JOURNAL OF BACTERIOLOGY, May 1993, p. 3195–3203
0021-9193/93/103195-09$02.00/0

Vol. 175, No. 10

Component A2 of Methylcoenzyme M Reductase System from *Methanobacterium thermoautotrophicum* ΔH: Nucleotide Sequence and Functional Expression by *Escherichia coli*

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Received 9 November 1992/Accepted 3 March 1993

The gene for component A2 of the methylcoenzyme M reductase system from *Methanobacterium thermoautotrophicum* ΔH was cloned, and its nucleotide sequence was determined. The gene for A2, designated *atwA*, encodes an acidic protein of 59,335 Da. Amino acid sequence analysis revealed partial homology of A2 to a number of eucaryotic and bacterial proteins in the ATP-binding cassette (ABC) family of transport systems. Component A2 possesses two ATP-binding domains. A 2.2-kb *Xma*I-*Bam*HI fragment containing *atwA* and the surrounding open reading frames was cloned into pGEM-7Zf(+). A cell extract from this strain replaced purified A2 from *M. thermoautotrophicum* ΔH in an in vitro methylreductase assay.

The final step in the production of methane by methanogenic archaea is the reductive demethylation of 2-(methylthio)ethanesulfonate (CH_3-S-coenzyme M [CH_3-S-CoM]) (59). This reaction is catalyzed by a methylreductase (MR); two isoenzymes (MRI and MRII) have been detected, and recently it has been demonstrated that the expression of these enzymes varies with cell growth conditions (6, 7, 50). The formation of CH_4 by active MR requires *N*-7-(mercaptoheptanoyl)-L-threonine-O^3-phosphate (HS-HTP) as the reductant and produces CoM-S-S-HTP, the disulfide of 2-mercaptoethanesulfonate (HS-CoM) and HS-HTP (5, 15):

$$CH_3\text{-S-CoM} + \text{HS-HTP} \xrightarrow{\text{MR}} CH_4 + \text{CoM-S-S-HTP} \quad (1)$$

MRI (and presumably MRII) binds two mol of coenzyme F_{430} (14), an Ni-tetrahydrocorphin, per mol of enzyme (18, 38, 47, 48). There is considerable evidence that F_{430} is involved in catalysis and that active enzyme contains the nickel(I) species (4, 33, 45, 49).

The MR enzymes purified from *Methanobacterium thermoautotrophicum* Marburg catalyze reaction 1 with no additional protein components or ATP, although titanium(III) citrate [Ti(III)C] and dithiothreitol (in both the presence and absence of aquocobalamin) have been shown to be stimulatory (16). The reaction carried out by *Methanosarcina thermophila* can also proceed in the absence of additional protein components. However, Jablonski and Ferry (32) have demonstrated that the in vitro MR reaction catalyzed by *M. thermophila* requires a second source of reducing equivalents, such as Ti(III)C or reduced ferredoxin, and ATP is stimulatory.

In contrast to the reactions in the Marburg strain and *M. thermophila*, the reaction carried out by *M. thermoautotrophicum* ΔH has historically been found to require a minimum of two protein components in addition to the MR, as well as ATP and a second reductant (51, 55):

$$CH_3\text{-S-CoM} + \text{HS-HTP} \xrightarrow[\text{Ti(III)C, ATP}]{\text{MR, A2, A3a}} CH_4 + \text{CoM-S-S-HTP} \quad (2)$$

It has been proposed that the MR from *M. thermoautotrophicum* ΔH is purified in an inactive form and that it is activated by reduction of the nickel of F_{430} to Ni(I) (53). According to this model, components A2 and A3a would be involved in electron transfer from Ti(III)C to F_{430}. Component A3a is a brown, oxygen-sensitive protein of apparent M_r 500,000 which is inhibited by bathophenanthrolinedisulfonate, a chelator of Fe(II) (55). Thus, A3a is thought to be an Fe:S protein which accepts electrons from Ti(III)C. Component A2 is a colorless protein of M_r 59,000 which binds to ATP-agarose affinity resin (52). It has been postulated that A2 might supply ATP to A3a and that the energy of ATP hydrolysis could be used to induce a conformational change, thereby bridging the potential gap between the Fe:S centers of A3a and the Ni(II)-Ni(I) couple of F_{430}. Component A3a is inhibited by ATP analogs (54) and is a likely site of ATP hydrolysis.

Although component A2 and the MR have been purified to homogeneity, component A3a remains a crude fraction. We have therefore been unable to define roles for the A components in reaction 2. For the study described here, *atwA* was cloned into *Escherichia coli* in an effort to obtain information about the function of A2 from the nucleotide sequence. We demonstrate that functional A2 can be purified from *E. coli* and that A2 has extensive homology to the ATP-binding domains of the ATP-binding cassette (ABC) superfamily of transport systems (26, 31).

Purification of component A2. *M. thermoautotrophicum* ΔH was cultured in a 200-liter fermentor as described previously (37). Cells were suspended in buffer I (20 mM potassium phosphate [pH 7.0], 10 mM β-mercaptoethanol) plus 0.5 mM CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate), and cell extracts were prepared anaerobically as described before (44).

Component A2 was purified anaerobically to homogeneity by a modification of previously published protocols (52, 55). Cell extract (1,000 mg [total] of protein) was loaded onto 300

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TABLE 1. Bacterial strains, phage, and plasmids

Strain, phage, or plasmid	Description	Reference or source
M. thermoautotrophicum ΔH	Wild type	64
E. coli		
XL1-Blue	*recA1 supE44 hsdR17 endA1 gyrA46 relA1 thi lac* F' [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10* (Tet^r)]	New England Biolabs
JM109	*recA1 supE44 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F' [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	Promega
Phage M13K07		Pharmacia
Plasmids/phagemids		
pTZ18R	Amp^r *lacZ'* polylinker (pUC18/19)	Pharmacia
pGEM-7Zf(+)	Amp^r *lacZ'* polylinker (Promega)	Promega
pBDL1	861-bp *Pst*I fragment of ΔH in pTZ18R	This work
pBDL2	861-bp *Pst*I fragment of ΔH in pTZ18R, inverted	This work
pBDL3	2.2-kb *Xma*I-*Bam*HI fragment of ΔH in pGEM-7Zf(+)	This work

ml of DEAE-Sepharose which had been equilibrated in buffer I supplemented with 50 mM potassium acetate (KAc) and 0.5 mM CHAPS. The column was washed with 500 ml of equilibration buffer, and the brown fractions which contained A2 were eluted with buffer I plus 1.5 M KAc and 0.5 mM CHAPS. These fractions were desalted with buffer I plus 0.1 M KAc by ultrafiltration (PM30 membrane; Amicon Corp., Danvers, Mass.). Fractions from DEAE-Sepharose columns (1,300 to 1,500 mg of protein) were then loaded onto 1,100 ml of Sephacryl S300 HR equilibrated in buffer I plus 0.1 M KAc. Protein was eluted with buffer I plus 0.1 M KAc, and pool S3 (as described by Rouviere and Wolfe [55]) was collected and concentrated.

Component C (the MR enzyme) was then removed from pool S3 by chromatography on phenyl-Sepharose CL-4B. Approximately 350 mg of protein was loaded onto 55 ml of phenyl-Sepharose which had been equilibrated in buffer I plus 1.0 M KAc. Component C was removed by washing the column with 3 volumes of buffer I plus 1.0 M KAc and then with 3 volumes of buffer I plus 0.8 M KAc. The remaining protein was eluted with 4 volumes of buffer I.

Component A2 (100 mg [total] of protein) was further purified by affinity chromatography with ATP-agarose (Sigma) as described before (52). The ATP-binding fraction was concentrated in 10 mM potassium phosphate, pH 7.0, and applied to 18 ml of hydroxylapatite Bio-Gel HTP (Bio-Rad Laboratories, Richmond, Calif.) which had been equilibrated in 10 mM potassium phosphate. The hydroxylapatite was washed with 1 volume of 10 mM potassium phosphate and eluted with a linear gradient of 10 to 200 mM potassium phosphate (pH 7.0). Fractions containing A2 activity (as determined by the CH_4 assay [55]) were further purified by fast protein liquid chromatography on a MonoQ HR 5/5 column as described previously (51). Component A2 eluted in 0.9 M KAc in buffer I. Component A2 was purified to apparent homogeneity by anaerobic preparative polyacrylamide gel electrophoresis (PAGE) as described previously (55). Component A2 was identified by assaying slices from a 10% polyacrylamide gel for the ability to complement protein components A3a and C in a CH_4 assay.

Synthesis of the A2 probe. Component A2 from the preparative gel was separated from any contaminants by sodium dodecyl sulfate (SDS)–10% PAGE. Protein from the SDS gel was transferred to an Immobilon P membrane (Millipore Corp., Bedford, Mass.) according to the manufacturer's instructions. The band corresponding to A2 was excised from the membrane, and the amino-terminal sequence was determined by using a gas-liquid phase protein sequenator from Applied Biosystems at the University of Illinois Genetic Engineering Facility. An oligonucleotide (5'-AAGCTI GAGA ATGTIACIAA GAAGTTTAAG GGIGTIGAGG T-3') based on residues 4 through 17 of the amino-terminal sequence was synthesized with an Applied Biosystems model 380A DNA synthesizer at the Genetic Engineering Facility. Wobble positions were chosen according to codon frequency usage for other genes from *M. thermoautotrophicum* (8, 13) and by using inosine to lower base-pairing stringency (40). The oligonucleotide was purified by reverse-phase high-performance liquid chromatography with a μBondapak C18 column (3.9 mm by 15 cm; Waters, Milford, Mass.). The column was equilibrated in 0.1 M triethylamine, pH 7.0, and probe was eluted with a linear gradient of 5 to 20% acetonitrile.

Synthetic probe (20 pmol) was labeled with 60 pmol of [γ-^{32}P]ATP (5,000 Ci/mmol) by using bacteriophage T4 polynucleotide kinase by the method of Sambrook et al. (56). Labeled probe was purified by chromatography on Sephadex G-50 (NICK column).

Preparation of genomic DNA. *M. themoautotrophicum* cells were lysed, and DNA was isolated in a fourfold scale-up modification of the procedure of Meile et al. (42). Nucleic acids were precipitated with 2-propanol and resuspended in 2 ml of 10 mM Tris-HCl (pH 8.0) plus 1 mM EDTA. A contaminating yellow color, presumably a cofactor(s), was removed by passing the sample through a Chromaspin-1000 size exclusion column (Clontech Corp., Palo Alto, Calif.) per the manufacturer's instructions.

Cloning of *atwA*. A Southern blot containing various restriction digests of *M. thermoautotrophicum* ΔH genomic DNA was hybridized to a ^{32}P-labeled probe designed from the amino terminus of the A2 protein. An intense signal was observed only with an 861-bp *Pst*I fragment, which was subsequently ligated into pTZ18R. *E. coli* XL1-Blue (Table 1) was transformed, and clones which contained the *Pst*I fragment in opposite orientations relative to the vector (pBDL1 and pBDL2) were identified (Fig. 1). Both pBDL1 and pBDL2 contained DNA sequences corresponding to that predicted for the amino terminus of the purified protein.

A digoxigenin-labeled probe synthesized from a 288-bp *Eco*RV-*Pst*I fragment of pBDL1 was used to identify a 2.2-kb *Xma*I-*Bam*HI fragment (Fig. 1) which, based on its molecular mass of 52 to 59 kDa (52), was large enough to encode the A2 protein. The *Xma*I-*Bam*HI fragment was unidirectionally ligated into pGEM-7Zf(+), and the recombinant phagemid (pBDL3) was transformed into *E. coli* JM109 (Table 1).

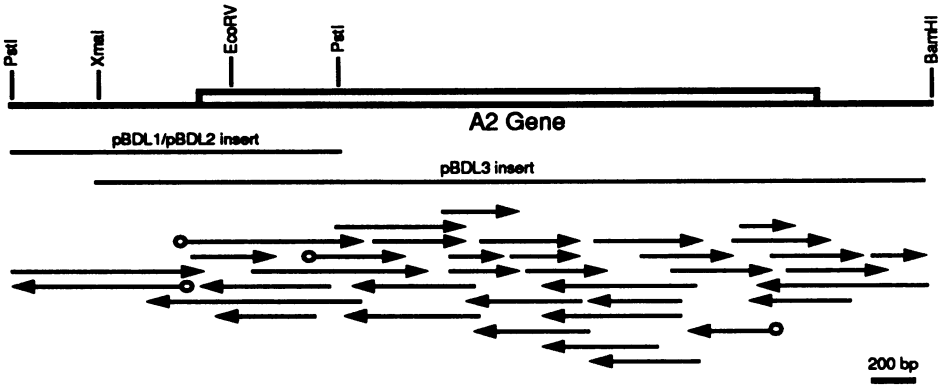

FIG. 1. Physical map of the 2.4-kb fragment containing the A2 gene. The arrows depict the sequencing strategy for the cloned DNA. Open circles denote the use of synthetic primers.

Sequence of *atwA*. The nucleotide sequence of *atwA* and its flanking regions, shown in Fig. 2, was determined by using nested deletions created from pBDL3. The open reading frame (ORF) corresponding to *atwA* consists of 1,590 nucleotides and encodes a peptide of 530 amino acids. The region upstream of the ATG start site contains sequences starting at −79 (TTTAATAG) and −43 (ATGT) which resemble the consensus box A (TATA box) and box B sequences, respectively, of methanogenic promoters (8, 24). Potential transcription termination sites (8) are located immediately downstream of *atwA*. A putative ribosome-binding site (GAGG GTG) begins at −12. The nucleotide sequence of *atwA* has been given GenBank accession number L11748.

The primary structure of the encoded A2 polypeptide was derived from the nucleic acid sequence. The predicted molecular mass of A2 is 59,335 Da, which correlates well with the molecular mass (59 kDa) calculated from SDS-PAGE analyses (52). The isoelectric point, 4.88, is consistent with chromatographic evidence that it is an acidic protein (44). Generally, codon usage frequency corresponds to that observed previously (8, 13, 34); the sole exception is the predominant use of CCC rather than CCA or CCU for proline (data not shown).

The A2 peptide contains seven cysteine residues, five of which are clustered in the amino-terminal portion of the peptide (Fig. 2). These residues show no similarity to consensus patterns for 2Fe:2S or 4Fe:4S centers (3). Rouviere et al. observed that homogeneous component A2 is purified from *M. thermoautotrophicum* as a colorless protein which does not catalyze the reduction of flavin adenine dinucleotide, NAD, NADP, coenzyme F_{420}, or methylviologen (52). Thus, it appears unlikely that A2 functions as an electron carrier in the MR system.

In an effort to account for the observation that A2 binds to ATP-agarose affinity resin, models proposing a function for A2 have included an A2-ATP complex. Amino acid sequence analysis reveals partial homology of A2 to a number of bacterial and eucaryotic proteins (1, 9, 46), all of which bind ATP and have roughly 30% sequence identity over a 200-amino-acid cassette (26, 29, 31). Most of the members of the ABC family of proteins are involved in energy-dependent uptake or secretion processes. In active transport systems, ABC proteins belong to a four-domain, membrane-associated complex (for a review, see references 26 and 29). Two of these domains are highly hydrophobic, integral membrane proteins, usually consisting of six transmembrane α-helices per domain. The other two ABC domains are located on the inner face of the cytoplasmic membrane. It is thought that ATP hydrolysis by the ABC domains induces conformational changes, resulting in pore formation and the movement of substrate across the membrane.

The conserved region common to ABC proteins includes two short sites (Walker motifs) which are thought to form part of the nucleotide-binding fold (28, 61). The sequences used to identify members of this family are indicated in Fig. 2 as ATP A (i.e., Walker site A) and ATP-binding transport (which precedes Walker site B). Component A2 possesses two ABCs, one in the amino-terminal portion of the protein and a second in the carboxyl-terminal portion.

The tandem ABCs of A2 have been aligned with other proteins that have primary sequence homology (Fig. 3). As expected, the regions of similarity are centered around the conserved motifs. The ATP A site of ATP-binding proteins is composed of a flexible glycine-rich loop (GXXXGXG) which lies between a hydrophobic β-strand and an α-helix (20). It has been suggested that the glycine loop binds the phosphoryl group of ATP (57) and that the conserved lysine residue is involved in ATP hydrolysis (60). The highly conserved ATP-binding transport site precedes a region of four hydrophobic amino acids (LFLA [N-terminal] and IIIL [C-terminal]) followed by an aspartate residue; the hydrophobic residues are thought to exclude water from the nucleotide-binding pocket, while the aspartate residue binds Mg-ATP (20, 27, 61).

Our hypothesis for the functions of A2 and A3a in the MR reaction has been modeled in a general way on the nitrogenase system, in which electrons are passed from hydrogen to the MoFe protein (dinitrogenase) for the reduction of N_2 to NH_3 (43). The reduced Fe protein (dinitrogenase reductase) forms a complex with the MoFe protein, and an electron is passed to the MoFe protein in a reaction that is coupled to ATP hydrolysis (39). Active MR from *M. thermoautotrophicum* Marburg loses activity rapidly (49). However, inactive MR from *M. thermoautotrophicum* ΔH can continuously catalyze the formation of CH_4 after the addition of components A2 and A3a, ATP, and Ti(III)C (51). Because the active form of the MR is believed to be the Ni(I) form, it was postulated that the oxidation of Ni(I) to Ni(II) inactivated the MR. Placing A2 on the inner face of the cytoplasmic membrane, where it would facilitate the transfer of electrons from A3a to the MR, is only inconsistent with the ABC transport model in that electrons have not been shown to be substrates for ABC transport systems.

Upstream and downstream sequence. In addition to *atwA*,

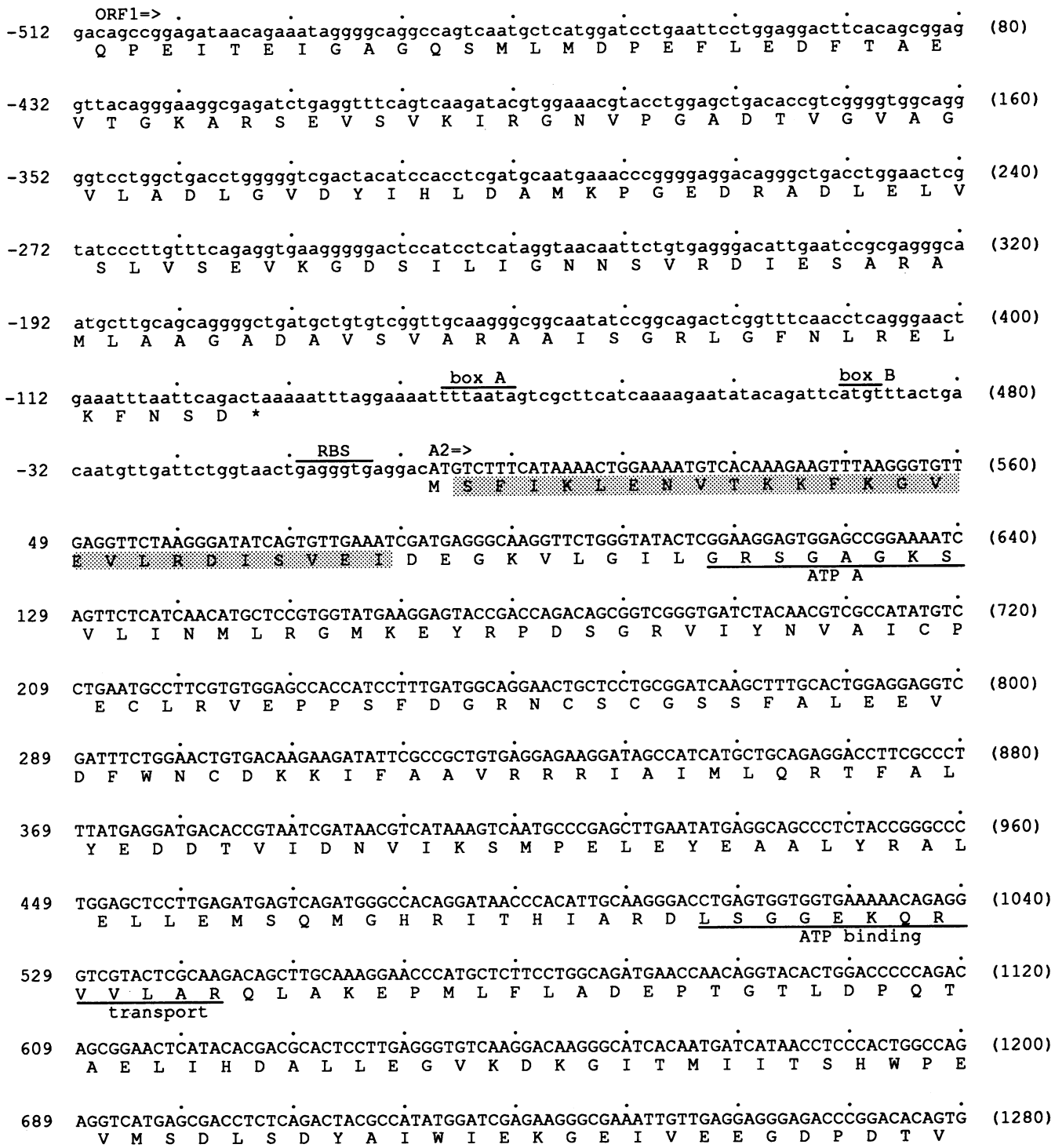

FIG. 2. Nucleotide and derived amino acid sequence of *atwA* and the surrounding ORFs. Numbers on the left reflect nucleotide position relative to the *atwA* ATG start site. A putative promoter (box A and box B) and ribosome-binding site (RBS) for *atwA* are indicated. Potential transcription terminators are shown: ——loop——, possible stem-loop; - - - -, oligo(dT) stretch. The amino acid sequence corresponding to that obtained from the N-terminal sequence of the purified protein is shaded. Numbers on the right in parentheses indicate positions relative to the sequence start site. The *atwA* sequence is indicated by capital letters.

the nucleotide sequence contains additional ORFs upstream (ORF1, >138 amino acids) and downstream (ORF2, >35 amino acids) of *atwA* (Fig. 2). No consensus transcription termination site downstream of ORF1 or promoter upstream of ORF2 is obvious. The results of codon usage analysis (23) with known preferences (8, 13, 34) support protein coding in these regions. The period-three constraint technique (19) also suggests that ORF1 encodes a protein, whereas ORF2 is

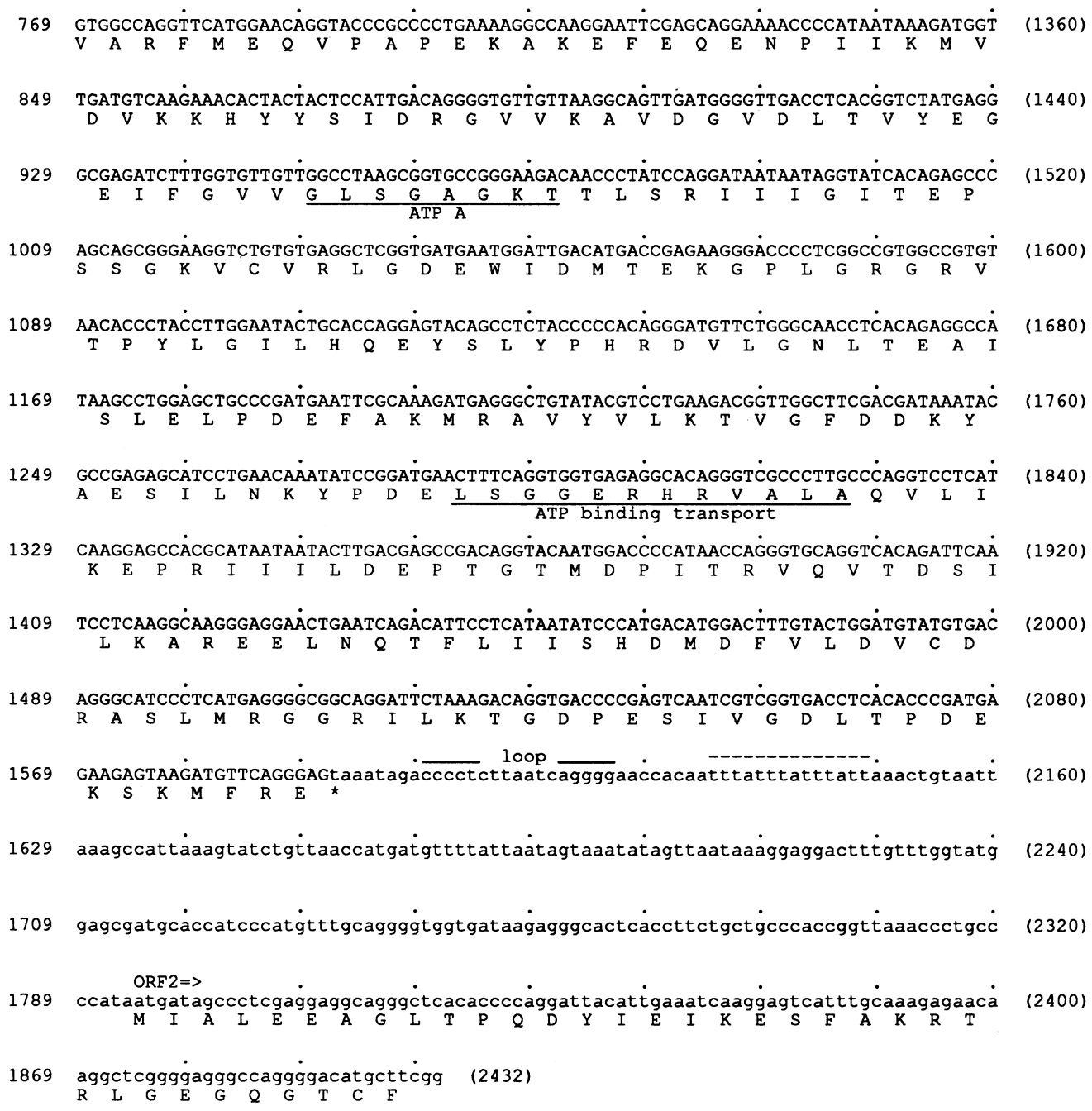

FIG. 2—*Continued.*

too short for this method to be used reliably. The oligopeptides encoded by these ORFs were compared with proteins in the major protein data bases (1, 9, 46), but no significant homologous sequence was found. A visual inspection of undescribed methanogen ORFs in the literature also revealed no matches.

Genes involved in ABC transport processes are generally grouped together on the chromosome (28). If A2 belongs to an analogous archaeal ABC transport system, the upstream or downstream DNA sequence might be expected to encode two integral membrane domains. The 138 amino acids encoded by ORF1 include three hydrophobic regions, but only one of these appears to be potentially membrane spanning (36). We are currently determining the nucleotide sequences of ORF1 and ORF2 as well as the size of the A2 transcript. Component A3a elutes at low salt concentrations from phenyl-Sepharose resin, suggesting that it is a hydrophobic protein.

Expression of A2 by *E. coli*. To measure the in vivo production of component A2, *E. coli* JM109(pBDL3) was grown aerobically in 10 ml of Luria-Bertani (LB) medium (56). Each 200-μl sample from the culture was added to 10 μCi of Trans^{35}S-label and incubated for 30 min. The cells were lysed, and protein was denatured as described before

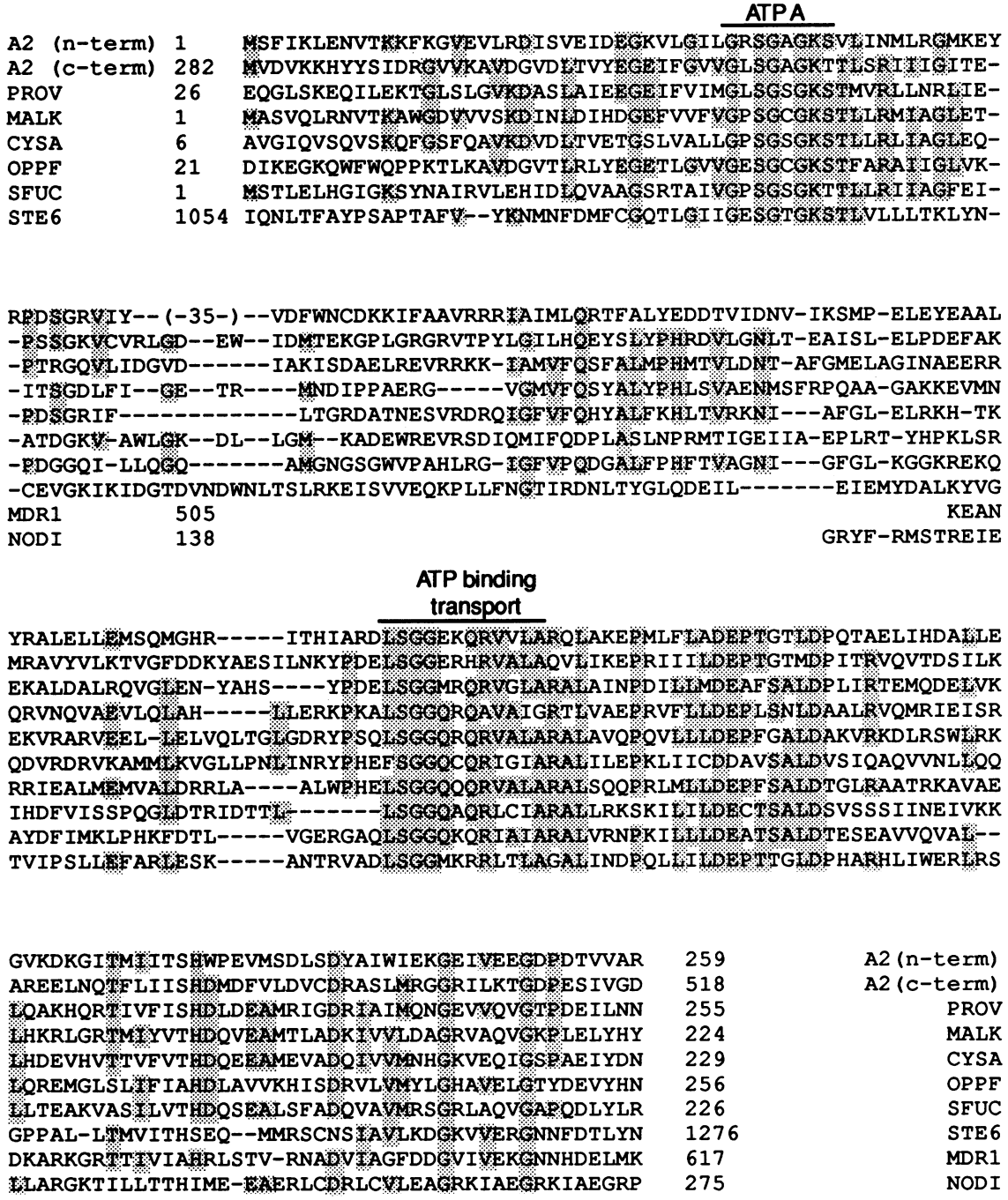

FIG. 3. Alignment of the amino acid sequences of several proteins with homology to the N- and C-terminal domains of A2 (46). Numbers reflect the amino acid sequence within the protein. Amino acids in columns that have at least 50% identical residues are shaded. Only the relevant regions of Mdr1 and NodI are shown, as they do not contain the ATP A motif. The proteins illustrated are ProV from *E. coli* (21, 58), MalK and OppF from *Salmonella typhimurium* (12, 30), CysA from *Synechococcus* sp. (22), SfuC from *Serratia marcescens* (2), Ste6 from *Saccharomyces cerevisiae* (35, 41, 62), human Mdr1 (10, 11), and NodI from *Rhizobium leguminosarum* (17).

(25). Peptides were separated by SDS–10% PAGE, and ^{35}S-containing peptides were visualized by autoradiography. A peptide of approximately 59 kDa was synthesized which was not present in *E. coli* JM109[pGEM-7Zf(+)] (data not shown). A 59-kDa band was also produced by pBDL3 in a coupled in vitro transcription-translation system (Promega, Madison, Wis.); this band was not produced by pGEM-7Zf(+).

To measure the expression of functional protein, *E. coli* JM109(pBDL3) was grown aerobically to the stationary phase in 8 liters of LB supplemented with carbenicillin (50 mg/liter). Cells were harvested by centrifugation, brought into an anaerobic chamber, and resuspended in buffer I. Cell extracts were prepared anaerobically (44), and component A2 was assayed (55) as described above. *E. coli* JM109 containing pGEM-7Zf(+) served as a negative control. With

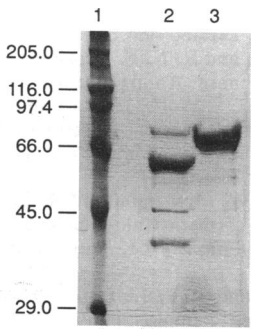

FIG. 4. Expression of 59-kDa peptide by *E. coli* JM109(pBDL3). After ATP-agarose affinity chromatography, peptides were separated by SDS–10% PAGE. Lane 1, molecular size standards (numbers on the left represent mass in kilodaltons); lane 2, *E. coli* JM109(pBDL3); lane 3, *E. coli* JM109[pGEM-7Zf(+)].

purified component A2 from *M. thermoautotrophicum*, CH_4 was produced at a rate of 24 nmol/min/mg of protein. When cell extract from *E. coli* JM109(pBDL3) was used as the source of A2, CH_4 was produced at a rate of 5 nmol/min/mg of protein. No CH_4 was produced by a cell extract from *E. coli* lacking *atwA*.

The proteins in cell extracts from *E. coli* JM109(pBDL3) and *E. coli* JM109[pGEM-7Zf(+)] were then further resolved by ATP-agarose affinity chromatography (as described for the purification of A2) and separated by SDS–10% PAGE. A band of approximately 59 kDa was present in the ATP-agarose eluate from *E. coli* JM109(pBDL3) but absent from the strain lacking the insert (Fig. 4).

Component A2 is the first archaeal protein to be added to the ABC family, and therefore it appears that the primary sequence of the ATP-binding pocket is highly conserved among all three kingdoms (63). With the exception of the UvrA protein, in all systems for which a function of the ABC proteins is known, ATP hydrolysis by ABC proteins is linked to the formation of some sort of channel. It is possible that A2 functions differently from the ABC transport proteins. However, the two ABC domains constitute most of the A2 peptide, and it is likely that A2 acts at the cytoplasmic membrane. It is conceivable that A2 is involved in electron transport from a membrane-bound electron carrier for activation of the MR. It will be interesting to determine the location of *atwA* relative to other genes of methanogenesis and also to sequence the ORFs surrounding *atwA* to determine whether the four-domain model of ABC transporters applies to this archaeal system. However, homogeneous A3a will be required to reconstitute this system and to determine the function of each A component.

We gratefully acknowledge the counsel and interest of Anthony DiMarco and Pierre Rouviere in the early stages of this work, as well as the technical assistance of A. Mantia, C. McMahon, and V. Gabriel. We are also indebted to J. Kansy, S.-J. Li, K. Magnuson, K. Reed, and A.-Y. Wang for many helpful discussions.

This work was supported by National Institute of Health grant AI-12277 and Department of Energy grant DE-FG02-87ER13651.

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