The *Escherichia coli* htrP Gene Product Is Essential for Bacterial Growth at High Temperatures: Mapping, Cloning, Sequencing, and Transcriptional Regulation of htrP

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We identified and characterized a new *Escherichia coli* gene, htrP. The htrP gene was identified because its insertional inactivation by the Tn10 transposon results in the inability of *E. coli* to form colonies at temperatures above 37°C and a slow growth phenotype at 30°C. The htrP gene was cloned and mapped to 66.3 min on the *E. coli* genetic map, 4 kbp clockwise from the tolC gene. The htrP gene was sequenced and shown to code for an acidic, 27,471-Da polypeptide and to be transcribed counterclockwise with respect to the genetic map. The predicted HtrP protein has two potential transmembrane segments and shares an identity of 64.4% over a length of 210 amino acids with the LuxI protein. Despite the fact that the htrP gene is essential for *E. coli* growth exclusively at high temperatures, the levels of htrP-specific transcripts decrease with increasing temperature.

*Escherichia coli* can survive and grow within a wide range of temperatures (10). To adapt to such a diverse range of growth temperature conditions, a variety of cellular processes are involved that allow *E. coli* to efficiently perform metabolic functions without losing viability (10). A sudden shift-up in temperature is known to induce the accelerated synthesis of a set of proteins, collectively known as heat shock proteins. The transient elevated expression of these proteins is highly conserved and is referred to as the heat shock response (22). It has been suggested that the heat shock response is part of the adaptive tolerance mechanism required for survival at high temperatures (37). Besides the induction of heat shock proteins, certain other functions are also affected. These include changes in membrane protein composition and lipid composition, transient inhibition of cell division, as well as altered turnover of many other proteins (10, 20). To gain an understanding of genes that may be uniquely required by *E. coli* to survive at high temperatures, we screened libraries of various transposon insertion mutations. Those insertion mutations that enable *E. coli* to grow well at low temperatures but result in inability to form colonies at high temperature should potentially define functions that are uniquely involved in bacterial survival at high temperature. Using this approach, we have defined thus far four such high temperature requirements (htr) genes. Of these, htrA and htrC define two new heat shock genes. The htrA gene encodes a periplasmic endopeptidase (17, 18) and is transcribed by the EsE4 RNA polymerase (6). The htrC gene is under the transcriptional regulation of the EsE4 RNA polymerase, similar to other classical heat shock genes (27). The htrB gene is transcribed by the EsE0 RNA polymerase, which transcribes housekeeping genes (10). The htrM gene has at least two promoters, one transcribed by EsE0 and another transcribed by EsE2 (28). Here, we present the identification, mapping, and sequence analysis of a fifth htr gene, designated htrP.

MATERIALS AND METHODS

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are described in Table 1. The inability to form colonies at high temperatures is referred to as the temperature-sensitive (Ts') phenotype.

**Bacteriophages.** Bacteriophage λ1105 mini-Kan::Tn10 (35) was used to insertionally inactivate the htrP gene, as previously described (17, 27). Phage P1L4, used in transductions, was from our collection. The *E. coli* genomic library, prepared in the λ2001/EMBL4 vector, was obtained from Y. Kohara (14).

**Media.** The LB rich and M9 minimal media were prepared as described by Maniatis et al. (19). LA is LB medium containing 1% agar. M9 medium was supplemented with glucose (0.2%), thiamine (2 μg/ml), MgSO₄ (1 mM), MgCl₂ (3 mM), CaCl₂ (0.1 mM), and FeCl₃ (0.3 μM). For [35S]methionine labeling experiments, the M9 high-sulfur medium, supplemented with a mixture of defined amino acids, was used as previously described (34). When necessary, the media were supplemented with ampicillin (100 μg/ml), tetracycline (15 μg/ml), spectinomycin (50 μg/ml), or kanamycin (50 μg/ml).

**Enzymes.** Restriction enzymes, T4 DNA ligase, and exonuclease III were purchased from Bethesda Research Laboratories. Sequenase enzyme was purchased from United States Biochemical Corporation.

**Cloning of htrP gene.** A library of wild-type *E. coli* chromosomal DNA, digested with *Pst*I and ligated in the corresponding site of the low-copy, spectinomycin-resistant (Sp') vector pGB2 (4), was used to isolate wild-type htrP clones. The previously described *E. coli* chromosomal DNA library, prepared in cosmid vector pREG153 (23), was also used to isolate cosmids carrying the htrP' gene. The abilities to complement the Ts' phenotype and recombine htrP mutations onto the plasmid were taken as preliminary evidence that the clone carried the bona fide htrP gene. The gene was further subcloned by standard DNA manipulation techniques (19). All initial subcloning was done with pGB2 as the vector.

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Mapping of htrP gene. Plasmid pSR589 DNA (htrP<sup>+</sup>) or its various subclones carrying the htrP gene were nick translated with [α-<sup>32</sup>P]dCTP (3,000 Ci/mmol; Dupont, NEN Research Products) and used to probe the λ *E. coli* genomic library as previously described (17).

Mapping of Kan::Tn10 insertion in htrP gene. The exact nucleotide position of the Tn10 insertion in htrP698::Tn10 was determined by first isolating clones carrying the htrP mutant allele. To do this, chromosomal DNA from htrP mutant bacteria was digested to completion with PstI. The digested DNA was ligated to pGB2 and pSK<sup>+</sup> (Bluescript; Stratagene) plasmids, similarly digested with PstI, and used to transform *E. coli* DH5α to kanamycin resistance (Kan<sup>r</sup>). The plasmid DNA from such Kan<sup>r</sup> clones was compared by restriction enzyme digestion with pSR589 (htrP<sup>+</sup>), placing the Tn10 insertion between the unique *BglII* and *NdeI* sites. To identify the junction between the chromosomal DNA and the Kan::Tn10 insertion, we used plasmid pSR604, carrying the region from the *HindIII* site of Kan::Tn10 (35) to the *BglII* site of the htrP gene, to sequence the junction by using both the universal reverse primer from the *BglII* site and a 24-nucleotide primer which reads out from the ends of the Tn10 insertion element. The sequence of this oligonucleotide was 5'-ATTGTGATCATATGACAAGATGTGT-3' (36).

Construction of htrP::Ω Kan<sup>r</sup> insertion mutation. An additional *htrP* null allele was constructed by in vitro insertion of the kanamycin resistance cassette, the Ω Kan<sup>r</sup> element (7, 26). To achieve this, we excised the 2.1-kb kanamycin resistance element of plasmid pH455 Ω Kan<sup>r</sup> by digestion with *BamHI* and inserted it into the unique *BglII* site of pSR589 within the *htrP* coding region (Fig. 1). The resulting plasmid, pSR603, was used to transfer the mutant *htrP* allele to the chromosome by linear transformation of the *recB recC sbaA* strain JC8679 (27). Ampicillin-sensitive kanamycin-resistant transformants were retained and transferred by P1 transduction back to a wild-type *E. coli* background. The closely linked Tet<sup>r</sup> marker from CAG12184 (*tolC200::Tn10*) was used to further verify that the new *htrP* allele was located at its expected map location.

RNA isolation and Northern (RNA) blots. RNA was isolated by the hot-phenol extraction procedure (19). RNA was...
isolated from cells grown at 30°C and from cells which were shifted to 42 or 50°C for the desired time. Northern blots (5) of total RNA (5 to 10 μg per lane) were probed with 200 ng of the radiolabeled 1.6-kbp Clal-NdeI DNA fragment, carrying the entire htrP coding sequence, isolated from plasmid pSR591. Labeling was done by nick translation, using [α-32P]dCTP (3,000 Ci/mmol; NEN) (19).

Construction of htrP-lacZ fusions. To clone the promoter region of the htrP gene, we cloned the 830-bp Clal-AsuII fragment from pSR590 into the SK + vector (Bluescript) at the Clal site, resulting in plasmid pSR712. The direction of the cloned fragment in pSR712 was verified by sequencing, and then the insert was transferred to the lacZ operon fusion vector pRS528 (30). This was done by using the HincII and EcoRI flanking restriction sites from the vector to reisolate the Clal-AsuII promoter-containing fragment and clone it into the Smal and EcoRI sites of pRS528. This orientation of cloning resulted in an in-frame lacZ operon fusion. To reduce the amount of upstream sequences, we constructed a smaller clone containing only 536 bp upstream of the AsuII site (Fig. 1). The plasmid pSR712 was digested with AarII and Clal and then treated with T4 DNA polymerase to make the ends blunt. The reaction mixture was then treated with T4 DNA ligase and used to transform DH5α. The new construct, pSR723, was used to reisolate the htrP promoter-containing AarII-AsuII fragment by digesting with HincII-EcoRI and cloning into the pRS528 vector. The Lac + constructs pRS528(htrP-lacZ)830 and pRS528(htrP-lacZ)536 were first verified in strain DH5α and then subsequently used to transform MC4100 to the Lac + phenotype. To make single-copy htrP-lacZ fusions in the chromosone, we used the λ phage vector λRS45 (30) to transfer the fusion by homologous recombination in the lac reca + host MC4100 (Table 1). The recombinant λ(htrP-lacZ) phage were used to infect E. coli strain MC4100. The lysogens were first plated on LB plates as blue colonies and then checked by induction of the λ lysisogen and assayed for the phenotype of the released plase.

Assay of β-galactosidase. β-Galactosidase activities were determined by the method of Miller (21). The overnight bacterial cultures grown in either M9 minimal or LB rich medium (19) at 22°C (room temperature) were diluted 1:100 and allowed to grow to an optical density of 0.2 at 600 nm. The cultures were subsequently shifted to either 30 or 37°C for further incubation. Parallel cultures were also grown at 30 and 37°C in the same way. Protein synthesis was inhibited by the addition of chloramphenicol (30 μg/ml), and samples (50 to 100 μl) were added to Z buffer. Sodium dodecyl sulfate (SDS) (0.1%) and chloroform were used to disrupt the cell membrane. Samples were assayed in duplicate, and the data presented here are the average of six independent experiments.

Sequencing. Nucleotide sequence analysis of the 1.6-kbp Clal-NdeI chromosomal insert in pSR591 was performed by either subcloning various restriction fragments or using nested sets of exonuclease III-generated subclones. Both strands were sequenced by the dideoxyribonucleotide chain termination reaction technique. The sequencing reactions were done with a Sequenase kit (U.S. Biochemicals, Cleveland, Ohio).

Computer analysis of sequence data. The predicted amino acid sequence of the HtrP protein was compared by the FASTA algorithm of Pearson and Lipman (25) against release 16 of SWISS-PROT and release 63 of GenPept. Potential transmembrane segments were evaluated by the method of Klein et al. (13), using PC Gene software.

Nucleotide sequence accession number. The GenBank accession number for the htrP gene is M64472.

RESULTS

A library consisting of at least 5 × 10⁴ mini-Kan::Tn10 insertional events was constructed at 30°C in the wild-type strain B178 by infecting the strain with λ1105 at a multiplicity of infection of 0.1 phage per bacterium. This library was subsequently screened for IS3 of GenPept, potential inserts associated with a temperature-sensitive phenotype (Ts’), i.e., inability to form colonies at 42°C. One such Ts’ isolate, subsequently designated htrP698::Tn10, was chosen for the present study. The htrP mutation was first shown, by P1 transduction, to confer a Ts’ phenotype in various wild-type E. coli genetic backgrounds. In all such genetic backgrounds tested, htrP mutant bacteria exhibited the following phenotypes: (i) inability to form colonies above 37°C in either rich (LB) or minimal (M9) medium, and (ii) a longer doubling time at 30°C compared with the isogenic wild-type parental strain, resulting in small colonies at 30°C. The fact that both these phenotypes cotransduce 100% (100 of 100 tested) with the kanamycin resistance marker shows that both phenotypes are the consequence of a single mutational event.

Cloning of htrP gene. Clones carrying the wild-type htrP gene were identified on the basis of complementation of the Ts’ phenotype from two libraries of cloned wild-type chromosomal DNA. The use of a library of E. coli DNA digested with PsiI and cloned in the low-copy-number vector pBG2 resulted in the identification of a 6.5-kb PsiI fragment that complemented both slow growth at 30°C and the Ts’ phenotype of htrP698::Tn10 mutant bacteria. We also used an E. coli genomic library cloned in cosmid vector pREG153 (23) to isolate cosmids clones that corrected the Ts’ phenotype. All subclones from these cosmids were shown to share the same 6.5-kb PsiI fragment. Further subcloning experiments resulted in the identification of a 1.6-kbp Clal-NdeI minimal DNA fragment that rescued all mutant phenotypes exhibited by the htrP698::Tn10 mutation (Fig. 1).

Mapping of htrP gene. To map the htrP gene, we used the 32P-labeled 6.5-kb PsiI fragment derived from plasmid pSR589 (htrP+) to hybridize to the λ E. coli DNA library (14). Hybridization was observed with λ clones 507 and 508 (data not shown), which carry the region corresponding to kbp 3,220 to 3,250 of the E. coli physical map.

To further confirm the map position, we first lysogenized htrP mutant bacteria with phage λ imm4ind and then infected them with λ transducing phages 505 to 509. Only recombinant phage λ 507 was found capable of (i) rescuing the Ts phenotype and (ii) recombinating the λactE::Tn10::Kanr marker from E. coli DNA library (14). Hybridization was observed with λ clones 507 and 508 (data not shown), which carry the region corresponding to kbp 3,220 to 3,250 of the E. coli physical map.

The htrP gene was also mapped genetically by using tolC::Tn10 as a marker for P1 transduction experiments. Results of such transduction experiments showed that the Tet’ marker from tolC and the Kanr’ marker from htrP are 85% cotransducible. These data are consistent with the rest of the mapping results presented here. The TolC phenotype...
was monitored by sensitivity to SDS and inability to form colonies on MacConkey agar plates.

We also looked into the possibility that the htrP698::Tn10 mutation lies in the ribB gene (2), previously mapped close to tolC (1, 2). However, all known insertions in the ribB gene exhibit a riboflavin auxotrophy, whereas all of our htrP mutant constructs were prototrophic.

Construction of htrP::Ω Kan' insertion mutant. Since we isolated only a single htrP allele, htrP698::Tn10, which was shown by sequencing to map close to the carboxy terminus of the protein (see below), an additional null allele was constructed as follows. First, the Ω Kan' cassette was inserted in the BglII site of plasmid pSR603 (Fig. 1). This DNA was used for linear transformation and recombination of the insertion mutation into the chromosome. The htrP510::Ω Kan' strain thus constructed exhibited the same phenotype as htrP698::Tn10. We conclude that the htrP gene is dispensable at low temperature but is absolutely required for E. coli bacterial viability at temperatures above 37°C.

Identification of HtrP protein. The htrP gene product was identified by in vivo expression from its own promoter in the maxicell system (29) as well as in the T7 promoter expression system (32) (Fig. 2). Introduction of the multicopy plasmid pSR590 (htrP+) or pSR591 (htrP−) resulted in the overproduction of a 27-kDa protein in both wild-type and htrP mutant bacteria. Since a protein of similar size is missing in htrP mutant bacteria (Fig. 3), we tentatively concluded that the 27-kDa protein is the product of the htrP gene. The htrP gene, when exclusively transcribed from the T7 promoter with BL21 (DE3) (32) as the host strain, resulted in the production of an identical 27-kDa acidic protein, which migrated at the expected isoelectric charge (24) (pI = 4.55) predicted from the DNA sequence analysis (see below). By the known orientation of the NdeI-ClaI fragment in plasmid pSR591 or the NdeI-PstI fragment in plasmid pSR590, with respect to the direction of T7 RNA polymerase transcription, it was found that the htrP gene, encoding the 27-kDa protein, is transcribed toward the NdeI site, which is counterclockwise, vis-à-vis the conventional E. coli genetic map. As can be seen in two-dimensional polyacrylamide gel electrophoresis (PAGE) gels (Fig. 2 and 3), the HtrP protein appears to possess multiple isoelectric forms, suggesting that it is posttranslationally modified.

Sequencing the htrP gene. The htrP gene was sequenced from DNA subcloned in both the low-copy-number vector pGB2 and Bluescript vectors. (Both strands were sequenced with either derived subclones or exonuclease III-generated overlapping subclones.) Sequence analysis of a 1.6-kb DNA region from the ClaI to NdeI restriction sites revealed a 756-nucleotide open reading frame (ORF) (Fig. 4). The ORF starts at an ATG, at position 794, and terminates at a TGA, at position 1550. The sequence predicts a 252-amino-acid polypeptide of 27,471 Da, possessing a pl of 4.55. A putative Shine-Dalgarno sequence was located at positions 780 to 783.

The position of the mini-Kan::Tn10 insertion was determined by sequencing the DNA from pSR604. The site of the mini-Kan::Tn10 insertion was found within the deduced htrP gene at position 1397 of the sequence (Fig. 4). This places the Tn10 insertion at the position coding for amino acid 202 of the HtrP protein.
Based on the sequence analysis and complementation by the minimal clone carrying 1.6-kb Clal-NdeI DNA fragment, it is concluded that the htrP gene is not part of an operon and hence that all the phenotypes associated with htrP~96:~Tn10 insertion mutation are due to lack of htrP function rather than due to any polar effects on a cotranscribed gene. These results were further confirmed by analysis of the transcripts (see below), which showed that the htrP gene encodes a monocistronic message.

Transcriptional regulation of htrP gene. Since the htrP gene is essential for E. coli growth at temperatures above 37°C, we looked at the relative abundance of htrP-specific transcripts by Northern analysis. RNA extracted from wild-type cells grown at 30, 42, and 50°C was used. Under all conditions tested, a single transcript of about 850 nucleotides was observed, using the 1.6-kb Clal-NdeI nick-translated DNA as the probe in the Northern analysis. Unlike heat shock genes, the accumulation of htrP-specific transcripts was observed to decline with a shift-up in temperature (Fig. 5). We also looked for the presence of htrP-specific transcripts in an isogenic rpoH null mutant. The htrP gene was found to be actively transcribed in the rpoH null mutant KY1621 (38). Thus, it is concluded that transcription of the htrP gene does not require the presence of the rpoH gene product (rpoH~18) and is not subject to any other type of heat shock regulation.

Expression of htrP promoter as judged by lacZ operon fusions. The putative promoter region of the htrP gene was cloned in the promoter probe operon fusion vector pRS528 (30) as described in Materials and Methods (Fig. 6). The cloning of either the 830-bp AsuII-Clal fragment or the 536-bp AsuII-AarII fragment resulted in a Lac' phenotype, indicating that an active promoter is present within this

FIG. 4. Nucleotide sequence of the htrP gene and its flanking regions. The site of the Tn10 insertion at nucleotide position 1397 is indicated by an arrow (\textdagger). The putative Shine-Dalgarno (SD) sequence is overlined.

FIG. 5. Northern analysis of htrP transcripts. RNA was extracted from isogenic rpoH~2 (KY1621) and rpoH~3 (SR407) strains grown at 30°C (lanes 1 and 4, respectively) or shifted to either 42°C (lanes 2 and 5, respectively) or 50°C (lanes 3 and 6, respectively) for 10 min before extraction. Total RNA (5 μg per lane) was analyzed by the Northern blot technique and probed with 200 ng of the 32P-labeled 1.6-kb Clal-NdeI DNA fragment that carries the entire htrP~2 gene.
region. To avoid complications arising from multicopy plasmid effects, the cloned promoter region from pSR590 was transferred onto the λRS45 transducing phage by homologous recombination. Such recombinant phage were used to introduce a single copy of the htrP-lacZ fusion into the chromosome (30). The level of lacZ expression was measured by monitoring β-galactosidase activity under different growth conditions. Consistent with the Northern analysis of htrP transcription, lacZ expression was found to decrease upon a shift-up in temperature. The steady-state levels measured under different growth-phase conditions (Table 2) were three- to fourfold lower at 37°C than those at 22°C. No significant differences in htrP promoter-driven lacZ expression were observed in the isogenic htrP+ or htrP mutant genetic backgrounds at 22 or 30°C, indicating the absence of self-regulation.

**htrP cells filament above 37°C.** Introduction of the htrP mutation into an otherwise wild-type *E. coli* background resulted in a decrease in viability at temperatures above 37°C (Fig. 7). To account for the decrease in viability at high temperature (37°C and above), we studied htrP bacteria, along with isogenic htrP+ strains, by light microscopy. Cells at different stages of growth in either minimal or LB medium were analyzed over a wide range of temperatures. No major differences were observed for either colony morphology or generation time at 22°C. The htrP bacteria grew as motile rods at 30°C in the early stages of growth, similar to the wild-type parent (Fig. 8). However, when htrP bacteria reached the late log phase, approximately 5 to 10% of the

**TABLE 2. β-Galactosidase activity of lacZ operon fusions to the htrP promoter**

<table>
<thead>
<tr>
<th>Strain and temp (°C)</th>
<th>β-Galactosidase activity (Miller units) with the following optical density at 600 nm of culture at time of sampling:</th>
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<th>0.8</th>
<th>1.5</th>
<th>≥2.4</th>
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<td>706</td>
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<td>652</td>
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<td>30</td>
<td></td>
<td>781</td>
<td>746</td>
<td>ND</td>
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<tr>
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<td>735</td>
<td>790</td>
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<td>695</td>
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* ND, not determined.

**FIG. 6.** Construction of plasmid-borne htrP-lacZ fusions. (a) The 830-bp Clal-AsuII and 536-bp AatII-AsuII fragments were isolated from htrP+ plasmid pSR590 and cloned into pBluescript SK+ (b and c, respectively). These htrP promoter-containing inserts were then recloned into the pRS528 operon fusion vector (d and e, respectively). Cross-hatched bars indicate pBluescript SK+ vector DNA. AsuII* denotes the hybrid Clal-AsuII site. SmaI* denotes the hybrid Clal-SmaI or HincII-SmaI site.

**FIG. 7.** Growth curves of htrP+ and htrP698::Tn10 isogenic bacteria. Exponentially growing cultures at 22°C were diluted 1:10 into prewarmed LB and incubated at 22, 30, 37, or 42°C. Bacterial growth was monitored by measuring CFU per milliliter at various times after the temperature shift by plating serial dilutions. ◆, 22°C; ◆, 30°C; ■, 37°C; •, 42°C.
cells were anucleated at 30°C. At 30°C, the htrP bacteria grew slowly, with a mean generation time of 75 min compared with 42 min observed for the isogenic htrP+ strains. In accordance with this, colony size was reduced in htrP bacteria compared with the htrP+ parental strain. When htrP cells grown at 22°C were shifted to 37 or 42°C, they either underwent filamentation or exhibited aberrant cell morphology. Filamentation started within 2 h at 42°C following a shift from 22°C. When the cultures were shifted to 37°C, the extent of filamentation varied, probably owing to the comparatively leaky nature of the phenotype at this temperature. We also examined the phenotype in low-phosphate medium (Fig. 8E). In this medium, filamentation was more pronounced at both 37 and 42°C than that observed in LB or minimal medium. The low-phosphate medium was M9 medium without added extra inorganic phosphates but supplemented with 5 mM KCl and Casamino Acids (0.4%). When htrP bacteria were incubated in saline solution (50 mM Tris [pH 7.5], 0.84% NaCl) at 37 and 42°C, they lost the normal rod shape and instead appeared round or formed ghostlike heads (Fig. 8F). Under identical conditions, the wild-type bacteria retained both the normal rod shape as well as viability. These results suggest that htrP mutant bacteria possess defective cell walls, implicating a direct or indirect effect of the HtrP protein in proper cell wall biosynthesis and/or assembly.

Computer analysis of HtrP protein sequence. Using the method of Kyte and Doolittle (16), we predicted the HtrP protein to be overall hydrophilic in nature. This observation was verified by isoelectric focusing experiments (Fig. 2 and 3) with [35S]methionine-labeled extracts prepared from cells expressing the HtrP protein by the T7 promoter system. Based on the method of Klein et al. (13), using PC Gene software from Intelligenetics, Inc., the HtrP protein was predicted to be an integral membrane protein, with two transmembrane helices spanning amino acid residues 92 to 109 and 231 to 251, respectively. Since the HtrP protein is not processed and lacks an obvious signal peptide, it is likely that it is either an inner membrane protein or membrane associated. To determine whether HtrP protein is an integral membrane protein or not, we labeled cells with [35S]methionine and fractionated the sonicated whole-cell extracts on sucrose step gradients (11) or by centrifugation at 220,000 × g for 2 h. When HtrP protein was overproduced by using pSR591 with the htrP gene expressed from T7 promoter, most of the HtrP protein was found in the soluble fraction (Fig. 9). This result argues against HtrP protein being an integral membrane protein.

Homology to other proteins. Searches of the protein sequence data bases, using the predicted HtrP sequence, revealed significant homology to the known LuxH protein of Vibrio harveyi. These two proteins share an identity of 64.4% over a length of 210 amino acids (Fig. 10). The luxH gene is the last gene in the luxAH operon (33). The function of the luxH gene is not yet known. However, it is known that the expression of luxH is not essential for light production (33). Another protein sequence found in the database, RibA (GenBank X51510), presumably involved in riboflavin bio-

FIG. 8. htrP mutants exhibit filamentation at high temperature. Bacterial cell morphology at 22°C of htrP+ (A) and htrP mutant (B) cells. Cultures were grown in LB medium to an optical density of 0.2 (595 nm) and examined under the light microscope. The htrP mutant culture was then shifted to 37°C (C) or 42°C (D) for 2 h and examined. The htrP mutant cultures were also shifted to 42°C in low-phosphate medium (E) or saline medium (F) for 2 h and examined. Under identical conditions, htrP+ bacterial cell morphology remained essentially as that at 22°C (see panel A). The bar in panel F represents 5 μm.
FIG. 9. Fractionation of HtrP protein. Bacterial cultures (25 ml) of strains carrying plasmid pSK* alone or pSR91 with the htrP gene cloned under the T7 RNA polymerase promoter were labeled with \(^{35}\)S]methionine for 10 min as described in the legend to Fig. 2A. The labeled cells were centrifuged at 2,000 \(\times \) g for 10 min, washed in M9 medium, and resuspended in 0.5 ml of 20% sucrose, followed by the addition of DNase and RNase (10 \(\mu\)g/ml). Cells were broken by sonication, and unbroken cells were removed by two cycles of centrifugation at 2,000 \(\times \) g for 10 min. The supernatant was then centrifuged at 220,000 \(\times \) g for 2 h. The supernatant containing the soluble proteins was precipitated with trichloroacetic acid (10%) and resuspended in 200 \(\mu\)l of sample buffer. The pellet (membrane fraction) was dissolved in 200 \(\mu\)l of SDS sample buffer. The total cell extracts (T) and soluble (S) and membrane (M) fractions were then analyzed by 12.5% SDS-PAGE. The arrow indicates the position of the HtrP protein.

synthesis in Bacillus subtilis, also shares significant homology (49.4% identity over a length of 198 amino acid residues) with the HtrP protein. However, the RibA protein is much larger than HtrP, consisting of 398 amino acid residues.

DISCUSSION

We identified, cloned, and mapped the htrP gene of E. coli. The htrP gene was identified on the basis of an insertion mutation, htrP698::Tn10, which confers a Ts-s growth phenotype. Since the htrP insertion mutation does not allow growth of E. coli cells at temperatures above 37°C, we used this phenotype to clone the wild-type htrP gene by complementation. The htrP gene maps at 66.3 min, corresponding to the 3,238- to 3,240-kbp region of the physical map of the E. coli chromosome. The htrP gene is located 4 kbp clockwise from the toIC gene, its direction of transcription being counterclockwise relative to the genetic map and in the direction opposite to that of the toIC gene (9).

The htrP gene was sequenced and found to possess an ORF coding for a protein with a predicted molecular weight of 27,471 and a predicted isoelectric point of 4.55. The evidence that this ORF indeed encodes the HtrP protein is as follows, (i) The predicted and observed sizes (~27,000 Da) (Fig. 2 and 3) of the protein product agree with each other. (ii) There is no other ORF of a similar size within the sequenced area. (iii) The predicted isoelectric charge of HtrP is pH 4.55, in agreement with its observed migration on two-dimensional SDS-polyacrylamide gels (Fig. 3).

(iv) When the wild-type gene was replaced by either the htrP698::Tn10 or htrP510::\(\Delta\)Kan mutant allele, the 27,900-Da protein was no longer made. (v) Sequencing of the htrP698::Tn10 mutation confirmed that the Tn10 insertion was present in this particular ORF, and (vi) the strong homology between the HtrP protein predicted from this ORF and LuxH protein runs through the length of this ORF, particularly in the first 220 amino acids.

The facts that the minimal htrP* clone (1.6-kb ClaI-NdeI) (i) complements all the phenotypes and (ii) possesses a single ORF and that (iii) a single 850-nucleotide-long htrP-specific transcript is found show that the htrP gene is not organized as an operon. This implies that all the phenotypes associated with the htrP698::Tn10 mutation arise because of the absence of htrP function and are not due to any polar effects on the expression of some other gene.

Analysis of RNA transcripts shows that (i) transcription of the htrP gene is not subject to regulation by Er32, since the htrP gene is transcribed in the rpoH null mutant, and (ii) the accumulation of the htrP-specific gene transcripts declines upon a shift-up in temperature. These results clearly indicate that the htrP gene is not a classical heat shock gene. Using htrP-lacZ operon fusions, we further confirmed that the level of transcription from the htrP promoter is reduced at high temperatures. However, it is quite interesting that even a shift up from 22°C to 30 or 37°C leads to a progressive decline in the rate of htrP promoter-driven lacZ expression. Since the reduction was observed at all growth stages, including the stationary phase, the transcription does not appear to be growth rate regulated, at least in the wild-type background used (MC4100). Although transcription from the htrP promoter is reduced at 37°C vis-à-vis that observed at 22°C, the transcription rate is still fairly high compared with other known promoters.

The HtrP protein is predicted to be overall hydrophilic in nature, although it has two potential transmembrane segments. Both the lack of a signal peptide sequence and the hydrophilic nature of the protein suggest that the HtrP protein either spans the inner membrane or is membrane associated. However, when the HtrP protein was overproduced from the T7 promoter expression system, it was found that most of the HtrP protein was present in the soluble fraction. It is thus likely that the major fraction of the HtrP protein is cytosolic in nature. Clearly, more experiments are
needed to pinpoint the exact location of the HtrP protein in a system in which the htrP gene is present in a single copy. The potential association of the HtrP protein with the E. coli inner membrane helps explain the unusual shapes exhibited by htrP mutants under certain experimental conditions, i.e., it could be that the lack of HtrP protein causes an inner membrane perturbation, which in turn somehow leads to abnormal cell wall formation. The HtrP protein may be subject to posttranslational modifications, since multiple isoelectric forms were seen in two-dimensional polyacrylamide gels. The nature of these modification(s) is not known at present. It may be interesting to know whether these modifications are temperature regulated, a possibility suggested by the need for HtrP protein only at high temperatures.

The HtrP protein shares a striking homology to the LuxH protein from V. harveyi. Since the function of LuxH is not known, the significance of such homology is difficult to interpret. However, another protein, RibA from B. subtilis, with which HtrP shares significant homology has been implicated in riboflavin biosynthesis. Our htrP mutants exhibit no riboflavin auxotrophy, and their Ts phenotype is not affected by the exogenous addition of riboflavin. As pointed out earlier, some riboflavin auxotrophs in E. coli have been shown to be tightly linked (59 to 96% by P1 transduction) to the tolC gene (2). Clearly, more studies are needed to establish a functional and genetic relationship between the HtrP protein and LuxH and to determine whether the HtrP protein is somehow involved in the biosynthetic pathway of riboflavin.

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