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

Satish Raina and Costa Georgopoulos

Department of Cellular, Viral and Molecular Biology, University of Utah School of Medicine, Salt Lake City, UT 84132, USA

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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 E σ^{70} -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 E σ^{32} -recognized consensus sequences. The σ^{32} 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 manoheptose 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 σ^{32} 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 σ^{24} (σ^E) (4, 6, 7) and σ^{32} (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 E σ^{32} -transcribed heat shock promoter. Another promoter, designated as P1, is similar to a consensus E σ^{70} -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 manoheptose. 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 μ g/ml), MgSO₄ (1 mM), MgCl₂ (3 mM), CaCl₂ (0.1 mM), and FeCl₃ (0.3 μ M). 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 μ g/ml), tetracycline (15 μ g/ml), spectinomycin (50 μ g/ml), or kanamycin (50 μ g/ml).

Cloning of the *htrM* gene

The previously described *Sau3A* partially-digested *E. coli* chromosomal DNA library (5, 10), prepared in the low copy vector pREG153 (10), was used to isolate cosmids carrying the *htrM*⁺ gene. Since *htrM* mutant bacteria do not adsorb phage λ well and, in addition, transform poorly, they were infected at a very high multiplicity of infection with the λ cosmid phage library (~100 phage per bacterium). The ability to (i) complement the Ts⁻ phenotype, (ii) allow colony formation on MacConkey agar plates, and (iii) recombine with the *htrM* chromosomal insertion mutations was taken as preliminary proof that a particular cosmid clone indeed carried the *htrM* gene. Further subcloning was done using standard DNA manipulation techniques (8).

Mapping of the *htrM* gene

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

Sequencing of *htrM*

To sequence *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 exonuclease III- or DNaseI-generated clones (8) spanning the *htrM* gene and its flanking sequences in pEMBL18⁺ (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 *htrM* clones or subcloned derivatives on the low copy vector, pGB2 (15).

Cloning of *htrM* mutant alleles

To clone the *htrM*::Tn5 mutant alleles, chromosomal DNA was prepared from *htrM*378::Tn5 and *htrM*401::Tn5 mutant bacteria and digested to completion by the restriction enzymes *Eco*RI or *Eco*RV. Neither of these restriction enzymes cuts within Tn5 or the coding sequence of the *htrM* gene. The digestion mixture was ligated to appropriately digested pEMBL18⁺ DNA and used for transformation, simultaneously selecting for resistance to ampicillin (Amp^R; 100 μ g/ml) and kanamycin (Kan^R; 50 μ g/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 *htrM* mutations.

The two *htrM* mutant alleles were also cloned by *in vivo* recombination onto the *htrM*⁺-carrying plasmids, pSR31 and pSR164. The DNA restriction digestion pattern of the resulting plasmids, carrying the various *htrM* alleles, was compared to the earlier constructs. The exact site of Tn5 insertions in the *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 μ g) were analyzed by the northern technique as described by Colman (16), using Hybond-N membrane (Amersham Corp.). To probe for *htrM* message, 200

ng of the 1.2 kb *Asu*II-*Mlu*I DNA fragment, carrying the entire *htrM* coding sequence, was isolated from pSR164, and ³²P-labeled with [α -³²P]-dCTP (3000 Ci/mmol) by nick-translation (8).

Primer Extension

To define the 5' terminus of the *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 *htrM* sequence was annealed to 10 μ g of RNA. RNA was extracted either from wild type cells or from the isogenic *rpoH*^Δ (KY1621) and *rpoH*⁺ (SR407) strains grown at 30°C, or following a 10 min shift from 30° 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 dideoxy 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 5' end *htrM*-specific sequences. To achieve this, a 650-bp *Asu*II-*Stu*I fragment was first cloned into the *Sma*I 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 μ g of M13-SR1 single-stranded DNA was extended from the universal primer position with T7 DNA polymerase in the presence of [α -³²P]-dCTP (20 mCi) containing 5 μ M of each nucleotide in a 10- μ l reaction. The replicated, double-stranded DNA was digested with *Ssr*I, the resulting 650 bp fragment was gel purified and hybridized to 40 μ g 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 *Msp*I-digested DNA on a denaturing gel as well as a dideoxy sequencing reaction using M13 mp19 (*Asu*II-*Stu*I) single-stranded DNA as the template.

Construction of *htrM*:: Ω Tet^R deletion mutations in the *htrM* gene

To confirm the nonessentiality of the *htrM* gene at low temperatures, an additional null allele was constructed by *in vitro* substitution of the *htrM* structural gene by a tetracycline-resistance cassette, the Ω -Tet^R element (17). To achieve this, the Tet^R element from pHP45 Ω -Tet^R was inserted between the *Asu*II and *Bgl*II sites of the *htrM* gene. The resulting plasmid, pSR440, was linearized and used to transform the JC8678 *recBrecCsbca* mutant strain (5). Amp^s Tet^R transformants were screened for and examined further. Phage P1 was grown on such transformants and used to transduce the Tet^R allele back into the B178, MC4100, and CA8000 wild type strains. All such transductants exhibited the same phenotypes as the original *htrM* insertion mutations. The new *htrM* mutant allele was designated as *htrM*:: Ω Tet^R.

RESULTS

Isolation of *htrM* null mutants

From a pool of Tn5 insertions in CA8000 bacteria (5), two mutant bacteria, *htrM*378::Tn5 and *htrM*401::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 P1-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 Amp^R 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*-*SmaI* fragment (Fig. 1a to 1d). The 2.5-kb *EcoRI*-*SmaI* 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 *AccI*-*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 *mtl* markers. This

Figure 1. Restriction map of the *htrM* gene and construction of *htrM* insertion and deletion derivatives. (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 ($\rightarrow$) orientation of the two *Tn5* insertions; (g) map of pSR440 which carries an Ω Tet^R deletion-substitution in the *htrM* gene between the *AsuII* and *BglII* sites. Abbreviations: RI, *EcoRI*; RV, *EcoRV*; BII, *BglII*; Ac, *AccI*; M, *MluI*; S, *SstI*; Sm, *SmaI*.

Figure 2. Identification of the *htrM* gene product. Cultures of strains carrying plasmid pBluescript SK⁺ alone, pSR45 or pSR164, with the *htrM* gene cloned under the T7 RNA polymerase promoter, were grown at 37°C in M9 minimal medium supplemented with 0.2% glucose and all amino acids except met and cys as previously described (4). Expression of T7 RNA polymerase was induced by addition of IPTG (0.5 mM). Following a 30 min incubation, cells were treated with rifampicin (200 µg/ml) for another 15 min (to block transcription by the *E. coli* RNA polymerase) and then labeled with ³⁵S-methionine (50 µCi/ml) for 10 min. The proteins were resolved by 12.5% SDS-PAGE. An autoradiogram of the dried gel is shown. The arrow indicates the position of the HtrM protein.

placed 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 *mtlA-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

Figure 3. Identification of the HtrM protein by two-dimensional electrophoresis. (A) Cultures of *htrM378::Tn5* mutant bacteria, carrying pSR45 (containing the *htrM* coding region and 130 nucleotides upstream but lacking the σ^{32} heat shock promoter) were grown at 30°C, shifted to 50°C for 10 min, then labeled for 10 min with ³⁵S-methionine (50 µCi/ml), and (B) a sample of extract, prepared as described in (A) above, was mixed with extracts overproducing the HtrM protein under the control of the T7 RNA polymerase system. The proteins were resolved in the first dimension on 1.6% (pH 5.0 to 7.0) and 0.4% (pH 3.5 to 10.0) ampholines (Pharmacia) and by 12.5% SDS-PAGE in the second dimension. An autoradiogram of the dried gel is shown. The shaded arrow points to the position of the 35 kDa HtrM protein and the open arrows indicate the position of the 70 kDa (DnaK) and 63 kDa (GroEL) heat shock proteins.

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^r 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 *Sma*I fragment, carrying the Ω-Tet^r cassette, between the *Asu*II and *Bgl*II sites of plasmid pSR31 (Fig. 1g). The resulting *htrM*::ΩTet^r allele was transferred onto the chromosome as described in Methods. The new *htrM*::ΩTet^r allele removes the entire *htrM* coding region except for that coding for the 32 carboxy terminal amino acids. The *htrM*::ΩTet^r 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 at permissive temperatures. 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 Δ .

(*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 (pI=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.

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*

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*^Δ (KY1621) and *rpoH*⁺ (SR407) strains at 30°C (lanes 4 and 5, respectively) and after a shift from 30° 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 *Asu*II-*Mlu*I DNA fragment that carries the entire *htrM*⁺ gene.

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 *hrrM* 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 Eo⁷⁰ housekeeping RNA polymerase. Two other transcriptional

Figure 6. Mapping of 5' termini of *htrM* transcripts. Primer extension reactions of total cellular RNA hybridized to ^{32}P -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 $E\sigma^{32}$ consensus heat shock promoters (see Discussion). In agreement with the assignment of P3(hs) as a $E\sigma^{32}$ -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 $E\sigma^{32}$ 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 $+1$

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, 530-nucleotide *AsuII*-*StuI* DNA fragment as a probe. The probe (10^6cpm) 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 (*AsuII*-*StuI*), 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.

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 *htrM*378::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 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 *htrM*378::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.

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 GalT 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-X-G-(X₁₀)-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 kbp region of the physical map of the *E. coli* chromosome. The *htrM* gene lies clockwise to the *kbl* and *tdh* 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 coding for a protein 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 *htrM*378::Tn5 or *htrM*401::Tn5 mutations are recombined onto the *htrM*-carrying plasmids. In addition, sequencing of the *htrM*378::Tn5 and *htrM*401::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 $E\sigma^{70}$ -transcribed housekeeping gene. It is interesting that the P2 promoter has at the –12 region a sequence CTTGCA which perfectly matches a sequence found in *rpoN*(σ^{54})-transcribed promoters. All promoters recognized by $E\sigma^{54}$ are characterized by an invariant GC doublet that lies at nucleotides –11 to –14 upstream of the transcriptional start, and a GG doublet that lies exactly 10 nucleotides upstream (24). The resemblance of P2 promoter to the *rpoN*-recognized promoters is limited only to the –12 region (Fig. 4).

Analysis of the *htrM* mRNA start site corresponding to P3(hs) shows that the –10 (CCCAAATA) and –35 (CGCCATGAA) putative promoter sequences bear close resemblance to the consensus sequence of $E\sigma^{32}$ -dependent heat shock promoters (3). In addition, the spacing between the –10 and –35 sequences is 13 nucleotides, similar to other known heat shock promoters

(2, 3). Since the *htrM* transcripts are relatively more abundant in *rpoH*⁺ 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) $E\sigma^{32}$ -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 $E\sigma^{32}$ -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-glycero-D-mannoheptose-6-epimerase, an enzyme required for the biosynthesis of lipopolysaccharide precursor ADP-L-glycero-D-mannoheptose. In their study (23), the putative transcriptional start site reported corresponds to our nucleotide position 179, the start site of our putative P1 promoter. These authors did not detect the putative $E\sigma^{32}$ -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 $E\sigma^{32}$ -directed start site. In order to clearly identify the σ^{32} -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-CGAAAGT while theirs reads TCGCAAGATCG. 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

1. Morimoto, R., Tissieres, A. and Georgopoulos, C. (1990) In Stress Proteins in Biology and Medicine, R. Morimoto, et al., eds., pp. 1–36, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
2. Neidhardt, F.C. and VanBogelen, R.A. (1987) In *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology, Neidhardt, F.C., et al., eds., pp. 1334–1345, American Society for Microbiology, Washington, D.C.
3. Cowing, D.W., Bardwell, J.C.A., Craig, E.A., Woolford, C., Hendrix, R. and Gross, C. (1985) Proc. Natl. Acad. Sci. U.S.A. **80**, 2679–2683.
4. Lipinska, B., Fayet, O., Baird, L. and Georgopoulos, C. (1989) J. Bacteriol. **171**, 1574–1584.
5. Raina, S. and Georgopoulos, C. (1990) J. Bacteriol. **172**, 3417–3426.
6. Lipinska, B., Sharma, S. and Georgopoulos, C. (1988) Nucleic Acids Res. **16**, 10053–10067.
7. Erickson, J.W. and Gross, C.A. (1989) Genes Dev. **3**, 1462–1471.
8. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. Tilly, K., McKittrick, N., Zylicz, M. and Georgopoulos, C. (1983) Cell **34**, 641–646.
10. O'Connell, M., Gesteland, R.F. and Atkins, J.F. (1989) EMBO J. **8**, 4315–4323.
11. Kohara, Y., Akiyama, K. and Isono, K. (1987) Cell **50**, 495–508.
12. Wahl, G.M., Stern, M. and Stark, G. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 3683–3687.
13. Sanger, F., Nicklen, S. and Coulson, A. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467.
14. Dente, L. and Cortese, R. (1987) Method Enzymol. **155**, 111–119.
15. Churchwood, G., Belin, D. and Nagamine, Y. (1984) Gene **31**, 165–171.
16. Colman, A. (1984) In Transcription and Translation: A Practical Approach, B.D. Hames and S.J. Higgins, eds., pp. 49–69, IRL Press, Washington, D.C.
17. Fellay, R., Frey, J. and Krisch, H. (1987) Gene **52**, 147–154.
18. Aiba, H., Adhya, S. and de Crombrughe, B. (1981) J. Biol. Chem. **256**, 11905–11910.
19. Malo, M.S. (1990) Nucleic Acids Res. **18**, 6159.
20. Austin, E.A., Graves, J.F., Hite, L.A., Parker, C.T. and Schnaitman, C.A. (1990) J. Bacteriol. **172**, 5312–5325.
21. Aronson, B.D., Ravnkar, P.D. and Somerville, R.L. (1988) Nucleic Acids Res. **16**, 3586.
22. Studier, F.W. and Moffatt, B.A. (1986) J. Mol. Biol. **189**, 113–130.
23. Pegues, J.C., Chen, L., Gordon, A.W., Ding, L. and Coleman, W.G. Jr. (1990) J. Bacteriol. **172**, 4652–4660.
24. Kustu, S., Santero, E., Keener, J., Popham, D. and Weiss, D. (1989) Microbiol. Rev. **53**, 367–376.
25. Pearson, W.R. and Lipman, D.J. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 3444–3448.
26. Wyk, P. and Reeves, P. (1989) J. Bacteriol. **171**, 5687–5693.
27. Wierenga, R.K., Terpstra, P. and Hoi, W.G.J. (1986) J. Mol. Biol. **187**, 101–107.
28. Lutkenhaus, J. (1990) Trends Genet. **6**, 22–25.