Gene Expression Analysis of Methylo trophic Oxidoreductases Involved in the Oligotrophic Growth of *Rhodococcus erythropolis* N9T-4

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*Rhodococcus erythropolis* N9T-4 shows extremely oligotrophic growth requiring atmospheric CO$_2$ without any additional carbon or energy source. We performed a gene expression analysis of the oxidoreductases, which are involved in methanol metabolism, under various growth and induction conditions in N9T-4. A real-time PCR analysis revealed that the genes encoding NAD-dependent formaldehyde dehydrogenase (nFADH) and *N,N''-dimethyl-4-nitrosoaniline*-dependent methanol dehydrogenase (MDH) were strongly expressed under the oligotrophic conditions at levels of 20–100 fold those under heterotrophic conditions, in which *n*-tetradecane was used as the sole carbon source, while glucose did not affect the gene expression pattern in a minimum medium. The genes encoding mycothiol-dependent formaldehyde dehydrogenase (mFADH) and formate dehydrogenase were not induced under oligotrophic conditions, although mFADH expression was observed when formaldehyde was added to the induction medium. These results suggest that N9T-4 had three distinct formaldehyde oxidation systems, and that MDH and nFADH were the key enzymes in its oligotrophic growth.

Key words: oligotroph; *Rhodococcus erythropolis*; carbon dioxide; formaldehyde dehydrogenase; methanol dehydrogenase

An oligotroph is a microorganism that can grow at very low concentrations of carbon and has been defined as one that develops at the first cultivation from nature on a medium with minimal organic matter content of about 1–15 mg of carbon per liter. Many researchers have focused on their growth at extremely low carbon concentrations and have actually isolated many oligotrophs from various natural environments, although biochemical and genetic studies on these organisms have been limited. Although it is thought that oligotrophs use a trace amount of nutrients from the atmosphere, little attention has so far been given to the CO$_2$ requirement for oligotrophs. However, it is possible that some oligotrophs fix CO$_2$ under such nutritionally limited conditions. We have previously isolated an extremely oligotrophic bacterium, *Rhodococcus erythropolis* N9T-4, from crude oil stored in a Japanese oil stockpile. This bacterium could grow on a minimal salt medium solidified by using agar or silica gel without any additional carbon or energy source. N9T-4 did not grow under CO$_2$-limiting conditions, but could grow on an NaHCO$_3$-containing medium under the same conditions, suggesting that the oligotrophic growth of N9T-4 depended on CO$_2$. We also found that some laboratory strains and isolates from nature, belonging to the genera *Rhodococcus* and *Streptomyces*, showed similar growth to N9T-4, suggesting that such extremely oligotrophic growth was a common feature in actinomycetes.

It should be noted that we have not used the term “autotrophic” but “oligotrophic” for the growth of N9T-4, because N9T-4 cells have no detectable key enzyme activities in the four microbial CO$_2$ fixation systems known so far. Although the CO$_2$ fixation system of this bacterium is still unknown, a proteomic analysis has revealed that two methylo trophic enzymes, *N,N''-dimethyl-4-nitrosoaniline* (NDMA)-dependent methanol dehydrogenase (MDH) and NAD-dependent formaldehyde dehydrogenase (nFADH), were highly expressed under oligotrophic conditions. NDMA-dependent MDH found in Gram-positive methylo trophic bacteria catalyzes the oxidation of methanol by using NDMA as an artificial electron acceptor, but this enzyme has shown remarkable formaldehyde dismutase activity without NDMA. In fact, the activities of these two enzymes were dramatically increased when N9T-4 cells were cultivated in a medium without any carbon source, and a high level of formaldehyde-removing activity was consequently observed in the cells. These results suggest that the metabolic flow from CO$_2$ to formaldehyde occurred in the oligotrophic growth of N9T-4 cells.

Since oligotrophic growth, including formaldehyde metabolism, was observed without any additional carbon or energy source in the medium, N9T-4 cells have been suggested to have a novel and effective CO$_2$ fixation pathway.

We analyze in this present study the gene expression of the oxidoreductases, including the foregoing two enzymes, that are involved in methanol metabolism by using a real-time PCR technique to elucidate the CO$_2$ fixation pathway in N9T-4.

Materials and Methods

*Bacterial strain and media for cultivation and induction. R. erythropolis* N9T-4, which was isolated from crude oil, was used throughout this study. The basal medium (BM) described previously

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1. Abbreviations: BM, basal medium; FDH, formate dehydrogenase; FMH, *5-formyl-mycothiol* hydrolase; MDH, *N,N''-dimethyl-4-nitrosoaniline*-dependent methanol dehydrogenase; nFADH, mycothiol-dependent formaldehyde dehydrogenase; NDMA, *N,N''-dimethyl-4-nitrosoaniline*; nFADH, NAD-dependent formaldehyde dehydrogenase
was used for the oligotrophic culture of N9T-4. To examine the carbon sources, 1% glucose, 0.5 mM formaldehyde, 1% methanol, or 1% n-tetradecane was added to BM. When n-tetradecane was added to BM plate solidified by agar, 2 mL of liquid n-tetradecane was soaked in two filter papers on the lid of a Petri dish to provide a vapor. The 2 × LB medium, in which the concentrations of tryptone (Nippon Becton Dickinson Company, Tokyo, Japan) and a yeast extract (Nippon Becton Dickinson Company) were twice those of a traditional LB medium, was also used to reproduce the heterotrophic conditions.

**Assay of the formaldehyde-removing activity.** N9T-4 cells were disrupted with glass beads in 100 mM Tris–HCl (pH 8.0) containing 1 mM DTT and 5 mM MgCl2, and centrifuged at 10,000 g for 10 min. The cell-free extract was used for the subsequent enzyme assays. A reaction mixture containing 50 mM potassium phosphate (pH 7.0), 0.8 mM formaldehyde, and 100 μL of the cell-free extract was incubated at 30 °C for 10–60 min. The reaction was started by adding formaldehyde, and the residual amount of formaldehyde was measured by a Formaldehyde-Test Wako kit (Wako Pure Chemical Industries, Osaka, Japan).

**Preparation of the induced cells.** *R. erythropolis* N9T-4 was cultivated at 30 °C for 3 d on BM plates with or without a carbon source. The cells were collected from 5–10 plates by scratching with a spreader in 0.85% KCl and centrifuged at 5,800 g for 10 min. The collected cells were placed on ice as quickly as possible and used immediately for RNA extraction.

**Induction in the liquid medium used N9T-4 cells grown at 30 °C for 20 h in the 2 × LB medium that were inoculated into 200 mL of the same medium to an OD600 of 0.02 and cultivated for 12 h. The cells were harvested, washed with 0.85% KCl, and resuspended in various media to an OD600 of 5.0. Five mL of the cell suspension was immediately transferred into a 16.5 × 165-mm test tube and incubated at 30 °C with reciprocal shaking at 150 rpm. The cells were collected by centrifugation after induction of 0.5 or 1 h and then used for total RNA extraction.

**Real-time PCR.** Total RNA was prepared from the induced cells by homogenizing with Iogen (Nippon Gene Co., Tokyo, Japan) in a glass bead mixer (Mini-Bead Beater; Biospec Products, Bartlesville, OK, USA). After a DNase I treatment, the RNA was reverse-transcribed by a high-capacity cDNA reverse transcription kit (Life Technologies Japan, Tokyo, Japan).

The reaction mixture for real-time PCR contained 20 ng of cDNA, 2.5 pmol of each primer, and 12.5 μL of SYBR Green PCR Master Mix (Life Technologies Japan, Tokyo, Japan) in a total volume of 25 μL. The PCR conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The 16S rRNA gene was used as an internal control to normalize the levels of expression between the samples, and all samples were analyzed in triplicate. The primers were designed by using Primer Express (Life Technologies Japan, Tokyo, Japan) with the following sequences: 16S rRNA gene, 5′-AAT CCG TGC CTT AGC TAA CG (forward), 5′-TGA GCC TTG CCG CGC TAC T-3′ (reverse); MDH gene, 5′-GAG GCC ACC GGA GGT GAA-3′ (forward), 5′-GGA ATG ATG AAC GCG CAG TA-3′ (reverse); nFADH gene, 5′-TGG CCA AAG CCC CAA CAT-3′ (forward), 5′-TGT CCT GGT AGT CGT CTT GGT A-3′ (reverse); N9T-4 and PR4 genomes did not have such FDH genes in this region. The genes encoding the α- and δ-subunits of FDH were respectively present downstream of the FMH gene in the RHA1 genome (ro02585 and ro02584 in Fig. 1a), while the N9T-4 and PR4 genomes did not have such FDH genes in this region. The genetic organization around the FDH gene of N9T-4 was similar to that of PR4, and the S-formyl-myo-inositol hydratase (FMH) gene was located next to the nFADH gene which catalyzes the formation of formate from S-formyl-myo-inositol. Although the FMH gene in the RHA1 genome was found to be located next to the nFADH gene, as in N9T-4, the other genetic organization around these two genes differed in RHA1. Genes encoding the α- and δ-subunits of FDH were respectively present downstream of the FMH gene in the RHA1 genome (ro02585 and ro02584 in Fig. 1a), while the N9T-4 and PR4 genomes did not have such FDH genes in this region. The genetic organization around the FDH gene of N9T-4 was also similar to that of PR4 (Fig. 1b). The gene encoding the α-subunits of FDH was not found in this region of the RHA1 genome, but next to the FMH gene as just described. There was no gene encoding the other subunit of FDH in the genome of N9T-4, and a frame-shift mutation was found near the 3′-end of the FDH gene of N9T-4. These results suggest that the formate-oxidizing system was inactive or incomplete in N9T-4, in agreement with the fact that no FDH activity was detected in the cell-free extract of N9T-4 grown oligotrophically (data not shown). The genetic organization around the MDH and nFADH genes was similar among the three *Rhodococcus* strains, and no other methylotrrophic gene was observed near the two genes (data not shown).

Results and Discussion

**Comparison of genetic organization involved in methanol oxidation between the three Rhodococcus genomes**

Our previous proteomic analysis revealed that MDH and nFADH were strongly expressed in the oligotrophic growth of N9T-4. Interestingly, when N9T-4 was grown on BM, these enzyme activities in the cells were higher than those on BM containing n-tetradecane as the carbon source. MDH catalyzes the dehydrogenation of methanol with the artificial electron acceptor, NDMA, to produce formaldehyde, and nFADH oxidizes formaldehyde with NAD as a cofactor. These enzymes are known to be involved in methanol oxidation in methylotrrophic bacteria,6) and NDMA-dependent MDHs have only been found in Gram-positive methylotrrophic bacteria.5,7) These results led us to think that oligotrophic growth would involve methanol metabolism in N9T-4, although this bacterium cannot utilize methanol as the sole carbon source. We attempted to search for other genes involved in methanol metabolism in the draft genome sequence of N9T-4 which has recently been determined by our group. We found mycothiol-dependent formaldehyde dehydrogenase (mFADH) and formate dehydrogenase (FDH) genes, besides MDH and nFADH. Mycothiol is a thiol compound found in Gram-positive bacteria and has similar functions to glutathione in Gram-negative bacteria.8) S-Hydroxymethyl-mycothiol is formed non-enzymatically from mycothiol and formaldehyde in Gram-positive methylotrophs; mFADH then catalyzes NAD-dependent dehydrogenation of S-hydroxymethyl-mycothiol to produce S-formyl-mycothiol.9) The genetic organization in the vicinity of the mFADH gene in the N9T-4 genome was compared with that in the *R. erythropolis* PR4 and *R. jostii* RHA1 genomes10) which have been completely sequenced (Fig. 1a). The genetic organization of N9T-4 was found to be quite similar to that of PR4, and the S-formyl-myo-inositol hydratase (FMH) gene was located next to the mFADH gene which catalyzes the formation of formate from S-formyl-myo-inositol. Although the FMH gene in the RHA1 genome was found to be located next to the mFADH gene, as in N9T-4, the other genetic organization around these two genes differed in RHA1. Genes encoding the α- and δ-subunits of FDH were respectively present downstream of the FMH gene in the RHA1 genome (ro02585 and ro02584 in Fig. 1a), while the N9T-4 and PR4 genomes did not have such FDH genes in this region. The genetic organization around the FDH gene of N9T-4 was also similar to that of PR4 (Fig. 1b). The gene encoding the α-subunits of FDH was not found in this region of the RHA1 genome, but next to the FMH gene as just described. There was no gene encoding the other subunit of FDH in the genome of N9T-4, and a frame-shift mutation was found near the 3′-end of the FDH gene of N9T-4. These results suggest that the formate-oxidizing system was inactive or incomplete in N9T-4, in agreement with the fact that no FDH activity was detected in the cell-free extract of N9T-4 grown oligotrophically (data not shown). The genetic organization around the MDH and nFADH genes was similar among the three *Rhodococcus* strains, and no other methylotrrophic gene was observed near the two genes (data not shown).
Gene expression of methylotrophic oxidoreductases on a solid-state medium

We first performed a gene expression analysis for MDH, nFADH, mFADH, and FDH during the growth of N9T-4 on a solid-state medium, because N9T-4 only showed oligotrophic growth on such a medium. As shown in Fig. 2, the expression of the MDH and nFADH genes in a BM-solid medium without any additional carbon source was two to three times higher than that in a BM-solid medium containing \( n\)-tetradecane as the sole carbon source. No marked change in the expression of the mFADH or FDH genes was apparent under this condition, but the expression levels of the MDH and nFADH genes on BM were not as high as expected. We conclude that the gene expression analysis using a solid-state medium produced misleading results, because it was difficult to obtain quantitative growth on the solid-state medium and the collecting the cells from this medium was very tedious.

Gene induction of methylotrophic oxidoreductases using a liquid medium

It was shown in our previous study that N9T-4 cells grown on BM had high formaldehyde-scavenging activity due to the formaldehyde dismutase activity of MDH.\(^3\) We found that this activity was markedly repressed in cells grown in the \( 2 \times \) LB medium (Fig. 3). Since the activity was increased when the original (1 \( \times \)) LB medium used, the nutrient conditions may have affected the expression of enzymes involved in the formaldehyde dismutase activity. We therefore attempted a gene expression analysis in a liquid medium using \( 2 \times \) LB-grown cells. The cells were washed with 0.85% KCl and immediately transferred into a BM-liquid medium with or without an additional carbon source, and the gene for the methylotrophic oxidoreductases already described was induced with shaking. A dramatic 20- to 100-fold increase in both nFADH and MDH gene expression was observed after

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Fig. 1. Comparison of the Genetic Organization around the mFADH (a) and FDH (b) Genes between the Three Rhodococcus sp.

The methylotrophic genes identified in the text are shown as black boxes. The homologous genes that were found in two or three strains are shown as gray boxes. PER- and ro-series numbers respectively represent the gene numbers in the R. erythropolis PR4 and R. jostii RHA1 genomes. The genetic annotation in the R. erythropolis N9T-4 genome is as follows: 1, arylformamidase; 2, transcriptional regulator; 3, aminoglycoside phosphotransferase; 4, FMH; 5, mFADH; 6, transcriptional regulator Lux family; 7, hypothetical protein; 8, tetrahydrofolypolyglutamate synthase; 9, valyl-tRNA synthetase; 10, hypothetical protein; 11, dipeptidyl aminopeptidase; 12, puromycin N-acetyltransferase; 13, ABC Fe\(^{3+}\) transporter periplasmic binding protein; 14, FDH \( \alpha\)-subunit.

Fig. 2. Gene Expression of Methylotrophic Oxidoreductases on a Solid-State Medium.

N9T-4 cells grown for 3 d on BM or BM containing \( n\)-tetradecane were collected, and total RNA extracted from the cells was used for real-time PCR. The relative expression rate (y-axis) represents the expression of each gene from the cells grown on BM relative to that from the cells grown on BM containing \( n\)-tetradecane and normalized by the value of 16S rRNA gene expression. Each sample was examined in three replicates and was repeated by at least three independent preparations of RNA. The standard deviation is displayed by an error bar.
We next examined the effects of various carbon sources on methylotrophic gene expression. We found for the first time in this study, after already finding nFADH, another formaldehyde dehydrogenase, mFADH, which required mycothiol and NAD as cofactors. We have described that MDH had formaldehyde dismutase activity without NDMA as a cofactor. N9T-4 therefore had three oxidoreductases that acted on formaldehyde, although only mFADH was induced by the addition of formaldehyde. Although it is still unknown how formaldehyde is involved in the CO₂ fixation pathway of N9T-4, given the previous results and those in this study, it is obvious that nFADH and MDH both play important roles in the oligotrophic growth of N9T-4.

a 1-h induction in BM (Fig. 4a), although no significant expression occurred in the mFADH and FDH genes. We next examined the effects of various carbon sources on the methylotrophic gene expression. n-Tetradecane added to BM resulted in none of the methylotrophic genes being expressed (Fig. 4b). The gene expression patterns were not changed by supplementing glucose to BM as the carbon source (Fig. 4c), suggesting that the expression of the MDH and nFADH genes was not affected by catabolite repression. These results are consistent with the fact that n-tetradecane supported the growth of N9T-4 as a carbon source under CO₂-limiting conditions, while glucose did not. Interestingly, methanol induced none of the genes tested, despite the fact that these genes are thought to be involved in methanol oxidation (data not shown). Figure 5 shows the gene expression patterns in BM and the LB medium containing formaldehyde. Marked expression of the MDH and nFADH genes was apparent in BM containing formaldehyde, but the levels were the same as or lower than those in BM without an additive, indicating that there was no effect of additional formaldehyde on the expression of these genes (Fig. 5a). However, it is noteworthy that the mFADH expression was increased 10-fold after a 1-h induction in BM with formaldehyde. The effect of formaldehyde addition was clearly observed in the LB medium, and only the mFADH gene was expressed in the LB medium containing formaldehyde (Fig. 5b).

We found for the first time in this study, after already finding nFADH, another formaldehyde dehydrogenase, mFADH, which required mycothiol and NAD as cofactors. We have described that MDH had formaldehyde dismutase activity without NDMA as a cofactor. N9T-4 therefore had three oxidoreductases that acted on formaldehyde, although only mFADH was induced by the addition of formaldehyde. Although it is still unknown how formaldehyde is involved in the CO₂ fixation pathway of N9T-4, given the previous results and those in this study, it is obvious that nFADH and MDH both play important roles in the oligotrophic growth of N9T-4.
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