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Biochemistry

Cloning and expression of *Bradyrhizobium japonicum* uptake hydrogenase structural genes in *Escherichia coli*

(cosmid pHU52/hydrogenase subunits/*Escherichia coli* "maxicells"/high level expression/immunological cross-reaction)

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ABSTRACT **To identify the structural genes for the components of *Bradyrhizobium japonicum* uptake hydrogenase (M_r 60,000 and 30,000), we have expressed these genes in *Escherichia coli* and shown that the products cross-react with antibodies to the respective hydrogenase subunits. We constructed subclones of overlapping DNA fragments from an uptake hydrogenase-complementing cosmid, pHU52 [Lambert, G. R., Cantrell, M. A., Hanus, F. J., Russell, S. A., Haddad, K. R. & Evans, H. J. (1985) *Proc. Natl. Acad. Sci. USA* 82, 3232–3236], in pMZ 545, a plasmid expression vector. DNA fragments inserted into one or more of the four cloning sites downstream from the *E. coli lac* operon promoter (P_{lac}) on pMZ 545 generate transcriptional, but not translational, fusions. Two subclones that directed the synthesis of M_r 60,000 and 30,000 proteins in *E. coli* "maxicells" were identified. The DNA inserts from these subclones were then inserted downstream of the bacteriophage λ P_L promoter on a transcriptional fusion vector. When the P_L promoter was activated *in vivo* by heat inactivation of the temperature sensitive *c*I repressor of λ in an appropriate *E. coli* strain, the respective fragments expressed higher levels of M_r 60,000 and 30,000 proteins that could be detected in immunoblots. These data provide direct evidence for the presence of uptake hydrogenase structural genes on the uptake hydrogenase-complementing cosmid pHU52.**

Hydrogen evolution mediated by the nitrogenase reaction is estimated to consume at least 25% of the energy that otherwise could be used to support N_2 fixation and other processes in *Bradyrhizobium*–legume symbioses (1). Hydrogen uptake (Hup) systems that participate in the oxidation of the evolved H_2 and conservation of some of the energy lost through H_2 evolution are particularly active in the slow-growing rhizobia (2, 3). Evidence supporting the proposed benefits of the H_2 recycling system has been provided by an experiment that showed that total weight and nitrogen content of soybean plants inoculated with a Hup^+ strain of *Bradyrhizobium japonicum* and grown to maturity were significantly greater than those of plants inoculated with an otherwise isogenic Hup^- strain of *B. japonicum* (4). The uptake hydrogenase, which is an essential component of the H_2 recycling system, has been purified from chemolithotrophically grown *B. japonicum* cells. The homogeneous protein is comprised of two subunits of M_r 60,000 and 30,000 (60- and 30-kDa subunits) (5).

A gene bank of *B. japonicum* USDA 122DES DNA was constructed in this laboratory and used to isolate a cosmid, pHU1, which complements the Hup^- point mutant PJ17 (6). Studies on the site-directed Tn*5* insertion mutagenesis of pHU1 indicated that more *hup*-specific DNA remained to be isolated (7). Lambert *et al.* (8) reported the isolation of a cosmid (pHU52) apparently encoding all essential Hup determinants. This cosmid conferred Hup activity and autotrophic growth capability to Hup^- *B. japonicum* strains, *Rhizobium meliloti* and *Rhizobium leguminosarum*. Conjugal transfer of pHU52 into these Hup^- rhizobia also conferred the ability to synthesize both the 60- and 30-kDa polypeptide components of the uptake hydrogenase (9). These results suggest that the determinants for hydrogenase subunit synthesis are present on pHU52. In this paper, we report cloning of the structural genes for the 60- and 30-kDa subunits of the uptake hydrogenase from pHU52, and their expression in *Escherichia coli*.

MATERIALS AND METHODS

General Methodology. Strains of *E. coli* and plasmids used in this study are listed in Table 1. All DNA manipulations *in vitro* were conducted according to Maniatis *et al.* (10). Transformations of the ligation mixtures were routinely carried out in *E. coli* DC 646. After restriction analysis, the appropriate clones were transformed into *E. coli* SS 3228c for "maxicell" experiments and into MZ 1 for high-level expression. *E. coli*, unless otherwise stated, was cultured on L broth (10 g of bacto-tryptone/5 g of bacto-yeast extract/5 g of $NaCl/H_2O$ to 1 liter) or LB agar plates [L-broth containing 1.5% (wt/vol) Noble agar] at 32°C. M9 medium (11) supplemented with glucose or fructose (0.4%)/$CaCl_2$ (0.1 mM)/$MgSO_4 \cdot 7H_2O$ (1 mM)/casamino acids (1%)/thiamine·HCl (2 μg/ml)/tryptophan (25 μg/ml) was used to grow *E. coli* SS 3228c for analysis of the plasmid-encoded proteins (12). Final concentration of ampicillin used in L-broth or LB agar plates was 50 μg/ml.

Analysis of Plasmid-Encoded Proteins. The maxicell procedure described by Sancar *et al.* (12) was followed with the modification of Roberts *et al.* (13) of adding cycloserine after UV irradiation. The *lac* promoter on pMZ 545 (Fig. 1) that was used in the maxicell procedure was activated by replacing glucose in the medium with fructose (14).

Cell Culture for Expression of 60- and 30-kDa Hydrogenase Subunits in *E. coli*. Subclones of the pHU52 insert DNA constructed in pDH 511 or pMZ 666 were transformed into MZ 1 *E. coli* strain for high-level expression of 60- and 30-kDa hydrogenase subunits. L broth (5 ml) containing ampicillin at 50 μg/ml was inoculated with fresh 14-hr cultures of MZ 1 containing the appropriate plasmids, and cultures were grown to midlogarithmic phase at 32°C. One set of cultures was then shifted quickly to a shaking water bath at 42°C, and incubation was continued for 1 hr to inactivate the temperature-sensitive *c*I repressor of bacteriophage λ in MZ 1 and thus activate the λ P_L promoter on the plasmids. The uninduced control cells were allowed to grow at 32°C for an additional hour. Each cell pellet obtained from these cultures was suspended in 150 μl of $NaDodSO_4$-lysis buffer [0.0625 M

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Abbreviation: kb, kilobase pair(s).

Table 1. *E. coli* strains and plasmids

	Relevant characteristics	Source
Strains		
DC 646	C600 λ*c*I$^+$ r$^-$ m$^+$	D. Court
SS 3228c	*lacI* 22, *lacZ*, *pro*-48, *met*-90, *trpA*, *trpR*, *his*-85, *rpsL*, *azi*-9, *gyrA*, *recA* 56, Δ*rlc*, *appD*, λ- (general maxicell strain)	T. Patterson
MZ 1	SA 500 *his ilv* Δ*8 galKam* (λcI 857 Δ*Bam* Δ*HI*)	M.Z. and D. Court
Plasmids		
pMZ 545	Amp^R	This study
pDH 511	Amp^R	D. Court
pMZ 666	Amp^R	This study

Tris·HCl, pH 6.8/2.3% (wt/vol) $NaDodSO_4$/5% (vol/vol) 2-mercaptoethanol/10% (vol/vol) glycerol], boiled for 5 min, and centrifuged in an Eppendorf microfuge at room temperature for 5 min. This supernatant (15 μl) was loaded onto 10% $NaDodSO_4$/polyacrylamide gels.

Expression of the 30-kDa Subunit in *E. coli*. L broth (100 ml) containing ampicillin at 50 μg/liter was inoculated with 2 ml of a fresh 14-hr culture of MZ 1 containing the appropriate plasmid, and the cells were grown at 32°C to an OD_{650} of 0.3–0.5. The culture was immersed in a 65°C water bath for 1 min, shaken vigorously, and then transferred to a 42°C shaking water bath; and the incubation was continued for 2 hr. Each cell pellet obtained from these cultures was resuspended in 1 ml of 60 mM Tris·HCl, pH 7.0/0.1% Triton X-100/0.1 mM phenylmethylsulfonyl fluoride/5 μM antipain, and the lysate was sheared through a hypodermic syringe. After addition of a few drops of toluene, the cell suspension was Vortex mixed vigorously to ensure complete lysis. Toluene was evaporated by shaking the cell suspension at 37°C for 40 min, and then the suspension was stored at −20°C until further use in anti-30-kDa affinity gel chromatography to be described elsewhere.

Purification of the 30-kDa Subunit. Anti-30-kDa rabbit antiserum was bound to Affi-Gel 15 (Bio-Rad) as described (9). A 0.2-ml sample of this Affi-Gel 15 with bound antibody was incubated with 1 ml of whole cell lysate in an Eppendorf tube at room temperature for 3 hr, and then the gel was washed 10 times with Tris/saline buffer (10 mM Tris·HCl, pH 7.4/0.9% NaCl), 3 times with 2 M NaCl, and once with Tris/saline buffer. The gel was suspended in 50 μl of $NaDodSO_4$-lysis buffer, heated for 5 min in a boiling water bath, and centrifuged in a microfuge for 5 min at room temperature. Supernatant (15 μl) was loaded onto 10% $NaDodSO_4$/polyacrylamide gels.

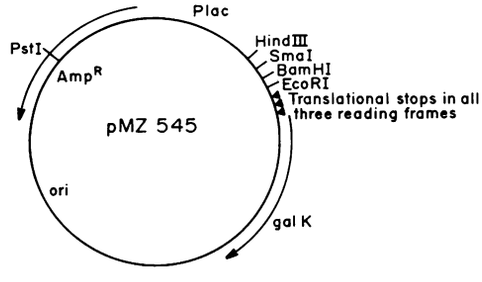

FIG. 1. Plasmid expression vector pMZ 545. Transcription initiated at the P_{lac} yields an untranslated "naked" mRNA of 17 nucleotides up to the first cloning site *Hin*dIII. Downstream from the four cloning sites, there are translational stops in all three reading frames. Therefore, insertion of DNA fragments into one or more of these cloning sites results only in transcriptional fusions.

Electrophoresis and Immunoblots. The conditions for $NaDodSO_4$/polyacrylamide gel electrophoresis with a minislab gel apparatus (Idea Scientific, Corvallis, OR) and electroblotting of the proteins from the gel onto nitrocellulose sheets were described (9). Immunological detection of hydrogenase subunit polypeptides bound to nitrocellulose was performed following the immunoblot ELISA as described by Harker *et al.* (5, 9).

RESULTS

Expression of *B. japonicum* Genes from pHU52 in *E. coli* Maxicells. As an effort to provide direct evidence for the presence of uptake hydrogenase structural genes (60 and 30 kDa) on the Hup-complementing cosmid pHU52, we have adopted the strategy of expressing these genes in *E. coli* and then showing immunological cross-reaction with antibodies to the respective hydrogenase subunits (5, 9). Thus proteins synthesized from the insert DNA of pHU52 in *E. coli* maxicells, were used to identify the clones that direct the synthesis of 60- and 30-kDa polypeptides in maxicells, to maximize their expression in *E. coli*, and then to show immunological cross-reaction. For this strategy, it is essential that subclones of pHU52 constructed in a plasmid expression vector do not generate translational fusions resulting in the expression of large hybrid proteins. A transcriptional fusion vector, pMZ 545 was constructed (Fig. 1) for this purpose.

Fig. 2*A* shows subclones constructed from the *B. japonicum* insert DNA of pHU52 in pMZ 545. The plasmid-encoded proteins from these subclones are shown in Fig. 2*B*. Proteins encoded by pMZ 545 include the 42-kDa galactokinase protein and a 29-kDa doublet (β-lactamase) conferring ampicillin resistance. Both pMZ 550 and pMZ 555 [the 5.9-kilobase (kb) *Hin*dIII subclones] synthesized a 60-kDa protein (Fig. 2*B*) in maxicells, indicating the presence of sequences functioning as transcription initiation signals on the DNA insert. Another protein of about 65 kDa (Fig. 2*B*) was expressed only in pMZ 575 and pMZ 590, but not in pMZ 574 (Fig. 2*A*). This clearly suggests that the structural gene encoding the 65-kDa protein is present on a 2.7-kb *Sma* I–*Bgl* II fragment and that the gene is transcribed from right to left (Fig. 2*A*). pMZ 600 (Fig. 2*A*) directed the synthesis of a 30-kDa protein (Fig. 2*C*) in maxicells. Absence of this protein in maxicells containing pMZ 603 (Fig. 2*C*) confirms that the 2.9-kb *Eco*RI fragment contains a deletion of the promoter-like sequences controlling the expression of 30-kDa protein. Also it is obvious that the structural gene for this 30-kDa protein is transcribed from left to right on the 2.9-kb *Eco*RI DNA insert in pMZ 600 (Fig. 2*A*).

Thus *B. japonicum* proteins synthesized from the *hup* locus on pHU52 are expressed in *E. coli* maxicells. However, one should not necessarily presume that these proteins are full-length polypeptides, because proteins from truncated genes terminate randomly at one of the translational stops distal to the four cloning sites on the plasmid vector pMZ 545 and yield truncated polypeptides in *E. coli*. Evidence of efficient transcription of the *B. japonicum* genes under the control of their own promoter-like sequences in *E. coli* is convincing. Since pMZ 545 is a transcriptional fusion vector, structural genes located on the DNA inserts can be expressed in *E. coli* only when their mRNAs are being actively translated. This clearly indicates that the *B. japonicum hup* locus has DNA sequences that are being efficiently recognized by *E. coli* ribosomes leading to effective translation initiation.

High-Level Expression of the 60-kDa Hydrogenase Subunit in *E. coli*. To show immunological cross-reaction of the 60-kDa protein with anti-60-kDa antiserum (5), we decided to maximize its expression in *E. coli* and to monitor its expression by use of immunoblots (9). The 5.9-kb *Hin*dIII fragment that directed the synthesis of the 60-kDa protein in *E. coli*

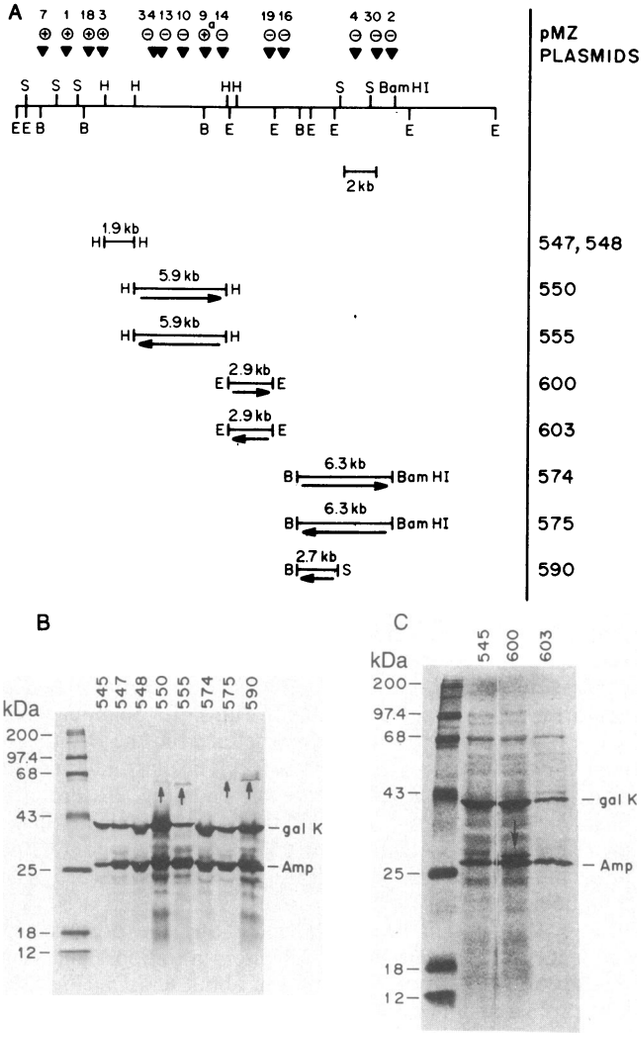

FIG. 2. (*A*) Restriction map of pHU52 and subclones constructed from DNA fragments of the *hup* locus on this cosmid. Triangles, positions of Tn*5* insertions. Insertions are numbered, and + or − below each number indicates whether that insertion resulted in a Hup$^+$ or Hup$^-$ phenotype in a complementation test. (See ref. 7.) B, *Bgl* II; E, *Eco*RI; S, *Sma* I; H, *Hin*dIII. (*B*) Expression of plasmid-encoded proteins in maxicells. Proteins labeled by ^{35}S-methionine in maxicells were analyzed by electrophoresis in vertical 10% acrylamide gels. The subclones are identified by their plasmid numbers, as shown in *A*. Arrows point to proteins expressed from cloned DNA fragments. (*C*) Plasmid-encoded proteins from pMZ 545, pMZ 600, and pMZ 603.

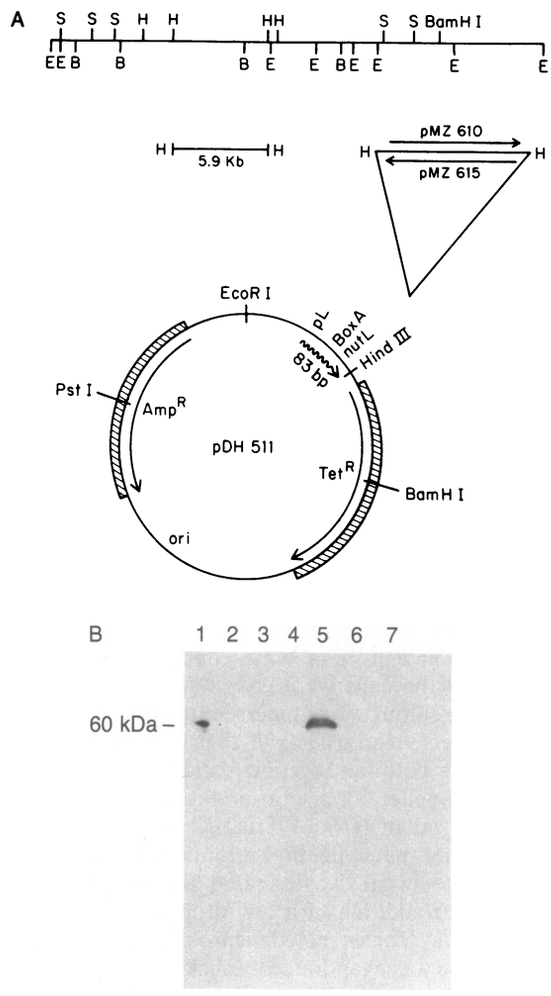

FIG. 3. (*A*) Subclones of the 5.9-kb *Hin*dIII fragment constructed in both orientations relative to the initiation of transcription from the P_L promoter of λ phage on pDH 511. B, *Bgl* II; E, *Eco*RI; S, *Sma* I; H, *Hin*dIII. (*B*) Specific detection in immunoblots of the 60-kDa hydrogenase subunit in MZ 1 cells containing 5.9-kb *Hin*dIII subclones in pDH 511. Affinity-purified anti-60-kDa antibody was used as a probe. Lanes: 1, purified hydrogenase (80 ng); 2 and 3, pDH 511; 4 and 5, pMZ 610; 6 and 7, pMZ 615. Lanes 2, 4, and 6 were loaded with crude extracts prepared from MZ 1 cells grown at 32°C. Lanes 3, 5, and 7 were loaded with crude extracts prepared from heat-induced cultures.

maxicells was cloned in both orientations relative to the bacteriophage λ P_L promoter in pDH 511 (Fig. 3*A*). The P_L promoter in pDH 511 is known to transcribe an 83-nucleotide untranslated "naked" mRNA ending at the *Hin*dIII cloning site. As a result, DNA inserts at this *Hin*dIII site yield only transcriptional fusions and do not express hybrid proteins. The *box*A and *nut*L sites located distal to the P_L promoter on pDH 511 are the cis-acting elements that are required in conjunction with λN protein (supplied in *trans* in MZ 1 at 42°C) to continue transcription at termination signals, if present, on the DNA inserts (15).

MZ 1 cells containing the plasmid pMZ 610 (Fig. 3*A*) when heat induced to activate the P_L promoter, expressed detectable quantities of the 60-kDa protein (compare Fig. 3*B*, lanes 1 and 5). This protein was absent in the crude extracts prepared from the cultures maintained at 32°C to repress the P_L promoter in pMZ 610 (Fig. 3*B*, lane 4). Although no efforts were made to quantify the expression of 60-kDa protein relative to the total cellular protein, crude extracts prepared from 0.5 ml of the heat-induced MZ 1 cells containing pMZ 610 contained greater amounts of this protein than the 80-ng sample of purified hydrogenase (compare Fig. 3*B*, lanes 5 and 1). Neither the vector (pDH 511) nor the plasmid pMZ 615 (where the 5.9-kb *Hin*dIII fragment was cloned in opposite orientation relative to the transcription initiation at the P_L promoter) showed the expression of 60-kDa protein in MZ 1 cells regardless of the repression or induction conditions (Fig. 3*B*, lanes 2, 3, 6, and 7). From these results we conclude that the structural gene for the 60-kDa protein is present on the 5.9-kb *Hin*dIII fragment of pHU52 (Fig. 3*A*) and is transcribed from left to right on the DNA insert in pMZ 610 (Fig. 3*B*).

Maximizing the Expression of 30-kDa Hydrogenase Subunit in *E. coli*. An experiment analogous to that described for detecting the structural gene for the 60-kDa subunit of hydrogenase was carried out to show expression of the 30-kDa hydrogenase subunit in *E. coli*. These experiments with subclones pMZ 672 and pMZ 670 (Fig. 4*A*) were negative. Failure to detect the expression of 30-kDa protein

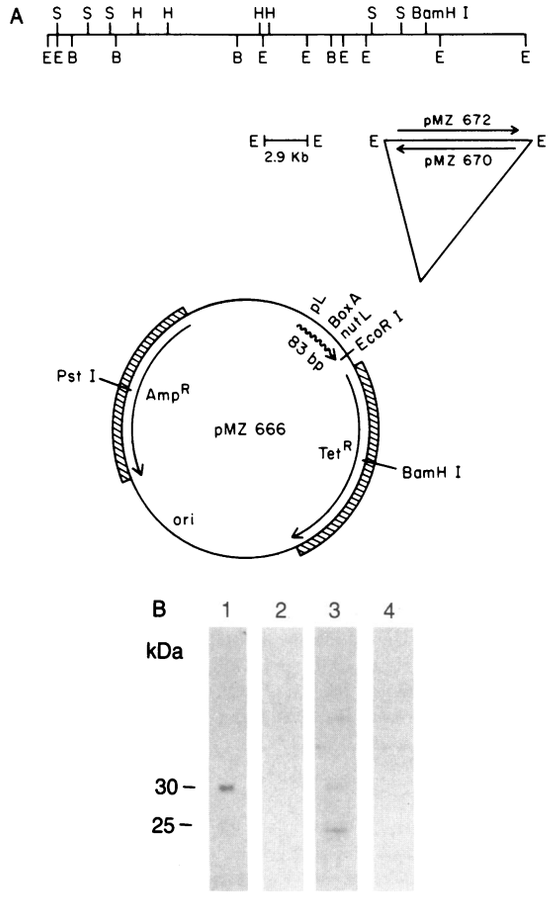

FIG. 4. (*A*) Subclones of the 2.9-kb *Eco*RI fragment constructed in both orientations relative to the initiation of transcription from the P_L promoter of λ phage on pMZ 666. (pDH 511 was cleaved with *Eco*RI, mung bean nuclease treated, and blunt-end ligated. This derivative of pDH 511 was then cleaved with *Hin*dIII, mung bean nuclease treated, and ligated with *Eco*RI linkers to generate pMZ 666.) B, *Bgl* II; E, *Eco*RI; S, *Sma* I; H, *Hin*dIII. (*B*) Specific detection in immunoblots of the 30-kDa hydrogenase subunit in MZ 1 cells containing 2.9-kb *Eco*RI subclones in pMZ 666. Affinity-purified anti-30-kDa antibody was used as a probe. Lanes: 1, purified hydrogenase (80 ng); 2, pMZ 666; 3, pMZ 672; 4, pMZ 670. Samples loaded onto these lanes were from anti-30-kDa Affi-Gel-purified crude extracts prepared from heat-induced cultures of MZ 1 cells containing the plasmids.

in *E. coli* is possibly due to the inefficient translation of the structural gene despite its transcription from the strong P_L promoter, the sensitivity of the immunoblot procedure (5, 9), or the susceptibility of 30-kDa polypeptide to degradation by proteases (5).

Assuming that the failure to detect 30-kDa subunit expression in *E. coli* was due to a low level of protein synthesis, MZ 1 cells containing the plasmids pMZ 666, pMZ 672, and pMZ 670 (Fig. 4*A*) were induced in 100-ml cultures. Crude extracts were prepared from these cell pellets in nondenaturing lysis buffer, which also contained 0.1% Triton X-100 to solubilize the membrane-bound proteins. Antigenic proteins from these whole cell lysates were affinity purified, and the antigenic proteins that cross-reacted with anti-30-kDa antiserum were analyzed on an immunoblot. The very faint anti-30-kDa cross-reacting protein of about 45 kDa in pMZ 666, pMZ 672, and pMZ 670 (Fig. 4*B*) likely originates from *E. coli*. Only MZ 1 cells containing pMZ 672 expressed detectable levels of the 30-kDa protein and of a strongly cross-reacting truncated polypeptide of about 25 kDa (Fig. 4*B*). These results suggest that the 30-kDa subunit is perhaps selectively degraded in *E. coli* and that protease sensitivity could not be overcome by the addition of phenylmethylsulfonyl fluoride and antipain. No detectable 30-kDa protein or the truncated 25-kDa polypeptide was found (Fig. 4*A*, lanes 2 and 4), in lanes loaded with antigenic proteins from MZ 1 (pMZ 666) and MZ 1 (pMZ 670) that cross-reacted with anti-30-kDa antibody in affinity gels. We conclude from these results that the structural gene for the 30-kDa hydrogenase subunit is present on the 2.9-kb *Eco*RI fragment of pHU52 (Fig. 4*A*) and is transcribed from left to right.

DISCUSSION

We have shown the expression of *B. japonicum* proteins from the *hup* locus of pHU52 in *E. coli* maxicells and have presented evidence that the expression of these *B. japonicum* genes in *E. coli* is under the control of their own sequences that are recognized by *E. coli* RNA polymerase and ribosomes.

The relative location of the structural genes encoding the hydrogenase 60- and 30-kDa subunits has been defined in our maxicell experiments. However, the nature and significance of a 65-kDa protein synthesized in *E. coli* maxicells (Fig. 2 *A* and *B*) is not known.

A 5.9-kb *Hin*dIII fragment and an adjacent 2.9-kb *Eco*RI fragment (located distal to the 5.9-kb *Hin*dIII DNA fragment, Fig. 2*A*) directed the synthesis of 60- and 30-kDa proteins, respectively, in *E. coli* maxicells (Figs. 2 *B* and *C*). High-level expression of 60- and 30-kDa hydrogenase subunits in *E. coli* was demonstrated when the 5.9-kb *Hin*dIII and 2.9-kb *Eco*RI fragments, respectively, were placed under the transcriptional control of a strong λ P_L promoter (Figs. 3 *A* and *B* and 4 *A* and *B*). In addition, it is evident that 60- and 30-kDa structural genes were transcribed from left to right on pHU52 (Figs. 3*A* and 4*A*). These results clearly provide direct evidence for the presence of hydrogenase structural genes on the *B. japonicum* insert DNA of the Hup-complementing cosmid pHU52.

The observed degradation of the 30-kDa protein in *E. coli* possibly mediated by proteases yielding a strongly cross-reacting truncated polypeptide of about 25 kDa in immunoblots (Fig. 4*B*) is not surprising because Harker *et al.* (5, 9) from this laboratory reported the protease susceptibility of the 30-kDa subunit. The protease sensitivity of the 30-kDa subunit appears to have been the main reason why the earlier investigators (16, 17) identified only one subunit of the *B. japonicum* hydrogenase.

Strains of *E. coli* take up hydrogen when grown under aerobic or anaerobic conditions. Two polypeptides of 60 and 30 kDa that cross-react with antibodies to the 60- and 30-kDa *B. japonicum* hydrogenase subunits, respectively, were reported to be present in *E. coli* in amounts proportional to measurable Hup activity (18). However, the MZ 1 *E. coli* strain (Table 1) used in our experiments for high-level expression of *B. japonicum* hydrogenase subunits did not have immunodetectable 60- and 30-kDa polypeptide components.

While the hydrogenase activity was found only in *B. japonicum* cells grown under defined conditions in the free-living state (19, 20) or in nodule bacteroids during symbiosis (16), the structural genes for the hydrogenase subunits expressed immunologically cross-reactive proteins in *E. coli* when they were transcribed from the λ P_L promoter. Therefore, it will be of interest to determine whether coordinate expression of 60- and 30-kDa subunits under the λ P_L promoter control in *E. coli* will result in the reconstitution of uptake hydrogenase activity. In addition, it should now be possible to monitor the levels of *hup*-specific mRNA in bacteroids or in the heterotrophically and chemoautotrophically grown rhizobial cells using the 60- and 30-kDa structural genes as specific DNA probes.

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