# Nucleotide Sequences of Membrane-Bound Hydrogenase Gene in *Alcaligenes hydrogenophilus*

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**The nucleotide sequences of membrane-bound hydrogenase small (*hupS*) and large (*hupL*) subunit genes of hydrogen bacterium *Alcaligenes hydrogenophilus* were determined. The *hupS* and *hupL* genes encoded polypeptides of 363 and 619 amino acids, respectively. The *hupS* was located upstream of *hupL* with 35 bp of intergenic region. The consensus ribosome-binding sequences were identified upstream of the start codons of *hupS* and *hupL*. Amino acid sequence of *hupS* is very similar to that of *Rhodobacter capsulatus*, *Bradyrhizobium japonicum*, and *Azotobacter vinelandii* at amino acid levels of 82%, 77%, and 81%, respectively. Similarly, amino acid sequence of HupL is similar to that of *R. capsulatus*, *B. japonicum*, and *A. vinelandii* at amino acid levels of 63%, 65%, and 68%, respectively. Northern hybridization analysis showed that *hupS* and *hupL* were co-transcribed, and addition of fructose to the culture medium remarkably decreased the amount of mRNA transcribed from *hupS* and *hupL*.**

**Keywords** hydrogenase; hydrogen bacteria; *Alcaligenes hydrogenophilus*; gene; nucleotide sequence

## Introduction

Hydrogen-oxidizing bacteria can grow with $H_2$ as an energy source and $CO_2$ as the only carbon source. The bacteria have attracted industrial attention because they accumulate poly-$\beta$-hydroxybutyrate, which can be used as a raw material of biodegradable plastic.[1] Moreover, transfer of hydrogen-oxidizing and $CO_2$-fixing abilities (Hox and Cfx) into various bacteria enables the development of a $CO_2$-consuming type industrial production system. We have been trying to clone genes encoding Hox and Cfx. *A. hydrogenophilus* is a gram-negative facultative hydrogen bacterium isolated from soil in this laboratory.[2] Autotrophically grown cells have two types of hydrogenases. One is a membrane-bound enzyme which consists of large (HupL) and small (HupS) subunits linked to an energy-producing respiratory chain, and the other is a soluble enzyme reducing $NAD^+$ as a physiological electron acceptor. The genetic information of Hox is encoded by a megaplasmid pHG 21-a.[3] In our previous work a gene bank of pHG 21-a was constructed using a broad host-range cosmid vector pVK102 in *Escherichia coli*.[4] Recombinant cosmids containing *hox* genes were identified by transferring the bank into $CO_2$-fixing *Pseudomonas oxalaticus* OX1 by conjugation. The cosmid pYM11, which had three times higher membrane-bound hydrogenase activity than *A. hydrogenophilus*, was isolated from $Hox^+$ transconjugants. The size of inserted DNA in pYM11 was 29 kb, and the locus of *hupS* and *hupL* structural genes was found by DNA hybridization analysis using oligonucleotide probes corresponding to the conserved sequences of HupS and HupL in *B. japonicum* and *R. capsulatus*.[5]

In this work we determined the complete nucleotide sequences of *hupS* and *hupL*, and examined the effect of an organic compound on the transcription by Northern blot analysis.

## Materials and Methods

**Bacterial Strains and Growth Conditions** Wild-type *A. hydrogenophilus* was cultivated autotrophically in a mineral-salt medium[2] at 30 °C under a gas mixture of 80% $H_2$, 10% $CO_2$, and 10% $O_2$. *A. hydrogenophilus* was also cultivated mixotrophically and heterotrophically in a mineral-salt medium containing 0.2% fructose under the gas mixture (80% $H_2$, 10% $CO_2$, and 10% $O_2$) and air, respectively. *P. oxalaticus* OX1 containing recombinant cosmid was cultivated autotrophically at 30 °C in a mineral-salt medium containing 50 mg/l of kanamycin. *E. coli* JM109 was cultivated in LB medium at 37 °C.

**Isolation of Plasmid, Cosmid and Phage** The recombinant cosmid pYM11 was isolated from autotrophically grown *P. oxalaticus* OX1 by the method of Yano and Nishi[6] and purified by equilibrium centrifugation in cesium chloride. Plasmid pUC18 was used as a cloning vector, and bacteriophages M13 mp 18 and mp 19 were used for cloning and DNA sequencing. Plasmid and phages were isolated by the method of Birnboim and Doly.[7]

**Determination of Nucleotide Sequences** Nucleotide sequences were determined by dideoxy chain termination method[8] using [$\alpha$-$^{35}$S]dCTP. A deletion kit for kilo-sequence (Takara Shuzo Co.) was used in the construction of deleted subclones. Sequencing reactions were performed using a Sequenase kit with 7-deaza-dGTP (Sequenase Version 2.0, United States Biochemical Co.).

**Southern and Northern Hybridization** Southern hybridization was done by the method described previously.[5] Total RNA was extracted for Northern blot analysis from *A. hydrogenophilus* grown autotrophically, mixotrophically, and heterotrophically by hot phenol method. RNA was separated with 1.0% agarose gel containing 2.2 M formaldehyde and transferred to a nylon membrane (Hybond N, Amersham Co.). Synthetic oligonucleotides were labeled at the 5′ end with [$\gamma$-$^{32}$P]ATP by T4 polynucleotide kinase (Takara Shuzo Co.). Hybridization was done in the mixture of 50% formamide, 50 × SSPE (1 × SSPE is 0.18 M NaCl, 10 mM sodium phosphate, and 2 mM sodium EDTA), 5 × Denhardt's solution, and 0.5% sodium dodecyl sulfate (SDS) at 45 °C for 16 h. The nylon membrane was washed with 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 25 °C, then washed with 2 × SSC containing 0.1% SDS at 37 °C for 15 min, 45 °C for 15 min, and 65 °C for 15 min.

## Results

**Nucleotide Sequencing** In our previous work we found that *hupS* and *hupL* were located in an 8.3 kb *Hin*dIII fragment (Fig. 1) derived from pYM11 using two oligonucleotide probes for amino acid sequences in HupS and HupL.[5] The probe for HupS and HupL hybridized to 1.5 kb and 2.8 kb *Sal*I fragments in the 8.3 kb fragment,

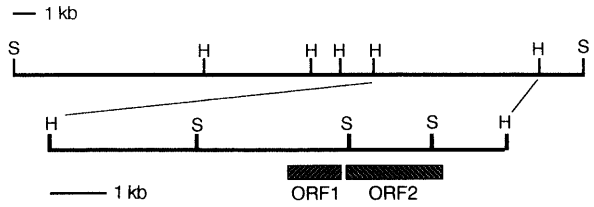


Fig. 1. Restriction Map of Fragment Inserted into pVK102 in pYM11 and the 8.3 kb *Hin*dIII Fragment Containing *hupS* and *hupL*

Restriction sites: H, *Hin*dIII, S; *Sal*I.

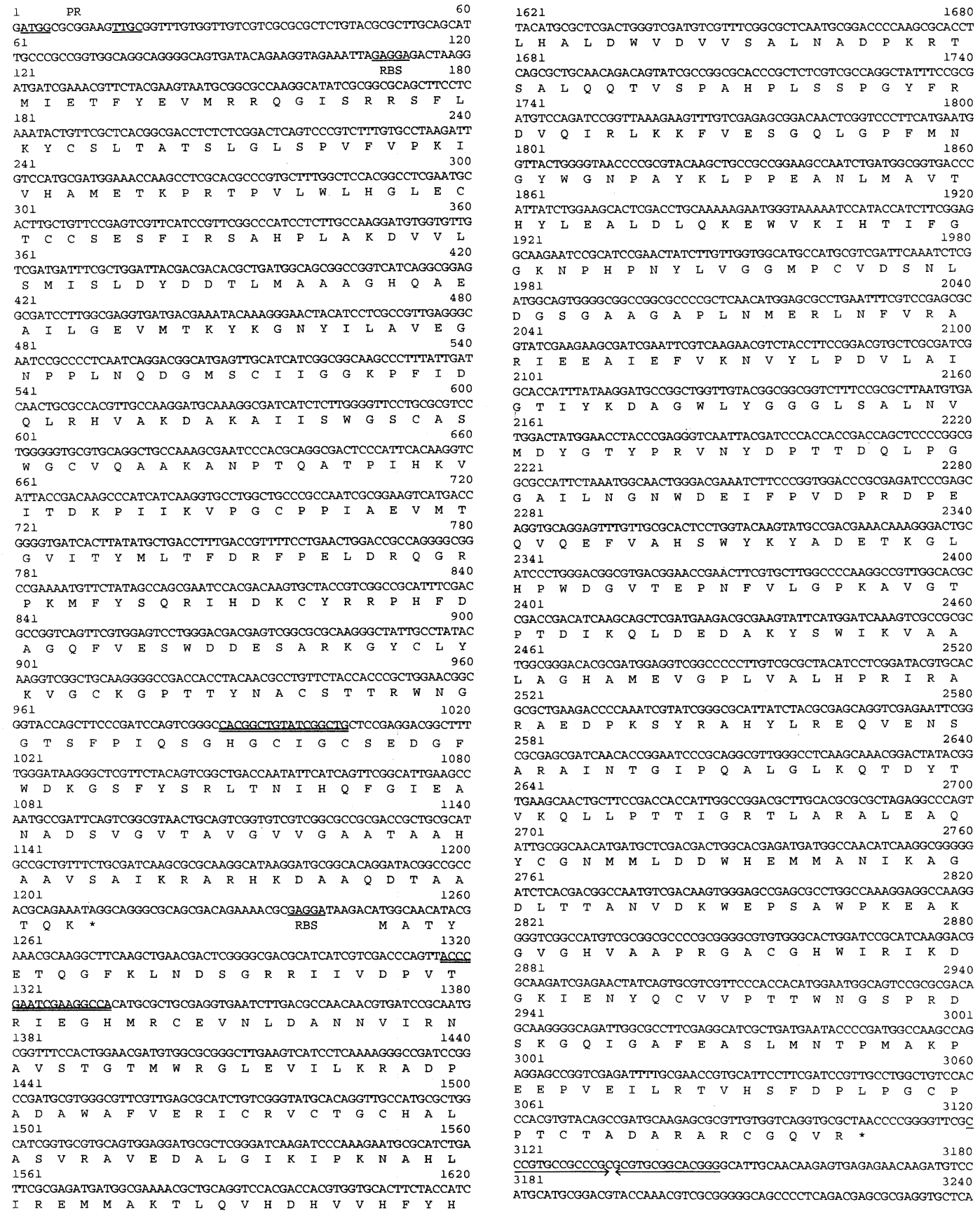


Fig. 2. Nucleotide and Deduced Amino Acid Sequence of *hupS* and *hupL* Genes in *A. hydrogenophilus*

Both genes are preceded by ribosome-binding sites (RBS). Possible *rpoN*-dependent promoter sequence (PR), discussed in the text, is indicated. Inverted repeat, which could function in transcription termination (→←), is also indicated. Sequences which seem to be hybridized with the probes for *HupS* and *HupL* are double-underlined.

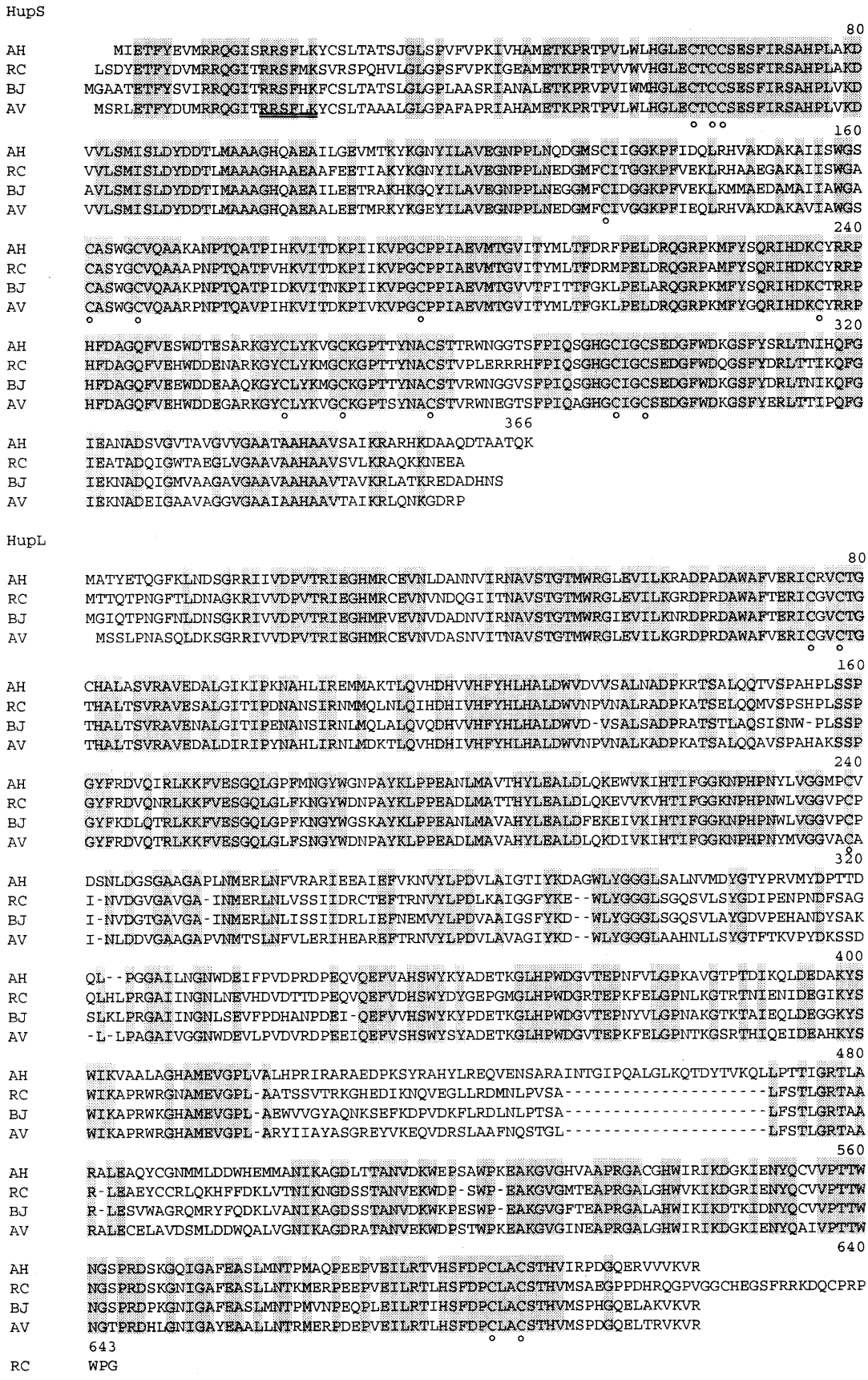


Fig. 3. Comparison of the Deduced Amino Acid Sequences of *hupS* and *hupL* Genes from *A. hydrogenophilus* (AH), *R. capsulatus* (RC), *B. japonicum* (BJ), *A. vinelandii* (AV)

Regions conserved in all 4 sequences are shaded. Conserved cysteine residues are marked. Sequences found in many signal peptides are double-underlined. Amino acids are numbered using amino-terminal amino acid of *B. japonicum* as No. 1.

respectively. Those fragments and a 1.4 kb *Sal*I/*Hin*dIII fragment were subcloned in M13 mp 19 for sequencing. Figure 2 shows the results of DNA sequencing; two large open reading frames, ORF 1 and 2, extended from the 1.5 kb *Sal*I fragment to the 1.4 kb *Sal*I/*Hin*dIII fragment. ORF1 was located upstream of ORF 2 with 35 bp of intergenic region. The consensus ribosome-binding sequences were identified upstream of the start codons of ORF 1 and 2. ORF1 and 2 are capable of encoding polypeptides of 363 and 619 amino acids, respectively. Deduced amino acid sequence of ORF1 is very similar to HupS of *R. capsulatus*, *B. japonicum*, and *A. vinelandii* at amino acid levels of 82%, 77%, and 81%, respectively. Similarly, deduced amino acid sequences of ORF2 is similar to HupL of *R. capsulatus*, *B. japonicum*, and *A. vinelandii* at amino acid levels of 63%, 65%, and 68%, respectively. Based on these amino acid sequence homologies, ORF 1 and 2 were identified as *hupS* and *hupL* in *A. hydrogenophilus*. Calculated sizes of the two subunits were 39.7 and 68.5 kDa. The coding region of *hupS* and *hupL* had an average G+C content of 59.0%. The frequency of the use of G or C in the third position of codons was 72.6%. Thirteen out of 14 cysteine residues in HupS were conserved among *A. hydrogenophilus*, *R. capsulatus*, *B. japonicum*, and *A. vinelandii*. Five out of 10 cysteine residues HupL were conserved among the four bacteria.

**Effect of an Organic Compound on the Transcription** *A. hydrogenophilus* was cultivated autotrophically, mixotrophically, and heterotrophically. From each culture total RNA was extracted and used for the Northern blot analysis. We synthesized a 20-mer oligonucleotide probe complementary to the sequence from nucleotide 131 to 150 (Fig. 2). Figure 4 shows the results of Northern hybridization. Laveled oligonucleotide probe hybridized with RNA extracted from autotrophically grown cells. The size of the band was estimated to be about 3 kb from the bands of 23S and 16S ribosomal RNA. Only a faint band was found in the lane of RNA extracted from mixotrophically grown cells. No signal could be seen in the lanes of heterotrophically grown cells.

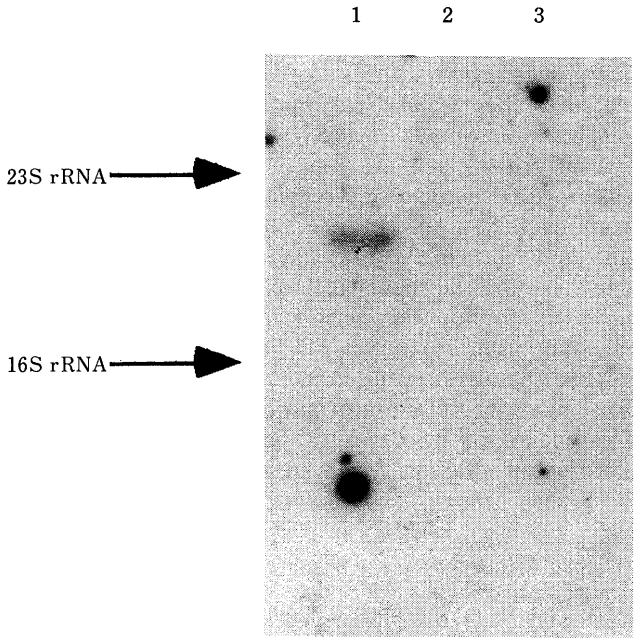


Fig. 4. Northern Blot Analysis of RNA from *A. hydrogenophilus*

Twenty μg of total RNA extracted from autotrophically (lane 1), mixotrophically (lane 2), and heterotrophically (lane 3) grown cells were electrophoresed into 0.8% agarose gel containing 2.2 M formaldehyde. Arrows are directed to the position of 23S and 16S ribosomal RNA markers.

## Discussion

Sequences of genes for small and large subunits of membrane-bound and periplasmic hydrogenases were determined in various bacteria, such as *B. japonicum*,[9] *R. capsulatus*,[10] *A. vinelandii*,[11] *Desulfovivrio vulgaris*,[12] and *E. coli*.[13] There is a signal peptide like sequence including –R–R–X–F–X–K–(where X is a variable amino acid residue) at amino acid 19 (Fig. 3), which is found in the amino-terminal region of small subunits in all other hydrogenases. The region may be important in directing the enzyme to the membrane or periplasm. Two subunits of *A. hydrogenophilus* contained 24 cysteine residues, and 18 residues were conserved in the 4 bacteria listed in Fig. 3. The small subunit has 13 conserved residues, while the large subunit has only 5. However, 4 out of the 5 conserved residues are involved in the motif –C–X–X–C–S/T– which occurs twice in each subunit near the amino and carboxy termini. These conserved residues appear to be involved in the construction of iron-sulfur clusters seen in these types of hydrogenases and ferredoxins.[14]

Coding regions of *A. hydrogenophilus hupS* and *hupL* had an average G +C content of 59%. This is very low value compared to the G+C content of 70% in total DNA of this bacterium. The use of codons with G or C in the third position is only 72.6%. we expected that there would be an extreme bias toward the use of G or C at the third position of the codons in DNA with high G+C content. In the *R. capsulatus* genes the usage is more than 86%.[9] The value of *rbc* genes in *A. eutrophus*, which is closely related to *A. hydrogenophilus*, is more than 90%.[15] The G+C content in total DNA is about 67%. The two genes in *A. hydrogenophilus* sequenced in this study are located in megaplasmid pHG 21-a. A difference in codon usage between plasmid and chromosomal DNA might exist in this bacterium.

Northern blot analysis of RNA extracted from autotrophically grown cells of *A. hydrogenophilus* showed that size of the transcript hybridizing the oligonucleotide probe was about 3 kb. This result indicated that *hupS* and *hupL* were co-transcribed, because the total size of the two genes was about 3 kb. The upstream non-coding region did not contain a possible promoter region homologous to the consensus *E. coli* −35 and −10 elements. But there is a sequence resembling the promoter element found in −24 and −12 regions of *rpoN*-dependent promoters at nucleotides 2 to 16 in Fig. 2. The similar region was found in the upstream of the genes encoding soluble hydrogenase in *A. eutrophus*.[16] Besides hydrogen oxidation, the transcription of genes relating nitrate assimilation, denitrification, and various substrate transport systems were found to be *rpoN*-dependent in *A. eutrophus*.[17] Many organic compounds are known to repress the expression of hydrogenase activity.[18,19] The regulation has heretofore been analyzed in protein or activity levels. We demonstrated here the effect in transcriptional level by Northern hybridization. As shown in Fig. 4, addition of fructose

remarkably decreased the amount of *mRNA* transcribed from *hupS* and *hupL*. In *A. eutrophus*, the repressive effects of organic carbon sources on hydrogenase expression are related to their ability to support rapid growth.[18] There may be some sensor proteins which recognize the energy state in cells. When the supply of energy is limited, the sensor would activate the regulator which binds near the promoter region and, in turn activate the transcription of *hupS* and *hupL* in the presence of *rpoN*-like protein. The gene encoding the regulator HoxA has been isolated in *A. eutrophus*,[20] but the sensor protein mediating signal transduction in *hox* regulation has not yet been identified. Further analysis is required to understand the complicated *hox* regulation, and the information will enable us to transfer Hox phenotype into various bacteria to develop a new microbial production system in an inorganic environment.

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