The HtrA (DegP) Protein, Essential for Escherichia coli Survival at High Temperatures, Is an Endopeptidase

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As a preliminary step in the understanding of the function of the Escherichia coli HtrA (DegP) protein, which is indispensable for bacterial survival only at elevated temperatures, the protein was purified and partially characterized. The HtrA protein was purified from cells carrying the htra gene cloned into a multicopy plasmid, resulting in its overproduction. The sequence of the 13 N-terminal amino acids of the purified HtrA protein was determined and was identical to the one predicted for the mature HtrA protein by the DNA sequence of the cloned gene. Moreover, the N-terminal sequence showed that the 48-kilodalton HtrA protein is derived by cleavage of the first 26 amino acids of the pre-HtrA precursor polypeptide and that the point of cleavage follows a typical target sequence recognized by the leader peptidase enzyme. The HtrA protein was shown to be a specific endopeptidase which was inhibited by diisopropylfluorophosphate, suggesting that HtrA is a serine protease.

Both procaryotic and eucaryotic cells respond to a variety of stresses by accelerating the rate of synthesis of a group of proteins called heat shock proteins. This response in Escherichia coli is positively regulated by the product of the rpoH (htpR) gene, σE, which enables RNA polymerase to recognize promoters of heat shock genes (21). The heat shock genes all belong to the rpoH (htpR) regulon, are absolutely necessary for bacterial survival at high temperatures, and perform various, although not very well understood, functions in the cell. One of the processes in which the heat shock proteins are involved is cellular proteolysis. For example, the Lon protease belongs to the group of heat shock proteins (7, 8). It is known that the accumulation of abnormal proteins leads to the induction of the rpoH (htpR)-dependent heat shock response (1, 12) and that aberrant proteins are frequently subject to proteolysis (13, 14, 28). It has been shown that, to a large extent, this proteolysis is rpoH (htpR)-dependent; i.e., rpoH (htpR) mutant cells are defective in the proteolysis of abnormal proteins such as canavanine-containing proteins, puromycyl peptides, and the X90 fragment of β-galactosidase (2, 11). This decrease occurs even in rpoH htrA mutant bacteria, suggesting that other members of the heat shock regulon are also involved. Indeed, it has been observed that mutations in some of the other heat shock genes result in decreased proteolysis: dnaJ mutants are defective in the degradation of puromycyl peptides and a fragment of β-galactosidase, groEL mutants are defective in the proteolysis of puromycyl peptides, and dnaK mutants are defective in the degradation of canavanine-containing proteins and puromycyl peptides (16, 25).

Recently, the existence of a second heat shock regulon whose transcription is induced at a high temperature independently of rpoH (htpR) by a newly discovered sigma factor, σE (σE), was demonstrated (9, 10, 18, 27). So far, two genes belonging to this regulon have been identified, i.e., the rpoH (htpR) and htra genes. The htra gene product is a 48-kilodalton (kDa) cell envelope protein which is synthesized as an unstable 51-kDa precursor and is indispensable for bacterial survival at elevated temperatures (above 42°C) (17). Recently, it was shown that the htra gene is identical to the independently identified degp gene (24). Mutations in the degp gene result in a decreased ability to degrade chimeric membrane and periplasmic proteins (23, 24). This finding suggests that the HtrA (DegP) protein may itself be a protease or control the activity of another protease(s).

To examine the biological role of the Htra protein in the heat shock response, we purified this protein and searched for its exact enzymatic activity. In this report, we present the purification of the Htra protein and show that the mature protein is formed by cleavage of the first 26 amino acids of the pre-HtrA polypeptide. The purified HtrA protein was shown to be a specific endopeptidase by tests with various nonphysiological substrates.

MATERIALS AND METHODS

Bacteria, plasmids, and media. The bacterial strains, plasmids, and media used during the course of this work have been described previously (17, 18).

Buffers. Buffer A is 50 mM Tris hydrochloride (pH 7.3)–10% (wt/vol) sucrose–1 mM EDTA. Buffer B is 50 mM Tris hydrochloride (pH 7.3)–1 M NaCl–1.49 M ammonium sulfate–39 mM dithiothreitol. Buffer C is 50 mM Tris hydrochloride (pH 7.3)–40 mM KCl–1 mM EDTA–10 mM β-mercaptoethanol–10% (vol/vol) glycerol. Buffer D is 50 mM imidazole hydrochloride (pH 6.8)–0.1 mM EDTA–10 mM β-mercaptoethanol–10% (vol/vol) glycerol. Buffer E is 10 mM Tris hydrochloride (pH 7.3)–0.2 mM EDTA–10 mM β-mercaptoethanol–10% (vol/vol) glycerol. Buffer F is 20 mM potassium phosphate (pH 6.5)–10 mM β-mercaptoethanol–10% (vol/vol) glycerol.

Proteolytic activity assay with resorufin-labeled casein as a substrate. Resorufin-labeled casein was dissolved in H2O at a concentration of 4 mg/ml, and the solution was stored in the dark at −20°C. For the standard assay, 10 μl of the casein solution was mixed with 20 μl of 100 mM Tris hydrochloride (pH 8.0)–9 μl of H2O–1 μl of HtrA preparation and incubated at 37°C in the dark for 4 to 10 h. Subsequently, 96 μl of 5% trichloroacetic acid was added.

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The sample was incubated at 37°C for 10 min and centrifuged for 5 min. Next, 100 μl of the supernatant containing the liberated peptides labeled with resorufin was neutralized with 150 μl of Tris hydrochloride (pH 9.5). The fluorescence of the sample was measured at 574 nm (excitation) and 602 nm (emission).

Protein assay. The protein concentration was estimated following staining with amido black (Schwarz/Mann). Protein solution (1 to 5 μl; equivalent to 0.5 to 10 μg of protein) was applied to nitrocellulose (if more than 1 μl was loaded, the solution was applied in 1-μl portions and the nitrocellulose was dried between applications). The nitrocellulose was stained for 1 min with 0.1% amido black solution (wt/vol) in isopropanol-acetic acid-H₂O at 25:10:65. Subsequently, it was destained in the same mixture without amido black. Control protein standards were assayed along with the samples tested. For qualitative rapid assays, the intensity of color, proportional to the amount of protein, was estimated visually. For quantitative assays, the stained fragments were cut out and eluted with 88% formic acid (1 h at room temperature), and the A₆₃₀ of the eluates was measured.

Electrophoresis of proteins. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (17). Gels containing 12.5% (wt/vol) acrylamide were used, unless otherwise stated. Two-dimensional gel electrophoresis was performed as previously described (17).

Protein sequencing. Proteins were resolved by SDS-PAGE (10% acrylamide) with a model 360 mini-vertical electrophoresis apparatus (Bio-Rad Laboratories). All reagents for electrophoresis were purchased from Bio-Rad. SDS (Bio-Rad) was recrystallized twice from ethanol. To decrease the blocking of N-terminal amino acids, we electrophoresed the gels for 2 h at 3 mA with electrophoresis buffer containing 0.05 mM glutathione (Sigma Chemical Co.) prior to loading the samples. During the actual run, the electrophoresis buffer was supplemented with 0.1 mM sodium thioglycolate (Sigma). Following electrophoresis, proteins were transferred from the gel to polyvinylidene difluoride membrane (Immobilon; Millipore Corp.) as previously described (19). Membrane fragments containing the desired protein were cut out and used for sequencing in a model 477A sequencer (Applied Biosystems) in accordance with manufacturer instructions. The analysis was performed by R. Schackmann, University of Utah Biotechnology Center.

Proteolytic activity assay with synthetic peptides as substrates. Peptides carrying a fluorescent chromogenic 4-methylcoumaryl-7-amide (MCA) were used in a reaction mixture containing 100 μM peptide, 100 mM Tris hydrochloride buffer (pH 7.8), and 2 μg of HtrA protein in a final volume of 100 μl. Following overnight incubation at 37°C, 200 μl of ethanol was added and fluorescence was measured at 380 nm (excitation) and 400 nm (emission). The control reactions contained no HtrA.

Immunoblotting procedures. Following SDS-PAGE, proteins were transferred to 0.45-μm-pore-size nitrocellulose (5). The nitrocellulose was treated with 1% bovine serum albumin to block unoccupied protein-binding sites, exposed to rabbit antiserum prepared against purified HtrA protein, and incubated with hors eradish peroxidase-conjugated goat anti-rabbit immunoglobulin G. The visualization of immunoblot bands was performed as described by Hawkes et al. (15).

Reagents. All protease inhibitors (see Table 1) were purchased from Sigma. Resorufin-labeled casein was purchased from Boehringer Mannheim Biochemicals. The synthetic peptides used for the proteolytic activity assay (see Results) and distributed by Peninsula Laboratories were a gift from M. Rechsteiner, Department of Biochemistry, University of Utah. Protein molecular weight standards were purchased from Pharmacia/LKB Chemicals. The cyclohexylamino-1-propanesulfonic acid (CAPS), N,N-methylenedisarcosylamide (BIS)-Tris-propane, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), and morpholinoethanesulfonic acid (MES) buffers were purchased from Research Organics.

Purification of the HtrA protein. To purify the HtrA protein, we used the high-copy-number plasmid pBL133 carrying the entire htrA gene in the pEMBL8⁺ vector (17). E. coli recA(pBL133) mutant bacteria were grown overnight at 37°C in L broth supplemented with 50 μg of ampicillin per ml. The culture was harvested by centrifugation, and the cell pellet (25 g) was rinsed briefly with ice-cold buffer A, suspended in 25 ml of buffer B, and frozen in liquid nitrogen. Cells were allowed to thaw slowly in a 4 to 8°C water bath, followed by the addition of 10 ml of cold buffer B. Subsequently, 6.4 ml of lysozyme solution (10 mg/ml, freshly prepared) was added, and the mixture was brought to 100 ml with buffer A. After 5 min at 0°C, the final volume of the mixture was transferred to 37°C for 3 min and then returned to 0°C. Lysed cells were centrifuged for 30 min at 45,000 rpm and 0°C in a 50.2 Ti rotor (Beckman Instruments, Inc.). Granulated ammonium sulfate (0.34 g/ml; Schwarz/Mann) was slowly added to the supernatant over a 20-min period at 0°C with continuous stirring. Stirring was continued for an additional 20 min at 0°C. The solution was centrifuged at 30,000 rpm for 15 min at 0°C in a Beckman 50.2 Ti rotor. The pellet was transferred to a dialysis bag and dialyzed against buffer C. The resulting solution (7.5 ml) was passed through a DE52 cellulose column (11 by 2.5 cm; Whatman, Inc.), equilibrated with buffer C, at a flow rate of 2.8 ml/10 min...
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FIG. 2. Western blot analysis of purified HtrA protein. Total protein from E. coli recA(pEMBL8) cells (lane 1) and E. coli recA(pBL133) cells (lane 2) (approximately 50 μg of protein per lane) and purified HtrA protein (10 μg) (lane 3) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with rabbit antiserum prepared against the gel-purified 48-kDa HtrA protein. The arrow indicates the position of the processed HtrA 48-kDa protein.

(this flow rate was used in all steps involving column chromatography). The flowthrough fractions were tested for the presence of protein by the amido black staining procedure (described above), and the fractions containing detectable amounts of protein were analyzed by SDS-PAGE. Fractions containing HtrA (as judged by electrophoresis) were pooled and dialyzed against buffer D. After dialysis, the solution (30 ml) was applied to a BioRex 70 column (11 by 1.5 cm; Bio-Rad) as follows. The upper two-thirds of the column, previously equilibrated with buffer D, was removed from the column and mixed with the protein solution on a rotary shaker at 4°C for 30 min to improve the adsorption process. Next, the suspension was poured into the column. The column was washed with 5 volumes of buffer D, and the HtrA protein was eluted with a 400-ml linear gradient of 0 to 1 M KCl in buffer D. Fractions containing protein were analyzed by SDS-PAGE, and those containing HtrA were pooled (19.6 ml). HtrA protein was eluted in the range of 40 to 70 mM KCl, with a peak at 60 mM KCl. The protein solution was dialyzed against buffer E, and 15 ml of the solution was loaded onto a heparin-Sepharose column (type 1; 8.5 by 1.2 cm; Sigma) equilibrated with buffer E. HtrA protein was eluted with a 200-ml linear gradient of 0 to 0.7 M KCl in buffer E. Fractions containing the HtrA protein (as judged by SDS-PAGE) were eluted in the range of 30 to 70 mM KCl, pooled (8.4 ml), and dialyzed against buffer F before being applied (7 ml) to a hydroxylapatite (Bio-Rad) column (3.5 by 0.9 cm) equilibrated with buffer F. (This step was necessary to remove some low-molecular-weight proteins not easily seen on the stained gel [see Fig. 1, lane 7].) The column was washed with 40 ml of buffer F containing 70 mM phosphate. The HtrA protein was subsequently eluted with buffer F containing 0.3 M phosphate. Fractions containing HtrA (as judged by SDS-PAGE) were pooled (5.6 ml). The various steps used in the HtrA purification scheme are shown in Fig. 1.

In was previously shown that the HtrA protein, when overproduced from a plasmid, is present in both the membrane and the soluble fractions (17). In the procedure described above, only soluble HtrA was purified, since it represented approximately 80% of the total HtrA protein in the overproducing cells (results not shown).

### TABLE 1. Influence of protease inhibitors on the proteolytic activity of HtrA

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<th>Inhibitora</th>
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<th>Activity (%)</th>
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*Abbreviations: DFP, diisopropylfluorophosphate; E-64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane; NEM, N-ethylmaleimide; pHMB, p-hydroxy mercuribenzoic acid; PMSF, phenylmethylsulfonyl fluoride; TLCK, N-tosyllysine chloromethyl ketone; TPCK, N-tosylphenylalanine chloromethyl ketone.

a Proteolytic activity was estimated as the ability to release resorufin-labeled peptides, soluble in trichloroacetic acid, from resorufin-labeled casein. Samples containing the indicated concentrations of inhibitors were incubated for 5 h under the conditions described in Materials and Methods.

### RESULTS

**Purification of HtrA.** The HtrA protein was overproduced and purified by the procedure described in Materials and Methods. The final 48-kDa HtrA protein preparation (Fig. 1, lane 8) was approximately 90% pure, as judged by SDS-PAGE. Most of the protein contamination was due to two proteins of approximately 43 kDa which copurified with HtrA during all the steps (Fig. 1, lanes 5 to 8; Fig. 2). This persistent copurification and the fact that both contaminating proteins were, like HtrA, highly basic (based on two-dimensional gel electrophoresis; results not shown) suggested that the contaminants were degradation products of HtrA. To demonstrate this, we gel purified the 48-kDa HtrA protein following SDS-PAGE and raised anti-HtrA rabbit antisera. These antisera were used to monitor the presence of HtrA-related polypeptides during the purification by the Western blot (immunoblot) technique (5, 15). It was indeed found that...


FIG. 3. Casein as a substrate for the HtrA protease. Approximately 10 µg of casein (lane 1), α-casein (lane 3), and β-casein (lane 5) was incubated with 0.5 µg of purified HtrA protein in 50 mM Tris hydrochloride (pH 8.0) at 37°C for 30 min in a final volume of 25 µl, and the reaction mixtures were resolved by SDS-PAGE. Lanes 2, 4, and 6 (casein, α-casein, and β-casein, respectively) represent control reactions without HtrA. β-Casein (0.4 mg/ml) in 50 mM Tris hydrochloride (pH 8.0) was incubated with purified HtrA protein (20 µg/ml) at 37°C, and 25-µl samples were withdrawn after 5, 10, 20, 30, and 90 min (lanes 9 to 14, respectively) of incubation, placed into an equal volume of twofold-concentrated electrophoresis buffer, and frozen in liquid nitrogen. Subsequently, samples were resolved by SDS-PAGE. Lane 8 represents a control sample without HtrA. The arrow indicates the position of the HtrA protein. The protein molecular weight standards (MWS) (lane 7) were 94,000, 67,000, 43,000, 30,000, 20,100, and 14,400.

the 43-kDa proteins reacted very efficiently with the antibodies raised against the 48-kDa HtrA protein (Fig. 2). It was concluded that these protein species were the degradation products of HtrA. These degradation products already existed in cells overproducing the HtrA protein (Fig. 2, lane 2; unpublished results); thus, inhibition of the degradation processes during purification is of little use.

HtrA protease activity. The htrA gene has been shown to be identical to the degP gene (24). Mutations of the degP gene were previously shown to block the proteolysis of chimeric proteins located in the bacterial inner membrane (23, 24). Since the HtrA protein was shown to be present in the inner membrane or the periplasm, we suspected that it was a protease acting on some membrane and/or periplasmic proteins. We tested the proteolytic activity of HtrA with various nonphysiological substrates and found that HtrA degraded casein and that β-casein was a better substrate than α-casein (Fig. 3, lanes 1 to 6). HtrA action resulted in the appearance of several large polypeptide fragments, suggesting that it is an endopeptidase. To show that the demonstrated proteolytic activity of the HtrA preparation was indeed due to the presence of the HtrA protein and not to the presence of a minor contaminant, we assayed the ability to degrade β-casein in all fractions eluting from the BioRex 70 and heparin-Sepharose columns. Proteolytic activity coincided with the presence of the HtrA protein (Fig. 4). This result supports our conclusion that the HtrA protein is the actual protease. It is a highly specific protease, since it did not degrade bovine serum albumin, ovalbumin, globin, or insulin (results not shown) or any of the synthetic peptides which are routinely used as protease substrates (B-butyloxycarbonyl [BOC]-Val-Pro-Arg-MCA, BOC-Phe-Ser-Arg-MCA, BOC-Leu-Ser-Thr-Arg-MCA, succinyl [SUC]-Ala-Pro-Ala-MCA, SUC-Ala-Ala-Pro-Pro-MCA, SUC-Gly-Pro-Leu-Gly-Pro-MCA, SUC-Leu-Leu-Val-Tyr-MCA, carboxbenzoxyl [Z]-Phe-Arg-MCA, Z-Gly-Gly-Arg-MCA, Glu-Gly-Gly-Arg-MCA, Gly-Ala-Ala-Phe-MCA, SUC-Ala-Ala-Phe-MCA, Gly-Pro-MCA, Lys-Ala-MCA, Leu-MCA, Pro-Phe-MCA-MCA, SUC-Gly-Pro-MCA, Arg-MCA, Pyr-MCA, and Z-Arg-Arg-MCA).

It was found that HtrA proteolytic activity was largely independent of the pH of the reaction. Degradation products of β-casein (Fig. 5) and casein could be detected within a pH range of 4.8 to 10 (results not shown). Furthermore, the HtrA protease activity was independent of ATP, reducing agents (B-mercaptoethanol, dithiothreitol), and divalent cations such as Mg²⁺, Mn²⁺, and Zn²⁺ (results not shown).

We tested the HtrA protease activity in the presence of several inhibitors known to affect serine proteases (diisopropylfluorophosphate, phenylmethylsulfonyl fluoride, N-tosyllysine chloromethyl ketone, and N-tosylyphenylalanine chloromethyl ketone) cysteine proteases (p-hydroxy mercuribenzoic acid, N-ethylmaleimide, iodoaceticamide, and trans-epoxy-succinyl-1-1-kelylamido-(4-guanidino) butane), aspartic acid proteases (pepsatin A), or metalloproteins (EDTA and 1,10-phenanthroline) (3) and found that only diisopropylfluorophosphate efficiently blocked the ability of HtrA to degrade casein (Table 1). This result suggests that HtrA is a serine protease.

Sequencing of the N-terminal end of the HtrA protein. We previously showed (17) that HtrA is synthesized as an unstable 51-kDa precursor protein which is processed by the removal of an N-terminal fragment, resulting in a stable 48-kDa protein. The DNA sequence predicts that the N-terminal end of the pre-HtrA protein includes a sequence typical for a leader peptide, including a consensus cleavage site for leader peptidase (18). To confirm this prediction concerning the processing of pre-HtrA, we sequenced the first 13 N-terminal amino acids of the gel-purified 48-kDa HtrA protein. We found that the amino acid sequence matched perfectly the predicted sequence for pre-HtrA; namely, it was produced by the removal of the 26 N-terminal residues, with the cleavage occurring at the end of the sequence Ala-Thr-Ala, a typical target sequence for the E. coli leader peptidase enzyme (Fig. 6).

DISCUSSION

We have purified the 48-kDa HtrA protein from bacterial cultures carrying a multicopy plasmid overproducing the HtrA protein. During the purification steps, we monitored the presence of the HtrA protein by SDS-PAGE, since at that time no specific activity had been assigned to it. Our final preparation was approximately 90% pure (Fig. 1) and contained, besides HtrA, two minor proteins of approximately 43 kDa. These two contaminants may be degradation products of HtrA, as suggested by their antigenic relationship to HtrA. Another possibility, not excluded by our data, is that these two shorter polypeptides result from the initiation of translation at internal sites on the htrA mRNA.

The identity of our purified protein with the HtrA protein was established by demonstrating that (i) it comigrated with
overproduced HtrA on SDS-polyacrylamide gels (Fig. 1), (ii) it comigrated with overproduced HtrA on two-dimensional gels (results not shown), and (iii) the sequence of the first 13 N-terminal amino acids of the purified mature HtrA protein agreed with that predicted by the nucleotide sequence of the cloned htrA gene.

Previously, we demonstrated that the 48-kDa HtrA protein is synthesized as an unstable 51-kDa precursor which is processed by the cleavage of an N-terminal fragment (17). Sequencing of the N-terminal portion of purified HtrA showed that the first 26 amino acids of pre-HtrA are removed and that the cleavage occurs following the Ala-Thr-Ala consensus target sequence for the E. coli leader peptidase enzyme. These results are in agreement with the recently published data of Strauch et al. (23) showing that the DegP (HtrA) protein, when overproduced in a maxicell system, is synthesized in a precursor form. The apparent molecular mass of the DegP protein, when expressed in maxicells, has been estimated by SDS-PAGE to be 50 kDa (23), reasonably close to our estimate for HtrA (48 kDa).

Since the mutations in the degP (htrA) gene prevented the degradation of certain periplasmic fusion proteins and mutant forms of the maltose-binding protein (24), we suspected that the HtrA protein is a protease. We showed that purified HtrA is an endopeptidase that degrades casein to smaller polypeptides. It is highly specific, since it was unable to degrade all other proteins and peptides tested. This proteolytic activity of HtrA was blocked efficiently by diisopropylfluorophosphate, a potent inhibitor of serine proteases, but not by any of the inhibitors typical for the cysteine or aspartic acid proteases or metalloproteases. Based on this evidence, we postulate that HtrA is a serine protease. HtrA protein contains the amino acid sequence Gly-Asn-Ser-Gly-Gly-Pro-Lys, starting at amino acid position 208 of the processed protein (18). This sequence is very similar to the consensus sequence Gly-Asp-Ser-Gly-Pro-Lys, which usually surrounds the active serine residues of the catalytic domains of known serine proteases (4). Thus, it is possible that the serine present at amino acid position 210 of the mature HtrA protein is the active serine residue of the catalytic domain.

The physiological substrate(s) for the HtrA protease re-
mainly to be identified. When extracts prepared from htrA mutant bacteria were treated with purified HtrA protein, we were unable to detect any proteolytic protein cleavages in one or two-dimensional SDS-PAGE (results not shown). We cannot exclude the possibility that some minor protein(s) may have been processed during the reaction. In a very recent paper, Cavard et al. (6) showed that in vivo the acetylated precursor form of the colicin A lysis protein is degraded in htrA+ (degP+) but not in htrA (degP) mutant bacteria, suggesting that this protein may be a natural substrate for the HtrA (DegP) protease.

The htrA gene is essential for E. coli viability only at elevated temperatures (17). In addition, its transcription is rapidly increased following heat shock (18). Elevated temperatures may cause the formation of abnormal proteins. It is known that aberrant proteins (like canavanine-labeled proteins or β-galactosidase fragments) are degraded by cellular proteases at a higher rate than are normal proteins (13, 20, 22, 28). Since the HtrA protein is an envelope protein (17, 23, 24), it may recognize improperly folded or denatured proteins accumulated during heat shock in the inner membrane and/or periplasmic space. Strauch and Beckwith observed that the temperature sensitivity of degP (htrA) mutants was decreased in an lpp degP (htrA) mutant when compared with the isogenic lpp+ degP (htrA) mutant (24). Since the lpp mutation causes the release of periplasmic proteins into the medium (26), this observation supports the hypothesis that the HtrA protease is necessary for the cell to remove abnormal, possibly toxic proteins. The concentration of such abnormal proteins may increase at higher temperatures.

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LITERATURE CITED


