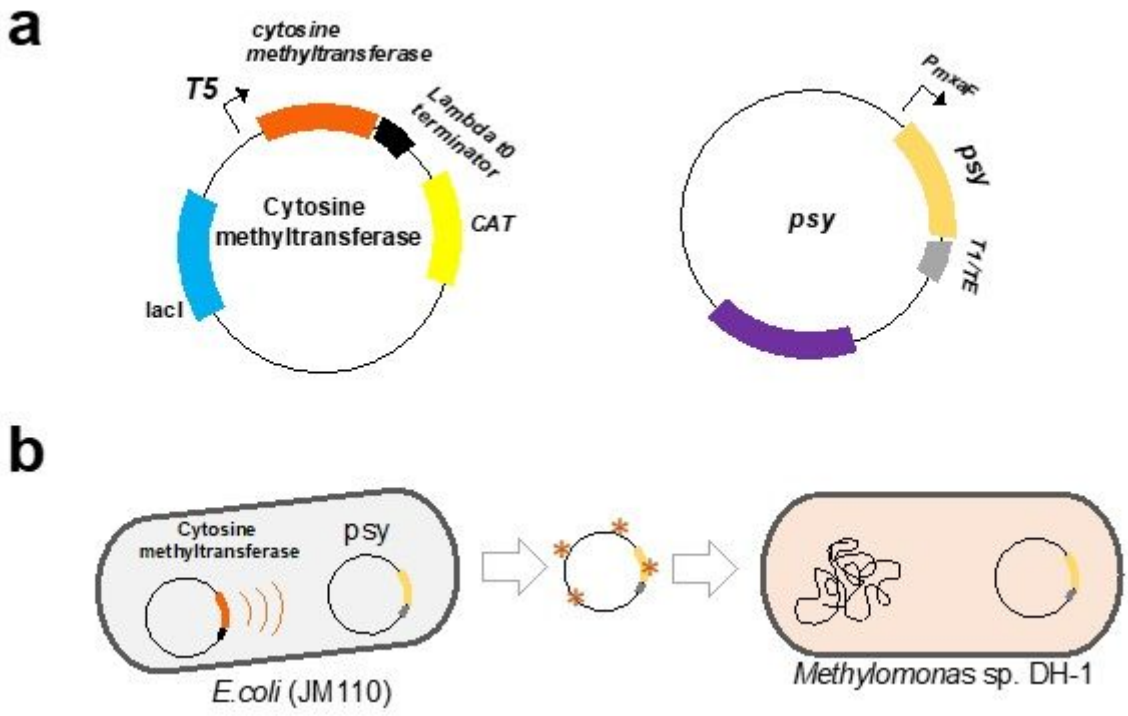

Figure 3

Plasmid maps of the constructed plasmids. (a) The cytosine methyltransferase-containing plasmid and psy-containing plasmid are shown. The psy-containing plasmid was constructed in a proof-of-concept metabolic engineering process to increase the metabolic flux towards carotenoid, and the cytosine methyltransferase-containing plasmid was used to methylate the psy-containing plasmid. The psy gene was under the control of the maxF promoter, which was predicted by using Promoter Hunter [31]. (b) The overall strategy of plasmid methylation in E. coli (JM110) and transformation into Methylomonas sp. DH-1. The first step was to methylate the target plasmid by using cytosine methyltransferase, and the second step was to transform it into Methylomonas sp. DH-1 after separation from the methylase-containing plasmid.

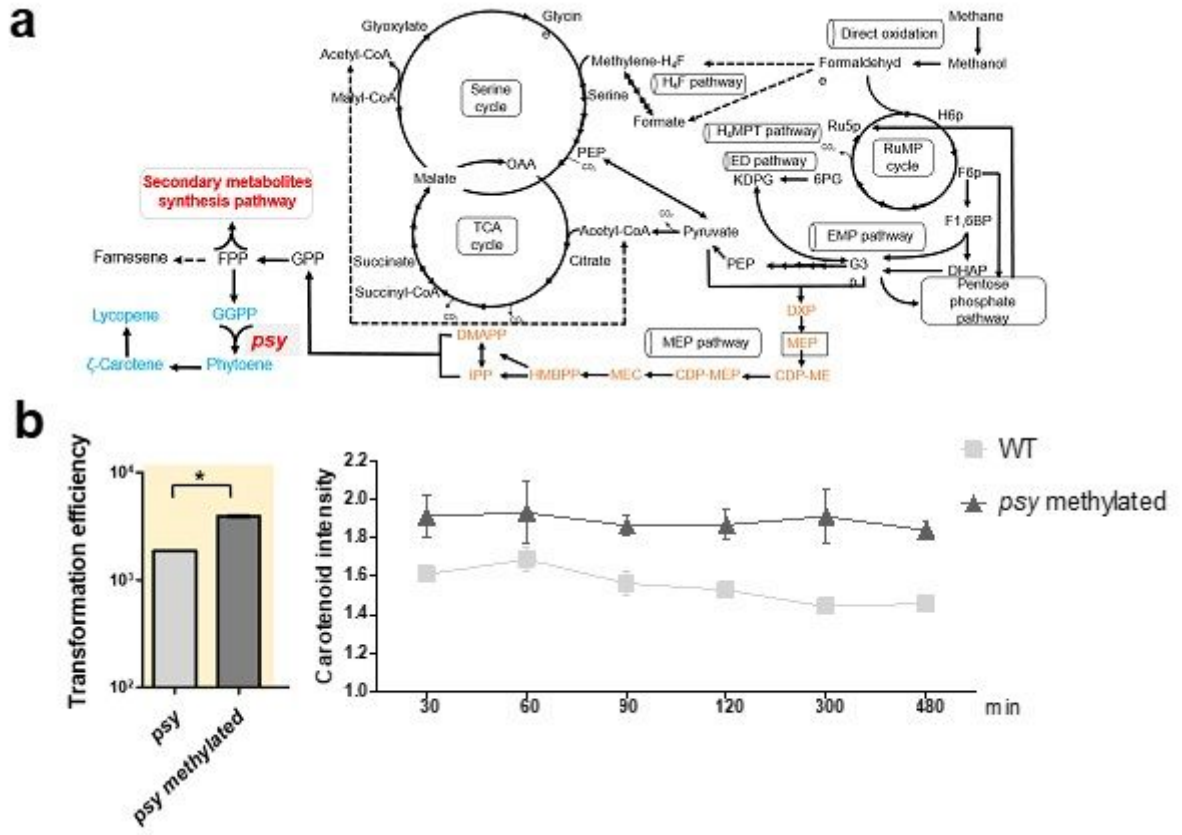

Figure 4

The transformation efficiency of methylated plasmid DNA. (a) The overall biosynthetic pathway towards carotenoid. The psy gene is indicated. (b) Transformation efficiency of non-methylated plasmids (light gray bar) and methylated plasmid (dark gray bar) in Methylomonas sp. DH-1. The map of the two plasmids is shown in Fig. 3a. Standard deviations were calculated from triplicates. The asterisk (*) denotes p-values < 0.01. (c) Carotenoid intensity in Methylomonas sp. DH-1 cells after transformation with the methylated plasmid. The intensity was measured by using multi-detection micro-plate reader, and the carotenoid intensity was obtained 8 h after cultivation. Standard deviations were calculated from triplicates.