An Extremely Oligotrophic Bacterium, *Rhodococcus erythropolis* N9T-4, Isolated from Crude Oil[▽]

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Rhodococcus erythropolis N9T-4, which was isolated from crude oil, showed extremely oligotrophic growth and formed its colonies on a minimal salt medium solidified using agar or silica gel without any additional carbon source. N9T-4 did not grow under CO₂-limiting conditions but could grow on a medium containing NaHCO₃ under the same conditions, suggesting that the oligotrophic growth of N9T-4 depends on CO₂. Proteomic analysis of N9T-4 revealed that two proteins, with molecular masses of 45 and 55 kDa, were highly induced under the oligotrophic conditions. The primary structures of these proteins exhibited striking similarities to those of methanol: *N,N'*-dimethyl-4-nitrosoaniline oxidoreductase and an aldehyde dehydrogenase from *Rhodococcus* sp. These enzyme activities were three times higher under oligotrophic conditions than under *n*-tetradecane-containing heterotrophic conditions, and gene disruption for the aldehyde dehydrogenase caused a lack of growth on the minimal salt medium. Furthermore, 3-hexulose 6-phosphate synthase and phospho-3-hexuloisomerase activities, which are key enzymes in the ribulose monophosphate pathway in methylotrophic bacteria, were detected specifically in the cell extract of oligotrophically grown N9T-4. These results suggest that CO₂ fixation involves methanol (formaldehyde) metabolism in the oligotrophic growth of *R. erythropolis* N9T-4.

Many countries, including Japan, have built large stockpiles on land or in the sea for the long-term storage of petroleum oil imported from oil-producing countries. It is natural that microorganisms exist in the long-term-stored crude oil, since various bacteria have been found in petroleum oil fields (18) and microorganisms may enter into crude oil during transportation. It is necessary to investigate microorganisms in crude oil for quality control of the stored crude oil and the oil-storing facilities, and the physiology of microorganisms in crude oil is of interest. However, little is known about these microorganisms.

We have examined the bacterial communities in stored crude oil in Japanese oil stockpiles, and a method for the molecular analysis of the bacterial community in crude oil has been established (32). In this method, the crude oil samples were washed with isooctane to concentrate the microbial fraction, DNA was extracted from the concentrated sample, and the 16S rRNA gene was amplified by PCR, followed by denaturing gradient gel electrophoresis analysis. The denaturing gradient gel electrophoresis profiles revealed that five sequences related to *Burkholderia cepacia*, *Ochrobactrum anthropi*, *Stenotrophomonas maltophilia*, *Propionibacterium acnes*, and *Brevundimonas diminuta* were frequently observed. Furthermore, the sequences related to *O. anthropi* were observed

in all of the 15 crude oil samples from various Japanese oil stockpiles.

We also isolated bacteria from the crude oil samples by the traditional culture-based method. However, only three of the five predominant bacteria in crude oil described above were isolated on agar plates; these were related to *Burkholderia*, *Stenotrophomonas*, and *Propionibacterium*. An isolate related to *S. maltophilia* grew well on *n*-eicosane (C₂₀) as a sole carbon source under microaerobic conditions, indicating that bacteria existing in crude oil have the potential to degrade some components of crude oil.

In this study, we isolated an oligotrophic bacterium, *Rhodococcus erythropolis* N9T-4. This bacterium showed extremely oligotrophic growth on a minimal salt agar plate without any additional carbon source. The growth conditions examined, a proteomic analysis, and the behavior of a mutant strain derived from N9T-4 identified proteins involved in the extreme oligotrophic growth, which we propose is based on CO₂ fixation by a pathway that is different from those known for other bacteria.

MATERIALS AND METHODS

Crude oil samples. Crude oil samples used in this study were collected from the oil stockpiles in Japan as follows. Khafji crude oil samples (heavy oil) were collected from the facilities at Tomakomai-tobu Oil Stockpiling (Hokkaido), Fukui Oil Stockpiling (Fukui), and Shibushi Oil Stockpiling (Kagoshima). Arabian light crude oil samples (medium oil) were collected from the Tomakomai and Shibushi facilities. Upper Zakum crude oil samples (medium oil) were collected from the facilities at the Shibushi facility and Japan Underground Oil Stockpiling (Ehime). These samples were stored at 4°C until analysis.

Bacterial strains, cultivation media, and growth conditions. *R. erythropolis* NBRC12320, *R. erythropolis* NBRC15567, and *R. erythropolis* NBRC16296, which were obtained from the Biological Resource Center of the National Institute of Technology and Evaluation (NBRC), Chiba, Japan, were used as type cultures. *Escherichia coli* DH5α [F[−] φ80lacZΔM15 Δ(lacZYA-argF)U169 deoR recA1

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hsdR17(r_K^- m_K^+) *phoA* *supE44* λ^- *thi-1* *gyrA96* *relA1*] was used for DNA cloning. *E. coli* DH5 α was grown at 37°C on Luria-Bertani medium. Appropriate antibiotics were added to the medium if necessary.

Basal salt medium (BSM) used throughout this study was composed of 1.0 g of NaNO₃, 1.0 g of K₂HPO₄, 1.0 g of KH₂PO₄, 0.5 g of MgSO₄ · 7H₂O, 0.1 g of CaCl₂ · 9H₂O, 1% (vol/vol) metal solutions, 0.1% (vol/vol) vitamin mixture in 1,000 ml of deionized water (pH 7.0). The composition of the metal solutions was as follows: 2.0 g MnSO₄, 2.2 g ZnSO₄ · 7H₂O, 0.26 g CuSO₄, 0.22 g CoCl₂, 0.26 g Na₂MoO₄ · 2H₂O, 0.4 g H₃BO₃, and 0.06g KI in 1,000 ml of deionized water. The vitamin mixture had the following composition: 1 mg thiamine-HCl, 2 mg riboflavin, 2 mg Ca-pantothenate, 2 mg pyridoxine-HCl, 0.1 mg biotin, 1 mg *p*-aminobenzoic acid, 2 mg nicotinic acid, and 0.1 mg folic acid in 100 ml of deionized water. Purified agar (Nacalai Tesque, Kyoto, Japan) was used to prepare agar plates.

A silica gel plate was prepared according to the method of Funk and Krulwich (9), which was modified as follows. Ten grams of powdered Silica gel 60 (0.063 to 0.200 mm; Merck Ltd., Kyoto, Japan) was dissolved in 100 ml of 7% KOH. Each 10 ml of the potassium silicate solution and autoclaved 2× BSM was added to a 30-ml vial and mixed several times upside down. The solution was poured into a petri dish in which 1.75 ml of 20% *o*-phosphoric was added beforehand. After the petri dish was mixed gently, gelation began after about 1 min and was complete after about 15 min. The plate was placed upside down at 30°C overnight, and the water oozing to the lid was removed. Just before inoculation, the plates were sterilized by UV light irradiation for 30 min on a clean bench.

To make CO₂-limiting conditions, 15 g of a CO₂ absorbent (Soda Sorb; W. R. Grace S. A., Epervon Cedex, France) was added to a petri dish and placed in a plastic pouch with an inoculated plate. For the examination of carbon sources, 1% glucose, 1% glycerol, 1% succinic acid, 1% citric acid, 1% ethanol, 1% methanol, *n*-tetradecane, and *n*-hexadecane were added to BSM. Each *n*-alkane solution was soaked in two filter papers on the lid of a glass petri dish and provided as a vapor.

Cloning of the 16S rRNA gene. To identify the isolated bacteria, 16S rRNA gene fragments were amplified by colony PCR with the following set of primers: 341F (5'-CCT ACG GGA GGC AGC AG-3', *E. coli* 16S rRNA gene positions 341 to 357) and 907R (5'-CCG TCA ATT CMT TTG AGT TT-3', *E. coli* 16S rRNA gene positions 907 to 926). The PCR conditions were as follows: 3 min of denaturing at 95°C followed by 35 cycles consisting of 1 min at 95°C, 1 min at 56°C, and 1 min at 72°C and finally by 10 min of extension at 72°C. The PCR products were cloned directly into pCR2.1-TOPO by use of a TOPO TA cloning kit (Invitrogen Japan K.K., Tokyo, Japan) according to the manufacturer's instructions. The ligates were transformed into competent cells of *E. coli* TOP10 (Invitrogen) by electroporation. Database searches of the 16S rRNA gene sequences determined were conducted by a BLAST program using the GenBank database.

Two-dimensional gel electrophoresis (2-DE). N9T-4 cells were collected by a spatula from 10 to 50 plates and washed in 0.85% KCl. The washed cells were disrupted with glass beads in 60 mM Tris-HCl (pH 8.8) containing 5 M urea, 1 M thiourea, 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 1% Triton X-100, and a tablet of a protease inhibitor (Complete, Mini, EDTA; Roche Diagnostics K. K., Tokyo, Japan). After centrifugation at 10,000 × *g* for 10 min, the supernatant was subjected to isoelectric focusing. The cell extract (0.1 mg) was loaded on an agarGEL (10 cm; ATTO Corp., Tokyo, Japan) with a linear pH gradient between 4 and 7 and focused at 300 V (constant) for 150 min, followed by second-dimension analysis using a sodium dodecyl sulfate (SDS)-polyacrylamide gel (10 by 10 cm) (17). The gel was blotted onto a polyvinylidene difluoride membrane and stained with Coomassie brilliant blue R-250. Each spot was excised from the membrane and subjected to amino acid sequencing with a protein sequencer (model 476A; Applied Biosystems Japan Ltd., Tokyo, Japan).

Measurement of enzyme activities. N9T-4 cells were disrupted with glass beads in 100 mM Tris-HCl (pH 8.0) containing 1 mM dithiothreitol and 5 mM MgCl₂ and centrifuged at 10,000 × *g* for 10 min. The cell extract was used for the following enzyme assays. 3-Hexulose-6-phosphate synthase (HPS) and phospho-3-hexuloisomerase (PHI) activities were measured using recombinant PHI and HPS, respectively, according to the methods of Arfman et al. (2). Formaldehyde dismutase activity was measured as formaldehyde-removing activity without cofactor as follows. A reaction mixture containing 50 mM potassium phosphate (pH 7.0), 0.8 mM formaldehyde, and 100 μl of cell extract was incubated at 30°C for 10 to 60 min. The reaction was started by the addition of formaldehyde, and the residual amount of formaldehyde was measured by Formaldehyde-Test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). NAD-dependent formaldehyde dehydrogenase activity was measured at 30°C by following the increasing absorbance at 340 nm in a reaction mixture containing 100 mM

Tris-HCl (pH 9.0), 1 mM NAD, 1 mM formaldehyde, and 20 μl of the cell extract. One unit of each enzyme activity described above was defined as the amount of enzyme which converted 1 μmol of each substrate per min. Partial purification of the proteins involved in the formaldehyde-removing activity was performed by Resource Q column chromatography in a linear gradient of 0 to 0.5 M KCl in 50 mM Tris-HCl buffer (pH 8.0). Active fractions were concentrated by ultrafiltration (exclusion was at a molecular weight of 10,000) and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (10% gel, 10 by 10 cm). The gel was transferred to a polyvinylidene difluoride membrane, and amino acid sequencing was carried out as described above.

Gene disruption. A gene disruption cassette was constructed by replacing the middle region of the ALDH gene (*ald*) in N9T-4 with the kanamycin resistance (Km^r) gene by a three-step PCR method (15). Amplification of the Km^r fragment was done with 50 ng of Tn5 transposon (Epicenter Biotechnologies, WI) as a template with the primers 5'-CAA CCA TCA TTC GAT GAA TTG TGT C-3' and 5'-GTT GAT GAG AGC TTT GTT GTA GGT G-3'. Amplification of the 5' upstream region of *ald* was performed by using 50 ng of N9T-4 genomic DNA/ml with the primers 5'-CAA TGA CCG TGT ACG CCC-3' and 5'-GAC ACA ATT CAT CGA TGA TGG TTG GAC GCG TAC TGC ATG ATC AGG-3'. The 3' downstream region of *ald* was amplified using 50 ng of N9T-4 genomic DNA/ml with the primers 5'-CAC CTA CAA CAA AGC TCT CAT CAA CGC AGA ACC TTA TCC CCG TCA-3' and 5'-TCA GAA GAA GCC CTG AGC C-3'. All PCR conditions described above were as follows: 95°C for 5 min followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min and finally by 72°C for 3 min. Subsequent fusion PCR was done by using 133 ng/ml of each of the three fragments amplified above with the primer set used for amplification of the *ald* structural gene. The PCR conditions were as follows: 95°C for 2 min followed by 30 cycles of 94°C for 20 s, 55°C for 30 s (increasing temperature at the rate of 0.1°C/s), and 68°C for 2 min and finally by 72°C for 3 min. The disruption cassettes were introduced into N9T-4 competent cells by electroporation (26).

Nucleotide sequence accession numbers. The nucleotide sequence data obtained in this study have been submitted to GenBank (accession numbers AB286677 and AB286678).

RESULTS

Oligotrophic growth of *R. erythropolis* N9T-4. The crude oil samples were treated with 2,2,4-trimethylpentane (isooctane) to remove the hydrophobic fraction and to concentrate the hydrophilic microbial fraction in crude oil (32). The samples after isooctane treatment were spread onto BSM plates without any additional carbon source and incubated at 30°C for several days. As a result, the only bacterium found, N9T-4, was isolated from a Khafji crude oil sample, and the 16S rRNA gene sequence showed 100% identity to that of *R. erythropolis*. The morphological and biochemical properties of N9T-4 were also consistent with those of *Rhodococcus* spp. Strain N9T-4 showed extremely oligotrophic growth, forming clear colonies within 5 days at 30°C on a BSM plate without any additional carbon source. To examine the requirement of CO₂ for oligotrophic growth, we constructed CO₂-limiting conditions using a CO₂ absorbent in a plastic bag. Gas chromatographic analysis showed that CO₂ was not detected in the air in the plastic bag (data not shown). As shown in Fig. 1A, N9T-4 did not grow on BSM with the CO₂ absorbent in a plastic bag, while the strain grew well on BSM without the CO₂ absorbent under the same conditions. When the inoculated plate that had been incubated for several days under the CO₂-limiting conditions, where no growth was observed on the plate, was placed outside of the plastic bag and further incubated in air, colonies were observed on the plate after several days. Furthermore, N9T-4 grew on BSM containing 0.1% NaHCO₃ even under the CO₂-limiting conditions. These results suggest that the oligotrophic growth of N9T-4 depends on CO₂. It is of interest that N9T-4 did not grow on BSM containing glucose under the CO₂-limiting con-

FIG. 1. Oligotrophic growth of *R. erythropolis* N9T-4. (A) Strain N9T-4 was cultivated at 30°C for 5 days on BSM agar plates containing various carbon sources in plastic bags with or without CO₂ absorbent. The carbon sources used are 1% glucose, 0.5% NaHCO₃, and *n*-tetradecane. *n*-Tetradecane was soaked in two filter papers on the lid of a glass petri dish and provided as a vapor. (B) The inorganic growth of N9T-4 was verified using a BSM silica gel plate. Strain N9T-4 was cultivated at 30°C for 5 days on a silica gel plate containing BSM in a plastic bag with or without CO₂ absorbent. A plate containing water was also put into each plastic bag to prevent the silica gel from drying.

ditions (Fig. 1A). To examine the carbon sources sustaining the growth of N9T-4 under the CO₂-limiting conditions, various carbon sources, such as sugars (fructose, galactose, mannose, and starch), organic acids (succinic acid, citric acid, and malic acid), alcohols (methanol, ethanol, and propanol), alkanes (*n*-hexane, *n*-decane, *n*-tetradecane, and *n*-hexadecane), and an aromatic hydrocarbon (naphthalene), were used for BSM. As a result, N9T-4 could grow on only *n*-tetradecane and *n*-hexadecane among the carbon sources tested under the CO₂-limiting conditions. It is of interest that sugars, such as glucose, did not sustain the growth of N9T-4, and *n*-tetradecane was used for the subsequent experiments as the carbon source under the CO₂-limiting conditions.

One might say that the oligotrophic growth of N9T-4 was due only to the utilization of agar in the BSM plates. To counter this suggestion, we prepared BSM plates solidified by the use of inorganic silica gel. Figure 1B shows the inorganic growth of N9T-4 on a BSM silica gel, while diffusion of the colonies was observed because it was difficult to dry silica gel plates appropriately. Complete drying of the silica gel plates caused cracks on the surface. Under the CO₂-limiting conditions, N9T-4 did not grow on the BSM silica gel, suggesting that this bacterium requires and utilizes CO₂ for its growth.

Analysis of proteins induced under CO₂-limited conditions. To analyze the proteins involved in the oligotrophic growth of N9T-4, differential 2-DE was performed using the cells grown

on BSM without any additional carbon source (oligotrophic growth) and on that with *n*-tetradecane as the sole carbon source (heterotrophic growth). The protein profiles of N9T-4 are shown in Fig. 2, and the identified proteins induced specifically under each condition are listed in Table 1. Six proteins were highly induced in the cells grown oligotrophically, and four of them were similar to chaperones. As for the other two proteins with apparent molecular masses of 45 kDa and 55 kDa, the N-terminal sequence of the 45-kDa protein was identical to that of ThcE from *Rhodococcus* sp. strain NI86/21 (23), and that of the 55-kDa protein matched perfectly to that of a rhodococcal aldehyde dehydrogenase (ALDH), except for only one amino acid (13). Recently, the whole-genome sequencing of *Rhodococcus* sp. strain RHA1 has been completed (19). Two putative genes corresponding to the two oxidoreductases described above were found in the strain RHA1 genome. Therefore, we synthesized two sets of primers to amplify the genes encoding these proteins and amplified 1,275- and 1,524-kb DNA fragments corresponding to the 45- and the 55-kDa proteins, respectively, by PCR. When the amino acid sequences deduced from the amplified DNA fragments were compared with those of other oxidoreductases from various origins (data not shown), the 45-kDa protein was identical to ThcE from *Rhodococcus* sp. NI86/21 (23), which showed a high similarity to methanol:*N,N'*-dimethyl-4-nitrosoaniline (NDMA) oxidoreductase (MNO) from the gram-positive methylotrophic bac-

FIG. 2. 2-DEs of the cell extract of N9T-4 grown under oligotrophic and heterotrophic conditions. One-hundred-microgram portions of protein prepared from the cells grown on BSM (A) or on BSM containing *n*-tetradecane with CO₂ absorbent in a plastic bag (B) were separated by use of a two-dimensional polyacrylamide gel. The spots surrounded by circles on each gel represent highly expressed proteins under each condition. The spot numbers correspond to those in Table 1.

teria *Amycolatopsis methanolica* and *Mycobacterium gastr* MB19 (5). The 55-kDa protein exhibited striking similarities to an ALDH from *R. erythropolis* UPV-1 (99.6% identity) (13) and ThcA from *Rhodococcus* sp. strain NI86/21 (99% identity), which is similar to an aliphatic ALDH (22).

Since the 45- and 55-kDa proteins were expected to be MNO and ALDH, the enzyme activities of N9T-4 grown under oligotrophic growth conditions were examined. The cell extract prepared from the N9T-4 cells grown on BSM was subjected to Resource Q column chromatography, and ALDH and MNO

activities were measured as NAD-dependent formaldehyde dehydrogenase and formaldehyde dismutase activities in each fraction, respectively. As shown in Fig. 3A, these enzyme activities were separated by Resource Q column chromatography, and two protein bands corresponding to the enzyme activities were detected by SDS-PAGE (Fig. 3B). N-terminal amino acid sequences of the protein bands in the fraction that showed the remarkable MNO and ALDH activities were completely identical to those of the 45- and 55-kDa proteins detected on the 2-DE, respectively. Furthermore, the partially

TABLE 1. Identification of proteins of N9T-4 by 2-DE

Condition of specific protein expression and protein spot no.	MM1 ^a (kDa)	N-terminal sequence ^b	Description of protein	MM2 ^c (kDa)	Accession no. of closest protein
Oligotrophic					
O-1	97	MDTGSLTEKSREALQEAQN MDIGKFTEKSQQALAEQN	Chaperone ClpB	97.2	YP_706636
O-2	70	ATAVGIDLGTNNX ARAVGIDLGTNNX	Chaperone DnaK (HSP70)	67.3	P80692
O-3	70	ATAVGIDLGTNNX ARAVGIDLGTNNX	Chaperone DnaK (HSP70)	67.3	P80692
O-4	55	TVYARPGTADAIMSFQSRYP TVYARPGTADAIMSFQSRYP	ALDH	55.2	AAZ14956
O-5	45	AIELNQIWDFFIWEF AIELNQIWDFFIKEF	MNO	46.5	AAB80771
O-6	35	ARDYYEVLGVPR-RAARDDI ARDYYEVLGVSRASQDEI	Chaperone DnaJ3	33.4	Q0S1V6
Heterotrophic					
H-1	60	AKITAFDEEARRQLERGLNA AKTIAYDEEARRGLERGLNA	Chaperonin GroEL	56.6	YP_702111
H-2	40	MDLFEYQAKELFAEHXV MDLFEYQAKELFAKHEV	Succinyl-CoA synthetase β -subunit	40.8	YP_880328
H-3	36	TVRVGVNGFGRIGRNFFRAV TVRVGINGFGRIGRNFFRAV	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	35.9	YP_707099
H-4	33	AEVLVLVEHAEGALKKVVTE AEVLVLVEHAEGALKKVSTE	Electron transfer flavoprotein α -subunit	32.0	YP_706396
H-5	33	ANYTAADVKKRLRELTGSGMM ANYTAADVKKRLRELTGSGMM	Elongation factor EF1B	29.3	YP_706510
H-6	30	TNIVVLIKQVPDTSERKLTDG TNIVVLIKQVPDTSERKLTDG	Electron transfer flavoprotein β -subunit	28.0	YP_706395

^a MM1, molecular mass obtained by the experimental data in the study.

^b Upper sequences, experimental data; lower sequences, N-terminal sequences retrieved from the databases.

^c MM2, molecular mass calculated based on the amino acid sequences on the databases.

FIG. 3. Identification of MNO and ALDH activities in the cell extract of N9T-4 grown under oligotrophic conditions. (A) The cell extract prepared from N9T-4 cells grown on BSM plates was subjected to Resource Q column chromatography, and cofactor-independent formaldehyde dismutase and NAD-dependent ALDH activities were measured in each fraction. Filled circles, ALDH activity; triangles, MNO activity; solid line, protein concentration at 280 nm; dotted line, conductivity. (B) SDS-PAGE pattern of the concentrated fractions shown in panel A. The N-terminal amino acid sequences of bands b and c were completely identical to those of the 45- and 55-kDa proteins detected on the 2-DE, respectively. The N-terminal amino acid sequence of band a was STTGTPKTAELQDDDTN, which was close to that isocitrate lyase of *Rhodococcus* sp. strain RHA1.

purified MNO produced almost equal molar of methanol and formate from formaldehyde, confirming the formaldehyde dismutase reaction of MNO of N9T-4 (Fig. 4).

Inducible MNO and ALDH activities were also observed under the oligotrophic growth of N9T-4, and these activities were three times higher than those under heterotrophic growth (Table 2). Interestingly, induction of the ALDH activity was not observed when formaldehyde was added to the medium, while MNO was induced by formaldehyde.

Disruption of the ALDH gene in *R. erythropolis* N9T-4. To evaluate whether ALDH was essential for the oligotrophic

growth of N9T-4, disruption of the corresponding gene (*ald*) was attempted by homologous recombination using a Km^r gene as a selective marker. A 1,131-bp Km^r gene was inserted to the middle of *ald* (1,524 bp), and the gene cassette was introduced to N9T-4 cells by electroporation. As a result, four *ald* disruptants were obtained from 420 Km^r mutants. A 2.7-kb DNA fragment was amplified using the genomes of each disruptant as a template and a primer set which was used for amplification of the *ald* gene, suggesting Km^r gene insertion (data not shown). As shown in Fig. 5, the disruptants did not grow on BSM unless an additional carbon source, such as *n*-tetradecane, was provided. We also attempted to obtain the *mno* disruptant by the method described above; however, none of the disruptant was obtained, though about 1,000 Km^r mutants were examined for *mno*, and all the Km^r mutants were due to nonhomologous recombination of the Km^r gene.

Enzyme activities involved in formaldehyde fixation in *R. erythropolis* N9T-4. As described above, two enzymes involved in formaldehyde oxidation were induced under oligotrophic growth conditions, suggesting that N9T-4 produced formaldehyde in the cells and that oligotrophic growth involves formaldehyde fixation. Since two homologs of HPS and PHI, which are the key enzymes for formaldehyde fixation in the ribulose monophosphate (RuMP) pathway in methylotrophic bacteria,

FIG. 4. Formaldehyde dismutase reaction of partially purified MNO. Fraction 31 shown in Fig. 3 (0.27 mg) was incubated with 70 mM formaldehyde in 50 mM potassium phosphate (pH 7.0), and the amounts of formaldehyde, methanol, and formic acid in the reaction mixture were measured at several times. The amount of formaldehyde was measured by a colorimetric method as described in Materials and Methods, while those of methanol and formic acid were determined by gas chromatography and an enzymatic method, respectively (21). Circles, triangles, and squares represent the concentrations of formaldehyde, methanol, and formic acid, respectively.

TABLE 2. MNO and ALDH activities in the cell extract of *R. erythropolis* N9T-4^a

Medium	Sp act ($\times 10^{-2}$ U/mg) of:	
	ALDH	MNO
LB	2.05	0.524
LB + formaldehyde	1.81	1.84
BSM	109.8	31.0
BSM + <i>n</i> -tetradecane	31.9	10.0

^a N9T-4 was cultivated at 30°C for 2 days on LB with or without 1 mM formaldehyde and for 5 days on BSM with or without *n*-tetradecane vapor.

FIG. 5. Growth of wild-type and *ald* disruptant strains of *R. erythropolis* N9T-4. The wild type and four *ald* disruptant strains of *R. erythropolis* N9T-4 were streaked onto two BSM plates and incubated at 30°C for 3 days under oligotrophic and heterotrophic conditions as indicated. For heterotrophic conditions, two filter papers soaked with *n*-tetradecane were placed on the lid of one of the plates. "W" indicates the wild-type strain, and the disruptants are represented by numbering.

are found in the *Rhodococcus* sp. strain RHA1 genome, the HPS and PHI activities of N9T-4 were measured. As a result, HPS and PHI activities (3.18 and 1.35 mU/mg, respectively) were observed for the cell extract of N9T-4 grown under oligotrophic conditions, while no formaldehyde fixation activity was detected for that under heterotrophic conditions.

Growth of other *R. erythropolis* strains. To examine whether the oligotrophic growth of N9T-4 is a general characteristic of *R. erythropolis*, some type cultures of *R. erythropolis* obtained from NBRC were cultivated on BSM plates as described above. All NBRC strains showed growth similar to that of N9T-4, while the colonies of NBRC15567 appeared on the part of the BSM plate at a high cell density (Fig. 6). Among the NBRC strains, the largest colonies on the BSM plates were observed for N9T-4. Furthermore, the ortholog genes corresponding to the 45- and 55-kDa proteins of N9T-4 were amplified by PCR using the genomes of all the NBRC strains as templates (data not shown).

DISCUSSION

R. erythropolis N9T-4 could grow on an inorganic minimal medium without any additional carbon and energy sources and required CO₂ for growth. However, we are not using the term "autotrophic" but "oligotrophic" for its growth, since the CO₂ fixation system of this bacterium is still unknown. Kuznetsov et al. have defined oligotrophic bacteria as those that develop at first cultivation on media with the minimal content of organic matter of about 1 to 15 mg of C per liter (16). There may be no information of the CO₂ requirement in their definition or in those of other researchers who have studied oligotrophs, but it is possible that some oligotrophs fix CO₂ using low concentrations of other carbon sources. Since N9T-4 grew well on a BSM agar plate but not in a liquid medium consisting of BSM, we could not exclude the possibility that the oligotrophic growth of N9T-4 was due to the utilization of agar in the BSM plates. However, it did not grow in a liquid BSM containing agar and could grow on BSM solidified using silica gel. Although further investigation is needed to understand the extremely oligotrophic growth of N9T-4, we consider that N9T-4 has a novel and effective type of CO₂ fixation system or a specific carbon metabolism to utilize CO₂ as the sole carbon source. Until now, four CO₂ fixation pathways or cycles have been known for autotrophic bacteria: the Calvin cycle (4), the acetyl coenzyme A (acetyl-CoA) pathway (30), the reductive tricarboxylic acid (TCA) cycle (8), and the 3-hydroxypropionate cycle (11). The Calvin cycle, in which ribulose 1,5-bisphosphate carboxylase/oxygenase is a key enzyme, is well known as a CO₂ fixation system in plants and also functions in many phototrophic and chemotrophic prokaryotes. The reductive TCA cycle is found in strictly anaerobic and microaerobic autotrophs, and the key enzymes are ATP citrate lyase, 2-oxoglutarate:acceptor oxidoreductase, and pyruvate:acceptor oxidoreductase. In the 3-hydroxypropionate cycle, acetyl-CoA and propionyl-CoA are the acceptors of CO₂, and malonyl-CoA reductase, forming 3-hydroxypropionate, and propionyl-CoA synthase are the key enzymes. The acetyl-CoA pathway is only noncyclic CO₂ fixation system, and carbon monoxide dehydrogenase functions in acetyl-CoA synthesis in this pathway. It should be noted that there were no key enzyme activities in the four microbial CO₂

FIG. 6. Growth of other *Rhodococcus erythropolis* strains on BSM agar plates under the oligotrophic conditions. *R. erythropolis* strains NBRC12320, NBRC15567, and NBRC16296 were cultivated at 30°C for 5 days under conditions the same as those for N9T-4.

FIG. 7. Postulated pathway of CO₂ fixation system in *R. erythropolis* N9T-4. FDH, formate dehydrogenase.

fixation systems in the cell extract and membrane fractions of N9T-4 (data not shown). Recently, Jahn et al. proposed a novel CO₂ fixation pathway, which seems to be an incomplete reductive TCA cycle, for the autotrophic archaeon *Ignicoccus hospitalis* (12). They suggest that acetyl-CoA is the primary CO₂ acceptor catalyzed by the pyruvate:acceptor oxidoreductase; however, the regeneration system of acetyl-CoA has not been clarified.

R. erythropolis N9T-4 was isolated from a crude oil sample, and so the question arises as to whether the characteristics of N9T-4 are due to the specific environmental conditions, i.e., crude oil, or are widely found in nature. In this study, it was elucidated that other *R. erythropolis* strains preserved in a Japanese microorganism stock center showed growth similar to that of N9T-4, suggesting that this oligotrophy is a common feature in *Rhodococcus* spp. We also found that the oligotrophic growth of N9T-4 was the best among the *Rhodococcus* spp. tested in this study. It will be of interest to screen various environmental samples for the same type of oligotroph as N9T-4, and this screening is now in progress.

Two proteins (45 kDa and 55 kDa) were highly induced in the cell extract prepared the N9T-4 cells grown under the oligotrophic conditions. MNOs to which the 45-kDa protein showed similarity have been found in gram-positive bacteria and catalyze the oxidation of methanol by use of NDMA as an artificial electron acceptor (5). Furthermore, MNOs contain a tightly but noncovalently bound NADP(H) per subunit (6) and also have the formaldehyde dismutase activity without endogenous NADP. Other types of nicotinoprotein containing a bound NAD(H) as a cofactor have been found in gram-positive methylotrophic bacteria. Alcohol:NDMA oxidoreductase from *A. methanolicus* and *R. erythropolis* DSM 1069 has a broad substrate specificity for aliphatic alcohols but not for methanol and contains a tightly but noncovalently bound NAD(H) (27, 29). NAD-dependent methanol dehydrogenase from *Bacillus methanolicus* C1 also contains a tightly bound NAD(H) (6, 7), and an activator protein facilitated the oxidation of the reduced NADH cofactor (3). Except for MNOs, however, these nicotinoproteins did not show the formaldehyde dismutase activity, and the non-MNO nicotinoproteins had a relatively low similarity to the 45-kDa protein N9T-4 compared with MNOs. The best alignment with the 55-kDa protein of N9T-4 was obtained with ALDH from *R. erythropolis* UPV-1, which was able to grow on phenol as the sole carbon and energy

sources and to remove formaldehyde from industrial wastewater (10). The ALDH had a broad substrate specificity for aliphatic aldehydes such as *n*-hexanal and *n*-octanal and also acted toward formaldehyde, although the affinity was quite low. In this study, we confirmed that the 45- and 55-kDa proteins of N9T-4 had formaldehyde dismutase and NAD-dependent ALDH activities, respectively, and both enzyme activities were induced under the oligotrophic growth of N9T-4. Interestingly, the ALDH of N9T-4 was not induced by formaldehyde in the medium, while MNO activity was increased under the same conditions. Furthermore, gene disruption experiments revealed that *ald* was essential for the oligotrophic growth of N9T-4 and suggested that *mno* is indispensable even for heterotrophic growth, so that *mno* disruptant could not be obtained. These results suggested that ALDH is involved in formaldehyde formation from CO₂ and that MNO plays a role for the detoxification of formaldehyde produced in the cells.

In methylotrophic bacteria, formaldehyde is oxidized to CO₂ by ALDH and formate dehydrogenase (25); meanwhile, formaldehyde is assimilated in the RuMP pathway or the serine pathway in methylotrophic bacteria and in the xylulose monophosphate pathway in methylotrophic yeasts (1). Since *hps* and *phi* were found in the *Rhodococcus* sp. strain RHA1 genome, it was expected that RuMP pathway also functions in *R. erythropolis* N9T-4. Actually, HPS and PHI activities were detected under the oligotrophic growth of N9T-4, suggesting that oligotrophically produced formaldehyde is fixed by RuMP pathway. As with results for strain RHA1, recent genome projects with various microorganisms have revealed that the *hps* and *phi* orthologs are widely distributed among bacterial and archaeal genomes (14). One of the physiological roles of the RuMP pathway is known to be the detoxification of formaldehyde. *Bacillus subtilis* produces HPS and PHI only when the growth medium contains formaldehyde and detoxifies the formaldehyde through enzyme reactions (31). In the case of the vanillin-degrading bacterium *Burkholderia cepacia* TM1, formaldehyde is released from the methoxyl group of vanillic acid with its hydroxylation to protocatechuic acid, and HPS and PHI is involved in the detoxification and assimilation of formaldehyde in this strain (20). Recently, the reverse RuMP pathway has been proposed as the novel physiological role of formaldehyde fixation in some archaea. The archaeal RuMP pathway is involved in the synthesis of ribulose 5-phosphate from fructose 6-phosphate through the reverse reaction of formaldehyde fix-

ation by HPS and PHI to compensate for the incomplete pentose phosphate pathway in several archaea (24, 28). We believe that oligotrophic formaldehyde fixation by the RuMP pathway in *R. erythropolis* N9T-4 sheds light on the physiological role of the RuMP pathway.

Taking the observations in this study together, we propose the mechanism for the oligotrophic growth of N9T-4, which involves methanol (formaldehyde) metabolism, as follows (Fig. 7): (i) CO₂ is converted to formaldehyde by an unknown reaction which may involve ALDH; (ii) temporarily, toxic formaldehyde is dismutated by MNO to methanol and formate, which show lower toxicity (or consequently, methylformate, which is even less toxic, is produced from these compounds); and (iii) formaldehyde is occasionally produced from methanol and formate by the reverse reaction of MNO and fixed by the RuMP pathway.

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