Organization and Regulation of the Genes Involved in the Ribulose Monophosphate Pathway in Methylotrophic Bacteria

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CONTENTS

ABBREVIATIONS

bp	Base pair(s)
C_1-	One-carbon
Da	Dalton
DEAE	Diehtlaminoethyl-
DHAP	Dihydroxyacetone phosphate
DTT	Dithiothreitol
E4P	Erythlose 4-phosphate
F6P	Fructose 6-phosphate
GAP	Glyceraldehyde phosphate
HPLC	High-performance liquid chromatography
HuMP	3-Hexulose 6-phosphate
HPS	3-Hexulose-6-phosphate synthase
IPTG	Isopropyl-β-D-thiogalactopyranoside
IS	Insertion sequence
kb	Kilobase(s)
kDa	Kilodalton
*K*m	Michaelis constant
*M*r	Molecular mass
ORF	Open reading frame
PHI	Phospho-3-hexuloisomerase
Ri5P	Ribose 5-phosphate
RuMP	Ribulose monophosphate
Ru5P	Ribulose 5-phosphate
S7P	Sedoheptulose 7-phosphate
Tris	Tris(hydroxymethyl)aminomethane
X5P	Xylulose 5-phosphate
X-gal	5-Bromo 4-chloro 3-indolyl β-D(-)-galactopyranoside

INTRODUCTION

A huge quantity of one-carbon (C_1-) compounds such as methane methanol and CO_2 of several oxidation levels exist in the nature. Microbes growing on reduced C_1-compounds such as methane, methanol and methylated amines, as their sole source of carbon and energy, must form every carbon-carbon bond. These microbes are called methylotrophs by analogy of autotrophs that are able to use CO_2 as their sole source of carbon. Methanotrophs able to grow on methane constitute a subgroup of the methylotrophic bacteria. Methylotrophs are those microorganisms able to grow at the expense of reduced carbon compounds containing one or more carbon atoms but containing no carbon-carbon bonds. While facultative methylotrophs are able to grow on a variety of other organic multi-carbon compounds, obrigate methylotrophs can not grow on carbon sources other than C_1-compounds. The ecology of methane-utilizing bacteria has been reviewed by Quayle (74), Whittenbury *et. al.* (101, 102) and Hanson (36). Methane is produced anaerobically by methanogenic bacteria. Methanol arises in nature by the oxidation of methane and by the hydrolysis of methyl ethers and esters present in pectin and lignin which are major structural components of plants. And as such, methylotrophs are abundant in nature and they are readily isolated from almost any sample of soil, water or sewage.

Most of the methylotrophs that have been well studied are Gram-negative bacteria, but an increasing number of Gram-positive methylotrophs are now being isolated and characterized (55). These organisms growing on

C_1-compounds have to form these bonds in order to synthesize cell constituents and to oxidize these substrates to obtain energy required for growth. In methanol-utilizer, at first, methanol is oxidized to formaldehyde by methanol dehydrogenase. Methanol dehydrogenase present in Gram-negative bacteria is PQQ-dependent. This well-characterized enzyme (6-8, 29, 31, 38), which constitutes about 10-15 % of the total soluble protein of the cell-free extract, is located in the periplasmic space (2, 43, 75, 101, 102). On the other hand, in Gram-positive bacteria, though only a limited number of methanol dehydrogenases were reported, thermotolerant, methanol-utilizing strains of *Bacillus methanolicus* were found to possess a cytoplasmic NAD-dependent methanol dehydrogenase (10, 11, 28). The producing formaldehyde by their methanol dehydrogenase was then led to the assimilation pathway for producing cell constituents, or to the further oxidation pathway.

Two pathways for the assimilation of formaldehyde in methylotrophic bacteria are known. One is the serine pathway, which initiates with the condensation of methylenetetrahydrofolate and glycine to form serine. This 3-carbon compound then undergoes a series of transformations to phosphoenolpyruvate, which is carboxylated to form malate. The malate is cleaved into two 2-carbon compounds which are then converted back into glycine, thus completing the cycle (6). Another is the ribulose monophosphate (RuMP) pathway (41, 42, 47-49). In this thesis, the author firstly describe the genetic study on the RuMP pathway. The RuMP pathway can be divided into three stages (Fig. 1). Stage 1 (fixation), the condensation of three molecules of ribulose 5-phosphate to yield three molecules of fructose 6-phosphate.

This stage is most unique to this pathway (Fig. 2). Formaldehyde is condensed with the acceptor (ribulose 5-phosphate) by the 3-hexulose-6-phosphate synthase to produce 3-hexulose 6-phosphate which is then isomeraized to fructose 6-phosphate by phospho-3-hexuloisomerase. They are key enzymes in the RuMP pathway. Stage 2 (cleavage) involves the splitting of one molecule of fructose-6-phosphate to produce two C_3-compounds. In some methylotrophs,

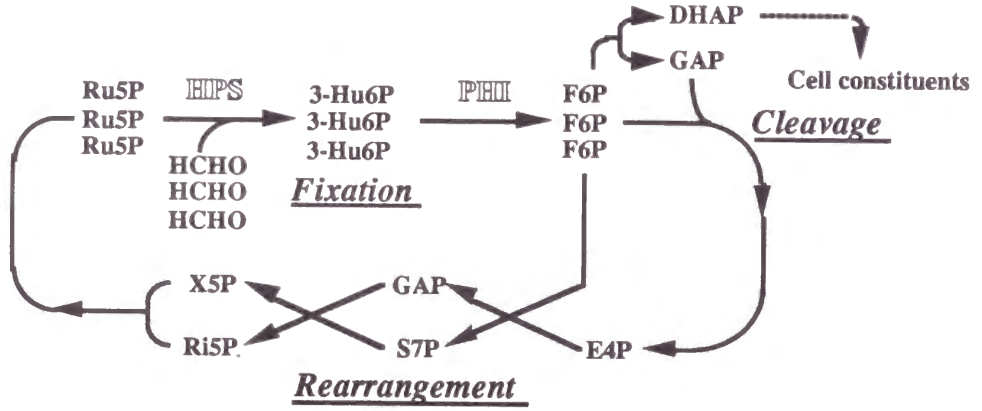

Fig. 1. The RuMP pathway of methylotrophic bacteria.
This pathway composed three stages (fixation, cleavage and rearrangement).
Ru5P; Ribulose 5-phosphate, 3-Hu6P; 3-Hexulose 6-phosphate, DHAP;
Dihydroxyacetone phosphate, GAP; Glyceraldehyde phosphate, S7P;
Sedoheptulose 7-phosphate, X5P; Xylulose 5-phosphate.

such as *Bacillus* spp. (25) and *Arthrobacter globiformis* (56), this is achieved by enzyme in the glycolytic pathway as originally investigated by Kemp and Quayle (49). In other methylotrophs, such as *Pseudomonas* W6 (13), and *Pseudomonas oleovorans* (56) this split is catalyzed by enzymes in the Entner-Doudoroff pathway. Stage 3 (rearrangement) involves the regeneration of three molecules of ribulose 5-phosphate from the two molecules of fructose 6-phosphate and one molecules of glyceraldehyde 3-phosphate produced in stage 1 and 2. These sugar phosphate interconversions are catalyzed by

transaldorase and transketolase in *M. methanica* and *M. capsultus*. On the other hand *Bacillus* spp. PM6 and S2A1, lack transaldorase, and their sugar phosphate interconversions involve sedoheptulose 1,7-diphosphatase and fructose diphosphate aldorase instead (24).

Application of genetic techniques to the methylotrophic bacteria has greatly enhanced studies on these important organisms. Two methylotrophic systems have been studied in some detail, the serine pathway for formaldehyde

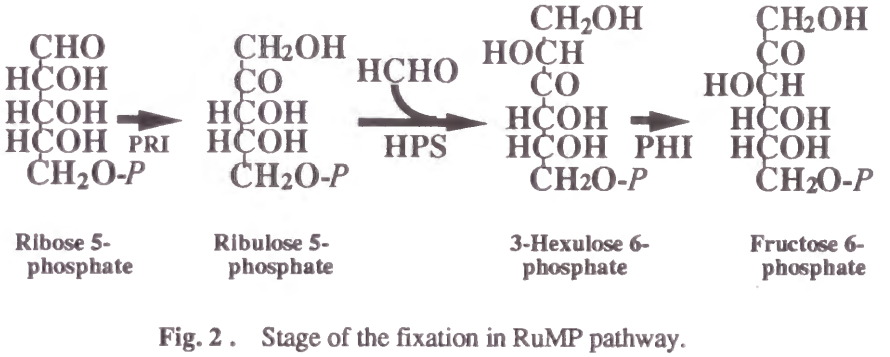

Fig. 2 . Stage of the fixation in RuMP pathway.

assimilation and methanol oxidation system. In both cases, genes have been cloned and mapped in *Methylobacterium* species (the facultative serine pathway methanol-utilizers). In addition, methanol oxidation genes have been studied in some methylotrophs (58). Studies on genetic regulation of the RuMP pathway enzymes had been unsuccessful, since most bacteria having the RuMP pathway is obrigate C_1-utilizer.

In this thesis, the author first cloned the RuMP pathway gene clusters on the basis of protein informations from two characteristic strains, *Me. aminofaciens* 77a and *My. gastri* MB19.

Chapter 1 describe the organization of the RuMP pathway gene cluster from a Gram-negative obrigate methylotrophic bacteria, *Methylomonas*

aminofaciens 77a which only grown on methanol as sole carbon source. The cloned fragment from *Me. aminofaciens* 77a contained four ORFs (*rmpA*, *rmpB*, *rmpD* and *rmpI*). *rmpA* and *rmpB* coded for HPS and PHI respectively, which are key enzymes for formaldehyde fixation in stage 1. *rmpD* could code for transaldolase participating in the stage 3 of RuMP pathway. *rmpI* and neighboring sequences coincide with IS*10*-R. Furthermore, a homologous gene for *rmpA* was eventually found in another chromosomal region of *Me. aminofaciens* 77a. The duplicated gene contained high similarity sequence to *rmpA* .

Chapter 2 describes organization of the RuMP pathway gene cluster from a Gram-positive facultative methylotrophic bacteria, *Mycobacterium* gastri MB19. This strain could grown on several carbon source (glucose, glycerol, ethanol and methylated amines) but not methane. The cloned fragment from *My. gastri* MB19 contained three complete and two partial ORFs (*rmpA*, *rmpB*, *rmpC*, *rmpR* and *orf1*). The four of five ORFs products were known to function in the RuMP pathway. *rmpA*, *rmpB*, *rmpC* and *rmpR* could code for HPS, PHI, G6PDH and a putative regulatory protein respectively. Primary structure of *rmpA* and *rmpB*, organization of RuMP gene cluster and regulation for *rmpA* and *rmpB* expression in *Me. aminofaciens* 77a and *My. gastri* MB19 more discussed.

Chapter 3 describes over production of HPS and PHI in *E. coli* and *C. boidinii,* which form the basis for further application of these enzymes in biotechnology.

CHAPTER 1

The RuMP Pathway Gene Cluster from an Obrigate Methylotroph *Methylomonas aminofaciens* 77a

SECTION 1

Cloning and sequence analysis of the gene encoding 3-hexulose-6-phosphate synthase from *Methylomonas aminofaciens* 77a

HPS catalyzes the aldol condensation of formaldehyde with Ru5P to give HuMP, and participates in the RuMP pathway for formaldehyde fixation in some methylotrophic bacteria. The enzymes that have been purified from a methane-utilizer and obligate or facultative methanol-utilizers had the different characteristic in some properties, e.g. substrate specificity, molecular weight and macro structure.

As the first step, to study the gene organization and regulation of the RuMP pathway, author cloned the gene encoding for HPS from an obligate Gram-negative methylotroph, *Me. aminofaciens* 77a.

MATERIALS AND METHODS

Bacterial strains, culture conditions and plasmids

Me. aminofaciens 77a was used as a source of HPS gene (*rmpA*),

and were grown at 28 °C on minimal salts medium containing 1.0 % methanol (46). *E. coli* JM109 was the host for pUC118 and pKK223-3 plasmids, and was usually grown at 37 °C on LB broth (89) in the presence of ampicillin (10 μg/ml) when necessary. If necessary, 0.1 mM IPTG and 0.05 mM X-Gal were added to the medium.

Preparation of cell-free extract and HPS assay

Me. aminofaciens 77a grown under the conditions described above, was used as a source of cell-free extract, harvested by centrifugation at 8,000 x g at 4 °C, and washed twice with 50 mM potassium phosphate buffer (pH 7.5). The washed cells were suspended in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT, 5 mM $MgCl_2$ and 0.15 mM PMSF, disrupted by sonication for 20 min (19 kHz, Insonator model 201M; Kubota, Tokyo), and centrifuged at 12,000 x g for 20 min at 4 °C. The resulting supernatant was then used as cell-free extract.

The HPS activity was determined by measuring the rate of Ru5P-dependent disappearance of formaldehyde according to Kato *et. al* (46). One unit of the activity was defined as the amount of enzyme that fixes 1 μmol of formaldehyde into Ru5P per min.

HPS purification and amino-acid sequence

Purification of HPS from *Me. aminofaciens* 77a was performed as described previously (46), and the enzyme expressed in *E. coli* JM109 carrying pUH1 was purified by DEAE-toyopearl 650M column chromatography. At

the first, HPS was eluted by step-wise method with 10-40 mM HEPES buffer (pH 8.0) containing 1 mM DTT, 5 mM $MgCl_2$ and 0.15 mM PMSF. The next step was done with 10-20 mM HEPES buffer in a similar way of the 1st step.

The purified enzyme (1.2 mg) was digested with *Achromobacter* Lysyl Endopeptidase (Wako chemicals) at the enzyme-substrate ratio of 1/200 for 12 h at 37 °C in 0.1 M ammonium bicarbonate, and the resulting peptide mixtures were separated by reversed-phase HPLC with a column of Cosmosil 5C18-P (4.6 mm x 25 cm ; Nacalai Tesque). Gradient elution was done at 1 ml/min with 0.1 % TFA in water as solvent A and 0.1 % TFA in 80 % acetonitrile as solvent B (50). Amino acid sequences of the amino-terminal region of purified enzyme and of peptides were determined with a protein analyzer (Applied Biosystems model 4701A) with an on-line HPLC apparatus (model 120A).

Amplification of partial *hps*

To amplify a partial *rmpA* fragment from the chromosomal DNA of *Me. aminofaciens* 77a by PCR, upstream and downstream primers were designed from the N-terminus amino acid sequence of the native enzyme and of a proteolytic peptide, respectively. The sequences of the primer used as follows: N terminal (N), 5'-AA(A/G)GTIGCICCICA(T/C)GTIGA(T/C)AT-3'; internal (B9), 5'-ATIGCIGCICCIGCIAC(A/G/T)A T-3'. Chromosomal DNA from *Me. aminofaciens* 77a extracted by the modified method of Saito and Miura (80) was used as template for amplification of a portion of *hps* by PCR. The conditions of PCR were according to the standard procedure

suggested by Perkin-Elmer/Cetus. The PCR product was purified, and cloned into the *Sma* I site of pUC118.

Southern hybridization analysis

The *Me. aminofaciens* 77a genomic DNA digested with various restriction enzymes was separated and transferred to Hybond-N^+ filter (Amersham). Hybridization was carried out with the PCR product that was ^{32}P- labeled by using a random primer DNA labeling kit (TaKaRa Shuzo Co.).

Colony hybridization

Colonies of *E. coli* transformats were transferred to Hybond-N^+ filters and lysed. The liberated DNA was fixed on the membrane and hybridization was carried out at 42 °C as described previously (89).

Nucleotide sequence analysis

DNA sequencing was performed by the dideoxy chain-termination method using an automated DNA sequencer (Applied Biosystem, model 373A). The sequencing reaction was carried out according to the manual of the *taq* dye terminator cycle sequencing kit (Applied Biosystem). Synthetic oligonucleotides used for sequencing the structure gene are based on the sequence of the PCR product, as follows (number in parentheses refer to sequence):1 5'-dCTTACCCTTCCGGAAGAACTTG-3' (1-22), 2 5'-dAAG GTAGCTCCACACGTTGACA-3' (441-462), 3 5'-dCTGCTAACAAGTAC

GGCAAGAA-3' (674-696), 4 5'-dGGTGGTGTTAAGCCTGCTACTGTT-3' (867-890), 5 5'-dCTTGATACCGTTGTGCTTGAT-3' (507-487), 6 5'-dCTTCTTGCCGTACTTGTTAGCAG-3' (647-622), 7 5'-dAACAGTAGC AGGCTTACACCACC-3' (890-867), 8 5'-dGGTGGCAGGTCGGCCCAG TTCG-3' (1307-1285). The nucleotide sequence data reported appeared in DDBJ, EMBL and GenBank nucleotide sequence databases (accession number D64136).

RESULTS

N-terminal and internal amino acid sequences of HPS from *Me. aminofaciens* 77a

The first 47 amino acid residues of the purified enzyme were ALTQMALDSLDFDATVALAEKVAPHVDILEIGTPXIKHNGIKLLETL. Three major peptides, B1, B6, and B9, from an *Achromobacter* lysyl endopeptidase digest had the sequences of YGK, MDAGFYEAEPFYK, and DAGATIIVAGAAIYGAADPA, respectively (Fig. 1).

Cloning the *Me. aminofaciens hps* gene

Amplification of a *Me. aminofaciens* 77a DNA fragment mediated by PCR with primers N1 and B9 gave single PCR product of approximately 0.5-kb in length. This PCR product was subcloned into pUC118, and its nucleotide sequence was determined. Author concluded that the 507 bp fragment was a partial of *hps*, since amino acid sequences of four peptide fragments derived from the purified enzyme were found in the amino acid

sequence deduced from the nucleotide sequence of the PCR product.

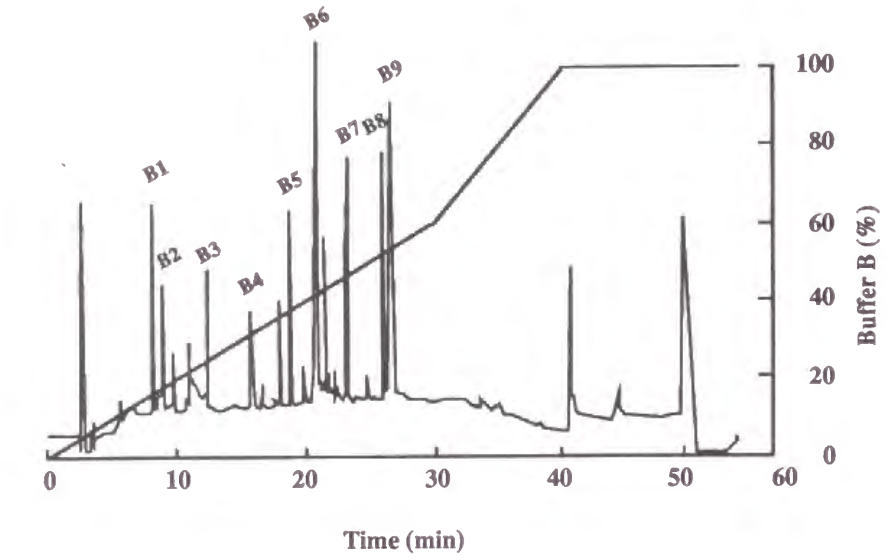

Fig. 1 Preparation of peptide fragments by the reversed-phase HPLC. Column: Cosmosil 5C18-P, 4.6 mm x 25 cm. Buffer A: 0.1 % TFA in H_2O, Buffer B: 0.1 % TFA in 80 % acetonitrile. Flow rate: 1 ml/min. Detection: UV215.

Me. aminofaciens 77a chromosomal DNA was digested with *Bam*H I, *Eco*R I, *Hin*d III, *Kpn* I, *Pst* I, *Sac* I and *Sal* I. The enzyme digests were fractionated on agarose gel and transferred to Hybond-N^+ filters. Hybridization was carried out with the ^{32}P-labeled PCR product as the probe (Fig. 2). The *Pst* I fragments of approximately 5-kb were extracted, ligated into the *Pst* I site of pUC118, and used to transform *E. coli* JM109. The resultant recombinant *E. coli* library was screened by colony hybridization with the same probe. Restriction analyses revealed that all of the positive clones had identical insert in pUC118 at the *Pst* I site, a recombinant plasmid designated pUH1.

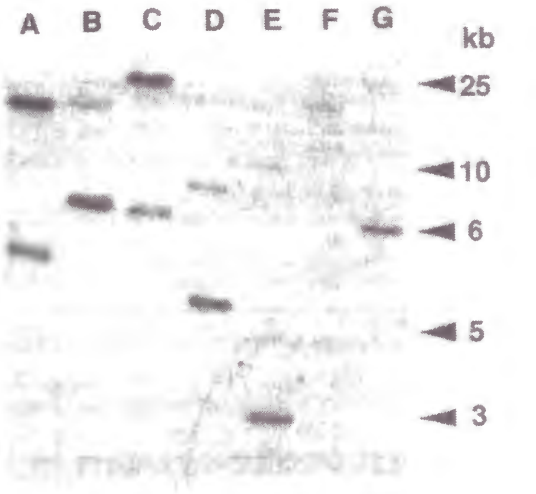

Fig. 2 Genomic southern analysis of *M. aminofaciens* 77a
A DNA ladder (λ-*Hin*d III) was used as a size marker. The probe used in the experiment are ^{32}P-labeled partial *hps* probe. LaneA: *Sal* I - digested, laneB: *Sma* I - degested, laneC: *Eco*R I - degested, laneD: *Hin*d III - digested, laneE: *Pst* I - digested, laneF: *Kpn* I - digested, laneG: *Sac* I - digested. Each sample was separated on 0.8 % agarose gel electrophoresis with TAE buffer.

This plasmid contained partial digested *Pst* I fragments of about 4-kb and 0.5-kb from chromosomal DNA from *Me. aminofaciens* and this result is in accordance with Southern hybridization analysis.

To determine the existence of the *hps* in the 4.5-kb fragment, the HPS activity of the clone carrying pUH1 was measured. The specific activity of cell-free extracts prepared from the cells grown in the presence of IPTG was 6.2 units mg^{-1} while HPS activity was not detected in the host *E. coli* strain, which was in agreement with the value (6.2 units mg^{-1}) for that of *Me. aminofaciens* 77a (Table 1). Therefore, *hps* was in the cloned 4.5-kb fragment. Its own promoter seemed to function in *E. coli* cells, because the expression of HPS activity (5.9 units mg^{-1}) was independent of induction of the *lac*

promoter in pUC118 by the addition of IPTG.

Table 1. Expression of the cloned HPS gene from *Me. aminofaciens* 77a. *Me. aminofaciens* 77a was grown on basal medium with methanol (28) as sole carbon source. One unit (U) of enzyme is the amount of catalyzing the disappearance of 1 μmol formaldehyde per min.

Strain	Plasmid	Inserted (kb)	S.A.(U/mg) IPTG +	S.A.(U/mg) IPTG -
Me. aminofaciens 77a	-	-	-	6.2
E.coli JM109	pUC118	-	N.D.	N.D.
	pUH1	4.0	5.8	6.2

N.D. Not detected

Purification of HPS expressed in *E. coli*

The recombinant HPS was purified 39-fold from cells of *E. coli* carrying pUH1, with a yield of about 17 %. The specific activity of final preparation was 188 units mg^{-1}, which was the same level as that purified from *Me. aminofaciens* 77a (199 units mg^{-1}) (46). The homogeneity of the enzyme preparation was confirmed to be 41,000 on gel filtration, while SDS-PAGE analysis gave a value of 24,000, indicating that the enzyme exists in a dimetric form. The first 20 N-terminal amino acids, ALTEMALDSLDFDATV LAEK-, of the enzyme were identical to those of the enzyme from the parent strain.

Nucleotide sequence of *hps*

The total nucleotides of 1,307 bases including the 507 bp of PCR product in pUH1 were sequenced. An ORF that started from GTG codon at the nucleotide position 379 and ended with a TAA termination codon at the position 1,008 was found (Fig. 3).


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                                        -35 region
GTTTCAATCGCCTAGATGCCAGCGCCTCATTGGCGTTGACATCCGGCGCGCTTGGCGATT 1260
                                -10 region
ACCATTGCCGTTCCGCTTTTGTATAAAAAGTAGTTTTGATTTTTAGTAGTACTCAATTTG 1320
                    SD
TAACCCTTTTGGAGGAAGTATCGTGGCATTGACACAAATGGCATTAGATTCACTGGATTT 1380
                                (V) A L T Q M A L D S L D F
CGACGCAACTGTTGCGCTGGCTGAAAAGGTAGCTCCACACGTTGACATTCTTGAAATCGG 1440
  D A T V A L A E K V A P H V D I L E I G
TACACCATGCATCAAGCACAACGGTATCAAGTTGCTGGAAACTCTGCGCGCAAAGTTCCC 1500
  T P C I K H N G I K L L E T L R A K F P
TAACAACAAGATCCTGGTTGACCTGAAGACTATGGATGCTGGCTTCTACGAAGCTGAGCC 1560
  N N K I L V D L K T M D A G F Y E A E P
TTTCTACAAGGCTGGTGCTGATATCACTACCGTTCTGGGCGTAGCTGATCTGGGTACAAT 1620
  F Y K A G A D I T T V L G V A D L G T I
CAAAGGCGTAATCGACGCTGCTAACAAGTACGGCAAGAAGGCACAGATCGACCTGATCAA 1680
  K G V I D A A N K Y G K K A Q I D L I N
TGTTGGTGATAAGGCTGCTCGTACTAAGGAAGTTGCTAAGCTGGGCGCGCACATCATTGG 1740
  V G D K A A R T K E V A K L G A H I I G
CGTTCACACTGGTCTGGACCAACAAGCTGCTGGTCAAACTCCTTTTGCTGACCTGGCAAC 1800
  V H T G L D Q Q A A G Q T P F A D L A T
TGTAACTGGCCTGAACCTGGGTCTGGAAGTTTCCGTAGCTGGTGGTGTTAAGCCTGCTAC 1860
  V T G L N L G L E V S V A G G V K P A T
TGTTGCACAAGTTAAAGACGCTGGTGCTACCATCATCGTTGCTGGCGCTGCTATCTACGG 1920
  V A Q V K D A G A T I I V A G A A I Y G
TGCTGCTGACCCAGCTGCTGCTGCTGCTGAAATCACTGGCCTGGCTAAGTAATTTTTCGC 1980
  A A D P A A A A A E I T G L A K *
TTAAGCTAAGCTAAAAAAATCCACCATACATAGCTCTGATGAATCCCCTAATGATTTTGG 2040
TAAAAATCATTAAGTTAAGGTGGATACACATCTTGTCATATGATCAAATGGTTTCGCGAA 2100
AAATCAATAATCAGACAACAAGATGTGCGAACTCGATATTTTACACGACTCTCTTTACCA 2160
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Fig. 3. Nucleotide sequence and the deduced amino acid sequence for HPS gene.
The deduced amino acid sequence of ORF are shown below the nucleotide sequence in one-letter code. The amino acid sequences consistent with those found on HPS protein are underlined. Potential ribosome-binding sequences is marked as SD.

This ORF consisted of 624 bp and coded for a protein with a calculated molecular mass of 21,224. This value was similar to the molecular mass of the HPS subunit (24,000). The first 47 amino acids on the N-terminal predicted from the nucleotide sequence were identical with those of HPS found by the Edman degradation procedure. The amino acid sequences of the peptide

fragments produced by an *Achromobacter* lysyl endopeptidase were found, starting at position 568 and ending at 606 for B6, starting at position 685 and ending at 693 for B1, starting at position 913 and ending at 972 for B9, respectively. Author concluded that this ORF encoded *hps*.

DISCUSSION

Genetic studies of methylotrophic bacteria have been performed mainly for methanol oxidation systems (5, 9, 33, 53, 59, 62, 63, 78, 98). However, studies on genetic regulation of RuMP pathway enzymes had been unsuccessful, since most bacteria having the RuMP pathway are obrigate methylotrophy. In obrigate methylotroph, mutations in C1 metabolism genes are assumed to be lethal.

In this section, author first cloned the gene coding HPS which is key enzyme of RuMP pathway on the basis of protein informations. The *hps* gene consisted of 624 bp and coded for a protein with molecular weight calculated to be 21,224. A putative Shine-Dalgarno (SD) sequence (AGGA) was located 7 to 10 bases upstream of the GTG triplet on the gene. Since the cloned *hps* was expressed in *E. coli* cells is IPTG-independent manner, the promoter region was expected to be present in the upstream of *hps*. Sequences resembling the *E. coli* consensus are found upstream of the ORF (26, 73).

The enzymatic properties of HPS were shown in Table. 2 (45, 46). Although the enzymes from several methylotrophic bacteria are different in the molecular weight and macro structure each others, alignment of the N-terminus sequence of three enzymes shows high similarity (Fig. 4), suggesting that HPS was derived from a common ancestor.

Table 2. Enzymatic properties of HPSs from some methylotrophic bacteria. Enzyme activities were measured under the standard conditions as described as Materials and Methods.

Properties	Value with enzyme from		
	Methylomonas aminofaciens 77a	*Mycobacterium gastri* MB19	*Bacillus* C1
Distribution	Soluble	Soluble	Soluble
Specific activity of final preparation	199 U/mg (30 °C, pH 7.5)	135 U/mg (30 °C, pH 7.5)	480 U/mg (50 °C, pH 7.0)
Molecular weight (Gel filtration)	45,000	43,000	32,000
Subunit structure type	homodimer	homodimer	monomer
Molecular weight	24,000	24,000	27,000
pI	5.1	-	-
Heat stability	80 °C,10 min	-	65 °C, 1 hr
pH optimum	pH 8.0	pH 7.5 - 8.0	pH 7.0
Apparent Km value for			
HCHO	0.29 mM	1.4 mM	0.15 mM
D-ribulose 5-phosphate	0.06 mM	-	0.70 mM
$MgCl_2$	0.17 mM	-	0.13 mM

Fig. 4. Comparison N-terminal amino acid sequences of HPS from some methylotrophic bacteria.

SECTION 2

Genetic organization of RuMP pathway gene cluster in *Me. aminofaciens* 77a

Application of genetic techniques to the methylotrophic bacteria has greatly enhanced studies on these important organisms. Two methylotrophic systems have been studied in some detail, the serine pathway for formaldehyde assimilation and the methanol oxidation system. In both cases, genes have been cloned and mapped in *Methylobacterium* species (facultative serine cycle methanol-utilizers). In addition, methanol oxidation genes have been studied in some methylotrophs (65, 66). In some cases in methanol oxidizing enzyme genes are clustered on chromosome, but little or no operon appears. But genetic studies on the RuMP pathway enzymes have not been made. In the section 1, the author first cloned the *hps* which can code the key enzyme of the RuMP pathway.

In this section, to reveal the gene organization surrounding *hps* (*rmpA*) region, sequenced the 4.4-kb insert of pUH1 was sequenced, which contained four ORFs, and identified these products.

MATERIALS AND METHODS

Nucleotide sequence analysis

The clone ligated with pUC118 are cut with *Bam*H I and *Kpn* I of

pUC118 multi cloning site, or *BstX* I and *Bgl* II, and then deletion mutant strains were constructed with a deletion kit (TaKaRa shuzo, Co., Ltd.). DNA sequencing was performed by the dideoxy chain termination method (90) using an automated DNA sequencer (Applied Biosystem, model 373A).

Northern blot hybridization

The culture of *Me. aminofaciens* 77a was carried out as described previously (46). Total RNA was extracted by the AGPC (Acid-Guanidium-Phenol-Chloroform) method (89) using ISOGEN (NIPPON GENE CO., LTD.), and RNA samples (20 μg/lane) were electrophoresed on a 1.0 % agarose gel containing 20 mM MOPS buffer containing 1 mM EDTA and 2.2 M formaldehyde and transferred to a nylon membrane filter (Gene screen) in 20 x SSC. Prehybridization and hybridization were carried out at 42 °C in a solution consisting of 30 % formamide, 5 x SSC, 0.1 % SDS and 100 μg of calf thymus DNA per ml. The probe DNAs were labelled by random primed DNA labeling kit (Boehringer Mannheim).

Computer analysis

The DNA sequence was analyzed by the DNASIS (HITACHI software engineering co. LTD.). The National Center for Biotechnology Information (NCBI) was searched for homologous amino acid sequences with BLAST or FASTA programs (data base: GenBank, EMBL and SWISS-PROT).

Construction of vectors

The recombinant gene expression utilized the *tac* promoter on pKK223-3. To determine the *rmpB* expression product, its fragment were amplified by PCR from pUH1 and *Me. aminofaciens* 77a chromosomal DNA as the template. Upstream and downstream primers were designed from the obtained sequence. N-terminal 5'-GGAATTCCTATTTAAGGTGAATGAA C-3'; and C-terminal, 5'-GGAATTCCTTACTCGAGGTTAGCATGAAT-3'. The PCR product was purified and cloned into the *Eco*R I site of pKK223-3, and named plasmid pKP1, and then transformed into *E. coli* JM109.

Enzyme assays and purification

Transcription of insert DNAs were driven by the *tac* promoter present on the expression vectors, pKH1 and pKP1. The overexpressed enzymes were purified by one-step of DEAE-sepharose column chromatography. The enzymes were eluted with a linear gradient between 10 mM and 100 mM Tris-HCl buffer (pH 8.2) containing 1 mM DTT, 5 mM $MgCl_2$ and 0.15 mM PMSF.

The HPS activity was assayed by measuring the rate of Ru5P-dependent disappearance of formaldehyde as described in the section 1. The PHI was assayed discontinuously by following the formation of Fu6P from HuMP. The produced Fu6P determined after isomerization to G6P by glucose-6-phosphate isomerase immediately. One unit of PHI activity was defined as the amount of enzyme which produce 1 μmol of NADPH with the oxidation of glucose 6-phosphate to 6-phosphogluconate by glucose-6-phosphate

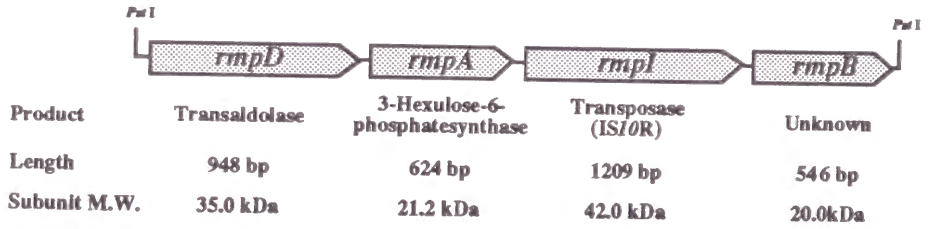
dehydrogenase per minute under the conditions described previously (91).

RESULTS

Nucleotide sequence of the clone from pUH1

The sequence of the pUH1 insert revealed four open reading frame in the same direction as shown in Fig. 1.

The first open reading frame (*rmpD*), starting at the nucleotide position of 248 on Fig. 3 and ending at nucleotide position of 1,196 encodes a putative product of 316 amino acids and the calculated molecular mass of the polypeptide



Product	Transaldolase	3-Hexulose-6-phosphatesynthase	Transposase (IS*10*R)	Unknown
Length	948 bp	624 bp	1209 bp	546 bp
Subunit M.W.	35.0 kDa	21.2 kDa	42.0 kDa	20.0kDa

Fig. 1 Structure of *rmpA* surrounding region of *Me. aminofaciens* 77a. The entire 4.45-kb region shown has been sequenced in this section. All of ORFs in this region located in same direction. The arrow boxes indicate direction of a transcription. The transcriptional stop codon was included in the length. Subunit M. W. was calculated by the based on sequence.

was 34,850 Da. A putative SD sequence (AGGA) was located 6 to 9 base upstream of the ATG triplet on the gene, This putative product has significant similarity to transaldolase (TALs) from *Haemophilus influenzae, E. coli* (accession number P45055, P30148 respectively) and them other sequences (Fig. 2).

Fig. 2. Comparison of the deduced amino acid sequences of *rmpD* product from *Me. aminofaciens* 77a and other homologous proteins. Amino acid sequences of RmpD from *Me. aminofaciens* 77a, Hae from *H. influenzae* and Eco from *E. coli* were aligned by introducing gaps (hyphens) to achieve maximum homology. Hae and Eco had been reported as TAL, elsewhere. Residues in black boxes indicate identical sequences.

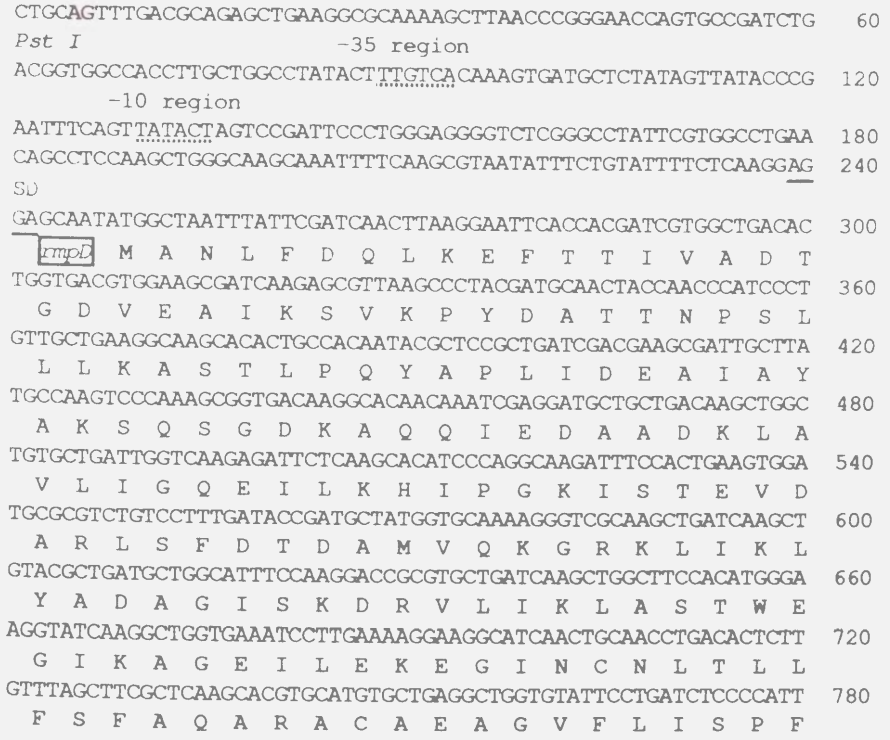
TAL is an enzyme among the pentose-phosphate pathway. TAL catalyses the transformation of sedoheptulose 7-phosphate and glyceraldehyde 3-phosphate to erythrose 4-phosphate and fructose 6-phosphate and plays an important role of regeneration of ribulose 5-phosphate in RuMP pathway.

The second open reading frame (*rmpA*) started from the GTG codon at the nucleotide position of 1,343 and ending at the nucleotide position of 1,970, encoded a putative product of 206 amino acids and the calculated molecular mass of the polypeptide was 21,224 Da. This open reading frame encoded HPS (accession number D64136) as described in the section 1.

The third open reading frame (*rmpI*), starting at the nucleotide position of 2,123 termination at the nucleotide position of 3,329, encoded a putative product of 401 amino acids and the calculated molecular mass of the polypeptide

was 46,025 Da. and the surrounding region completely coincide with transposable element, IS*10*-R. A target sequences (TACATAGCT) were shown as underline (Fig 3), located at the nucleotide position of 2,007 and of 3,345 respectively.

The fourth open reading frame (*rmpB*) starting at the nucleotide position of 3,428 and terminating at the nucleotide position of 3,971, and encoded a putative product of 181 amino acids and the calculated molecular mass of the polypeptide was 19,344 Da. A putative SD sequence (AAGGT) was located 8 to 12 base upstream of ATG triplet on the gene. A long inverted repeat sequence was observed in downstream of the transcriptional termination triplet (TAA) of *rmpB* (Fig. 3). This putative product does not show significant identity to previously known protein.



```
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  D  P  G  V  L  S  V  R  K  I  Y  A  Y  Y  K  E  H  G  Y  K
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  T  V  V  M  G  A  S  F  R  N  T  G  E  I  T  A  L  A  G  C
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  D  R  L  T  V  S  P  N  L  L  E  R  A  E  G  Y  R  R  Y  L
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GCGACTCGCTGTATACCGTTGGCATGCTAGCTTTATCTGTTCGGGCAATACGATGCCCAT 2400
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```

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  N  L  E  *
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TAGCGCTGCAG                                                   4451
      Pst I
```

Fig. 3. Nucleotide sequence of the 4,451-base *Pst* I fragment containing the *rmp* genes of *Me. aminofaciens* 77a.
Solid line and dotted line under the sequences indicate putative SD and promoter sequences respectively. The target sequences of transposon were marked reversed boxes. The bold arrow was described in this section. The narrow arrows indicated transcriptional terminator as inverted repeat.

Identification and purification of the *rmpB* product

The *rmpA* expression in *E. coli* was under the control of the own promoter of *Me. aminofaciens* 77a. Since the PHI activity was also detected in *E. coli* carrying pUH1 together with HPS activity (data not shown), the PHI gene was assumed to be located on the 4.4-kb DNA fragment. Deletion analysis of this fragment from the 5'-end followed by determination of the PHI activity with pUH1d5'-29, pUH1d5'-46 and pUH1d5'-4) located the PHI activity to the region of *rmpB* (Fig. 4). To determine the *rmpB* product, the *rmpB* gene was expressed under the *tac* promoter as described Materials and Methods. Whole cell extract from *E. coli* carrying pKP1 had a strong activity of PHI, and a coomassie blue staining of this extract 15% SDS-polyacrylamide gel gave single intense band. The first 20 N-terminal amino acids, MNKYQELVVSKLTNVINNTA- of the enzyme were identical to the *rmpB* putative product. From the results obtained author concluded that *rmpB*

encoded PHI.

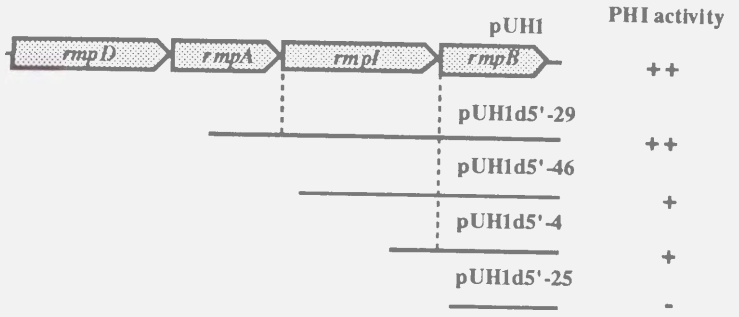


Fig. 4. Deletion analysis of 4.4-kb DNA fragment.
These fragments were cloned into pUC118, and transformed *E. coli* JM109. PHI activity was determined under the IPTG-independent conditions as described Materials and Methods. These transformants grown on LB-broth at 37 °C.

Enzymatic properties of PHI

The expressed PHI was purified from *E. coli* carrying pKP1 as one-step on DEAE-sepharose column chromatography (Table 1), and the homogeneity was confirmed on SDS-PAGE (Fig. 5). The molecular weight was estimated to be 45,000 on gel-filtration, while SDS-PAGE analysis gave Mr. 20,000, indicating that the expression product exists a dimeric form. The estimated molecular weight of *rmpB* 19,344 from the sequence analysis was close to the molecular weight of the subunit of this purified enzyme. Final preparation of PHI showed high specific activity (U/mg) and calculated as 15,400.

Table 1. Purification of PHI from *E. coli* carrying pKP1.
The reaction proceeded under the standard conditions. One unit (U) of the enzyme was defined as the amount of enzyme which produce 1 μmol of NADPH with the oxidation of glucose 6-phosphate to 6-phosphogluconate by G6PDH per minute.

Step	Total protein (mg)	Total activity (U x 10^3)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell-free extract	597	436	7360	100	1
DEAE sepharose	141	217	15400	24	2.1

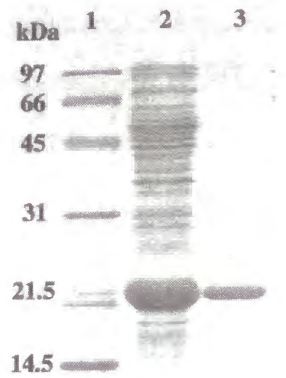


Fig. 5. SDS-PAGE of the *rmpB* product.
Lane 1 was loaded with the following molecular mass standards: phospholyase (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soy bean trypsin inhibitor (21.5 kDa), carbonic anhydrase (14.5 kDa). Lane 2 was supernatant of sonicate (20 μg protein) from *E. coli* JM109 carrying pKP1 was induced IPTG. Lane 3 was purified PHI (3 μg protein). Acrylamide concentration was 15 % in the gel.

Northern Blot analysis

The DNA fragments which contains whole of the coding region were used as probes against total RNA from *Me. aminofaciens* 77a. When *rmpA* region was used the probe, single hybridization band was observed and calculated to be 0.6-kb corresponding to *rmpA* translation product. When the *rmpB* region was used the probe, hybridization band was detected at the size of ca. 0.55-kb, corresponding to *rmpB* translation product. These band were observed different intensities each hybridization, indicating the each ORF was regulated different at transcription level and to be monocistronic (Fig. 6).

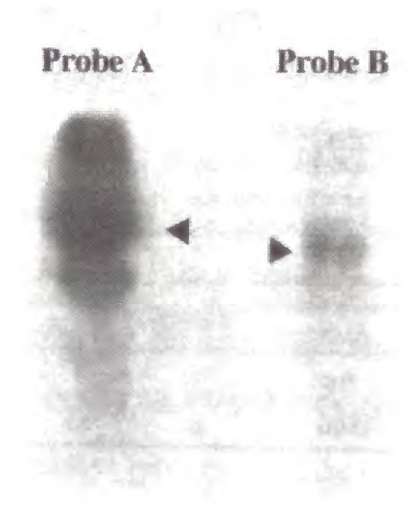


Fig. 6. Northern blot analysis of *Me. aminofaciens* 77a.
Molecular weight was calculated on based by RNA markers (Perfect RNA Markers, 0.2-10 kb, Novagen). Whole length of *rmpA* and *rmpB* were used as the probe 1 and 2, respectively. Each lane was loaded 20 μg RNA which extracted from *Me. aminofaciens* 77a cultured under the standard conditions described as Materials and Methods, containing 1.0 % methanol.

DISCUSSION

Organization of the genes involved in for formaldehyde metabolism was studied in an obrigate methylotroph, *Me. aminofaciens* 77a. The insert DNA of pUH1 contain four ORF (from leftmost *Pst* I site in Fig. 1, *rmpD*, *rmpA*, *rmpI* and *rmpB*). Sequencing analysis revealed that the *rmpD*, *rmpA*, *rmpI* and *rmpB* contained 948, 624, 1,208, and 543 bp respectively, and these ORFs revealed that could code for a transaldolase, HPS, transposase (IS*10*-R) respectively. Though the putative *rmpB* (from rightmost *Pst* I site in Fig. 1) product does not have significant identity to any known protein, the expression analysis revealed a product of *rmpB* as PHI and enabled us to characterize the purified PHI. The molecular weight was estimated to 45,000 by gel-filtration, while SDS-PAGE analysis gave a value of 20,000, indicating that the expression product exists as dimeric form. Although the key-, or related enzymes in the RuMP pathway was revealed to constitute the gene cluster, the *rmpI* was not involved in assimilation pathway of formaldehyde directly. This insertion sequence which located between *rmpA* and *rmpB* encoded transposase of IS*10*-R. The author assumed that the *rmpI* did not exist primarily, and the *rmpA* and *rmpB* were arranged side by side in this region, and *rmpI* was inserted later. This assumption was based on following reasons: 1) codon usages (Table 2) and termination codon of *rmpI* differ from those of the others genes, 2) the transcriptional terminator was observed downstream of the *rmpB*, but was not observed downstream of the *rmpA*. However, if the *rmpI* was excised from the first target sequences

(TACATAGCT) at the nucleotide position of 2007 to end of 3345, transcriptional terminator of downstream *rmpA* could be observed (showed in Fig. 3 at bold arrow). The influence of *rmpI* on *rmpA* expression and *rmpB* is described in the next section.

Table 2. Codon usage of *rmp* genes from *Me. aminofaciens* 77a.
The upside table I show the calculating codon usage by adding *rmpA*, *rmpB* and *rmpD* up. The codon usage of *rmpI* is shown under-table II. A %age indicate that divide total count of cottons by the one by one codon.

I

Codon	Count	%age	Codon	Count	%age	Codon	Count	%age	Codon	Count	%age
TTT-Phe	7	0.99	TCT-Ser	5	0.70	TAT-Tyr	3	0.42	TGT-Cys	2	0.28
TTC-Phe	16	2.26	TCC-Ser	14	1.98	TAC-Tyr	16	2.26	TGC-Cys	2	0.28
TTA-Leu	2	0.28	TCA-Ser	1	0.14	TAA-***	2	0.28	TGA-***	0	0.00
TTG-Leu	14	1.98	TCG-Ser	0	0.00	TAG-***	0	0.00	TGG-Trp	3	0.42
CTT-Leu	5	0.70	CCT-Pro	8	1.13	CAT-His	3	0.42	CGT-Arg	12	1.69
CTC-Leu	6	0.84	CCC-Pro	1	0.14	CAC-His	6	0.84	CGC-Arg	7	0.99
CTA-Leu	1	0.14	CCA-Pro	12	1.69	CAA-Gln	19	2.68	CGA-Arg	1	0.14
CTG-Leu	44	6.22	CCG-Pro	1	0.14	CAG-Gln	4	0.56	CGG-Arg	0	0.00
ATT-Ile	15	2.12	ACT-Thr	24	3.39	AAT-Asn	6	0.84	AGT-Ser	2	0.28
ATC-Ile	32	4.52	ACC-Thr	10	1.41	AAC-Asn	17	2.40	AGC-Ser	8	1.13
ATA-Ile	1	0.14	ACA-Thr	12	1.69	AAA-Lys	5	0.70	AGA-Arg	1	0.14
ATG-Met	15	2.12	ACG-Thr	1	0.14	AAG-Lys	49	6.93	AGG-Arg	1	0.14
GTT-Val	25	3.53	GCT-Ala	58	8.20	GAT-Asp	22	3.11	GGT-Gly	37	5.23
GTC-Val	4	0.56	GCC-Ala	6	0.84	GAC-Asp	21	2.97	GGC-Gly	22	3.11
GTA-Val	8	1.13	GCA-Ala	22	3.11	GAA-Glu	33	4.66	GGA-Gly	0	0.00
GTG-Val	16	2.26	GCG-Ala	8	1.13	GAG-Glu	9	1.27	GGG-Gly	0	0.00

II

Codon	Count	%age	Codon	Count	%age	Codon	Count	%age	Codon	Count	%age
TTT-Phe	4	0.99	TCT-Ser	7	1.73	TAT-Tyr	6	1.48	TGT-Cys	5	1.24
TTC-Phe	5	1.24	TCC-Ser	1	0.24	TAC-Tyr	7	1.73	TGC-Cys	4	0.99
TTA-Leu	14	3.47	TCA-Ser	10	2.48	TAA-***	0	0.00	TGA-***	1	0.24
TTG-Leu	7	1.73	TCG-Ser	4	0.99	TAG-***	0	0.00	TGG-Trp	9	2.23
CTT-Leu	12	2.97	CCT-Pro	4	0.99	CAT-His	9	2.23	CGT-Arg	6	1.48
CTC-Leu	8	1.98	CCC-Pro	3	0.74	CAC-His	10	2.48	CGC-Arg	4	0.99
CTA-Leu	12	2.97	CCA-Pro	4	0.99	CAA-Gln	10	2.48	CGA-Arg	13	3.22
CTG-Leu	5	1.24	CCG-Pro	4	0.99	CAG-Gln	4	0.99	CGG-Arg	2	0.49
ATT-Ile	8	1.98	ACT-Thr	7	1.73	AAT-Asn	7	1.73	AGT-Ser	5	1.24
ATC-Ile	9	2.23	ACC-Thr	5	1.24	AAC-Asn	9	2.23	AGC-Ser	8	1.98
ATA-Ile	1	0.24	ACA-Thr	10	2.48	AAA-Lys	22	5.45	AGA-Arg	3	0.74
ATG-Met	8	1.98	ACG-Thr	3	0.74	AAG-Lys	7	1.73	AGG-Arg	2	0.49
GTT-Val	9	2.23	GCT-Ala	11	2.72	GAT-Asp	5	1.24	GGT-Gly	5	1.24
GTC-Val	3	0.74	GCC-Ala	3	0.74	GAC-Asp	10	2.48	GGC-Gly	9	2.23
GTA-Val	6	1.48	GCA-Ala	6	1.48	GAA-Glu	9	2.23	GGA-Gly	3	0.74
GTG-Val	2	0.49	GCG-Ala	6	1.48	GAG-Glu	7	1.73	GGG-Gly	1	0.24

SECTION 3

A possible role of insertion sequence IS*10*-R (*rmpI*) in the RuMP pathway gene cluster

Transposons are normal constituents of most bacterial genomes and of many extrachromosomal plasmids and bacteriophages. They can alter both the organization and the expression of prokaryotic genomes at frequencies comparable to or greater than spontaneous mutation rates.

Most prokaryotic transposons promote transposition and rearrangements at frequencies of 10^{-4} to 10^{-7} per generation (51). These low frequencies are attributable to stringent regulation and some transposons are self- regulating. Most transposons exert strong polar effects on expression of the neighboring genes (1, 15, 95, 96). For example insertion elements could modify the gene expression by blocking transcription to inhibit gene expression or by acting as mobile promoters to activate transcription of flanking genes.

In this section, the *rmpI* was found to have regulatory for HPS and PHI activities and physiological role of *rmpI* in RuMP pathway gene cluster.

MATERIALS AND METHODS

Strains and plasmids

E. coli JM109 was used as the host strain for propagation of recombinant plasmids which are listed in Table 1. *E. coli* transformats were

grown at 37 °C on LB broth in the presence of ampicillin (10 μg/ml).

Table 1. Strains and plasmids used in this section.

Strain or plasmid	Genotype or description	Source or reference
Bacterial strains		
E. coli JM109	*recA*, *supE*, *endA*, *hsdR*, *gyrA*, *relA*, *thi*, D(*lac-proAB*), F'*traD*, *proAB*, *lacI*^{q}, *lacZ*ΔM15	Lab stock
Me. aminofaciens 77a		28
Plasmids		
pUC118	Amp^r	Lab stock
pUH1	4.4-kb *Pst* I fragment containing the *rmpA* around region of *Me. aminofaciens* 77a cloned into pUC118, Amp^r	Section 1
pUH1d5'-4	Deletion clone of pUH1; 1.1-kb, lack of *rmpA*, *rmpD* and *rmpI* (partial), Amp^r	This study
pUH1d5'-25	Deletion clone of pUH1; 0.8-kb, lack of *rmpA*, *rmpB* (partial), *rmpD*, *rmpI*, Amp^r	This study
pUH1d5'-29	Deletion clone of pUH1; 2.5-kb, lack of *rmpA* (partial) and *rmpD*, Amp^r	This study
pUH1d5'-46	Deletion clone of pUH1; 1.8-kb, lack of *rmpA*, *rmpD* and *rmpI* (partial), Amp^r	This study
pUH1d3'-15	Deletion clone of pUH1; 2.3-kb, lack of *rmpB*, and *rmpI* (partial), Amp^r	This study
pUH1d3'-21	Deletion clone of pUH1; 4.3-kb, Amp^r	This study
pUH1d3'-36	Deletion clone of pUH1; 2.0-kb, lack of *rmpB*, and *rmpI*, Amp^r	This study
pUH1d3'-37	Deletion clone of pUH1; 1.7-kb, lack of *rmpA* (partial), *rmpB*, and *rmpI*, Amp^r	This study
pUH1d3'-46	Deletion clone of pUH1; 3.3-kb, lack of *rmpB* and *rmpI* (partial), Amp^r	This study

Construction of deletion clones

The clone , pUH1 are double digested with *Bam*H I and *Kpn* I on pUC118 multi cloning site for deleting from 3'-end, and with *Bst*X I and *Bgl* II of insert of pUH1 for deletion of 5'-end, and then deletion mutant were constructed by the method of Henikoff (37) and Yanisch *et. al.* (105) with a deletion kit (TaKaRa shuzo, Co., Ltd.).

Preparation of cell-free extract and the enzyme assay

E. coli JM109 harboring deletion plasmids derived from pUH1 were grown at 37 °C for 12 h, harvested by centrifugation at 5,000 x g at 4 °C, and washed twice 50 mM potassium phosphate buffer (pH 7.5). The washed cells were suspended in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT, 5 mM $MgCl_2$ and 0.15 mM PMSF, disrupted by sonication for 10 min (19 kHz, Insonator model 201M; Kubota, Tokyo), and centrifuged at 12,000 x g for 20 min at 4 °C. The resultant supernatant were dialyzed for 16 h against 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT, 5 mM $MgCl_2$ and 0.15 mM PMSF. The resulting dialysates were assayed for enzyme.

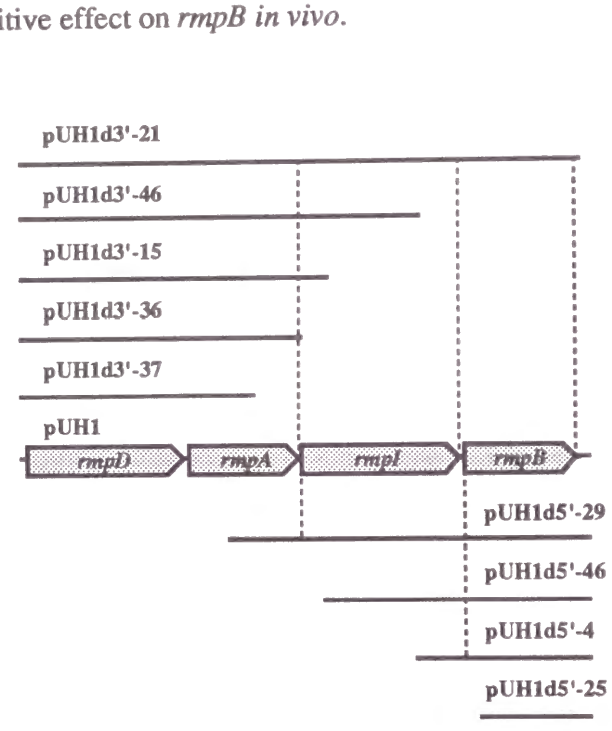
The HPS and PHI activities were determined as described in section 2. The relative activities were expressed as the specific activities (U/mg) of cell-free extract from *E. coli* carrying pUH1 defined as 100 %.

RESULTS AND DISCUSSION

Deletion analysis

The activities of HPS and PHI were assayed to several deletion clones of pUH1-derivatives in *E. coli*, and their relative activities were shown in Fig. 2. As expected, no detectable activity of HPS was found in *rmpA* deleted strains (pUH1d3'-37, pUH1d3'-29, pUH1d3'-46, pUH1d5'-4, pUH1d5'-25). Interestingly, deletion of *rmpI* (pUH1d3'-46, pUH1d3'-15, pUH1d3'-36) caused activation of HPS activity. The clones of partially-deleted

for *rmpI* (pUH1d3'-46, pUH1d3'-15) or completely lacking *rmpI* (pUH1d3'-36) was about 2.5-fold and 6-fold higher than the control strain (harboring pUH1), respectively. On the other hand, the deletion of *rmpI* resulted in a dramatic decrease in PHI activity (pUH1d5'-46, pUH1d5'-4). These experiments suggested that the *rmpI* had a negative effect on *rmpA* and a positive effect on *rmpB in vivo*.



	Relative activity (%)	
	HPS	PHI
pUH1d3'-21	99.0	94.0
pUH1d3'-46	237.0	0
pUH1d3'-15	251.0	0
pUH1d3'-36	570.0	0
pUH1d3'-37	0	0
pUH1	100	100
pUH1d5'-29	0	84.0
pUH1d5'-46	0	9.3
pUH1d5'-4	0	1.6
pUH1d5'-25	0	0

Fig. 1 Relative activities of HPS and PHI from several deletion clones of 4.4-kb pUH1 insert.
Relative activities were calculated from supernatant of *E. coli* carrying pUH1 as 100%.

The role of *rmpI* for RuMP gene cluster

In section 2, the author described that the insertion sequence IS*10*-R was found in the RuMP pathway gene cluster from *Me. aminofaciens* 77a. IS*10*-R have been studied in some detail. IS*10*-R was classified as Class I from the basis on 1) mechanistic feature of transposition, 2) genetic

organization, and 3) DNA sequence homology.

In certain cases, insertion of an IS element can transcriptionally activate expression of an adjacent chromosomal gene. Orientation-dependent turn-on of distal genes by ClassI insertion elements IS2 and IS*3*, have been reported (16, 18, 34, 71, 79, 100). When IS2 insertion occurs at the *arg* locus, weak turn-on gave in both orientations has been reported. On the other hand, IS2 insertions near *int* gave activation of *int* expression while other activation was not observed (16, 34, 71).

A structure of IS*10*-R was shown in Fig. 2. In IS*10*-R (a part of Tn*10*), two promoters (pIN and pOUT) locate upstream from the coding region in an opposite direction, and IS*10*-R acts negatively control expression of its own transposase protein at the translational level (27, 35, 93). Furthermore, the third promoter (pIII) exist near the inside-end of IS*10*-R of unknown genetic importance (92).

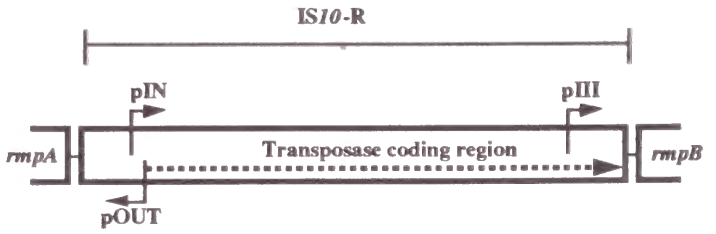


Fig. 2. Structure of IS*10*-R.
This transposable element have possible three promoter sequences in this region indicate as arrows (pIN, pOUT and pIII).

The RuMP gene cluster of *Me. aminofaciens* 77a was found in contain IS*10*-R (*rmpI*), Deletion of *rmpI* caused activation of *rmpA* expression, since pOUT present in an opposite direction to the *rmpA* promoter that conflict

with each other. It means *rmpI* play a role as a repression element of upstream *rmpA* expression *in vivo*. On the other hand, deletion of *rmpI* caused repression for *rmpB* expression, since pIII extend outward and *rmpB* caused activation by pIII at translational level *in vivo*.

SECTION 4

Cloning and sequence analysis of the homologous *rmp* genes

As described in the previous section, studies on RuMP pathway gene cluster in *Me. aminofaciens* 77a revealed the existence of four ORFs (*rmpA*, *rmpB*, *rmpI* and *rmpD*) that including transposable element (IS*10*-R). The transposable element cause deletions, inversions, duplications, and replicon fusions, by transposition within the genome (93).

The author found two hybridization bands which showed different intensities in Southern blot analysis with the probe of the partial *hps*. It suggests that the chromosomal DNA of *Me. aminofaciens* 77a has two *hps* genes of high similarity.

In this section, to make clear the relationship between the duplicated genes and the transposable element. The homologous gene cluster in the cloned RuMP pathway gene cluster was studied. In addition, the author compared the structure of *hps* and *hpsII,* and of the organization around the *hpsII*.

MATERIALS AND METHODS

Strains, culture conditions, and vectors

Me. aminofaciens 77a was used as a source of HPS homologous gene which designated *hpsII* and growth conditions as described previously

(46). *E. coli* XL1-blue MRA (STRATAGENE, California, USA) was used as the host strain for recombinant λ-EMBL3 vector, and was grown on LB broth (89) containing 0.2 % maltose.

Southern hybridization

Southern hybridization was performed by the method of Southern (94). The digested chromosomal DNAs with several restriction enzymes were separated on a 0.7 % agarose gel in TAE buffer and then transferred to a nylon membrane (Pall Bio Support), and hybridized at 42 °C in the presence of 50 % formamide. Hybridization probes are shown in Fig. 1A.

Genomic library construction

Chromosomal DNA from *Me. aminofaciens* 77a was extracted by the modified method of Saito and Miura (80). A library of *Sau*3A I partial-digested chromosomal DNA from *Me. aminofaciens* 77a fractionated by sucrose density gradient centrifugation (5-20 %) (68). The fractionated DNAs were ligated with the λ-EMBL3, and were packaged into λ phage by *In vitro* packaging kit (Gigapac III Gold; STRATAGENE, California, USA).

Plaque hybridization

The ^{32}P-labeled *rmpA* fragment was used as the probe to obtain the *hpsII* clone. DNA was labeled with [α-^{32}P]dCTP using the random primed DNA labeling kit (Boehringer Mannheim, Germany). Plaque hybridization was carried out by the method of Sambrook *et al.* (89).

DNA sequencing

Sequencing of the DNA fragment subcloned to pUC118 or pBR322 was carried out. DNA sequencing was performed by the dideoxy chain termination method (90) using an automated DNA sequencer (Applied Biosystem, model 373A) or DSQ-1000L DNA sequencer (SHIMADZU).

RESULTS

Southern blot analysis of *Me. aminofaciens* 77a chromosomal DNA.

The chromosomal DNA isolated from *Me. aminofaciens* 77a was digested with several restriction enzymes and separated on 0.7 % agarose gel electrophoresis. When *rmpA* was used the probe, two hybridization bands of different sizes and slightly different intensities were found in all digests (Fig. 1.B.I). For example, *Eco*R I fragments of about 19.3-kb and 5.2-kb were found to hybridize to the probe. The 19.3-kb band of *Eco*R I digest was the original *hps* (*rmpA*) gene, but the 5.2-kb was unknown. Since the used probe does not contain any restriction site recognition sequences of the enzymes used by digestion, this result indicates that *Me. aminofaciens* 77a has another homologous gene to *hps*. In the same way, when a *rmpB*- or *rmpI*-fragment was used as the probe, two hybridization bands were found as the same pattern when a *rmpA* was used as the probe. But the band intensities of hybridizing bands were weak (Fig. 1.B.II and 1.B.III). These result suggest possibility that *Me. aminofaciens* 77a has two homologous gene cluster of the RuMP pathway.

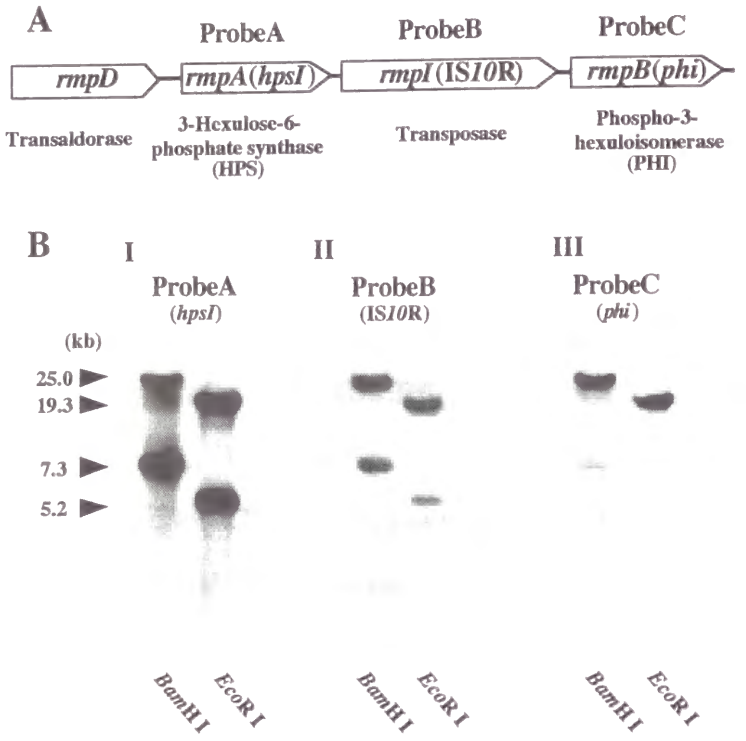


Fig. 1. Southern blot analysis of *Me. aminofaciens* 77a with several probes. A: Structure of 4.4-kb DNA insert of pUH1. Probe A, B and C indicate that Southern blot analyses are used these region as the probes. B: Hybridizing pattern when several probes were used. An DNA ladder (λ-*Eco*T22 I) was used as a size marker.

Cloning and partial nucleotide sequence of the *hpsII* from *Me. aminofaciens* 77a

The genomic library was screened by plaque hybridization method with ^{32}P-labeled *rmpA* probe. The positive clone had the 5.2-kb insert fragment, in accordance with Southern hybridization analysis (data not shown). The nucleotide sequence revealed the 5.2-kb fragment containing a homologous gene the *hps* designated as *hpsII*. The nucleotide sequence was determined partially (Fig.2). Two short inverted repeat sequences were observed downstream the translational stop codon for *hpsII*. The first inverted repeat

located 30 bp downstream the stop codon, and the second inverted repeat located 68 bp downstream the stop codon of *hpsII*.

Another ORF was found downstream the *hpsII* . This ORF showed high similarities to *hisB* gene which can code on imidazole glycerol-phosphate dehydratase from *Azospirillum brasilense* and other sequences.

```
GCTGAGCCATTCTTCAAGGCTGGCGCTGACATCGTTACCGTATTGGGTACTGCTGACATC  60
 A  E  P  F  F  K  A  G  A  D  I  V  T  V  L  G  T  A  D  I
GGCACTATCAAGGGTGTAATTGATGTAGCTAACAAGTACGGCAAGAAGGCACAGATCGAC 120
 G  T  I  K  G  V  I  D  V  A  N  K  Y  G  K  K  A  Q  I  D
CTGATCAACGTAGTTGACAAGGCTGCTCGTACCAAGGAAGTTGCTAAGCTGGGTGCGCAC 180
 L  I  N  V  V  D  K  A  A  R  T  K  E  V  A  K  L  G  A  H
ATCATCGGCGTTCACACTGGTCTGGACCAACAAGCTGCTGGTCAAACTCCTTTCGCTGAC 240
 I  I  G  V  H  T  G  L  D  Q  Q  A  A  G  Q  T  P  F  A  D
CTGGGTCTGGTATCCGGTCTGAACCTGGGCGTGGATATTTCCGTTGCAGGTGGCGTGAAG 300
 L  G  L  V  S  G  L  N  L  G  V  D  I  S  V  A  G  G  V  K
GCAACTACTGCTAAGCAAGTGGTTGATGCAGGCGCGACTATCGTTGTTCGTGGCGCTGCT 360
 A  T  T  A  K  Q  V  V  D  A  G  A  T  I  V  V  R  G  A  A
ATCTACGGTGCGGCTGATCCAGCTGCTGCTGCTGCTGAAATCAGTGCTGCTGCTAAGGTA 420
 I  Y  G  A  A  D  P  A  A  A  A  A  A  E  I  S  A  A  A  K  V
CCCAAAGCAGTGGTGGTGTGTTCGGCTGGCTGAAGAAGCTGTTCAGCTAATCAAGCTGTC 480
 P  K  A  V  V  V  C  S  A  G  *
TGTGGCATATCCGTAGATATGCCAATATTTAGCTTGTCTGGAACCGTTGCAAGACTGTGT 540
                                                  M  T  A
TGACTGTTTTGCAACGGTTTTGATTTTATGACTCAATGATTTGTGATGTGAATGACTGCC 600
ATGCGTAACGCTGAAGTGAGCCGCAATACTCTAGAAACCAAGATTGCTGTCGCCATCAAT 660
 M  R  N  A  E  V  S  R  N  T  L  E  T  K  I  A  V  A  I  N
CTGGATGGCACCGGTGTATCCAAGCTAAATAGCGGGGTTGGTTTTTTTGATCATATGCTG 720
 L  D  G  T  G  V  S  K  L  N  S  G  V  G  F  F  D  H  M  L
GACCAAATCGCCCGACATGGCATGATGGATATTAACGTTGAGTGTCAGGGCGACCTGCAT 780
 D  Q  I  A  R  H  G  M  M  D  I  N  V  E  C  Q  G  D  L  H
ATTGACGCTCACCACACCGTTGAGGATGTGGGCATTGCCTTGGGTCAGGCCTTTAGTCGG 840
 I  D  A  H  H  T  V  E  D  V  G  I  A  L  G  Q  A  F  S  R
GCATTGGGCGACAAAAAAGGTATACGCCGTTATGCACATGCGTATGTGCCATTGGATGAA 900
 A  L  G  D  K  K  G  I  R  R  Y  A  H  A  Y  V  P  L  D  E
GCTTTGTCGCGGGTTGTGCTGGATATTTCCGGGCGTCCAGGGCTGGAGTTCAATGTGGAC 960
 A  L  S  R  V  V  L  D  I  S  G  R  P  G  L  E  F  N  V  D
TTTACCCGTGCTCGCATTGGCGAATTCGTAATCAT                           997
 F  T  R  A  R  I  G  E  F  V  I
```

Fig. 2 Nucleotide sequence and deduced amino acid sequence of *hpsII* (up) and *hisB* (below). The arrows indicate the stem-loop structure.

Southern blot analysis using *hpsII* probe

Southern blot hybridization was probed with a 0.4-kb *Hinc* II restriction fragment from *hpsII* (Fig. 3). As shown in Fig. 4, two hybridization bands

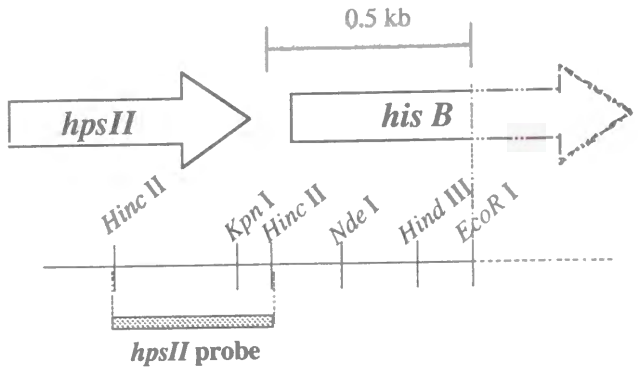


Fig. 3. Restriction map of *hpsII*.
A dotted box indicate that 0.4-kb *Hinc* II fragment was used the Southern analysis (Fig. 4) as the *hpsII* probe.

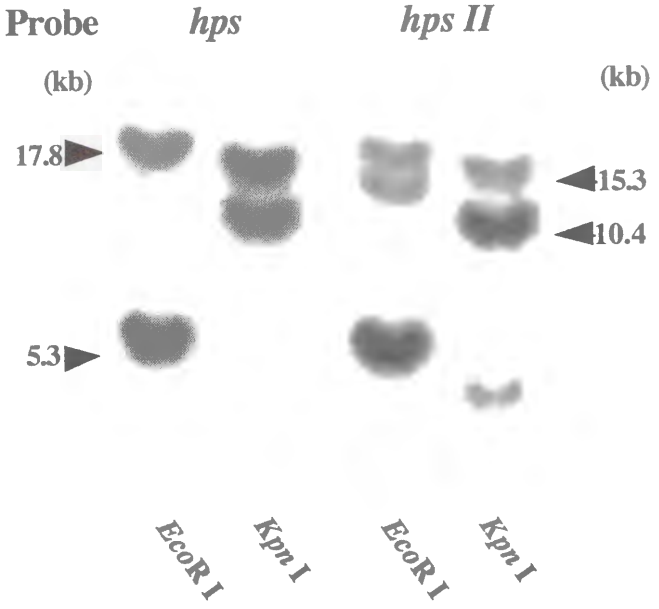


Fig. 4. Southern blot analysis of *Me. aminofaciens* 77a chromosomal DNA with ^{32}P-labeled *hpsII* probe.
Left: the *hps* from pUH1 was used as the probe. Right: the *hpsII* which described in this section, was used as the probe. DNA ladder (λ-*Eco*T22 I) was used as a size marker.

both different size and equal size were found, but on the reversed intensities in all digestions to when *hps* probe was used. Furthermore, the third band was found in all digestions with *hpsII* probe. Author guessed that the third band also has similarity to the *hpsII* probe, when hybridization was carried out under low stringency conditions, *hps* probe also hybridizes to the third band. These suggest that a chromosomal DNA from *Me. aminofaciens* 77a contain three loci showing similarity to *hps*.

DISCUSSION

Southern hybridization analysis revealed that the chromosomal DNA of *Me. aminofaciens* 77a contain another region showing homology of *hps*. Therefore the author cloned this region using λ-EMBL3 system.

Comparison of partial nucleotide sequence of *hps* and *hpsII* are shown in Fig. 5. The sequences of *hps* and *hpsII* are ca. 90 % identical, though there are no significant homology downstream of these ORFs, and the deduced amino acid sequences of *hps* and *hpsII* also showed high similarity of ca. 87 % (Fig. 6), which suggests that the genes originated relatively recently from common ancestor gene by duplication.

Similar duplications of the structural genes in *E. coli* were reported previously. *E. coli* K-12 has two structural genes (*argF* and *argI*) for ornithine carbamoyl-transferase (14, 54, 99). High homology was observed for *argF* and *argI* (78.1% at the nucleotide level, 86% at the amino acid level). The two gene products associate to form four functional catalytic trimers, designated

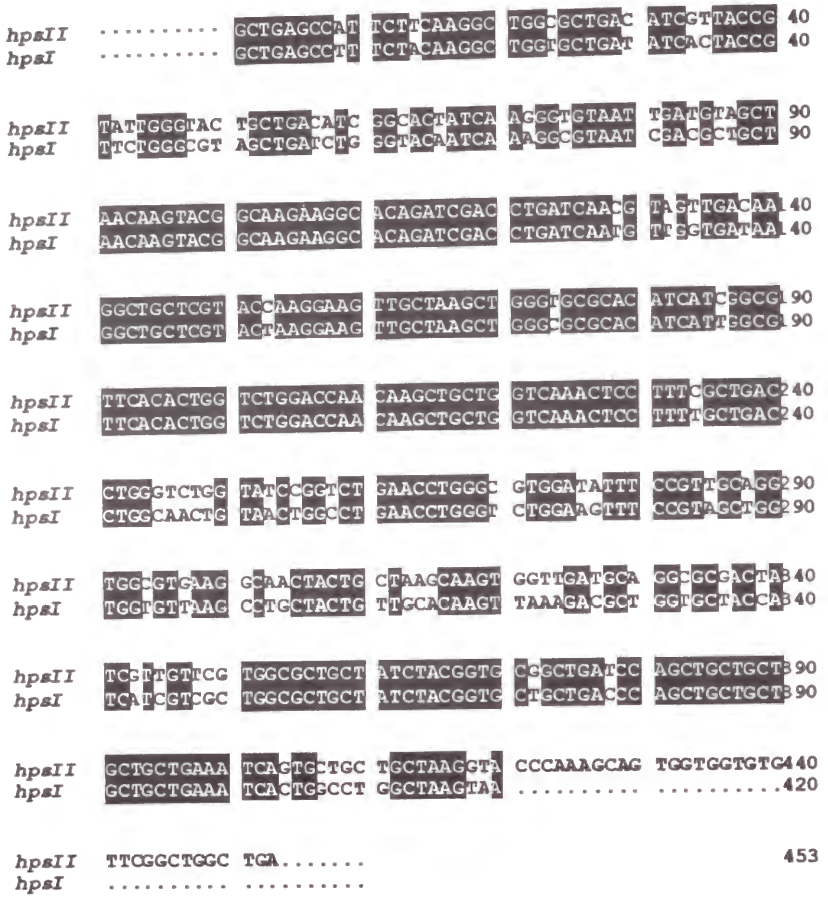


Fig. 5 Comparison of the nucleotide sequences of *hps* and *hpsII*.
Residues in black boxes indicate identical sequences.

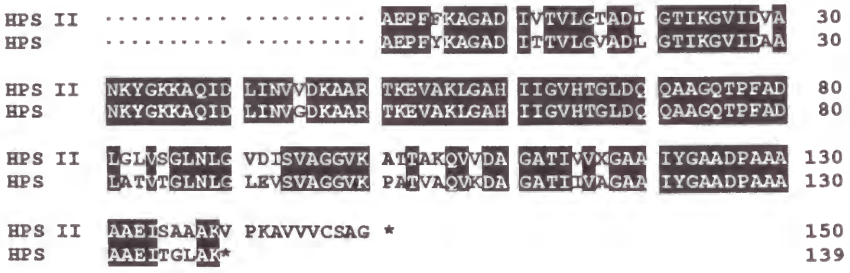


Fig. 6 Alignment of the deduced amino acid sequences of the *Me. aminofaciens* *hps* and *hpsII*.
Residues in black boxes indicate identical sequences.

FFF, FFI FII, and III. The FFF and III isozymes exhibit nearly identical kinetic parameters but differ in physical characteristics such as heat stability. Translation elongation factor EF-Tu of *E. coli* is encoded by two structural genes, *tufA* and *tufB* (4, 106). The deduced amino acid sequences of these two gene products are identical except for several C-terminal amino acids. Since the two EF-Tu genes are functionally and structurally indistinguishable (32, 69), it was suggested that the additional *tuf* gene is required to supply extra EF-Tu for emergency requirements. Since a significance that these two genes existence differ among organisms. In the case of *Me. aminofaciens* 77a, insertion element, *rmpI* existed in the RuMP pathway gene cluster, and Southern blot analysis also suggested another homologous region to *hps* hybridized to *rmpB*, and *rmpI* DNA. This raises some questions, why does *Me. aminofaciens* 77a have these two closely related gene clusters?, Are they expressed equally?, Are there any functional differences between the two gene products?.

A mobile DNA elements can duplications by transposition within the genome (51). Since the author surmise the genes duplicate owing to genome arrangement by the transposable element. To answer these questions, the primary sequence of around *hpsII* are now under investigation.

SUMMARY

Organization of the genes involved in the formaldehyde assimilation was studied on the obligate methylotroph, *Me. aminofaciens* 77a. The 3-hexulose-6-phosphate synthase and phospho-3-hexuloisomerase activities were detected in *E. coli* carrying pUH1 which contain a 4.4-kb DNA fragment from the *Me. aminofaciens* 77a chromosomal DNA. The DNA sequence for a 4.4-kb DNA fragment contain four genes (*rmp A, B, D* and *I*). Sequencing analysis revealed that the *rmpA*, *rmpB* ,*rmpD* and *rmpI* contained 624, 543, 948 and 1206 bp open reading frames respectively. *rmpA* encoded 3-hexulose-6-phosphate synthase. Putative *rmpD* product have significant similarity to transaldolases. *rmpI* and its close region was identified with transposable element (IS*10*-R) which located between *rmpA* and *rmpB*. But, putative *rmpB* product does not have significant identity to any previously known protein. To determine the *rmpB* product, *rmpB* was driven under the *tac* promoter was expressed in *E. coli.* Whole cell extract carrying pKP1 had a strong activity of phospho-3-hexuloisomerase.

Deletion analysis of the cloned region revealed that *rmpI* and neighboring regions affected on activities of 3-hexulose-6-phosphate synthase and phospho-3-hexuloisomerase. The *rmpB* expression was repressed when *rmpI* was deleted, on the other hand, 3-hexulose-6-phosphate synthase activity (*rmpA*) was ca. 6 fold higher when *rmpI*-region was deleted. It seems that *rmpI* play regulatory function in the RuMP pathway of *Me. aminofaciens* 77a.

Me. aminofaciens 77a has two homologous gene cluster of the RuMP pathway. To appear the relationship the duplicated genes and transposable element, the homologous gene (*hpsII*) was cloned, and sequencing. These genes showed high similarities at DNA and amino acid levels. The author suggests that the RuMP genes duplicated by the transposable element IS*10*-R present in the RuMP clusters during evolution.

CHAPTER 2

The RuMP Pathway Gene Cluster from a Facultative Methylotroph *Mycobacterium gastri* MB19

SECTION 1

Cloning and sequence analysis of the gene encoding 3-hexulose-6-phosphate synthase from *Mycobacterium gastri* MB19

Facultative methylotrophie bacteria can be found abundantly among organisms employing the Calvin cycle and the serine pathway for the assimilation of C_1-compounds. But most of the methanol-using bacteria by way of RuMP pathway exhibit obligate methylotrophy (57). Recently, only a few facultative methylotrophic bacteria employing RuMP pathway was isolated (28, 30, 40). These facultative RuMP pathway methylotrophs are found almost exclusively among Gram-positive bacteria. Most of these organisms grow on methylated amines. *Mycobacterium gastri* MB19 is Gram-positive methanol-using facultative methylotrophic bacteria isolated from a soil sample and can also grow on methylated amines (45), such as methylamine, as sole carbon-, and nitrogen source.

In this section, *My. gastri* MB19 was studied metabolic regulation of the RuMP pathway. The author cloned the gene encoding for HPS and compared of a primary structure of HPS and PHI, and their genes organization of these

from *Me. aminofaciens* 77a.

MATERIALS AND METHODS

Bacterial strains, culture conditions and plasmids

My. gastri MB19 isolated from a soil sample from Tottori Prefecture (45). This strain was cultivated at 28 °C on minimal salts medium containing 1.0 % methanol or other carbon sources as described previously (44). *E. coli* JM109 was the host for pUC118. *E. coli* was grown on LB broth (89) in the presence of ampicillin (10 μg/ml), when necessary. If necessary, 0.1 mM IPTG and 0.05 mM X-Gal were added to the medium.

Enzyme assays

The HPS activity was assayed by measuring the rate of Ru5P-dependent disappearance of formaldehyde as described previously (46, 64). One unit of the activity was defined as the amount of enzyme that fixes 1 μmol of formaldehyde into Ru5P per min. The PHI activity was assayed by following the formation of Fu6P from HuMP, and produced Fu6P was isomeraized to G6P by glucose-6-phosphate isomerase. One unit of the enzyme was defined as the amount of enzyme which produces 1 μmol of NADPH with the oxidation of glucose 6-phosphate to 6-phosphogluconate by glucose-6-phosphate dehydrogenase per minute under the conditions described previously (91).

HPS purification and amino-acid sequence

Purification of HPS from *My. gastri* MB19 was performed as described below.

Step 1: Preparation of cell-free extract. My. gastri MB19 was grown under the conditions described above, harvested by centrifugation at 6,700 x g for 20 min at 4 °C, and washed twice with 50 mM potassium phosphate buffer (pH 7.5). The washed cells were suspended in 50 mM potassium phosphate buffer (pH 7.5), disrupted by sonication for 20 min (19 kHz, Insonator model 201M; Kubota, Tokyo), and centrifuged at 12,000 x g for 20 min at 4 °C. The resulting supernatant was then used as cell-free extract.

Step 2: DEAE-sepharose chromatography. The obtained cell-free extract was dialyzed against 10 mM Tris-HCl pH 8.2. The dialyzed enzyme solution was applied to a DEAE-Sepharose column (ϕ 5.0 x 15 cm) previously equilibrated with the Tris-HCl buffer (pH 8.2), and then the column was washed with 900 ml of the buffer. The enzyme was eluted with 6-bed volume of a linear gradient between 10 mM and 100 mM Tris-HCl buffer (pH 8.2). The active fractions were collected, and then dialyzed against 10 mM potassium phosphate buffer (pH 7.0) containing 3 M NaCl.

Step 3: Phenyl-sepharose chromatography. The dialyzed enzyme solution was put on a phenyl-sepharose column (ϕ 2.6 x 13 cm) previously equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 3 M NaCl. After washing the column with 240 ml of the equilibration buffer, elution is carried out with a gradient of decreasing NaCl concentration and increasing ethylene glycol concentration (the final concentrations being 0

and 50 %, respectively; total volume, 480 ml). The active fractions are collected, and then dialyzed against 10 mM Tris-HCl buffer (pH 8.2).

Step 4: Second DEAE-sepharose chromatography. The dialyzed enzyme solution was dialyzed against 10 mM Tris-HCl pH 8.2. The dialyzed enzyme solution was applied to a DEAE-Sepharose column (ϕ 1.0 x 15 cm) previously equilibrated with Tris-HCl buffer (pH 8.2), then the column was washed with 45 ml of the buffer. The enzyme was eluted with 20-bed volume of a linear gradient between 10 mM and 100 mM Tris-HCl buffer (pH 8.2). The active fractions were collected, and then dialyzed against 10 mM potassium phosphate buffer (pH 7.0).

All procedures are performed at 0 - 4 °C. The buffer solution contained 1 mM DTT, 5 mM $MgCl_2$ and 0.15 mM PMSF.

Amino acid sequence of the amino-terminal region of purified enzyme and of peptides were determined on a protein analyzer (Applied Biosystems model 476A). The peptides preparation are described in chapter 1.

The *hps* cloning

To amplify a partial *hps* fragment from the chromosomal DNA of *My. gastri* MB19 by PCR, upstream and downstream primers were designed based on the N-terminus (MKLQVAIDLLSTEAALELAGKVAEYVDIIE LGTPLI) and the internal amino acid sequence (I-6: shown in fig. 2), respectively. Sequences of the primers used were as follows: N-terminal, 5'-ATGAAA(G)C(T)TICAA(G)GTC(A/G/T)GCIATC(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 from *My. gastri* MB19 extracted by the modified method of Marmur (61) was used as template for amplification of *hps*-fragment by PCR. The conditions for PCR were those for the standard procedure suggested by Perkin-Elmer/Cetus. The PCR product was purified and cloned into the pT7Blue (Novagen).

The *My. gastri* MB19 chromosomal DNA digested with various restriction enzymes was separated and transferred to Biodyne nylon membranes (Pall Bio Support). Hybridization was carried out with the PCR product that was radio-labeled with a random primed DNA labeling kit (Boehringer Mannheim).

Colony hybridization was carried out as follows. Colonies of *E. coli* transformats were transferred to membranes and lysed. The liberated DNA was fixed and hybridization was carried out as described previously (89).

Nucleotide sequence analysis

The clones, pUHM1 were cut with *Kpn* I and *Xba* I, and then deletion mutants were produced with a deletion kit (TaKaRa Shuzo, Co., Ltd.). DNA sequencing was performed by dideoxy chain-termination method using an automated DNA sequencer (Applied Biosystem, Model 373A). The sequencing reaction was carried out according for the manuals for the *Taq* dye terminator cycle sequencing kits (Applied Biosystem).

RESULTS

Purification of HPS from *My. gastri* MB19

HPS was purified 42 fold from the cell-free extract (Table 1). The purified HPS had a specific activity of 135 units/mg of protein. The purified enzyme showed a single band on SDS-PAGE, indicating an apparent homogeneity of the protein (Fig. 1). Enzymatic properties were described as previous report (45).

Table 1. Purification procedure of HPS from *My. gastri* MB19.
One unit (U) of the activity was defined as the amount of enzyme that disappearance 1μmol of formaldehyde per minute. The reaction proceed under the standard conditions as described Materials and Methods.

Step	Total Protein (mg)	HPS activity Total (U)	HPS activity S.A. (U/mg)	Fold	Yield (%)
Cell-free extract	4210	13500	3.2	1	100
DEAE-Sepharose	252	15900	63.1	19	118
Phenyl-Sepharose	83	8240	99.3	31	61
DEAE-Sepharose	47	6300	135.0	42	47

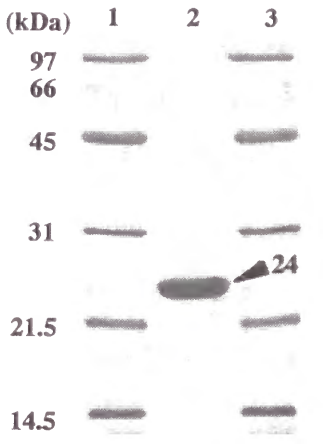
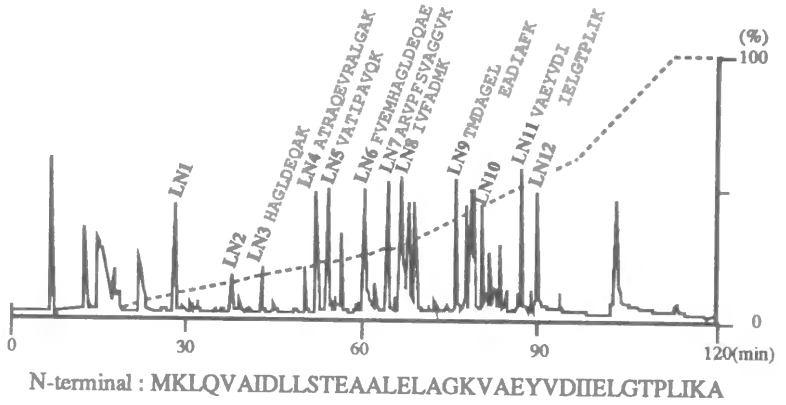


Fig. 1. SDS-PAGE of the purified HPS from *My. gastri* MB19
Lane 1 and 3 were loaded with the following molecular mass standards: phospholyase (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soy bean trypsin inhibitor (21.5 kDa), carbonic anhydrase (14.5 kDa). Lane 2 was purified HPS (5 μg protein) from *My. gastri* MB19. Acrylamide concentration was 15 % in the gel.

N-terminal and internal amino acid sequence of *My. gastri* MB19

Both N-terminal and internal region of the purified enzyme were determined. The elution pattern and peptide sequences obtained *Achromobacter* lysylendpeptidase digestion was shown in Fig. 2.



N-terminal : MKLQVAIDLLSTEAALELAGKVAEYVDIIELGTPLIKA

Fig. 2. Preparation of peptide fragments by the reversed-phase HPLC. Column: Cosmosil 5C18-AR300 4.6 x 250, Flow rate: 0.5 ml/min, Buffer A: 0.06 % TFA in H_2O, Buffer B: 0.052 % TFA in 80 % acetonitrile, Detection : UV210

Selection of *My. gastri hps* clone

Amplification of a *My. gastri* MB19 DNA fragment mediated by PCR with primers N1 and I6 gave a single PCR product of approximately 0.4-kb in length. This PCR product was subcloned into pT7Blue by TA cloning, and its nucleotide sequence was determined. The Author concluded that the 0.4-kb fragment was a portion of *hps*, since the amino acid sequences of four peptide fragments and N-terminal sequence derived from the purified enzyme were found in the amino acid sequence deduced from the nucleotide sequence of this PCR product.

My. gastri MB19 chromosomal DNA was digested with *Bam*H I,

*Eco*R I, *Hin*d III, and *Kpn* I. The enzyme digests were fractionated and transferred to nylon membranes. Hybridization was carried out with the ^{32}P-labeled PCR product as the probe (Fig.3). A *Pst* I fragments of approximately 4-kb were extracted from agarose gel, ligated to the *Pst* I site of pUC118, and then used to transform *E. coli* JM109. The resultant recombinant *E. coli* library was screened by colony hybridization with the same probe. The author selected positive clone, which carried a recombinant plasmid designated pUHM1. This plasmid contained the 4.2-kb insert. HPS activity was assayed of *E. coli* clone carrying pUHM1 under the several growing conditions such

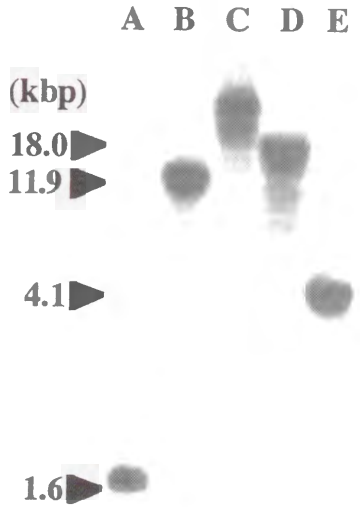


Fig. 3. Genomic southern analysis of *My. gastri* MB19.
A DNA ladder (λ-*Eco*T14 I) was used as a size marker. The probe used in the experiment are ^{32}P-labeled partial *hps* probe. LaneA: *Bam*H I - digested, laneB: *Eco*R I - digested, laneC: *Hin*d I - digested, laneD: *Kpn* I - digested, laneE: *Pst* I - digested, Each sample was separated on 0.7 % agarose gel electrophoresis with TAE buffer.

as addition of methanol, IPTG for culture. However, the HPS activity was not detected. This implies that a promoter or codon usage, that could not be recognized by *E. coli* translation systems.

Nucleotide sequence of *hps*

The DNA sequence of the *hps* on the 4.2-kb *Pst* I fragment was

analyzed. The DNA sequence and deduced amino acid sequence of *hps* from *My. gastri* MB19 are shown in Fig. 4. ORF consisted of 624 bp, which could code for a protein of a molecular mass of 20,935 Da. This value was similar

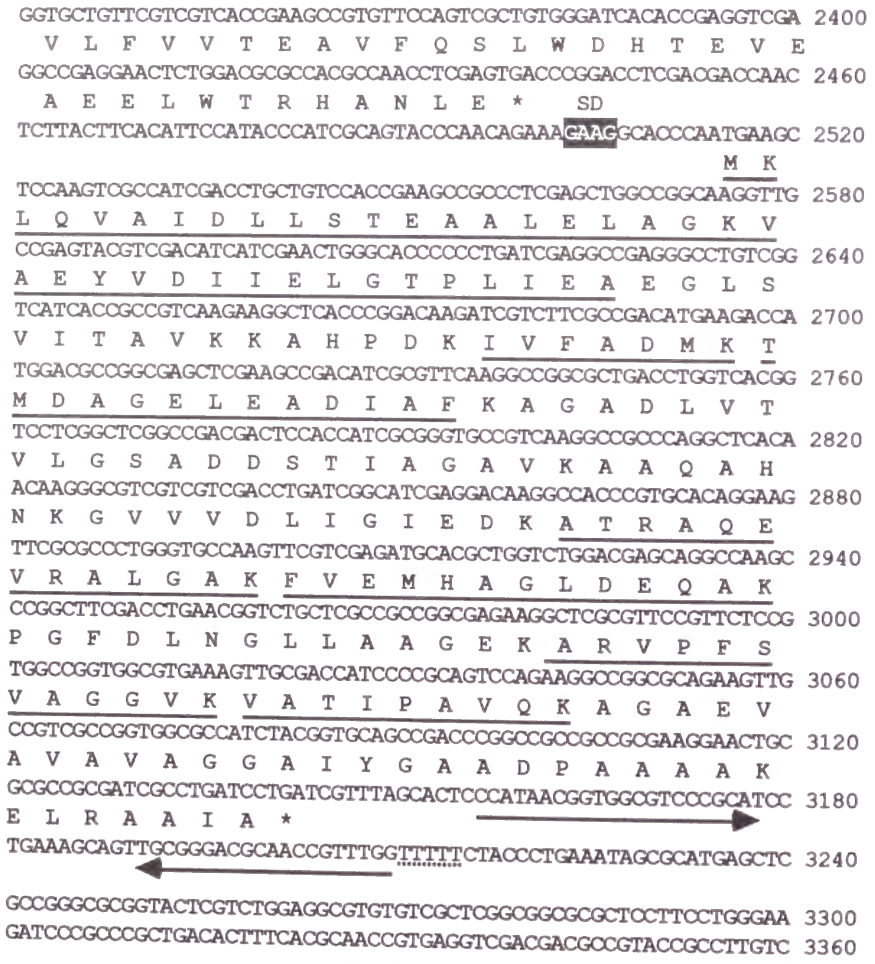


Fig. 4. Nucleotide sequence and the deduced amino acid sequence for HPS gene from *My. gastri* MB19.
The deduced amino acid sequence of ORF are shown below the nucleotide sequence in one-letter code. The amino acid sequences consistent with those found on HPS protein are underlined. Potential ribosome-binding sequences is marked as SD. A putative transcriptional terminator was indicated allows and followed by a series of T residues present characteristic rho-independent transcriptional terminator which marked as dotted line.

to the molecular mass of the HPS subunit (24,000) by SDS-PAGE. Thirty-six N-terminal amino acids of the purified HPS were identical with the DNA-deduced amino acid sequence from residue 36 onward. An ORF that started from ATG codon at the nucleotide position at 2,514 and ended with a TGA termination codon at position at 3,141 were found. The amino acid sequences of the peptide fragments produced with *Achromobacter* lysyl endopeptidase were all found in the sequence. The author concluded that this ORF encoded *hps* from *My. gastri* MB19. A putative SD sequence (GAAG) was located 8 to 11 bases upstream of the ATG triplet on the gene, so a transcription terminator was observed in downstream of the translational termination triplet (TAA) on the gene and these sequences were followed by a series of T residues present characteristic rho-independent transcriptional terminator. But a putative promoter sequence was not observed in upstream of the ATG and putative SD sequence. Analysis of sequence in upstream region revealed another ORF was located close region of ATG triplet of *hps*. Organization of *hps* surrounding region described as next section.

DISCUSSION

Methanol dehydrogenase is present in Gram-negative bacteria are PQQ-dependent. The well-characterized enzyme, which constitutes about 10-15 % of the total soluble protein of the cell, is located in the periplasmic space (2, 43, 75). On the other hand, in Gram-positive bacteria, though only a limited number of methanol dehydrogenases were reported, thermotolerant,

methanol-utilizing strains of *Bacillus methanolicus* studied were found to possess a cytoplasmic NAD-dependent methanol dehydrogenase (12, 19-21) Although, methanol oxidation system is clearly different between Gram-positive and -negative methylotrophic bacteria, the primary structure of HPS of Gram-positive and -negative methanol-utilizer has been shown to be very similar in enzymatic properties, structures and function. These suggest that HPS might be derived from a common ancestor, but methanol oxidation system in Gram-positive and -negative methyrotorophic bacteria have originated independently in different evolutionary lineages.

SECTION 2

Genetic organization of RuMP pathway gene cluster in *My. gastri* MB19

Genetic study on mathylotrophic bacteria were enhanced mainly for methanol oxidation systems (3, 55). But the regulatory systems were reported very little.

In this section, the RuMP pathway gene cluster was revealed in a facultative methylotrophic bacterium, *My. gastri* MB19. This strain could grown on several multi-carbon sources besides methanol, and related to formaldehyde assimilation genes (*rmpA* and *rmpB*) expression were repressed growing on multi-carbon sources. The author described regulation of the genes expression system in *My. gastri* MB19.

MATERIALS AND METHODS

Nucleotide sequencing analysis and computer analysis

The clone ligated on pUC118 were sequenced with an automated DNA sequencer described as section 1. The DNA sequence was analyzed using DNASIS (HITACHI software engineering co. Ltd.). The National Center for Biotechnology Information (NCBI) was searched for homologous amino acid sequences with BLAST or FASTA programs (data base: Gen Bank, EMBL and SWISS-PROT).

Enzyme assays

The HPS and PHI activities were determined as described in the section 1. The relative activities were calculated for based on specific activities (U/mg) of cell-free extract from methanol grown *My. gastri* MB19 as defined 100 %.

Northern blot assay

The culture of *My. gastri* MB19 was cultivated at 28 °C on minimal salts medium containing 1.0 % methanol or other carbon sources and harvested by centrifugation at 6,700 x g for 20 min at 4 °C. Total RNA was extracted by the AGPC (Acid-Guanidium-Phenol-Chloroform) method (89) using ISOGEN (NIPPON GENE CO., LTD.), and RNA samples (20 μg/lane) were electrophoresed on a 1.0% agarose gel containing 20 mM MOPS buffer containing 1 mM EDTA and 2.2 M formaldehyde, and transferred to a nylon membrane filter (Gene screen) in 20 x SSC. Prehybridization and hybridization were carried out at 42 °C in a solution containing 30 % formamide, 5 x SSC, 0.1 % SDS and 100 mg of calf thymus DNA per ml. The probes which contains whole of coding region were labelled by random primed DNA labeling kit (Boehringer Mannheim).

RESULTS

Nucleotide sequences around the *hps* (*rmpA*) region

The sequence of the pUHM1 insert revealed three complete and two

partial ORFs in the direction as shown in Fig. 1. *rmpA* was coded by the third ORF.

Sequence analysis of the upstream region of *rmpA* revealed that ORF was located with the spacer of 73-bp, and starting at nucleotide position of 1,841, on Fig. 2 and ending at nucleotide position of 2,438. A putative SD sequence (GAAG) was located 10 to 14 bases upstream of the ATG triplet

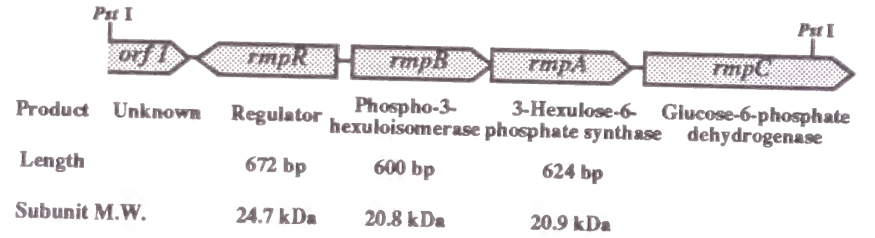


Fig. 1. Structure of rmpA surrounding region of *My. gastri* MB19. The entire 4.2-kb region was sequenced in this section. Three complete ORFs and two partial ORFs were shown. The arrowboxes indicate a direction of transcription. The transcriptional stop codon was included in the length. Subunit M.W. was calculated on the based of sequence.

on the gene. This putative product does not have significant identity to any known protein on databases, but this ORF showed similarity with a *rmpB* from *Me. aminofaciens* 77a described as chapter 1 (Fig. 3). The author concluded the product of this ORF could code phospho-3-hexuloisomerase (PHI) and the gene correspond to *rmpB* in RuMP gene cluster.

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GTCAGGAGGACTGACGGCTGGTGCTGTAGATCAACGAGAGCCCCAGGTGCGGAACATCGA   60
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GAGTCACTCGATGCTGATCGGGCGTACCGGATTTGATGCGCTCACCCACCAAATACATCC  120
  S  H  S  M  L  I  G  R  T  G  F  D  A  L  T  H  Q  I  H  P
CGGAATTGCCGAGGATGACGCGGTTACTGGCGTTGACCAGGGCGGCATCGCCCGGAATCA  180
  G  I  A  E  D  D  A  V  T  G  V  D  Q  G  G  I  A  R  N  Q
ACAGCAGATCCGGCATGATGATGGGCTCCAGGTCCGTGAGCCGGATATCGCAGCCGGCCA  240
  Q  Q  I  R  H  D  D  G  L  Q  V  R  E  P  D  I  A  A  G  H
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  K  N  G  S  F  S  *
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TCGGGAGAGCTCGTCCAATCCGGTAAGCGCCGCCCGGCTCACCTTCGATTTGCCCGCGAG  600
ATTTCGATCGAGCAGCGCGGCAACGTCTTTGGCGAGGCTCTTGGTTTCGGTGGCGACGCC  660
GTTGATCCACGAGGTGAGGGCGTCGACAGCCCGGGAGACTTCAGTGGTCGAGTTCATTGG  720
                    *  R  L  F  Q  R  R  T  R  D  R  S  Q

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                    SD                  O_E
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GCGCCGCGATCGCCTGATCCTGATCGTTTAGCACTCCCATAACGGTGGCGTCCCGCATCC 3180
E  L  R  A  A  I  A  *
TGAAAGCAGTTGCGGGACGCAACCGTTTGGTTTTTCTACCCTGAAATAGCGCATGAGCTC 3240

GCCGGGCGCGGTACTCGTCTGGAGGCGTGTGTCGCTCGGCGGCGCGCTCCTTCCTGGGAA 3300
GATCCCGCCCGCTGACACTTTCACGCAACCGTGAGGTCGACGACGCCGTACCGCCTTGTC 3360
GAGAGCTGGAATTCCACCATGTCCGCTGACCACGGTGATTCGAGTGTGAGGCCCGGACGC 3420
        rmpD    M  S  A  D  H  G  D  S  S  V  R  P  G  R
AACCTGCTCCGGGATCCGCGCGATCGTCGCTTGAACCGCATCGCCGGTCCGTCCTCCCTT 3480
 N  L  L  R  D  P  R  D  R  R  L  N  R  I  A  G  P  S  S  L
GTCCTGTTCGGAGTCACCGGCGATCTCGCCCCGGAAGAAACTCGTGCCCGCGGTGTACGAC 3540
 V  L  F  G  V  T  G  D  L  A  R  K  K  L  V  P  A  V  Y  D
CTCGCCAACCGGGGGTCTGTTGCCGCCGAGCTTTGCCTTGGTGGGCTTCGGCCGGCGCGAA 3600
 L  A  N  R  G  L  L  P  P  S  F  A  L  V  G  F  G  R  R  E
TGGACGAACGAGGACTTCGCCGCCGAGGTCAAGGCGAACGTGAAGGCTTACGCCCGAACA 3660
 W  T  N  E  D  F  A  A  E  V  K  A  N  V  K  A  Y  A  R  T
```

```
CCTTTCGACGAGGCCGTGTGGGAGCAACTCTCCGAGGGCATCCGCTTCGTCCAAGGCGCG 3720
 P  F  D  E  A  V  W  E  Q  L  S  E  G  I  R  F  V  Q  G  A
TTCGACGACGAGACGGCGTTCAAACGGCTGCGCGCCACGCTGGAGGATCTCGACGAGCAG 3780
F  D  D  E  T  A  F  K  R  L  R  A  T  L  E  D  L  D  E  Q
CGCGGCACGCGCGGCAATTACGCCTTCTACCTTTCGATCCCACCCAAGGCCTTCGAACAG 3840
R  G  T  R  G  N  Y  A  F  Y  L  S  I  P  P  K  A  F  E  Q
GTCTGCCGCCAGCTCTCCGAATCCGGGCTGGCGCAGGCCGAGAACGACAAGTGGCGCCGG 3900
V  C  R  Q  L  S  E  S  G  L  A  Q  A  E  N  D  K  W  R  R
GTGGTCATCGAGAAGCCGTTCGGACACGACCTCGAGTCGGCCCGCCAACTCAACGACGTC 3960
 V  V  I  E  K  P  F  G  H  D  L  E  S  A  R  Q  L  N  D  V
GTCGAGTCCGTGTTCCCGCCGGACGCCGTGTTCCGGATCGACCATTACCTGGGCAAGGAG 4020
 V  E  S  V  F  P  P  D  A  V  F  R  I  D  H  Y  L  G  K  E
ACGGTCCAGAACATCCTGGCCCTGCGCTTCGCGAACCAGCTCTTCGAGCCGCTGTGGAAC 4080
 T  V  Q  N  I  L  A  L  R  F  A  N  Q  L  F  E  P  L  W  N
GCGAATTACGTTGACCACGTACAGATCACGATGGCCGAATCCATCGGCACCGGCGGCCGG 4140
 A  N  Y  V  D  H  V  Q  I  T  M  A  E  S  I  G  T  G  G  R
GCAGGTTACTACGACGGTGTCGGCGCGGCCCGCGACGTCATCCAGAACCACCTGCTGCA  4199
 A  G  Y  Y  D  G  V  G  A  A  R  D  V  I  Q  N  H  L  L
```

Fig. 2. Nucleotide sequence of the 4,199-base *Pst* I fragment containing the *rmp* genes of *My. gastri* MB19.
Residues in black boxes indicate putative ribosome-binding sequences is marked as SD. The arrows indicate inverted repeat structure, and putative operator marked as O_E and O_I indicate external operator and internal operator, respectively.

```
MB19 MTQAAEADGA V-KVVGDDIL NNLSLVRDEV AD-TAAKVDP EQVAVL-ARQ  50
77a  MNKYQEL--V VSKL-----T N-V-INNT-- AEGY----DD KILSLVDA--  50

MB19 IVQPGRVFVA GAGRSGLVL- -RMAAMRLMH FGLTVHVAGD TTTPAISAGD 100
77a  ---AGRTFIG GAGRS-L-LV SRFFAMRLVH AGYQVSMVGE VVTPSICAGD 100

MB19 LLLVASGSGT TSGVVKSAET AKKAGARIAA FTTNPDSPLA GLADAVVIIP 150
77a  LFIVISGSGS TETLMPLVKK AKSQGAKIIV ISMKAQSPMA ELADLVV--P 150

MB19 AAQKTL-HGS HISRQYA-GS LFEQ-VLFVV TEA-VFQSLW DH---TEVE- 200
77a  VGGN-DANAF DKTHGMPMGT IFELSTLWFL -EATIAKLV- DQKGLTP-EG 200

MB19 --AEELWTRH ANLE*..... .......... .......... .......... 214
77a  MRA--I---H ANLE*..... .......... .......... .......... 214
```

Fig. 3. Alignment of the deduced amino acid sequences of PHIs from *My. gastri* MB19 and *Me. aminofaciens* 77a.
Residues in black boxes indicate identical sequences. Amino acid sequence of PHIs from *My. gastri* MB19 and *Me. aminofaciens* 77a was abbreviated MB19 and 77a respectively. These sequences were aligned by introducing gaps (hyphens) to achieve maximum homology.

Two stem-loop structure suggesting the binding signal of regulatory protein as an operator were observed upstream translational start codon of *rmpB*.

The transcriptional terminator was not observed downstream of the *rmpB*.

The second ORF (*rmpR*) of the reversed orientation to *rmpA* and *rmpB* encodes a putative product of 223 amino acids and calculated molecular mass of the polypeptide was 24,666 Da. This putative product showed similarity with a number of DNA-binding regulatory protein from *Streptomyces coelicolor* and *Bacillus subtilis* (76) (Fig. 4).

```
RmpR   MFGVGGATLREALSELRERGVVETRRGRSGGT  32
GntR   EFSVSRSPIREALKILASEKIIRLERMGAVVI  54
YhcK   FFNVGRPSVREALAALKRKGLVQINNGERARV  63
GlcC   KLGFSRSALREGLTVLRGRGIIETAQGRDSRV  81
ScpR     FGIAASTAQKALAHLRTGEVRTELG        55
KorS     YGVSGGTIRKAMVEVRASGLVETRHGK      72
```

Fig. 4. Amino acid sequence alignment of *rmpR* putative product and other regulatory proteins.
These proteins have DNA-binding HTH motif in this region and classified gntR family. RmpR: putative *rmp* operon regulatory protein from *My. gastri* MB19. GntR: gluconate operon regulatory protein from *Bacillus subtilis*. YhcK: Hypothetical transcriptional regulator protein from *E. coli* K-12. GlcC: Glc operon transcriptional activator from *E. coli*. ScpR: putative transcriptional regulator from *Streptomyces coelicolor*. KorS: regulatory protein, coded on the plasmid from *Streptomyces ambofaciens*. Residues in black boxes indicate identical and similarity sequences.

The putative product of the fifth partial ORF (*rmpC*) have significant similarity with gulucose-6-phosphate dehydrogenase (G6PDH) to Zwf2 gene from *Mycobacterium leprae*, *Synechococcus* sp. strain PCC 7942 (Fig. 5). A putative SD sequence (GAGA) was located 15 to 18 bases upstream of ATG triplet on the gene. G6PDH is an enzyme in the Entner/Doudoroff pathway. It catalyses the conversion glucose 6-phosphate and to 6-phosphoguluconate coupled with oxidation of NADPH. G6PDH is assumed to play an important role in the stage for cleavage to produce the cell constituents in the RuMP pathway.

The partial ORF in Fig. 2, show some similarities to hypothetical proteins from *Bacillus subtilis* and *Methanococcus jannaschii* (22) on database, but function is unknown.

Fig. 5. Comparison of amino acid sequences of *rmpC* product from *My. gastri* MB19 and other homologous proteins.
Amino acid sequences of RmpC from *My. gastri* MB19, Zwf2 from *Mycobacterium leprae* and Syne from *Synechococcus* PCC 7942 were aligned by introducing gaps (hyphens) to achieve maximum homology. Zwf2 and Syne had been reported as G6PDH, elsewhere. Residues in black boxes indicate identical sequences.

Transcriptional regulation of formaldehyde fixation enzymes

To compare the activities of HPS and PHI, *My. gastri* MB19 was grown on various carbon and nitrogen sources (Fig.6). Both enzymes activities showed the highest in methanol-grown cell, but ethanol- and glucose-grown cells did not show there enzyme activities when the ammonium sulfate as a nitrogen source. When a methylamine was used as a nitrogen source, HPS and PHI activities were detected in glucose- and ethanol-grown cells. Comparison the levels for regulatory pattern of HPS and PHI was similar,

suggesting that both genes were regulated under the same control.

To reveal the *rmpA* and *rmpB* expression mechanism at the mRNA level, Northern blot analysis was carried with cells grown on various carbon and nitrogen sources in *My. gastri* MB19 was shown in Fig. 7. Total RNAs

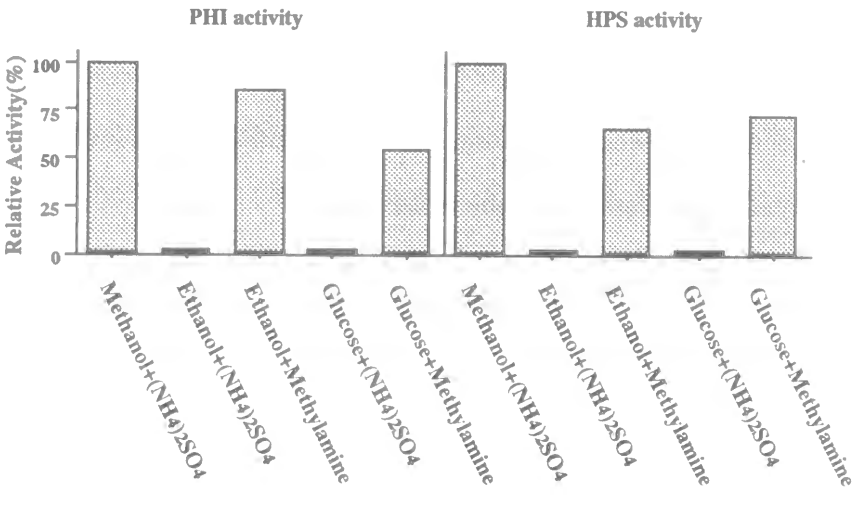

Fig. 6. Relative activities of PHI and HPS.
Activities were measured on the sonicated supernatant from various carbon-, and nitrogen-sources grown cells of *My. gastri* MB19. Enzyme assays are performed under the standard conditions, and relative activity was described as Materials and Methods.

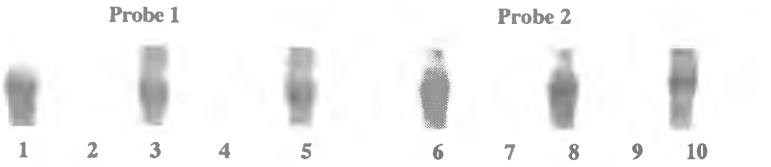

Fig. 7. Northern blot analysis of *My. gastri* MB19.
Probe 1 and 2 were used *rmpA* and *rmpB* of pUHM1 from *My. gastri* MB19, respectively. Lane 1 and 6: hybridizing was performed with total RNA was extracted from methanol-ammonium sulfate grown cell as carbon- and nitrogen-source respectively, lane 2 and 7: from ethanol-ammonium sulfate grown cell, lane 3 and 8: from ethanol-methylamine chloride grown cell, lane 4 and 7: from glucose-ammonium sulfate grown cell, lane 5 and 8: from glucose-methylamine chloride grown cell. 20 μg total RNA was loaded on the each lane, and separated denatured 1.0 % agarose gel.

were extracted from cells grown on the substrates described above, and were compared by Northern analysis with the ^{32}P-labeled whole genes of *rmpA* and *rmpB* were prepared by PCR as the probe. The bands hybridisable to *rmpA* or *rmpB* were detected and these reflected the enzyme activity profiles. Total RNAs of methanol- or methylamine-induced cells gave hybridizing the band, but ethanol- or glucose-induced cells did not. And the size of hybridizing bands from both probes were similar (ca. 1.5-kb). These results suggested that expression of *rmpA* and *rmpB* regulated at the mRNA level, and these genes were regulated as a polycistronic operon.

DISCUSSION

Organization of the RuMP pathway gene clusters in two strains

In this chapter, the author described the RuMP pathway gene cluster in Gram-positive facultative methanol-utilizing bacteria, *My. gastri* MB19. Five ORFs were observed in the cloned gene. Four of five ORFs products could be subscribed to the RuMP pathway. *rmpA*, *rmpB*, *rmpC* and *rmpR* could code for HPS, PHI, G6PDH and regulatory protein respectively. HPS and PHI are the key enzymes in RuMP pathway and play a role of the fixation stage of formaldehyde, G6PDH act at the stage of cleavage of 6-carbon sugar phosphate to 3-carbon compounds for produce a cell constituents. In the stage of cleavage, advocated two variants in RuMP pathway (6); Fructose 6-phosphate produced by PHI and then one of the molecules is converted to either fructose 1,6-bisphosphate by phosphofructokinase, or to 2-keto 3-deoxy

6-phosphogluconate by the Entner/Doudoroff enzymes. G6PDH is the enzyme of Entner/Doudoroff pathway and a possible coding sequence existed the RuMP gene cluster of *My. gastri* MB19. It suggest this strain use the latter pathway in the stage of cleavage.

The RuMP pathway gene clusters from *Me. aminofaciens* 77a and *My. gastri* MB19 (Fig. 8). *rmpA* and *rmpB* coded for key enzymes HPS and PHI, respectively, Both of these genes showed high similarities at amino acid sequence level (Fig. 3 and 9) though G+C contents were very different between both strains (data not shown). It suggests that two enzymes

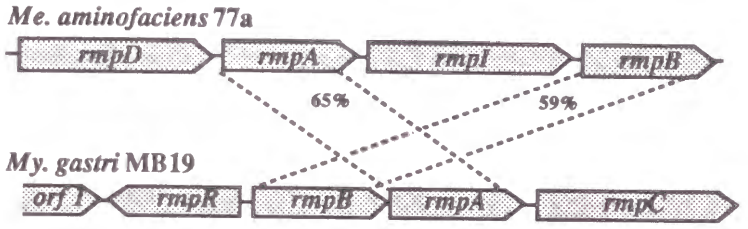

Fig. 8. Organization of the gene clusters involved in the of RuMP pathway of *Me. aminofaciens* 77a and *My. gastri* MB19.
Amino acid sequence similarity (percentages) between equivalent genes of both gene clusters are indicated. *rmpA*; 3-Hexulose-6-phosphate synthase, *rmpB; Phospho-3-hexuloisomerase, rmpC*; Glucose-6-phosphate dehydrogenase, *rmpD*; Transaldolase, *rmpI*; Transposase (IS*10*-R), *rmpR*; Regulator protein, *orf1*; Unknown.

were derived from a common ancestor. However, *rmpB* located downstream of *rmpA* in *Me. aminofaciens* 77a and further the insertion sequence which located between *rmpA* and *rmpB* encoded transposase of IS*10*-R. On the other hand, *rmpA* located downstream of *rmpB*, and both genes had arranged by side by side. *rmpC* and *rmpD* could code for G6PDH and TAL, respectively. G6PDH acts the stage of cleavage of 6-carbon sugar phosphate to 3-carbon

compounds for produce a cell constituents as described above, and TAL plays an important role in the RuMP pathway for the stage of rearrangement.

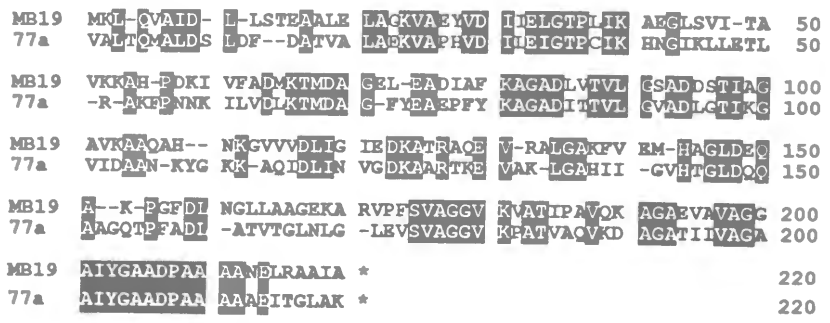

Fig. 9. Alignment of the deduced amino acid sequences of HPSs from *My. gastri* MB19 and *Me. aminofaciens* 77a.
Residues in black boxes indicate identical sequences. Amino acid sequence of HPSs from *My. gastri* MB19 and *Me. aminofaciens* 77a was abbreviated MB19 and 77a respectively. These sequences were aligned by introducing gaps (hyphens) to achieve maximum homology.

These suggest that this cluster contain enzymes them each stage of the RuMP pathway (fixation, cleavage and rearrangement, see introduction). Further study for searching the genes around region of these clusters will lead to clear into the genetic organization of RuMP pathway.

Comparison of regulation for *rmpA* and *rmpB* expression in two strains

rmpR showed similarity with a number of DNA-binding regulatory proteins containing a-helix-turn-a-helix (HTH) DNA recognition motif from amino acid residues 1 to 32, which belongs to the *gntR* family (39, 70, 77, 97, 107, 108) (Fig.4). The author assumes the *rmpR* product binds two stem-loop structures observed upstream of the translational start codon of *rmpB*. These structures are the binding signal of the regulatory protein as an operator.

Northern analyses in *rmpA* and *rmpB* suggested that expression of *rmpA* and *rmpB* are under the same control at the mRNA level, and these genes were regulated as operon. The model for regulation *rmp* operon for *rmpA* and *rmpB* expression was shown in Fig. 10. The expression of *rmpA* and *rmpB* in *My. gastri* MB19 is regulated at the mRNA level by methanol-, formaldehyde- or sugar phosphate derivatives-dependent regulatory protein of *rmpR* product-operator interaction.

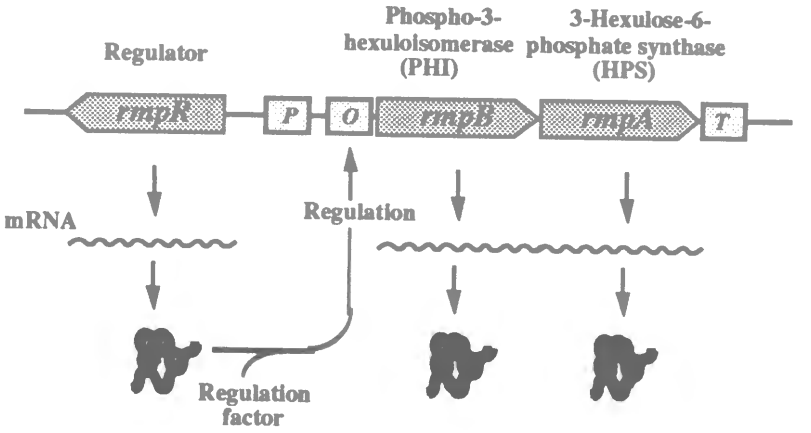

Fig. 10. The model of *rmp* operon.
An O, P and T indicate the promoter , operator and terminator structure, respectively.

In an obrigate methylotroph *Me. aminofaciens* 77a was *rmpI* (IS*10*-R) located between *rmpA* and *rmpB*, and *rmpI* behaves like regulatory element. Deletion of *rmpI* caused activation of *rmpA* expression, on the other hand, *rmpB* expression was dramatic decrease. It suggest that the *rmpI* as repression element for *rmpA* expression and as activation element for *rmpB* expression. It were caused by promoters in *rmpI* (see section 3 in chapter 1).

These differences in regulation between *rmpA* and *rmpB* expression

may be based on characteristics of methylotrophy. A facultative methylotroph *My. gastri* MB19, to grow on various carbon sources, the strict regulation system was got on a evolutionary.

SUMMARY

In this chapter, the author described the RuMP pathway gene cluster in a Gram-positive facultative methanol-utilizing bacterium, *My. gastri* MB19. Five ORFs were found the cloned fragment. Four of five ORFs are suggested to be of known functions in the RuMP pathway. *rmpA*, *rmpB*, *rmpC* and *rmpR* could code for HPS, PHI, G6PDH and regulatory protein respectively. *Me. aminofaciens* 77a and *My. gastri* MB19 have different organization in the RuMP pathway gene cluster. Both Northern and enzymatic analysis from *My. gastri* MB19 suggested that expression of *rmpA* and *rmpB* are under the same control at the mRNA level, and these genes were regulated as an polycistronic operon. On the other hand, expression of *rmpA* and *rmpB* was regulated as monocistronic in *Me. aminofaciens* 77a. The author suggests differences in their regulating mechanism in the RuMP pathway was based on characteristics of their methylotrophies, i.e. an obrigate methylotroph and a facultative methylotroph.

CHAPTER 3

Application of The Enzymes in RuMP Pathway of Methylotrophic Bacteria

SECTION 1

Overexpression of the genes encoding HPS and PHI in *E. coli*

The assimilation of methanol by RuMP pathway is the most cost-effective route in terms of requirement for ATP and NADH. Biochemical substrates with labelling by ^{13}C at specific sites are useful for studies of metabolic pathways (23, 72), and tracing with ^{13}C NMR of a fate of a metabolite *in vivo* is becoming a routine clinical technique for the diagnosis of a variety of diseases. For such ^{13}C NMR techniques, less expensive ^{13}C-labeled substrates involving enzymatic modifications of precursors that are chemical enriched with ^{13}C. From these viewpoint, authors focused on aldol condensation reaction of HPS and isomerization of PHI that key enzymes of formaldehyde fixation from RuMP pathway, and established the enzymatic process to prepare the ^{13}C-labeled sugars which are value as a clinical diagnosis (60, 103, 104).

As described previously, the author was first cloned genes for these key enzymes, and established the system of more easily purification of HPS and PHI from *E. coli* in this section. These results indicate to develop an enzymatic method to produce the ^{13}C-labeled sugars, such as glucose or fructose.

MATERIALS AND METHODS

Bacterial strains and culture conditions

E. coli JM109 was the host for pT7blue and pKK223-3. *E. coli* was grown on LB broth or 2 x YT medium in the presence of ampicillin (10 μg/ml) when necessary. If necessary, 0.1 mM IPTG and 0.05 mM X-Gal were added to the medium.

Expression vector modification

The recombinant genes expression was utilized *tac* promoter of *E. coli*. To construct the overexpression plasmids, the fragments were amplified by PCR from pUH1 and *Me. aminofaciens* 77a chromosomal DNA as the template. Upstream and downstream primers were designed from the sequences. The sequences of the primers were as follows: for HPS, N-terminal 5'-GGAATTCCTTTTGGAGGAAGTATCGTGGCAT-3'; and C-terminal, 5'-GGAATTCCTTACTTAGCCAGGCCAGTGATTT-3', for PHI , N-terminal, 5'-GGAATTCCTATTTAAGGTGAATGAAC-3'; and C-terminal, 5'-GGAATTCCTTACTCGAGGTTAGCATGAAT-3', the PCR products were purified and cloned into pT7blue by the method of TA cloning and sequenced. Resultant plasmids were digested by *Eco*R I and purified insert cloned into the *Eco*R I site of pKK223-3, which were named the plasmids pKH1 and pKP1 respectively, and then transformed into *E. coli* JM109.

Enzyme assays and purification

The HPS was assayed by measuring the rate of Ru5P-dependent disappearance of formaldehyde as described in the previous chapters. The PHI was assayed discontinuously by following the formation of Fu6P from HuMP, and produced Fu6P was isomeraized to G6P by glucose-6-phosphate isomerase immediately. One unit was defined as the amount of enzyme which produce 1 μmol of NADPH with the oxidation of glucose 6-phosphate to 6-phosphogluconate by glucose-6-phosphate dehydrogenase per minute under the conditions described previously.

The purification of HPS and PHI from *E. coli* carrying pKH1 and pKP1 were performed with same procedure as described below.

Step 1: Preparation of cell-free extract. *E. coli* carrying pKH1 and pKP1 were used as a source of cell-free extract, and were grown on LB broth containing 10 μg/ml ampicillin at 37 °C and shaking well, after cultivation for 2 h, 0.1 mM (final concentration) IPTG was added and continued the enrichment for 14 h, harvested by centrifugation at 5,000 x g for 10 min at 4 °C, and washed twice with 50 mM potassium phosphate buffer (pH 7.5). The washed cells were suspended in 50 mM potassium phosphate buffer (pH 7.5), disrupted by sonication for 10 min (19 kHz, Insonator model 201M; Kubota, Tokyo), and centrifuged at 12,000 x g for 20 min at 4 °C. The resulting supernatant was used as cell-free extract.

Step 2: DEAE-sepharose chromatography. The cell-free extract was dialyzed against 10 mM Tris-HCl pH 8.2. The dialyzed enzyme solution was applied to a DEAE-Sepharose column (ϕ 5.0 x 15 cm) previously

equilibrated with Tris-HCl buffer (pH 8.2), then the column was washed with 900 ml of the buffer. The enzyme was eluted 6-bed volume with a linear gradient between 10 mM and 100 mM Tris-HCl buffer (pH 8.2). The active fractions were collected, and then dialyzed against 10 mM potassium phosphate buffer (pH 7.5).

All procedures are performed at 0 - 4 °C. All the buffer solution contain 1 mM DTT, 5 mM $MgCl_2$ and 0.15 mM PMSF. Protein concentration was determined by method of Bradford (17) using bovine serum albumin as the standard with the Bio-Rad protein assay kit. Sodium dodecyl sulfate (SDS)- gel electrophoresis and Coomassie blue staining of gels were done according to Laemmli (52).

RESULTS AND DISCUSSION

Effective expression *rmpA* and *rmpB* in *E. coli*

Me. aminofaciens 77a was used as a source of the genes for overexpression. Because the codon usage of *rmpA* and *rmpB* from *My. gastri* MB19 exhibit GC rich, so the genes were not expect effective expression in *E. coli*. The expression vector modification described as Materials and Methods. The insert genes (*rmpA*, *rmpB*) were driven by the *tac* promoter of pKK223-3. The resultant plasmids named pKH1 and pKP1 in which *rmpA* and *rmpB* has been cloned into *Eco*R I site of pKK223-3 respectively. To set up the most effective expression conditions, various induction periods and concentrations of IPTG in medium were tested (Fig. 1). When IPTG was added 0.1 mM

after 2 h from the start, and then cultivation were continued for 12 h at 37 °C, the highest HPS activity was observed in *E. coli* JM109 harboring pKH1 and pKPI.

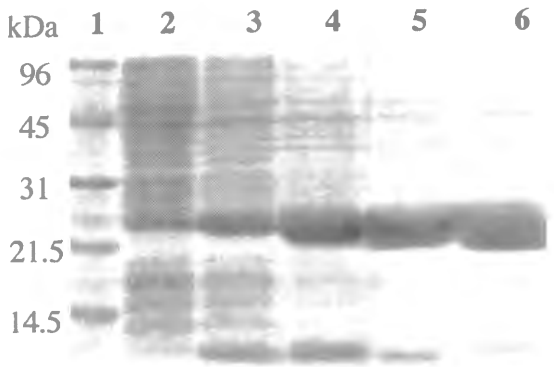

Fig. 1. SDS-PAGE of HPS from *E. coli* carrying pKH1.
Lane 1: molecular mass standard. Lane 2: cell-free extract from *E. coli* carrying pKH1 without IPTG induction . Lane 3, 4, 5 and 6 cell-free extracts from *E. coli* carrying pKH1 which cultivated 4, 6, 8 and 12 hours after 0.1 mM IPTG added, respectively.

The level of HPS and PHI activities in the supernatant of the sonicated cell-free extracts of the transformats were 121 U/mg and 7,360 U/mg respectively. These specific activities indicate that HPS and PHI occupy about 80 % and 50 % of all soluble proteins in *E. coli*, respectively. When these transformants cultivated under the above conditions, a protein band corresponding to the subunit was also observed in precipitates fraction (data not shown). The precipitates had no activity, and so they may be inclusion bodies.

Purification of HPS and PHI from *E. coli* transformant

Through the purification procedures described in Materials and Methods, HPS and PHI were purified with yields of 32 % and 24 % respectively (Table 1). The purified enzymes showed only one band on SDS-PAGE (Fig. 2A snd B). Thus, the effective expression and more easily purification were

established by the use of *E. coli* transformants. These results extended the application of HPS and PHI for enzymatic process to prepare the ^{13}C-labeled sugars which are value as a clinical diagnosis.

Table 1. Purification of HPS and PHI from *E. coli* carrying pKH1 and pKP1. Upper table show the summary of HPS purification , and summary of PHI purification was shown under table. The reaction proceeded under the standard conditions as described Materials and Methods.

HPS

Step	Total protein (mg)	Total activity (U x 10^3)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell-free extract	758	159	209	100	1
DEAE sepharose	242	77	319	32	1.5

PHI

Step	Total protein (mg)	Total activity (U x 10^3)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell-free extract	597	436	7360	100	1
DEAE sepharose	141	217	15400	24	2.1

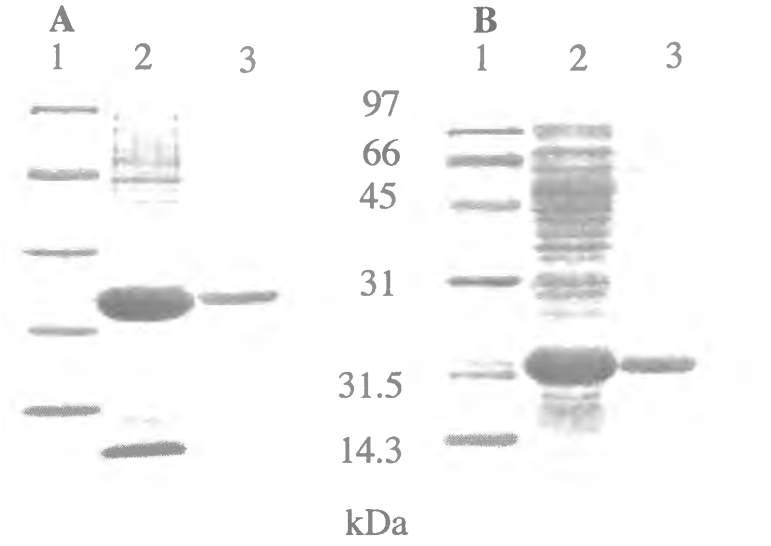

Fig. 2. SDS-PAGE of the purified products from *E. coli* carrying pKH1 and pKP1. Lane 1 was loaded with the following molecular mass standards: phospholyase (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soy bean trypsin inhibitor (21.5 kDa), carbonic anhydrase (14.5 kDa). Lane 2 was supernatant of sonicate (20 μg protein) from *E. coli* JM109 carrying pKH1 and pKP1 was induced IPTG. Lane 3 was purified PHI (3 μg protein). Acrylamide concentration was 15 % in the gel.

SECTION 2

Expression of *rmpA* and *rmpB* in a methylotrophic yeast, *Candida boidinii*

A methylotrophic yeasts are able to grow at a high rate and to a high density during laboratory culture on methanol. The study of the utilization of methanol by yeasts began with the investigations of *Candida boidinii*, isolated on methanol by Ogata *et. al.* (67).

A transformation system of exogenous gene had been established for *C. boidinii* (82) in our laboratory, and high-level expression were performed with a strong alcohol oxidase promoter, since a lot of useful enzymes effective expression were reported (81, 85, 88).

In this section, to extend the application of HPS and PHI, the author attempted expression of these genes in *C. boidinii*.

MATERIALS AND METHODS

Microorganisms, culture conditions and plasmids

E. coli JM109 was used as the host for pUC118, pT7Blue and pNOTeI (81) which is *E. coli* - *C. boidinii* integrated shuttle vector contained alcohol oxidase (*AOD1*) promoter (87) and orotidine-5'-phosphate decarboxylase gene (*URA3*). *E. coli* was grown at 37 °C on LB broth or 2 x YT medium in presence of ampicillin. *C. boidinii* TK62 (83) was used as the host for pNOTeI

modification vectors. *C. boidinii* and its transformants were cultivated on a YPD medium, YNB medium or synthetic MI-medium at 28 °C which were previously described (84).

Construction of expression vectors

The recombinant genes expression in *C. boidinii* was performed as follows. *rmpA* and *rmpB* were amplified by PCR from pUH1 and *Me. aminofaciens* 77a chromosomal DNA as the template. Upstream and downstream primers were designed from the sequences and *Not* I recognition site was added. In addition, the peroxisomal targeting signal (PTS-1: -AKL) was added to C-terminal of HPS and PHI elsewhere. The sequences of the primers were as follows: for HPS, N-terminal 5'-ATAAGAATGCGGCCGCT AAAATGGCATTGACACAAATGGCAT-3'; and C-terminal, 5'-ATAAGA ATGCGGCCGCTTACTTAGCCAGGCCAGTGA-3', for PHI, and C-terminal for HPS-PTS-1, 5'-ATAAGAATGCGGCCGCTTATAATTTAGCC TTAGCCAGGCCAGTGATTT-3', for PHI, N-terminal, 5'-ATAAGAATGC GGCCGCTAAAATGAACAAATATCAAGAGCTC-3'; and C-terminal, 5'-ATAAGAATGCGGCCGCTTACTCGAGGTTAGCATGAAT-3', and C-terminal for PHI-PTS-1, 5'-ATAAGAATGCGGCCGCTTATAATTTAGCC TCGAGGTTAGCATGAATCG-3',the PCR products were purified and cloned into pT7blue by the method of TA cloning and sequenced. Resultant plasmids were digested by *Not* I and purified insert cloned into the *Not* I site of pNOTeI, there were named the plasmids pNH1 and pNP1, and pNHP1 and pNPP1 were added PTS-1 to C-terminus, respectively, and then transformed

into *C. boidinii* transformation were performed as described previously (82).

Enzyme assays

The HPS and PHI activity was determined as described in the section 1. The relative activities were calculated for based on specific activities (U/mg) of cell-free extract from methanol-grown *Me. aminofaciens* 77a as defined 100 %. *C. boidinii* were grown on the methanol-medium as described above, and was harvested by centrifugation at 5,000 x g for 10 min at 4 °C. The cells were then disrupted with a Beadbeater (model 3110BX; Biospec Products, Bartlesville, Okla). The cell-free extracts were obtained by centrifugation at 12,000 x g for 10 min at 4 °C.

RESULTS AND DISCUSSION

Expression of HPS and PHI in *C. boidinii*

The expression vectors were constructed (Fig. 1) as describes under Materials and Methods. These insert DNA fragments were driven by the

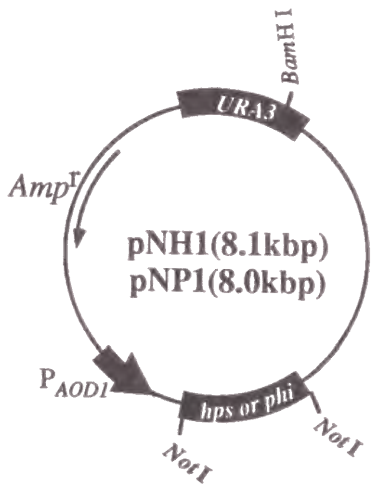

Fig. 1 HPS and PHI expression vector (pNH1, pNP1) in *Candida boidinii*.
These vectors derived from pNOTeI (81) which integrated into the *URA3* locus at a unique restriction site on the *C. boidinii URA3* gene.

AOD1 promoter, and induced by methanol. *C. boidinii* transformants were cultivated on methanol as a sole carbon source. The effective production of HPS and PHI in all of *C. boidinii* transformants were observed (Fig. 2). The HPS and PHI activities of transformants (pNH1 and pNP1) was higher about 1.2-fold and 10-fold than that of *Me. aminofciens* 77a, respectively, and expression product of pNP1 transformants could detected coomassie blliliant blue R-250 staining on SDS-PAGE (Fig. 3). To extend the application, the PTS-1 was added to the C-terminus of both enzymes. The PTS-1 was consensus sequence for

peroxisome targeting and PTS-1-enzymes were accumulated into this organelle. Attention on this phenomenon was focused in the production-viewpoint of useful exogenous proteins in our laboratory, because the expression products were protected to the proteases by peroxisomal membrane,

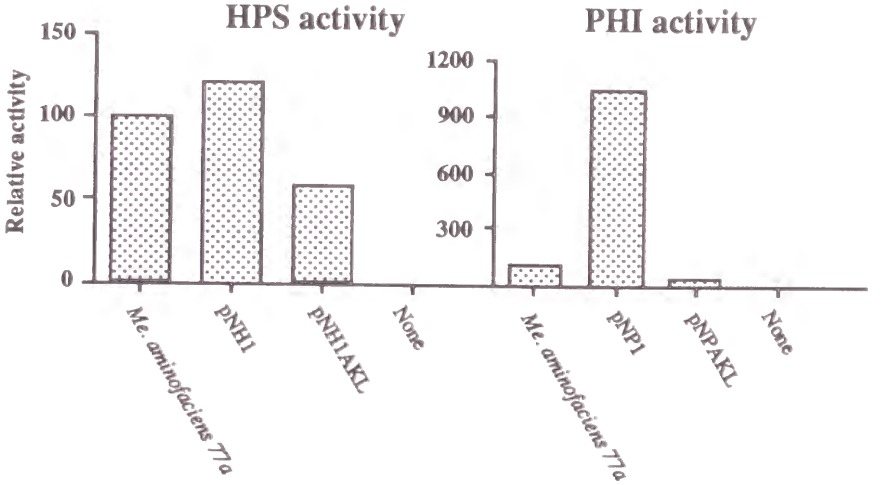

Fig. 2. Relative activities of HPS and PHI of *C. boidinii* transformants with pNH1, pNP1, pNH1AKL and pNP1AKL.
The relative activities were calculated for based on specific activities (U/mg) of cell-free extract from methanol-grown *Me. aminofaciens* 77a as defined 100 %. *C. boidinii* AOU-1 was used as a control, indicated as None. pNH1AKL and pNP1AKL were added the consensus peroxisome targeting signal to C-terminus of HPS and PHI, respectively.

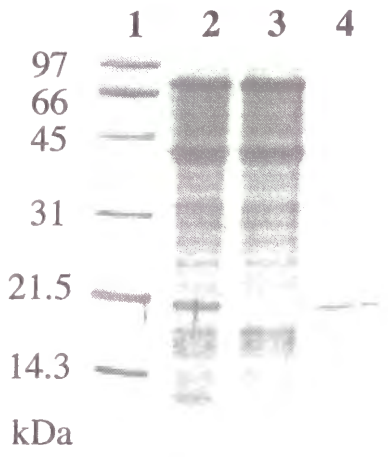

Fig. 3. SDS-PAGE of cell-free extract from *C. boidinii* transformants. Lane 1: molecular mass standard. Lane 2: *C. boidinii* carrying pNP1. Lane 3: *C. boidinii* AOU-1 as control strain. Lane 4: purified PHI from *E. coli* carrying pKH1.

furthermore accumulating proteins fractionated easily, since purification of useful enzymes were easily and getting high yields. However, effective expression of addition PTS-1 to HPS and PHI were not up to the expectation in this cases (Fig. 2). HPS was half of activity when in cytosolic expression, and very little activity of PHI was observed. The peroxisome of *C. boidinii* grown on methanol are occupied by a large amount of enzymes related to methanol metabolism such as alcohol oxidase, dihydroxyacetone synthase and so on, since accumulation space of exogenous proteins were not enough. This problem have been solving, the gene targeting disruption system (86) had constructed and applied the major peroxisomal enzymes in our laboratory. Furthermore, level of expression in transformant was very closely related to the copy number of integration of plasmid as investigated. These subjects are under consideration to confirm more effective expression in *C. boidinii.*

SUMMARY

To establish the system of more easily purification of HPS and PHI, the author used the hosts with *E. coli* with the *tac* promoter system. These transformants expressed dramatically, and HPS and PHI occupied about 80 % and 50 %, respectively of all soluble proteins in each *E. coli* transformants. And the easily purification with one-step chromatography was performed. To extend the application of HPS and PHI, the author attempted expression of these genes in *C. boidinii.* These transformants also expressed well under the *AOD1* promoter. These results indicate to develop an enzymatic method to produce the ^{13}C-labeled sugars, such as glucose or fructose, and are value as a clinical diagnosis.

CONCLUSION

In this thesis, this is the first time, to reveal the organization and regulation of the genes involved in the RuMP pathway in methylotrophic bacteria. The results described in each chapter are summarized as follows:

CHAPTER 1 This chapter described the gene organization and regulation of RuMP gene cluster from an obrigate methylotroph *Me. aminofaciens* 77a. The HPS gene (*rmpA*) was cloned and sequenced the from the basis of the protein information (SECTION 1).

The HPS and PHI activities were detected in *E. coli* carrying pUH1 which contain a 4.4-kb DNA fragment. The DNA sequence for a 4.4-kb DNA fragment contain four genes (*rmp A, B, D* and *I*). Sequencing analysis revealed that the *rmpA, rmpB ,rmpD* and *rmpI* encoded HPS, PHI, TAL and transposase (IS*10*-R), respectively (SECTION 2).

Deletion analysis of the cloned region revealed that *rmpI* and neighboring regions affected on activities of HPS and PHI. The *rmpB* expression was repressed when *rmpI* was deleted, on the other hand, HPS activity (*rmpA*) was ca. 6-fold higher when *rmpI*-region was deleted. It seems that *rmpI* play regulatory function in the RuMP pathway of *Me. aminofaciens* 77a (SECTION 3).

Me. aminofaciens 77a has two homologous gene cluster of the RuMP pathway. To clarify the relationship between the duplicated genes and transposable element, the homologous gene (*hpsII*) was cloned, and

sequencing. These genes showed high similarities at DNA and amino acid levels. The author suggests that the RuMP genes duplicated by the transposable element IS*10*-R present in the RuMP clusters during evolution of the organism (SECTION 4).

CHAPTER 2 This chapter described the gene organization and regulation of RuMP gene cluster from a facultative methylotroph *My. gastri* MB19, and compared to *Me. aminofaciens* 77a. HPS was purified from *My. gastri* MB19, and *rmpA* was cloned from the basis of the protein information (SECTION 1).

Five ORFs were found the cloned fragment. Four of five ORFs are suggested to be of known functions in the RuMP pathway. *rmpA*, *rmpB*, *rmpC* and *rmpR* could code for HPS, PHI, G6PDH and regulatory protein respectively. *Me. aminofaciens* 77a and *My. gastri* MB19 have different organization in the RuMP pathway gene cluster. Both Northern and enzymatic analysis from *My. gastri* MB19 suggested that expression of *rmpA* and *rmpB* are under the same control at the mRNA level, and these genes were regulated as an polycistronic operon. On the other hand, *rmpA* and *rmpB* expression was regulated as monocistronic in *Me. aminofaciens* 77a. The author suggests differences in their regulating mechanism in the RuMP pathway was based on characteristics of their methylotrophies (SECTION 2).

CHAPTER 3 This chapter described the application of enzymes in the RuMP pathway. To establish the system of more easily purification of HPS

and PHI, the author used the hosts with *E. coli* with the *tac* promoter system. These transformants expressed dramatically, and HPS and PHI occupied about 80 % and 50 % of all soluble proteins in *E. coli*, respectively. And the easily purification could performed with one-step chromatography (SECTION 1).

To extend the application of HPS and PHI, the author attempted expression of these genes in *C. boidinii*. These transformants also expressed well under the *AOD1* promoter. These results promote the further development an enzymatic method to produce the ^{13}C-labeled sugars, such as glucose or fructose, and are value as a clinical diagnosis (SECTION 2).

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