A *Lactococcus lactis* gene encodes a membrane protein with putative ATPase activity that is homologous to the essential *Escherichia coli* *ftsH* gene product

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A gene, encoding a protein homologous to an essential *Escherichia coli* protein, FtsH, was identified adjacent to the *hpt* gene and the *trnA* operon in the Gram-positive bacterium *Lactococcus lactis*. The deduced amino acid sequence of the gene product showed full-length similarity to FtsH of *E. coli*, Yme1p of *Saccharomyces cerevisiae* and a conserved region found in a new family of putative ATPases. In-frame fusions of *L. lactis* *ftsH* and *phoA1* in *E. coli*, and immunodetection of the *L. lactis* FtsH protein in cell fractions using anti-*E. coli* FtsH serum showed that *L. lactis* *ftsH* was expressed and encodes a membrane protein. When contained on a high copy number plasmid, the *L. lactis* *ftsH* gene complemented the lethality of a Δ *ftsH3::kan* mutation in *E. coli* at 37 °C and below, indicating that the *L. lactis* *ftsH* gene can functionally replace the *E. coli* *ftsH* gene to some extent. The resulting *E. coli* strain showed temperature sensitivity and salt sensitivity. A *L. lactis* mutant with an insertion into *ftsH* was salt-, heat- and cold-sensitive. These results suggest that FtsH is somehow involved in stress responses. Southern hybridization analysis indicated that genes homologous to *ftsH* of *L. lactis* were also present in *Bacillus subtilis*, and several *Lactobacillus* and *Leuconostoc* species, suggesting high conservation of *ftsH* in bacterial species.

Keywords: *Lactococcus lactis*, *ftsH*, (*tma*), membrane protein, AAA-protein family

INTRODUCTION

The facultative anaerobic Gram-positive bacterium *Lactococcus lactis* subsp. *lactis* is widely used as an industrial organism for homolactic fermentation. *L. lactis* has become a model organism for fundamental genetic research on plasmids and plasmid-encoded functions involved in the production of fermented food products. However, the genetic characterization of chromosomally encoded functions in *L. lactis* has only recently been started. We initiated a genetic approach to study purine metabolism in *L. lactis* and have characterized the *hpt* gene encoding hypoxanthine guanine phosphoribosyl-transferase, an enzyme involved in the salvage of purine bases to the corresponding nucleotides (Nilsson & Lauridsen, 1992). During this work we detected part of an

open reading frame adjacent to *hpt*, transcribed in the same direction, encoding a putative membrane protein.

In this report we describe the cloning and characterization of the *L. lactis* gene, previously named *tma*, adjacent to *hpt* and the tRNA operon *trnA* (Nilsson & Johansen, 1994). We show that the gene is homologous to the *Escherichia coli* *ftsH* gene, and that this gene is conserved in several Gram-positive bacteria, including lactic acid bacteria. We suggest that the gene be designated *ftsH*.

The *ftsH* genes of *E. coli* and *L. lactis* encode members of a new family of ATPases, the AAA-protein family (Kunau *et al.*, 1993). The family includes both eukaryotic and prokaryotic members which have been reported to be involved in diverse cellular activities, e.g. cell cycle control, protein secretion, peroxisome biogenesis and proteolysis.

E. coli *ftsH* mutants show pleiotropic phenotypes: defects in cell growth and cell viability (Ogura *et al.*, 1991;

The GenBank accession number for the nucleotide sequence reported in this paper is X69123.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype/relevant features	Source or reference
<i>Escherichia coli</i>		
XL1-Blue	<i>endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1</i> $\Delta(lac)$ [F' <i>proAB lacI^qZAM15 Tn10</i>]	Stratagene, La Jolla
DH5 α	$\phi 80lacZAM15 \Delta(lacZYA-argF)$ U169 <i>recA1</i> <i>endA1 hsdR17 supE44 thi-1 gyrA96 relA1</i>	Hanahan (1983)
SØ609	<i>ara thi rpsL</i> $\Delta(pro-gpt-lac)$ <i>hpt deoD purD</i>	Jochimsen <i>et al.</i> (1975)
CC118	<i>araD139</i> $\Delta(ara-leu)$ 7697 $\Delta lacX74$ <i>phoA</i> Δ 20 <i>galE</i> <i>galK thi rpsE rpoB argEam recA1</i>	Manoil & Beckwith (1986)
AR423	<i>met gal supE hsdR sfiC</i> $\Delta(srl-recA)$ 306::Tn10 $\Delta ftsH3::kan$ [pAR171 <i>ftsH rep^{ts} Cm^R</i>]	Akiyama <i>et al.</i> (1994a)
AR3120	<i>met gal supE hsdR sfiC</i> $\Delta(srl-recA)$ 306::Tn10 $\Delta ftsH3::kan$ [pLN32 <i>L. lactis ftsH</i>]	This study
<i>Lactococcus lactis</i>		
CHCC285	Wild-type	Nilsson & Lauridsen (1992)
MG1363	Plasmid-free	Gasson (1983)
DN4302	MG1363 <i>ftsH::pLN43</i>	This study
Plasmids		
pBluescript KS+	Cloning vector (Ap ^R)	Stratagene
pBluescript SK-	Cloning vector (Ap ^R)	Stratagene
pVS2	Ery ^R Cm ^R	von Wright (1987)
pV2	pBluescript KS+ with a 1.3 kb <i>Clal</i> - <i>Hpa</i> II fragment (Ery ^R) from pVS2	Svend E. W. Hansen (personal communication)
pAR171	<i>ftsH rep^{ts} Cm^R</i>	Akiyama <i>et al.</i> (1994a)
pLN2	pBluescript SK- with a 1.9 kb <i>Eco</i> RI- <i>Mbo</i> I fragment (<i>ftsH'</i>) from CHCC285	Nilsson & Lauridsen (1992)
pLN3	pBluescript SK- with a 1.3 kb <i>Hind</i> III- <i>Mbo</i> I fragment (<i>ftsH'</i>) from CHCC285	Nilsson & Lauridsen (1992)
pLN22-27	pLN2 <i>ftsH::Tn5phoA1</i>	This study
pLN30	pBluescript KS+ with a 7.3 kb <i>Spe</i> I fragment (<i>hpt ftsH</i>) from CHCC285	This study
pLN32	pBluescript KS+ with a 3.2 kb <i>Eco</i> RI fragment (<i>ftsH</i>) from pLN30	This study
pLN39	pBluescript KS+ with a 1.0 kb <i>Hind</i> III- <i>Eco</i> RI fragment (<i>ftsH'</i>) from pLN32	This study
pLN43	pV2 with a 1.3 kb <i>Eco</i> RV fragment (<i>ftsH'</i>) from pLN32	This study

Tomoyasu *et al.*, 1993a), defects in protein assembly into and through the membrane (Akiyama *et al.*, 1994a, b; Tomoyasu *et al.*, 1993a) and a defect in a proteolytic pathway (Herman *et al.*, 1993). A *Bacillus subtilis ftsH* mutant shows salt sensitivity (Geisler & Schumann, 1993). However, it has not satisfactorily been demonstrated how *ftsH* mutations cause these diverse defects, and what the function of FtsH is. In this respect, it is interesting to know the structural similarity and functional relationship of FtsH in Gram-negative and Gram-positive bacteria. Comparative studies on the FtsH proteins in Gram-positive and Gram-negative bacteria

will provide better understanding of the function of this highly conserved putative membrane ATPase.

METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used are listed in Table 1. *L. lactis* was grown in M17 medium (Oxoid), containing 0.5% glucose, routinely at 30 °C. *E. coli* and *B. subtilis* were grown in L-broth (Miller, 1972) at 37 °C unless otherwise stated. The phosphate-buffered minimal medium of Clark & Maaløe (1967) was used for selection of SØ609 Hpt⁺ as described previously (Nilsson & Lauridsen, 1992). For growth of plasmid-containing cells, the

media contained appropriate antibiotics: *E. coli*, ampicillin (50 mg l⁻¹), kanamycin (300 or 30 mg l⁻¹), chloramphenicol (10 mg l⁻¹); *L. lactis*, erythromycin (1 mg l⁻¹). For identification of blue PhoA⁺ colonies on agar plates, 40 mg XP 1⁻¹ (5-bromo-4-chloro-3-indolyl phosphate, Sigma) was added.

DNA manipulations and sequencing. *L. lactis* chromosomal DNA was isolated according to Johansen & Kibbenich (1992). *E. coli* and *B. subtilis* chromosomal DNA was isolated as described by Silhavy *et al.* (1984) and *E. coli* plasmids as described by Birnboim & Doly (1979). The use of restriction enzymes, T4 DNA ligase and calf intestine alkaline phosphatase were as recommended by the suppliers (Boehringer Mannheim, Promega, Stratagene). Plasmid transformation of *E. coli* and *L. lactis* was performed according to Mandel & Higa (1970) and Holo & Nes (1989), respectively. The nucleotide sequence of both strands of DNA was determined by the dideoxy method (Sanger *et al.*, 1977). Universal primers from Stratagene or customized primers were used as sequencing primers. All nucleotide sequence data were processed and the deduced amino acid sequences compared using the GCG software package, version 7.0 (Devereux *et al.*, 1984) and EMBL nucleotide sequence database release 37.0.

Cloning of ftsH. A *L. lactis* strain CHCC285 chromosomal DNA library, containing 1–10 kb *SpeI* restriction fragments in the bacteriophage vector λ Zap II (Stratagene) was used. By selecting for Hpt⁺ colonies of *E. coli* strain SØ609 (Nilsson & Lauridsen, 1992), we isolated clone λ LN2 containing a 7.3 kb and a 1.3 kb *SpeI* fragment. The 7.3 kb fragment, containing the *hpt* and *ftsH* genes, was subcloned in the plasmid vector pBluescript KS⁺ (Stratagene) resulting in pLN30 (Table 1).

Construction of pLN2::Tn5phoA1. Plasmid pLN2 (Table 1), which contains the first 441 codons of the *ftsH* gene, was used to transform *E. coli* strain CC118. Following infection of CC118(pLN2) with λ rex::Tn5phoA1 b221 c1857 Pam3 (Gutierrez *et al.*, 1987), blue colonies on LB XP agar plates (PhoA⁺) that were Kan^R and Ap^R, were selected and pooled. Plasmids were isolated and used to transform CC118 to PhoA⁺, Ap^R and Kan^R. Six independent pLN2::Tn5phoA1 isolates were obtained.

Identification of L. lactis FtsH in E. coli. Various *E. coli* extracts from strains containing plasmids were used for SDS-PAGE and subsequently blotted semi-dry to nitrocellulose as described by Ipsen & Larsen (1988). The nitrocellulose was subsequently immunostained (Larsen *et al.*, 1992) using the anti-*E. coli* FtsH serum described previously (Tomoyasu *et al.*, 1993b).

Cell-fractionation of L. lactis. Cells were grown in 400 ml of M17 medium to an OD₆₀₀ of 0.9–1.0, harvested and resuspended in 5 ml 30 mM sodium phosphate (pH 6.8) containing 0.4 M sucrose and 1 mg lysozyme ml⁻¹. After incubation at 37 °C for 1 h, the cells were harvested, washed twice with 30 mM sodium phosphate containing 0.4 M sucrose, resuspended in 5 ml 10 mM sodium phosphate (pH 6.8) and sonicated. After ultracentrifugation of the cell extract (100 000 g, 1 h at 4 °C), the supernatant (cytoplasmic) fraction was collected. The pellet (membrane) fraction was resuspended in 10 mM sodium phosphate containing 1 M NaCl and ultracentrifuged as above. The supernatant and pellet were collected separately. The supernatant was desalted by gel filtration (Sephadex G-25M, Pharmacia).

The collected fractions were used for SDS-PAGE, blotted to nitrocellulose and immunostained with anti-*E. coli* FtsH serum as described above.

Southern hybridization analysis. A 1.0 kb *HindIII*-*KpnI* (the *KpnI* site originates from the polylinker of pBluescript SK⁻)

restriction enzyme fragment of plasmid pLN3 (Table 1) contains an internal part of the *L. lactis ftsH* coding region. This fragment was used as probe in Southern hybridization analysis with *ScaI*-digested chromosomal DNA from various bacterial strains, using the ECL Gene Detection System, version 2 (Amersham) as follows. In the hybridization buffer NaCl was added to a final concentration of 1.0 M; primary washes were in 2 × SSC, 1 M urea, at 25 °C. All other procedures including probe labelling, DNA transfer and signal detection were as described by Amersham. The molecular mass markers were obtained from Gibco BRL.

To verify the integration of pLN43 (Table 1) into the *ftsH* gene of *L. lactis* strain DN4302 *ftsH*::pLN43, a 1.3 kb *EcoRV* internal fragment of *ftsH* was used as probe in Southern hybridization analysis with *EcoRI*-digested chromosomal DNA from DN4302 and *L. lactis* strain MG1363 wild-type. Standard conditions as recommended by Amersham were used.

RESULTS

Nucleotide sequence of L. lactis ftsH

Adjacent to the *hpt* gene and *trnA* operon of *L. lactis*, a gene (*ftsH*) was found (Nilsson & Lauridsen, 1992; Nilsson & Johansen, 1994). The complete *ftsH* gene was cloned and the nucleotide sequence was determined. The sequence of the last 24 codons of *hpt* and the entire *ftsH* gene is shown in Fig. 1. A possible ribosome binding site (Ludwig *et al.*, 1985; Shine & Dalgarno, 1974) and a putative promoter (Koivula *et al.*, 1991) are located upstream of *ftsH* (Fig. 1). The putative promoter contains the sequence 5' ATATG 3' in the -16 region (consensus 5' RTRTG 3' where R = purine), which is found in strong *B. subtilis* promoters (Henkin *et al.*, 1988; Moran *et al.*, 1982). A potential stem-loop structure followed by five Ts (nucleotides 82–101) was located between the *hpt* and *ftsH* coding regions. The translation stop codon of the *ftsH* gene was located in the loop of a potential transcription terminator structure (nucleotides 2352–2391).

Analysis of the deduced amino acid sequence of the *ftsH* gene revealed a purine nucleotide binding site motif (residues 233–240 and 288–293) (Walker *et al.*, 1982). Two putative transmembrane sequences can be predicted (residues 1–29 and 136–158) (Kyte & Doolittle, 1982). The transmembrane sequence (residues 136–158) and the region containing the purine nucleotide binding site are separated by a very glycine rich sequence (residues 159–170, GGMGARGGGGGG).

Comparison of L. lactis FtsH with other proteins

Database searches for genes encoding similar amino acid sequences revealed that the deduced amino acid sequence of *L. lactis ftsH* was 47% identical to that of *E. coli ftsH* (Tomoyasu *et al.*, 1993a) and 36% identical to that of *Saccharomyces cerevisiae Yme1* (Thorsness *et al.*, 1993). The deduced *L. lactis* FtsH amino acid sequence contains a region of 200 amino acid residues that reveals similarity to conserved domains in the AAA-protein family of putative ATPases (Erdmann *et al.*, 1991; Kunau *et al.*, 1993). In this region the *L. lactis* FtsH amino acid

	TTGGACTACGAAGAAACTATCGTAATCTTCCATATGTCGGAGTTTTAAACCAGAAGTTTATAACAAATAATTCGTTGATTTTAGTCATCTAAAGTTTT	100
	L D Y E E N Y R N L P Y V G V L K P E V Y N K *	
	-35 -16 -10	
	TCTCAGACTAAAGTATGAAAAATCTCTGACCAATACTTACTATTAGCATTCCTCAAAAGGAATATGCTATAACTAGGTATAGCTGTTATTGACTAGCCTG	200
	RBS Tma	
1	TATCCTTTATTACCAGTGATACTAAGAAGTAAATCTTGGCTTTTCTAATAATAATAACAAAGATAAGGAAAATATGAATAACAACAACAACCAAAA	300
	M N N N K Q P K	
9	ACAAGGAAATTTTGTAATAATATCTTGATGTGGGTAATCTTGGCTATTGTTGTTGTTGTCGGTTTCAATTTCTTCTCAGTAGTAATCAATCAAGCGTG	400
	Q G N F V K N I L M W V I L A I V V V V G F N F F S S N Q S S V	
	/phoA(24) /phoA(26)	
42	GATAAAATTAGCTATTCACAATTGATGACGAACTTGACGGTAACAAGATTGAAAACGTCACAATGCAACCTTCTGATAGCTTAATTACTGTAACAGGTG	500
	D K I S Y S Q L M T K L D G N K I E N V T M Q P S D S L I T V T G E	
	/phoA(25)/phoA(27)	
76	AATATAAGAACCTGTAAAGTAAAGGAACAAATAATTTCCCACTTTTAGGCAATTCTAGTAGTGAAGTTAAAACTTCAAGCTTATATTATTCCAAC	600
	Y K E P V K V K G T N N F P L L G N S S S E V K N F Q A Y I I P T	
	EcoRV /phoA(22)/phoA(23)	
109	TGACAGTGTTGTCAAGGATATCCAAAATGCAGCTAAAAGTAATGATGTAACCTTAGTGTTGTTCAAGCTTCATCAAGTGGTATGTGGGTTCAAATCTC	700
	D S V V K D I Q N A A K S N D V K L S V V Q A S S S G M W V Q I L	
142	TCATACATCATTCCAATGCTTCTATTGTTGGTATCTTCTGGCTCATGATGGCGGAATGGGCGCTCGTGGCGGAGGCGGCGGTGGAATCCGATGTCCT	800
	S Y I I P M L L F V G I F W L M M G G M G A R G G G G G G N P M S F	
176	TCGGTAAATCTCGTGCTAAACAACAGATGGTAAACATCTAAAGTTCGTTTTGCTGACGTTGCCGGTCTGAAGAGGAAAAACAAGAGCTTGTAGAAGT	900
	G K S R A K Q Q D G K T S K V R F A D V A G S E E E K Q E L V E V	
209	TGTTGATTTCTTAAAAATCCGAAAAATATCATGATTTAGGAGCTCGTATCCAGCAGGTGTTCTTCTTGAAGGCCCTCCAGGTACAGGTAAAACATTG	1000
	V D F L K N P K K Y H D L G A R I P A G V L L E G P P G T G K T L	
242	CTTGCTAAGGCTGTTGCCGGTGAAGCAGGAGTTCCTTTCTATAGTATCTCAGGTTCTGATTTCTGTTGAAATGTTTGTGGTGTGGTGCCTCACGTGTCC	1100
	L A K A V A G E A G V P F Y S I S G S D F V E M F V G V G A S R V R	
276	GTGACTTATTTGAAATGCTAAGAAACTGCACCATCAATTATCTTTATGATGAAATTGATGCTGTTGGTGTGCTCAAGTGGTGCAGGTCTTGGTGGTGG	1200
	D L F E N A K K T A P S I I F I D E I D A V G R Q R G A G L G G G	
309	TAACGATGAACGTGAACAACCCCTTAACCAATTGCTCGTTGAAATGGATGGTTTCCAAGATGATGGCAACTCAGTAATCGTTATTGCTGCAACTAACCGT	1300
	N D E R E Q T L N Q L L V E M D G F Q D D G N S V I V I A A T N R	
342	TCAGATGTGCTTGACCCAGCGCTTTTACGTCCAGGTGCTTTTGACCGTAAAGTCTTGGTGGAGCTCCAGATGTTAAAGGTGTAAGCCGTTCTTAAAG	1400
	S D V L D P A L L R P G R F D R K V L V G A P D V K G R E A V L K V	
376	TTCATGCTAAAAACAACTTTAGCAAGTGATGTTGATTTGCACAATGTTGCTACACAACTCCAGGCTATGTCGGAGCTGATTTGGAAAATGTTTGA	1500
	H A K N K P L A S D V D L H N V A T Q T P G Y V G A D L E N V L N	
409	TGAAGCTGCACTTGTGCTGCACGTCAAAATAAAAAAGAAATCAATGCTGCTGACATTGATGAAGGAATGGACCGTGAATGGCTGGTCCAGCTAAGAAA	1600
	E A A L V A A R Q N K K E I N A A D I D E G M D R A M A G P A K K	
442	GATCGTATTCAATCAATGCGGAACTGAGATCGTGGCTTACCACGAAGCAGGTACGCTATTGTTGGACTCGTTCTTGAATGGATCTACTGTTGTA	1700
	D R I Q S M R E R E I V A Y H E A G H A I V G L V L E N G S T V R K	
476	AAGTTACCGTTGTTCCAGTGGACGCATCGGTGGTTACATGCTTGTCTTCCAGATGAAGAAATCATGCAACCACTAATTTCCATCTTCAAGACCAACT	1800
	V T V V P R G R I G G Y M L A L P D E E I M Q P T N F H L Q D Q L	
	EcoRV	
509	TGCCAGCCTTATGGGTGGACGACTTGGTGAAGAAATGTCTTTGGTGTAGCTACTCCAGGGGCATCAATGATATCGAAAAAGCAACACATTGCTCGT	1900
	A S L M G G R L G E E I V F G V A T P G A S N D I E K A T H I A R	
542	TCAATGGTAAGTGAATATGGGATGTCTAAGAACTTGGTATGGTATCTTATGAAGGAGACCATCAAGTATTTATTGGTCCGACTATGGTCAAACTAAGA	2000
	S M V T E Y G M S K K L G M V S Y E G D H Q V F I G R D Y G Q T K T	
576	CTTACTCAGAAGCACTGCTGTTATGATTGATGATGAAGTGCCTGATTCTCGGTGAAGCTTATGACCGTCTAAGAAAGCAATGAACACACCGTGA	2100
	Y S E A T A V M I D D E V R R I L G E A Y D R A K E A I E T H R E	
609	GCAACATAAAGCAATTGCGGAAGCTCTGCTTAAATATGAAACACTTGTGCGAAACAAATCATGTCACTCTTCAAACAGGAAAAATGCCTGATGAAGCA	2200
	Q H K A I A E A L L K Y E T L D A K Q I M S L F K T G K M P D E A	
642	GCGGCAGCAGAAGTACCAGAACCAAAACATTTGAAGAATCTCTCAAAGATGCAATGCGAATGTTGATGATTTTTCAAACATTAATATCTATAATGGTG	2300
	A A A E V P E P K T F E E S L K D A N A N V D D F S N I N I Y N G D	
676	ATGAAAAACAGATTCTAAACCAGAAGAAATAAGGAAAAATCAGAAGATGAACAGCCGAATAAGGCTGTTTTCTTTTTTTTATGTTTGAATAAGT	2400
	E K T D S K P E E N K E K S E D E T A E *	

Fig. 1. For legend see facing page.

sequence is 71 % identical to that of *E. coli* FtsH and 31 amino acid residues are identical among all sequences shown in Fig. 2.

Localization of *L. lactis* FtsH in the membrane

A system using Tn5phoA1 to generate protein fusions between the product of a target gene and alkaline phosphatase (AP) has been developed to detect genes encoding membrane and periplasmic proteins in *E. coli* (Manoil & Beckwith, 1985, 1986). Fusion of AP to a periplasmic protein or to a periplasmic domain of a membrane protein is essential for AP activity. Selection for Tn5phoA1 insertions in pLN2 (Table 1, contains the first 441 codons of *L. lactis* ftsH) resulted in six different plasmids (pLN22–pLN27, Table 1) expressing AP activity in *E. coli*. The fusion points between Tn5phoA1 and pLN2 in pLN22–27 were determined by DNA sequencing. All the insertions were located in the ftsH coding region between nucleotide 467 and 663 (codons 64–129) (Fig. 1).

To further analyse the localization of the *L. lactis* FtsH protein in the cell, we carried out Western blotting analysis of fractionated *L. lactis* cell extracts with anti-*E. coli* FtsH serum, which also reacts with FtsH of *L. lactis*. Sonicated cell extracts from *L. lactis* strain MG1363 and DN4302 ftsH::pLN43 (described below) were fractionated into cytoplasmic and membrane fractions by ultracentrifugation. The membrane fraction was washed with 1 M NaCl and separated into supernatant and pellet. In Fig. 3(a) and (b) (lanes 1–3) a SDS-polyacrylamide gel of the various fractions of cell extract of MG1363 and DN4302 blotted to nitrocellulose and gold-stained is shown. No significant differences in the protein patterns of the cytoplasmic or membrane fractions of MG1363 and DN4302 were found (Fig. 3a and b, lanes 1 and 3), whereas, the supernatants of the salt-washed membranes differed in protein patterns (Fig. 3a and b, lanes 2). The anti-*E. coli* FtsH serum only detected FtsH from MG1363 and a truncated form of FtsH from DN4302 in the pellets of the salt-washed membrane fractions (Fig. 3a and b, lanes 6).

It is concluded from the results described above, that FtsH of *L. lactis* is an integral membrane protein spanning the membrane twice with the region flanked by these hydrophobic stretches protruding outside the cell, and that it has a large cytoplasmic carboxy-terminal part with a putative ATP-binding domain (Fig. 4). Such overall features of the *L. lactis* FtsH topology are consistent with those of the *E. coli* homologue, FtsH (Tomoyasu *et al.*,

1993b), except that *L. lactis* FtsH has a larger outside domain than *E. coli* FtsH.

Complementation of Δ ftsH3::kan in *E. coli* with ftsH of *L. lactis*

Since the deduced amino acid sequences of ftsH from *L. lactis* and *E. coli* showed a high degree of identity, we were interested to know whether or not the *L. lactis* ftsH gene could complement a ftsH mutation in *E. coli*.

The *E. coli* strain AR423 Δ ftsH3::kan (pAR171 ftsH rep^{ts} Cm^R) shows temperature-sensitive growth, because the replication of the plasmid pAR171, containing the essential ftsH gene and a chloramphenicol (Cm) resistance marker, is defective at 42 °C (Akiyama *et al.*, 1994a).

The plasmid pLN32 contains the entire *L. lactis* ftsH gene, whereas the plasmids pLN2 and pLN39 contain various parts of the *L. lactis* ftsH gene, all cloned in the vector pBluescript KS+ or pBluescript SK— containing ampicillin resistance (Ap^R) markers (Fig. 5a, Table 1). These plasmids were used to transform strain AR423 by selection for Ap^R at 30 °C. The strains obtained were incubated in LB at 42 °C for 6 h, and then plated on LB agar at 30 °C. The plasmid content of the colonies was tested by streaking on LB agar containing Ap or Cm; and the possession of the Δ ftsH3::kan mutation was tested by streaking on LB agar containing Kan. From transformants containing the entire *L. lactis* ftsH gene on pLN32, Cm^S colonies could be isolated with a frequency of approximately 40–50 %, indicating that these had lost pAR171, containing the *E. coli* ftsH wild-type. Such colonies were all Ap^R and Kan^R, indicating that these had retained pLN32, containing the *L. lactis* ftsH gene, and the mutation Δ ftsH3::kan. Colonies of transformants with pLN2 and pLN39, containing only part of *L. lactis* ftsH, were all Ap^R, Cm^R and Kan^R, showing that no loss of pAR171 was obtained. Thus pAR171, and hereby the *E. coli* wild-type ftsH gene, can be lost only from strains containing the complete *L. lactis* ftsH gene. One strain, AR3120 Δ ftsH3::kan (pLN32 *L. lactis* ftsH), was saved for further studies.

Western blots with anti-*E. coli* FtsH serum were performed using extracts of AR423, AR423 containing the plasmids in Fig. 5(a) and AR3120 to detect the gene products of *E. coli* ftsH and/or *L. lactis* ftsH (Fig. 5b). The *E. coli* FtsH protein could be detected in the AR423 derivatives but not in AR3120, whereas the *L. lactis* FtsH protein was detected in AR423(pLN32) and AR3120. No *L. lactis* ftsH product was detected in AR423, AR423(pLN39), AR423(pLN2) or AR423(pKS+). This

Fig. 1. Nucleotide sequence of the *L. lactis* ftsH gene. The numbers to the right indicate nucleotide positions ('hpt, nucleotides 1–72; ftsH, nucleotides 278–2365, 696 codons). The deduced amino acid sequences of 'hpt and ftsH (FtsH) are shown in one-letter code below, with the translation stop codons indicated by asterisks. The numbers to the left indicate the deduced amino acid residue position of FtsH. The putative ribosome binding sites (RBS) and promoter regions (–35, –16, –10) are indicated. Two putative stem-loop structures are underlined. The Tn5phoA1 insertions in pLN22–27 are indicated above the sequence. The EcoRV sites used to construct the integration plasmid pLN43 are shown above the sequence.

Yme1p	MNVSKILVSPVTNTVLRIFAPRLPQIGASLLVQKKWALRSKKFYRFYSEKNSGEMPPKKEADSSGKASNKSTISSIDNSQPPPPSNTNDKT	92
LlFtsH	MNNKQPKQGNFVKNI <u>LMWVILAIVVVGFNFFSSNQSSVDKISYSQ</u> LMTKLDGNKIENVTMQPSDSLITVTGEYKEPVKVGKTNNFPLLGSSSEVK	100
EcFtsH	MAKNLILWLVI <u>AVVLSVFS</u> SGPSESNGRKYDYSTFLQEVNDQVREARINGREINVT.....KKDSNR	65
Yme1p	KQANVAVSHAMLATREQEANKDLTSPDAQAAFYKLLQSNPYQYVVSFRFETPGIASSPECMELYMEALQRIGRHSEADAVRQNLTTASSAGAVNPSSASS	192
LlFtsH	FQAYIIPTDSVVKDIQNAKSNVDKLSVVQASSSGMWQILSYIIPMLLVFGIWLMMGGMGARGGGGGGNPMSFGKSRKQQDGKTSKVRFADVAGSEE	200
EcFtsH	YTTYIPVQDPKLLD...NLLTKNVKVVGPEPEPSLLASIFISWFPMLLLIGVWIFFMRQM...QGGGKGAMSFGKSKARMLTEDQIKTTFADVAGCDE	159
Yme1p	SSNQSGYHGNFPMYSPLYGSRKEPLHVVVSESTFTVSRWVKWLLVFGILTYSFSEGFYITENTTLKSSEVADKS...VDVAKTN.VKFDDVCGCDE	288
TBP-1		QYSDIGGLDK 158
CDC1		GYDDIGGCRK 220
CDC2		TWDDVGGLE 493
Pas1p		KWGDIGALAN 704
NSF		EKMIGIGGLDK 233
LlFtsH	E.KQELVEVDFLKNPK.KYHDLGARIPAGVLLGPPGTGKTLAKAVAGEAGVPFYI.SGSDFVEMFVGVGASRVRLFENAKKTAPS.....II	289
EcFtsH	A.KEEVAELVEYLREPS.RFQKLGKIPKGVLMVGGPPGTGKTLAKAIAAGEAKVPFFTI.SGSDFVEMFVGVGASRVDMFEQAKKAAPC.....II	248
Yme1p	A.RAELEEIVDFLKDPT.KYESLGGKIPKGVLLTGPPGTGKTLARATAGEAGVDFFM.SGSEFDEVYVGGAIRDLFAQARSRAPE.....II	377
TBP-1	Q.IQELVEAIVLPMNHKEKFENLGIQPPKGVLMYGGPPGTGKTLARACAAQTATFLKL.AGPQLVQMFIGDGAKLVDAFALAKEKAPS.....II	248
CDC1	Q.MAQIREMVELPLRHPQLFKAIGIKPPRGVLMYGGPPGTGKTLARAVANETGAFFLI.NGPEVMSKMAGESNLKRAFEAEAKNAPA.....II	310
CDC2	I.KEELKETVEYVPLHPDQYTKFGLSPSKGVLYGPPGTGKTLAKAVATEVSANFISV.KGPELLSMWYGESESNIRIDFKARAAAPT.....VV	583
Pas1p	A.KDVLLETLEWPTKYEPYFVNCPLRLRSIGILLYGYPGCGKTLASAVAQCGLNFI.SV.KGPEILNKFIGASEQNIREFERAQSVKPC.....IL	794
NSF	EFSDIFRRASFASRVFPEIVEQMGCKHVKGILLYGPPGCGKTLARQIGKMLNAREPKVNVGPEILNKYVGESEANIRKLFADAEERQLRGANSGLHII	333
LlFtsH	FIDEIDAVGRQRGAGLGGGNDEREQTLNQLLVEMDGFQDDGNSVIVIAATNRSDVLDPALLRPGRFDRKVLVGPAPDVKGREAVLKVHA.....KN.K	380
EcFtsH	FIDEIDAVGRQRGAGLGGGNDEREQTLNQLLVEMDGFEGN.EGIIVIAATNRSDVLDPALLRPGRFDRQVVGGLPDVVRGREGIILKVMH.....RR.V	338
Yme1p	FIDEIDAIGGRKRP...KQAYAKQTLNQLLVLDGFSQT.SGIIIGATNFPEALDKALTRPGRFDRKVVNVLDLPVVRGRADILKHHM.....KK.I	464
TBP-1	FIDEIDAIGTKRFDSEKAGDREVQRTMLELLNQLDGFQNPQ.VKVIATNRVDILDPALLRSGRLDKRIEFMPNNEEARARIMQIHS.....RK.M	338
CDC1	FIDEIDSIAPKRDKTNG...EVERRVVSQLLTMDGMKARSN.VVVIATNRPNIDPALRRFRGRDREVDIGIPDATGRLEVLRIHT.....KN.M	397
CDC2	FLDEDSIAKARGGSLDAGGASDRVNVQLLTMDGMNAKKN.VFVIGATNRPDQIDPAILRPGRDLQIIVYPLDENARLSILNAQL.....RK.T	673
Pas1p	FFDEDSIAPKRGH...DSTGVTDRVNVQLLTMDGAEGLDG.VYILAATSRPDLIDSALLRPGRLDKSVICNIPTESERLDILQAIIVNSDKDKTGQKFF	890
NSF	IFDEIDAICKQRG.SMAGSTGVHDTVVNQLLSKIDGVEGLNN.ILVIGMTNRPDIDEALLRPGRLEVKMEIGLPDEKGRQLIHLIHTARM.....RGHQ	426
LlFtsH	PLASDVLDHNVATQTPGYVGADLENVLNEAALVAARQNKKEINAADIDEGMDRAMAGPAKKDRIQSMREREIVAYHEAGHAIIVGLVLENGSTVRKVTVP	480
EcFtsH	PLAPDIDAAIIARGTPGFSGADLANLVNEAALFAARGNKRVSVMVEFEKAKDKIMGAERRSMVMTEAQKESTAYHEAGHAIIGRLVPEHDPVHKVTIIP	438
Yme1p	TLADNVDPITIIARGTPGLSGAELANLVNQAAYVACQKNAVSVDMSHFEWAKDKILMGAERKTHVLTDAAARKATAFHEAGHAIMAKYTNGATPLYKATILP	564
TBP-1	NVSPDVNYEELARCTDDFNGAQCKAVCEA	368
CDC1	KLADDVDLEALAAETHGYVGADIASLSCSEA	427
CDC2	PLEPGLLETAIAKATQGFSGADLLIVQRA	703
Pas1p	ALEKNADKLIAEKTAGFSGADLQGLCYNA	920
NSF	LLSADVDIKELAVETKNFSGAELEGLVRAA	456
LlFtsH	RGRIGGYMLALPDEEIMQPTNFHLQDQLASLMGGRLGEEIVFGVA...TPGASNDIEKATHIARSMVTEYGMSSKLLGMVSY.EGDHQVFIGRDYGGTKTYS	577
EcFtsH	RGRALGVTFPLPEGDAISASRQKLESQISTLYGGRLAEEIYGPHEVSTGASNDIKVATNLARNMVTQWGFSEKLGPLLYAEEEGEVFLGRSVAKAKHMS	538
Yme1p	RGRALGITFQLPEMDKVDITKRECCARLDVCMGGKIAEELIYGKNDTSGCGSDLSQATGTARAMVTQYGMSSDDVGPVNLSEN...WESWS.NKIRD..	657
LlFtsH	EATAVMIDDEVRRILGEAYDRAKEAIEITHREQHKAIEALLKYETLDAKQIMSLFKTGKMPDEAAAAEVPPEPKTFEESLKDANANVDDF.SNINIYNGDE	676
EcFtsH	DETARIIDQEVKALIERNYNRARQLLTDNMDILHAMKDALKMYETIDAPQIDDL.....ARRDVRPPAGWEEP...GASNNSGDNQSPKAPRPVDE	627
Yme1p	IADNEVIELLDSEERARRLLTKKNVELHRLAAGLIEYETLDAHEIEQV.....CKGEKLD.....KLKTSTNTVVEGPDSDERDKIDG	736
LlFtsH	KTSKPEENKEKSEDETAE	695
EcFtsH	PRTPNPG.NTM.SEQ	640
Yme1p	DKPKIP...TMLNA	747

Fig. 2. Amino acid sequence alignment of *L. lactis* (Ll) FtsH, *E. coli* (Ec) FtsH, Yme1p and the conserved domains found in *L. lactis* FtsH (residues 191–410); TBP-1 (Nelbock *et al.*, 1990); CDC48p (CDC1, CDC2; CDC48p contains two conserved domains) (Fröhlich *et al.*, 1991); Pas1p (Erdmann *et al.*, 1991); and NSF (Wilson *et al.*, 1989). S4 of the 26S protease, M5S1 and SUG1 resembling TBP-1 (Dubiel *et al.*, 1992; Shibuya *et al.*, 1992; Swaffield *et al.*, 1992), VCP and p97 resembling CDC48p (Koller & Brownstein, 1987; Peters *et al.*, 1990), and Sec18p resembling NSF (Eakle *et al.*, 1988) also contain the conserved domain (not shown). Identical amino acid residues found in these sequences are indicated in boldface type above the sequences. Identical amino acid residues of *L. lactis* FtsH, *E. coli* FtsH and Yme1p are indicated by asterisks. The two putative membrane-spanning sequences of *L. lactis* FtsH and *E. coli* FtsH are underlined.

Fig. 3. Localization of *L. lactis* FtsH in cell fractions. Fractionation of extracts from MG1363 and DN4302, and treatment of membrane fractions with 1 M NaCl were done as described in Methods. Electrophoresis of the cell fractions on an SDS-polyacrylamide gel (a and b) was done, blotted to nitrocellulose and gold-stained (lanes 1–3) or subjected to Western blotting with anti-*E. coli* FtsH serum (lanes 4–6). Positions of molecular mass reference markers, FtsH and a truncated form of FtsH (FtsH') are indicated. All other bands are a result of unspecific binding of the secondary anti-serum used (control not shown). (a) Lanes: 1 and 4, cytoplasmic fraction of MG1363; 2 and 5, supernatant derived from membrane fraction of MG1363 washed with 1 M NaCl; 3 and 6, pellet derived from membrane fraction of MG1363 washed with 1 M NaCl. (b) Lanes: same as (a) except extract from DN4302 was used.

Fig. 4. Deduced topology of the *L. lactis* FtsH protein. Numbers indicate amino acid residue positions. Shaded boxes illustrate putative membrane-spanning regions. The encoded FtsH-PhoA fusions of pLN22–27 that results in AP activity are fused at positions indicated by triangles. The region with similarity to the conserved region of the AAA-protein family is indicated by an open box. This region contains the ATP binding motifs.

demonstrates that AR3120 was able to grow without the essential *E. coli* ftsH gene product when containing the *L. lactis* ftsH gene product. The plasmid pLN2 encodes a truncated *L. lactis* FtsH protein containing the epitope for the anti-*E. coli* FtsH serum, when transcribed and

Fig. 5. Immunological detection of *L. lactis* FtsH and *E. coli* FtsH. (a) The lines represent *L. lactis* chromosomal DNA fragments cloned in pBluescript KS+ or SK-. The restriction enzyme sites shown are: E, *EcoRI*; H, *HindIII*; M, *MboI*. The location of the *L. lactis* 'hpt' and ftsH genes is indicated by hatched and shaded areas, respectively. (b) Western blot. The FtsH proteins of *L. lactis* (LI) and *E. coli* (Ec) were detected using anti-*E. coli* FtsH serum. The positions of *L. lactis* FtsH and *E. coli* FtsH are indicated to the left. Lanes: 1, AR423(pKS+); 2, AR423(pLN39); 3, AR423(pLN2); 4, AR3120; 5, AR423(pLN32); 6, AR423. The molecular mass reference markers are indicated on the right.

translated *in vitro* (Nilsson & Lauridsen, 1992; data not shown). This truncated FtsH was not detected by the anti-*E. coli* FtsH serum in the Western blot, possibly due to rapid degradation *in vivo*.

E. coli AR3120 showed temperature-sensitive growth in that it could grow at 30 °C and 37 °C but not at 42 °C. A *B. subtilis* mutant with a salt-sensitive phenotype has been shown to be impaired in a putative homologue of ftsH (Geisler & Schumann, 1993). AR423, AR423(pLN32) and AR3120 were tested for growth on LB agar plates containing 4% (w/v) NaCl. AR423 and AR423(pLN32), but not AR3120, were able to grow on this medium.

Construction of a *L. lactis* ftsH mutant

A 1.3 kb *EcoRV* fragment of pLN32, containing an internal part of *L. lactis* ftsH (Fig. 1), was cloned into the *L. lactis* integration vector pV2 giving pLN43 (Table 1).

Fig. 6. Growth of DN4302 and MG1363 at various NaCl concentrations. MG1363 (a) and DN4302 (b) were grown exponentially in M17 medium and then diluted twofold in M17 medium containing 0% (■), 4% (●) or 7% (▲) (w/v) NaCl (final concentrations).

Transformation of pLN43 into *L. lactis* strain MG1363, selecting for erythromycin resistance, resulted in strain DN4302 *ftsH::pLN43*. The integration of pLN43 into *ftsH* of DN4302 was verified by Southern hybridization analysis (not shown). The construction of DN4302 indicates that the *ftsH* gene is not essential in *L. lactis* or that the truncated form of FtsH encoded by the disrupted *ftsH* gene (Fig. 3b, lane 6) is enough to retain any essential function. DN4302 and MG1363 were tested for growth on M17-agar plates containing 4% or 1% NaCl. MG1363 could grow at both 4% and 1% NaCl, whereas DN4302 grew slowly at 1% and not at all at 4% NaCl. Fig. 6 shows the growth of DN4302 and MG1363 at various salt concentrations in M17 broth. MG1363 grew at 4% NaCl after a lag phase of about 2 h, whereas DN4302 at 4% NaCl showed almost no growth. No growth of either strains occurred with 7% NaCl. DN4302 and MG1363 were also tested for growth on M17-agar plates containing 0.5 M sucrose. Both strains appeared to grow equally well on this medium.

Growth at 38 °C, 30 °C and 16 °C on M17-agar plates was also tested. DN4302 only grew at 30 °C, whereas MG1363 grew at all temperatures. However, incubating DN4302 anaerobically restored growth at 38 °C.

Homologous genes in other bacteria

A 1.0 kb *HindIII*–*KpnI* fragment (nucleotides 667–1600, Fig. 1) was used as probe in Southern hybridization analysis with *ScaI*-digested chromosomal DNA from

various bacterial strains (Fig. 7). Single chromosomal bands can be detected from the Gram-positive bacteria *L. lactis*, *B. subtilis*, *Leuconostoc* sp. and *Lactobacillus* sp. (Fig. 7, lanes 1, 3–8). With *E. coli* DNA only a very faint band was detected (Fig. 7, lane 2). Under the same experimental conditions, homology to *hpt*, using a *hpt* probe, was only detected in *L. lactis* (results not shown).

DISCUSSION

The *ftsH* gene of *L. lactis* was cloned and shown to encode a transmembrane protein with putative ATPase activity. The deduced amino acid sequence indicates that FtsH belongs to the AAA-protein family of putative ATPases containing several members of eukaryotic proteins (Erdmann *et al.*, 1991; Kunau *et al.*, 1993) and also includes the membrane protein FtsH of *E. coli* (Tomoyasu *et al.*, 1993a, b). FtsH from *L. lactis* and *E. coli* seem to be homologous proteins based on the high similarity of the amino acid sequences and that the *L. lactis* *ftsH* gene can complement certain defects of an *E. coli* Δ *ftsH3::kan* strain. Southern hybridization analysis of various Gram-positive bacteria using *L. lactis* *ftsH* as probe suggests that homologous genes exist. In *B. subtilis* a putative *ftsH* gene has been located on the chromosome of *B. subtilis* adjacent to the *hpt* gene (Geisler & Schumann, 1993; Ogasawara *et al.*, 1994), indicating a similar arrangement of *hpt* and *ftsH* as in *L. lactis*. It seems from these results that *ftsH* genes are widely conserved in bacteria.

The function of FtsH is unknown. Our *L. lactis* *ftsH* mutant and the *B. subtilis* *ftsH* mutant (Geisler & Schumann, 1993) were constructed by a Campbell-type integration with an internal gene fragment. This should inactivate the genes. In *E. coli*, *ftsH* is essential for growth. Since the resulting mutants are viable, it is possible that the *ftsH* genes are not essential in *L. lactis* and *B. subtilis*. However, in at least *L. lactis*, the truncated form of FtsH produced by the mutant DN4302 may still retain essential functions.

The *L. lactis* *ftsH* mutant DN4302 is impaired in salt tolerance and other stress responses, and shows a different pattern of membrane-associated proteins compared to the wild-type strain (Fig. 3, lanes 2). These phenotypes could be explained by an improper assembly of membrane proteins, some necessary for the salt tolerance, caused by the *ftsH* mutations in *L. lactis*. In *E. coli* the maturation of penicillin binding protein 3 (PBP3) and β -lactamase is dependent on FtsH function, in that post-translational processing at the C-terminal part of PBP3 seems to be defective and accumulation of the plasmid-encoded precursor of β -lactamase in the cytoplasm was observed in the thermosensitive *ftsH1* mutant of *E. coli* (Begg *et al.*, 1992; Tomoyasu *et al.*, 1993a). Analysis of several newly constructed *E. coli* *ftsH* mutants encoding FtsH variants, including C-terminally truncated forms with dominant phenotypes, suggests that FtsH is involved in assembly/folding of proteins into and through the membrane and that FtsH is needed to assure efficient stop-transfer of some membrane proteins (Akiyama *et al.*, 1994a, b).

Fig. 7. Southern hybridization of an internal part of the *L. lactis* *ftsH* gene to chromosomal DNA from various bacteria. Lanes: 1, *L. lactis* CHCC285; 2, *E. coli* XL-1 Blue; 3, *B. subtilis* 168; 4, *Leuconostoc mesenteroides* DB1165; 5, *Leu. oenos* LOP89005; 6, *Lactobacillus plantarum* LP2T1G; 7, *Lactob. acidophilus* LA2753; 8, *Lactob. lactis* CH-2. Molecular mass markers are shown on the right in kb.

However, how FtsH is functionally related to these phenotypes is not known. Recently it was shown that a *bfbB* mutation in *E. coli* causing increased lysogenization frequencies of bacteriophage λ through a reduced degradation of the α II protein, is located in *ftsH*, and the HflB/FtsH protein has been suggested to be an energy-dependent chaperone and/or protease (Herman *et al.*, 1993). Other members of the AAA-protein family are also involved in proteolysis, e.g. subunit 4 of the human ATP-dependent 26S protease, and it was proposed that other eukaryotic members such as Tbp1, Sug1 and MSS1 are also subunits of the 26S protease (Dubiel *et al.*, 1992). Taken together FtsH might function as a chaperone and/or a protease although no direct biochemical evidence for these functions has been reported so far. Comparative biochemical and genetic analysis of FtsH from *L. lactis* and *E. coli* will provide better understanding of the biological significance of FtsH, if it is a chaperone or a protease, and what are its substrates.

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