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Transcriptional Regulation of the Proton-Translocating ATPase (*atpIBEFHAGDC*) Operon of *Escherichia coli*: Control by Cell Growth Rate

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The F_0F_1 proton-translocating ATPase complex of *Escherichia coli*, encoded by the *atpIBEFHAGDC* operon, catalyzes the synthesis of ATP from ADP and P_i during aerobic and anaerobic growth when respiratory substrates are present. It can also catalyze the reverse reaction to hydrolyze ATP during nonrespiratory conditions (i.e., during fermentation of simple sugars) in order to maintain a electrochemical proton gradient across the cytoplasmic membrane. To examine how the *atp* genes are expressed under different conditions of cell culture, *atpI-lacZ* operon fusions were constructed and analyzed in single copy on the bacterial chromosome or on low-copy-number plasmids. Expression varied over a relatively narrow range (about threefold) regardless of the complexity of the cell growth medium, the availability of different electron acceptors or carbon compounds, or the pH of the culture medium. In contrast to prior proposals, *atp* operon expression was shown to occur from a single promoter located immediately before *atpI* rather than from within it. The results of continuous-culture experiments suggest that the cell growth rate rather than the type of carbon compound used for growth is the major variable in controlling *atp* gene expression. Together, these studies establish that synthesis of the F_0F_1 ATPase is not greatly varied by modulating *atp* operon transcription.

The proton-translocating ATPase of *Escherichia coli* is a member of the F_0F_1 class of ATPases that occur widely in bacteria and in the mitochondria and chloroplasts of eukaryotic organisms (7, 32). During aerobic cell growth, the ATPase of *E. coli* couples the energy derived from oxygen respiration to ATP synthesis by the process commonly known as electron transport-linked phosphorylation. During anaerobic growth, *E. coli* can also generate an electrochemical proton gradient by respiration using a variety of alternative electron acceptors, including nitrate, trimethylamine *N*-oxide (TMAO), dimethyl sulfoxide, and fumarate; the ATPase also functions to synthesize ATP under these conditions. However, when the cell performs anaerobic fermentations, it can generate ATP only by substrate-level phosphorylation reactions. During this growth mode, *E. coli* must employ the ATPase to hydrolyze ATP to generate the electrochemical proton gradient required to support other membrane functions, including solute transport and flagellar rotation (23).

The structure and function of the bacterial ATPase have been studied extensively (see reviews in references 7, 32, and 37). The ATPase of *E. coli* is composed of eight dissimilar polypeptides that are present in a stoichiometry of $a_1:c_{12}:b_2:\delta_1:\alpha_3:\gamma_1:\beta_3:\varepsilon_1$ (6). Its molecular mass is approximately 545 kDa for the combined F_0F_1 complex. The *atpIBEFHAGDC* genes that encode these subunits are located at 84 min on the *E. coli* chromosome map (2). An additional gene of unknown function, called *atpI*, precedes the other eight *atp* genes (3, 14, 37). The *atpI* gene product appears to be a hydrophobic protein, as judged from analysis of its predicted amino acid sequence. Analysis of the *atpIBEFHAGDC* mRNA revealed the location of the 5′ end that initiates at a position 73 nucleotides upstream of the start of *atpI* translation (13, 14, 28). All of the genes appear to be cotranscribed from a single promoter to give a 7-kb mRNA that terminates just following the *atpC* gene (9, 13, 28). Numerous investigations have documented the differential translation of the *atp* genes such that each subunit of the ATPase is produced in the appropriate amount for assembly into the mature F_0F_1 complex (1, 18, 19, 27, 29, 31, 35). This process involves an ordered endonucleolytic processing of the *atp* message to yield several stable intermediates that are then differentially translated. This processing involves the formation of complex mRNA secondary structures that allow different levels of ribosome binding and efficiencies of translation.

Given the relatively detailed understanding of *atp* mRNA translation, little is known about the control of *atp* operon transcription under the various conditions of *E. coli* growth. In this study, we examined the expression of *atpI-lacZ* operon fusions that were contained on the bacterial chromosome in a single copy or on low-copy-number plasmids and found that *atp* operon expression was remarkably constant under a variety of cell growth conditions.

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MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. The genotypes of the *E. coli* K-12 strains, bacteriophages, and plasmids used are given in Table 1. To construct the *arcA*, *fnr*, *himA*, and *fis* strains, an MC4100/λEK13 lysogen was infected with a P1 lysate which had been prepared from cells containing the specified deletion or mutation (21, 25). Mutant MC4100/λEK13 strains were selected by the transfer of antibiotic resistance. The Δ*arcA*::Kan^r phenotype was confirmed by the characteristic small colony size when cells were plated on

TABLE 1. *E. coli* K-12 strains, plasmids, and bacteriophages used

Strain, plasmid, or phage	Origin	Genotype or phenotype	Source
Strains			
MC4100		F$^-$ *araD139* (Δ*argF-lac*)*U169 rpsL150 relA1 flb5301 deoC1 ptsF25 rbsR*	33
PC2		Δ*fnr*	25
PC35	MC4100	Δ*arcA* Kan^r	25
SJP3	MC4100	*himA*Δ*82*	25
SJP4	MC4100	*fis767*	25
MRi93	MC4100	*pcnB*	16
Plasmids			
pHJUP1		*atpI*$^+$	13
pRS415		*lacZ*$^+$ *lacY*$^+$ *lacA*$^+$	34
pJM1	pRS415	*atpI′-lacZ lacY*$^+$ *lacA*$^+$	This study
pJM2	pRS415	*atpI*$^+$*B′-lacZ lacY*$^+$ *lacA*$^+$	This study
pJM3	pRS415	*atp′IB′-lacZ lacY*$^+$ *lacA*$^+$	This study
pEK13	pRS415	*atpI′-lacZ*$^+$	This study
Phages			
M13mp18			20
13SJP1811	M13mp18	*atpI*	This study
M13SJP18102	M13SJP1811	M13SJP1811 with an additional *Bam*HI site	This study
λRS45		*lacZ*$^+$ *lacY*$^+$ *lacA*$^+$	34
λEK13	λRS45	*atpI′-lacZ*$^+$	This study

toluidine blue plates (25). The *himA*::Tet^r phenotype was confirmed by the inability of Mu phage to form clear plaques on a *himA* mutant (25).

Construction of *atpI-lacZ* operon fusions. An *atpI-lacZ* fusion (pEK13) containing the first 13 codons of *atpI* and the upstream 1.3 kb of DNA was constructed by digesting plasmid pHJUP1 (13) with *Hin*dIII. The resulting 1.3-kb *atpI* fragment was inserted into M13mp18 to give phage M13SJP11. A *Bam*HI restriction site was introduced into *atpI* at codon 13 by site-directed mutagenesis to give M13SJP102. The 1.2-kb *Bam*HI fragment that contains the 5′ end of *atpI* and the upstream region was transferred into plasmid pRS45 (34) to give pEK13. The correct orientation of the *atpI-lacZ* fusion in pEK13 was confirmed by restriction enzyme digestions with *Sca*I and *Hin*dIII and by DNA sequence analysis. The fusion was then transferred onto λRS45 (34) to give phage λEK13. Lambda phages containing the 1.3-kb *atpI-lacZ* fusion were isolated by their ability to confer the Lac$^+$ phenotype to MC4100. A high-titer lysate of λEK13 was used to lysogenize MC4100. Since λ phages integrate at the *att* lambda integration site, the wild-type *atp* operon is preserved intact at 84 min on the chromosome. Single lysogens were identified by β-galactosidase assay and stocked for subsequent analysis.

To examine if additional promoter(s) are located within the *atpI* gene, various regions of the *atp* operon (Fig. 1) were PCR amplified from DNA isolated from MC4100, to introduce an upstream *Eco*RI site and a downstream *Bam*HI site. These fragments were then inserted into the corresponding sites of plasmid pRS415 to generate the *atpI-lacZ*, *atpI*$^+$*B-lacZ*, and *atpIB-lacZ* plasmids, pJM1, pJM2, and pJM3, respectively. The constructions were verified by DNA sequence analysis. Expression of the plasmid-borne fusions was monitored in a *pcnB* strain to maintain the fusions at low copy number in the cell (16).

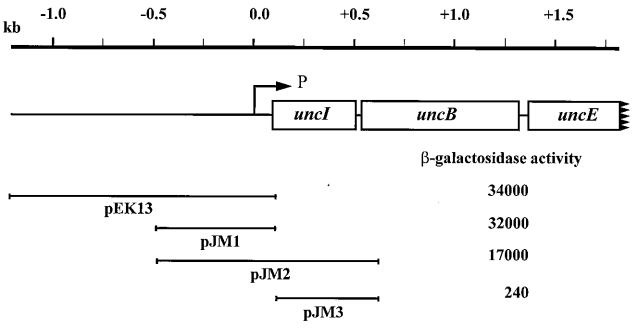

FIG. 1. Physical map of *atpI-lacZ* operon fusions used in this study. The *atpI-lacZ*$^+$ fusion plasmid pEK13 contains a 1.3-kb *Bam*HI fragment with the first 13 codons of the *atpI* gene and the associated upstream regulatory region from pHJUP1; this fragment was inserted into the *Bam*HI site of pRS415 to generate pEK13. Plasmid pJM1 is identical to pEK13 except that it contains only the 554 bp before *atpI*, while pJM2 contains *atp* DNA extending to codon 29 of *atpB*. Plasmid pJM3 contains DNA from the bp 18 of *atpI* to codon 29 of *atpB*. The boxed regions represent the indicated *atp* genes. The arrow indicates the direction of *atpIBE* transcription from the *atp* promoter. β-Galactosidase activity is expressed as in Table 2.

Cell growth. For strain, phage, and plasmid constructions, cells were grown on Luria broth (LB) or solid media (5, 25). When required, ampicillin, kanamycin, and tetracycline were added to the medium in concentrations of 80, 40, and 20 mg/liter, respectively. For β-galactosidase assay, cells were grown in a glucose-containing minimal medium (pH 7.0) unless otherwise indicated (4). Carbon compounds were added to the minimal medium at 40 mM. Buffered LB (5) was used to evaluate the effect of medium richness. Anaerobic cell growth was performed in 10-ml anaerobic tubes fitted with butyl rubber stoppers as previously described (5). Aerobic growth was performed by incubating 10-ml culture volumes in 150-ml flasks with loose-fitting caps shaken vigorously (380 rpm). All cultures were incubated at 37°C.

For anaerobic growth with the alternative electron acceptor nitrate, fumarate, or TMAO, a glucose- or a glycerol-containing minimal medium was supplemented with each of the respiratory compounds at a final level of 40 mM (5). Flasks were inoculated from overnight cultures of the medium grown under identical conditions, and the cultures were allowed to double four to five times to mid-exponential phase (optical density at 600 nm of 0.40 to 0.45; Kontron Uvikon 810 Spectrophotometer) before harvest. To determine the effect of pH on *atpI-lacZ* transcription, buffered media were adjusted to pH 5.7, 6.0, 6.3, 7.0, and 7.5 as previously described (4). The pH values were measured before and after cell growth; at the end of the experiment, the pH did not drop by more than 0.2 units.

Growth of cells in a continuous culture was performed as previously described (36). A Queue Mouse bioreactor (Queue Corporation, Parkersburg, W.V.) was fitted with a 2-liter vessel and operated at a 1-liter liquid working volume (36). The medium was Vogel-Bonner medium (pH 6.5) modified by addition of Casamino Acids (0.25 mg/liter) and by using glucose at a concentration of 2.25 mM to limit cell growth (i.e., a carbon-limited medium). Aerobic continuous culture conditions were maintained by saturating the culture medium with sterile air at a rate of 2.0 liters/min. Anaerobic conditions were maintained by continuously sparging the vessel with oxygen-free nitrogen at a rate of 200 ml/min. To vary cell growth rate (k), the medium addition rate was adjusted accordingly: the medium addition rates ranged from 2 to 16 ml/min (k = 0.12 to 0.96/h), which corresponded to cell doubling times of 350 and 58 min, respectively (23, 36). When cells were shifted to a new rate of growth, steady state was generally achieved in five reactor residence times.

β-Galactosidase assay. β-Galactosidase assays were performed as previously described (5). β-Galactosidase values represent the values of at least four experiments. Values usually did not vary by more than 6% from the mean. Protein concentration was estimated by assuming that a cell optical density of 1.4 at 600 nm is equal to 150 μg of protein per ml (21). Units of β-galactosidase are expressed as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolyzed per minute per milligram of protein. An extinction coefficient of 0.0045 was used for ONPG.

Molecular biology techniques. Transformation of *E. coli* and chromosomal and plasmid isolations were performed as described previously (17). DNA sequencing, using a Sequenase kit (version 2.0; U.S. Biochemical), and PCR am-

TABLE 2. Effects of carbon compounds on *atpI-lacZ* expression

Addition(s)[a]	β-Galactosidase activity (nmol of ONPG hydrolyzed/min/mg of protein)	
	$+O_2$	$-O_2$
Glucose	11,300	15,000
Galactose	15,000	14,800
Xylose	16,700	17,300
Pyruvate	23,500	21,500
Glycerol	21,500	NG[b]
Acetate	18,100	NG
Succinate	21,700	NG
Fumarate	23,400	NG
Malate	18,700	NG
LB	9,820	11,500
LB, glucose	7,600	8,300
LB, pyruvate	8,800	8,000

[a] MC4100/λEK13 cells were grown either in a minimal medium aerobically or anaerobically or in a LB with supplements as described in the text. Carbon compounds were added at a final concentration of 40 mM.
[b] NG, no growth.

plification, using a GeneAmp PCR system 2400 (Perkin-Elmer Cetus), were performed according to the manufacturer's instructions.

Materials. ONPG, ampicillin, kanamycin, and tetracycline were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS AND DISCUSSION

Effect of oxygen and carbon substrates on *atpI-lacZ* expression. To examine how the composition of the cell culture medium affects *atp* operon expression, an *atpI-lacZ* fusion containing the first 13 codons of *atpI* and the 1.3-kb upstream region was constructed (Fig. 1, pEK13). Following its insertion into the chromosome in single copy (see Materials and Methods), cells were grown under aerobic and anaerobic conditions in rich media or in minimal salts medium that contained single carbon compounds. During aerobic cell growth, *atpI-lacZ* expression did not vary by more than threefold (Table 2). Expression was highest when the tricarboxylic acid (TCA) cycle intermediates fumarate, malate, and succinate were used or when acetate and pyruvate, metabolic precursors of the cycle, were used. Expression was lowest when a rich medium (LB or a LB plus glucose) was used. During anaerobic cell culture conditions, *atpI-lacZ* expression did not vary significantly from that seen during aerobic conditions. Anaerobic cell growth on a glucose minimal salts medium led to expression about 30% higher than that seen during aerobic growth.

In light of the findings of Futai et al. (7, 15, 22), we investigated the possibility that there were additional weak *atp* promoters located within the *atpI* gene. To do this, we constructed a series of plasmid-borne *atpI-lacZ* fusions (Fig. 1). These plasmids were then introduced into an *E. coli* strain harboring a *pcnB* (11) mutation, and the β-galactosidase activity was determined in batch cultures grown aerobically in buffered LB. The strain with plasmid pJM3 that contained all of *atpI* except its first 13 codons displayed approximately 0.7% of the activity measured from pEK13, which has 1.3 kb of DNA upstream of *atpI*. This result indicates that there is little to no transcription originating from within the *atpI* open reading frame. The levels of β-galactosidase activity in cells containing either plasmid pEK13 or plasmid pJM1 were about the same (ca. 32,000 to 34,000 U). However, the level of β-galactosidase directed from the *atpI*$^+$*B-lacZ* fusion on pJM2, which includes all of *atpI* and the first 29 codons of *atpB*, was approximately 50% of that measured from pJM1. This difference is most likely due to the instability of the mRNA containing the *atpI* reading frame, since there is proposed to be a site for endonucleolytic cleavage within *atpI* (19). Thus, *atp* operon expression appears to be the result of transcription from a single promoter located 73 bp upstream of *atpI* (13, 14, 28). All subsequent studies were performed with the λEK13 *atpI-lacZ* operon fusion.

TABLE 3. Effects of alternative electron acceptors on *atpI-lacZ* expression

Electron acceptor added[a]	β-Galactosidase activity (nmol of ONPG hydrolyzed/min/mg of protein)	
	Glucose	Glycerol
None	15,000	NG[b]
Oxygen	11,300	21,500
Nitrate	10,400	18,900
Fumarate	12,400	21,100
TMAO	13,800	23,400

[a] MC4100/λEK13 cells were grown on minimal medium as described in the text. The electron acceptors were present at 40 mM except for oxygen, in which case the medium was saturated with air by vigorous shaking.
[b] NG, no growth.

Effects of respiratory substrates on *atpI-lacZ* expression. As *E. coli* can respire by using oxygen, nitrate, TMAO, or fumarate as an electron acceptor, we tested how the presence of each of these compounds affected *atpI-lacZ* expression (Table 3). When glucose was used as a carbon source, *atpI-lacZ* expression varied by no more than about 30% with the different electron acceptors. Interestingly, expression was highest when no electron acceptor was provided (i.e., conditions in which the cell must ferment). When glycerol was used in place of glucose, *atpI-lacZ* expression was elevated by almost twofold for each respiratory condition tested. Thus, the type of carbon substrate used for cell growth appears to have a greater influence on the level of *atp* gene expression than does the presence of a respiratory substrate.

Effect of medium pH on *atpI-lacZ* expression. Since the cell maintains a relatively constant internal pH, we examined whether changes in the pH of the cell culture medium affect *atpI-lacZ* expression. Cells were grown in a buffered glucose minimal medium at pH values ranging from pH 5.7 to 7.5. Aerobically, *atpI-lacZ* expression was remarkably constant, not varying more than +5% (Table 4). During anaerobic cell culture, cells did not grow well below pH 6; however, above this pH value, gene expression increased modestly. Gene expression was higher under anaerobic than aerobic conditions for each pH value tested. Together, these findings suggest that the hydrogen ion concentration of the bacterial culture medium is not an important variable for control of *atp* gene expression in *E. coli.*

TABLE 4. Effect of medium pH on *atpI-lacZ* expression[a]

Medium pH	β-Galactosidase activity (nmol of ONPG hydrolyzed/min/mg of protein)	
	$+O_2$	$-O_2$
5.7	11,700	ND
6.0	11,700	ND
6.3	11,000	13,800
7.0	11,300	15,000
7.5	11,100	15,800

[a] MC4100/λEK13 cells were grown in a glucose minimal medium adjusted to different pH values as previously described (4). ND, not determined.

TABLE 5. Effects of oxygen and mutations in the *arcA*, *fnr*, *fis*, and *himA* genes on *atpI-lacZ* expression

Strain[a] (relevant genotype)	β-Galactosidase activity (nmol of ONPG hydrolyzed/min/mg of protein)	
	$+O_2$	$-O_2$
MC4100 (wild type)	11,300	15,000
SJP1 (Δ*arcA*)	10,800	14,500
PC2 (Δ*fnr*)	10,500	10,900
SJP4 (Δ*fis*)	15,000	16,000
SJP3 (Δ*himA*)	8,700	12,400

[a] Cells containing λEK13 were grown in a glucose minimal medium either aerobically or anaerobically as described in the text.

Effects of *arcA* and *fnr* mutations on *atpI-lacZ* expression. The regulation of many of genes in *E. coli* involved with aerobic and anaerobic metabolism, including respiratory pathway genes, the TCA cycle genes, and fermentation-related genes, is modulated by the *arcA* and *fnr* gene products (8, 10, 12). We tested whether mutations in either of these two regulatory genes affect *atpI-lacZ* expression. Little to no difference was observed during either aerobic or anaerobic growth in a glucose minimal medium of *fnr*, *fnr arcA*, or *arcA* strains (Table 5). Similar results were observed when strains defective in the *himA* (encoding integration host factor) and *fis* genes were examined.

Effect of cell growth rate on *atpI-lacZ* expression. When cells were grown in batch culture in different types of media, we noted that *atpI-lacZ* expression was higher in a minimal medium containing two-, three-, or four-carbon compounds than in a rich medium (Table 2). Cell growth rates were determined on each type of medium, and when the generation times were graphed versus the level of *atpI-lacZ* expression, an inversely proportional relationship was seen (Fig. 2).

To determine if the variation in *atp* gene expression was due to the type of carbon used or to differences in cell growth rate, we also examined *atpI-lacZ* expression in cells grown in continuous culture (Fig. 3). Expression also varied by threefold and was higher at slow ($k = 0.12\ h^{-1}$) that at fast ($k = 0.96\ h^{-1}$) cell doubling times. Anaerobiosis had little effect on *atp* gene expression. Since cell growth in these experiments was carbon limited (i.e., by glucose), it appears that the rate of cell growth rather than the type of carbon used is the major determinant in controlling *atp* operon expression. In support of this proposal, Sakai-Tomita et al. (30) noted that the synthesis of the ATPase of *Vibrio parahaemolyticus* was catabolite repressed, whereas it was not in *E. coli.*

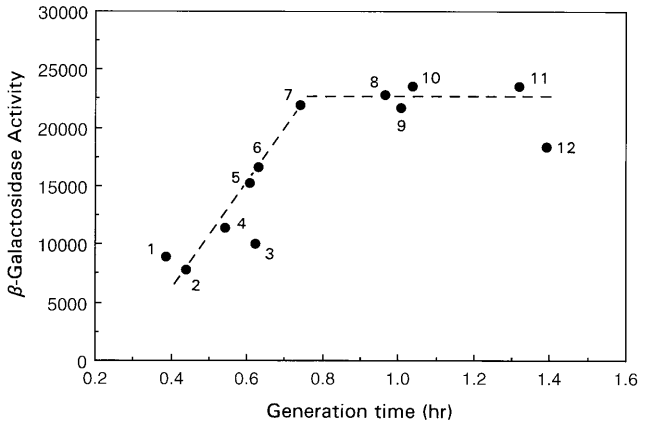

FIG. 2. Effect of cell growth rate on *atpI-lacZ* expression in batch culture. Cells were grown in the indicated medium, and the cell generation time (hours) was recorded. Medium or carbon compound used for cell growth: 1, LB plus pyruvate; 2, LB plus glucose; 3, LB; 4, glucose; 5, galactose; 6, xylose; 7, succinate; 8, malate; 9, glycerol; 10, fumarate; 11, pyruvate; 12, acetate.

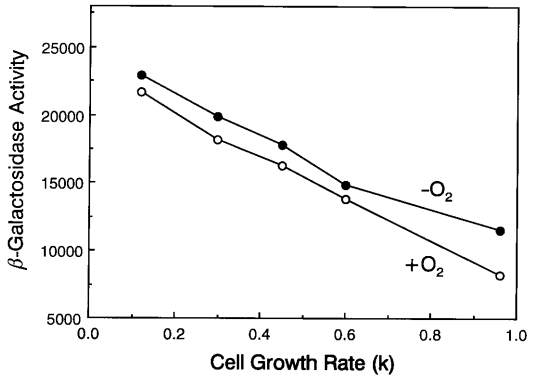

FIG. 3. Effect of cell growth rate on *atpI-lacZ* expression in continuous culture during aerobic and anaerobic growth conditions. Cells were grown at the indicated growth rates as described in Materials and Methods. For aerobic growth, air was maintained at 100% saturation in the culture medium throughout the experiment. For anaerobic growth, the vessel was sparged with O_2-free nitrogen (200 ml/min). β-Galactosidase activity is expressed as nanomoles of ONPG hydrolyzed per minute per milligram of protein. Cells were grown aerobically (○) or anaerobically (●) as described in Materials and Methods.

Regulatory implications. During most conditions, the proton-translocating ATPase of *E. coli* is used for cellular energy generation via electron transport-linked phosphorylation reactions, as is typical of the ATPases of many bacterial species and of eukaryotic cells (7, 32). Although there have been extensive studies addressing translational regulation of the *atp* operon that encodes the ATPase, little was known about how the *atp* genes are transcribed in response to cell growth under different environmental conditions. In this study, we demonstrate that *atp* operon expression, as measured by using *atpI-lacZ* fusions, remains within a remarkably narrow range regardless of the medium composition, extracellular pH, or the presence of respiratory substrates (Tables 2 and 3). Gene expression did not vary by more than threefold. Expression was highest in a medium containing carbon compounds that do not yield ATP by substrate-level phosphorylation (i.e., TCA cycle intermediates or acetate), whereas it was lowest when a glucose-containing minimal medium or a rich medium (i.e., LB) was used. The reduced level of *atpI* and *atpB* gene expression under these latter conditions correlates with a reduced need for cellular ATP synthesis when the biosynthetic intermediates are available from the LB medium.

Since the ATPase complex is involved in proton translocation across the cytoplasmic membrane, we were interested in determining whether any changes in external H^+ concentration or anaerobiosis affected ATPase gene expression. No effect on *atpI* expression when the medium pH was varied (Table 5). It appears that transcription of the *atp* operon in *E. coli* is not dynamically modulated as a strategy to regulate ATPase capacity within the cell. Rather, the results suggest that the number of ATPase complexes remains relatively constant under different cell culture conditions that include anaerobic respiration or fermentation (Tables 2 and 3). The ATPase capacity of the cell is apparently sufficient to maintain the electrochemical proton gradient during changes in extracellular pH encountered under different cell growth conditions.

Such conditions include those in which the cell must use the ATPase to hydrolyze ATP rather than synthesize it. Since *atp* operon expression was modestly higher under anaerobic fermentative conditions, ATP hydrolysis does not require significantly elevated levels of the ATPase complex.

Gene expression in batch cell culture correlates well with *atp* expression occurring during continuous culture (Fig. 2 and 3). An inverse pattern of *atp* gene expression was observed with cell growth rate and was similar to the pattern observed for expression of the *gltA* (citrate synthase) and *sdhCDAB* (succinate dehydrogenase) genes of the TCA cycle (24–26). Expression of the *atp*, *sdhCDAB*, *mdh*, and *gltA* genes was highest in a minimal medium when cells were grown on TCA cycle intermediates or with acetate, in which case cells grew more slowly than they did in rich medium. The cells must expend more energy in order to synthesize cellular intermediates needed for macromolecule production. Since *E. coli* only modestly varies the level of *atpIBEFHAGDC* operon transcription, it will be of interest to determine whether other types of bacteria regulate ATPase genes in a similar way.

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