Stimulation of methanotrophic growth in co-cultures by cobalamin excreted by rhizobia

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ABSTRACT

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 caused positive effects on the methanotroph, co-cultures were constructed using *Methylovulum miyakonense* HT12 and each of nine non-methanotrophic 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 co-cultures. We purified the stimulating factor produced by *Rhizobium* sp. Rb122, and identified it as cobalamin. Growth stimulation by cobalamin was also observed for three other γ-proteobacterial methanotrophs. These results suggest that microbial interactions through cobalamin play an important role in methane oxidation in various ecosystems.

INTRODUCTION

Methane is the second most important greenhouse gas, and mitigating emissions of methane has become a major global concern (17). Aerobic methanotrophs are the major terrestrial methane sink and are widespread in a large variety of ecosystems (26). They belong to the *γ-Proteobacteria* (type I), *α-Proteobacteria* (type II) and *Verrucomicrobia* (26). Methanotrophs utilize methane as a single source of carbon and energy, but only some methanotrophic strains in the class *α-Proteobacteria* can assimilate substrates with C-C bonds (8).

Mutual interactions that occur between methanotrophs and other organisms, ranging from microbes to plants and animals, may affect the global methane cycle in various ways. Stable isotope probing (SIP) experiments revealed that methane-derived carbon was incorporated into methylotrophic or heterotrophic bacteria when they were incubated with methanotrophs (5, 14, 20, 22-23), indicating an important role of methanotrophs in supplying nutrients in the forms of carbon sources to other non-methanotrophic organisms. At deep-sea hydrothermal vents and cold seeps, invertebrates form symbiotic associations with γ-proteobacterial methanotrophs living in their tissues (21, 26). Some invertebrates can derive most of their carbon nutrition from methane, indeed acquiring it from methane-derived metabolites of methanotrophs or by digestion of methanotrophs. Inversely, the hosts provide methanotrophs with simultaneous access to methane

55 and oxygen by positioning themselves appropriately and also by providing a stable
56 environment. In peat bogs, *Sphagnum* mosses associate with α-proteobacterial
57 methanotrophs and utilize carbon dioxide that is generated from methane by
58 methanotrophic symbionts (18, 24). Although previous studies have demonstrated
59 that methanotrophs serve as food suppliers for other organisms, less attention has
60 been paid to the specific benefits that methanotrophs acquire from such
61 interactions.

62 Previously, we established a microbial consortium from forest soil utilizing
63 methane as the single carbon and energy source, from which we isolated a new
64 obligate methanotroph *Methylovulum miyakonense* HT12 (15). The aim of the
65 present study is to clarify the beneficial factors for methanotrophic growth and
66 methane oxidation provided by other bacteria in the methane-grown microbial
67 consortium to further understand interactions between methanotrophs and
68 microorganisms.

MATERIALS AND METHODS

Methanotrophic strains and growth conditions. *M. miyakonense* HT12 was a lab stock (15). *Methylococcus capsulatus* Bath, *Methylobacter luteus*, *Methylomonas methanica* S1 and *Methylosinus trichosporium* OB3b were obtained from the culture collection of NCIMB (No. 11132, No. 11914, No. 11130 and No. 11131, respectively). Details of the isolation of methanotrophic strains OS501, R4F, B3R, SH31p and SS2C will be described elsewhere: Iguchi H, Sato I, Sakakibara M, Yurimoto H and Sakai Y, in preparation. The methanotrophic strains were grown in nitrate mineral salts (NMS) medium (28) under a 20 : 80 methane to air atmosphere at 28ºC with shaking in 25 ml vials capped with butyl-rubber stoppers. In order to compensate for the 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% (w/v).

Isolation of bacterial strains grown in the consortium culture. The methane-enriched culture of forest soil (15) was subcultured every month over two 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 Soya Broth (OXOID, Cambridge, UK), 5-fold diluted Nutrient Broth (Becton, Dickinson and Company) and NMS medium with 0.1% methanol. A single colony grown on the plate at 28ºC

89 was isolated. The 16S rRNA gene was amplified by PCR using the 27f-1492r primer
90 set (27) and sequenced. A similarity search for the nucleotide sequence of the 16S
91 rRNA gene of the isolates was carried out using the BLAST program

92 **Mixed culture experiments.** The cells of the heterotrophic isolates were
93 prepared from liquid cultures grown in modified LB broth. Because of the inability
94 to grow in this medium, strain Rb122 was grown in TY medium (0.5% tryptone,
95 0.3% yeast extract, 0.083% calcium chloride). Methanotroph cells were prepared
96 from liquid cultures in NMS medium with methane. Cells of the methanotroph and
97 the heterotroph were washed with water, and then mixed to adjust the OD_{600} ratio
98 to 10 : 1 (OD_{600} values, 0.0025 and 0.00025, respectively), and cultivated in NMS
99 medium with methane. For the second generation culture, the cells were harvested
100 by centrifugation from the 8 day-old mixed culture, washed with water and
101 inoculated into fresh NMS medium at an OD_{600} value of 0.0025. Triplicate samples
102 were prepared for each culture and for each time point, because after the methane
103 concentration was measured the vial was opened to measure the OD_{600}.

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

Quantitative PCR (qPCR). qPCR was carried out using a LightCycler system (Roche Diagnostics, Tokyo, Japan) and SYBR Premix ExTaq (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Genomic DNA, which was extracted from the culture by a method using SDS and proteinase K as described previously (16), was used as template. Primers specific for the 16S rRNA gene of each strain were designed. The primer sets were ht-fw (TGGCCCCAATTATGGGGTAA) and ht-re (AGGGATCTCTGCCGAATCCA) for *M. miyakonense* HT12, and rb-fw (GTCGGGCAGTTGACTGTTCG) and rb-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 co-culture of *M. miyakonense* HT12 and strain Rb122 were grown in NMS medium with methane. Strain Rb122 was grown in Rhizobium 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 ºC.

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

Stimulatory activity assay on methanotrophic growth (the 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 48-72 hr. 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 standard (Wako Pure Chemical Industries, Osaka, Japan).

Bioassay of cobalamin (the *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 Difco & BBL Manual.

Purification of cobalamins from the Rb122 culture. Four 500 ml batches of stationary-phase cultures of strain Rb122 were prepared by growing 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. Then, the elution was

149 evaporated, dissolved in water and applied to a Sep-Pak C18 cartridge column
150 (Waters, Milford, MA). The column was eluted with 30% acetonitrile after washing
151 with 10% acetonitrile.

152 **Nucleotide sequence accession numbers.** The determined partial 16S
153 rRNA gene sequences have been deposited in GenBank under accession numbers
154 AB636288 to AB636303.

155

RESULTS

Isolation of bacterial strains from the 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 over two 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 *Methylocystis* sp. and ten heterotrophic bacteria in the consortium (Table 1). Nine out of these ten heterotrophic strains were isolated in pure culture (Table 1). They are all gram-negative bacteria belonging to either *Proteobacteria* or *Bacteroidetes*. Strain NS1203 was a facultative methylotroph that grew on methanol while the other eight strains were non-methylotrophic heterotrophs. Among the strains identified in the consortium, *Methylocystis* sp. and *Sphingobacterium* sp. could not be isolated (Table 1).

Methane oxidation and growth of *M. miyakonense* HT12 were stimulated in the mixed culture. 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

176 members of the order *Rhizobiales*. We used the representative rhizobial strain
177 Rb122, which is closely related to the genus *Rhizobium* (Table 1), for an in-depth
178 analysis of the stimulatory effect on methane oxidation by *M. miyakonense* HT12.

179 **Analysis of the co-culture consisting of *M. miyakonense* HT12 and**
180 ***Rhizobium* sp. Rb122**

181 Growth of the co-culture of *M. miyakonense* HT12 and *Rhizobium* sp.
182 Rb122 was analyzed by qPCR. Although we could not quantify the cell mass during
183 the early growth phase, the cell mass of both strains certainly increased in the
184 mixed culture, and the number of cells was estimated to be one- to two-orders of
185 magnitude higher for *M. miyakonense* HT12 than for *Rhizobium* sp. Rb122 (Fig. 2B).
186 *M. miyakonense* HT12 exhibited slow growth in pure culture (Fig. 3A), while the
187 growth of the co-culture with *Rhizobium* sp. Rb122 was stable even after repetitive
188 subculturing with methane. These results indicate that *Rhizobium* sp. Rb122 plays
189 a critical role in stimulating the growth of *M. miyakonense* HT12.

190 ***Rhizobium* sp. Rb122 produces a methanotrophic growth-stimulating factor.**
191 We hypothesized that the observed positive effect on *M. miyakonense* HT12 growth
192 by *Rhizobium* sp. Rb122 was due to the production of some growth-stimulating
193 factor(s). To verify this, we examined filtered spent medium from *Rhizobium* sp.
194 Rb122 for a stimulatory effect. The quantitative assay method to evaluate the
195 growth-stimulatory effect on *M. miyakonense* HT12 was established as described

196 Materials and Methods (designated as the HT12 assay).

197 Both the spent medium from the co-culture of *M. miyakonense* HT12 and
198 *Rhizobium* sp. Rb122 and the spent medium from the pure culture of *Rhizobium* sp.
199 Rb122 (Fig. 4A) showed stimulatory activity, whereas the spent medium from the *M.*
200 *miyakonense* HT12 culture did not. These results indicated that *Rhizobium* sp.
201 Rb122 produces growth-stimulating compounds in the medium for *M. miyakonense*
202 HT12.

203 Next, we characterized the chemical properties of the growth-stimulating
204 factor present in the spent medium of *Rhizobium* Rb122. Growth stimulation for *M.*
205 *miyakonense* HT12 was observed by addition of the spent medium at 0.01% (v/v)
206 concentration. Neither autoclaving (121°C, 20 min) nor enzyme treatments with
207 proteinase K or lipase significantly changed the stimulatory activity. The activity
208 could be extracted from the spent medium with methanol or butanol, but not with
209 ethyl acetate. These observations suggested that the factor is heat-stable, relatively
210 polar microelement for cells.

211 Based on these data, we tested the following bioactive compounds for
212 stimulation of the growth of *M. miyakonense* HT12: thiamine, riboflavin, pyridoxine,
213 cyanocobalamin, niacin, calcium pantothenate, biotin, folic acid, inositol, ascorbic
214 acid, pyrroloquinoline quinone, glutathione and amino acids. Among them,
215 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_{12} 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 the stimulatory effect equivalent to CN-Cbl (data not shown).

Bacto tryptone, which is used to stimulate the growth of methanotrophs in pure culture, was also found to contain cobalamins (93 ng/g) by the *Lactobacillus* assay (12).

Cobalamin is the growth-stimulating factor produced by *Rhizobium* sp. Rb122. The cobalamin content in the spent culture was quantified by the *Lactobacillus* assay. Cobalamin was detected from both the co-culture and the Rb122 culture, and the amount detected in the co-culture was two-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 release 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 confirm its growth-stimulatory activity, we purified cobalamin compounds according to the standard method for vitamin B_{12} (29). *Rhizobium* sp. Rb122 was grown in RMM with glucose, and the culture supernatant was incubated

236 with potassium cyanide to convert cobalamins to CN-Cbl. The resultant solution
237 was evaporated, extracted with methanol, and purified using an Amberlite XAD-2
238 column and a Sep-Pak C18 column. The purified compounds exhibited stimulatory
239 activity based on the HT12 assay (Table 3). The activity detected in the HT12 assay
240 and the cobalamin content assessed by the *Lactobacillus* assay were nearly
241 identical throughout the purification step (Table 3), indicating that cobalamin is the
242 principal methanotrophic growth-stimulating compound produced by *Rhizobium* sp.
243 Rb122.

244 The final preparation was subjected to LC-MS analysis with CN-Cbl as the
245 standard. The final preparation generated a peak at 10.5 min that was identical to
246 the CN-Cbl standard based on the retention time (Fig. S1A). The MS profile showed
247 that this peak yielded a *m/z* 678.90 fragment ion, which corresponded to
248 $[CN\text{-}Cbl+2H]^{2+}$ (Fig. S1B). The LC elution of the purified compounds was examined
249 for growth-stimulating activity for *M. miyakonense* HT12, and only the fraction
250 including the peak at 10.5 min was found to possess the activity. Based on these
251 results, we concluded that cobalamin is the growth-stimulating factor for *M.*
252 *miyakonense* HT12 produced by *Rhizobium* sp. Rb122.

253 **Stimulatory activity for methanotrophs.** *M. miyakonense* HT12 is in a
254 new phylogenetic lineage (15), so we raised the question of whether *Rhizobium* sp.
255 Rb122 as well as cobalamin exerts a stimulating effect on other methanotrophs.

256 Growth and methane consumption rates were compared between three cultures of
257 methanotrophs: addition of *Rhizobium* sp. Rb122 (co-culture), addition of
258 cyanocobalamin and no additive. Four methanotrophic strains from the culture
259 collection and five methanotrophic isolates obtained by our laboratory were used in
260 this experiment.

261 Both *Rhizobium* sp. Rb122 and cobalamin stimulated three methanotrophic
262 strains, OS501, R4F and *M. methanica* S1 (Table 4 and Fig. 3). The growth profiles
263 revealed that the slow growth and the deficient growth of strain OS501 and strain
264 R4F, respectively, was significantly stimulated by *Rhizobium* sp. Rb122 (Fig. 3B, C).
265 No growth-stimulating effects were observed for the other methanotrophs by either
266 *Rhizobium* sp. Rb122 or cobalamin, although *M. luteus* showed a slight stimulation
267 in growth and methane oxidation by *Rhizobium* sp. Rb122.

268 Cobalamin production was quantified for the methanotrophs grown on
269 methane by the *Lactobacillus* assay. All of the tested methanotrophs except strain
270 OS501 produced cobalamins (Table 4). Accordingly, cobalamin was determined to be
271 the stimulating factor in the co-culture with *Rhizobium* sp. Rb122 for strain OS501,
272 strain R4F and *M. methanica* S1, but some other factors are likely to be provided for
273 *M. luteus* in the co-culture. We screened stimulating factors from the bioactive
274 compounds as performed for *M. miyakonense* HT12, and found that thiamine,
275 pyridoxine and calcium pantothenate slightly stimulated the growth of *M. luteus*.

276 The phylogenetic tree based on the 16S rRNA gene sequences showed that
277 the methanotrophs tested (Table 4) were divergently located in the tree, and no
278 obvious phylogenetic correlation could be found with respect to the stimulatory
279 effect or cobalamin production of methanotrophs, except that the methanotrophs for
280 which growth stimulation was observed belong to the γ-*Proteobacteria* (Fig. S2).
281

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 of *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 the other four strains NS1202, NL123, NL127 and NL124 in pure cultures (Table S1), indicating that the pivotal stimulatory mechanism in these co-cultures is mediated by cobalamin. The degree of variation in the stimulatory effect observed in the co-culture experiments (Fig. 1) is thought to be dependent on the growth of the co-cultured bacterium and its cobalamin productivity (Table 2 and S1). Strains Rb122, NS1202 and NL123 excreted a considerable amount of cobalamin into the medium while strains NL127 and NL124 did not. Such an 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 *Methylobacillus*

302 *flagellatus* KT is deficient in cobalamin synthesis but can grow on methanol without
303 cobalamin (11). However, cobalamin auxotrophy related to methanotrophic growth
304 has not been investigated. It is worthwhile to note that among the tested
305 methanotrophs, methanotrophic growth of four γ-proteobacterial methanotrophs
306 that employ the RuMP pathway were stimulated by cobalamin (Table 4). And their
307 slow or deficient growth in pure culture was correlated with the ability to
308 synthesize cobalamin *de novo* (Fig. 3 and Table 4). On the other hand,
309 α-proteobacterial methanotrophs and methylotrophs, which employ the serine cycle,
310 require ethylmalonyl-CoA mutase, an Ado-Cbl dependent enzyme, for
311 methylotrophic growth (1, 6). Indeed, all of the tested α-proteobacterial
312 methanotrophs were shown to produce cobalamin *de novo* (Table 4). Therefore,
313 although the positive effect of cobalamin on methanotrophic growth seems to be
314 limited to γ-proteobacterial methanotrophs, cobalamin should have significant
315 functions in methanotrophic metabolism.

316 Bowman *et al.* (3) reported that cobalamin increases the sMMO activity of
317 *M. trichosporium* OB3b. However, activation of sMMO cannot explain the
318 growth-stimulation of *M. miyakonense* HT12 since cobalamin also stimulates the
319 growth of this strain on methanol. In some organisms (4, 7), cobalamin auxotrophy
320 was reported to be stimulated by addition of methionine, because methionine
321 synthase (MetH) requires cobalamin as a cofactor (25). However, addition of

322 methionine had no effect on the growth of *M. miyakonense* HT12 on methane.
323 Further work is needed to elucidate the specific function of cobalamin in
324 methanotrophic metabolism.
325 Interestingly, *M. miyakonense* HT12 seemed to obtain some growth factors
326 from *Rhizobium* sp. 122 other than cobalamin, since the culture supernatant of
327 *Rhizobium* sp. 122 exhibited higher cobalamin activity in the HT12 assay than in
328 the *Lactobacillus* assay (Table 3). The presence of another growth-stimulatory
329 factor was also suggested for *M. luteus* (Table 4). Vitamins and organic acids were
330 reported to enhance the growth rate of the methanotroph (30), and amino acids
331 could be utilized as a nitrogen source for methanotrophs (10). Production of such
332 compounds by *Rhizobium* sp. 122 may support methanotrophic growth in
333 co-cultures.
334 In natural environments, organisms lacking the ability to synthesize
335 cobalamin must rely on cobalamin producers such as bacteria and archaea (25).
336 Cobalamin availability in the environment may thus restrict the growth of such
337 organisms (2). The four tested methanotrophic strains were isolated from different
338 habitats (HT12 forest soil; OS501, lake water; R4F, rice root; S1, aquatic plant), and
339 cobalamin-producing microorganisms including rhizobia are found ubiquitously in
340 these environments (19, 25). Therefore, we suggest that microbial interactions with
341 methanotrophs through cobalamin are distributde in various natural environments,

342 and play an important role in the methane cycle.

343

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Figure Legends

FIG. 1. Stimulatory effect of the bacterial inocula on the 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 co-cultures, mixed cultures grown on methane for eight days were transferred to fresh medium. Shown are the data for these second cultures. The data are the means from triplicate samples.

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

FIG. 3. Comparison of the growth on methane between the pure methanotroph culture (circles) and the co-culture of the methanotroph with *Rhizobium* sp. Rb122 (triangles). Methanotroph: (A), *M. miyakonense* HT12; (B), strain OS501; (C), strain R4F; (D), *M. methanica*. Open symbols indicate growth. Methane consumption

463 (closed symbols) in the co-culture demonstrates that growth was dependent on the
464 methanotrophic activity. The data are the means of triplicate samples.
465
466 **FIG. 4.** Stimulatory activity of the Rb122 culture supernatant (A) and
467 cyanocobalamin (B) on the growth of *M. miyakonense* HT12. The cells were grown
468 on methane in NMS medium containing the indicated concentrations of additives.
469 Plotting OD_{600} value of the culture measured at a time point (cultivated for 48-72 h)
470 with respect to the concentrations of the additives showed dose-dependent
471 growth-stimulatory activity.

TABLE 1. Bacterial strains identified in the consortium culture

Strain/clone (Accession number)	Phylum	Closest relative[b] (Accession number)	Similarity[b] (%)
HT12 (AB501287)	*Proteobacteria* (gamma)	*Methylovulum miyakonense* HT12 (AB501287)	100
NM120[a] (AB636288)	*Proteobacteria* (alpha)	*Methylocystis* sp. 18-2 (AB007841)	99
NL123 (AB636289)	*Proteobacteria* (alpha)	*Mesorhizobium* sp. WSM3872 (FJ827044)	99
Rb122 (AB636290)	*Proteobacteria* (alpha)	*Rhizobium* sp. BZ3 (HQ588847)	97
NS1202 (AB636291)	*Proteobacteria* (alpha)	*Sinorhizobium* sp. J1 (DQ294628)	100
NL127 (AB636292)	*Proteobacteria* (alpha)	*Xanthobacter flavus* (EF592179)	100
NL121 (AB636293)	*Proteobacteria* (beta)	*Hydrogenophaga* sp. AH-24 (AB300163)	99
NS1203 (AB636294)	*Proteobacteria* (beta)	*Ideonella* sp. B513 (AB049107)	99
NS1204 (AB636295)	*Proteobacteria* (beta)	*Lutiella nitroferrum* 2002 (AY609199)	93
NL124 (AB636296)	*Bacteroidetes*	*Flavobacterium* sp. WG2 (FN547416)	95
NL128 (AB636297)	*Bacteroidetes*	*Emticicia ginsengisoli* Gsoil 085 (AB245370)	98
NC22[a] (AB636298)	*Bacteroidetes*	*Sphingobacterium* sp. P-7 (AM411964)	94

[a] Not isolated.

[b] Closest relative and similarity are based on 16S rRNA gene sequences.

TABLE 2. Cobalamin production determined by the *Lactobacillus* assay

Culture	Cultivation time (days)	OD_{600}	Cobalamin (pg/ml culture)[a]	
			Extracellular	Intracellular
Co-culture of HT12 and Rb122				
	1	0.041	3.79	2.77 x 10
	2	0.520	1.34 x 10	6.70 x 10
	3	0.918	9.56	1.15×10^2
	4	0.873	1.29 x 10	1.28×10^2
Rb122 culture				
	1	0.142	7.68 x 10	1.53×10^2
	2	0.738	1.24×10^3	4.91×10^3
	3	0.714	2.52×10^3	4.82×10^3
	4	0.689	2.39×10^3	4.24×10^3
HT12 culture				
	7	0.108	ND	ND
	12	0.301	ND	ND

[a] ND, not detected.

TABLE 3. Cobalamin activity of the purified compound from the culture of *Rhizobium* sp. Rb122

Sample	Cobalamin (μg)	
	HT12 assay	*Lactobacillus* assay
Culture supernatant	7.52	4.38
Amberlite XAD-2	2.75	2.03
Sep-Pak C_{18}	1.16	1.03

TABLE 4. Stimulatory activity and cobalamin production for methanotrophs

Strain	Proteobacterial subgroup	Stimulatory activity[a]		Cobalamin production[c] (ng/ml)
		Co-culture with Rb122	Cobalamin[b]	
Methylovulum miyakonense HT12^T	Gamma	++	++	ND
Methylococcaceae bacterium strain OS501	Gamma	++	++	ND
Methylomonas sp. R4F	Gamma	++	++	NT
Methylomonas methanica S1^T	Gamma	+	+	2.76
Methylobacter luteus^T	Gamma	+	-	2.58
Methylococcus capsulatus Bath	Gamma	-	-	2.16
Methylosinus trichosporium OB3b^T	Alpha	-	-	4.32
Methylosinus sp. B3R	Alpha	-	-	6.26
Methylocystis sp. SS2C	Alpha	-	-	5.05
Methylocystis sp. SH31p	Alpha	-	-	3.65

[a] Stimulatory activity was evaluated by growth and methane oxidation. ++, strong stimulation; +, weak stimulation; -, no stimulation.

[b] Cyanocobalamin was added to the medium at a final concentration of 100 pg/ml.

[c] Cobalamin in the whole culture at the stationary phase was quantified by the *Lactobacillus* assay. ND, not detected; NT, not tested due to the inability to grow without cobalamin.

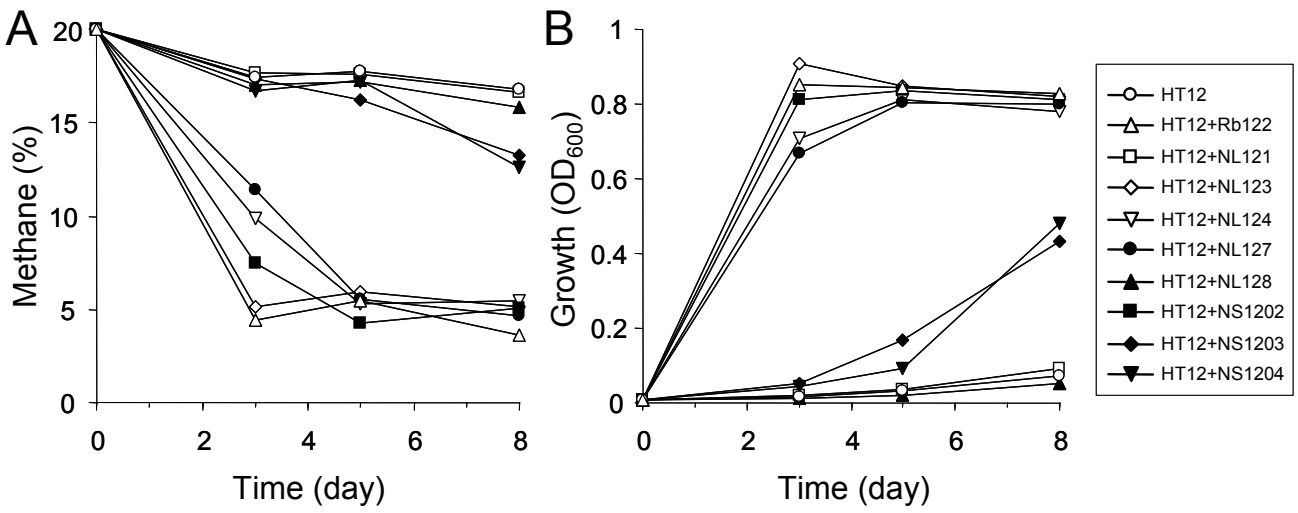
A
Methane (%)
20
15
10
5
0
0
2
4
6
8
Time (day)
B
Growth (OD600)
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
Time (day)
HT12
HT12+Rb122
HT12+NL121
HT12+NL123
HT12+NL124
HT12+NL127
HT12+NL128
HT12+NS1202
HT12+NS1203
HT12+NS1204

Figure 1

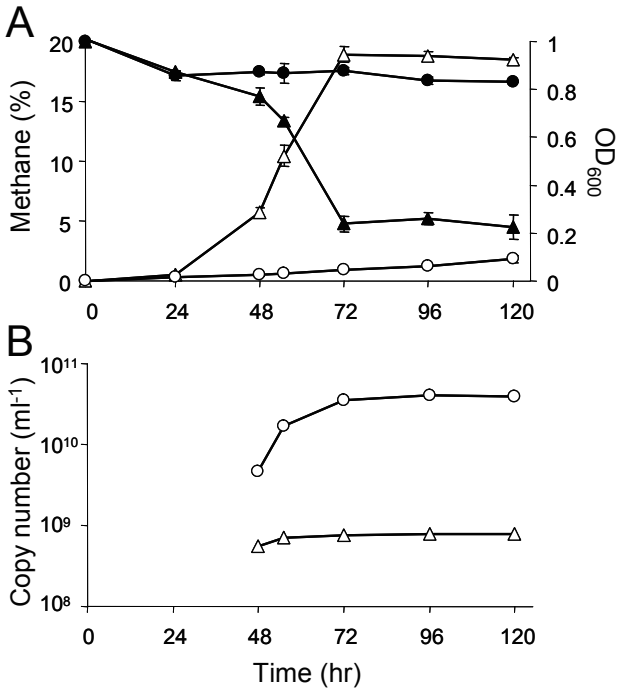

Figure 2

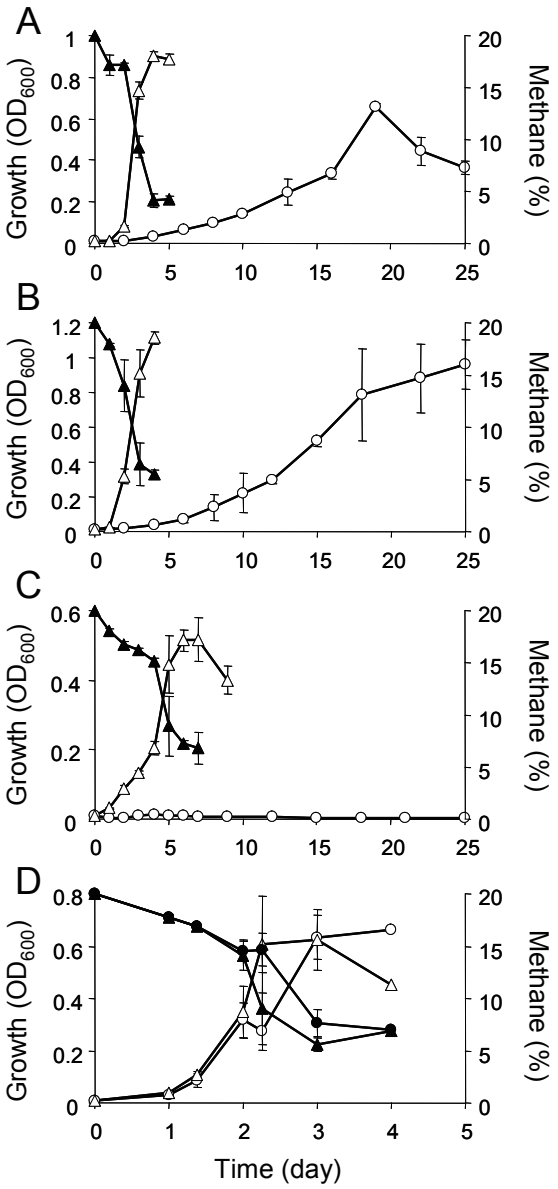

Figure 3

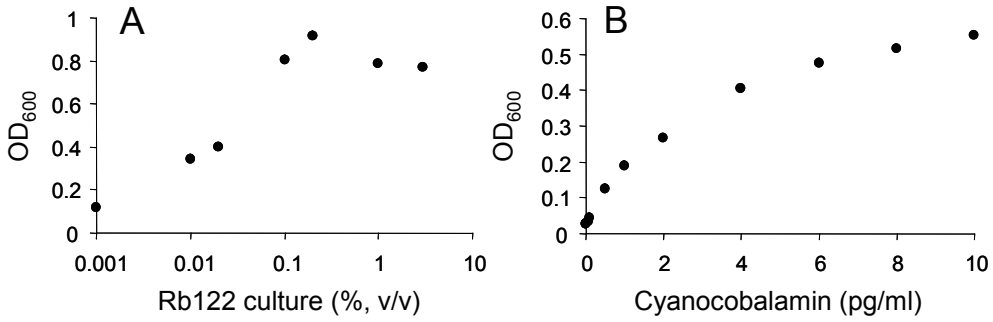
A
B
OD600
OD600
1
0.8
0.6
0.4
0.2
0
0.6
0.5
0.4
0.3
0.2
0.1
0
0.001
0.01
0.1
1
10
0
2
4
6
8
10
Rb122 culture (%, v/v)
Cyanocobalamin (pg/ml)

Figure 4

Supplemental material

Materials and Methods

LC-MS analysis. LC-MS analysis was performed on Shimadzu LC-20 series HPLC system (Shimadzu) and 4000 Q TRAP MS system (Applied Biosystems, Foster city, CA). Hydrosphere C18 column (2 mm x 150 mm, 3 mm [YMC, Kyoto, Japan]) was used and the column flow was maintained at 0.2 ml/min with a linear gradient of 5 to 40% acetonitrile in 20 mM ammonium acetate for 20 min. The UV detector was set at 361 nm. The MS spectra were acquired by scanning Q1 from 500-1400 Da in the positive electrospray ionization mode. The following conditions were used for MS quantification: declustering potential, 60; entrance potential, 10; curtain gas, 30; ionSpray voltage, 5500; temperature, 300°C; ion source gas 1, 50 L/min; ion source gas 2, 80 L/min.

TABLE S1. Cobalamin production of heterotrophic strains determined by the *Lactobacillus* assay.

Culture[a]	Cultivation time (hr)[b]	OD_{600}	Cobalamin (pg/ml culture)	
			Extracellular	Intracellular
NL123	95	1.032	1.30×10^3	4.93×10^3
	168	1.928	2.96×10^3	3.75×10^3
NL124	56	1.696	3.71×10	7.09×10^3
	80	2.044	4.13×10	8.98×10^3
NL127	56	1.644	3.43×10	6.04×10^3
	80	2.060	4.55×10	6.67×10^3
NS1202	32	1.648	8.50×10^2	5.96×10^3
	48	3.780	1.69×10^3	5.59×10^3

[a] The culture media used were RMM supplemented with glucose for strains NL123 and NS1202, and with succinate for strains NL124 and NL127.

[b] Shown are the data of the time points in the exponential and stationary growth phase, respectively.

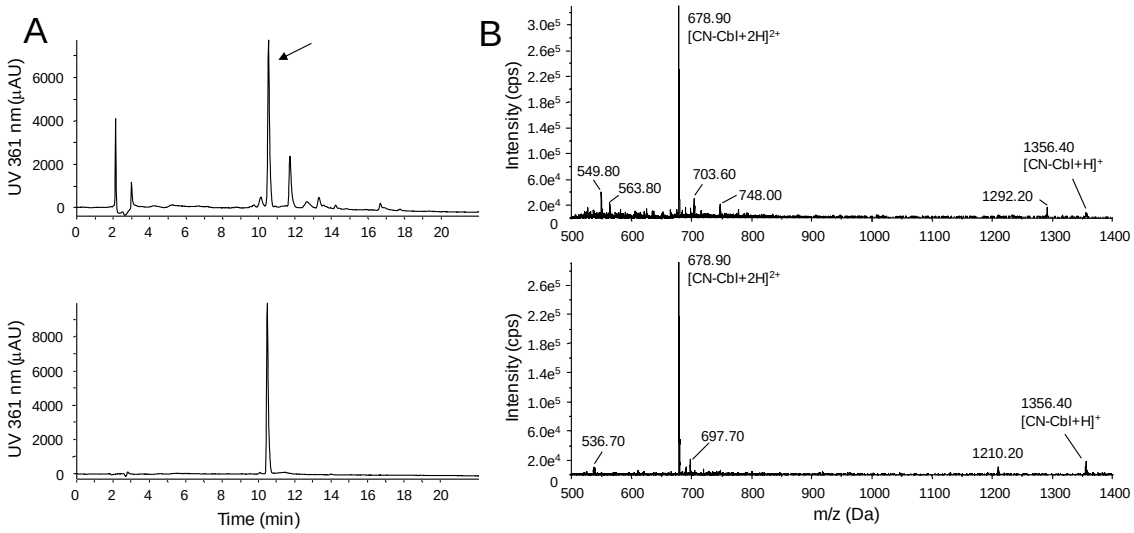

FIG. S1. LC-MS analysis of the purified compounds from the Rb122 culture (top panels) and CN-Cbl standard (bottom panels). (A) UV spectrum at 361 nm. The peak at 10.5 min marked with an arrow is identical to CN-Cbl based on the retention time. (B) MS spectrum of the peak with retention time of 10.5 min. The purified compounds yielded the major ion at *m/z* 678.90, which corresponds to CN-Cbl.

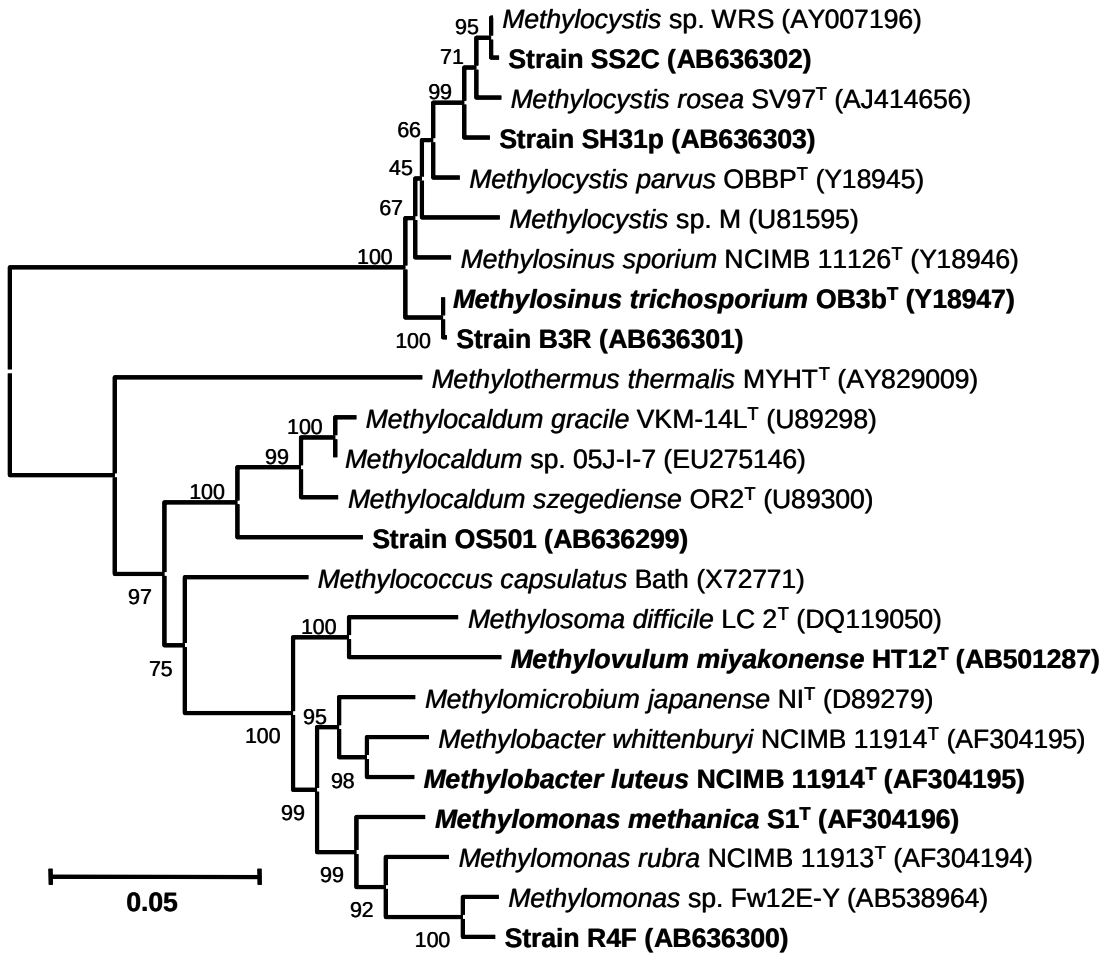

FIG. S2. Phylogenetic tree of 16S rRNA gene sequences. GenBank accession numbers are given in parentheses. Bootstrap values based on 100 resamplings are shown at branch points. Methanotrophs tested in Table 4 are shown in bold letters. Sequence alignment of the 16S rRNA gene was performed using the CLUSTAL W program. The tree was constructed using the neighbor-joining method with Kimura two-parameter model.