Stimulation of Methanotrophic Growth in Cocultures by Cobalamin Excreted by Rhizobia

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Methanotrophs play a key role in the global carbon cycle, in which they affect methane emissions and help to sustain diverse microbial communities through the conversion of methane to organic compounds. To investigate the microbial interactions that cause positive effects on methanotrophs, cocultures were constructed using Methylovulum miyakonense HT12 and each of nine nonmethanotrophic bacteria, which were isolated from a methane-utilizing microbial consortium culture established from forest soil. Three rhizobial strains were found to strongly stimulate the growth and methane oxidation of M. miyakonense HT12 in cocultures. We purified the stimulating factor produced by Rhizobium sp. Rb122 and identified it as cobalamin. Growth stimulation by cobalamin was also observed for other three gammaproteobacterial methanotrophs. These results suggest that microbial interactions through cobalamin play an important role in methane oxidation in various ecosystems.

MATERIALS AND METHODS

Methanotrophic strains and growth conditions. M. miyakonense HT12 was a laboratory stock (15). Methylococcus capsulatus Bath, Methylobacter luwos, Methylomonas methylalis S1, and Methylomus trichosporium OB3b were obtained from the culture collection of NCIMB (strain no. 11132, 11914, 11130, and 11131, respectively). Details of the isolation of methanotrophic strains OS501, R4F, B3R, SH31p, and SS2C will be described elsewhere (H. Iguchi, I. Sato, M. Sakakibara, H. Yurimoto, and Y. Sakai, unpublished data). The methanotrophic strains were grown in nitrate mineral salts (NMS) medium (28) under a 20:80 methane-to-air atmosphere at 28°C with shaking in 25-ml vials capped with butyl rubber stoppers. In order to compensate for slow growth, M. miyakonense HT12, strain OS501, and strain R4F were subcultured in medium supplemented with Bacto tryptone (Becton Dickinson and Company) at 0.01% (wt/vol). Isolation of bacterial strains grown in the consortium culture. The methane-enriched culture of forest soil (15) was subcultured every month over 2 years. The culture was serially diluted and spread onto agar plates: the media used were modified Luria-Bertani (LB) broth (0.2% tryptone, 0.1% yeast extract, 0.1% sodium chloride, 0.1% glucose), 10-fold-diluted tryptone soy broth (Oxoid, Cambridge, United Kingdom), 5-fold-diluted nutrient broth (Becton Dickinson and Company), and NMS medium with 0.1% methanol. A single colony grown on a plate at 28°C was isolated. The 16S rRNA gene was amplified by PCR, using the 27I-1492r primer set (27), and then sequenced. A similarity search for the nucleotide sequences of the 16S rRNA genes of the isolates was carried out using the BLAST program.

Mixed culture experiments. The cells of the heterotrophic isolates were prepared from liquid cultures grown in modified LB broth. Because of its inability to grow in this medium, strain Rb122 was grown in TY medium (0.5% tryptone, 0.5% yeast extract, 0.083% calcium chloride). Methanotroph cells were prepared...
from liquid cultures in NMS medium with methane. Cells of the methanotroph and the heterotroph were washed with water, mixed to adjust the ratio of optical densities at 600 nm (OD_{600} values) to 10:1 (OD_{600} values of 0.0025 and 0.00025, respectively), and cultivated in NMS medium with methane. For the second-generation culture, the cells were harvested by centrifugation from the 8-day-old mixed culture, washed with water, and inoculated into fresh NMS medium at an OD_{600} value of 0.0025. Triplicate samples were prepared for each culture and each time point, because after the methane concentration was measured, the vial was opened to measure the OD_{600}.

Methane concentrations were determined using a Shimadzu GC-14B gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a Porapak Q column (Shimawa Chemical Industries, Kyoto, Japan). Nitrogen gas was used as the carrier. Analytical temperatures of the oven, injector, and detector were 100, 120, and 225°C, respectively.

qPCR. Quantitative PCR (qPCR) was carried out using a LightCycler system (Roche Diagnostics, Tokyo, Japan) and SYBR Premix ExTaq (Takara Bio, Shiga, Japan) according to the manufacturers’ instructions. Genomic DNA, which was extracted from the culture by a method using SDS and proteinase K as described previously (16), was used as the template. Primers specific for the 16S rRNA gene of each strain were designed. The primer sets were htr-fw (TGGCCCAACATATGGGCGTA) and htr-re (AGGGATCTCTGCCGAATTATAGG) for strain HT12 and htr-fw (TGTGGGCACTTGACTGCTTCG) and htr-re (TACCGTCTCCGGTAACCGCGA) for strain Rb122. A standard curve for copy number calculation was generated with plasmid DNA (pGEM-T Easy [Promega, Madison, WI] harboring the 16S rRNA gene).

Preparation of spent medium and cell extracts. The pure culture of M. miyakonense HT12 and the culture of M. miyakonense HT12 and strain Rb122 were grown in NMS medium with methane. Strain Rb122 was grown in Rzobiobium minimal medium (RMM) with glucose (13). Bacterial cells were removed from the culture by centrifugation, and the spent medium was filter sterilized using a 0.45-μm filter. Until use, the samples were stored at −80°C.

For quantification, cobalamins were twice extracted from the hophylized culture or the harvested cells by using methanol with incubation at 80°C. The extracts were hophylized, dissolved in water, and filter sterilized using a 0.45-μm filter.

Stimulatory activity assay of methanotrophic growth (HT12 assay). M. miyakonense HT12 was grown on methane in NMS medium containing a test sample. The stimulatory activity of the sample was evaluated based on the OD_{600} value after cultivation for 48 to 72 h. We confirmed that the addition of NMS medium, RMM, or methanol extracts of the media did not stimulate growth. For quantification of the stimulatory activity, a calibration curve was established using a cyanocobalamin (CN-Chl) standard (Wako Pure Chemical Industries, Osaka, Japan).

Bioassay of cobalamin (Lactobacillus assay). Cobalamin was quantified by the growth of Lactobacillus delbrueckii NBRC 3073 on B_{12} assay medium (Becton Dickinson and Company) according to the procedures in the Dilco and BBL manual.

Purification of cobalamins from Rb122 culture. Four 500-ml batches of stationary-phase cultures of strain Rb122 were prepared by growth in RMM with glucose at 28°C with shaking. The cells were removed by centrifugation, and the resulting 1.9-liter supernatant was incubated with potassium cyanide to convert cobalamins to cyanocobalamin. This was performed to facilitate purification and detection because cobalamins may occur in different forms in cells and are sensitive to light. The solution was evaporated with ethanol and extracted with methanol.

The extracts were evaporated and dissolved in water. The sample was applied to an Amberlite XAD-2 column (Sigma-Aldrich, St. Louis, MO). The column was eluted with 50% methanol after washing with water. The eluate was evaporated, dissolved in water, and applied to a Sep-Pak C_{18} cartridge column (Waters, Milford, MA). The column was eluted with 30% acetonitrile after washing with 10% acetonitrile.

Nucleotide sequence accession numbers. The partial 16S rRNA gene sequences determined in this study have been deposited in GenBank under accession numbers AB636280 to AB636303.

### RESULTS

Isolation of bacterial strains from methane-grown consortium culture. Previously, we established a stable microbial consortium utilizing methane as the single carbon and energy source from a forest soil sample which has been maintained for over 2 years (15). From this consortium culture, the methanotroph M. miyakonense HT12 was isolated (15). Sequence analysis based on the 16S rRNA gene identified another methanotroph (a Methylocystis sp.) and 10 heterotrophic bacteria in the consortium (Table 1). Nine of these 10 heterotrophic strains were isolated in pure culture (Table 1). They were all Gram-negative bacteria belonging to either the Proteobacteria or Bacteroidetes. Strain NS1203 was a facultative methylotroph that grew on methanol, while the other eight strains were nonmethylotrophic heterotrophs. Among the strains identified in the consortium, Methylocystis sp. and Spliobacterium sp. could not be isolated (Table 1).

Methane oxidation and growth of M. miyakonense HT12 were stimulated in mixed cultures. Each of the isolated strains was added to the culture of M. miyakonense HT12 to test for positive effects on growth and methane oxidation. Five strains, Rb122, NL123, NS1202, NL124, and NL127, caused significant effects on methane oxidation (Fig. 1). The concomitant increase in methane consumption with the cell density indicated that M. miyakonense HT12 showed improved growth on methane in the mixed culture. Interestingly, three of the five strains were members of the order Rhizobiales. We used the representative rhizobial strain Rb122, which is closely related to the genus Rhizobium (Table 1), for an in-depth analysis of the
stimulatory effect on methane oxidation by *M. miyakonense HT12*.

**Analysis of coculture consisting of *M. miyakonense HT12* and *Rhizobium* sp. Rb122.** Growth of the coculture of *M. miyakonense HT12* and *Rhizobium* sp. Rb122 was analyzed by qPCR. Although we could not quantify cell mass during the early growth phase, the cell masses of both strains certainly increased in the mixed culture, and the number of cells was estimated to be 1 to 2 orders of magnitude higher for *M. miyakonense HT12* than for *Rhizobium* sp. Rb122 (Fig. 2B). *M. miyakonense HT12* exhibited slow growth in pure culture (Fig. 3A), while the growth of the coculture with *Rhizobium* sp.

**FIG. 1.** Stimulatory effects of bacterial inocula on growth (A) and methane oxidation (B) of *M. miyakonense HT12*. Mixed cultures were constructed by inoculating *M. miyakonense HT12* together with each of the bacterial strains into the medium. To generate stable bacterial cocultures, mixed cultures grown on methane for 8 days were transferred to fresh medium. Values shown are the data for these second cultures. The data are the means for triplicate samples.

**FIG. 2.** Growth profiles of *M. miyakonense HT12* in pure culture and coculture with *Rhizobium* sp. Rb122. (A) Methane oxidation (closed symbols) and growth (open symbols) were compared between the pure culture (circles) and the coculture (triangles). The data are the means for triplicate samples. (B) Cell masses of *M. miyakonense HT12* (circles) and *Rhizobium* sp. Rb122 (triangles) in the coculture were determined by quantitative PCR targeting the 16S rRNA gene. Representative data from triplicate samples are shown.

**FIG. 3.** Comparison of growth on methane between pure methanotroph culture (circles) and coculture of a methanotroph with *Rhizobium* sp. Rb122 (triangles). (A) *M. miyakonense HT12*; (B) strain OS501; (C) strain R4F; (D) *M. methanica*. Open symbols indicate growth. Methane consumption (closed symbols) in the coculture demonstrates that growth was dependent on the methanotrophic activity. The data are the means for triplicate samples.
Rhizobium from the pure culture of *M. miyakonense* HT12 and compounds for growth-stimulating factor present in the spent medium of Rhizobium showed stimulatory activity, whereas the spent medium from *M. miyakonense* HT12 culture did not. These results indicate that Rhizobium sp. Rb122 releases cobalamin into the medium, but *M. miyakonense* HT12 does not produce it.

In order to identify the cobalamin compound produced by *Rhizobium* sp. Rb122 and to confirm its growth-stimulatory activity, we purified cobalamin compounds according to the standard method for vitamin B12 (29). *Rhizobium* sp. Rb122 was grown in RMM with glucose, and the culture supernatant was incubated with potassium cyanide to convert cobalamins to CN-Cbl. The resultant solution was evaporated, extracted with methanol, and purified using an Amberlite XAD-2 column and a Sep-Pak C18 column. The purified compounds exhibited stimulatory activity based on the HT12 assay (Table 3). The activity detected in the HT12 assay and the cobalamin content assessed by the *Lactobacillus* assay were nearly identical throughout the purification step (Table 3), indicating that cobalamin is the principal methanotrophic growth-stimulating compound produced by *Rhizobium* sp. Rb122.

The final preparation was subjected to liquid chromatography-mass spectrometry (LC-MS) analysis with CN-Cbl as the reference compound. The cobalamin content in the spent medium of *Rhizobium* sp. Rb122 (Fig. 4A) was 6.70 pg/ml, which was 2 orders of magnitude lower than that in the Rb122 culture (Table 2). On the other hand, cobalamin was not detected in the *M. miyakonense* HT12 culture (Table 2). These results show that *Rhizobium* sp. Rb122 releases cobalamin into the medium, but *M. miyakonense* HT12 does not produce it.

### Table 2. Cobalamin production determined by the *Lactobacillus* assay

<table>
<thead>
<tr>
<th>Culture</th>
<th>Cultivation time (days)</th>
<th>OD&lt;sub&gt;600&lt;/sub&gt;</th>
<th>Cobalamin concn (pg/ml)</th>
<th>Extracellular</th>
<th>Intracellular</th>
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<tbody>
<tr>
<td><strong>Coculture of HT12 and Rb122</strong></td>
<td>1</td>
<td>0.041</td>
<td>3.79</td>
<td>2.77 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
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<td></td>
<td>2</td>
<td>0.520</td>
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<td>6.70 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.918</td>
<td>9.56</td>
<td>1.15 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>4</td>
<td>0.873</td>
<td>1.29 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.28 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
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</tr>
<tr>
<td><strong>Rb122 culture</strong></td>
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<td>0.142</td>
<td>7.68 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
<td>1.53 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>ND</td>
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<td></td>
<td>2</td>
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<td>4.91 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>3</td>
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<td>2.52 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
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<td></td>
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<td><strong>HT12 culture</strong></td>
<td>7</td>
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<td>ND</td>
<td>ND</td>
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</tr>
<tr>
<td></td>
<td>12</td>
<td>0.301</td>
<td>ND</td>
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</table>

* ND, not detected.

### Notes
- Table 2 shows the cobalamin concentration in the spent medium of *Rhizobium* sp. Rb122 and *M. miyakonense* HT12 cultures.
- Cobalamin is the growth-stimulating factor produced by *Rhizobium* sp. Rb122. The cobalamin content in the spent culture was quantified by the *Lactobacillus* assay.
- Neither autoclaving (121°C, 20 min) nor enzyme treatments with proteinase K or lipase significantly changed the stimulatory activity. The activity could be extracted from the spent medium with methanol or butanol but not with ethyl acetate. These observations suggested that the factor is a heat-stable, relatively polar microelement for cells.
- Based on these data, we tested the following bioactive compounds for stimulation of the growth of *M. miyakonense* HT12: thiamine, riboflavin, pyridoxine, cyanocobalamin, niacin, calcium pantothenate, biotin, folic acid, inositol, ascorbic acid, pyrroloquinoline quinone, glutathione, and amino acids.
- Among them, cyanocobalamin was found to have a clear stimulatory activity, in a dose-dependent manner (Fig. 4B), whereas the other compounds had no activity. Vitamin B<sub>12</sub> is used to describe compounds of the cobalamin group, which include cyanocobalamin (CN-Cbl), methylcobalamin (Me-Cbl), hydroxocobalamin (OH-Cbl), and adenosylcobalamin (Ado-Cbl). Me-Cbl and OH-Cbl also exhibited a stimulatory effect equivalent to that of CN-Cbl (data not shown).

**FIG. 4.** Stimulatory activity of the Rb122 culture supernatant (A) and cyanocobalamin (B) on the growth of *M. miyakonense* HT12. The cells were grown on methane in NMS medium containing the indicated concentrations of additives. Plotting the OD<sub>600</sub> values of the culture measured at a time point (48 to 72 h) with respect to the concentrations of the additives showed dose-dependent growth-stimulatory activity.
Cobalamin production was quantified for the methanotrophs grown on methane by the Lactobacillus assay. All of the tested methanotrophs except for strain OS501 produced cobalamins (Table 4). Accordingly, cobalamin was determined to be the stimulating factor in the coculture with Rhizobium sp. Rb122 for strain OS501, strain R4F, and M. methanica S1, but some other factors were likely provided for M. luteus in the coculture. We screened stimulating factors from the bioactive compounds as performed for M. miyakonense HT12 and found that thiamine, pyridoxine, and calcium pantothenate slightly stimulated the growth of M. luteus.

The phylogenetic tree based on the 16S rRNA gene sequences showed that the methanotrophs tested (Table 4) were located divergently in the tree, and no obvious phylogenetic correlation could be found with respect to the stimulatory effect or cobalamin production of methanotrophs, except that the methanotrophs for which growth stimulation was observed belonged to the Gammaproteobacteria (see Fig. S2 in the supplemental material).

**DISCUSSION**

We investigated the microbial interaction that caused a positive effect on methanotrophic growth and methane oxidation of M. miyakonense HT12 and showed that five bacterial strains related to the genera Rhizobium, Sinorhizobium, Mesorhizobium, Xanthobacter, and Flavobacterium stimulated the growth of M. miyakonense HT12 (Fig. 1 and Table 1). Further analysis of the stimulatory mechanism exerted by Rhizobium sp. Rb122 identified cobalamin as the growth-stimulating factor. We also detected cobalamin production by four other strains in pure cultures, namely, NS1202, NL123, NL127, and NL124 (see Table S1 in the supplemental material), indicating that the pivotal stimulatory mechanism in these cocultures is mediated by cobalamin. The degree of variation in the stimulatory effect observed in the coculture experiments (Fig. 1) is thought to be dependent on the growth of the cocultured bacterium and its cobalamin productivity (Table 2; see Table S1). Strains Rb122, NS1202, and NL123 exerted a considerable amount of cobalamin into the medium, while strains NL127 and NL124 did not. Such extracellular production of cobalamin may be the cause
of the strong stimulatory effect on methanotrophic growth exerted by rhizobia.

With respect to methylotrophs (methanol utilizers incapable of growth on methane) employing the ribulose monophosphate (RuMP) pathway, *Methylophaga* species were reported to show cobalamin auxotrophy (9), while *Methylbacinlus flagellatus* KT is deficient in cobalamin synthesis but can grow on methanol without cobalamin (11). However, cobalamin auxotrophy related to methanotrophic growth has not been investigated. It is worthwhile to note that among the tested methanotrophs, methanotrophic growth of four gammaproteobacterial methanotrophs that employ the RuMP pathway was stimulated by cobalamin (Table 4), and their slow or deficient growth in pure culture was correlated.

RuMP pathway was stimulated by cobalamin (Table 4), and among the tested methanotrophs, methanotrophic growth of KT is deficient in cobalamin synthesis but reported to show cobalamin auxotrophy (9), while *M. miyakonense* is capable of growth on methane) employing the ribulose monophosphate (RuMP) pathway by *Rhizobium* was reported to be stimulated by addition of methionine, methanol. For some organisms (4, 7), cobalamin auxotrophy since cobalamin also stimulates the growth of this strain on vitamin B12, through a symbiotic relationship with bacteria. *Nature* 438:90–93.

Bowman and Sayler (3) reported that cobalamin increases the soluble methane monoxygenase (sMMO) activity of *M. trichosporium OB3b*. However, activation of sMMO cannot explain the growth stimulation of *M. miyakonense* HT12, since cobalamin also stimulates the growth of this strain on methanol. For some organisms (4, 7), cobalamin auxotrophy was reported to be stimulated by addition of methionine, because methionine synthase (MetH) requires cobalamin as a cofactor (25). However, addition of methionine had no effect on the growth of *M. miyakonense* HT12 on methane. Further work is needed to elucidate the specific function of cobalamin in methanotrophic metabolism.

Interestingly, *M. miyakonense* HT12 seemed to obtain some growth factors from *Rhizobium* sp. 122 other than cobalamin, since the culture supernatant of *Rhizobium ex planta* sp. 122 exhibited higher cobalamin activity in the HT12 assay than in the *Lactobacillus* assay (Table 3). The presence of another growth-stimulatory factor was also suggested for *M. luteus* since the culture supernatant of *Rhizobium* sp. 122 may support *M. miyakonense* HT12 on methane.

In natural environments, organisms lacking the ability to synthesize cobalamin must rely on cobalamin producers such as bacteria and archaea (25). Cobalamin availability in the environment may thus restrict the growth of such organisms (2). The four tested methanotrophic strains were isolated from different habitats (HT12 from forest soil, OS501 from lake water, R4F from rice root, and S1 from an aquatic plant), and cobalamin-producing microorganisms, including rhizobia, are found ubiquitously in these environments (19, 25). Therefore, we suggest that microbial interactions with methanotrophs through cobalamin are distributed in various natural environments and play an important role in the methane cycle.

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