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The Membrane-Bound H^+-ATPase Complex Is Essential for Growth of *Lactococcus lactis*

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The eight genes which encode the (F_1F_o) H^+-ATPase in ‘ øΩ±Ω±ΩΩ ≠ ¥öΩ∑ subsp. ΩÆ≥ ±Æ∑ MG1363 were cloned and sequenced. The genes were organized in an operon with the gene order ø˙∞¤fi⁄ ÿ flŸ‹ › ; i.e., the order of ø˙∞¤ and ø˙∞fi is reversed with respect to the more typical bacterial organization. The deduced amino acid sequences of the corresponding H^+-ATPase subunits showed significant homology with the subunits from other organisms. Results of Northern blot analysis showed a transcript at approximately 7 kb, which corresponds to the size of the ø˙∞ operon. The transcription initiation site was mapped by primer extension and coincided with a standard promoter sequence. In order to analyze the importance of the H^+-ATPase for ‘ Ú¥öΩ∑ physiology, a mutant strain was constructed in which the original ø˙∞ promoter on the chromosome was replaced with an inducible nisin promoter. When grown on GM17 plates the resulting strain was completely dependent on the presence of nisin for growth. These data demonstrate that the H^+-ATPase is essential for growth of ‘ Ú¥öΩ∑ under these conditions.

The (F_1F_o) H^+-ATPase complex plays an important role in the free energy metabolism of virtually all living cells. The structures of F_1F_o-ATPase complexes from different sources are very similar and consist of two parts: a membrane integral part, F_o, which forms a proton channel, and a soluble part, F_1, which contains the catalytic site for ATP hydrolysis. In bacteria, the enzyme is located in the cytoplasmic membrane, where it catalyzes the interconversion of ATP and the transmembrane proton gradient. Depending on the particular organism and on the conditions for growth, the enzymes function in the direction of either ATP synthesis or ATP hydrolysis (14). In organisms which contain a respiratory chain, such as *Escherichia coli* and *Bacillus subtilis*, the primary role of the enzyme is to synthesize ATP driven by the proton gradient that results from respiration, when these organisms are supplied with an electron acceptor. In organisms that lack a respiratory chain, or in the absence of electron acceptors, the enzyme generates a transmembrane proton gradient, and this process is then driven by ATP hydrolysis. The anaerobic bacterium *Lactococcus lactis* also possesses an F_1F_o-ATPase complex. This bacterium lacks the respiratory chain, and the enzyme here is involved in the extrusion of protons driven by ATP hydrolysis to generate the necessary driving force for solute transport and to maintain an acceptable intracellular pH value (21, 38). The latter function is supported by the fact that the activity of the F_1F_o-ATPase in these anaerobic bacteria is enhanced at low external pH (2, 23).

The anaerobic bacteria have an alternative route to generate a proton gradient across the cytoplasmic membrane, namely, through end product excretion. In the so-called energy recycling model, which was first demonstrated by Michels et al. (27), it was suggested that carrier-mediated excretion of end products can occur in symport with protons, and this contributes to the generation of the transmembrane proton gradient. This mechanism has been thoroughly investigated in *Lactococcus lactis* by Otto et al. (30), and ten Brink et al. (40), who demonstrated that the energy recycling by lactate efflux makes a significant contribution to the generation of the proton gradient in this organism, particularly at high external pH and low external lactate concentrations. An interesting question is then whether this contribution would be sufficient to allow growth of *L. lactis* in the absence of the H^+-ATPase.

In this paper we report the cloning, sequencing, and characterization of the genes that encode the H^+-ATPase in *L. lactis* subsp. *cremoris* MG1363. A mutant strain was constructed in which the expression of H^+-ATPase on the chromosome is under control of the *nisA* promoter. The strain was completely dependent on nisin for growth on GM17 plates, which demonstrates that the H^+-ATPase is an essential enzyme for growth of *L. lactis*.

MATERIALS AND METHODS

Bacterial strains. The plasmid-free *L. lactis* subsp. *cremoris* strain MG1363 (16) was used to study the *atp* operon in *L. lactis*. *E. coli* K-12 strain BOE270 is highly competent with respect to transformation and was derived from strain MT102, which is an *hsdR* derivative of strain MC1000 [*araD139* (*ara-leu*)*7679 galU galK* (*lac*)*174 rpsL thi-1*] (7). BOE270 was used as a host for plasmids in the cloning procedures and for propagation of plasmid DNA in *E. coli*.

Oligonucleotides and enzymes. Oligonucleotides were obtained from Hobolth DNA Synthesis (Hillerød, Denmark). Restriction enzymes (Gibco BRL, Pharmacia), *Taq* and *Pfu* DNA polymerases (Pharmacia and AH Diagnostics, respectively), calf intestine alkaline phosphatase (Pharmacia), and T4 DNA ligase (Gibco BRL) were used as recommended by the manufacturers.

Sequencing and sequence analysis of the H^+-ATPase operon. The DNA sequencing was carried out either by the dideoxy nucleotide chain termination method (33) with [α-^{33}P]ddNTP (500 Ci/mmol) (Pharmacia) or by autosequencing by capillary electrophoresis with the Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer).

The alignments of DNA and amino acid sequences were performed on the BLAST server at the National Center for Biotechnology Information (NCBI). The numbers given below refer to the numbering used in the GenBank sequence.

Transformation. Cells of *E. coli* were made competent by the Ca^{2+} method (32). Plasmid DNA was used to transform the cells by a standard transformation procedure (28), and the transformation mixtures were plated at 30°C on Luria-

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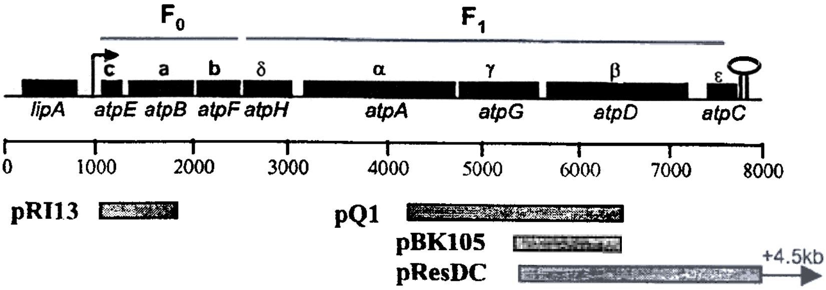


FIG. 1. Genetic organization of the *L. lactis atp* operon and DNA fragments used in this work. The open reading frames are shown as boxes, and the designations of the *atp* genes are shown below the boxes in italic letters. The designations of the H⁺-ATPase subunits are shown above the boxes. The arrow indicates the direction of transcription of the *atp* operon, and the stem loop indicates the putative terminator. The cloned fragments, which are referred to in the text, are indicated in the boxes below the scale.

Bertani agar plates supplemented with either ampicillin (100 μg/ml) or erythromycin (200 μg/ml). Cells of *L. lactis* (16) were made competent by growth in GM17 medium containing 1% glycine and resuspended in 10% glycerol and 0.5 M sucrose as described by Holo and Nes (18). Plasmid DNA was used to transform the cells by electroporation (18), and the cells were allowed to regenerate in SGM17 medium for 2 h and then plated onto Schmidt-Ruppin plates containing the appropriate selective antibiotic.

Cloning of the ø¨∞ operon from ' Ú¥ΩΣ subsp. Ω£≥ ±¥Σ MG1363. Fragments of the *atp* operon were cloned as PCR products or by the plasmid rescue technique (see below). Chromosomal DNA from *L. lactis* MG1363 was used as a template for amplification of DNA. Several primer sets were used to amplify different regions of the *atp* operon (Fig. 1). Here we took advantage of the fact that in an unrelated project, the first part of the *atp* operon from the closely related bacterium *L. lactis* subsp. *lactis* B1014 was accidentally discovered in a clone from a gene library, which allowed us to design primers for the amplification of the genes that encode the F₀ part of the enzyme complex. PCR amplification was carried out in a total volume of 100 μl and in the presence of 0.4 mM concentrations of each deoxynucleoside triphosphate (Boehringer), 3 to 5 μM concentrations of each primer DNA, 0.1 μg of chromosomal DNA, 2.5 U of *Taq* polymerase, and the buffer recommended by the manufacturer (Pharmacia). The reactions were carried out for 25 cycles (1 min at 94°C for denaturing, 1 min at 55°C for annealing, and 2 min at 72°C extension step) by use of a DNA thermal cycler. The resulting PCR products were cloned in pMOSBlue (Amersham) and sequenced. To confirm the correctness of the cloned product, the sequence was also determined directly on the PCR products.

Cloning of ø¨∽ by plasmid rescue. Plasmid pQ1, which harbors the DNA sequence from position 4177 to position 6394 (the C-terminal part *atpG* of the product and the N-terminal part *atpD* of the product) and was obtained by cloning a PCR fragment obtained with primers 3987 (5′-TTGGTGGTGGATCAATGACGGC) and 3991 (5′-TTNCCNTCACGAGTACGNTCNCC), was inserted into pMOSBlue. This plasmid was used to construct a plasmid for cloning the remaining part of the *atp* operon by the plasmid rescue technique as follows. A 3.2-kb *Eco*RI fragment from pCP12 carrying the *erm* gene and the strong artificial constitutive promoter CP12 (19) was cloned into pQ1 digested with *Eco*RI, resulting in pBK105, in which the *atpGD′* genes (sequence from position 5268 to 6394) had been placed under control of the CP12 promoter. The plasmid pBK105 was then used to transform *L. lactis* MG1363 to erythromycin resistance (2 μg/ml). This plasmid is unable to replicate in *L. lactis*, and only cells with the plasmid integrated into the chromosome will become resistant to erythromycin. If the plasmid integrates into the *atp* operon by a Campbell-type event, the genes *atpDC* will come under control of the CP12 promoter. Chromosomal DNA of some transformants was prepared, and the appropriate integration of pBK105 was verified by PCR techniques. The chromosomal DNA was digested with *Sal*I, ligated at a low DNA concentration, and transformed in *E. coli*, which resulted in pRESDC, in which approximately 4.5 kb downstream of the *atp* operon was cloned. Plasmid pRESDC was more extensively characterized and sequenced.

Primer extension. Total RNA was extracted from exponentially growing *L. lactis* (30°C, optical density at 600 nm [OD_{600}] = 0.5) in GM17 (1% glucose) by the FastRNA kit, BLUE (Bio 101), as recommended by the manufacturer.

Total RNA (10 μg) and ^{33}P-labeled primer (10 pmol) were heated for 2 min at 80°C in 5 μl of hybridization buffer (100 mM KCl, 50 mM HEPES, pH 7.0), followed by a gradual cooling to 30°C over a 60-min period. Three microliters of a solution containing 250 mM Tris-HCl (pH 8.4), 20 mM $MgCl_2$, 20 mM dithiothreitol (DTT), 0.1 mM concentrations of each deoxynucleoside triphosphate, and 0.75 U of avian myeloblastosis virus reverse transcriptase (Life Sciences)/μl was added, and the mixture was incubated at 40°C for 30 min. The extension product was precipitated with ethanol and resuspended in 6 μl of formamide loading buffer, preheated at 85°C for 3 min, and loaded onto a polyacrylamide gel with a set of dideoxy sequencing reactions (33) prepared on a PCR product as a marker. The sequence of the primer used in the 5′-3′ direction was 5′-GACCGATAGCAATTGCTCC-3′ (primer 5264).

Northern blotting. A single-stranded RNA probe labeled with [α-^{32}P]CTP was derived from a PCR product (primer 5883, 5′-CAACGTGTCCTTCAACGC, and primer T7atpC, 5′-TAATACGACTCACTATAGATAAACCACACCAGCAGGGG), which contains *atp′DC′* (position 6918 to 7462) and the T7 promoter, by in vitro transcription using T7 RNA polymerase (Promega). A total RNA preparation (12 μg) was dried in a vacuum drier and resuspended in 4.5 μl of H_2O, 2 μl of 5× formaldehyde gel running (FGR) buffer (0.1 M MOPS [morpholinepropanesulfonic acid] [pH 7], 40 mM sodium acetate, 5 mM EDTA), 3.5 μl of formaldehyde (final concentration, 7% [vol/vol]), and 10 μl of formamide (final concentration, 50% [vol/vol]). The RNA molecules were denatured by incubation for 15 min at 60°C and separated by electrophoresis in a 1.2% (wt/vol) agarose gel containing 2.2% formaldehyde, which was run at 5 V/cm with FGR buffer as the electrophoresis buffer. The gel was then washed in H_2O for 20 min at room temperature. The RNA was transferred to a Zeta-Probe GT membrane (Bio-Rad) by overnight capillary blotting with 50 mM NaOH as the transfer buffer. The membrane was air dried and prehybridized for 2 h at 42°C in hybridization buffer (1 mM NaCl, 4 mM $Na_4P_2O_7$, 5× Denhardt's solution, 1% sodium dodecyl sulfate [SDS], 10% [wt/vol] polyethylene glycol 6000, 50 mM Tris-HCl [pH 7.5], 50% [vol/vol] formamide) before the α-^{32}P-labeled riboprobe was added. After overnight hybridization at 42°C, the membrane was washed twice for 5 min at room temperature in 2× SSC, twice at 30 min at 65°C in 0.2× SSC–1% SDS, and twice for 30 min at 65°C in 0.1× SSC before being used for autoradiography (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The 0.24- to 9.5-kb RNA ladder from Gibco BRL was used as a molecular size standard.

Replacement of the chromosomal ø¨∞ promoter in ' Ú¥ΩΣ by the nisin-inducible ≤Σl promoter. A PCR fragment that harbors the DNA sequence from position +998 to position 1850 (the *atpEB′* genes) was amplified using *Taq* polymerase. After polishing the DNA ends with *Pfu* polymerase, the fragment was cloned into the *Sfr*I1 site on the vector pCR-Script Amp SK(+) (Stratagene) (Fig. 2). A plasmid was isolated in which the fragment was inserted in the orientation opposite to that of *lacZ* (pRI13). A 1.5-kb *Sal*I-*Pst*I fragment from pNZ8010 (12) that carries the *cat-194* gene and the *nisA* promoter was then cloned into pRI13 digested with *Sal*I-*Pst*I, which yielded the plasmid pATP1, in which the *atpEB′* genes had been placed under the control of the (nisin-inducible) *nisA* promoter. A 2.4-kb *Apa*I-*Not*I fragment from pATP1, which contains the *cat-194* gene, the *nisA* promoter, and the *atpEB′* genes, was cloned into pRC1 digested with *Apa*I-*Not*I, which gave rise to plasmid pNIS-ATP2. pRC1 is a 3.5-kb derivative of pBluescript II KS in which the *bla* gene has been replaced by the *ermAM* genes to allow for selection of erythromycin resistance in *L. lactis* (25). The strain NZ9000 (12) is a derivative of strain MG1363 (16) in which the *nisR* and *nisK* genes (required for induction of the *nisA* promoter) are integrated into the *pepN* locus on the chromosome. Plasmid pNIS-ATP2 was introduced into strain NZ9000 with selection for erythromycin resistance (2 μg/ml) on plates that contained nisin (5 ng/ml). Since this plasmid is unable to replicate in *L. lactis*, only cells in which the plasmid has integrated into the chromosome should become resistant to erythromycin. If the plasmid integrates into the *atpEB* locus, the transcription of the entire *atp* operon will be placed under the control of the *nisA* promoter. The clones were verified by PCR with primers positioned upstream of the *nisA* promoter and immediately downstream of position 1850.

Nucleotide sequence accession number. The sequence of the *lipA* gene, the sequence of the *atp* operon of *L. lactis* subsp. *cremoris* strain MG1363, and the sequence downstream of the *atp* operon (8,912 bp) have been deposited in the NCBI data bank with the accession no. AF059739, and the numbers used in the present paper refer to the numbering used in this sequence.

RESULTS AND DISCUSSION

The genes encoding the H⁺-ATPase in ' Ú¥ΩΣÚ The genes encoding the subunits of the H⁺-ATPase were cloned on a series of overlapping fragments, and the complete sequence of

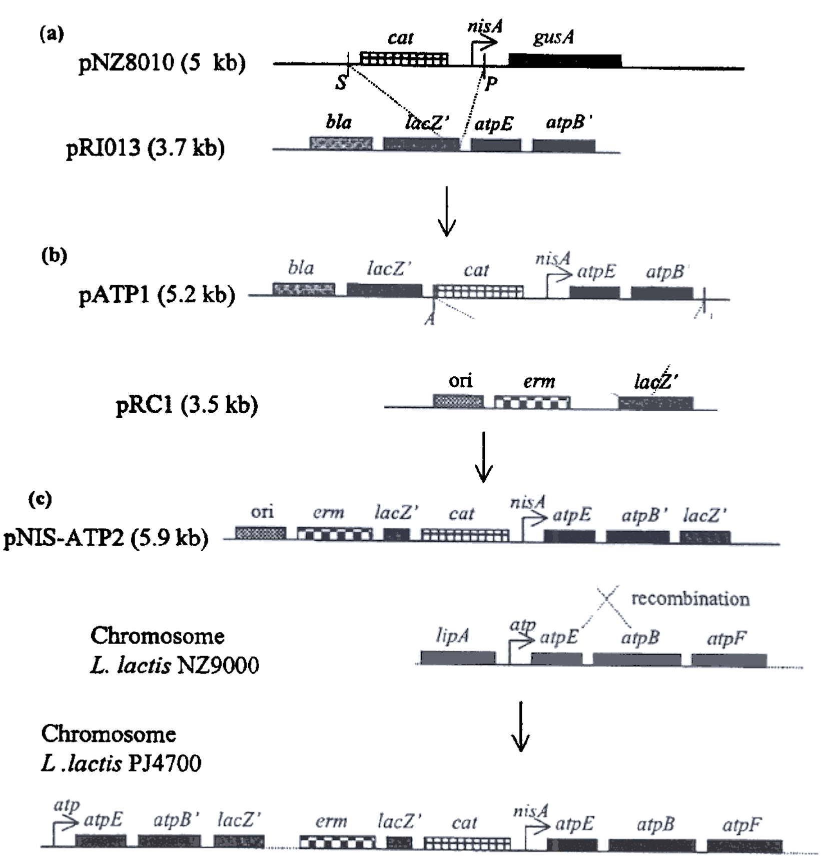


FIG. 2. Cloning strategy used in the replacement of the native *atp* promoter with the nisin-inducible *nisA* promoter. (a) A 1.5-kb *Sal*I-*Pst*I fragment from pNZ8010 (12) carrying the *cat-194* gene and the *nisA* promoter was cloned into pRI13 digested with *Sal*I-*Pst*I (pATP1). (b) A 2.4-kb *Apa*I-*Not*I fragment from pATP1, containing the *cat-194* gene, the *nisA* promoter, and the *atpEB'* genes, was then cloned into pRC1 digested with *Apa*I-*Not*I (pNIS-ATP2). (c) Plasmid pNIS-ATP2 was integrated into the *atp* operon in *L. lactis* strain NZ9000 with selection for erythromycin resistance (2 μg/ml) on plates containing nisin (5 ng/ml), resulting in replacement of the native *atp* promoter with the inducible *nisA* promoter. The designation of the genes is shown above the boxes in italic letters. See Materials and Methods for further details. *S*, *Sal*I; *P*, *Pst*I; *A*, *Apa*I; *N*, *Not*I.

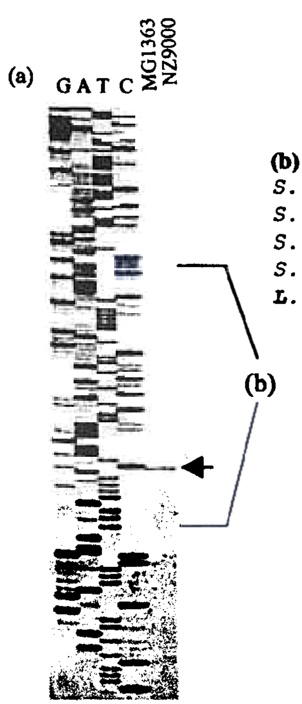
the *atp* operon was determined and analyzed for the presence of open reading frames (Fig. 1). Within a 7-kb region we identified eight open reading frames with putative ribosome binding sites. The deduced amino acid sequences of the eight gene products of the *L. lactis atp* operon were aligned with the corresponding amino acid sequences from other organisms, and the sequences of the *L. lactis* ATPase subunits showed good homology with those of other bacteria (Table 1). The homologies were particularly high between *L. lactis*, *Streptococcus mutans*, and *Streptococcus bovis*, which confirms the close evolutionary relationships of these bacteria. Among the ATPase subunits, the α, β, and γ subunits from the cytoplasmic domain, F_1, were especially highly conserved. The consensus nucleotide-binding domains, Walker motifs A (GXXXXGKT) and B (L-hydrophobic-hydrophobic-hydrophobic-D) (1, 42), were also conserved in the deduced sequences of the α and β

TABLE 1. Homology between the deduced amino acid sequences of the eight *L. lactis atp* gene products and ATPase subunits from other bacteria

Source of ATPase[a]	% Identity (% similarity) of subunits (gene, size [aa])[b]							
	c (*atpB*, 71)	a (*atpE*, 237)	b (*atpF*, 168)	δ (*atpH*, 175)	α (*atpA*, 500)	γ (*atpG*, 289)	β (*atpD*, 469)	ε (*atpC*, 141)
B. megaterium								
E. coli								
S. mutans								
Streptococcus faecalis								
S. bovis								
PS3								
Synechococcus sp.								

[a] References of bacteria are as follows: *B. megaterium* (6), *E. coli* (43), *S. mutans* (37), *S. faecalis* (36), *S. bovis* DDBJ/EMBL/GenBank database accession no. AB009314), thermophilic bacterium PS3 (29), *Synechococcus* sp. (9).

[b] The genes encode the respective subunits, and the sizes of the subunits are given in amino acids (aa).



(b)

S. bovis	5′	ATTTCG**TTGAC**A**A**TTCCTTAAAA**TAG**GAC**T**A**A**A**A**T**T**AACGA**AGTAA**AAATT	3′
S. pneumoniae	5′	TAAAAG**TTGAC**A**A**ATTTAAATTT**TAG**GAC**T**A**A**A**C**T**T**AAGTA**AGTAA**ATTTT	3′
S. sanguis	5′	AAAAGA**TTGAC**T**A**TTCGATATTT**TAG**GAG**T**A**A**A**C**T**T**AAGGA**AGTAA**TTTAA	3′
S. mutans	5′	TTTTCA**TTGAC**A**A**ACCTGCAAAA**TAG**GAC**T**A**A**A**C**T**T**AAACA**AGTAA**AAAAA	3′
L. lactis	5′	TTGTCT**TTGAC**A**A**AAACTACGAA**TAG**TAG**T**A**A**G**A**A**T**TTCTT**AGTAA**AATAA	3′

-35 -10 * TS

FIG. 3. The *atp* promoter and the transcriptional initiation site for the *atp* operon. (a) Determination of the transcription initiation site of the *atp* operon. Primer extension analysis was carried out using primer 5264 labeled by ^{33}P as described in Materials and Methods. A sequence ladder was made by sequencing with primer 5264 on a PCR product as described in Materials and Methods. (b) Comparison of the promoter region of related organisms. Letters in bold indicate conserved bases. The putative −35 and −10 consensus boxes of the *atp* promoter for *L. lactis* upstream of *atpE* are underlined. The transcription initiation site (TS) at +1 bp is indicated with an asterisk. Note the extensive homology, particularly around position +1.

subunits. Significantly lower homologies were seen for the subunits of the membrane-bound domain, F$_o$. The δ subunit, a part of the F$_1$ domain, exhibited the lowest subunit homology in the comparison.

The order of the genes was found to be *atpE*, *atpB*, *atpF*, *atpH*, *atpA*, *atpG*, *atpD*, and *atpC*, which encode the subunits c, a, b, δ, α, γ, β, and ε, respectively (Fig. 1). This organization is virtually identical to what is found in most bacteria (34, 35, 36, 43), though the c and a subunits were reversed in this instance, as has also been observed for other *Streptococcus* species (13, 37). The functional implications, if any, of this gene reversal in *L. lactis* are not known.

Three of the genes (*atpF*, *atpA*, and *atpD*) appeared to use UUG as the initiation codon instead of the more frequently used AUG start codon, and it was indeed shown previously that *L. lactis* can initiate translation at the initiation codons UUG and GUG (41). The gene products encoded by *atpF*, *atpA*, and *atpD* should be produced two, three, and three times more frequently, respectively, than the other gene products, and it is therefore somewhat surprising that the more highly expressed genes, *atpF*, *atpA*, and *atpD*, would use the UUG start codon.

The gene encoding the b subunit, *atpF*, overlaps with the Shine-Dalgarno sequence of the gene for the δ subunit, *atpH*, which suggests translational coupling between these genes. Interestingly, such overlap was also reported for *atpF* and *atpH* of the *atp* operon in *Anabeana* sp. strain PCC 7120 (26), *Bacillus megaterium* (6), *Enterococcus hirae* (36), and *Clostridium thermoaceticum* (10).

In most bacteria, such as *E. coli* (43) and *B. megaterium* (6), the *atp* operon starts with the gene *atpI* as the first structural gene, but such a gene appears to be absent in *L. lactis* (although we cannot rule out the possibility that *atpI* is positioned elsewhere on the chromosome). The function of the polypeptide encoded by the *atpI* gene in these organisms is unknown; the polypeptide is not an essential part of the H$^+$-ATPase complex, and the *atpI* gene has been demonstrated to be dispensable for growth (17, 20).

We also determined the sequences up- and downstream of the *atp* operon in *L. lactis*. Preceding the first gene in the *atp* operon, *atpE*, the *lipA* gene, which encodes an esterase, was identified (G. Fernandes et al., submitted for publication). Downstream of *atpC* there was a long noncoding region before the next open reading frame. A homology search at NCBI showed no homology of the putative polypeptide to known proteins.

Transcription of the ø∞ genes. A standard promoter with −35 (TTGACA) and −10 (TAGAAT) consensus boxes separated by 17 nucleotides was identified in the region upstream of *atpE* (Fig. 3). The presence of the −35 and −10 consensus sequences suggests that the promoter is recognized by the *L. lactis* σ^{39} transcription factor (3), and primer extension analysis confirmed the existence of a transcript that corresponds to this promoter (Fig. 3). In comparison with other lactococcal promoters, the similarities were particularly high between the *atp* promoter and the *rrnA* promoter (rRNA operon) (8). The region upstream of the −35 region of the *atp* promoter sequence (position 853 to 963) has a higher A+T content (75%) than the average value reported for *L. lactis* DNA (62.8%), which may contribute to the activity of the *atp* promoter, due to curvature of the A+T-rich sequences (5, 15).

Comparison of the promoter region with *atp* promoters from related bacteria (Fig. 3) showed homology, not only in the −35 and −10 boxes but also directly preceding the −10 box and

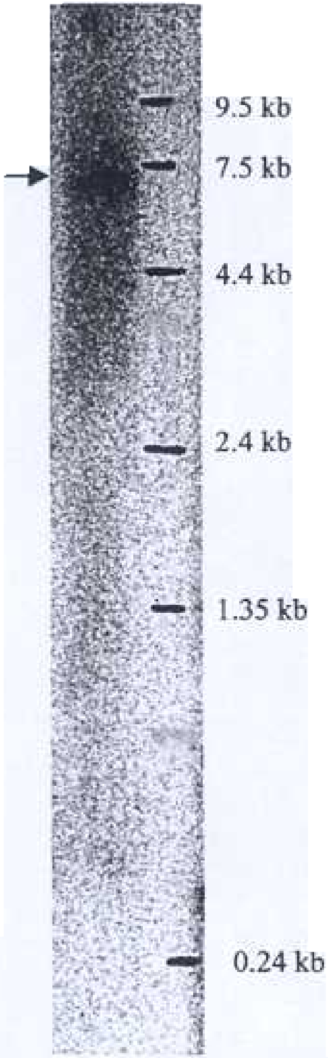


FIG. 4. Northern blot analysis. Total RNA was extracted from *L. lactis*, and Northern blot analysis was performed as described in Material and Methods. A ribonucleotide probe labeled with [α-^{32}P]CTP containing the C-terminus-encoding part of *atpD* and the N-terminus-encoding part of *atpD* (position 7915 to 3459) was used as a probe. The 0.24- to 9.5-kb RNA ladder from Gibco BRL was used as a molecular size standard.

around the transcription start site. The region upstream of the *atp* promoter contains several inverted and direct repeats. Such repeats were also observed in *Enterococcus faecalis* (36), and it was suggested that they may be involved in the regulation of the expression of the *atp* operon at low external pH (2, 22, 23) in order to keep the intracellular pH at an acceptable level.

The size of the *atp* mRNA was determined by Northern blot analysis (Fig. 4), which identified mRNA at approximately 7 kb, which demonstrates that the eight genes are transcribed as a single polycistronic message. Other transcripts could not be identified in the present analysis, in which the 3′ end of the *atp* operon was used as a probe. But smaller transcripts might still occur if other probes are employed.

An inverted repeat in the region immediately after *atpC* was recognized, followed by a T-string (7 bp), a structure that resembles a rho-independent terminator (31). The location of a terminator at this position is also supported by the transcript size found in the Northern analysis.

The F_1F_0-ATPase is essential for growth of ‘ Ú¥ΩΣÚ In the anaerobic bacterium *L. lactis*, the role of the H$^+$-ATPase is to maintain the electrochemical proton gradient across the cytoplasmic membrane, and it has been proposed that the H$^+$-ATPase functions to regulate the internal pH (4, 11, 21, 24). Is the H$^+$-ATPase then essential for growth? In principle, the anaerobic bacteria have the option to generate a proton gradient through carrier-mediated excretion of end products in symport with protons (30).

The electrochemical proton gradient (Δp) is composed of an electrical component, the transmembrane potential difference ($\Delta\psi$), and a chemical component, the transmembrane pH difference (ΔpH). The magnitude of the energy produced by lactate excretion depends strongly on the H$^+$-lactate stoichiometry (n) during the excretion process. If n *is* 1, the excretion process is electrochemically neutral and only a chemical gradient of protons (ΔpH) can be generated. If n *is* 2, the translocation is electrogenic and both a ΔpH and a membrane potential ($\Delta\psi$) can be formed. At high pH (6.8) and a low external lactate concentration (<5 mM), ten Brink and Konings determined the H$^+$-lactate stoichiometry (n) in *L. lactis* to be 1.9 (39). Thus, in principle the contribution of H$^+$-lactate efflux may suffice so that the H$^+$-ATPase would be dispensable for growth under these conditions.

One way to test how important the H$^+$-ATPase is for growth of *L. lactis* would be to replace the chromosomal *atp* promoter with an inducible promoter. In order to replace the original *atp* promoter with an inducible nisin promoter (12), a plasmid, pNIS-ATP2, was constructed, which carries the *atpE* gene and part of the *atpB* gene under the control of the *nisA* promoter. This plasmid, which cannot replicate in *L. lactis*, was integrated into the chromosome of *L. lactis* as described in Materials and Methods (Fig. 2). The resulting strain contained an inducible nisin promoter upstream of the entire chromosomal *atp* operon. When the strain was grown at 30°C on GM17 plates (buffered at pH 7) with different concentrations of nisin, we observed that at very low nisin concentrations the growth of the strain decreased dramatically and in the absence of nisin, growth was completely abolished (Fig. 5). This demonstrates

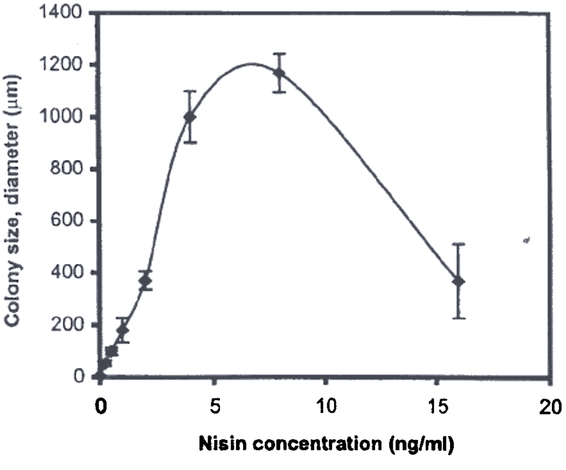


FIG. 5. Colonies of strain *L. lactis* PJ4700, in which the native *atp* promoter had been replaced by a *nisA* promoter. The strain was streaked on GM17 plus 2 µg of erythromycin/ml at various nisin concentrations (0, 0.25, 0.5, 1, 2, 4, 8, and 16 ng of nisin/ml), and the graph illustrates the average diameter of colonies obtained with the different nisin concentrations.

that the H^+-ATPase is essential for growth of *L. lactis* under these conditions, presumably because it is essential for maintaining the proton gradient necessary for solute transport and for maintaining the cytoplasmic pH at an acceptable level. This is also in agreement with the observation that the activity of the F_1F_o-ATPase in related anaerobic bacteria is enhanced at low external pH (2, 23).

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