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Molecular Characterization of a Stress-Inducible Gene from *Lactobacillus helveticus*

ANDRÈAS SMEDS,[1] PEKKA VARMANEN,[2] AND AIRI PALVA[3]*

Agricultural Research Centre of Finland, Food Research Institute, Jokioinen 31600,[1] and Faculty of Veterinary Medicine/Department of Basic Veterinary Sciences, 00014 University of Helsinki,[3] Finland, and The Royal Veterinary and Agricultural University, Copenhagen, Denmark,[2]

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A gene (*htrA*) coding for a stress-inducible HtrA-like protein from *Lactobacillus helveticus* CNRZ32 was cloned, sequenced, and characterized. The deduced amino acid sequence of the gene exhibited 30% identity with the HtrA protein from *Escherichia coli*; the putative catalytic triad and a PDZ domain that characterize the HtrA family of known bacterial serine proteases were also found in the sequence. Expression of the *L. helveticus htrA* gene in a variety of stress conditions was analyzed at the transcriptional level. The strongest induction, resulting in over an eightfold increase in the *htrA* transcription level, was found in growing CNRZ32 cells exposed to 4% (wt/vol) NaCl. Enhanced *htrA* mRNA expression was also seen in CNRZ32 cells after exposure to puromycin, ethanol, or heat. The reporter gene *gusA* was integrated in the *Lactobacillus* chromosome downstream of the *htrA* promoter by a double-crossover event which also interrupted the wild-type gene. The expression of *gusA* in the stress conditions tested was similar to that of *htrA* itself. In addition, the presence of an intact *htrA* gene facilitated growth under heat stress but not under salt stress.

In their natural environments, bacteria spend most of their life in a starving or nongrowing state because of various growth-limiting conditions (14). To face starvation and other stresses, bacteria are able to rapidly and transiently express a characteristic set of proteins in order to survive and protect the cells from fatal damage (12, 20). Stress-inducible proteins can be divided into two main groups: specific stress proteins and general stress proteins (14).

The HtrA protein of *Escherichia coli* is located at the periplasmic side of the inner membrane (28) and is a member of the stress-inducible *rpoE* regulon, which responds to misfolded proteins in the extracellular compartment (23). In addition to the *E. coli* HtrA (DegP/Do) (15, 26, 30), in the last few years several other HtrA homologs have been identified in a variety of bacteria as well as some eukaryotes (21, 22, 34). With only a few exceptions, the same putative proteolytic active site can be found in all the HtrA homologs identified (21). The members of the growing HtrA family also commonly possess a PDZ domain (10, 21) and are characterized as trypsin-like serine proteases (16, 17). Degradation of abnormal proteins in the periplasm has been suggested to be the main physiological role of HtrA in *E. coli*, but regulatory functions have also been reported for this protein (21). In *Bacillus subtilis*, the deduced protein products of the genes *yyxA* 6 [accession no. P39668]) and *ykdA* (accession no. AJ002571) have recently been reported as HtrA-like proteins, but no HtrA homologs from lactobacilli have yet been described.

Lactobacillus helveticus is a lactic acid bacterium widely used as a starter in the manufacturing of Swiss-type cheeses and other fermented dairy products (11). During the manufacturing and ripening of these cheeses, the lactic acid bacteria starters are exposed to a variety of stresses, as the temperature is elevated in the cheese cooking process and the addition of NaCl increases osmolarity.

* Corresponding author. Mailing address: Faculty of Veterinary Medicine/Department of Basic Veterinary Sciences, P.O. 57, 00014 University of Helsinki, Finland. Phone: 358-9-70849531. Fax: 358-9-70849799. E-mail: airi.palva@helsinki.fi.

In this study, we describe the cloning, DNA sequence, and expression of a stress-regulated *htrA*-like gene of *L. helveticus* CNRZ32. The effects of different stresses on the *htrA* expression were analyzed at the transcriptional level.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *L. helveticus* CNRZ32 and *E. coli* DH5αF′ (Gibco BRL) were propagated in MRS broth (Difco) and Luria broth (Difco), respectively. When plasmid pUC19 was present, ampicillin (50 μg/ml) was supplied to the growth medium. For plasmid pSA3, tetracycline (12.5 μg/ml), chloramphenicol (100 μg/ml), or erythromycin (4 μg/ml) was used. *L. helveticus* cells first grown to the exponential phase (optical density at 600 nm [OD_{600}] of 0.8) at 37°C were studied in the following stress conditions: for heat shock, the growth temperature of the cells was shifted from 37 to 52°C; for salt stress, NaCl was added to a final concentration of 4% (wt/vol); for puromycin stress, 100 μg of puromycin per ml was supplied to the growth medium; for ethanol stress, cells were exposed to 5% (wt/vol) ethanol; and for oxidative stress, H_2O_2 was added to a final concentration of 0.005% (wt/vol).

Screening of an *L. helveticus* genome library. An *L. helveticus* genomic library was established in λgt10 by using the λ DNA in vitro packaging and cDNA rapid cloning module (λgt10; Amersham) (31). The library was screened by DNA hybridization, using an internal 1.2-kb fragment of the *L. delbrueckii* subsp. *bulgaricus* CNRZ397 *pepI* gene as a probe (1). The probe was labeled with digoxigenin-dUTP (Boehringer Mannheim).

DNA isolation and cloning methods. Plasmid DNAs of *E. coli* clones were isolated by alkaline lysis, using Wizard Minipreps (Promega) or FlexiPrep (Pharmacia) kits. Other standard DNA methods were performed as specified in reference 24. *E. coli* and *L. helveticus* strains were transformed by electroporation (3) using a Gene Pulser (Bio-Rad Laboratories).

DNA syntheses. Oligonucleotides were synthesized with a model 392 Applied Biosystems DNA/RNA synthesizer and purified by ethanol precipitation. DNA was amplified by PCR as recommended by the manufacturer of Dynazyme DNA polymerase (Finnzymes).

DNA sequencing and sequence analysis. DNA sequencing was performed on an A.L.F. DNA sequencer according to the manual for the AutoRead sequencing kit (Pharmacia). Both DNA strands were sequenced by using pUC19-specific primers and sequence-specific oligonucleotides for primer walking. DNA sequence data were assembled and analyzed with the PC/GENE set of programs (release 14.0; IntelliGenetics). The BLAST program was used for searching homologous protein sequences at the National Center for Biotechnology Information, and alignment studies were performed on the ExPASy server, using the SIM program. The comparison matrix used in the alignments was PAM400 (gap open penalty, 12; gap extension penalty, 4).

Construction of a *gusA* expression cassette. A 0.9-kb *Sal*I-*Bam*HI fragment from the upstream region of *htrA*, including the promoter and the 12 first nucleotides downstream of ATG, was ligated to the second codon of a promoterless *gusA* reporter gene with flanking *Bam*HI sites. A transcription terminator

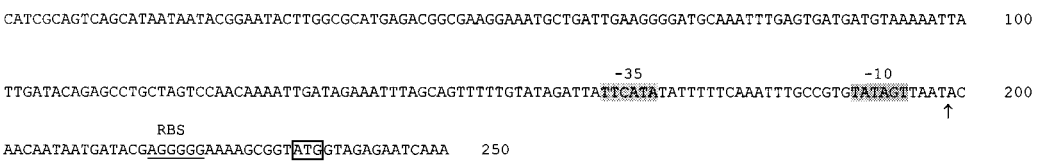

FIG. 1. Promoter region of the *htrA* gene from *L. helveticus*. The predicted −35 and −10 regions of the putative promoter are shadowed. RBS refers to the presumed ribosome binding site. The transcription start site of *htr*A, determined by primer extension, is marked with an arrow. The start codon is boxed.

(a hairpin with a free energy of −24.9 kcal mol^{-1}) from the *slpA* gene of *L. brevis* (33) was fused to the 3′ end of *gusA* at the *Bam*HI site. A 0.85-kb *Hin*dIII-*Sal*I fragment, carrying the 3′ end of *htrA*, was also ligated to the hairpin at the cleavage site for *Hin*dIII. The cassette was cloned into the shuttle vector pSA3 (7) at the *Sal*I site, thus disrupting the gene coding for tetracycline resistance. The pSA3::*gusA* construct was transformed into *E. coli* DH5αF′ cells, and plasmid DNA was isolated by alkaline lysis (FlexiPrep; Pharmacia).

Integration of pSA3::*gusA* into the *L. helveticus* CNRZ32 chromosome. Isolated plasmid pSA3::*gusA* DNA was introduced into *L. helveticus* CNRZ32 by electroporation (3) and cultured anaerobically for 72 h on MRS agar with 4 μg of erythromycin per ml (MRSE). Transformants were propagated for 30 generations in MRSE broth at 37°C, further cultured overnight at 45°C (1% inoculation), and plated on MRSE agar at 45°C.

Gene replacement at the chromosomal 5′ *htrA* locus. For the integration and curing of plasmid pSA3 and a double-crossover event (4), transformed *L. helveticus* colonies were picked from the MRSE agar at 45°C and grown at 37°C in MRS broth for 78 generations. The cells were plated and incubated on MRS agar for 48 h. In the screening for erythromycin-sensitive colonies, randomly chosen colonies were transferred to both MRS and MRSE plates. One colony of three erythromycin-sensitive colonies found was shown by PCR to contain the expected integrant (data not shown). As a consequence of the second crossover event, the reporter gene *gusA* was fused next to the *htrA* promoter on the chromosome of *L. helveticus* CNRZ32, generating the new strain GRL56.

Transcription analyses. Total RNA was isolated with RNeasy Midi kit according to instructions provided by Qiagen. For removing chromosomal DNA, the samples were treated with 45 U of DNase, phenol-chloroform extracted, and ethanol precipitated. RNA gel electrophoresis and Northern blotting were performed as described by Hames and Higgins (13). Total RNA isolated from wild-type *L. helveticus* CNRZ32 cells was hybridized with a 1.2-kb digoxigenin-labeled *htrA*-specific DNA probe, and total RNA isolated from GRL56 was hybridized with a 1.8-kb digoxigenin-labeled *gusA*-specific DNA probe. A DIG luminescent detection kit (Boehringer Mannheim) was used for hybrid detection. The level of transcripts was quantitated with a laser densitometer, and the specific induction ratios were calculated by dividing the signals from RNA of stressed cells by the signals from the control RNA. Primer extension was performed with total RNA in an A.L.F. DNA sequencer essentially as described earlier (19, 32), using the oligonucleotide 5′-TGGCACTCTTTTCTGAAACC-3′ with a fluorescein label as the primer.

Nucleotide sequence accession number. The nucleotide sequence described in this paper has been deposited in the EMBL sequence data bank under accession no. AJ005672.

RESULTS

Cloning and sequencing of the *L. helveticus htrA* gene. Screening of the λgt10-based genomic library of *L. helveticus* 53/7 by plaque hybridization, with an internal 1.2-kb fragment of the *L. delbrueckii* subsp. *bulgaricus* CNRZ397 *pepI* gene as the probe, gave the hybridization-positive clone gt10/cl30 of 6.5 kb (31). The clone was isolated and further subcloned, and the DNA was sequenced. A 1,239-bp open reading frame (ORF), encoding a gene product showing homology with heat shock proteins, was found in the same gene locus as the *pepI* operon in the sequence analysis of two of the subclones. Primers with flanking restriction sites for *Bam*HI and *Hin*dIII were designed up- and downstream of the ORF, followed by PCR with isolated chromosomal DNA from *L. helveticus* CNRZ32 as the template. The CNRZ32 PCR product was cloned into plasmid pUC19 (Pharmacia Biotech) and sequenced. *L. helveticus* CNRZ32 was chosen for further characterization of the *htrA* gene, since chromosomal modifications are more difficult to perform with strain 53/7. A search of protein databases with the BLAST program and alignment studies with the SIM program revealed significant homology (29.3% identity) with the HtrA/DegP protein from *E. coli* (16, 30). Other proteins showing high similarity with the CNRZ32 protein were Sphtra from *Streptococcus pneumoniae* (42.3% identity), YkdA from *B. subtilis* (38.7% identity), and YyxA from *B. subtilis* (37.6% identity).

The *L. helveticus* ORF encodes a protein with a calculated molecular mass of 42,647 Da. A putative Shine-Dalgarno sequence AGGGGG (29) was identified 9 nucleotides upstream of the ATG. Regions with reasonable homology to consensus −35 and −10 regions of bacterial promoters (TTCAT AN_{20}TATAGT) were also identified upstream of the start codon (Fig. 1). An inverted repeat structure (ΔG of −18.8 kcal mol^{-1}) was found 24 nucleotides downstream of the stop codon; this may be the transcriptional terminator of the gene.

The N-terminal amino acid sequence of the ORF revealed a strong hydrophobic region preceded by positively charged amino acids. However, a consensus sequence for the cleavage site of the signal peptidases could not be found downstream of the hydrophobic region.

The same active-site catalytic triad as previously noted in the HtrA/DegP family of bacterial serine proteases (2, 17, 21) was also present (Fig. 2). Furthermore, the substrate binding domain PDZ (10, 22) was found at the C-terminal end of the amino acid sequence (data not shown). Due to its homology to the HtrA protein family, the *L. helveticus* CNRZ32 gene product analyzed was designated the *L. helveticus* HtrA.

Analysis of *htrA* transcription. The size of the *htrA* transcript was determined by Northern blotting, using a 1.2-kb digoxigenin-labeled *htrA*-specific probe. Under heat shock conditions, the probe detected an 1.4-kb transcript, confirming that the gene coding for the HtrA like protein is a monocistronic transcriptional unit. Mapping of the 5′ end (data not shown) revealed that the transcription start site is located 32 nucleotides upstream of the ATG codon (Fig. 1).

Regulation of *htrA* gene expression. It is known that the HtrA protease from *E. coli* is a heat shock protein whose synthesis is induced both by various stress factors and by protein misfolding in general (9, 17, 21). To examine the possibility that the HtrA homolog from *L. helveticus* CNRZ32 could also be stress induced, we exposed exponentially growing *L. helveticus* CNRZ32 cells (OD_{600} of 0.8) to different stress conditions. The precise conditions chosen on the basis of their reduction of the bacterial growth rate (Fig. 3). Total RNAs isolated from CNRZ32 cells before and after stress were blotted on a nylon membrane and hybridized with a digoxigenin-labeled *htrA*-specific DNA probe (Fig. 4). The strongest induction, resulting in over an eightfold increase in the level of *htrA* transcription, was found in cells exposed to 4% (wt/vol) NaCl (Fig. 4B). Exposure of growing CNRZ32 cells to ethanol (5%, wt/vol) (Fig. 4C) or puromycin (100 μg/ml) (Fig. 4D) resulted in about a fivefold induction. The induction in cells exposed to a temperature upshift from 37 to 52°C was rapid but resulted in only a doubling of the amount of *htrA* mRNA (Fig. 4A). However, oxidative stress did not affect *htrA* transcription (Fig. 4E). As a control, total RNA isolated from heat-stressed wild-

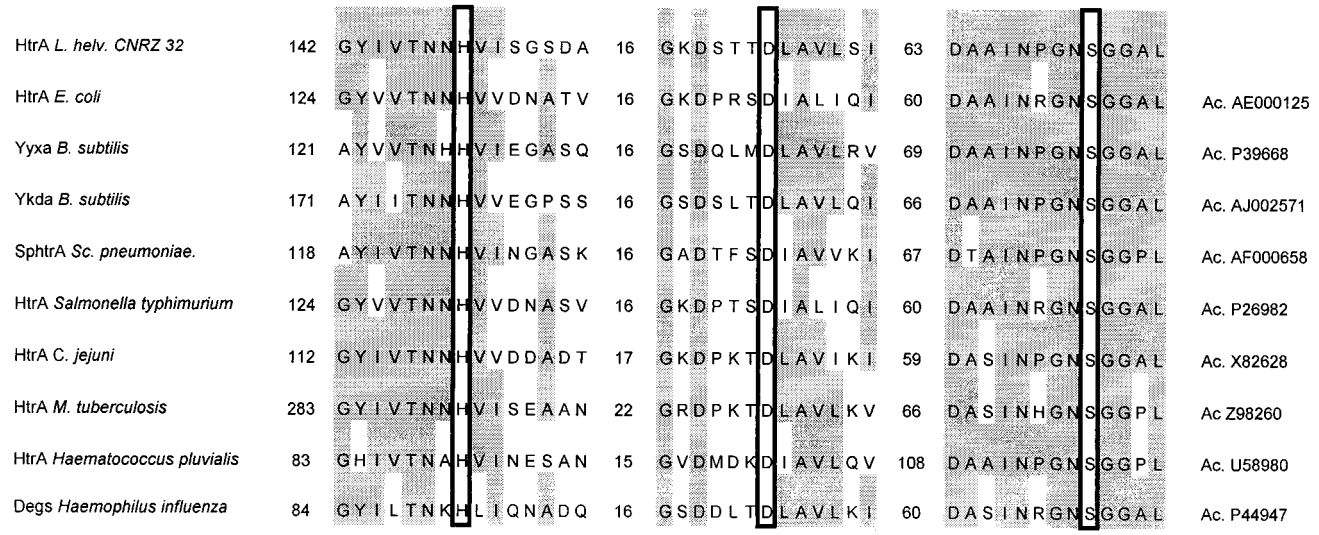

FIG. 2. Alignment of regions spanning the putative active-site catalytic triad with presumed members of the HtrA/DegP family of serine proteases. The numbers represent sequence gaps, and the putative catalytic triad residues (H, His; D, Asp; S, Ser) are boxed. The most conserved regions are shadowed. Accession numbers (Ac.) are taken from the GenBank, EMBL and SwissProt databases.

type *L. helveticus* CNRZ32 cells was hybridized with a digoxigenin-labeled *ldh*-specific DNA probe (25). Heat shock did not affect the level of *ldh* transcripts (data not shown).

To elucidate the function and essentiality of the *htrA* gene, we replaced the 5′ end of *htrA* with the *gusA* reporter gene as described in Materials and Methods (4). The fusion of the *gusA* reporter gene was downstream of the stress-inducible *htrA* promoter and also disrupted the *htrA* gene in the resulting strain GRL56. When total RNA from GRL56 cells exposed to heat and salt stress as described in Materials and Methods were analyzed, the induction of *gusA* transcripts was found to be similar to the induction of *htrA* transcripts in the wild-type *L. helveticus* CNRZ32 cells (data not shown).

Bacterial growth analysis. To examine the possibility that the HtrA protein from *L. helveticus* CNRZ32 is essential for growth in unfavorable environments, growth experiments with wild-type *L. helveticus* CNRZ32 cells and GRL56 cells with the interrupted *htrA* gene were performed (Fig. 5). The growth rate declined after heat shock (52°C), and the growth of both wild-type CNRZ32 cells and GRL56 cells ceased after 1 h of the provocation. However, the cell density of CNRZ32 was clearly higher within 1 h after the heat shock than that of GRL56 cells (Fig. 5A). This difference was not noticeable in cells exposed to temperature upshift from 37 to 48°C, although induction of *htrA* transcripts could be demonstrated (data not shown). After salt stress (4% NaCl), no distinctive difference in the growth profiles of the wild-type and mutant strains could be found (Fig. 5B).

DISCUSSION

In this work, we have identified and characterized a gene, *htrA*, coding for a putative member of the HtrA/DegP family of serine proteases (2, 16, 21). The *htrA* gene is located at the *pepI* locus (31) on the chromosome of *L. helveticus*. The *L. helveticus* HtrA protein carries a putative catalytic domain containing a triad of His, Ser, and Asp residues (Fig. 2). The domain is characteristic for trypsin-like serine proteases (16, 21), and mutating two of the catalytic triad residues has been shown to lead to a loss of protease activity in *E. coli* (27). We also found

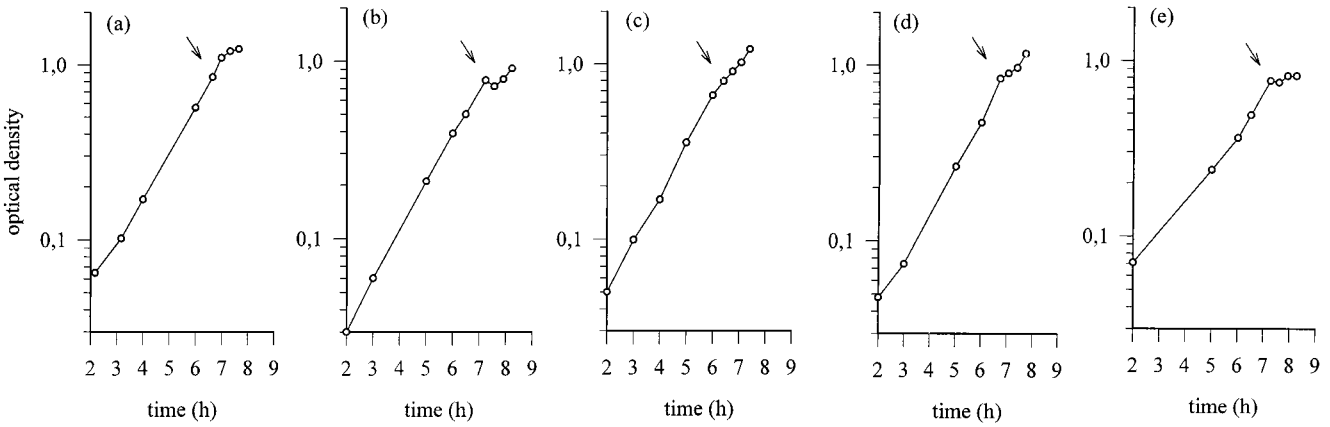

FIG. 3. Growth of *L. helveticus* CNRZ32 before and after stress. The cells were grown to an OD_{600} of 0.8 and exposed to heat shock (a) and to salt (b), ethanol (c), puromycin (d), and oxidative (e) stress. The time of initiation of the stress is indicated by an arrow.

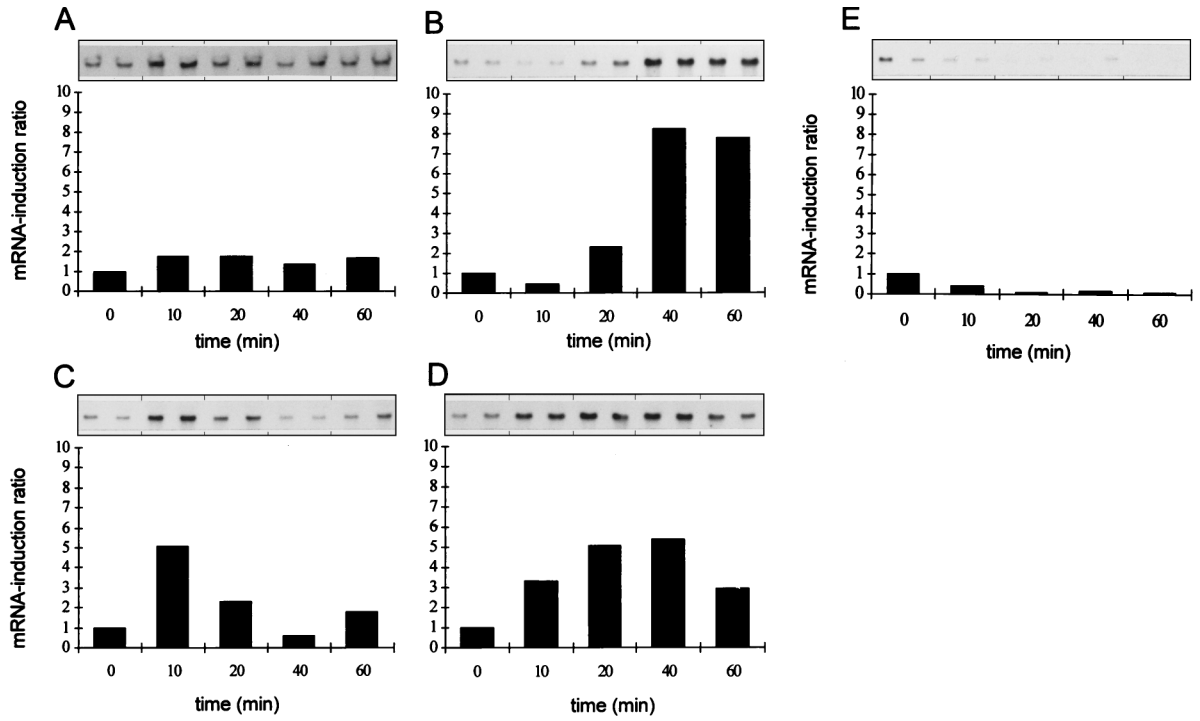

FIG. 4. Northern blotting analyses of the *htrA* transcripts under different stress conditions. Transcription was induced by heat shock (A), and by salt (B), ethanol (C), puromycin (D), and oxidative (E) stress. Samples (10 μg of total RNA per each lane from parallel cultures) were taken before (0) and 10 min, 20 min, 40 min, and 1 h after initiation of the stress. The mRNA induction ratio was calculated by dividing the signals from RNA of stressed cells by the signals from RNA of the control (0).

a putative PDZ domain at the C terminus of HtrA which is probably involved in protein-protein interactions (9, 22). Pallen and Wren (21) suggested that the recognition of target protein is carried out by the PDZ domain and that the recognition of the sites for cleavage is carried out by the catalytic domain (21). HtrA homologs from gram-positive bacteria generally have one PDZ domain, whereas other HtrA homologs mostly have two (21). The deduced protein sequence of the *htrA* gene from *L. helveticus* revealed an apparent identity with those from other gram-positive bacteria, described as serine proteases and/or putative members of the HtrA/DegP family (21). Secondary structure predictions from *L. helveticus* HtrA indicate a strong preference for β structure, as has also been experimentally found for *E. coli* HtrA (28).

Expression of *htrA* was induced at the transcriptional level as a response to environmental changes. The specific mRNA induction was not directly correlated to the decline of the bacterial growth rate after the stress given but rather was characteristic of the stress condition chosen (Fig. 3 and 4). The amount of *htrA* transcripts quite unexpectedly only doubled as

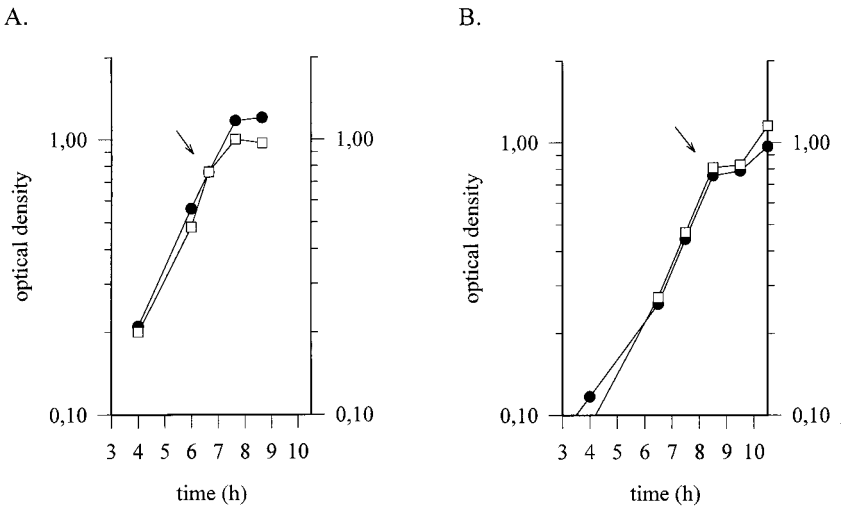

FIG. 5. Comparison of growth of wild-type and *htrA* mutant strains of *L. helveticus* under stress. Wild-type *L. helveticus* CNRZ32 (●) and mutant GRL56 (□) cells grown at 37°C were subjected to heat shock (A) and salt stress (B). The time of initiation of the stress is indicated by an arrow.

the response to the heat shock, possibly due to the slow temperature upshift rate in the growth medium used. However, the induction ratio varied between experiments, being occasionally much higher. Interestingly, the heat shock response in *L. helveticus* cells exposed to a less severe heat shock (temperature upshift from 37 to 48°C) exhibited an induction pattern quite distinct from that of the cells exposed to 52°C. At 48°C a twofold induction level was also obtained, but the amount of *htrA* transcripts rapidly declined between 10 and 20 min after heat shock and remained at a very low level thereafter (data not shown). This may suggest an involvement of a repressor in the regulation of the *htrA* gene. The lower temperature would allow the repressor to refold, thus regaining its binding ability to the putative operator sequences. However, no sequence homologous to the inverted repeat structure (CIRCE) typical of genes encoding heat shock proteins in gram-positive bacteria (5, 14) was found upstream of the *htrA* gene.

A clear *gusA*-specific mRNA induction was seen in GRL56 cells exposed to salt stress. The expression level and profile of *gusA* mRNA in GRL56 cells (which are mutant for *htrA*) were similar to those of *htrA* mRNA in the wild-type cells, indicating that the *htrA* structural gene in *L. helveticus* does not participate in the regulation of its own expression and can thus be successfully replaced. Furthermore, the *htrA* gene product from *L. helveticus* CNRZ32 appeared to facilitate growth at 52°C, whereas growth in salt stress conditions was not affected by the deletion of *htrA* (Fig. 5). HtrA from *E. coli* is thought to be essential for growth at elevated temperatures (15), but nonessential functions for the HtrA homologs of *Helicobacter pylori*, *E. coli* (HhoA and HhoB), and *Campylobacter jejuni* have also been reported (21). Preliminary Southern blotting analyses (data not shown) suggest that the *htrA* gene in *L. helveticus* is present in only one copy (data not shown).

L. helveticus GRL56 showed no β-glucuronidase activity as a response to the heat and salt stresses tested, possibly due to the instability of the enzyme under such conditions. β-Glucuronidase is thought to be rapidly degraded and/or inactivated under heat and salt stress (8).

On the basis of the amino acid sequence, we propose that HtrA from *L. helveticus* is located at the outer surface of the plasma membrane. Without further experimental evidence, it is difficult to predict whether the N-terminal region functions as a cleavable signal sequence (without an apparent cleavage site) or as a membrane anchor sequence. Interestingly, the closest homologs of HtrA, i.e., *S. pneumoniae* Sphtra and *B. subtilis* YyxA, appear to possess similar N-terminal sequences lacking an obvious leader peptide cleavage site. On the other hand, sequence analysis of YkdA from *B. subtilis* suggests an intracellular location. If the N-terminal sequences of these three proteins indeed function as membrane anchor domains, it represents a somewhat unusual way to locate proteins on the outer surface of the cytoplasmic membrane in gram-positive bacteria, which commonly use the lipoprotein type of attachment.

The regulation of HtrA in *E. coli* has recently been described as a complex network of signal transduction pathways (18). The alternative sigma factor RpoE, the anti-sigma factor RseA, the two-component regulatory system CpxRA, and two phosphoprotein phosphatases, PrpA and PrpB, are all components of the network (18). Homologs to these components exist also in several other gram-negative bacterial species, indicating similar regulatory systems (21). However, it is still unclear how expression of *htrA*-like genes from gram-positive bacteria is regulated and if there are conserved regulatory pathways at all. Indeed, SigB-like promoter sequences, typical for general stress-inducible genes from gram-positive bacteria (14), could not be detected upstream of the *htrA* gene in *L. helveticus*. So far, no alternative stress-inducible sigma factor has been reported for lactobacilli. The exact role of the HtrA protein and the regulation of its expression in *L. helveticus* remain to be elucidated.

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