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Composition and Primary Structure of the F_1F_0 ATP Synthase from the Obligately Anaerobic Bacterium *Clostridium thermoaceticum*

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The subunit composition and primary structure of the proton-translocating F_1F_0 ATP synthase have been determined in *Clostridium thermoaceticum*. The isolated enzyme has a subunit composition identical to that of the F_1F_0 ATP synthase purified from *Clostridium thermoautotrophicum* (A. Das, D. M. Ivey, and L. G. Ljungdahl, J. Bacteriol. 179:1714–1720, 1997), both having six different polypeptides. The molecular masses of the six subunits were 60, 50, 32, 17, 19, and 8 kDa, and they were identified as α, β, γ, δ, ε, and *c*, respectively, based on their reactivity with antibodies against the F_1 ATPase purified from *C. thermoautotrophicum* and by comparing their N-terminal amino acid sequences with that deduced from the cloned genes of the *C. thermoaceticum atp* operon. The subunits *a* and *b* found in many bacterial ATP synthases could not be detected either in the purified ATP synthase or crude membranes of *C. thermoaceticum*. The *C. thermoaceticum atp* operon contained nine genes arranged in the order *atpI* (*i*), *atpB* (*a*), *atpE* (*c*), *atpF* (*b*), *atpH* (δ), *atpA* (α), *atpG* (γ), *atpD* (β), and *atpC* (ε). The deduced protein sequences of the *C. thermoaceticum* ATP synthase subunits were comparable with those of the corresponding subunits from *Escherichia coli*, thermophilic *Bacillus* strain PS3, *Rhodospirillum rubrum*, spinach chloroplasts, and the cyanobacterium *Synechococcus* strain PCC 6716. The analysis of total RNA by Northern hybridization experiments reveals the presence of transcripts (mRNA) of the genes *i*, *a*, and *b* subunits not found in the isolated enzyme. Analysis of the nucleotide sequence of the *atp* genes reveals overlap of the structural genes for the *i* and *a* subunits and the presence of secondary structures (in the *b* gene) which could influence the posttranscriptional regulation of the corresponding genes.

ATP synthase is the key enzyme in the energy transduction processes which couples the transmembrane ion gradient generated by respiration (electron transport) to the synthesis of ATP from ADP and P_i (17, 39, 48, 49). The enzyme also catalyzes the hydrolysis of ATP, which generates an ion gradient. In most biological systems, the primary ions involved in the ion gradient are protons, but in some systems, sodium ions replace the protons (27, 43). ATP synthases from various sources have similar structures. They consist of two subcomplexes, a membrane-extrinsic F_1 part and a membrane-intrinsic F_0 part. The most investigated F_1F_0 complex is that of *Escherichia coli*, in which the F_1 has five subunits, α, β, γ, δ, and ε, and F_0 has three subunits, *a*, *b*, and *c* (16). From biochemical and genetic studies, it has been demonstrated that all eight subunits are essential for the function of *E. coli* ATP synthase (12, 18, 47).

The ATPase (*atp*) operons of various organisms have been sequenced (18). In *E. coli*, alkaliphilic bacterium *Bacillus firmus* OF4, and thermophilic *Bacillus* strain PS3, the *atp* operon consists of nine structural genes, *atpIBEFHAGDC*, encoding *i*, *a*, *c*, *b*, δ, α, γ, β, and ε subunits (25, 38, 55). These genes are grouped together to form a single transcriptional unit. However, in the purple nonsulfur photosynthetic bacteria *Rhodopseudomonas blastica* (52) and *Rhodospirillum rubrum* (14), the F_0 genes (*atpBEF*) are grouped separately from the F_1 genes (*atpHAGDC*). In the cyanobacteria *Synechococcus* strain PCC 6301 (6), *Synechococcus* strain 6716 (53), *Synechocystis* sp. strain PCC 6803 (28), and *Anabaena* strain PCC 7120 (7), the structural genes are arranged in two transcriptional units, one containing the *B*, *E*, *F*, *H*, *A*, and *G* genes and the other containing the *D* and *C* genes.

The homoacetogens *Clostridium thermoaceticum* and *Clostridium thermoautotrophicum* are thermophilic anaerobic gram-positive bacteria which are strikingly similar with respect to physiology, DNA composition, and the metabolism of carbon sources (2, 13, 29, 30, 42, 57, 58). One of the most distinctive features of these bacteria is their ability to produce acetate directly from CO_2 by using the acetyl coenzyme A pathway (13, 29, 42, 58). The acetyl coenzyme A pathway is also the major pathway involved during autotrophic growth on C_1 compounds like CO and methanol (13, 29, 30, 42, 57, 58). This pathway does not yield any net gain of ATP at the substrate level (13, 29, 30). Thus, to support growth, acetogens must generate energy through chemiosmosis (11, 21–23, 30). Evidence for this is the presence of an electron transport system (11, 21–23) and a proton-translocating ATP synthase (8, 9, 24, 33) in membranes of *C. thermoaceticum* and *C. thermoautotrophicum*.

The F_1 ATPase and ATP synthase from *C. thermoautotrophicum* (9) and the F_1 ATPase from *C. thermoaceticum* (24) have been purified. The F_1 ATPases from both clostridia have four subunits and identical subunit composition. The ATP synthase from *C. thermoautotrophicum* has six subunits with an apparent composition of $\alpha_3,\beta_3,\gamma,\delta,\varepsilon,c_{6-8}$ (9, 10), and it lacks the *a* and *b* subunits of F_0 found in many aerobic bacteria, including *E. coli* (16, 17, 48).

In this study, we describe the subunit composition of the ATP synthase from *C. thermoaceticum* and the primary structure of the *atp* operon (C*atp*) encoding the subunits of this enzyme complex. The subunit composition of the *C. ther-*

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TABLE 1. Oligonucleotide primers used in PCR for amplification of DNA probes used in Southern and Northern hybridization experiments

Primer[a]	Sequence (5′-3′)
FP	GA(AG)(CA)G(TCG)AC(TCG)(CA)G (TCG)GA(AG)GG(TCG)AA(TC)GA
RP	GT(CGA)A(GA)(GA)TC(GA)TC(GA) GC(CGA)GGIAC(AG)TA
IFP	ACAGTATCTTTAGTGGAC
IRP	AATAGATGGGGATAGGTC
BFP	TTGGGACTTCGGGCTCTG
BRP	CAGTGCCCGTGGACAAAG
FFP	CGGTCTTATTCGCCCCTG
FRP	GCTCACTCATTGCAGTCG
HFP	CGACTGCAATGAGTGAGC
HRP	CTTTGCCCGAACTCTCTT

[a] The nucleotide sequences of the degenerate primers FP and RP were designed from two highly conserved regions of amino acid sequences of the β subunit of *E. coli* F_1 ATPase as described in Materials and Methods. The nucleotide sequences of the remaining primers were designed directly from the DNA sequence of the C*atp* operon. The sequences of IFP and IRP were taken from the upstream and downstream regions of *atpI*. Similarly, the sequences of BFP and BRP, FFP and FRP, and HFP and HRP were taken from the upstream and downstream regions of *atpB*, *atpF*, and *atpH*, respectively.

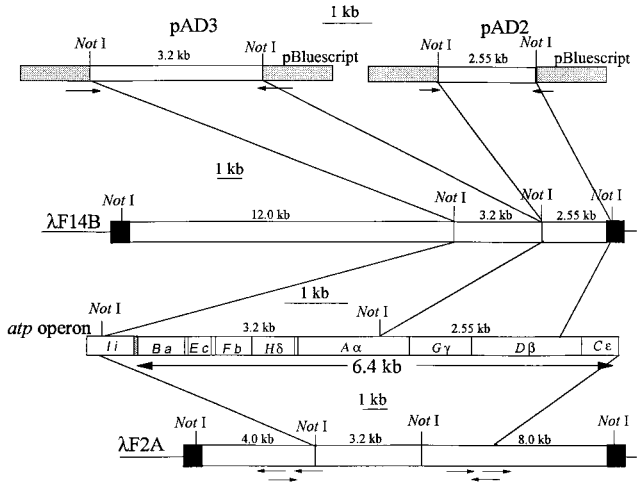

FIG. 1. Strategies used in cloning and sequencing experiments. Two ATPase-positive clones, λF2A and λF14B, were selected by screening the *C. thermoaceticum* genomic library in λFIX II. The *Not*I sites at both ends of the insert belong to the vector's multiple cloning site. The 2.5- and 3.2-kb *Not*I fragments of λF14B were cloned into pBluescript (SK−) to obtain the subclones pAD2 and pAD3, respectively. The DNAs from the two plasmids and that from λF2A were used as templates for sequencing as described in Materials and Methods.

moaceticum ATP synthase is identical with that of *C. thermoautotrophicum* (9). The C*atp* operon contains nine structural genes, including those encoding the *i*, *a*, and *b* subunits which are not found in the ATP synthases purified from both clostridia. Results of Western and Northern blotting experiments suggest the presence of polycistronic mRNAs, which include the transcripts of the genes encoding *i*, *a*, and *b* subunits, and the absence of the *a* and *b* subunits in membranes of *C. thermoaceticum*.

(A preliminary report of this work has been published elsewhere [10].)

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. thermoaceticum* (ATCC 39073) and *C. thermoautotrophicum* JW 701/5 were grown on 1% (wt/vol) glucose at 58°C under 100% CO_2 (31). *E. coli* XL1-Blue MRA and XL1-Blue MRA(P2) were used as hosts to screen the genomic library of *C. thermoaceticum* in λFIX II (described below). *E. coli* XL-Blue was used as a host for plasmids in transformation experiments (described below). All *E. coli* strains were grown and maintained in LB or 2×YT medium.

Preparation of membranes and purification of ATP synthases from *C. thermoautotrophicum* and *C. thermoaceticum*. The membranes were prepared from whole cells after the cells were broken in a French press (24). The ATP synthase was purified from cholate-washed membranes (9).

Antibodies against synthetic peptides. Synthetic peptides were designed from the protein sequences of the *a*, *b*, and δ subunits deduced from the cloned genes of *C. thermoaceticum* and used as antigens to raise antibodies in adult New Zealand White rabbits. The amino acid sequences of the synthetic peptides used were ^{2}GLRALGEIMTHVRPVEIF^{19} (for subunit *a*), ^{37}LGKVLADREARIEG NLND^{54} (for subunit *b*), and ^{1}MSEQNVARRYARALFNIARE^{20} (for subunit δ).

DNA source and synthetic oligonucleotides. The genomic DNA of *C. thermoaceticum* was isolated as described previously (37). The λ DNA was isolated by using the Wizard Lambda Preps DNA purification system (Promega Corp., Madison, Wis.). The plasmid DNA was isolated by using a QIAprep Spin Plasmid kit (Qiagen Inc., Chatsworth, Calif.).

The ATPase probe and PCR. The DNA probe used to screen the genomic library was a 390-bp PCR product amplified from *C. thermoaceticum* genomic DNA by using the degenerate primers FP (forward primer) and RP (reverse primer) (Table 1), designed from two highly conserved regions of amino acid sequences ^{191}ERTREGND^{198} and ^{311}YVPADDLTD^{319}, respectively, of the β subunit of *E. coli* F_1 ATPase (10, 50). The amplification was carried out for 30 cycles in a 480 Thermal Cycler (Perkin-Elmer Instruments Div., Norwalk, Conn.). Each cycle includes 1 min of melting at 94°C, 90 s of annealing at 49°C, and 2 min of extension at 72°C. The PCR product was cloned into pCRII vector (Invitrogen Corp., San Diego, Calif.), generating pAD1, and sequenced. The deduced amino acid sequence of the PCR product was found to be highly homologous with the corresponding sequences of the F_1β subunits from various sources (10). The PCR product was labeled with digoxigenin (DIG)-11-dUTP (Boehringer Mannheim Co., Indianapolis, Ind.) under conditions similar to those described above except that the nucleotide mixture was made with 5.3 mM DIG-11-dUTP and 2.7 mM Gene Amp deoxynucleoside triphosphates (Perkin-Elmer).

Genomic library and screening. A genomic library of *C. thermoaceticum* was constructed in λFIX II by Stratagene (La Jolla, Calif.). The library was screened for ATPase-positive clones by plaque hybridization using the 390-bp DIG-labeled PCR product (see above) as a probe. The hybridization experiments and the detection of ATPase-positive clones (plaques) were carried out by using a Genius kit (Boehringer Mannheim). The positive clones picked up after primary screening were purified by secondary or tertiary screening using the same protocol.

Plasmids and phages. Several ATPase-positive clones were obtained after secondary or tertiary screening of the library. Two clones, λF2A and λF14B, were selected and used for further study. The DNAs isolated from these clones were digested with *Not*I and subjected to electrophoresis on 1% (wt/vol) agarose gels (45). Three fragments were obtained from each clone: 3.2, 4.0, and 8.0 kb from λF2A and 2.5, 3.2, and 12.0 kb from λF14B. The 8.0- and 2.5-kb fragments hybridized to the 390-bp DNA probe. The 2.5- and 3.2-kb fragments were recovered from agarose gels, purified by using a Geneclean spin kit (Bio 101, Vista, Calif.), and cloned into pBluescript (SK−) (45). The resulting plasmids carrying the 2.5- and 3.2-kb *Not*I fragments were designated pAD2 and pAD3, respectively (Fig. 1). The plasmids carrying the 3.2-kb *Not*I fragment from either λF2A or λF14B were found to be indistinguishable with respect to restriction map and DNA sequence analysis (not shown), indicating that this fragment is common to both clones.

Isolation of mRNA and Northern hybridization experiments. Total RNA was isolated from freshly grown cells of *C. thermoaceticum* by using an RNeasy kit (Qiagen) and separated by electrophoresis on 1.2% (wt/vol) agarose gels under denaturing conditions in the presence of 2.3 M formaldehyde (45). The Northern (RNA) blot analyses were carried out by using a Genius kit as instructed by the manufacturer (Boehringer Mannheim). The DNA probes specific for *atpI*, *atpB*, *atpF*, and *atpH* genes were synthesized and labeled with DIG-11-dUTP by PCR using primer pairs IFP-IFP, BFP-BRP, FFP-FRP, and HFP-HRP (Table 1), respectively. The conditions for PCR were the same as used for the amplification of the 390-bp DNA fragment as described above. The DNA probes for all genes except *atpI* were amplified by using pAD3 DNA as a template. To amplify the *atpI* probe, *C. thermoaceticum* genomic DNA was used as a template; the amplified product (500 bp) was purified by using a Geneclean spin kit (Bio 101) and used as a template to reamplify the specific product by PCR.

Other methods. Proteins were estimated by a modified Lowry method as described previously (15). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (26) in the presence of urea (51). Twelve percent (wt/vol) acrylamide in the resolving gel and 4% (wt/vol) acrylamide in the stacking gel were used, and the proteins were stained with Coomassie brilliant blue 250. The Western blotting experiments were carried out as instructed by the manufacturer (Bio-Rad, Hercules, Calif.). The synthesis of peptides and oligonucleotides and the sequencing of protein and

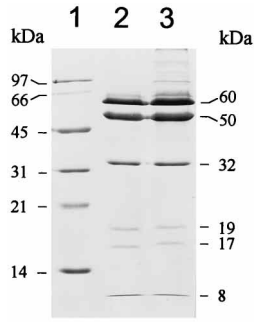

FIG. 2. SDS-PAGE with 8 M urea of the purified ATP synthases from *C. thermoaceticum* (10 μg; lane 2) and *C. thermoautotrophicum* (10 μg; lane 3) and of the protein standards (lane 1). The proteins were stained with Coomassie blue.

DNA samples were carried out at the Molecular Genetics Facility of the University of Georgia. The animal work, including the injection of antigens to rabbits and collection of blood samples, was carried out at the Animal Facility of the University of Georgia. The sequence data were analyzed with the Genetics Computer Group package (University of Wisconsin Biotechnology Center, Madison) on the VAX/VMS system of the BioScience Computing Resources at the University of Georgia.

Nucleotide sequence accession number. The nucleotide sequence reported here has been assigned accession no. U64318 in the GenBank, EMBL, and DDBJ libraries.

RESULTS

Subunit composition of the ATP synthase from *C. thermoaceticum* and comparison with that of *C. thermoautotrophicum*. Figure 2 shows the results of SDS-PAGE of the F_1F_0 ATP synthases purified from *C. thermoaceticum* and *C. thermoautotrophicum*. The enzymes from both bacteria have the same subunit composition, each having six different polypeptides with molecular masses of 60, 50, 32, 19, 17, and 8 kDa. These molecular masses are in close agreement with the calculated molecular masses of the α (55,357 Da), β (49,863 Da), γ (33,305 Da), δ (20,163 Da), ε (14,518 Da), and *c* (7,458 Da) subunits, respectively, deduced from the cloned genes of the Ct*atp* operon (Table 2). We previously showed that the N-terminal amino acid sequences of the six ATP synthase subunits of *C. thermoautotrophicum* matched the N-terminal amino acid sequences of the α, β, γ, δ, ε, and *c* subunits of the ATP synthase deduced from the cloned genes of the Ct*atp* operon (9). The F_1 ATPases purified from *C. thermoaceticum* (24) and *C. thermoautotrophicum* (9) have the same subunit composition, and immunoblot analyses of the membranes from both clostridia revealed similarities of the α, β, and γ subunits of the F_1 ATPases (9). Determinations of the N-terminal amino acid sequences of the 32-, 19-, 17-, and 8-kDa polypeptides of the *C. thermoaceticum* ATP synthase showed a complete match with the N-terminal amino acid sequences of the γ, δ, ε, and *c* subunits deduced from the cloned genes of the Ct*atp* operon. Therefore, the six subunits of the purified ATP synthase from *C. thermoaceticum* are α, β, γ, δ, ε, and *c*, and its composition is identical to that of the *C. thermoautotrophicum* ATP synthase, as shown in Fig. 2. The F_0 moiety of the ATP synthase of the acetogenic clostridia is composed of the δ and *c* subunits as suggested previously (9) and apparently lacks the *a* and *b* subunits present in several aerobic bacteria.

Western blotting experiments to test the presence of *a* and *b* subunits. Antibodies against synthetic peptides designed from the deduced protein sequences of the *a* and *b* subunits of the *C. thermoaceticum* ATP synthase failed to react with any protein in the purified F_1F_0 ATP synthase or in crude membranes or whole-cell extracts of *C. thermoaceticum* or *C. thermoautotrophicum*. Antibodies against a synthetic peptide designed from the deduced protein sequence of the *C. thermoaceticum* δ subunit used as a positive control reacted strongly with the corresponding subunit present in the ATP synthase purified from both clostridia (not shown). These results support the findings that the *a* and *b* subunits are not present in the F_1F_0 complex of *C. thermoaceticum* and *C. thermoautotrophicum.*

Cloning and sequencing of the *C. thermoaceticum* ATP synthase genes. The entire Ct*atp* operon is present in the clone λF2A, and plasmids pAD2 and pAD3 together have the complete DNA sequences for the genes encoding *a*, *c*, *b*, δ, α, and γ subunits of the ATP synthase and partial sequences of the *i* and β genes (Fig. 1). The sequences of the remaining portions of the *i* and β genes and that of the gene encoding the ε subunit were obtained by primer walking on λF2A DNA as template as outlined in Fig. 1.

Identification and analysis of the *atp* genes. Nine open reading frames (ORFs), each having a putative start codon and a ribosome binding site (Shine-Dalgarno [S/D] sequence), were identified within the 7.5-kb region sequenced (Fig. 3). The nine structural genes of the *atp* operon encoding nine subunits of the *C. thermoaceticum* ATP synthase were organized in the order *atpI* (*i*), *atpB* (*a*), *atpE* (*c*), *atpF* (*b*), *atpH* (δ), *atpA* (α), *atpG* (γ), *atpD* (β), and *atpC* (ε), which is similar to that found in most bacteria (3, 25, 38, 46, 55). The deduced protein sequences of the nine genes reveal similarities with the corresponding sequences of the nine ATP synthase subunits from different species (Table 2). The protein encoded by the first gene, *atpI*, is called the inhibitor protein or the *i* subunit. The *i* protein is the least homologous (21 to 24% identity) among

TABLE 2. Comparison of the Ct*atp* gene products with the corresponding *atp* gene products from *E. coli*, thermophilic bacterium PS3, *R. rubrum*, spinach, and *Synechococcus* strain PCC 6716[a]

Gene	Subunit	Mol wt	% Identity (% similarity)				
			E. coli	ThePS3	Rhoru	Spio1	SynP1
atpI	*i*	14,207	24 (49)	23 (51)	21 (49)		
atpB	*a*	25,411	28 (63)	34 (64)	31 (65)	36 (70)	36 (69)
atpE	*c*	7,458	25 (65)	34 (71)	29 (61)	56 (78)	58 (80)
atpF	*b*	19,019	31 (58)	38 (62)	29 (51)	22 (41)	29 (52)
atpH	δ	20,163	30 (58)	31 (56)	29 (53)	27 (54)	28 (55)
atpA	α	55,357	57 (76)	67 (81)	61 (77)	60 (76)	64 (81)
atpG	γ	33,305	40 (62)	46 (66)	43 (66)	42 (63)	45 (66)
atpD	β	49,863	70 (83)	73 (84)	69 (81)	72 (84)	72 (84)
atpC	ε	14,518	31 (62)	39 (62)	31 (54)	35 (55)	39 (63)

[a] References are as follows: *E. coli*, 55; thermophilic bacterium PS3 (TheP3), 38; *R. rubrum* (Rhoru), 14; *Synechococcus* (SynP1), 6; spinach subunits *a*, *c*, *b*, α, γ, β, and ε, database accession no. P06451, P00843, P06453, P06450, P05435, P00825, and P00833, respectively; and the δ subunit, 20.

the ATPase subunits of *C. thermoaceticum* compared with the corresponding subunits from *E. coli*, thermophilic bacterium PS3, and *R. rubrum* (Table 2). Cozens and Walker (6) and Brusilow et al. (3) used hydropathy profiles of the deduced amino acid sequences to characterize the inhibitor proteins of *Synechococcus* and *B. megaterium*, respectively. We performed a similar analysis of the protein encoded by the first structural gene of the Ct*atp* operon and found that it very closely resembles the inhibitor protein of thermophilic bacterium PS3 (Fig. 4), indicating that the first gene of the Ct*atp* operon is *atpI*. We did not find any ORF in the 680-bp region sequenced upstream of *atpI*.

The start codons for the genes encoding α, β, γ, δ, ε, and *c* subunits were identified from the N-terminal amino acid sequences of the respective proteins purified from *C. thermoautotrophicum* (9). Two types of start codons were found in the Ct*atp* operon. The start codons are ATG for *atpI*, *atpE*, *atpH*, *atpG*, and *atpC* and TTG for *atpB*, *atpF*, *atpA*, and *atpD*. Previously, a conserved S/D sequence of AGGAGt/g was proposed for several genes sequenced from *C. thermoaceticum* (36, 44). This sequence matched perfectly with the proposed S/D sequences of the genes encoding the *a*, *c*, *b*, β, and ε subunits. The N-terminal amino acid of the β subunit of the *C. thermoautotrophicum* ATP synthase is methionine (9), and the start codon of the β gene of the *C. thermoaceticum atp* operon is TTG, demonstrating the use of TTG for methionine. Thus, methionine is proposed as the first amino acid in the deduced protein sequences of *a*, *b*, and α subunits, as the genes for these subunits carry the TTG start codon. The structural genes *atpI* and *atpB* overlap by 50 bases, and the structural genes *atpF* and *atpH* overlap by 4 bases.

The α, β, and γ subunits show greater similarity to the corresponding subunits from other species than do the remaining subunits (18). The α, β, and γ subunits of *C. thermoaceticum* have 40 to 73% identities with the corresponding subunits from *E. coli*, thermophilic bacterium PS3, *R. rubrum*, spinach chloroplasts, and *Synechococcus* strain PCC 6716 (Table 2). The homology is relatively poor (22 to 39% identities) for other ATPase subunits except for the *c* subunit. It has higher homology with the *c* subunits of spinach chloroplasts and *Synechococcus* (56 to 58% identities) than with that of *E. coli*, thermophilic bacterium PS3, and *R. rubrum* (25 to 29% identities), and alignment of the protein sequences shows that the most conserved region of the subunit is the DCCD-binding pocket (Fig. 5), in which 18 of 22 residues are found to be identical.

The consensus nucleotide-binding domains, Walker motifs A (GXXXXGKT/S) and B (L-hydrophobic-hydrophobic-hydrophobic-D) (1, 56), are found to be conserved in the deduced protein sequence of the β subunit of *C. thermoaceticum* (Fig. 3). In the α subunit, the motif A sequence is conserved but the motif B sequence is altered by a single substitution of a cysteine residue for a hydrophobic residue (Fig. 3). Apparently, this difference does not affect the catalytic activity of the enzyme.

Northern blot analysis. The analysis of the Northern blots (Fig. 6) reveals the presence of the transcripts (mRNAs) of the genes encoding subunits *i*, *a*, and *b*. It also appears that initially long transcripts were synthesized, which subsequently degraded into smaller products, as indicated by the presence of smear in the blots. Within the smear of the blots hybridized to the *atpI* and *atpB* probes were found some pronounced bands of smaller fragments. The maximum size of the transcripts was approximately 7 kb, suggesting that all nine genes are transcribed into a single polycistronic mRNA. The presence of a smear in the Northern blots of *atp* mRNA and the presence of intense bands of smaller fragments in the Northern blots of *atpI* and *atpB* have also been reported for *E. coli* and have been suggested to be due to specific endonucleolytic cleavage of the transcripts of the corresponding genes (34, 35).

Regulatory sequences. The exact promoter structure of the Ct*atp* operon was not determined. Several *C. thermoaceticum* genes that have been cloned and sequenced have promoter structures similar to those of *E. coli* genes (36, 44). Two putative promoters (P1 and P2) with sequence similarities to the *E. coli* consensus promoter (41) were found in front of the first structural gene, *atpI* (Fig. 3). Due to the presence of several intergenic regions in the Ct*atp* operon, the presence of internal promoters as has been described for the *E. coli atp* operon (55) may be possible. A GC loop followed by a string of T residues, a structure resembling a putative terminator (40), was found immediately after the ε gene. It may serve as the putative transcription terminator of the *atp* operon.

There are three long and two short intergenic regions found in the Ct*atp* operon. The long regions are *atpB-atpE* (98 bp), *atpE-atpF* (88 bp), and *atpH-atpA* (49 bp). The two long intergenic regions around *atpE* are present also in other bacterial *atp* operons, including those of *E. coli* (55), *Bacillus subtilis* (46), *B. megaterium* (3), and thermophilic bacterium PS3 (38). The remaining long intergenic region (*atpH-atpA*) is not common to any bacterial *atp* operon. A long palindromic sequence which may protect the *atpE* mRNA from nuclease attacks (35) is found downstream of *atpE* (*c* gene) (Fig. 3). A similar finding is also reported for the *B. subtilis atp* operon (46).

DISCUSSION

The results of this study demonstrate that the ATP synthases purified from *C. thermoaceticum* and *C. thermoautotrophicum* have the same subunit composition and that they lack subunits *i*, *a*, and *b*, although the structural genes encoding these subunits are present in the Ct*atp* operon. Previously we have shown that the absence of these subunits does not influence the functional integrity of the ATP synthase purified from *C. thermoautotrophicum* (9).

The *i* subunit is also found to be absent in the ATP synthases purified from many bacteria (12, 16, 17, 27, 38, 43), but subunits *a* and *b* are generally present. In *E. coli*, the inhibitor protein can be synthesized in vitro and in minicells, but it has never been detected in vivo (12). The mutations in *atpI* do not affect the activity of the ATPase, and so the function of this gene and its protein is unknown (19, 54). In *C. thermoaceticum*, the transcripts (mRNAs) of the genes encoding the *i*, *a*, and *b* subunits are found, which shows that the absence of these subunits in the ATP synthase of this bacterium is not due to the lack of transcription. McCarthy et al. (34, 35) demonstrated that in *E. coli*, the mRNAs of *atpI* and *atpB* are comparatively less stable due to their fragmentation by endonucleolytic cleavage and have half-lives much shorter than those of the remaining genes of the *atp* operon. Fragmentation of the transcripts of *atpI* and *atpB* is also likely to occur in *C. thermoaceticum*, as is evident from the Northern hybridization experiments (Fig. 6), and could be one of the reasons for the lack of expression of these genes. It is interesting that the structural genes of subunits *i* and *a* overlap considerably in the Ct*atp* operon but are well separated in other bacteria (3, 25, 38, 46, 55).

The codon usage may also play an important role in differential expression of the *atp* genes. In *E. coli*, the presence of rare codons in *atpI* has been suggested to be one of the reasons for the poor expression of this gene (55). We have compared the codon usages of different genes in the Ct*atp* operon. A

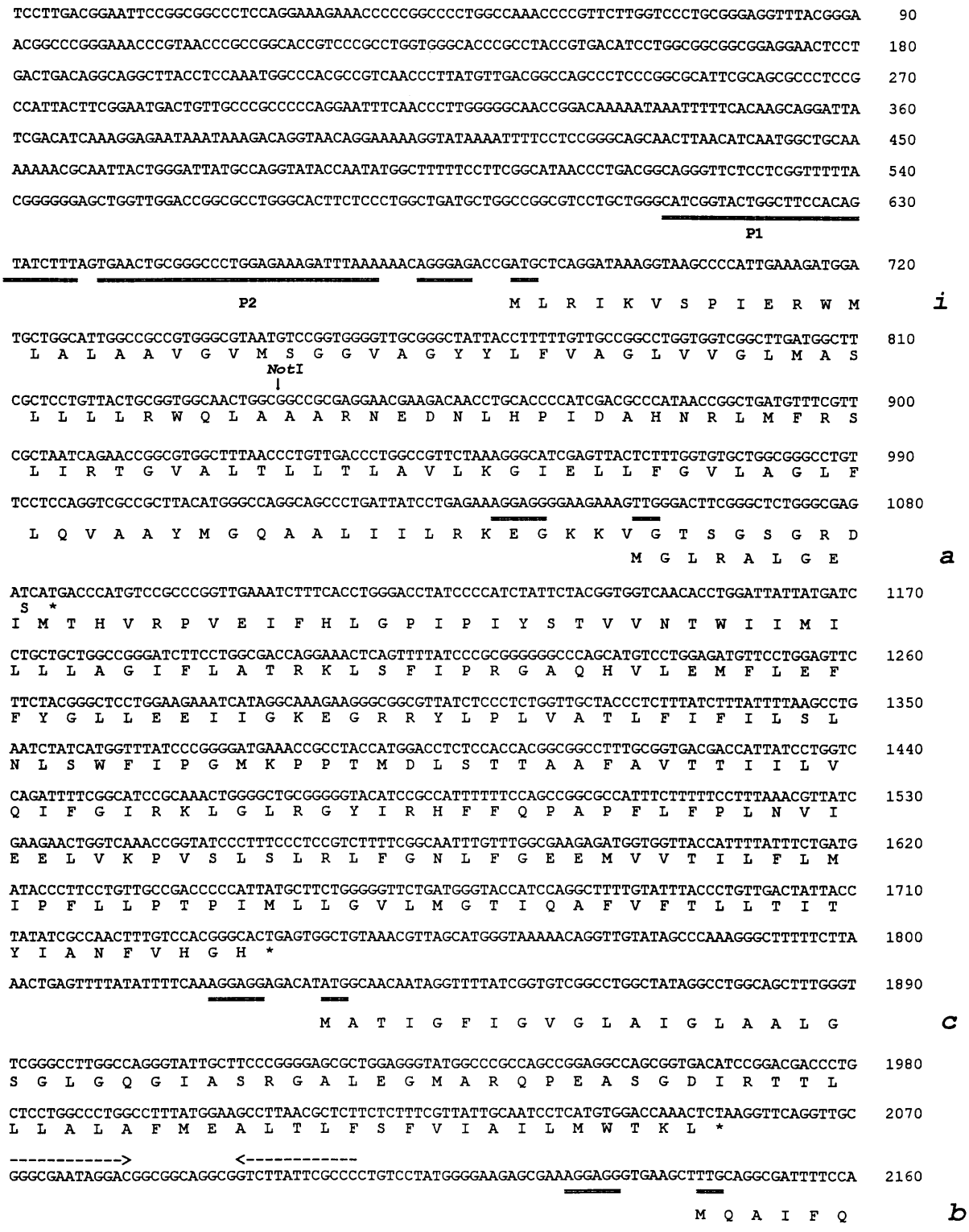

FIG. 3. Nucleotide and deduced protein sequences of the structural genes of the C*tatp* operon. Putative promoters P1 and P2 are indicated by labeled thick lines below the sequence; the terminator is marked by thick arrows above the sequence; start codons and S/D sequences are marked by unlabeled thick lines below the sequence; inverted repeats are marked by dashed single-line arrows above the sequence. The nucleotide-binding motifs of the α and β subunits are shown by underlined letters. The names of the encoded ATPase subunits are given at the right at the start of the ORFs; nucleotide numbering is also shown at the right.

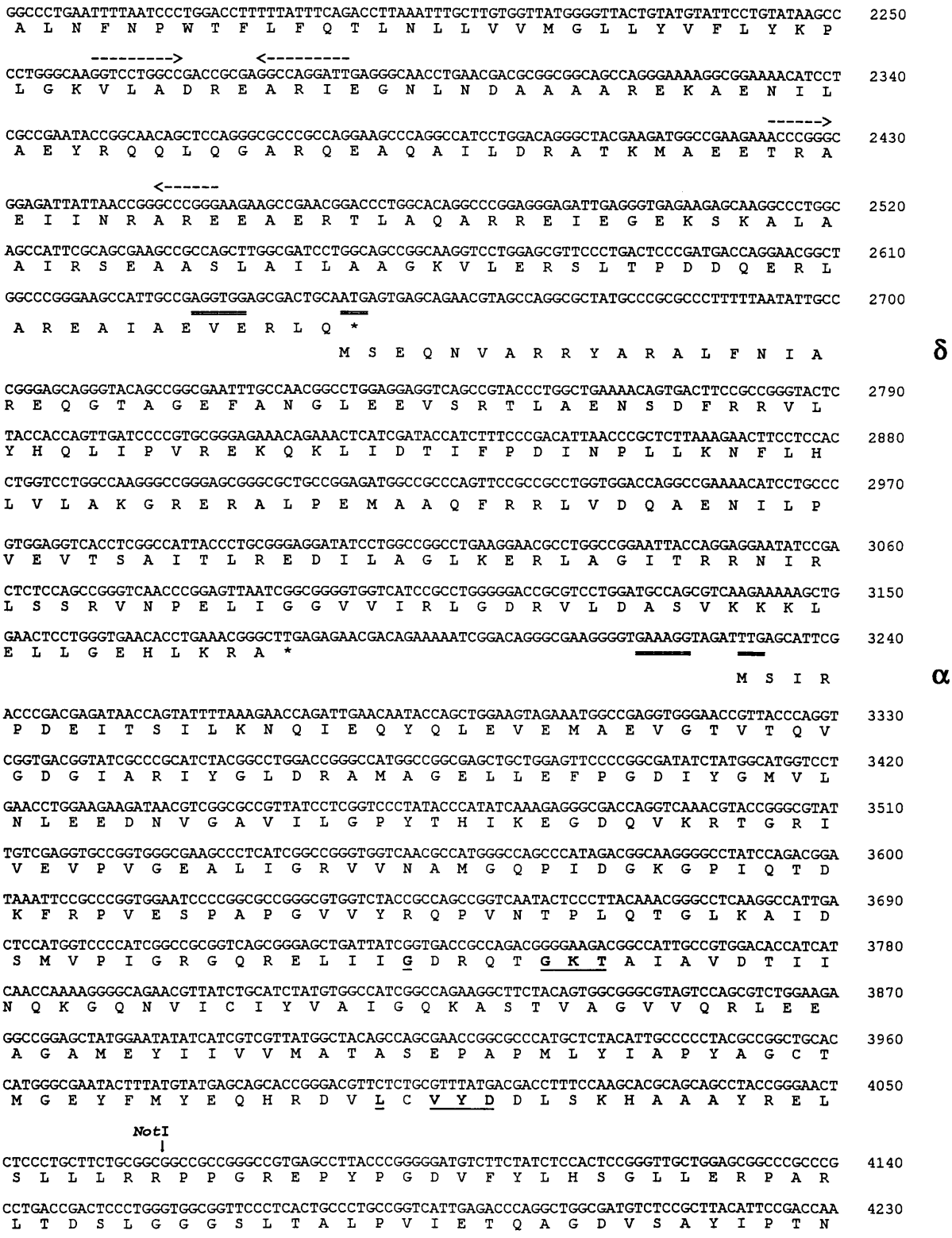

FIG. 3—*Continued.*

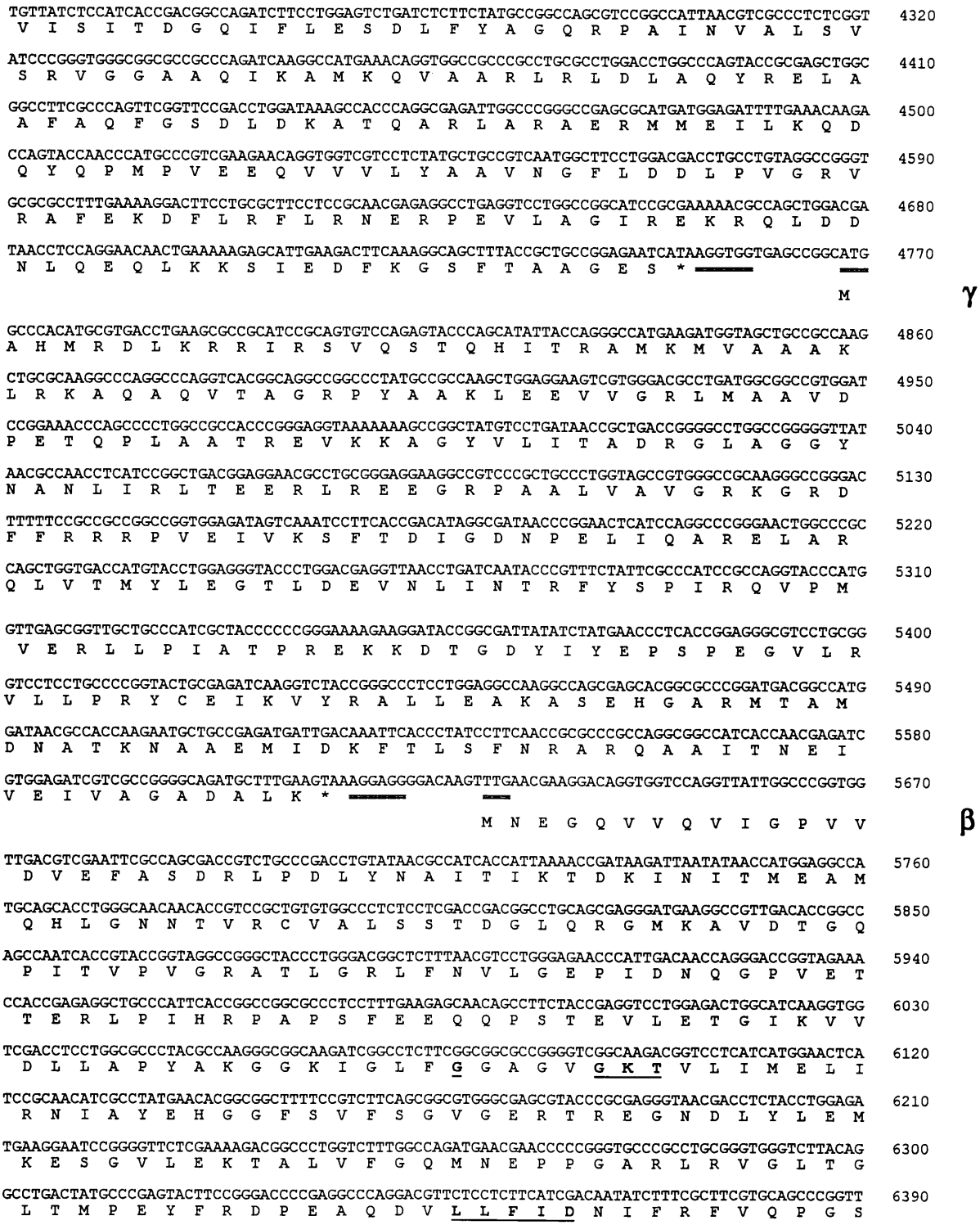

FIG. 3—*Continued.*

difference is observed in the relative use of codons for certain amino acids between *atpB* and the rest of the genes in the operon. These codons are TTT (Phe), GGG (Gly), CTT (Leu), and CAT (His). The numbers of these codons present in *atpB* are 14, 9, 5, and 3, which account for 40, 35, 43, and 42%, respectively, of the total number of the corresponding codons present in the entire C*tatp* operon. We have compared the use of these codons among other genes of *C. thermoaceticum* which have been sequenced, e.g., the formyltetrahydrofolate synthetase, carbon monoxide dehydrogenase, methylenetetrahy-

```
CCGAGGTTTCCGCCCTCCTGGCCCGGATGCCCTCGGCGGTGGGTTATCAGCCCACCCTGCCCACAGAGATGGGGCCCCTGCAGGAACGGA   6480
  E  V  S  A  L  L  A  R  M  P  S  A  V  G  Y  Q  P  T  L  P  T  E  M  G  P  L  Q  E  R  I

TTACCTCCACGAAAAAGGGTTCCATCACCTCCGTGCAAGCTATCTATGTGCCGGCCGACGACCTGACCGACCCGGCCCCGGCGACGACCT   6570
  T  S  T  K  K  G  S  I  T  S  V  Q  A  I  Y  V  P  A  D  D  L  T  D  P  A  P  A  T  T  F

TCGCCCATCTGGACGCCACCACGGTTCTGTCCCGGCAGATCGCTGAGCTGGGCATCTACCCGGCCGTCGACCCCCTGGACTCCACCTCCC   6660
  A  H  L  D  A  T  T  V  L  S  R  Q  I  A  E  L  G  I  Y  P  A  V  D  P  L  D  S  T  S  R

GTATCCTGGACCCGCGCGTCCTGGGAGAAGAGCACTACCAGGTGGTCCGGGGAGTCCAGCAGGTACTGCAGCGTTATAAAGAACTCCAGG   6750
  I  L  D  P  R  V  L  G  E  E  H  Y  Q  V  V  R  G  V  Q  Q  V  L  Q  R  Y  K  E  L  Q  D

ACATTATCGCCATCCTGGGAATGGATGAGCTGTCCGAAGATGATAAACTCATAGTAGCCCGGGCACGCAAGATCCAGCGTTTCCTCTCCC   6840
  I  I  A  I  L  G  M  D  E  L  S  E  D  D  K  L  I  V  A  R  A  R  K  I  Q  R  F  L  S  Q

AGCCCTTCCACGTAGCCGAGGCTTTTACCGGCCAGCCCGGGGTTTATGTGCCCCTGAAGGAAACCATTCGCGGTTTCAAAGAGATCCTGG   6930
  P  F  H  V  A  E  A  F  T  G  Q  P  G  V  Y  V  P  L  K  E  T  I  R  G  F  K  E  I  L  E

AGGGCCGCCACGACAACCTCCCCGAGCAGGCCTTCTATATGGTCGGGACCATCGACGAAGCCGTCAAGAAGGGCCAGGAGTTGATGTAGA   7020
  G  R  H  D  N  L  P  E  Q  A  F  Y  M  V  G  T  I  D  E  A  V  K  K  G  Q  E  L  M  *

                                                                                  M        ε

TGGCCTCCCTCAACCTGGAGATCATAACTCCCGAGCGGGTGGTCCTGCAGGCGGAAGCCGCCAGCGTCATAGCTCCAGGTATCCAGGGCT   7110
  A  S  L  N  L  E  I  I  T  P  E  R  V  V  L  Q  A  E  A  Q  S  V  I  A  P  G  I  Q  G  Y

ACCTGGGTGTCCTACCGGAGCACGCCCCTTTGATCACTCCCCTCCAGGCCGGGGTCGTCACCTGCCGCCGGCGGGAGAGAGCGGAGGAAC   7200
  L  G  V  L  P  E  H  A  R  L  I  T  P  L  Q  A  G  V  V  T  C  R  R  R  E  R  A  E  E  R

GTGTGGCTGTTTCCGGCGGTTTCCTGGAAGCCGGCCCGGACCAGGTAATTATCCTGGCCGATACAGCCGAACGGTCGGAAGAGATCGACG   7290
  V  A  V  S  G  G  F  L  E  A  G  P  D  Q  V  I  I  L  A  D  T  A  E  R  S  E  E  I  D  V

TCGAATGGGCCCGGCAGGCGCGGGAGCGGGCCGAGCGGCGCTTGCGGGAGCGCCCCCCGGGCCTGGACGTCGCCAGGGCCGAGGCCGCCC   7380
  E  W  A  R  Q  A  R  E  R  A  E  R  R  L  R  E  R  P  P  G  L  D  V  A  R  A  E  A  A  L

TGCGGCGAGCCGTAGCCCGCTTGAAGGCCGCCGGAGCTATTTAAGTAGTCTATTTTTCCAGTCCCCTGGCAAGAATAGGAATAACACTA   7469
  R  R  A  V  A  R  L  K  A  A  G  A  I  *

CCCCCTGCAAAGGGGGCTTTTCTTTTGTTCC                                                              7500
```

FIG. 3—*Continued.*

drofolate reductase (36, 37), corrinoid/iron sulfur protein (32), and methyltetrahydrofolate corrinoid/iron-sulfur protein (44) genes. Of the four above-mentioned codons, GGG and CTT are rarely used by these genes, which suggests that the presence of the higher number of these codons in *atpB* may reduce the translational efficiency of its mRNA in *C. thermoaceticum.*

It is not clear why the gene encoding the *b* subunit is not expressed in *C. thermoaceticum*. Computer analysis of the transcript (mRNA) of this gene reveals the presence of two appar-

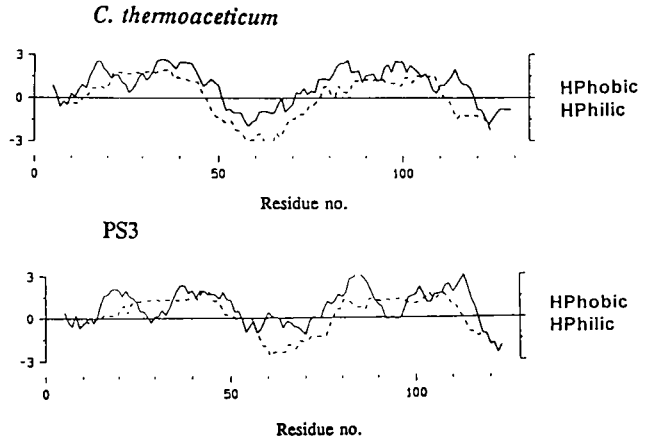

FIG. 4. Hydropathy profiles of the inhibitor protein of thermophilic bacterium PS3 and that of the predicted product (molecular weight, 14,207) of the inhibitor gene from *C. thermoaceticum*. The plots were generated by using the computer program PEPPLOT (Genetics Computer Group). The segments of the plots above the horizontal line represent hydrophobic (HPhobic) regions, and the segments below the line represent hydrophilic (HPhilic) regions. The dotted lines represent the alpha helix, and the solid lines represent the beta structures.

```
AtpC_Cthe     1 ....MATIGFIGVGLAIGLAALGSGLGQGIASRGALEGMARQPEASGD
AtpC_Synp1    1 MDPLVASASVLAAALAIGLASLGPGIGQGNASGQAVEGIARQPEAEGK
AtpC_Syny3    1 MDSTVAAASVIAAALAVGLGAIGPGIGQGNASGQAVSGIARQPEAEGK
AtpC_Spiol    1 MNPLIAAASVIAAGLAVGLASIGPGVGQGTAAGQAVEGIARQPEAEGK
AtpC_Pea      1 MNPLIAAASVIAAGLAVGLASIGPGVGQGTAAGQAVEGIARQPEAEDK
AtpC_Tobac    1 MNPLISAASVIAAGLAVGLASIGPGVGQGTAAGQAVEGIARQPEAEGK
AtpC_Eugrr    1 MNPIICAASVIGAGLAIGLGAIGPGIGQGTASGKAIEGIARQPEAEGK
AtpC_Thep3    1 .....MSLGVLAAAIAVGLGALGAGIGNGLIVSRTIEGIARQPELRPV
AtpC_Ecoli    1 MENLNMDLLYMAAAVMMGLAAIGAAIGIGILGGKFLEGAARQPDLIPL

AtpC_Cthe    42 IRTTLLLALAFMEALTLFSFVIAI.LMWTKL...
AtpC_Synp1   46 IRGTLLLTLAFMESLTIYGLVIALVLLFANPFAS
AtpC_Syny3   46 IRGTLLLTLAFMESLTIYGLVIALVLLFANPFA.
AtpC_SpIOL   46 IRGTLLLSLAFMEALTIYGLVVALALLFANPFV.
AtpC_Pea     46 IRGTLLLSLAFMEALTIYGLVVALALLFANPFV.
AtpC_Tobac   46 IRGTLLLSLAFMEALTIYGLVVALALLFANPFV.
AtpC_Eugrr   46 IRGTLLLSLAFMEALTIYGLVVALAIIFANPFV.
AtpC_Thep3   41 LQTTMFIGVALVEALPIIGVVFSFIYLGR.....
AtpC_Ecoli   46 LRTQFFIVMGLVDAIPMIAVGLGLYVMFAVA...
```

FIG. 5. Alignment of deduced protein sequences of the *C. thermoaceticum c* subunit (AtpC_Cthe) with that from *Synechococcus* strain PCC 6716 (AtpC _Synp1) (accession no. Q05366), *Synechocystis* strain PCC 6803 (AtpC_Syny3) (accession no. P27182), spinach chloroplasts (AtpC_Spiol) (accession no. P00843), tobacco (AtpC_Tobac) (accession no. P06286), *Euglena gracilis* (Atp C_Eugrr) (accession no. P10603), thermophilic bacterium PS3 (AtpC_Thep3) (accession no. P00845), and *E. coli* (AtpC_Ecoli) (accession no. P0084). The consensus DCCD-binding pocket of the *c* subunit is indicated by boxes, and highly conserved residues are in boldface.

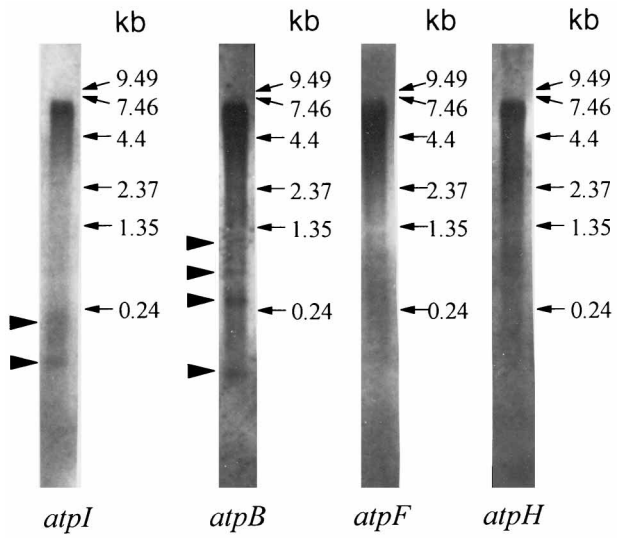

FIG. 6. Northern blots of total RNA from *C. thermoaceticum* after hybridization with different DNA probes. Total RNA extracts were subjected to electrophoresis in the presence of 2.3 M formaldehyde on 1.2% agarose gels. The RNA blots were hybridized to DNA probes specific for the genes *atpI* (lane 1), *atpB* (lane 2), *atpF* (lane 3), and *atpH* (lane 4) as described in Materials and Methods. The relative sizes of the RNA standards are indicated by arrows to the right of each lane. The positions of the smaller RNA fragments that hybridized to *atpI* and *atpB* probes are shown by arrowheads on the left.

ently stable secondary structures (inverted repeats) containing six and seven GC pairs, respectively (Fig. 3), which may reduce or inhibit the translational efficiency of mRNA (34, 40), but we do not have any data to support this possibility. It should be noted that the inverted repeats are also present in other *atp* genes (not shown).

TTG is found to be the start codon for *atpA* and *atpD* (Fig. 3), the two highly expressed genes of the C*tatp* operon. TTG has been described as one of the least preferred start codons in both *E. coli* (55) and *C. thermoaceticum* (36). These results indicate that the translational efficiencies of the *atp* genes in *C. thermoaceticum* are not influenced by the use of TTG as a start codon.

It should be noted that the Na-dependent ATP synthase from the obligately anaerobic gram-positive acetogenic bacterium *Acetobacterium woodii* has six different subunits (43) and a subunit structure identical to that of *C. thermoaceticum* (9). The simplest structure of the ATP synthase has been found in the gram-positive anaerobic bacterium *Clostridium pasteurianum*, which contains only four subunits, one in F_0 and three in F_1 (4, 5). These results suggest that in general, the ATP synthases from gram-positive anaerobic bacteria are simpler in subunit composition than those from other bacteria, including the anaerobic gram-negative bacterium *Propionigenium modestum* (27).

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