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# The *Lactobacillus plantarum ftsH* Gene Is a Novel Member of the CtsR Stress Response Regulon[▽]

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**FtsH proteins have dual chaperone-protease activities and are involved in protein quality control under stress conditions. Although the functional role of FtsH proteins has been clearly established, the regulatory mechanisms controlling *ftsH* expression in gram-positive bacteria remain largely unknown. Here we show that *ftsH* of *Lactobacillus plantarum* WCFS1 is transiently induced at the transcriptional level upon a temperature upshift. In addition, disruption of *ftsH* negatively affected the growth of *L. plantarum* at high temperatures. Sequence analysis and mapping of the *ftsH* transcriptional start site revealed a potential operator sequence for the CtsR repressor, partially overlapping the −35 sequence of the *ftsH* promoter. In order to verify whether CtsR is able to recognize and bind the *ftsH* promoter, CtsR proteins of *Bacillus subtilis* and *L. plantarum* were overproduced, purified, and used in DNA binding assays. CtsR from both species bound specifically to the *ftsH* promoter, generating a single protein-DNA complex, suggesting that CtsR may control the expression of *L. plantarum ftsH*. In order to confirm this hypothesis, a Δ*ctsR* mutant strain of *L. plantarum* was generated. Expression of *ftsH* in the Δ*ctsR* mutant strain was strongly upregulated, indicating that *ftsH* of *L. plantarum* is negatively controlled by CtsR. This is the first example of an *ftsH* gene controlled by the CtsR repressor, and the first of the low-G+C gram-positive bacteria where the regulatory mechanism has been identified.**

FtsH proteins are membrane-bound ATP- and $Zn^{2+}$-dependent metalloproteases belonging to the AAA (ATPase associated with different cellular activities) protein family, a distinct subfamily of the Walker-type ATPases (27). These ATP-dependent proteases also have intrinsic chaperone activity and have therefore been designated charonins (for reviews, see references 22 and 33). Consistent with its dual chaperone-protease function, FtsH plays an important role in the protein quality control network, which not only allows refolding or degradation of denatured and misfolded proteins generated under stress conditions but also enables the temporal control of many cellular processes by regulating the protein stability of specific, critical regulators (18, 19, 22). In *Escherichia coli*, *ftsH* is a heat-inducible gene (21), while in *Bacillus subtilis*, it is transiently induced at the transcriptional level upon osmotic and temperature upshifts (12). Recently, a similar pattern of expression was also observed in lactic acid bacteria (LAB) such as *Oenococcus oeni* and *Lactobacillus plantarum*. In *O. oeni*, *ftsH* expression was increased by high temperatures and osmotic shock, and its protective role against heat shock was demonstrated by heterologous expression in *E. coli* (3). In addition, genome-wide microarray-based expression profiling revealed that *ftsH* is upregulated by bile treatment in *L. plantarum* WCFS1 (5). Therefore, FtsH seems to be an important component of the stress response machineries developed by several bacteria in order to withstand harsh conditions and sudden environmental changes. Although *ftsH* is induced by stress in *B. subtilis*, *O. oeni*, and *L. plantarum*, no typical stress-responsive elements of gram-positive bacteria genes have been observed in its promoter. In *B. subtilis*, a model organism for gram-positive bacteria, in which the stress response has been extensively studied, heat-inducible genes are divided into different subclasses based on their regulatory mechanisms (34). In particular, class I genes encode classical chaperones such as DnaK and GroEL, whose expression involves a highly conserved operator sequence, which is the binding site for the HrcA repressor. Class II genes are regulated by the alternative $\sigma^B$ factor. Class III genes, such as *clp* genes, are controlled by the class III stress gene repressor CtsR (designation from "class three stress gene repressor"), which binds to a specific heptanucleotide direct repeat (RGTCADN NAN RGTCADN), referred to as the CtsR box (9). Genes regulated by as yet unknown mechanisms are grouped under class IV; these include *ftsH*, whose heat shock induction was shown to be CtsR independent in *B. subtilis* (9, 34).

*Lactobacillus plantarum*, a facultatively heterofermentative LAB, is one of the most widespread LAB in the environment. A natural inhabitant of the human gastrointestinal tract, it is also found in several food fermentation products for which stress conditions such as heat, cold, and acidity are common. In wine, although *L. plantarum* is capable of malolactic fermentation, it usually contributes to the production of undesirable products such as biogenic amines and precursors of ethyl car-

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TABLE 1. Oligonucleotides used in this study

| Oligonucleotide[a] | Sequence (5′–3′) | Application |
|---|---|---|
| FtsHP | CGTTTGTGAGCCGGAATTACCGC | Primer extension analyses |
| ldhDF | ACGCCCAAGCTGATGTTATATC | qRT-PCR |
| ldhDR | AGTGTCCCACGAGCAAAGTT | qRT-PCR |
| gyrAF | CATGCGGTTAGGCGATGAT | qRT-PCR |
| gyrAR | ATCGGCGTTGACGGTTTG | qRT-PCR |
| FtsH1F | TTACGGTCTTGACTATGCAGAACGCTATCGGAACT | Gel mobility shift assay |
| FtsH2F | TCGATGAAGGAGGCACATATGAACAATCGACGCA | Gel mobility shift assay |
| FtsH1R | GCTGGATACTAACGTTTTTAACGTTATTCTTATCTAATTG | Gel mobility shift assay |
| FB1ctsR | ATCTCGAGTTAAAATCCTGCGGTTAGTG | *ctsR* chromosomal deletion |
| RB1ctsR* | GACTTTGCATGTGCTTCACC | *ctsR* chromosomal deletion |
| FB2ctsR* | CGGGCCCGGATAATTATCGG | *ctsR* chromosomal deletion |
| RB2ctsR* | TCATCCGTAATCGTAACCCG | *ctsR* chromosomal deletion |
| FBctsR* | TGAACCGGCAACAAGGCATG | *ctsR* chromosomal deletion |
| CtsRrtF | AATTTGGTCGATGATGCTGATG | qRT-PCR |
| CtsRrtR | TAAGTCCCGGTCCGTTAATCC | qRT-PCR |
| CtsR1 | GGTGGTCTCCCATGCAAAGTCAAAATATC | *ctsR* overexpression |
| CtsR2 | CTCCTCGAGGCTTTCGTAACGCAAGTGGTT | *ctsR* overexpression |
| CatF | TCAAATACAGCTTTTAGAACTGG | *ctsR* chromosomal deletion |
| CatR | CCAGTAAATGAAGTCCATGGA | *ctsR* chromosomal deletion |
| ftsHKOF | ATGGTACCGGACTTATTCGAACAAGCTAAG | *ftsH* disruption |
| ftsHKOR | TAGGATCCGTAAGCTGCTTGTTGGTTG | *ftsH* disruption |
| pUCeryF | CCAGGCTTTACACTTTATGC | *ftsH* disruption |
| pUCeryF | TGGAAAGTTACACGTTACTAAAG | *ftsH* disruption |
| ftsHrtF | GCAGCTACCTTCGAAGAATCCA | qRT-PCR |
| ftsHrtR | GGGAAACTTGGTTCAGCAACA | qRT-PCR |
| ftsHF | AAAACTGCAGAATCGACGCAATGGAC | *ftsH* disruption |
| ftsHR | GCTCTAGACGCTCATAACCGAATTAACG | *ftsH* disruption |

[a] Asterisks indicate 5′-phosphorylated oligonucleotides.

bamate and is therefore generally considered a nuisance. Vinification generates multiple stress conditions, including an acidic pH, ethanol, extreme temperatures, and growth-inhibitory compounds such as fatty acids and tannins. The survival of *L. plantarum* in this stressful environment indicates that it has developed several tolerance and resistance mechanisms. Study of the stress response of *L. plantarum* is thus essential in order to understand the high adaptability of this microorganism to stress conditions.

The genome of *L. plantarum* WCFS1 has been completely sequenced (23). Analysis of the 5′ noncoding region of the *L. plantarum ftsH* gene allowed us to identify a putative operator sequence highly similar to the CtsR binding site. In this work, we report that the *ftsH* gene of *L. plantarum* is heat induced and is under the control of CtsR. To our knowledge, this is the first report identifying *ftsH* as a novel member of the CtsR stress response regulon, and this is the first *ftsH* gene in low-G+C gram-positive bacteria for which the regulatory mechanism has been identified.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** *E. coli* strains DH10B, TG1, and BL21 λ70 DE3 were used for DNA cloning and overexpression and were grown in Luria-Bertani (LB) broth or on LB agar plates at 37°C. *L. plantarum* WCFS1 (23) was routinely grown in MRS broth (7) (initial pH 6.2) at 28°C without shaking. When required, appropriate antibiotics were added at the following concentrations: ampicillin at 100 μg $ml^{-1}$ and erythromycin at 200 μg $ml^{-1}$ for *E. coli*; erythromycin at 10 μg $ml^{-1}$ and chloramphenicol at 10 μg $ml^{-1}$ for *L. plantarum*.

For heat, salt, and bile stresses, 0.1 ml of stationary-phase *L. plantarum* cells (optical density at 600 nm [$OD_{600}$], 2.6) was diluted in 30 ml of fresh MRS broth (pH 6.2), and growth was initiated at 28°C. When the $OD_{600}$ reached 0.6 (pH 6.1), the cultures were transferred to water baths maintained at 42°C for various times or at 50°C for a short (5-min) heat shock. For salt and bile stresses, *L. plantarum* cells were harvested by centrifugation (at 4,500 × *g* for 10 min) and resuspended in 30 ml of fresh MRS broth containing either 0.8 M NaCl or 0.15% (wt/vol) porcine bile extract; stresses were imposed for 10 min and 1 h. Aliquots were removed, and total RNA was extracted and used for quantitative real-time PCR (qRT-PCR) analysis. The control culture was grown at 28°C in MRS medium.

The expression of the *ftsH* gene in the wild type was monitored over a complete culture cycle (20 h) performed at 28°C in MRS. Growth was monitored by both OD measurement ($OD_{600}$) and direct plate counting. Total RNA used for qRT-PCR analyses was extracted at the various growth stages. In order to analyze *ftsH* expression in an *L. plantarum* Δ*ctsR* mutant, stationary-phase *L. plantarum* Δ*ctsR* cells were diluted in fresh MRS medium and allowed to grow to mid-exponential phase ($OD_{600}$, 0.6 to 0.8). Total RNA was then extracted and used for qRT-PCR analysis.

The growth rates of the *L. plantarum ftsH* mutant and wild-type strains were determined by diluting overnight cultures 1:1,000 in fresh MRS medium and monitoring growth by $OD_{600}$ measurement and direct plate counting. A complete cycle of growth at 28°C and at 42°C (heat stress condition) was monitored.

**DNA manipulation and analysis.** Standard methods were used for DNA manipulations, including isolation, restriction endonuclease analysis, and ligation (32). *Taq* polymerases, restriction enzymes, alkaline phosphatase, and T4 DNA ligase were purchased from Roche (Milan, Italy), Invitrogen (Milan, Italy), New England Biolabs (Hertfordshire, United Kingdom), Fermentas (Burlington, Ontario, Canada), and Promega (Milan, Italy) and were used as recommended by the suppliers.

Double-stranded plasmid DNA was isolated using QIAprep spin miniprep kits (Qiagen, Milan, Italy). PCR products and DNA restriction fragments were purified with the QIAquick PCR purification and gel extraction kits (Qiagen, Milan, Italy). *L. plantarum* chromosomal DNA was prepared using a microbial DNA extraction kit (Cabru, Milan, Italy) according to the manufacturer's procedure. For PCR experiments, 20 ng of genomic DNA from *L. plantarum* was added to a 50-μl PCR mixture and amplified with the Expand Long Template PCR system (Roche, Milan, Italy) by following the manufacturer's instructions. The reaction mixture was cycled through the following temperature profile: 94°C for 5 min, 5 cycles of 94°C for 1 min, 45°C for 1 min, and 68°C for 2 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, and 68°C for 2 min. The PCR was ended by incubation at 72°C for 5 min. The oligonucleotides used in this study are listed in Table 1.

**RNA isolation and analysis.** Total RNAs were extracted using the UltraClean microbial isolation kit (Cabru, Milan, Italy) according to the manufacturer's instructions. The quality of the RNA samples was verified by electrophoresis on 1.2% agarose gels, and RNA concentrations were calculated using Quantity One software (Bio-Rad, Milan, Italy). About 1 μg of total RNA was used to synthesize cDNA using Quantitect reverse transcription kit (Qiagen, Milan, Italy), which includes DNase I treatment. The absence of chromosomal DNA contamination was confirmed by real-time PCR on corresponding DNase I-treated RNA.

qRT-PCR was performed on an Applied Biosystems 7300 real-time PCR system using SYBR green I detection. The *ldhD* and *gyrA* genes of *L. plantarum* were used as internal controls for the analysis of *ftsH* gene expression during abiotic stresses (10, 15). Five microliters of 20-fold-diluted cDNA was added to 15 μl of a real-time PCR mixture containing the Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA) and 100 nM each primer (Table 1). Cycling conditions included an initial denaturation-enzyme activation at 95°C for 10 min, followed by 35 cycles of 20 s at 95°C, 30 s at 58°C, and 30 s at 72°C. Fluorescence was monitored during each extension phase, and a melting-curve analysis was performed after each run to confirm the amplification of specific transcripts. Data were analyzed using AB 7300 software, by applying the two-standard-curves quantification method. Each assay included triplicate PCR of the samples, negative no-template controls, and standard curves for both the internal-control and target genes, obtained by amplifying serial dilutions (ratio, 1:10) of the cloned target sequence.

**Primer extension analysis.** Total RNA was isolated as described previously (30) from *L. plantarum* cells grown in MRS medium to mid-exponential phase at 30°C or 42°C for 15, 30, or 60 min. Primer extension products of *ftsH* transcripts were obtained using oligonucleotide FtsHP (Table 1), and primer extension was performed as previously described (30).

**Overproduction and purification of CtsR.** *B. subtilis* CtsR was overexpressed and purified as previously described (9). The *L. plantarum ctsR* coding sequence was PCR amplified using primer pair CtsR1–CtsR2 (Table 1) and was cloned between the NcoI and XhoI sites of plasmid pET28/16 (6), generating a carboxy-terminal translational fusion with six histidine residues under the control of a T7 bacteriophage promoter. The recombinant pET2816CtsR vector was transformed into *E. coli* strain BL21 λDE3, in which the T7 RNA polymerase gene is under the control of the inducible *lacUV5* promoter (36).

The His-tagged recombinant protein was then overexpressed and purified as previously described (13). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 15% acrylamide gels was performed as described previously (25), and protein concentrations were determined using the Bio-Rad protein assay based on the method developed by Bradford (4).

**Gel mobility shift DNA binding assays.** DNA fragments corresponding to the promoter region of the *L. plantarum ftsH* gene were generated by PCR with biotin-labeled primers (Table 1). Primers FtsH1F, FtsH2F, and FtsH1R were used to amplify two DNA fragments of 343 bp and 202 bp, with or without the predicted CtsR binding site, respectively. Binding of CtsR to DNA was carried out in a 10-μl reaction volume containing 1 μg of poly(dI-dC) (Pharmacia, Milan, Italy), 25 mM $Na_2HPO_4/NaH_2PO_4$ (pH 7), 150 mM NaCl, 0.1 mM EDTA, 2 mM $MgSO_4$, 1 mM dithiothreitol, and 10% glycerol. The DNA binding reaction was initiated by the addition of CtsR, and the mixture was incubated at room temperature for 20 min. Samples were then loaded directly onto a 4% polyacrylamide gel (50 mM Tris, 400 mM glycine, 1.73 mM EDTA) for electrophoresis (14 V $cm^{-1}$). Electrophoresis was performed for 1 h at room temperature. DNA fragments were transferred by semidry electrotransfer to a nylon membrane (Amersham, Milan, Italy) and revealed with horseradish peroxidase-coupled streptavidin by ECL detection (Pierce, Rockford, IL).

**Construction of a chromosomal deletion mutant of the *ctsR* gene.** The deletion mutant for *L. plantarum ctsR* was constructed using the Cre-*lox*-based mutagenesis system (26). DNA fragments corresponding to the chromosomal regions upstream (800-bp fragment; primer pair FB1ctsR–RB1ctsR) and downstream (1,200-bp fragment; primer pair FB2ctsR–RB2ctsR) of *ctsR* were amplified by PCR using a proofreading DNA polymerase and *L. plantarum* WCFS1 chromosomal DNA. The amplicons were cloned between the XhoI-SmiI and Ecl136II restriction sites of the suicide vector pNZ5319 (26), and the recombinant mutagenesis vector, pNZ5319CTSR, was introduced into *L. plantarum* WCFS1 by electroporation. Chloramphenicol-resistant transformants were selected and replica plated to check for erythromycin sensitivity, reflecting loss of the plasmid vector. Candidate double-crossover mutant clones were analyzed by PCR, and correct integration of the *lox66-P32-cat-lox71* cassette into the genome was further verified by PCR using primer FBctsR, annealing uniquely to the genomic region, combined with the mutagenesis vector-specific primers (CatF and CatR) (Table 1). In order to excise the *P32-cat* selectable marker cassette, the *cre* expression plasmid pNZ5348 (26) was transformed into the *ctsR*::*lox66-P32-cat-lox71* gene replacement mutant. Erythromycin-resistant and chloramphenicol-sensitive colonies were checked by PCR for Cre-mediated recombination and correct excision of the *P32-cat* cassette by using primers spanning the recombination locus (FBctsR and RB2ctsR) (Table 1).

*ctsR* deletion was confirmed by genomic DNA sequencing, and the absence of the gene transcript was verified by qRT-PCR (primers CtsRrtF and CtsRrtR) (Table 1).

**Disruption of the *ftsH* gene.** The *ftsH* gene of *L. plantarum* WCFS1 was disrupted by single-crossover plasmid integration as reported previously (39). An 870-bp internal *ftsH* fragment was PCR amplified using primers ftshKOF and ftshKOR and was cloned into pUC18ery between the KpnI and BamHI restriction sites. The resulting recombinant plasmid, pUCFTSH, was transformed into *L. plantarum* by electroporation, and candidate integrants were obtained on MRS agar plates containing 10 μg erythromycin $ml^{-1}$. Correct integration of pUCFTSH in the *ftsH* locus was confirmed by PCR analysis using primers annealing to flanking genomic regions (ftsHF and ftsHR) combined with vector-specific primers (pUCeryF and pUCeryR) (Table 1). A single *ftsH* disruption mutant was selected and used in subsequent studies. The absence of the *ftsH* transcript was confirmed by qRT-PCR (primers ftsHrtF and ftsHrtR) (Table 1).

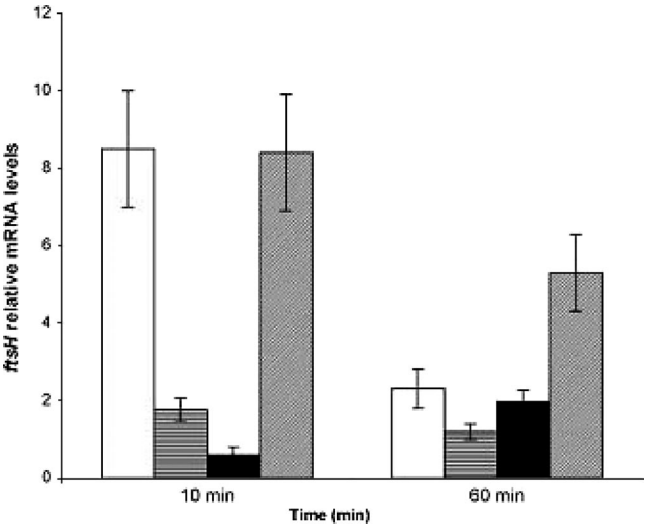


FIG. 1. Relative mRNA levels of *L. plantarum ftsH* in response to various types of stress as determined by qRT-PCR. mRNA levels were calculated relative to the transcript level detected in corresponding unstressed cultures and were normalized using *ldhD* as an internal control. Total RNA was extracted and analyzed in the same way 10 and 60 min after exposure to stress. The data presented are averages for three independent experiments; error bars indicate standard deviations. Stress conditions were a heat stress at 42°C (open bars), osmotic stress in 0.8 M NaCl (horizontally striped bars), bile stress (0.15% porcine bile) (filled bars), and combined heat and osmotic stresses (42°C and 0.8 M NaCl) (hatched bars).

## RESULTS

**Heat shock induces *ftsH* expression in *L. plantarum*.** Analysis of the *ftsH* mRNA level during growth under optimal conditions revealed that this gene is expressed at a basal level throughout growth, with a tendency for the level to increase progressively in the late-stationary phase (data not shown). Expression of *ftsH* was then analyzed following exposure of cells to a high temperature (42°C), an osmotic stress (0.8 M NaCl), bile (0.15% porcine bile), or heat and osmotic stresses combined (42°C and 0.8 M NaCl), imposed for 10 min and 1 h (Fig. 1). The constitutive *ldhD* gene was used as an internal control in the qRT-PCR experiments (10, 15). Results were

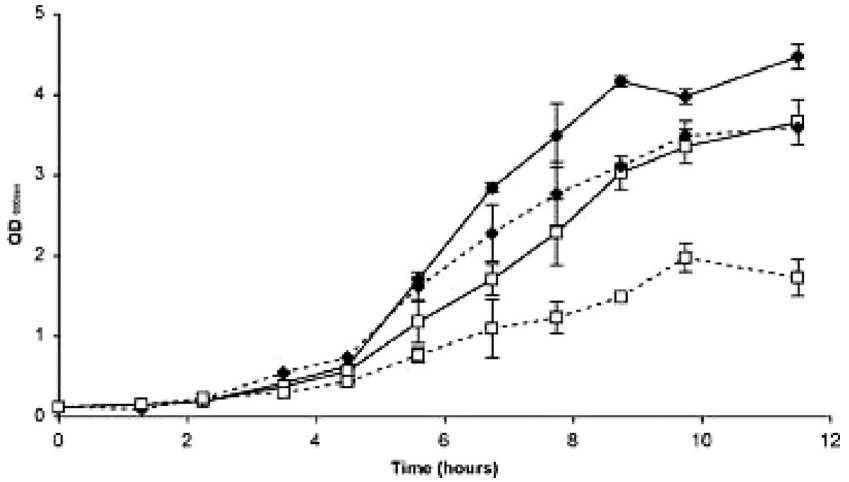


FIG. 2. Growth of *L. plantarum* wild-type and *ftsH* mutant strains at an optimal temperature and under heat stress conditions. Cells were cultivated either at 28°C (solid lines) or at a suboptimal temperature of 42°C (dashed lines). The increase in $OD_{600}$ is shown as a function of time (hours) and was monitored over 12 h for both the wild-type (◆) and *ftsH* mutant (□) strains. Data shown are means ± standard deviations for one of three independent experiments.

also confirmed by normalizing to *gyrA* mRNA levels. *ftsH* transcriptional levels were calculated relative to the mRNA level detected in the control unstressed cells. As reported in Fig. 1, strongly increased *ftsH* expression was observed 10 min after a temperature upshift to 42°C (eightfold increase). Further induction was observed after 1 h (twofold increase). In contrast, weaker induction was detected in response to osmotic (0.8 M NaCl) and bile (0.15% porcine bile) stresses after a 10-min exposure. Osmotic stress significantly induced expression of the *ftsH* gene after 10 min (twofold increase), while only a 1.6-fold increase was noted after 1 h for bile stress (Fig. 1). Marked induction (five- to eightfold) was observed in response to the combined heat and osmotic shocks.

***ftsH* deletion affects growth at a high temperature.** In order to elucidate the importance of FtsH in *L. plantarum*, a mutant strain was constructed by insertional mutagenesis. qRT-PCR analyses confirmed the absence of *ftsH* transcripts in the mutant strain (data not shown). The *ftsH* mutant displayed a lower growth rate than the wild type under optimal growth conditions (Fig. 2). The growth impairment of the mutant strain was much more marked when cells were cultivated under heat stress conditions (42°C) (Fig. 2). This finding suggests that, in comparison to its role in other bacteria, for which no viable null mutant could be obtained (34), FtsH plays a less critical role in *L. plantarum*. However, FtsH function becomes particularly crucial for coping with harsh conditions, such as high temperatures, indicating the involvement of this protease in heat stress response mechanisms.

**Promoter analysis of *ftsH* and identification of a putative CtsR binding site.** In order to identify regulatory elements controlling *ftsH* expression, the 5′ mRNA transcriptional start site of *ftsH* was determined by primer extension analysis on total RNA extracted from exponentially growing *L. plantarum* cells following incubation at 30°C and 42°C for 15, 30, and 60 min (Fig. 3). Extension products were obtained only from RNA extracted from heat-stressed cells (Fig. 3A). Indeed, a marked band corresponding to the primer extension product appeared after a 10-min temperature upshift, decreased in intensity between 10 and 30 min after the temperature upshift, and became extremely faint after 60 min of heat stress, corroborating the heat induction results obtained by qRT-PCR (see Fig. 1). No extension product was detected for RNA extracted from control cells harvested before stress treatment or from control cells incubated at 30°C for 15, 30, or 60 min. The transcription initiation site was identified at position −41 relative to the translational start codon. −35 (TTGGTC) and −10 (TATAAT) hexamers separated by 18 nucleotides, highly similar to the consensus of *L. plantarum* sigma A-dependent promoters, were identified at an appropriate distance from the transcriptional start site (Fig. 3B). Sequence analysis of the promoter also revealed a potential binding site for the CtsR

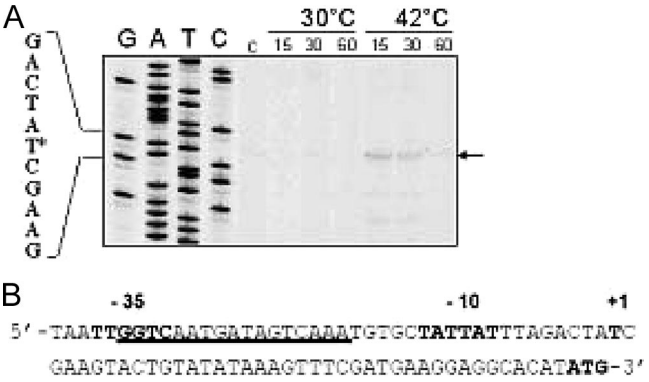


FIG. 3. Mapping and sequence analysis of the *L. plantarum ftsH* promoter. (A) Primer extension analysis of *ftsH* mRNA. Total RNA was isolated from *L. plantarum* WCFS1 cells grown to exponential phase at 30°C (control [C]) and after incubation at either 30°C or 42°C for 15, 30, or 60 min. Lanes G, A, T, and C show DNA sequencing products obtained on genomic DNA with the same primer used for primer extension. The corresponding nucleotide sequence is shown on the left. The transcription start site is indicated by an asterisk. (B) Nucleotide sequence of the *ftsH* promoter region. −10 and −35 sequences and the transcriptional start site are in boldface. The *ctsR* binding site is underlined. The translation initiation codon (ATG) is in boldface.

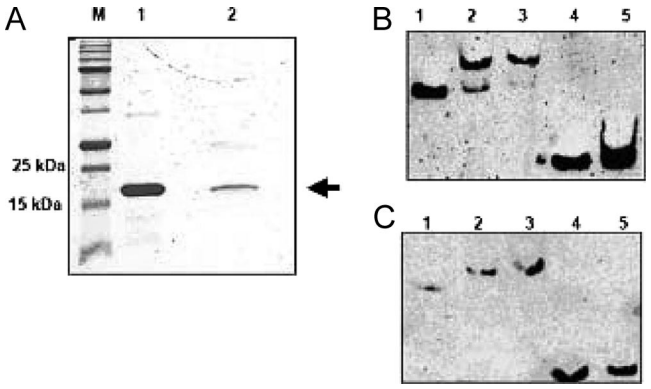


FIG. 4. Overexpression, purification, and gel mobility shift assays with purified CtsR. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of purified His-tagged CtsR from *E. coli* strain BL21 λDE3 carrying the recombinant pET2816CtsR plasmid. Purified recombinant CtsR proteins from *B. subtilis* (lane 1) and *L. plantarum* (lane 2) were used. The position of CtsR is indicated by an arrow. M, molecular mass standard. (B and C) Electrophoretic mobility shift assay. Biotin-labeled DNA fragments corresponding to the promoter region of *ftsH* were incubated with increasing amounts of purified CtsR from *B. subtilis* (B) and *L. plantarum* (C). Lanes 1, no CtsR; lanes 2 and 3, 100 and 250 ng of CtsR, respectively; lanes 4 and 5, promoter fragments lacking the CtsR box were preincubated with or without 250 ng of CtsR protein, respectively.

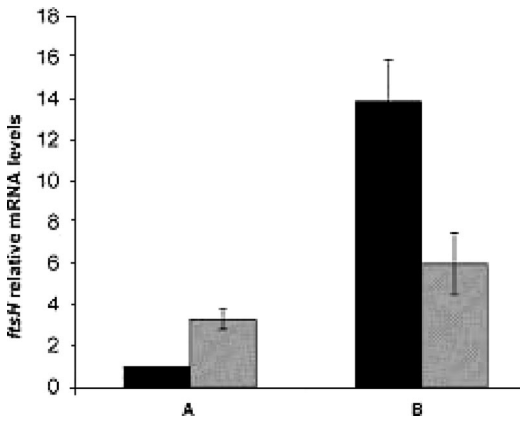


FIG. 5. Comparison of *ftsH* mRNA levels in wild-type and Δ*ctsR* mutant strains of *L. plantarum* by qRT-PCR. *ftsH* expression was analyzed in wild-type (filled bars) and Δ*ctsR* mutant (hatched bars) strains under optimal-temperature (30°C) growth conditions (A) and following a heat stress imposed for 5 min at 50°C (B). In all experiments, mRNA levels were normalized to that for the *ldhD* housekeeping gene. Under unstressed conditions (A), *ftsH* mRNA levels in the Δ*ctsR* mutant strain were calculated relative to the mRNA level of the wild-type strain, which was assigned a value of 1. Under heat shock conditions (B), *ftsH* mRNA levels in the wild-type and Δ*ctsR* mutant strains were calculated relative to the corresponding mRNA levels for the unstressed condition (A). Data are averages ± standard deviations of three independent experiments.

repressor, partially overlapping the −35 box and closely resembling the consensus heptad direct-repeat (RGTCADN NAN RGTCADN) CtsR binding site defined in several gram-positive bacteria (9). The potential CtsR box is located between the −35 and −10 boxes, consistent with the role of CtsR as a repressor and in agreement with the locations of previously characterized *ctsR* operators (9, 34).

**Purified CtsR binds specifically to the *ftsH* promoter region of *L. plantarum*.** An in vitro approach was used to demonstrate direct interaction of CtsR with its putative target site in the *ftsH* promoter. To verify whether CtsR was able to recognize and bind the *ftsH* promoter, a gel mobility DNA binding assay was performed (Fig. 4). The *B. subtilis* and *L. plantarum ctsR* genes were cloned and overexpressed in *E. coli* (see Materials and Methods). The overproduced proteins were purified (Fig. 4A) and used in DNA binding experiments with a biotin-labeled DNA fragment corresponding to the promoter region of *L. plantarum ftsH* (see Materials and Methods). CtsR from both species was able to bind specifically to the *ftsH* promoter, generating a single protein-DNA complex, with a gradual displacement of the DNA fragment as the protein concentration increased (Fig. 4B and C). This binding was specific, as shown by the absence of any protein-DNA complex when CtsR proteins were incubated with promoter fragments lacking the CtsR target sequence (Fig. 4B and C). The cross-reactivity between *B. subtilis* CtsR and the *L. plantarum ftsH* promoter confirmed the high degree of conservation of this transcriptional regulator and its corresponding target, even among distantly related bacteria. These results indicate that *L. plantarum* CtsR likely controls the expression of *ftsH* by interacting directly with its promoter region.

***ftsH* is derepressed in an *L. plantarum* Δ*ctsR* mutant.** In order to determine the role of CtsR in controlling the expression of *ftsH*, a Δ*ctsR* mutant strain of *L. plantarum* was generated (see Materials and Methods). The expression of *ftsH* in the Δ*ctsR* mutant strain was analyzed by qRT-PCR and compared to that of the wild type. As reported in Fig. 5, under optimal growth conditions, *ftsH* was more than threefold derepressed in the mutant strain relative to its expression in the wild-type control (Fig. 5A); the lack of *ctsR* gene expression in the mutant was confirmed by the absence of any *ctsR* transcript detectable by PCR (data not shown). This result indicates that *ftsH* expression is repressed by CtsR, in agreement with the data obtained by gel mobility shift assays. This is the first instance where the *ftsH* gene of a gram-positive bacterium has been shown to be under CtsR regulation, hence placing it among the class III stress genes.

When the *L. plantarum* culture was shifted to 50°C for 10 min, the amounts of *ftsH* transcript increased further both in the wild type (about 14-fold) and, to a minor extent, in the mutant strain (6-fold) relative to those of the corresponding unstressed control cultures (Fig. 5B). The residual induction observed in the mutant strain could suggest a heat shock control mediated by additional regulators other than CtsR and has been observed previously for many CtsR-dependent genes (6, 8, 9).

## DISCUSSION

Functional studies have revealed an important role for FtsH in the bacterial stress response. In several bacteria, including *E. coli*, *B. subtilis*, *Lactococcus lactis*, *O. oeni*, *Helicobacter pylori*, and *L. plantarum*, *ftsH* expression is induced in response to heat and other stress factors (3, 12, 14, 21). Moreover, in *E. coli*, FtsH controls the cellular levels of the heat shock sigma factor ($\sigma^{32}$) in response to varying temperatures (21, 37). Although the involvement of FtsH in protection against environ-

mental stress has been documented for various bacterial species, little is known about the mechanisms involved in its transcriptional regulation in response to stress. To date, no known typical stress-responsive *cis*-regulatory element has been found in its promoter that could substantiate its classification as a "stress response gene" (20). Therefore, the *ftsH* gene has recently been assigned to the heat-inducible class IV genes, whose regulatory mechanisms are still unknown (9, 34). We show that, as has been reported for other bacterial species, the *ftsH* gene of *L. plantarum* is significantly induced upon exposure to stress conditions, especially heat stress. The involvement of *ftsH* in the stress response was also confirmed by the behavior of the mutant strain. Indeed, compared to the wild type, the *ftsH* mutant strain displayed a significant growth defect when subjected to heat stress.

Interestingly, the consequences of the *ftsH* mutation are remarkably species specific, ranging from drastic growth impairment (2) to negligible/milder effects on sporulation, development, and the stress response (11, 16, 29). For some bacteria, such as *E. coli*, *L. lactis*, and *H. pylori*, the apparent impossibility of isolating any viable *ftsH*-null mutant indicates that this protease is essential (1, 17, 31, 38). In contrast, in species such as *B. subtilis* and *Caulobacter crescentus*, FtsH seems dispensable for growth under physiological conditions (11, 16). Furthermore, minor effects on normal growth and the cellular stress response were recently observed in a Δ*ftsH* strain of *Corynebacterium glutamicum* (28). Since we were able to isolate a viable insertional inactivation mutant strain, FtsH is clearly not essential in *L. plantarum*.

According to our results, the relevance of *ftsH* function in *L. plantarum* reflects the situation observed in bacteria such as *B. subtilis*, *C. crescentus*, and *C. glutamicum* but with a marked contribution to growth under heat shock conditions.

Sequence analysis of the *ftsH* promoter allowed us to predict a potential CtsR binding site. Primer extension experiments indicate that the putative CtsR operator overlaps the promoter and occupies a position consistent with a regulatory function by this transcriptional repressor. By adopting two different experimental approaches, we have shown that *L. plantarum ftsH* is indeed controlled by CtsR. Purified CtsR from both *B. subtilis* and *L. plantarum* recognized and bound specifically to the *ftsH* promoter. Moreover, using a recently developed Cre-*lox*-based mutagenesis system (26), we were able to obtain a Δ*ctsR* strain in which the basal transcriptional level of the *ftsH* gene was significantly derepressed compared to that of the wild type. To our knowledge, this is the first report demonstrating that an *ftsH* gene is under the control of CtsR. *ftsH* transcriptional induction was still evident upon heat shock in the Δ*ctsR* mutant strain, suggesting a control mechanism involving CtsR together with one or more additional regulators. Similar results were also obtained for *B. subtilis* (9, 24) and *L. lactis* (40), where the expression of some members of the CtsR regulon appears to be controlled by additional regulators.

In conclusion, the *ftsH* gene of *L. plantarum* is involved in protection against stress, mainly heat shock, and is controlled by the class III stress gene repressor CtsR. From an evolutionary point of view, CtsR control of *ftsH* might be either vestigial or a novel and exclusive acquisition of the *L. plantarum* gene by the CtsR regulon with respect to other, closely related bacterial species. We favor the latter hypothesis. Indeed, although in *Oenococcus oeni* CtsR acts as a master regulator of most of the known stress response genes, it does not appear to control *ftsH* expression in this wine bacterium (3, 20). Although little is known about the mechanisms used by *L. plantarum* to adapt to environmental fluctuations, preliminary analysis of the *L. plantarum* genome allowed the identification of potential CtsR binding sites upstream of several genes in addition to *ftsH*, including those encoding small heat shock proteins (*hsp18.5*) and subunits of the Clp ATP-dependent protease (35; G. Spano and D. Fiocco, unpublished data). This finding strongly suggests that the particular stress conditions encountered by *L. plantarum* have led to the coordinated CtsR-dependent expression of *ftsH* with that of other stress response genes, and this possibility will be the subject of further investigation.

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