The htrM gene, whose product is essential for *Escherichia coli* viability only at elevated temperatures, is identical to the rfaD gene

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ABSTRACT

We have identified a new *E. coli* gene, htrM. The htrM gene was identified because its insertional inactivation by the Tn5 transposon results in *E. coli*’s inability to form colonies at temperatures above 43°C. The corresponding htrM+ gene was cloned on the basis of its ability to correct the temperature-sensitive phenotype of the htrM::Tn5 insertion mutations. The htrM gene has been mapped to 81.2 min on the conventional *E. coli* genetic map. It was sequenced and shown to code for an acidic, 34,893-Da polypeptide. Three transcriptional starts were located 48, 90 and 123 nucleotides upstream of the ATG, initiation codon referred to as the P1, P2 and P3(hs) promoters, respectively. The −10 and −35 regions of the P1 promoter bear a close similarity to the Eo70-recognized consensus sequences, while the −12 region of the P2 promoter resembles the consensus promoter sequence transcribed by the rpoN gene product. Transcripts of the htrM gene accumulate with increasing temperature. The −10 and −35 regions of the P3(hs) promoter, represented by nucleotides 160 to 130 upstream of the ATG initiation codon, are similar to the Eo32-recognized consensus sequences. The α32 transcription factor is essential for maximal htrM gene transcription, since htrM RNA transcripts are made at reduced rates in a rpoH null mutant background. Surprisingly, the htrM gene turns out to be identical to rfaD, whose product is required for the biosynthesis of the ADP-L-glycero-D manohheptose lipopolysaccharide precursor [Pegues et al. (1990) J.Bacteriol. 172, 4652–4660].

INTRODUCTION

All living organisms undergo a stress response (reviewed in reference 1). In the case of *E. coli*, the stress response is best seen when an exponentially growing culture is suddenly shifted from 30°C to 42°C (reviewed in references 1 and 2). Under these conditions the rate of synthesis of a set of 20 or so polypeptides is suddenly accelerated. The stress or heat shock response has been shown to be transient and under the positive control of the α32 transcription factor (1, 2). About half of the heat shock proteins have been identified and their corresponding genes sequenced (1, 2, 3). In an effort to identify more heat shock genes, and potentially identify the genes coding for those heat shock proteins already identified, we have recently taken a different genetic approach. The new approach consists of (i) creating libraries of *E. coli* bacteria carrying various transposon insertions, and (ii) screening such libraries for the presence of those insertions which result in a temperature-sensitive (Ts−) phenotype. Such genes have been termed htr (high temperature requirement). Previously described members of this group of genes include htrA (4) and htrC (5), which are under the positive control of the α24 (α5) (4, 6, 7) and α32 (5) transcriptional factors, respectively.

In this paper, we describe the isolation, characterization, sequencing and transcriptional regulation of htrM, a new member of the group. The htrM gene has at least three promoters, one of which resembles a consensus Eo32-transcribed heat shock promoter. Another promoter, designated as P1, is similar to a consensus Eo32-transcribed promoter, used by housekeeping genes. While this work was in progress, Pegues et al. (23) published the sequence of the rfaD gene whose product is required for the biosynthesis of the lipopolysaccharide precursor ADP-L-glycero-D manohheptose. It turns out that the htrM and rfaD genes are identical.

METHODS

Bacterial and phage strains

Most of the *E. coli* and phage strains used in this study have been previously described (5); the rest are referred to in the text.

Media

The LB rich and M9 minimal media were prepared as described in (8). LA is LB medium containing 1% agar. The MacConkey agar medium (Difco laboratories) was prepared following the manufacturer’s recommended procedures. The 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 (9). When necessary, the media were supplemented with ampicillin (100 µg/ml), tetracycline (15 µg/ml), spectinomycin (50 µg/ml), or kanamycin (50 µg/ml).
Cloning of the \textit{htrM} gene

The previously described \textit{SaU3A} partially-digested \textit{E. coli} chromosomal DNA library (5, 10), prepared in the low copy vector pHGE153 (10), was used to isolate cosmids carrying the \textit{htrM} \textsuperscript{+} gene. Since \textit{htrM} mutant bacteria do not adsorb phage \textit{λ} well and, in addition, transform poorly, they were infected at a very high multiplicity of infection with the \textit{λ} cosmid phage library (~100 phage per bacterium). The ability to (i) complement the T\textit{S} \textsuperscript{−} phenotype, (ii) allow colony formation on MacConkey agar plates, and (iii) recombine with the \textit{htrM} chromosomal insertion mutations was taken as preliminary proof that a particular cosmid clone indeed carried the \textit{htrM} gene. Further subcloning was done using standard DNA manipulation techniques (8).

Mapping of the \textit{htrM} gene

The \textit{htrM}-carrying cosmId DNA, or its subclone, pSR164, was nick-translated using [\textit{α}-\textit{32P}]-dCTP (3000 Ci/mmol) (New England Nuclear/DuPont), and was used to probe the \textit{E. coli} genomic DNA library composed of 476 overlapping 1 transducing phages (11), under the conditions described by Wahi et al. (12).

Sequencing of \textit{htrM}

To sequence \textit{htrM}, the chain-termination method was used (13), employing the T7 DNA polymerase, Sequenase (USB), following the manufacturer’s recommended procedures. Nested sets of overlapping exconuclease III- or DNaseI-generated clones (8) spanning the \textit{htrM} gene and its flanking sequences in pEMBL18 \textsuperscript{+} (14) or pBlueScript (Stratagene) vectors were used as templates for sequencing. Whenever necessary, synthetic oligomers were used as primers to confirm the DNA sequence from the various \textit{htrM} clones or subcloned derivatives on the low copy vector, pGB2 (15).

Cloning of \textit{htrM} mutant alleles

To clone the \textit{htrM}:Tn5 mutant alleles, chromosomal DNA was prepared from \textit{htrM378}:Tn5 and \textit{htrM401}:Tn5 mutant bacteria and digested to completion by the restriction enzymes \textit{EcoRI} or \textit{EcoRV}. Neither of these restriction enzymes cuts within Tn5 or the coding sequence of the \textit{htrM} gene. The digestion mixture was ligated to appropriately digested pEMBL18 \textsuperscript{+} DNA and used for transformation, simultaneously selecting for resistance to ampicillin (\textit{Amp}\textsuperscript{R}; 100 \text{μg/ml}) and kanamycin (\textit{Kan}\textsuperscript{R}; 50 \text{μg/ml}). The authenticity of the resulting clones was confirmed by restriction digestion of the DNA, and by testing for the inability of the subclones to complement the various phenotypes of the \textit{htrM} mutations.

The two \textit{htrM} mutant alleles were also cloned by \textit{in vivo} recombination onto the \textit{htrM} \textsuperscript{+}-carrying plasmids, pSR31 and pSR164. The DNA restriction digestion pattern of the resulting plasmids, carrying the various \textit{htrM} alleles, was compared to the earlier constructs. The exact site of Tn5 insertions in the \textit{htrM} gene was determined by sequencing, using the Tn5 sequencing primer and strategy described earlier (5).

RNA isolation and northern blotting

Total cellular RNA was isolated by the hot SDS-phenol extraction procedure (8). RNA was isolated from cells grown at 30°C and from cells which were shifted to 42° or 50°C for the desired length of time. RNA samples (5 to 10 \text{μg}) were analyzed by the northern technique as described by Colman (16), using Hybond-N membrane (Amersham Corp.). To probe for \textit{htrM} message, 200 ng of the 1.2 kb \textit{AsuII}-\textit{MluI} DNA fragment, carrying the entire \textit{htrM} coding sequence, was isolated from pSR164, and \textit{32P}-labeled with [\textit{α}-\textit{32P}]-dCTP (3000 Ci/mmol) by nick-translation (8).

Primer Extension

To define the 5' terminus of the \textit{htrM} messenger RNA, approximately 10 ng of an oligonucleotide probe complementary to the nucleotide sequence extending from positions 202 to 221 or 213 to 246 of the \textit{htrM} sequence was annealed to 10 μg of RNA. RNA was extracted either from wild type cells or from the isogenic \textit{pOPlpH} (KY1621) and \textit{pOPlpH}\textsuperscript{+} (SR407) strains grown at 30°C, or following a 10 min shift from 30°C to 42°C. The annealed primer was extended by AMV reverse transcriptase essentially as previously described (5). The primer extension products were electrophoresed on the same gel as the diodeoxy sequencing reactions carried out with the same primer.

Mung Bean Nuclease Protection Assay

In order to define unambiguously the transcriptional start site, total cellular RNA was hybridized to a uniformly radiolabelled probe containing the 3' end \textit{htrM}-specific sequences. To achieve this, a 650-bp \textit{AsuII}-\textit{StuI} fragment was first cloned into the \textit{Smal} site of the M13 mp19 vector, to give rise to the M13-SR1 clone. The orientation of the insert was confirmed by restriction analysis and DNA sequencing. To label the antisense strand, 1 μg of M13-SR1 single-stranded DNA was extended from the universal primer position with T7 DNA polymerase in the presence of [\textit{α}-\textit{32P}]-dCTP (20 mCi) containing 5 μM of each nucleotide in a 10-μl reaction. The replicated, double-stranded DNA was digested with \textit{SstI}, resulting 650 bp fragment was gel purified and hybridized to 40 μg of RNA under the conditions described (18). The RNA:DNA hybrids were digested with 300 U of mung bean nuclease as described (19). The protected fragment was sized and compared with pBR322 \textit{Mspl}-digested DNA on a denaturing gel as well as a diodeoxy sequencing reaction using M13 mp19 (\textit{AsuII}-\textit{Stal}) single-stranded DNA as the template.

Construction of \textit{htrM}:\textit{ΩTet}\textsuperscript{−} deletion mutations in the \textit{htrM} gene

To confirm the nonessentiality of the \textit{htrM} gene at low temperatures, an additional null allele was constructed by \textit{in vitro} substitution of the \textit{htrM} structural gene by a tetracycline-resistance cassette, the \textit{Ω}-\textit{Tet}\textsuperscript{R} element (17). To achieve this, the \textit{Tet}\textsuperscript{R} element from pH45\textit{Ω}-\textit{Tet}\textsuperscript{R} was inserted between the \textit{AsuII} and \textit{BglII} sites of the \textit{htrM} gene. The resulting plasmid, pSR440, was linearized and used to transform the JC8678 \textit{recBrecCsbC} mutant strain (5). \textit{Amp}\textsuperscript{R} \textit{Tet}\textsuperscript{R} transformants were screened for and examined further. Phage P1 was grown on such transformants and used to transduce the \textit{Tet}\textsuperscript{R} allele back into the B178, MC4100, and CA8000 wild type strains. All such transductants exhibited the same phenotypes as the original \textit{htrM} insertion mutations. The new \textit{htrM} mutant allele was designated as \textit{htrM}:\textit{ΩTet}\textsuperscript{R}.

RESULTS

Isolation of \textit{htrM} null mutants

From a pool of Tn5 insertions in CA8000 bacteria (5), two mutant bacteria, \textit{htrM378}:Tn5 and \textit{htrM401}:Tn5 were identified because they shared the following properties: (i) inability to form colonies at temperatures above 43°C, (ii) aberrant cell division, manifested by the formation of minicells at all temperatures, but...
especially at 42°C, (iii) an extreme mucoid phenotype at all
permissive temperatures (phage λ does not form plaques on htrM
mutant bacteria, probably due to interference at the adsorption
and/or injection level), and (iv) inability to form colonies on
media containing bile salts, such as MacConkey agar plates.
Using phage PI-mediated generalized transduction, we were able
to show that the various htrM mutations can be easily moved
back into a wild type E. coli strain. All such transductants were
shown to possess all mutant phenotypes. These results prove that
the htrM mutations, by themselves, confer all of the enumerated
properties to the mutant host.

Cloning of the htrM* gene
The htrM* gene was isolated on a low copy pREG-derived
cosmid as follows: A library of E. coli DNA, prepared on the
pREG153 plasmid (10), was introduced, by λ-mediated
transduction, into htrM mutant bacteria. Following a 60 min
period for drug-resistance expression, bacteria that were
simultaneously temperature-resistant (Ts+) and AmpR were
isolated at 43°C. Because htrM bacteria are largely refractory
to phage λ adsorption and/or injection, a high titer of a cosmid
lysate was used to overcome this infection barrier. All cosmid
clones isolated this way were shown to carry the bona fide
htrM* gene because (i) the htrM378::Tn5 and htrM401::Tn5
mutations could be recombined onto them (see Methods and
below), (ii) they allowed colony formation of htrM~ bacteria on
MacConkey agar plates at all temperatures, and (iii) they
suppressed the mucoid phenotype of htrM~ bacteria, simultaneously allowing phage λ infection.

A low copy vector, pGB2 (15), was then used to subclone
various DNA fragments from the cosmid carrying the htrM* gene. A 5.2-kb EcoRV fragment was initially cloned and found to
complement all the phenotypes of htrM mutant bacteria. The
resulting plasmid, pSR31 (Fig. 1b) was used for further subcloning. Based on restriction mapping of derived subclones, the htrM* gene was found to reside on a 2.5-kb EcoRI-Smal
fragment (Fig. 1a to 1d). The 2.5-kb EcoRI-Smal fragment was
transferred from the pGB2 vector to the pEMBL18+ and
pBluescript vectors to further delineate the limits of the htrM* gene. Towards this goal, a series of DNase I and exonuclease
III deletions were constructed (8). The ability to complement the
defects of the htrM mutant alleles, as well as ability to recombine
these alleles was limited to a 1.6-kb Accl-MluI fragment (pSR164)
(Fig. 1d). By the orientation of this 1.6-kb DNA fragment in
the pBluescript vectors with respect to the direction of T7 RNA
polymerase transcription, it was determined that htrM
transcription occurs in a clockwise direction vis-a-vis the
conventional E. coli map, towards the MluI restriction site. These
results were further confirmed by sequencing and determination
of the transcriptional start sites in vivo (see below).

Mapping of the htrM gene
From preliminary Hfr mapping experiments, it was inferred that
the htrM gene is located close to the cysE and muti markers. This

![Figure 1: Restriction map of the htrM gene and construction of htrM insertion and deletion derivatives.](https://example.com/figure1.png)

(a) Location of the htrM gene on the physical map of E.
coli; (b and d) maps of pSR31 and pSR164, respectively, both of which carry an intact htrM gene; (c) restriction map of pSR45 which carries the entire coding
region of the htrM gene and 130 nucleotides upstream, but lacks the putative htrM heat shock promoter; (e and f) the location (Δ) and (—) orientation of the two
Tn5 insertions; (g) map of pSR440 which carries an nTetR deletion-substitution in the htrM gene between the AsuII and BglII sites. Abbreviations: RI, EcoRI; RV,
EcoRV; BII, RsII, AcI, Accl; M, MluI; S, SstI; Sm, Smal.)
the restriction sites in the derivative clones obtained from pSR31 rfaG gene in the operon, which maps near the 3910 kb region. The E. coli htrM gene in the operon [these clones were obtained from Dr. Carl rfaG and various lambdoid phages. Some of these phenotypes, e.g., lipopolysaccharide and are resistant to many phages, e.g., PI mtlA-cysE pyrE-rpmBG genes (20). and chromosome between the chromosome. This corresponds to 81.2 minutes on the E. coli chromosome. To precisely locate the htrM gene in the 81 minute region on the genetic map of the E. coli chromosome. To precisely locate the htrM gene on the physical and genetic maps, we used [32P]-labeled cosmid clones carrying the htrM gene or their derivative subclones, and hybridized them to the entire E. coli λ phage library (11). Membrane filters, bound with DNA from each of the 476 overlapping λ clones were used in the hybridization experiments, as described earlier (4). The probes derived from htrM+ cosmid clones hybridized to λ transducing phages 573, 574, and 575 (data not shown). When probed with smaller clones, such as pSR164 carrying the intact htrM+ gene, hybridization was observed only to λ clones 574 and 575 (data not shown). This last result placed the htrM gene within the 3910 to 3920 kilobase region of the E. coli chromosome. Comparison of the restriction maps of the htrM+ clones and clones carrying the htrM::Tn5 insertions placed the htrM gene closer to the 3920 kilobase region of the E. coli chromosome. This corresponds to 81.2 minutes on the E. coli genetic map.

The rfa gene cluster has been mapped to the E. coli chromosome between the mla-cysE and pyrE-rpmBG genes (20). The rfa mutants lack or have defective core-heptose lipopolysaccharide and are resistant to many phages, e.g., P1 and various lambdoid phages. Some of these phenotypes, e.g., resistance to phages and sensitivity to dyes, are common to certain rfa mutants, as well as the htrM mutants. We transformed htrM mutant bacteria with clones derived from pLC 10-7 carrying the rfaG operon [these clones were obtained from Dr. Carl Schnaitman, Department of Microbiology, Arizona State University]. The inability to complement the htrM mutations was taken as proof that the htrM gene does not correspond to any gene in the rfaG operon, which maps near the 3910 kb region.

We also confirmed our mapping results by a comparison of the restriction sites in the derivative clones obtained from pSR31 which carries the region upstream of the htrM gene. The region upstream of the EcoRI-EcoRV fragment (Fig. 1a and 1b) includes the kbl-tdh genes (21).

Construction of an htrM::ΩTet allele deletion-substitution mutation in the htrM gene
In order to confirm that the htrM gene is dispensable at 30°C in different wild type backgrounds, we substituted a 1.7-kb SmaI fragment, carrying the Ω-TetR cassette, between the AulI and BglII sites of plasmid pSR31 (Fig. 1g). The resulting htrM::ΩTet allele was transferred onto the chromosome as described in Methods. The new htrM::ΩTet allele removes the entire htrM coding region except for that coding for the 32 carboxy terminal amino acids. The htrM::ΩTet deletion-substitution allele was found to confer an identical Ts growth phenotype as the original htrM378::Tn5 and htrM401::Tn5 isolates. There was complete correspondence in the rest of the phenotypes as well, including the inability to form colonies on MacConkey agar plates and overproduction of capsular polysaccharide atmissive. Thus, it is concluded that the htrM gene is dispensable for E. coli growth only at low temperatures, and that the htrM mutations exhibit various phenotypic defects at all temperatures.

Identification of the HtrM protein
To unequivocally identify the HtrM protein, the T7 promoter expression system was used to direct the exclusive transcription-translation of the cloned htrM gene using BL21(DE3) as the host strain (22). Bacterial cultures carrying either the pSR164 (htrM+) minimal clone, pSR45 (htrM+) or vector alone were labeled with [35S]-methionine as described in the legend to Fig. 2. It can be seen that the presence of plasmid pSR164
Figure 4. Nucleotide sequence of the htrM gene and its flanking regions. The putative Shine-Dalgarno (SD), and the various -10 and -35 transcriptional regulation regions are overlined. The major transcriptional start sites are shown by bold arrows. The insertional sites of Tn5 are indicated by A.

(htrM') resulted in the large overproduction of a ~35,000 Da protein, possessing an isoelectric point very similar to that predicted by the htrM coding sequence (pl=4.56; Fig. 3, and see below).

**Sequencing of the htrM gene**

The htrM gene was sequenced from DNA subcloned in both the low copy pGB2 vector and the pUC-derived vector, pEMBL18+. Whenever the htrM' gene was sequenced from pGB2, synthetic oligonucleotide primers were used for the sequencing reactions. Sequencing from a low copy vector was done to confirm the htrM sequence, since the htrM gene carried on a high copy vector conferred a slow growth phenotype to host bacteria (data not shown). This growth disadvantage may result in the selection of plasmid-encoded htrM mutant variants, and may also explain the frequently detected transfer of the htrM null alleles from the chromosome onto the htrM-carrying plasmid (data not shown). Sequencing from either the low copy vector or from the various subclones on high number copy plasmids revealed the presence of a 930-nucleotide long open reading frame (ORF) (Fig. 4). The ORF starts at an ATG and terminates at a TAA. The sequence predicts a polypeptide of 34,893 Da.
In order to sequence the Tn5 insertion sites in htrM378::Tn5 and htrM401::Tn5, plasmids p5SR328 and p5SR329, carrying the two htrM::Tn5 mutant alleles, were constructed as described in Methods. The exact site of Tn5 insertion in the htrM gene was then determined by sequencing these plasmids using the Tn5 primer described earlier (5), located 20 nucleotides from the end of ISS0L of Tn5. DNA sequencing revealed that the htrM378::Tn5 and htrM401::Tn5 insertions were at nucleotide positions 378 and 401, respectively, of the htrM-coding region and in opposite orientations (Figs. 1 and 4).

Transcriptional regulation of the htrM gene

Since the htrM gene is essential for E. coli growth at high temperature, especially above 43°C, we looked at the relative abundance of the htrM transcripts over a wide range of temperatures. RNA extracted from wild type cells revealed that (i) a shift-up in temperature induced a rapid increase in the htrM gene transcript levels (Fig. 5), (ii) in analogy with other known heat shock genes, e.g., dnaK, transcription of the htrM gene continued at 50°C (Fig. 5). The relative abundance of htrM transcripts was higher at 50°C than at 42°C or 30°C, and (iii) unlike the classical dnaK heat shock gene, whose transcription continues at 50°C, the accumulation of htrM gene transcripts slowly declined at this temperature (results not shown).

Since the relative transcription of htrM increases with an increase in the incubation temperature, we looked at the possibility that its transcription is regulated by the Eo32 RNA polymerase holoenzyme; it is known that induction of the classical heat shock regulon is exclusively under the transcriptional regulation of Eo32 (2). To test this possibility, RNA preparations isolated from isogenic rpoH+ and rpoH null mutant bacterial strains (5) were probed for the presence of htrM-specific mRNA. The levels of htrM transcripts were found to be reduced in the rpoH null mutant. As expected, the isogenic rpoH+ strain showed a typical increase in the abundance of htrM transcripts with increasing temperature (Fig. 5). Under similar conditions, rpoH null mutants did show a normal transcriptional pattern of known non-Eo32-regulated genes, e.g., htrA (results not shown). Since htrM gene transcription was reduced but not totally abolished in the rpoH null mutant, it appears to be partly under the transcriptional control of Eo32. Further results confirm the presence of active non-Eo32 promoter(s) (see below).

Mapping the 5' termini of htrM transcripts

It is known that the E. coli Eo32 RNA polymerase holoenzyme transcribes genes whose promoters possess homology to the heat shock consensus sequence for the -10 and -35 regions. Most of the heat shock genes show inducible heat shock gene transcription starting only from such upstream promoter sequences (2, 3). To determine if the htrM gene has such a promoter, we defined its transcriptional start sites through both primer extension analysis and mung bean nuclease protection experiments. Such analysis revealed that one of the two major transcriptional start sites is located at nucleotide position 179 (Figs. 4 and 6). This site corresponds to the start site of the rfaD gene as defined by Pegues et al. (23). This promoter, designated as P1, is similar in sequence to promoters transcribed by the Eo32 housekeeping RNA polymerase. Two other transcriptional

Figure 5. Northern RNA analysis of htrM transcripts. RNA was extracted from htrM* bacteria grown at 30°C (lane 1) or shifted either to 42°C or 50°C for 10 min before extraction (lanes 2 and 3). RNA extracted from isogenic rpoH+ (KY1621) and rpoH+ (SR407) strains at 30°C (lanes 4 and 5, respectively) and after a shift from 30°C to 50°C for 10 min (lanes 6 and 7, respectively). Approximately 5 µg of total RNA per lane was analyzed by the Northern blot technique and probed with 32P-labeled, nick-translated 200 ng of 1.2 kb AsuII-Mul DNA fragment that carries the entire htrM* gene.

Figure 6. Mapping of 5' termini of htrM transcripts. Primer extension reactions of total cellular RNA hybridized to 32P-end-labeled DNA oligonucleotide probe, complementary to nucleotides 201 to 220 in the htrM sense strand. RNA was extracted from htrM+ bacteria grown at 30°C (lane 1); shifted to 42°C for either 10 min (lane 2) or 20 min (lane 3), or shifted to 50°C for 10 min (lane 4) or 20 min (lane 5). Lanes labeled G, A, T and C correspond to the dideoxy sequencing reactions carried out, using the same oligonucleotide as the primer. The shaded arrow points the transcriptional start corresponding to the P3(hs) promoter, while the open arrow points to that of the P1 promoter. The open arrow indicates the position of the P1-promoted transcriptional start site, and the shaded arrow indicates the position of the P3 (hs)-promoted transcriptional start site.
starts were located at nucleotide positions 137 and 103 (Figs. 4, 6 and 7), whose putative promoters are designated P2 and P3(hs), respectively. Prominent transcripts arising from P2 promoter were seen only in some of the experiments, such as those shown in Fig. 7. The −10 and −35 regions corresponding to the P3(hs) start site bear a close resemblance to the −10 (6/9 bases) and −35 (7/9 bases) regions of the Es32-consensus heat shock promoters (see Discussion). In agreement with the assignment of P3(hs) as a Es32-regulated promoter, its rate of transcription increased at high temperature (Fig. 6). One of the major differences between the P3(hs) promoter and the classical heat shock promoters recognized by Es32 is the absence of the conserved C nucleotide at position −14 (3). This may explain why transcription from P3(hs) is not induced appreciably at 42°C but is induced at 50°C.

Requirement of sequences upstream of the P1 promoter
Since three transcriptional start sites were found during promoter mapping, we looked at the suppression of htrM mutant bacteria by introducing plasmid pSR45 which carries an intact P1 promoter (including 50 nucleotides upstream of the putative +1 start site), a truncated P2 promoter, and totally lacks the P3(hs) promoter. Surprisingly, such constructs were not able to fully complement the HtrM mutant phenotype. The colony-forming ability on MacConkey agar plates was not restored at any temperature, although a weak suppression of the Ts− phenotype was observed. To account for these results, it is argued that transcription from the P3(hs) promoter (and P2?) is somehow required for the full expression of the htrM gene and the complete restoration of the wild type phenotype. To verify that pSR45 is capable of directing synthesis of the HtrM protein, we performed the following experiments: (i) protein synthesis directed by the T7 promoter expression system of the cloned htrM inserts in pSR45 and pSR164 [which carries the complete htrM gene including the P2 and P3(hs) promoters] were compared. Synthesis of an identical 35 kDa protein was directed from both plasmids (Fig. 2), (ii) RNA made from htrM37S::Tn5 mutant bacteria carrying either pSR164 or pSR45 was probed for the presence of htrM transcripts. The results from such northern blot analysis showed that the level of htrM transcripts was reduced in host strains carrying pSR45 (Fig. 8) as compared to those carrying pSR164. Considering these results, it is likely that, although the P1 promoter is partly functional in plasmid pSR45, this level of transcription is insufficient to completely suppress all of the known mutant phenotypes. Perhaps some of the upstream sequences are required for efficient initiation by RNA polymerase at the P1 promoter. The overall conclusion from these experiments is that transcription from the P2 and/or P3(hs) promoters is absolutely required to achieve the levels of htrM transcripts sufficient to suppress all of the htrM null mutant phenotypes.

Homology to other known proteins
To obtain clues about the function of the HtrM protein, we searched the Swiss Protein database (release 14) and GenBank

![Figure 7. Mapping of the 5' termini of htrM transcripts with Mung Bean nuclease protection assay. Mung Bean nuclease mapping, with a uniformly labeled, single-stranded, 330-nucleotide AsuI-Sai DNA fragment as a probe. The probe (10^6 cpm) was hybridized to RNA extracted from htrM+ bacteria grown at 30°C, digested with 300 U Mung Bean nuclease, and electrophoresed. Lanes labeled G, A, T and C correspond to the dideoxy sequencing reactions carried out using M13mp18 (AsuI-Sai), as the template, initiated by the −20 universal primer, which was also used to synthesize the probe. The shaded arrow points to the transcriptional start site corresponding to the P1 promoter, while the open arrow points to that of the P2 promoter.](image)

![Figure 8. Northern RNA analysis of htrM transcripts from plasmids carrying only the P1 promoter or all three putative promoters of the htrM gene. RNA was extracted from cultures of htrM37S::Tn5 mutant bacteria grown at 30°C, carrying either the plasmid pSR45 (containing the htrM coding region and 130 nucleotides upstream which include only the putative P1 promoter) or pSR164 (containing the htrM coding region and all three putative promoters) and analyzed as described in the legend to Figure 5.](image)
sequence database (release 64). Specific comparison of protein sequences was done with the Dayhoff MDM-78 matrix using the program, PCompare (25). A significant homology score (above 3) was observed with abequose synthase, which is an NAD-specific dehydrogenase [the rfbJ gene product of Salmonella (26)], asparaginase, a periplasmic protein of E. coli, the GaIT protein of Saccharomyces carlsbergensis and the NAD-specific glutamate dehydrogenase of Neurospora. The galT gene encodes the uridine diphospho-glucose-4-epimerase enzyme. The identity of HtrM protein with abequose synthase and UDP-glucose-4-epimerase is 20% and 26%, respectively, in a 172-amino acid residue overlap at the amino end of HtrM. This is interesting since the HtrM protein also contains a consensus ADP-binding site at its amino terminus, a feature shared by FAD- and NAD-binding proteins (27). This conserved sequence consists of G-X-G-X-G-(X<sub>10</sub>-G and is found at amino acid residues 6 to 22 from the amino terminus of the HtrM protein. Some homology was also found to E. coli ATPase B (encoded by uncD).)

**DISCUSSION**

We have identified, cloned and mapped the htrM gene of E. coli. It maps at 81.2 minutes, corresponding to the 3916-3918 kb region of the physical map of the E. coli chromosome. The htrM gene lies clockwise to the kbl and idh genes, with the direction of transcription being clockwise relative to the genetic map and in the opposite direction of that of the kbl gene (21).

The htrM gene was sequenced and found to possess an ORF with a predicted molecular weight of 34,978 Da. The predicted isoelectric point of this protein is 4.56. Both the molecular weight observed on SDS polyacrylamide gels and the isoelectric charge in two-dimensional gels match the predicted estimates.

The evidence that this ORF indeed encodes the HtrM protein is as follows: (i) the predicted and observed sizes (~35,000 Da) (Fig. 3) of the protein product agree with each other, (ii) there is no other ORF of a similar size within the sequenced area, (iii) a protein with the predicted molecular weight and isoelectric charge is no longer made when the htrM378::Tn5 or htrM401::Tn5 mutations are recombinated onto the htrM-carrying plasmids. In addition, sequencing of the htrM378::Tn5 and htrM401::Tn5 mutations confirmed that both Tn5 insertions reside in this particular ORF.

Analysis of RNA transcripts show that the transcription of the htrM gene requires at least two promoters, designated here as P1 and P3(hs), and possibly a third, designated P2. The P1 promoter resembles that of a typical E<sub>32</sub>-transcribed housekeeping gene. It is interesting that the P2 promoter has at the -12 region a sequence CTTGCA which perfectly matches the -12 region a sequence CTTGCA which perfectly matches the -12 region a sequence. The -35 region a sequence is 13 nucleotides, similar to other known heat shock promoters (2, 3). Since the htrM transcripts are relatively more abundant in rpoH<sup>+</sup> as compared to isogenic rpoH null mutant bacteria, and the htrM gene is transcribed at 50°C it is concluded that transcription from the P3(hs) E<sub>32</sub>-dependent promoter must be physiologically important, particularly at high temperatures. However, the lower abundance of htrM transcripts in the rpoH null mutants is not entirely due to the lack of E<sub>32</sub>-directed transcription from the P3(hs) promoter, since transcription from the P1 promoter is also reduced, as seen from primer extension analysis (results not shown). Such reduction of P1 transcripts could be either due to an indirect consequence of the pleiotropic nature of the rpoH null mutation, or to a more direct requirement for P3(hs) transcription.

While this manuscript was in preparation, Pegues et al. (23) identified the rfaD gene, whose sequence turns out to be identical to that of the htrM gene. It was shown by these authors that the product of this gene encodes ADP-L-glyceride-D-manno-heptose-6-epimerase, an enzyme required for the biosynthesis of lipopolysaccharide precursor ADP-L-glyceride-D-manno-heptose. In their study (23), the putative transcriptional start site corresponded to our nucleotide position 179, the start site of our putative P1 promoter. These authors did not detect the putative E<sub>32</sub>-regulated heat shock promoter, or the putative P2 promoter, probably because the primer used for the reverse transcriptase reaction is located far (220 nucleotides downstream) from the E<sub>32</sub>-directed start site. In order to clearly identify the E<sub>32</sub>-directed start site, we used primers approximately 100 nucleotides away from the start site. The htrM transcripts show an unusually long leader sequence which may have further complicated the transcriptional analysis. The DNA sequence reported by Pegues et al. (23) is in perfect agreement with our sequence, with the exception of nucleotides 1214 to 1224. At these positions, our sequence reads unambiguously as ATGT-CGAAGAT while theirs reads TCGCAAGATC. We do not know the reason for this discrepancy; it could be due to the use of different strains.

It is interesting that the htrM gene identified by us is the same as rfaD. Obviously, htrM mutants will be defective in producing wild type lipopolysaccharide. Such a defect explains the inability of htrM mutants to grow on bile salt-containing media, e.g., MacConkey agar plates, and their resistance to infection by various E. coli phages. The more extreme phenotypes we observed for our htrM mutants as opposed to the rfaD mutants, such as temperature-sensitivity, overproduction of capsular polysaccharides, and resistance to phage λ, may be due to the fact that our htrM mutants carry null mutations in the htrM gene rather than the putative point mutations used in the Pegues et al. studies (23).

The basis for the minicell-forming phenotype of htrM mutants is not understood. It is known that overproduction of the cell division activator FtsZ leads to a similar phenotype (28). One of the indirect effects of the htrM mutation on cell physiology could result in the overproduction of FtsZ protein.

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REFERENCES


