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Bacillus subtilis F_0F_1 ATPase: DNA Sequence of the *atp* Operon and Characterization of *atp* Mutants

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We cloned and sequenced an operon of nine genes coding for the subunits of the *Bacillus subtilis* F_0F_1 ATP synthase. The arrangement of these genes in the operon is identical to that of the *atp* operon from *Escherichia coli* and from three other *Bacillus* species. The deduced amino acid sequences of the nine subunits are very similar to their counterparts from other organisms. We constructed two *B. subtilis* strains from which different parts of the *atp* operon were deleted. These *B. subtilis atp* mutants were unable to grow with succinate as the sole carbon and energy source. ATP was synthesized in these strains only by substrate-level phosphorylation. The two mutants had a decreased growth yield (43 and 56% of the wild-type level) and a decreased growth rate (61 and 66% of the wild-type level), correlating with a twofold decrease of the intracellular ATP/ADP ratio. In the absence of oxidative phosphorylation, *B. subtilis* increased ATP synthesis through substrate-level phosphorylation, as shown by the twofold increase of by-product formation (mainly acetate). The increased turnover of glycolysis in the mutant strain presumably led to increased synthesis of NADH, which would account for the observed stimulation of the respiration rate associated with an increase in the expression of genes coding for respiratory enzymes. It therefore appears that *B. subtilis* and *E. coli* respond in similar ways to the absence of oxidative phosphorylation.

ATP plays a central role in energy transduction in living organisms. Although some ATP is synthesized in aerobiosis by soluble enzyme systems like glycolytic enzymes that perform substrate-level phosphorylation, most is synthesized by membrane-bound enzyme complexes through oxidative phosphorylation. The energy-transducing membranes, where these complexes are found, are the plasma membrane of prokaryotic cells, the inner membrane of mitochondria, and the thylakoid membrane of chloroplasts. ATP synthesis from ADP and P_i is catalyzed by the ATP synthase complex and is driven by the proton gradient. This gradient is generated by respiration in mitochondria and respiring bacteria and by photosynthesis in chloroplasts and photosynthetic bacteria.

ATP synthases from different sources have very similar structures. They consist of two main subcomplexes: F_1, the extrinsic membrane subcomplex, and F_0, the integral membrane subcomplex. In bacteria, the F_1 portion consists of five subunits, α, β, γ, δ, and ε, and the F_0 portion consists of three subunits, a, b, and c (16, 18, 23, 30). The stoichiometry of *Escherichia coli* F_1 and F_0 was determined (3, 65, 17): F_1 has the composition $\alpha_3\beta_3\delta\gamma\varepsilon$, and F_0 has the composition ab_2c_{10-12}. Kagawa (29) defined three functions for the various subunits of ATPase: (i) the synthesis or hydrolysis of ATP was attributed to the F_1 subunits α, β, and γ; (ii) the transmembrane proton transport was assigned to the three F_0 subunits, a, b, and c, which form a proton channel, and (iii) the gate function, connecting the ATPase with channel activity, was attributed to F_1 subunits γ, δ, and ε and to F_0 subunits a and b.

The sequences of the ATPase structural genes from a variety of bacteria have been determined. F_1 *atp* genes are designated *atpHAGDC* and code for subunits δ, α, γ, β, and ε, respectively. The F_0 *atp* genes are *atpBEF* and code for subunits a, c, and b. In some species, such as the purple nonsulfur photosynthetic bacteria *Rhodopseudomonas blastica* (63) and *Rhodospirillum rubrum* (14), the F_0 genes are not adjacent to the F_1 genes. In the cyanobacteria *Synechococcus* strain PCC6301 (10) and *Anabaena* strain PCC7120 (11), the F_0 genes are adjacent to the genes for the δ, α, and γ subunits, but the genes for the β and ε subunits are not. However, in *E. coli* (68), *Bacillus megaterium* (4), *Bacillus firmus* (27), the thermophilic bacterium *Bacillus* strain PS3 (47), and *Mycoplasma gallisepticum* (51), all ATPase genes are arranged in a single operon in the order *atpIBEFHAGDC*. In *E. coli*, *atpI*, the first gene of the operon, encodes a 14-kDa protein of unknown function and which is not required for ATPase activity (65).

ATP synthase is dispensable in *E. coli*. However, mutations in the ATP synthase operon lead to several metabolic alterations. *atp* mutants have been isolated by using various selections, including loss of growth on a nonfermentable carbon source such as succinate, without impairing growth on glucose or glycerol (13); resistance to neomycin (31, 52); and resistance to *N,N*-dicyclohexylcarbodiimide (DCCD) in the presence of succinate as the sole energy and carbon source (15, 57). Mutants which could not synthesize ATP through oxidative phosphorylation were identified among both mutants unable to grow on succinate and those resistant to neomycin. Neomycin is accumulated in cells by an energy-dependent process which is impaired in these *atp* mutant strains. F_0F_1 ATPase from mutants resistant to DCCD showed altered interaction with this inhibitor but were apparently not defective in oxidative

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phosphorylation (57). Recently, an *E. coli* mutant in which the entire *atp* operon was deleted was studied. It displayed changes in carbon and energy metabolism in the absence of oxidative phosphorylation (28). The mutant presents an increased flow through the tricarboxylic acid cycle and the glycolytic pathways. These changes lead to both increased substrate-level phosphorylation and increased production of reducing equivalents. Respiration in this strain is stimulated, which neutralizes this excess of reducing equivalents and maintains a redox equilibrium. Nevertheless, respiration is not coupled to ATP synthesis (28).

Bacillus subtilis is the most extensively studied gram-positive bacterium. However, very little is known about oxidative phosphorylation in this aerobic organism. Recently Sutherland et al. localized F_1 ATPase like genes at the *Not*I site at position 3780 on the *Sfi*I-*Not*I physical map of *B. subtilis* (60). In the course of the *B. subtilis* genome sequencing project, we cloned and sequenced an operon of nine genes highly similar to other *Bacillus atp* operons and to the *E. coli atp* operon. This operon includes the *atp*-like genes found by Sutherland et al. (60). We constructed *B. subtilis* strains in which part of this operon is deleted. These are to our knowledge the first well-characterized mutants of oxidative phosphorylation from a gram-positive bacterium. To investigate energy and carbon flow in *B. subtilis*, we compared various features such as growth rate, growth yield, by-product formation, and respiration rate in the *atp* mutants and the wild-type strain.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All *B. subtilis* strains used throughout this work are derivatives of strain 168. The *narA* locus was cloned by complementation of the *narA1 sacA321* strain QB692 (34). *E. coli* P2392 (58) was used as the host for propagation of lambda. *E. coli* strains used for DNA sequencing were XL1-blue (5) and TG1 (19). Plasmid rescue cloning was performed in *E. coli* TP611 (*pcnB*) (21).

Luria-Bertani (LB) medium was used for standard cultures of *B. subtilis* and *E. coli* (40). Sporulation medium (SP) was prepared as described by Schaeffer et al. (56). 2YT (yeast-tryptone) medium was used for M13 recombinant phage preparation. The minimal salts medium used for *B. subtilis* growth was C medium (43), ferric ammonium citrate being replaced by ferric chloride as the iron source. The carbon source, glucose or succinate, was added to a final concentration of 4 g/liter. Tryptophan was added to a final concentration of 20 mg/liter. Nucleotide labeling was performed in a low-phosphate medium as described by Msadek et al. (44) except that the nitrogen source was glutamine at a final concentration of 20 mM. Antibiotics were added when necessary to the following concentrations: ampicillin, 100 mg/liter; chloramphenicol, 5 mg/liter; and kanamycin, 5 mg/liter. Bacteria were grown at 37°C in all experiments. The optical density (OD) of bacterial cultures was measured at 600 nm with a Hitachi U-1100 spectrophotometer.

Phages and plasmids. A *B. subtilis* gene bank was constructed in lambda FixII (Stratagene) (32). DNA sequences were determined from subclones in phage M13mp8 (39) and plasmid pUC18 (69). Plasmid rescue cloning was performed after subcloning in the integrative vector pDIA5304 (21). Transcriptional fusions with the *E. coli lacZ* gene were constructed by using integrative plasmids pJM783 (50) and pDIA5307 (6). The *Cla*I DNA fragment containing the kanamycin resistance gene *aphA3* from plasmid pAT21 (62) was cloned in plasmid vector pMTL22 (8) to introduce the convenient restriction sites on both ends of the cassette. The resulting plasmid was named pDIA5337.

DNA methods and genetic techniques. *E. coli* was transformed as described by Chung and Miller (9). Shotgun libraries in M13mp8 or in pUC18 were introduced into *E. coli* XL1-blue by transformation as described by Hanahan (22). Recombinant plasmids were transferred to *E. coli* TP611 by calcium chloride transformation (53). *B. subtilis* cells were transformed as described by Kunst et al. (33). Southern blotting and plaque or colony transfers were performed as described by Sambrook et al. (53). Membranes were further hybridized with nonradioactively labeled probes (Boehringer digoxigenin-UTP labeling or Amersham enhanced chemiluminescence).

The plasmid rescue cloning method (45) was used to clone the DNA region adjacent to the chromosomal λ*narA4* insert (described in Results). After a first cloning step in pUC18, the 1.1-kb-long *Sal*I-*Hin*dIII DNA fragment at one end of the lambda insert was converted to a *Hin*dIII fragment and then cloned in pDIA5304. The resulting plasmid was integrated into the chromosome by a Campbell-type event. Chromosomal DNA of this strain was prepared, and the appropriate integration of the plasmid was checked by Southern blotting. Chromosomal DNA was digested by *Not*I, ligated at a low DNA concentration (5 ng/μl), and used to transform *E. coli* TP611. Six independent transformants were analyzed; they all contained plasmids with the same identical restriction map. One of them (pDIA5329) was more extensively characterized and sequenced (Fig. 1).

The sequencing strategy used has been extensively described elsewhere (21, 41). DNA sequences were compiled by using the program XBAP of Dear and Staden (12). Sequences were analyzed with DNA Strider 1.1 software (35). To search for similar sequences, the FASTA (48) (in Swissprot release 29) and BLAST (1) programs were used. The search with the BLAST program was performed at the National Center for Biotechnology Information in the nonredundant protein library from the National Center for Biotechnology Information. Sequences were compared by using the Wisconsin Genetics Computer Group sequence analysis software package, version 6.0 (University of Wisconsin Biotechnology Center, Madison). The SubtiList database (42) was used to search for sequence patterns in *B. subtilis* sequences.

Construction of fusion and mutant strains. *B. subtilis* strains containing a transcriptional fusion between the *E. coli lacZ* gene and the *atp* operon as well as the *ctaA*, *ctaB*, and *ctaD* genes from the cytochrome *c* oxidase locus (55) were constructed as follows. The 3.3-kb *Eco*RI-*Kpn*I DNA fragment from plasmid pDIA5329, encompassing the 5′ end of the *upp* gene and the proximal part of the *atp* operon, was inserted between the *Eco*RI and *Kpn*I sites in pDIA5307 to give pDIA5330 (Fig. 1). The *lacZ* gene was thus placed after the 169th codon of the *atpH* gene, creating a transcriptional fusion. To construct fusions with *cta* genes, the 6-kb *Sal*I-*Eco*RI DNA fragment encompassing *ctaA*, *ctaB*, *ctaC*, and the first 51 codons of *ctaD* was first subcloned in pBluescript to give pDIA5331 (Fig. 1). The 3.5-kb *Bgl*II-*Bam*HI DNA fragment was then inserted at the *Bam*HI site of pJM783 to obtain a transcriptional fusion between *ctaA* and *lacZ* (plasmids pDIA5332) or between *ctaD* and *lacZ* (plasmid pDIA5333) according to the orientation of the cloned insert (Fig. 1). A transcriptional fusion with *ctaB* was constructed by inserting the *Bgl*II-*Nco*I DNA fragment from plasmid pDIA5331 encompassing the *ctaA-ctaB* intergenic region and the first 299 codons of *ctaB* into the polylinker of pMTL22 (8). This DNA fragment was then excised as an *Eco*RV-*Bgl*II fragment and

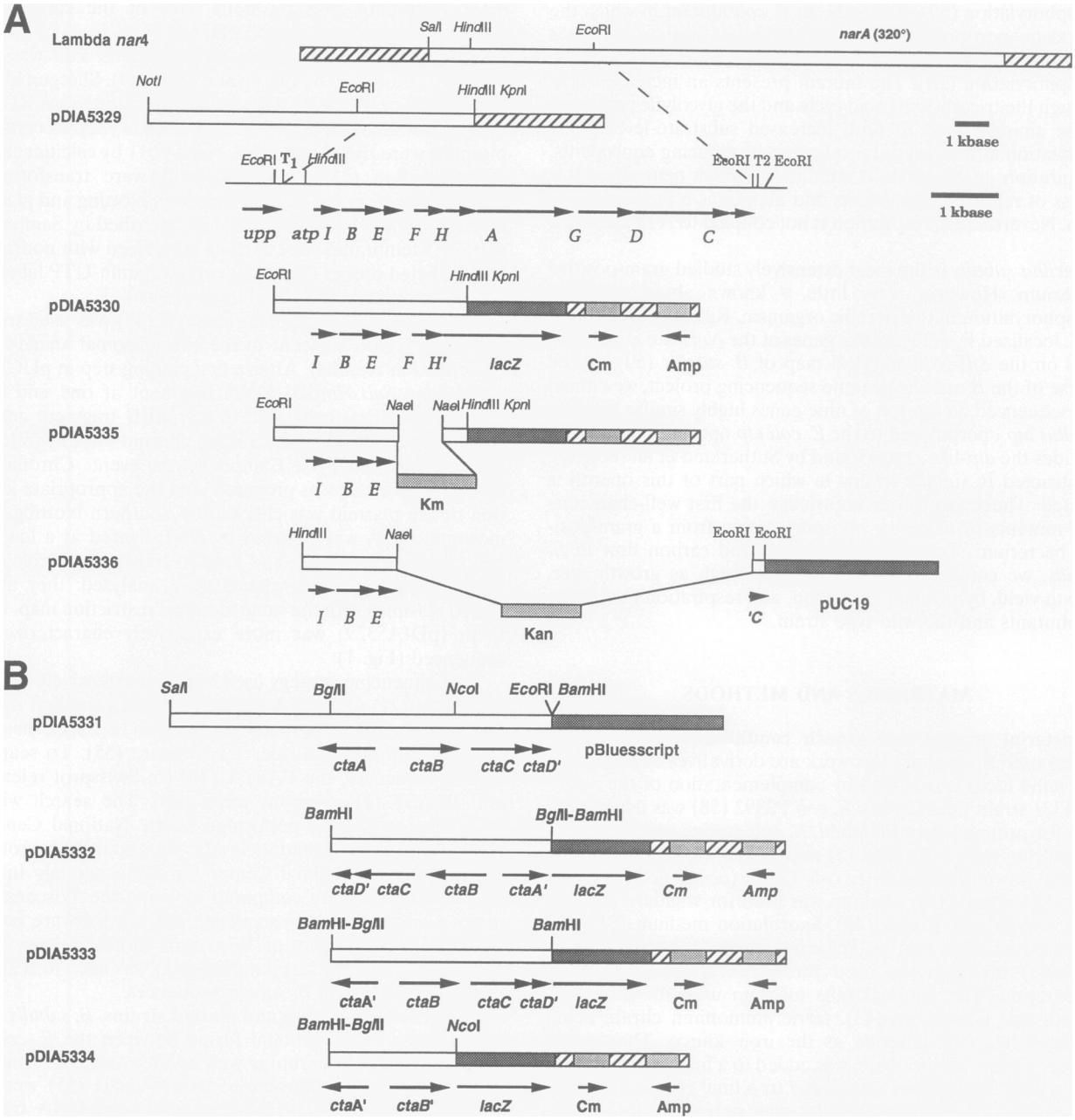


FIG. 1. (A) Restriction maps of λ*narA4* and of plasmids pDIA5329, pDIA5330, pDIA5335, and pDIA5336. (B) Restriction maps of plasmids pDIA5331, pDIA5332, pDIA5333, and pDIA5334. The *B. subtilis* DNA inserts are presented as open bars. Genes are represented by arrows. T1 and T2 are two putative transcription terminators. The initiation codon and the RBS of the *lacZ* gene originate from the *B. subtilis spoVG* gene (50). Ap, Cm, and Km refer to the ampicillin resistance gene from pBR322 (53), the chloramphenicol resistance gene from pC194 (25), and the kanamycin resistance gene *aphA3* (62) from pDIA5337 (this work). The vector part was not drawn to scale.

cloned in pJM783 digested with *Bam*HI and *Sma*I to give pDIA5334 (Fig. 1).

The four transcriptional fusions were introduced into the *B. subtilis* chromosome by Campbell-type recombination events. The previously described transcriptional fusion between *qoxA* and *lacZ* was also used (54).

Two *B. subtilis* strains from which part of the *atp* operon was deleted were constructed by homologous recombination using the following constructions. The *Nae*I restriction fragment containing *atpF* and the first 53 codons of *atpH* was replaced in plasmid pDIA5330 by a kanamycin resistance cassette (*aphA3*), yielding pDIA5335 (Fig. 1). To obtain a large deletion of the *atp* operon, pDIA5336 (Fig. 1) was constructed as follows: the *Eco*RI fragment encompassing the 3′ end of *atpC* gene was first cloned in pUC18; then the *Hin*dIII-*Nae*I fragment from pDIA5330 containing the beginning of the *atp* operon was inserted in the resulting plasmid digested by *Hin*dIII and *Hin*cII; finally, the kanamycin cassette was inserted between the two cloned fragments to give pDIA5336. Plasmids pDIA5335 and pDIA5336 were linearized and used to transform *B. subtilis*. Strains in which the wild-type *atp* operon was replaced by the disrupted copy, 168 Δ*atp*1 (pDIA5335) and

168 Δ*atp*2 (pDIA5336), were selected as kanamycin-resistant transformants.

Southern blotting was used to confirm the appropriate substitution of the wild-type *atp* genes by the mutated copy in mutant strains and that only a single copy of the *lacZ* fusion had integrated.

Respiration rate. Respiration rates of wild-type and mutant strains were determined with a Clark-type electrode as described by Jensen and Michelsen (28).

By-product and glucose measurement. Production of overflow metabolites was detected and measured by high-pressure liquid chromatography (HPLC) using a Perkin-Elmer series 3B liquid chromatograph (Perkin-Elmer, Norwalk, Conn.). Elutions were followed with a Perkin-Elmer LC25 refractive index detector equipped with a Sigma 15 integrator. Samples were filtered through SJHV 0.45-μm-pore-size filter units (Millipore Corp., Bedford, Mass.). Products were separated on an Aminex HPX 87H strong cation-exchange resin column (catalog no. 125-0140; Bio-Rad Laboratories, Richmond, Calif.); the column was protected with a cation H microguard column (catalog no. 125-0129; Bio-Rad). The column was eluted at 40°C with 0.7 ml of 0.01 N H_2SO_4 per min. Soluble fermentation products were identified by comparison with the retention time of the corresponding standards.

Glucose was assayed enzymatically, using a commercial test kit supplied by Merck (Merckotest 14365) and by HPLC. Dry weight was deduced from the determination of total soluble protein: the dry weight was calculated as double the soluble protein weight.

ATP/ADP ratios. In vivo labeling of nucleotides with $^{33}P_i$ was performed during early exponential phase (OD = 0.1 to 0.15) in minimal medium containing 0.1 mM phosphate. Bacteria were lysed by addition of an equal volume of 2 M formic acid to an aliquot of the culture. After precipitation of bacterial debris, nucleotides were separated by two-dimensional chromatography on polyethyleneimine-cellulose plates (polygram CEL 300 PEI; Macherey Nagel) as described by Cashel and Gallant (7). The radioactivity of each spot was quantified with a PhosphorImager (Molecular Dynamics). To localize unambiguously ATP and ADP spots, a mixture of ^{32}P-labeled ATP and ADP was separated under the same conditions.

β-Galactosidase assays. β-Galactosidase activity was assayed as previously described (43) and expressed in Miller units per milligram of protein (40). Protein concentrations were determined with a Bio-Rad protein assay kit.

Nucleotide sequence accession number. The DNA sequence reported here has been assigned GenBank TM/EMBL accession number Z28592.

RESULTS

Cloning and DNA sequencing of the *atp* operon. In the framework of the *B. subtilis* genome sequencing project, the DNA region between *gerB* and *sacXY* was assigned to us (from 314° to 333° on the *B. subtilis* genetic map [2]). Within this chromosomal region, we cloned the *narA* gene by complementation of the *narA1* marker (320°) of *B. subtilis* QB692 (20). Plasmid pDIA5338 (20) harboring *narA* was used as a probe to screen the lambda library constructed in λ FixII (32). Six positive lambda clones were isolated. Their restriction maps suggested that they contain largely overlapping chromosomal fragments. λ*narA4*, containing the largest insert (Fig. 1), was retained for DNA sequencing. Southern blotting showed the absence of cocloning and of rearrangement in the insert. The nucleotide sequence of the 18-kb insert was determined on both strands in a single shotgun cloning experiment.

Within this 18-kb DNA fragment, five adjacent coding sequences, highly similar to bacterial *atpHAGDC* genes, were identified. It appears, therefore, that this fragment contains the 3′ end of the *B. subtilis atp* operon. The 5′ end of the operon was cloned by the plasmid rescue method described in Materials and Methods. Using this strategy, we obtained plasmids harboring an insert ending at a *Not*I site located 9.5 kb from the end of the lambda insert. The 10.5-kb insert from the resulting plasmid, pDIA5329 (Fig. 1), was completely sequenced on both strands. This plasmid contains the beginning of the *atp* operon, as shown in Fig. 1.

Analysis of the DNA sequence and of the predicted proteins. The complete nucleotide sequence of the *atp* operon and the deduced protein sequences encoded by the nine *atp* genes are presented in Fig. 2. The start codon of each gene was designated according to data from the purified proteins from other organisms such as *E. coli* (67) and *Bacillus* strain PS3 (47), alignments of *atp* gene product sequences (see below), and positions of potential ribosome binding sites (RBSs). TTG start codons are proposed for *atpB* and *atpG*, GTG codons are proposed for *atpA*, and ATG codons are proposed for the other six genes in the operon. The deduced protein sequences of the nine *atp* gene products were aligned with sequences of ATPase subunits of various origins, using the local homology algorithm of Smith and Waterman (59). The alignment scores with ATPase subunit sequences of three other *Bacillus* strains (*B. megaterium* [4], thermophilic bacterium PS3 [47], and *B. firmus* [27]) and two more distantly related bacteria (*E. coli* [68] and *Synechococcus* strain PCC6716 [64]) are presented in Table 1. Similarities with *B. firmus* subunits are lower than with *B. megaterium* and PS3 subunits, as expected both from the phylogenetic trees of the *Bacillus* strains and from the extreme alkaliphile growth conditions of *B. firmus*. The product of *E. coli aptI* is not essential for the activity of the complex (66), and in *B. megaterium*, transcription of the *atpB* gene seems to initiate within the *atpI* gene (4). However, amino acid sequence conservation between the *atpI* gene products from the four *Bacillus* strains is high. Similarly, the general features of the eight subunits are conserved in *B. subtilis* ATPase. Subunits α, β, and c are the best conserved, and the DCCD binding pocket in subunit c is particularly well conserved.

Upstream from the *atp* operon is a palindromic sequence followed by seven T residues. This may be a transcription terminator for the preceding *upp* gene (36). A similar structure is found just after the *atpC* gene and may be the transcription terminator of the *atp* operon. We searched for other putative secondary structures in the DNA sequence. A number of short and three longer palindromes were identified. These three longer sequences are in the three long intergenic regions: *atpB-atpE*, which is 45 bp long, *atpE-atpF*, which is 162 bp long, and *atpA-atpG*, which is 76 bp long. Analysis of *atp* operons from other organisms showed that these three intergenic regions are also long in *atp* operons from *E. coli* and other *Bacillus* species. We have compared the noncoding regions of the four *Bacillus atp* operons available. The motif found in the *atpE-atpF* intergenic region and the surrounding sequences are highly conserved in the four *atp* operons (Fig. 3). We searched for similar structures in known *B. subtilis* DNA sequences by using the SubtiList database (42). Box A (Fig. 3) is also found at the 5′ end of some tRNA genes and at the 5′ end of the 5S rRNA, and box B (Fig. 3) is similar to the 3′ end of tRNA genes including the mature tRNA terminal sequence CCA. However, this sequence seems not to be conserved in the *E. coli atpE-atpF* intergenic region. We did not identify any other conserved sequences in the noncoding region of the *atp* operon: no sequences were similar to the DNA region con-

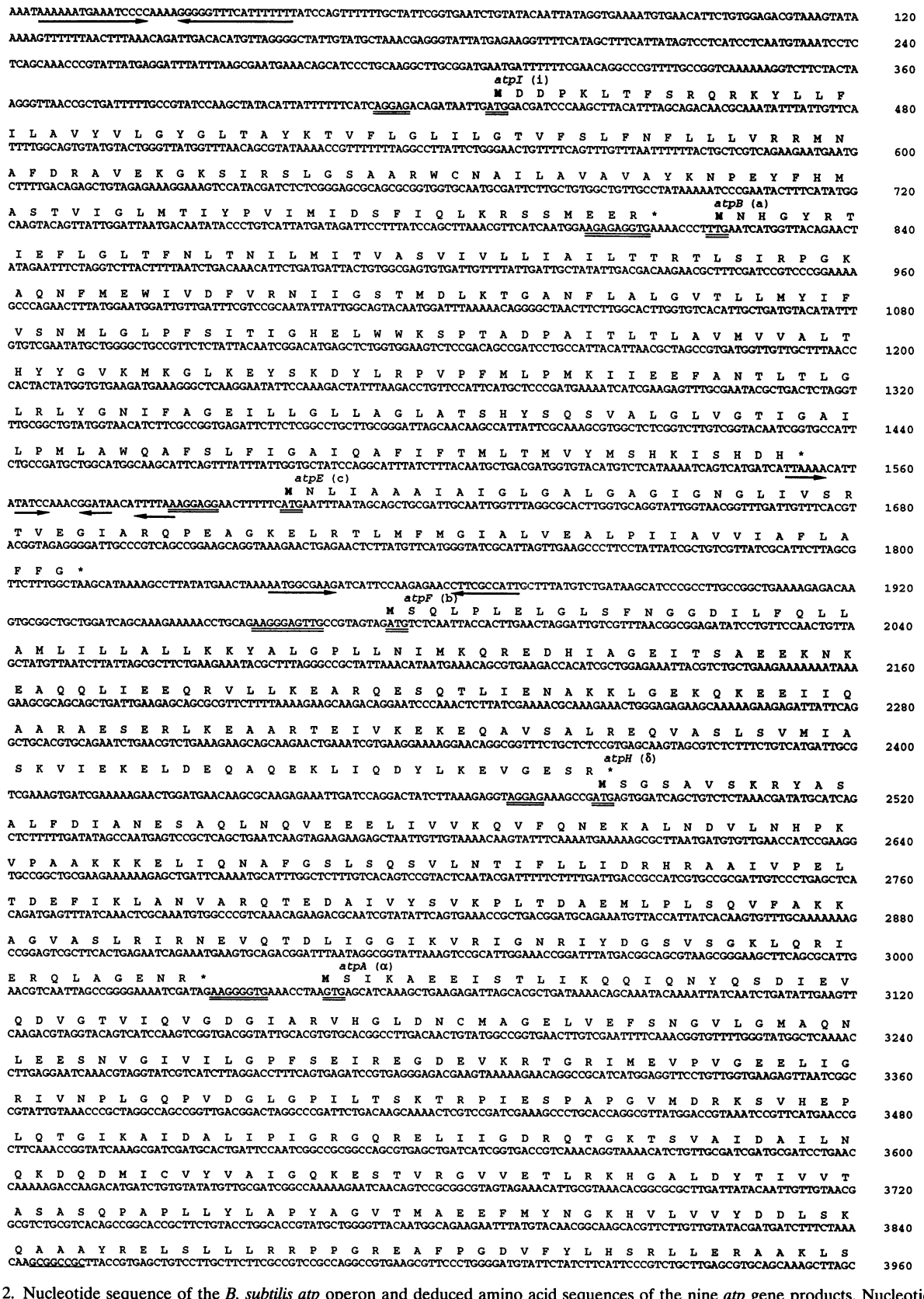


FIG. 2. Nucleotide sequence of the *B. subtilis atp* operon and deduced amino acid sequences of the nine *atp* gene products. Nucleotides are numbered from the 5′ end. Putative transcription termination signals and other palindromic sequences are represented by arrows. The proposed RBSs and initiation codons from the nine *atp* genes are underlined twice. The *Not*I site at position 3780 kb on the *Sfi*I-*Not*I physical map of *B. subtilis* (26) located at position 3844 in this sequence is underlined.

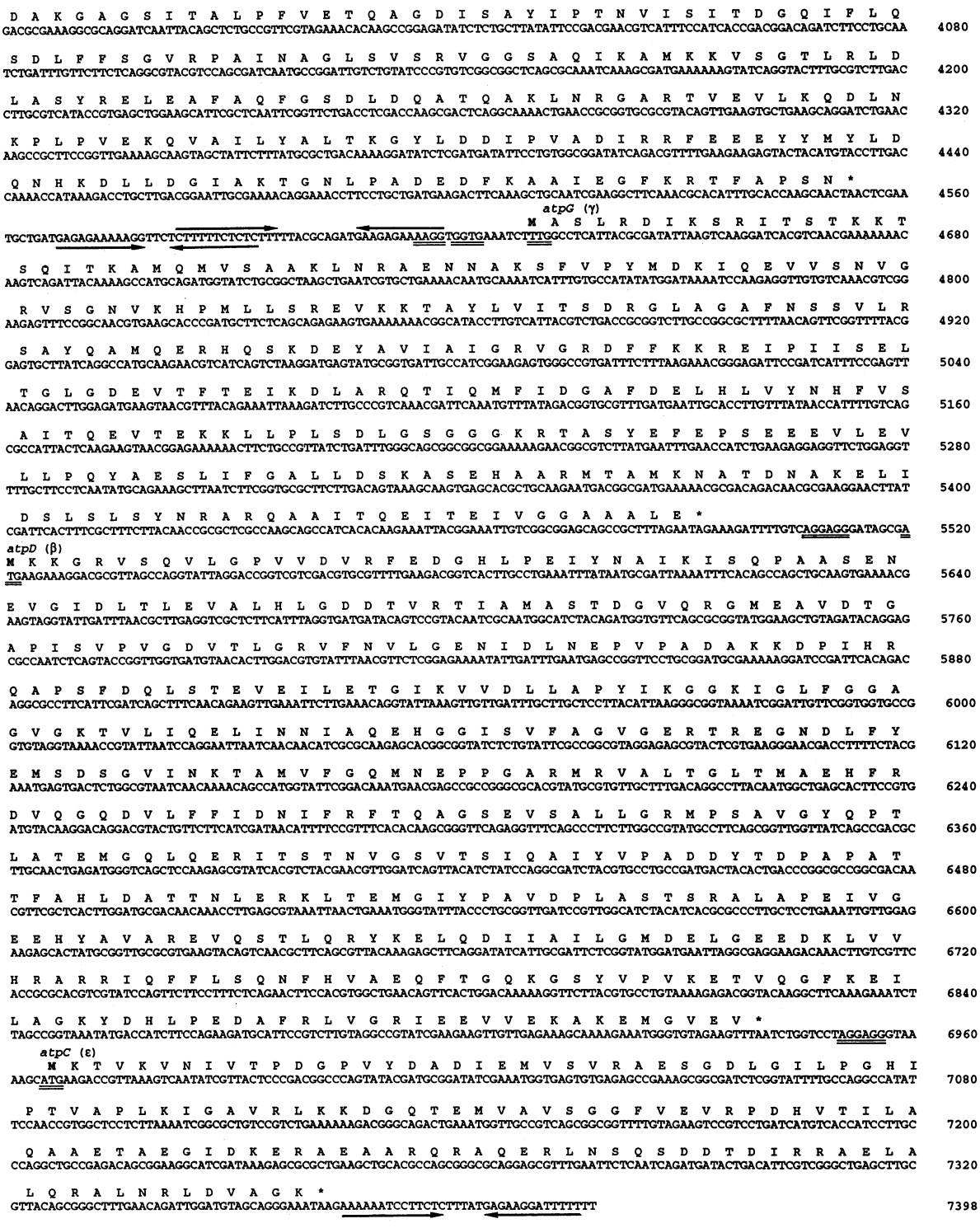


FIG. 2—*Continued.*

taining the putative promoter in the PS3 *atp* operon (47), the promoter in the *atpI* gene from *B. megaterium*, or the region in *B. megaterium atp* operon similar to the putative *E. coli* translation enhancer found in the *atpB-atpE* intergenic region (4). However, we should mention that these intergenic regions of *B. firmus* and *B. megaterium* are similar (55% identity over 79 bases). Finally, potential secondary structures are found in the four *Bacillus* and the *E. coli atpA-atpG* intergenic regions.

Expression of the *atp* operon. ATP synthase is a key enzyme in bacterial energy metabolism. We investigated the expression of the *atp* operon in the wild type under two different growth conditions (in glucose minimal medium and in succinate

TABLE 1. Similarities between the deduced amino acid sequences of the nine *B. subtilis atp* gene products and homologous proteins from five other bacteria

Gene	Subunit	Size (codons)	% identity (% similarity)[a]				
			B. megaterium	PS3	*B. firmus*	*E. coli*	*Synechococcus* strain PCC6716
atpi	i	127	48 (67)	44 (68)	25 (54)	40 (20)	
atpB	a	244	73 (89)	68 (85)	58 (79)	34 (63)	33 (67)
atpE	c	70	76 (87)	71 (88)	50 (78)	41 (77)	46 (75)
atpF	b	170	63 (80)	62 (79)	58 (76)	33 (59)	26 (52)
atpH	δ	181	47 (66)	47 (67)	43 (64)	24 (51)	24 (51)
atpA	α	502	85 (94)	82 (90)	80 (90)	55 (73)	65 (79)
atpG	γ	286	74 (85)	69 (81)	65 (80)	37 (61)	13 (36)
atpD	β	473	86 (93)	88 (93)	84 (91)	65 (80)	68 (79)
atpC	ε	139	69 (84)	68 (81)	62 (81)	33 (64)	36 (56)

[a] Data for *B. megaterium* (4), thermophilic bacterium PS3 (47), *B. firmus* (27), *E. coli* (68), and *Synechococcus* strain PCC6716 (64) are from the indicated references.

minimal medium), using transcriptional fusions between the *atp* operon and *E. coli lacZ* gene (Table 2). The expression of the *atp* operon was not significantly different under the two growth conditions. However, the bacterial growth rate is lower in succinate minimal medium (0.16 h^{-1}) than in glucose (0.74 h^{-1}). In *E. coli*, expression of the *atp* operon does not appear to be subject to substrate or growth rate control (46, 49). Our results suggest that expression of the *B. subtilis atp* operon is also constitutive.

Growth parameters of *B. subtilis atp* mutants. We constructed two *B. subtilis atp* mutant strains: 168 Δ*atp*1 and 168 Δ*atp*2. In both strains, part of the *atp* operon has been replaced by a kanamycin resistance gene after double-crossover events between plasmid pDIA5335 or pDIA5336 and the chromosome (see Fig. 1 and Materials and Methods). After transformation, mutant strains were selected on LB and SP plates containing kanamycin. However, colonies on SP plates were small and unable to grow on the same medium after restreaking, even when supplemented with 10 g of NaCl per liter (as in LB medium). However, the mutants grew as in LB medium on SP supplemented with glucose to a final concentration of 4 g/liter. The *B. subtilis atp* mutants were unable to grow on minimal medium supplemented with succinate as the sole carbon source, the ATP biosynthesis being sustained only by substrate-level phosphorylation in these strains. Both deleted strains were resistant on LB plates to neomycin at concentrations up to 10 mg/liter. The wild-type strain 168 is sensitive to a concentration of 0.5 mg/liter.

TABLE 2. Growth parameters of wild-type and *atp* mutant strains in glucose minimal medium at 37°C

B. subtilis strain	Growth rate (h^{-1})	Growth yield[a]	Acetate production[b] (mmol/mmol of glucose consumed)	Respiration rate (nmol of O_2 consumed/min/OD unit)
Wild-type 168	0.74	83 (4.6)	0.5	48
168 Δ*atp*1	0.45	36 (2.0)	0.95	70
168 Δ*atp*2	0.49	47 (2.6)	0.98	73

[a] Expressed as grams of biomass per mole of substrate. Biomass was calculated from the determination of total soluble protein; the maximum OD reached is indicated in parentheses.
[b] At the end of the exponential phase.

The growth rates and the growth yields in glucose minimal medium at 37°C were determined for the wild type and the two mutant strains (Table 2). The growth rates of strains 168 Δ*atp*1 and 168 Δ*atp*2 were 61 and 66%, respectively, of the wild-type level, and the growth yields were 43 (168 Δ*atp*1) and 56% (168 Δ*atp*2) of the wild-type level when the cultures reached stationary phase.

ATP/ADP ratios. The ATP/ADP ratio was determined by two-dimensional chromatography of nucleotides labeled in vivo (Fig. 4). The ATP/ADP ratio in mutant 168 Δ*atp*2 was half of the wild-type ratio. Thus, deletion of the *atp* operon affects the free-energy state of the cells consistent with the decreased growth rate of strain 168 Δ*atp*2.

By-product formation. The carbon and energy flow in *B. subtilis* strains defective for oxidative phosphorylation was studied by analyzing the composition of the medium by HPLC. During both exponential and early stationary phases of growth in glucose minimal medium, acetate accounted for about 80% of the by-products in both the mutant and wild-type strains. However, the total acetate production by the mutant was double that of the wild type (Table 2). Furthermore, the *atp* mutant strains consumed twice the amount of glucose for the same increase in biomass. Stimulation of the conversion of glucose to acetate appears therefore to be coupled in these *atp* mutant strains to ATP biosynthesis.

Respiration rate and transcription of terminal oxidase genes. We measured the respiration rates of the wild type and

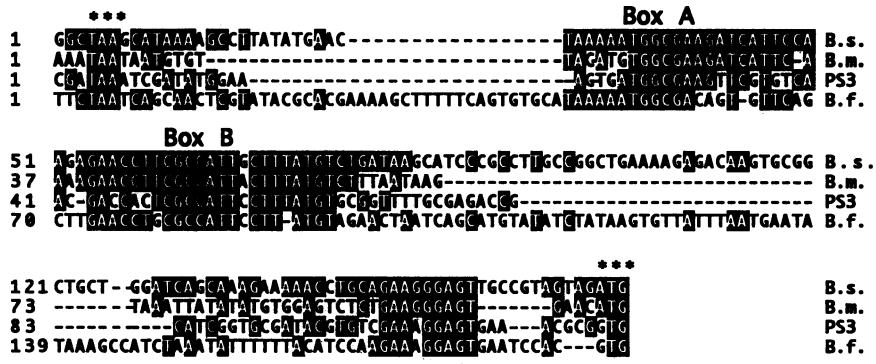
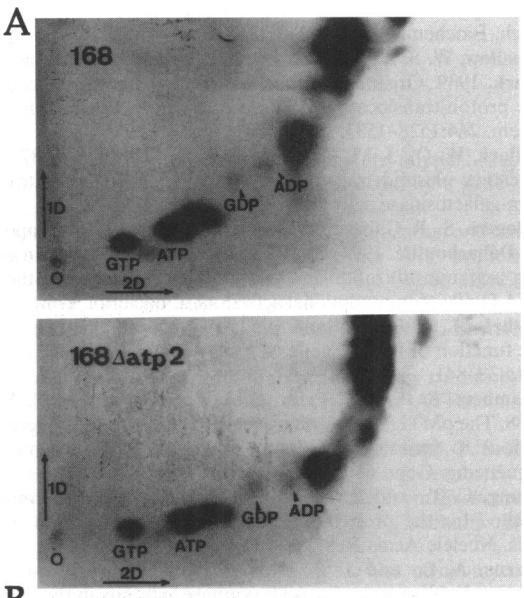


FIG. 3. DNA sequence alignments of the *atpE-atpF* intergenic regions from *B. subtilis* (B.s.), *B. megaterium* (B.m.), *B. firmus* (B.f.), and from the thermophilic bacterium PS3 (PS3). The multiple alignment was first performed by the CLUSTAL method (24) and then refined manually. The *atpE* stop codon and *atpF* start codon are indicated by asterisks.



B

Strain	GTP	ATP	GDP	ADP	Molar Ratio		
					ATP/GTP	ATP/ADP	GTP/GDP
168	135	632	14	23	4.7 (4±1)	18 (15±4)	6.4 (9±3)
168 Δatp	272	296	22	35	4.1 (4±1)	7 (8±2)	2.2 (3±1)

FIG. 4. Comparison of ^{33}P-labeled nucleotide pools from wild-type 168 strain and *atp* mutant 168 Δ*atp*2. (A) Nucleotides were chromatographed by elution in lithium chloride in the first dimension (1D) and elution in sodium formate in the second dimension (2D). (B) Quantification of GTP, ATP, GDP, and ADP spots from the chromatograph of panel A, using a PhosphoImager. Average ratios between four experiments and standard variations are indicated in parentheses.

those of the two *atp* mutants grown in glucose minimal medium at 37°C. The respiration rate of the mutant strains was 50% higher than in the wild type (Table 2). We investigated whether this increase in respiration correlated with an increase in the expression of the terminal oxidase structural genes. Transcriptional fusions between the *E. coli lacZ* reporter gene and *ctaA*, *ctaB*, *ctaD*, or *qoxB* were constructed (see Fig. 1 and Materials and Methods). *ctaA* and *ctaB* are two divergent genes whose products are involved in the biosynthesis of the *a*-type heme (61). *ctaD* is located downstream from *ctaB* and is the structural gene of cytochrome caa_3 oxidase subunit I (55). Finally, *qoxA* encodes the aa_3 quinol oxidase subunit II (54). The Δ*atp*1 deletion was introduced into each strain by homologous recombination, and β-galactosidase activity was assayed (Table 3). The absence of F_0F_1 ATPase activity led to an increase of expression of these four genes. It therefore seems that the increase in respiration rate is at least in part due to an increase in terminal oxidase synthesis.

DISCUSSION

B. subtilis is one of the most extensively studied bacteria. Surprisingly, information about oxidative phosphorylation in this species is limited. In the course of the *B. subtilis* chromosome sequencing project, we cloned and sequenced the *atp* operon encoding the nine subunits of the ATP synthase complex. Not surprising, it is highly similar to *atp* operons from both gram-positive and gram-negative bacteria.

We constructed two strains from which the *atp* operon was deleted. These two strains are unable to grow on minimal medium containing succinate as the sole carbon and energy source. Thus, they are deficient for ATP synthesis by oxidative phosphorylation. Consistent with the sequence data, it was therefore presumed that this operon encodes the eight subunits of the F_0F_1 ATPase complex. Although we cannot exclude the existence of a second ATP synthase, its activity does not appear to be sufficient for supporting growth of the mutants in the conditions tested.

Comparison of the *B. subtilis atp* operon with other *atp* operons revealed interesting features. On the basis of data from *E. coli* (3, 17, 65), the apparent stoichiometry of *B. subtilis* ATPase complex is assumed to be $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1a_1b_2c_{10-15}$. The regulatory mechanisms ensuring that the eight subunits are synthesized at the appropriate molecular ratios are not fully understood, and several nonexclusive mechanisms of regulation have been proposed. The relative translation initiation efficiencies of the eight genes could vary, and a translational enhancer upstream of *atpE*, encoding the c subunit present at 10 to 15 copies in the complex, has been proposed (38). Alternatively, the stability of the mRNA could allow higher expression of certain genes (37). Three intergenic regions of the *B. subtilis atp* operon are relatively long. Those flanking *atpE* and upstream of *atpG* (encoding the γ subunit) are, respectively, 45, 162, and 76 bases long. A similar pattern is found in *atp* operons in other bacilli and *E. coli*. We speculate that the two regions surrounding *atpE* could be involved in the higher expression of this gene. The noncoding sequence following *atpE*, highly conserved in all *Bacillus atp* operons (Fig. 3), could mimic a mini-tRNA structure. This conserved region may protect the mRNA from 3′-5′ degradation. ATPase subunit stoichiometry suggests that *atpG* is expressed three times less than *atpA* and *atpD*, its upstream and downstream genes. The two putative secondary structures predicted upstream from the *atpG* RBS sequence (Fig. 2) as well as a relatively poor translation start site (a TTG start codon and a T residue instead of an A at the middle position of the RBS sequence) are consistent with weaker expression.

B. subtilis and *E. coli* diverged more than 2 billion years ago.

TABLE 3. β-Galactosidase activities of fusion strains during exponential growth in minimal medium supplemented with the indicated carbon source at 37°C

Strain	Avg β-galactosidase activity (Miller units/mg of protein) ± SD[a] with indicated gene fusion					
	qoxA,′ glucose	*ctaA*,′ glucose	*ctaB*,′ glucose	*ctaD*,′ glucose	*atpH*′	
					Glucose	Succinate
Wild-type 168	616 ± 61	34 ± 4	384 ± 70	33 ± 6	1,317 ± 94	1,573 ± 148
168 Δ*atp*1	1,209 ± 210	238 ± 40	771 ± 86	444 ± 80		
Ratio, mutant/wild type	2	7	2	13		

[a] Standard variations are based on at least three independent cultures. For each experiment, an average of four samples were taken.

Their metabolisms are apparently very different, consistent with their different ecological niches: the mammalian gut for *E. coli* and the soil for *B. subtilis*. *B. subtilis* is an aerobic organism, whereas *E. coli* is able to grow under anaerobic conditions on fermentable carbon and energy sources. *E. coli* can rapidly adapt its metabolism from aerobiosis, during which oxidative phosphorylation is the main pathway for ATP synthesis, to fermentation, during which substrate-level phosphorylation is the only source of ATP. It is therefore interesting to compare oxidative phosphorylation in these two organisms.

The expression of the *E. coli atp* operon is constitutive (46). The expression of the *B. subtilis atp* operon does not differ significantly when *B. subtilis* is grown with glucose or succinate as the sole carbon and energy source. However, the metabolic pathway and the growth rate are different in these two conditions. It seems, therefore, that as in *E. coli*, the expression of the *atp* operon is not changed when the bacteria use different substrates for growth.

In the absence of oxidative phosphorylation, ATP can be synthesized only by substrate-level phosphorylation. In *E. coli*, deletion of the *atp* operon leads only to a small reduction of the growth rate: ATP is synthesized by an increase in the rate of glucose consumption concomitantly with an increase in acetate production (28). The increased flow of carbon through the glycolytic pathway and the tricarboxylic acid cycle observed in the *E. coli atp* mutant results in an increased generation of NADH and thus increased respiration observed in these cells. In *B. subtilis atp* deletion mutants, there was a similar slight reduction in the growth rate, an increase in the rate of glucose consumption, a twofold increase in acetate production, and an increase in the respiration rate associated with an increase in the expression of terminal oxidase genes.

This study shows that *B. subtilis*, like *E. coli*, adapts its metabolism in the absence of oxidative phosphorylation to maintain the growth rate as high as possible. This involves an unexpectedly high respiration rate uncoupled to ATP synthesis as suggested by Jensen and Michelsen (28). The similarity in carbon metabolism and energy flux in *E. coli* and *B. subtilis* demonstrates that this central element of metabolism and its regulation has been conserved during 2 billion years under different selective pressures.

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