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A Novel Operon Encoding Formaldehyde Fixation: the Ribulose Monophosphate Pathway in the Gram-Positive Facultative Methylotrophic Bacterium *Mycobacterium gastri* MB19

RYOJI MITSUI,[1] YASUYOSHI SAKAI,[1] HISASHI YASUEDA,[2] AND NOBUO KATO[1]*

Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa Sakyo-ku, Kyoto 606-8502,[1] and Fermentation and Biotechnology Laboratories, Ajinomoto Co., Inc., Kawasaki 210-0801,[2] Japan

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A 4.2-kb *Pst*I fragment harboring the gene cluster of the ribulose monophosphate (RuMP) pathway for formaldehyde fixation was identified in the chromosome of a gram-positive, facultative methylotroph, *Mycobacterium gastri* MB19, by using the coding region of 3-hexulose-6-phosphate synthase (HPS) as the hybridization probe. The *Pst*I fragment contained three complete open reading frames (ORFs) which encoded from the 5′ end, a DNA-binding regulatory protein (*rmpR*), 6-phospho-3-hexuloisomerase (PHI; *rmpB*), and HPS (*rmpA*). Sequence analysis suggested that *rmpA* and *rmpB* constitute an operon, and Northern blot analysis of RNA extracted from bacteria grown under various conditions suggested that the expression of the two genes is similarly regulated at the transcriptional level. A similarity search revealed that the proteins encoded by *rmpA* and *rmpB* in *M. gastri* MB19 show high similarity to the unidentified proteins of nonmethylotrophic prokaryotes, including bacteria and anaerobic archaea. The clusters in the phylogenetic tree of the HPS protein of *M. gastri* MB19 and those in the phylogenetic tree of the PHI protein were nearly identical, which implies that these two formaldehyde-fixing genes evolved as a pair. These findings give new insight into the acquisition of the formaldehyde fixation pathway during the evolution of diverse microorganisms.

Many C_1 compounds, including formaldehyde, methane, and formic acid, were present in the interstellar dust clouds of the primitive earth and were important precursors of more complex organic compounds. Among them, formaldehyde is the key intermediate for biological fixation of C_1 compounds.

The ribulose monophosphate (RuMP) pathway of formaldehyde fixation exists in a wide range of methylotrophic bacteria that can grow on C_1 compounds (14). The RuMP pathway can be divided into three phases. The first, the fixation phase, involves two reactions that are unique to the RuMP pathway: the condensation of formaldehyde with ribulose 5-phosphate and the subsequent isomerization of the product, D-*arabino*-3-hexulose 6-phosphate, to yield fructose 6-phosphate. These reactions are catalyzed by 3-hexulose-6-phosphate synthase (HPS) and 6-phospho-3-hexuloisomerase (PHI), respectively. The second phase involves cleavage of the hexose phosphate to form two triose phosphates; glyceraldehyde 3-phosphate (GAP) enters the third phase of formaldehyde fixation, which consists of rearrangement reactions, while dihydroxyacetone phosphate (DHAP) enters the central pathway for synthesis of cell constituents. The third, the rearrangement phase, contains the reactions that are necessary to regenerate the acceptor of formaldehyde fixation, ribulose 5-phosphate.

The lack of genetic studies on the RuMP pathway had prevented further insights into the physiological and evolutionary aspects of this pathway. We recently reported the gene cluster of the RuMP pathway of a methylotrophic bacterium, *Methylomonas aminofaciens* 77a (16). The *rmpA* and *rmpB* genes which encode HPS and PHI, respectively, are adjacent to *rmpI* which encodes IS*10*-R, whose promoter regions are involved in regulating the expression of *rmpA* and *rmpB*. The existence of IS*10*-R suggested that the RuMP pathway gene cluster was transposed during the evolution of methylotrophs. From this observation, the question has been raised as to whether such gene organization of the RuMP pathway is a common feature in other methylotrophic bacteria.

In this study, we cloned a DNA fragment that harbored the genes encoding HPS and PHI from the chromosomal DNA of another type of methanol-utilizing bacterium, a gram-positive and facultative methylotroph, *Mycobacterium gastri* MB19. We found that the genes that encode HPS and PHI in *M. gastri* MB19 constitute a formaldehyde-fixing operon and that the expression of these two genes is regulated in a parallel manner. Furthermore, a similarity search revealed that the chromosome of some nonmethylotrophic bacteria and archaea contains a pair of genes homologous to *rmpA* and *rmpB*, a finding which suggests that the formaldehyde fixation pathway is an evolutionary link between bacteria and archaea.

MATERIALS AND METHODS

Strains and culture conditions. *M. gastri* MB19 was grown on the methanol medium described by Kato et al. (8). *Escherichia coli* JM109 (18), which was used as the host for plasmid propagation, was grown in Luria-Bertani (LB) broth which contained 1% Bacto Tryptone (Difco Laboratories, Detroit, Mich.), 0.5% Bacto Yeast Extract (Difco Laboratories), 1% NaCl (pH 7.0), and ampicillin (50 μg/ml); when necessary, 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and 0.05 mM X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were added to the medium.

Analysis of HPS and PHI. The level of HPS activity and the PHI activity were assayed according to the methods of Arfman et al. (2). The amount of protein was determined by using a Bio-Rad protein assay kit (Japan Bio-Rad Laboratories, Tokyo, Japan) with bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 15% polyacrylamide gel (10). The apparent molecular mass of the native enzyme was determined by gel filtration with a fast protein liquid chromatography system on a Superdex 200 column (Pharmacia Biotech, Uppsala, Sweden) (15).

HPS was purified from *M. gastri* MB19 as described by Kato (7). The N-terminal and several internal amino acid sequences of the purified HPS were

* Corresponding author. Mailing address: Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa Sakyo-ku, Kyoto 606-8502, Japan. Phone: 81-75-753-6385. Fax: 81-75-753-6128. E-mail: nkato@kais.kyoto-u.ac.jp.

determined as previously described (21). The N-terminal amino acid sequence (N-1) was MKYLQVAIDLLSTEAALELAGKVAEYVDIIELGTPLIKA, and the sequences of several internal peptide fragments were as follows: I-1, HAG LDEQAK; I-4, ATRAQEVRALGAK; I-5, VATIPAVQK; I-6, FVEMHAGLD EQAE; I-7, ARVPFSVAGGVK; I-8, IVFADMK; I-9, TMDAGELEADIAFK; and I-11, VAEYVDIIELGTPLIK.

Selection of a DNA fragment containing the HPS gene. A DNA fragment containing the HPS gene (*rmpA*) was amplified by PCR from the chromosomal DNA of *M. gastri* MB19 by using the following procedure. Upstream and downstream primers were designed based on a part of the sequence of the N terminus (MKLQVAID) and a part of an internal amino acid sequence (I-6; FVEMHA GL) of HPS, respectively. The nucleotide sequences of the primers were as follows: N-terminal (N-1), 5′-ATGAAA(G)C(T)TICAA(G)GTC(A/G/T)GCIA TC(A/T)GA-3′, and internal (I-6), 5′-CCC(A/G/T)GCA(G)TGCATC(T)TCC (A/G/T)ACA(G)AA-3′. Chromosomal DNA was extracted from *M. gastri* MB19 by using the method of Marmur (11) and was used as a template for PCR amplification. The reaction mixture and conditions for PCR were those for the standard procedure suggested by Perkin-Elmer/Cetus (Takara Shuzo Co., Kyoto, Japan). The PCR product was electrophoresed on a 2.0% low-melting-temperature agarose gel. The primary band was extracted from the gel with SUPREC-01 (Takara Shuzo) and then ligated into pT7Blue vector (Novagen, Madison, Wis.). This PCR product was used as the *rmpA* probe.

The chromosomal DNA of *M. gastri* MB19 was digested with various restriction enzymes. The digests were electrophoresed on a 0.7% agarose gel in TAE buffer (18) and then transferred onto a Biodyne nylon membrane (Pall Bio Support Corp., New York, N.Y.). Hybridization was performed with the random primed ^{32}P-labeled *rmpA* probe under the highly stringent conditions as recommended by Southern (19). On the basis of the results of Southern analysis, a *Pst*I-digested genome library of *M. gastri* MB19 genomic DNA was constructed, and the *Pst*I fragment harboring the *rmpA* gene was ligated into pUC118 at the *Pst*I.

Colonies of *E. coli* transformants were transferred to the Biodyne nylon membrane and lysed with alkaline treatment. The liberated DNA was fixed on the membrane, and hybridization was carried out at 42°C with the ^{32}P-labeled *rmpA* probe. Restriction analysis suggested that all of the positive clones had an identical insert in pUC118, and the recombinant plasmid was designated pUHM-1.

Nucleotide sequencing. Several deletion DNA templates of the *Pst*I fragment containing the HPS gene were prepared for sequencing with a deletion kit (Takara Shuzo). DNA sequencing of the deletion DNA templates was performed by the dideoxy chain termination method by using a PE Applied Biosystems Model 373A Automated DNA Sequencer (Tokyo, Japan). Sequencing was performed according to the manual of the ABI Dye Terminator Cycle Sequencing Kit (Perkin-Elmer, Foster City, Calif.). The National Center for Biotechnology Information database was searched for homologous amino acid sequences with the BLAST and FASTA programs.

Expression of *rmpA* and *rmpB* in *E. coli*. The coding region of *rmpA* and that of *rmpB* were each amplified by PCR by using pUHM-1 as the template. The upstream and downstream primers for amplification of *rmpA* were designed from the obtained sequence of the *Pst*I fragment: 5′-AGAA*ATCGAT*AAATGAAG CTCCAAGTCGCCATCG-3′ (the translation start codon is underlined) at the N terminal and 5′-AATTCCAGC*TCTAGA*CAAGGCGGTACGGCG-3′ at the C terminal. For PCR amplification of *rmpB*, the following primer pair was used: N terminal, 5′-CGAA*ATCGAT*AAAAATGACGCAAGCCGCAGAAGCCGA CGGCG-3′, and C terminal, 5′-TGGG*TCTAGA*ATGTGAAGTAAGAGTTG GTCGTACGAGGTCCG-3′. The reaction mixture and conditions of PCR were as mentioned above. Each PCR product contained the open reading frame (ORF) of *rmpA* or *rmpB*, and the upstream and downstream ends of the PCR products of both *rmpA* and *rmpB*, and the upstream and downstream ends of the PCR products of both *rmpA* and *rmpB* contained the *Cla*I and *Xba*I sites (italicized sequence in the primer), respectively. The PCR product of *rmpA* and that of *rmpB* were each purified and cloned into the *Cla*I/*Xba*I site of pT13sNco (22), and the resultant plasmid was designated pT-rmpA or pT-rmpB, respectively. A pBR322-derived vector, pTTNco (22), was used as the control plasmid. *E. coli* JM109 was transformed with one of the three plasmids. The transformant *E. coli* strains were grown on a M9 Casamino Acids medium containing 1 μg of thiamine-HCl and 100 μg of ampicillin per ml as described by Sambrook et al. (18) at 37°C for 12 h. The *E. coli* cells were then washed with 50 mM potassium phosphate buffer (pH 7.6), suspended in the same buffer, and sonicated (150 W, 10 min). The cell extract was used for measurement of the level of HPS and PHI activity and for SDS-PAGE.

Northern blot analysis. *M. gastri* MB19 was cultured at 28°C on a minimal salts medium (8) that contained 1.0% (vol/vol) methanol, ethanol, or glucose as a carbon source and 0.4% (wt/vol) $(NH_4)_2SO_4$ or methylamine as a nitrogen source until the middle log phase. The *M. gastri* MB19 cells were then harvested by centrifugation at 6,700 × *g* for 20 min at 4°C. Some samples were used for the determination of the level of HPS activity and PHI activity. Total RNA was extracted from other samples by the acid-guanidinium thiocyanate-phenol-chloroform method using ISOGEN (Nippon Gene Co., Ltd., Tokyo, Japan). Northern blot analysis was performed as described previously (16). Briefly, RNA samples (20 μg/lane) were electrophoresed on a 1.0% agarose gel containing 20 mM MOPS (morpholine propane sulfonic acid) buffer, 1 mM EDTA, and 2.2 M formaldehyde. Perfect RNA Markers, 0.2 to 10 kb (Novagen, Madison, Wis.), was used for the nucleotide size measurement. After electrophoresis, capillary transfer to a nylon membrane (GeneScreen Plus; NEN Life Science Products, Boston, Mass.) in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) was performed. Prehybridization and hybridization were carried out at 42°C in a solution containing 30% formamide, 5× SSC, 0.1% SDS, and 100 μg of calf thymus DNA per ml, as previously described (17). The DNA probe consisted of the entire coding region of *rmpA* or *rmpB* and was labeled with a Random Primed DNA Labeling Kit (Roche Diagnostics K.K., Mannheim, Germany).

Nucleotide sequence accession number. The sequence reported in this paper has been deposited in the GenBank-DDBJ database (accession no. AB034913).

RESULTS

Nucleotide sequence and structural analysis of pUHM-1. Determination of the entire nucleotide sequence of the 4.2-kb *Pst*I insert in pUHM-1 revealed three complete and two partial ORFs; from the 5′ end, they are *orf1*, *rmpR*, *rmpB*, *rmpA*, and *rmpC*. Figure 1A shows the gene diagram of the insert, which includes some important elements between the ORFs. *rmpA* consists of 624 bp, and the deduced amino acid sequence includes 209 amino acid residues with a theoretical molecular mass of 20,953 Da. This value is close to the molecular mass of the purified HPS from *M. gastri* MB19, as determined by SDS-PAGE, of 24 kDa. The N-terminal and internal amino acid sequences of the purified HPS were found in the deduced amino acid sequence. From this and the results of the gene expression studies, we concluded that this ORF encodes the HPS gene. *rmpB* is located upstream of *rmpA* and encodes a protein of 223 amino acid residues; the theoretical molecular mass is 24,666 Da. The deduced amino acid sequence of *rmpB* is similar to the deduced amino acid sequence of PHI of *M. aminofaciens* 77a (66% similarity) (16). *rmpR* was located upstream of *rmpB* in the reverse orientation. The putative product of this gene which encodes 223 amino acid residues (theoretical molecular mass, 24,666 Da) shows similarity to many DNA-binding regulatory proteins and contains a DNA-binding α-helix–turn–α-helix (HTH) motif in the N-terminal region that is common in the *gntR* family (6, 13). These data indicate that the *rmpR* product most likely acts as a DNA-binding regulatory protein. The deduced amino acid sequence of the partial ORF (*rmpC*) was similar to that of glucose-6-phosphate dehydrogenase in *Mycobacterium leprae* (84% similarity) (13) and the same enzyme in *Synechococcus* sp. strain PCC7942 (82%) (12). This enzyme could play a critical role in the cleavage stage of a variant of the RuMP pathway (14). The function and identity of *orf1* could not be determined.

Two short stem-loop structures exist upstream of the transcriptional start codon of *rmpB* and could encode a binding site for a DNA-binding regulatory protein (possibly the product of *rmpR*) as an operator (4). On the other hand, a putative transcriptional terminator with subsequent T residues (1) was observed downstream of the termination triplet of *rmpA*. This sequence is characteristic of a rho-independent transcriptional terminator (6). The structure and organization of these three genes (*rmpR*, *rmpB*, and *rmpA*) are similar to several operon structures in many bacteria (5).

Expression of *rmpA* and *rmpB* in *E. coli*. In order to confirm the products of *rmpA* and *rmpB*, RmpA and RmpB were each expressed in *E. coli* under the control of a *trp* promoter. The cell extract of *E. coli* which had been transformed with the pT-rmpA or pT-rmpB plasmid exhibited a high level of HPS activity (210 $\mu mol \cdot min^{-1} \cdot mg\ of\ protein^{-1}$) or PHI activity (240 $\mu mol \cdot min^{-1} \cdot mg\ of\ protein^{-1}$), respectively. SDS-PAGE of the cell extract of *E. coli* which had been transformed with the pT-rmpA or pT-rmpB plasmid showed a major distinctive protein band whose molecular mass was in close agreement with the theoretical mass of the deduced amino acid sequences

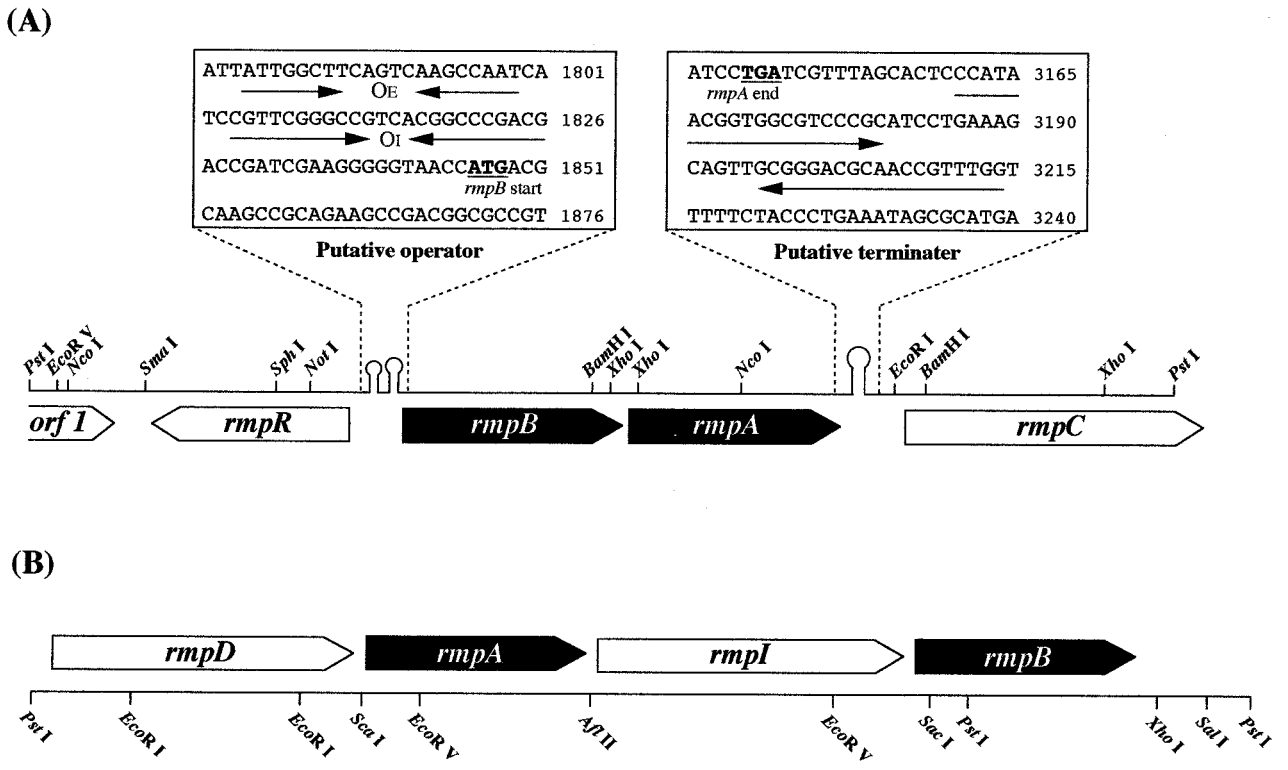

FIG. 1. Gene diagram comparing the RuMP ORFs of *M. gastri* MB19 (4,199 bp) (A) and *M. aminofaciens* 77a (4,451 bp) (B) (16). Genes: *rmpA*, a gene which encodes HPS; *rmpB*, a gene which encodes PHI; *rmpC*, a gene which encodes glucose-6-phosphate dehydrogenase; *rmpD*, a gene which encodes transaldolase; *rmpI*, a gene which encodes transposase (IS*10*-R); *rmpR*, a gene which encodes regulatory protein; and *orf1*, unknown. In the boxes, the arrows upstream of *rmpB* indicate inverted repeat structures, and the putative operators marked O_E and O_I indicate the external and internal operators, respectively. The arrows downstream of *rmpA* indicate the putative transcriptional terminator as an inverted repeat. The numbering of nucleotides starts from the beginning of the *Pst*I fragment.

of HPS and PHI, respectively (data not shown). The control strain harboring pTTNco exhibited neither HPS activity nor PHI activity. Judging from these findings, *rmpA* and *rmpB* encode HPS and PHI, respectively.

Transcriptional regulation of *rmpA* and *rmpB* in *M. gastri* MB19. *M. gastri* MB19 was grown on media containing various carbon and nitrogen sources until the middle log phase; the level of HPS and PHI activity in the cell extracts was then measured (Fig. 2A). In bacteria that had been grown on methanol as a carbon source, high HPS activity and high PHI activity were present. Negligible HPS activity and PHI activity were detected in cells that had been grown on a medium which contained ethanol or glucose as a carbon source and an inorganic nitrogen, $(NH_4)_2SO_4$. On the other hand, considerable HPS activity and PHI activity were present in cells that had been grown on a medium which contained methylamine as a nitrogen source even when glucose or ethanol was used as the carbon source. These data indicate that the expression of HPS and PHI is induced by methanol or methylamine.

To determine the transcriptional regulation of the *rmpA* and *rmpB* genes, Northern blot analysis was performed on the total RNA of *M. gastri* MB19 that had been grown on various media with the DNA fragment of *rmpA* or *rmpB* as the probe (Fig. 2B). Northern blot analysis of the methanol- and methylamine-grown cells revealed hybridizing bands; the size of the hybridizing band with the entire coding region of *rmpA* as the probe and that with the entire coding region of *rmpB* as the probe were identical and corresponded to the entire length of the transcription product of *rmpA* and *rmpB* (1.5 kb). On the other hand, the *rmpA* probe and *rmpB* probe did not hybridize against the total RNA of cells that had been grown on a medium which did not contain methanol or methylamine. These results are in fair agreement with the enzyme induction profile described above (Fig. 2A) and imply that expression of *rmpA* and *rmpB* is regulated at the mRNA level and that both

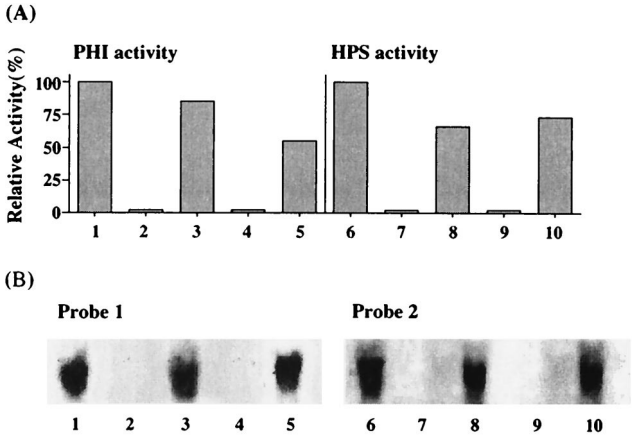

FIG. 2. Level of HPS and PHI activity (A) and results of Northern blot analysis of *rmpA* and *rmpB* gene expression (B) in *M. gastri* MB19 that had been grown on media with various nitrogen and carbon sources. *M. gastri* MB19 were grown on media containing methanol-$(NH_4)_2SO_4$ (lanes 1 and 6), ethanol-$(NH_4)_2SO_4$ (lanes 2 and 7), ethanol-methylamine (lanes 3 and 8), glucose-$(NH_4)_2SO_4$, or glucose-methylamine (lanes 5 and 10). Probes 1 and 2 used in Northern blot analysis are the entire coding regions of *rmpA* and *rmpB*, respectively, in pUHM-1. Total RNA was extracted from the cells grown on the media (1 to 10) described above. The hybridizing bands were observed at the size of 1.5 kb, which was determined by the comparison with the marker nucleotides.

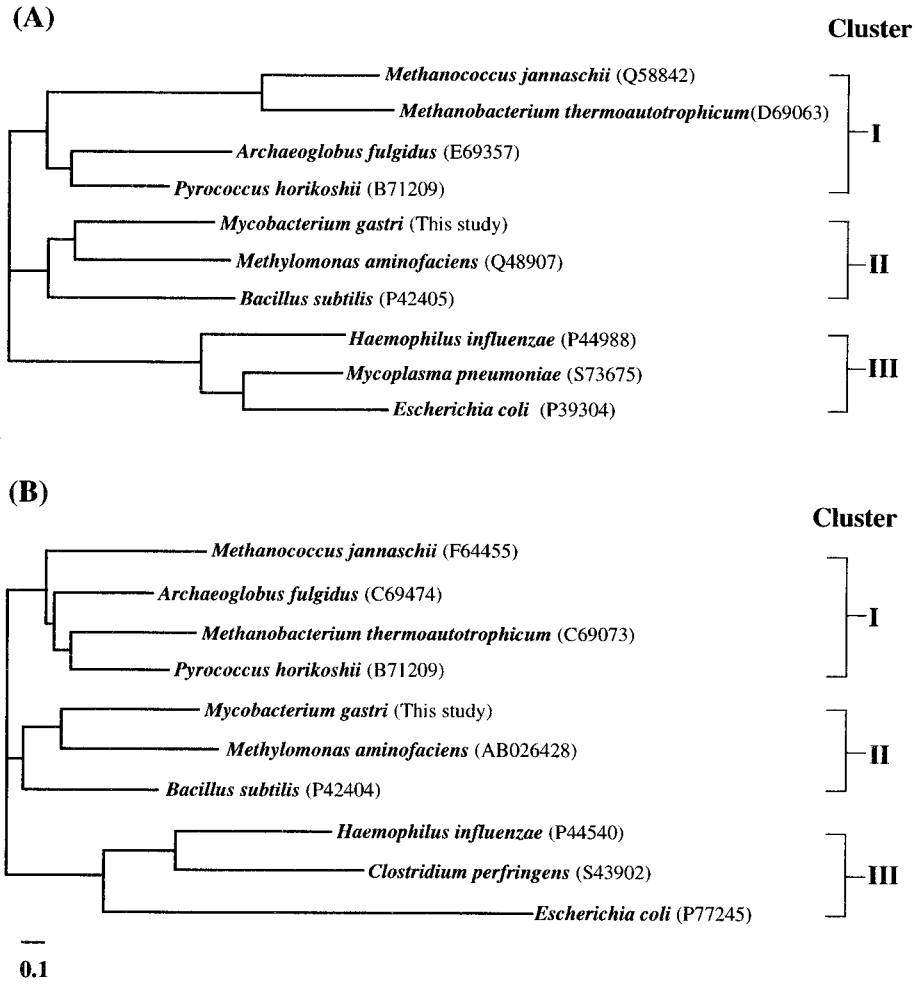

FIG. 3. Phylogenetic analyses of the amino acid sequence of the HPS protein (A) and PHI protein (B) by using the CLUSTAL W program via the DDBJ saver (20). The similarity between the amino acid sequence of the putative gene in the indicated organism found in the database and the amino acid sequences of HPS and PHI of *M. gastri* MB19 was as follows: *M. jannaschii* (HPS, 57.3%; PHI, 67.0%), *M. thermoautotrophicum* (HPS, 53.9%; PHI, 56.3%), *A. fulgidus* (HPS, 81.2%; PHI, 68.6%), *P. horikoshii* (HPS, 74.5%; PHI, 67.5%), *B. subtilis* (HPS, 76.2%; PHI, 72.1%), *H. influenzae* (HPS, 58.0%; PHI, 43.0%), *E. coli* (HPS, 62.8%; PHI, 36.0%), *M. pneumoniae* (HPS, 64.8%), and *C. perfringens* (PHI, 48.9%).

genes are transcribed as a polycistronic operon. Since methanol and methylamine are each initially oxidized to formaldehyde in *M. gastri* MB19, it is likely that formaldehyde induces the expression of both *rmpA* and *rmpB*.

Comparison of the RuMP pathway gene cluster of *M. gastri* and *M. aminofaciens* 77a. The organization and regulation of the genes of the RuMP pathway differ in *M. gastri* MB19 (Fig. 1A) and in *M. aminofaciens* 77a, a gram-negative and obligate methylotroph (Fig. 1B). The *rmpA* and *rmpB* genes are well conserved in both organisms as mentioned above, although the order of the *rmpA* and *rmpB* genes on the chromosome of one organism is opposite that of the other organism and the G+C content of *rmpA* and *rmpB* differs (67.3% in *M. gastri* MB19 and 49.3% in *M. aminofaciens* 77a). The characteristic difference between the two methylotrophs is the way in which *rmpA* and *rmpB* expression is regulated. In *M. aminofaciens* 77a, the insertion sequence, *rmpI*, is present between *rmpA* and *rmpB*, which are expressed monocistronically, and *rmpI* regulates the expression of the adjacent genes in a unique manner. Activation of the inside promoters of *rmpI* downregulates the expression of *rmpA* and upregulates the expression of *rmpB* (16). In contrast, in *M. gastri* MB19 the expression of *rmpA* and *rmpB* is regulated under the same control at the mRNA level, as a polycistronic operon. This specific regulation system is necessary for the facultative methylotrophy of *M. gastri* MB19 that can grow on diverse carbon and nitrogen sources (8). On the other hand, in an obligate methylotroph, it is physiologically important that the HPS and PHI enzymes are synthesized to result in an appropriate activity ratio for fixation of formaldehyde, the sole source of cell constituents, to occur.

DISCUSSION

In this study, we identified the gene cluster of the RuMP pathway in the gram-positive, facultative, methylotrophic bacterium, *M. gastri* MB19. In this region, the genes which encode a regulatory protein (*rmpR*), PHI (*rmpB*), and HPS (*rmpA*) are aligned in this order from the 5′ end. In addition, *rmpA* and *rmpB* were polycistronically transcribed in *M. gastri* MB19 that had been grown on a medium containing a C_1 compound such as methanol or methylamine. *rmpR* that codes a DNA-binding regulatory protein participates possibly in the regulation of the polycistronic transcription, although this has yet to be determined.

Database analyses revealed that the putative gene products of *rmpA* and *rmpB* of *M. gastri* MB19 are similar to those of diverse prokaryotes, not only among bacteria but also among archaea (Fig. 3). For example, the predicted genes *yckG* and *yckF* of *Bacillus subtilis* (9) show high similarity to the *rmpA* and *rmpB* genes, respectively, of both *M. gastri* MB19 and *M. aminofaciens* 77a. Recently, Yasueda et al. (23) found that the *yckG* and *yckF* genes encode the enzymatically active forms of HPS and PHI, respectively, and that they are expressed in the presence of formaldehyde.

A phylogenetic analysis of the amino acid sequence of the putative HPS gene of bacteria with a sequence similar to that of HPS of *M. gastri* MB19 revealed that the HPS sequences could be divided into three clusters: cluster I, archaea; cluster II, methylotrophs and *B. subtilis*; and cluster III, other bacteria. The phylogenetic tree of the amino acid sequence of the putative PHI gene of bacteria with a sequence similar to that of PHI of *M. gastri* MB19 is also made up of three clusters (Fig. 3). Cluster I of HPS and cluster I of PHI are composed of archaea, including the methanogens *Methanobacterium thermoautotrophicum* ΔH and *Methanococcus jannaschii*, the acetogen *Archaeoglobus fulgidus*, and the hyperthermophile *Pyrococcus horikoshii*. This implies that these two genes evolved as a pair. Recently, Chistoserdova et al. (3) reported the existence of a cluster of genes encoding anaerobic C_1 transfer enzymes, which had previously been thought to be unique to anaerobic metabolism in methanogens and acetogens, in the chromosome of an aerobic methylotroph, *Methylobacterium extroquens* AM1 (which has the serine pathway of formaldehyde fixation). Our present study shows that the formaldehyde fixation pathway in an aerobic methylotroph and that in an anaerobic archaeon share common genes across the boundary between bacteria and archaea.

Further studies on the RuMP gene cluster are necessary to elucidate the evolutionary history of methylotrophy and fixation of formaldehyde into organic compounds.

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