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Identification and Transcriptional Analysis of the *Escherichia coli htrE* Operon Which Is Homologous to *pap* and Related Pilin Operons

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We have characterized a new *Escherichia coli* operon consisting of two genes, *ecpD* and *htrE*. The *ecpD* gene encodes a 27-kDa protein which is 40% identical at the amino acid level to the pilin chaperone PapD family of proteins. Immediately downstream of the *ecpD* gene is the *htrE* gene. The *htrE* gene encodes a polypeptide of 95 kDa which is processed to a 92-kDa mature species. The HtrE protein is 38% identical to the type II pilin porin protein PapC. The *ecpD htrE* operon is located at 3.3 min on the genetic map, corresponding to the region from kbp 153 to 157 of the *E. coli* physical map. The *htrE* gene was identified on the basis of a Tn*5* insertion mutation which resulted in a temperature-sensitive growth phenotype above 43.5°C. The transcription of this operon is induced with a temperature shift from 22 to 37 or 42°C but not to higher temperatures, e.g., 50°C. Consistent with this result, the temperature-induced transcription was shown to be independent of the *rpoH* gene product (σ^{32}). The transcription of this operon was further shown to require functional integration host factor protein, since *himA* or *himD* mutant bacteria possessed lower levels of *ecpD htrE* transcripts. Among the three transcriptional start sites discovered, one, defined by the P2 promoter, was found to be under the positive regulation of the *katF* (*rpoS*) gene, which encodes a putative sigma factor required for the transcription of many growth phase-regulated genes.

Escherichia coli is a normal inhabitant of the intestinal tract of humans and has been recognized as an important pathogen involved in a variety of intestinal and extraintestinal diseases. These include urinary tract infections, bacteremia, bacterium-related traveler's diarrhea, and newborn meningitis (reviewed in references 4 and 15).

The initiation of many of these infections is thought to be mediated by molecular recognition between adhesins on bacterial cell surfaces and specific receptor molecules found on mucosal cells of the host (4). The majority of adhesins produced by extraintestinal *E. coli* are associated with structures known as fimbria or pili, whose genes are usually found on plasmids (15). The fimbrial adhesins are distinguished by their receptor specificity. Most uropathogenic *E. coli* strains express P pili that mediate Gal (α 1-4) Gal-sensitive hemagglutination (26). Type I pili are expressed by many pathogenic as well as nonpathogenic *E. coli* strains and are characterized by their D-mannose-sensitive agglutination (10, 13).

The genetic organization of both type I and P pilin loci is similar. Many genes necessary for the biosynthesis and expression of P and type I pili have been cloned. Of these, the *papC* and *papD* genes of the P pilin locus and their analogs, *fimD* and *fimC*, encode proteins which are involved in translocation and assembly of pili (21, 27, 35). PapC is an 88-kDa outer membrane protein thought to form the assembly center for pilus polymerization and act like a molecular usher in differentially recognizing various chaperone-subunit complexes (8). The 28.5-kDa PapD protein has been localized in the periplasmic space and is known to function as a chaperone mediating the assembly of pilin subunits (19, 24).

Another interesting feature of pilin operons is their intricate regulation. For example, it has been shown that the type I pili are subject to phase variation and that mutations in *himA* or *himD* reduce the frequency of this switch (1). Phase variation of the P pili locus is controlled at the transcriptional level by a methylation inhibition mechanism exerted near the Pap pilin transcriptional start site (46). The gene *lrp*, encoding the leucine-responsive regulatory protein, has been shown to be required for inhibition of this methylation, thereby positively regulating the *pap* P pilin operon (5).

Recently another class of pili, called curlins on the basis of their morphology as seen by electron microscopy, has been identified in *E. coli* (38). The structural genes encoding curlin adhesin pili have been shown to be expressed in both pathogenic and nonpathogenic *E. coli*. However, the genes involved in the assembly and transport of these pili have not been identified so far.

Over the last few years, we have been characterizing a group of transposon insertional mutations in *E. coli*, isolated following saturation mutagenesis with various transposons, such as Tn*5* and Tn*10*. These presumably null mutations were isolated on the basis of either inability to form colonies at 43°C or lethal hypersensitivity to a normally sublethal heat shock at 50°C (28, 39, 40). Here we report on one such Tn*5* insertion mutation which defines a new gene, *htrE*. The *htrE* gene maps at 3.3 min on the *E. coli* genetic map. It was sequenced and shown to encode an approximately 95-kDa protein which is highly homologous to the PapC protein involved in the assembly and transport of P type pili. Like the *pap* gene cluster, *htrE* was found to be part of an operon

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
CA8000	*Hfr*H *thi*	Laboratory collection
W3110	Wild type	Laboratory collection
MC4100	Δ(*argF-lac*)*U169 araD139 rpsL150 deoC1 relA1 ptsF25 flbB5501 rbsR*	Laboratory collection
JC8679	*recB21 recC22 sbcA23* λ^- F^- *supE*	A. J. Clark
SR10	CA8000 *htrE1394*::Tn*5*	This work
SR126	CA8000 *ponB*::Tn*10*	This work
SR532	CA8000 *htrE1353*::Ω-Tet^r	This work
SR335	CA8000 *htrE1467*::Ω-Kan^r	This work
SR381	CA8000 *htrE*Δ(*553-2224*)::Ω-Tet^r	This work
AMS150	*katF*::Tn*10*	32
DM555	MC4100 *katF*::Tn*10* constructed by P1 transduction	This work
K1299	Δ*himA82*	12
K1926	*himD7*	12
CV975	*lrp-35*::Tn*10*	5
SR1021	MC4100 Δ*himA82*	This work
SR1022	MC4100 *himD7*	This work
Plasmids		
pREG	Cosmid cloning vector	24
pSK/KS	Bluescript plasmid	Stratagene
pSR7	pSK carrying a 7.0-kb *Kpn*I fragment; *htrE*$^+$ *ecpD*$^+$	This work
pSR10	pSK carrying a 3.9-kb *Dra*I-*Hin*dIII fragment; *htrE*$^+$ *ecpD*$^+$	This work
pSR174	pSK carrying a 3.0-kb *Eco*RI-*Hin*dIII fragment; *htrE*$^+$	This work
pSR176	pSK carrying a 1.5-kb *Dra*I-*Ecor*V fragment; *ecpD*$^+$	This work
pDM655	pKS carrying 750-bp *Hinc*II-*Hin*dIII promoter region	This work

and cotranscribed with another gene, highly homologous to the *papD* gene, encoding a pilin chaperone protein. We designated this gene *ecpD* (*E. coli papD*) on the basis of its homology to the uropathogenic *E. coli papD* gene. The transcription of the *htrE* operon was found to be induced following a temperature shift from 22 to 37 or 42°C, but in an $E\sigma^{32}$-independent manner. We further show that the transcription of this operon requires the DNA-bending protein integration host factor (IHF) along with KatF, encoded by the *rpoS* gene and thought to be a sigma factor for growth phase-regulated genes.

MATERIALS AND METHODS

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

Media. M9 minimal medium was prepared as described by Miller (32) and was supplemented with glucose (0.2%), thiamine (2 μg/ml), $MgSO_4$ (1 mM), $MgCl_2$ (3 mM), $CaCl_2$ (0.1 mM), and $FeCl_3$ (0.3 μM). For [^{35}S]methionine labeling experiments, the M9 medium, supplemented with a mixture of the 20 amino acids (each at 20 μg/ml, except methionine and cysteine), was used. 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 *htrE* gene. A *Sau*3A *E. coli* chromosomal DNA library, prepared in cosmid vector pREG153 (23), was used to isolate cosmids carrying the *htrE*$^+$ gene. The ability to complement the TS phenotype and recombine *htrE* mutations onto the plasmid was taken as preliminary proof that the cosmid clone carried the *htrE*$^+$ gene. The *htrE*$^+$ gene was also subcloned from the transducing bacteriophage λ115 of Kohara's collection (22). The gene was further subcloned by standard DNA manipulation techniques (43).

Mapping of the *htrE* gene. Nick-translated cosmid DNA or subclones carrying the *htrE* gene were used to probe the λ *E. coli* genomic library as previously described (28).

Mapping of Tn*5* insertions in the *htrE* gene. The exact position of the Tn*5* insertions in *htrE1394*::Tn*5* was determined by first transferring these Tn*5* insertion mutations by homologous recombination onto plasmid pSR174 containing the *htrE*$^+$ gene. The Kan^r-containing sequences and the IS50L portion of Tn*5*, along with the adjacent *htrE* DNA, were recloned and sequenced as previously described (39).

Construction of *htrE*::Ω-Tet^r insertion or substitution mutations in the *htrE* gene. Additional null alleles were constructed as shown in Fig. 1, by in vitro DNA manipulations of pSR7 or pSR10. The plasmids were then linearized by appropriate enzyme digestions and used to transform strain JC8679 *recB recC sbcA*. The transformants were tested for ampicillin sensitivity to eliminate bacteria carrying undigested, intact plasmid. The *htrE1353*::Ω-Tet^r allele carries the Ω-Tet^r cassette (11) inserted at the unique *Asu*II restriction site within the *htrE* gene. The *htrE*::Ω-Kan^r allele was constructed by inserting the Ω-Kan^r cassette at the *Bgl*II site. A substitution derivative of the *htrE* gene was achieved by first constructing plasmid pSR535 *htrE*Δ(*553-2224*):: Ω-Tet^r. In this construct, the Ω-Tet^r cassette replaces most of the *htrE* gene sequence located between the *Eco*RV restriction sites.

Primer extension and mung bean nuclease protection assay of RNA transcripts. RNA was isolated by the hot-phenol extraction procedure (43). Approximately 5 ng of ^{32}P-end-labeled primer (5′TATATTACGCGAGTACCCGAAATG3′) complementary to nucleotide positions 83 to 106 of the *ecpD* coding region and 10 μg of RNA isolated from different isogenic bacteria were coprecipitated and resuspended in 10 μl of 50 mM Tris-HCl (pH 8.3)–100 mM KCl. The cDNA synthesis and resolution of primer extension products were carried out as previously described (39). The transcriptional start site(s) was also determined by hybridization of total

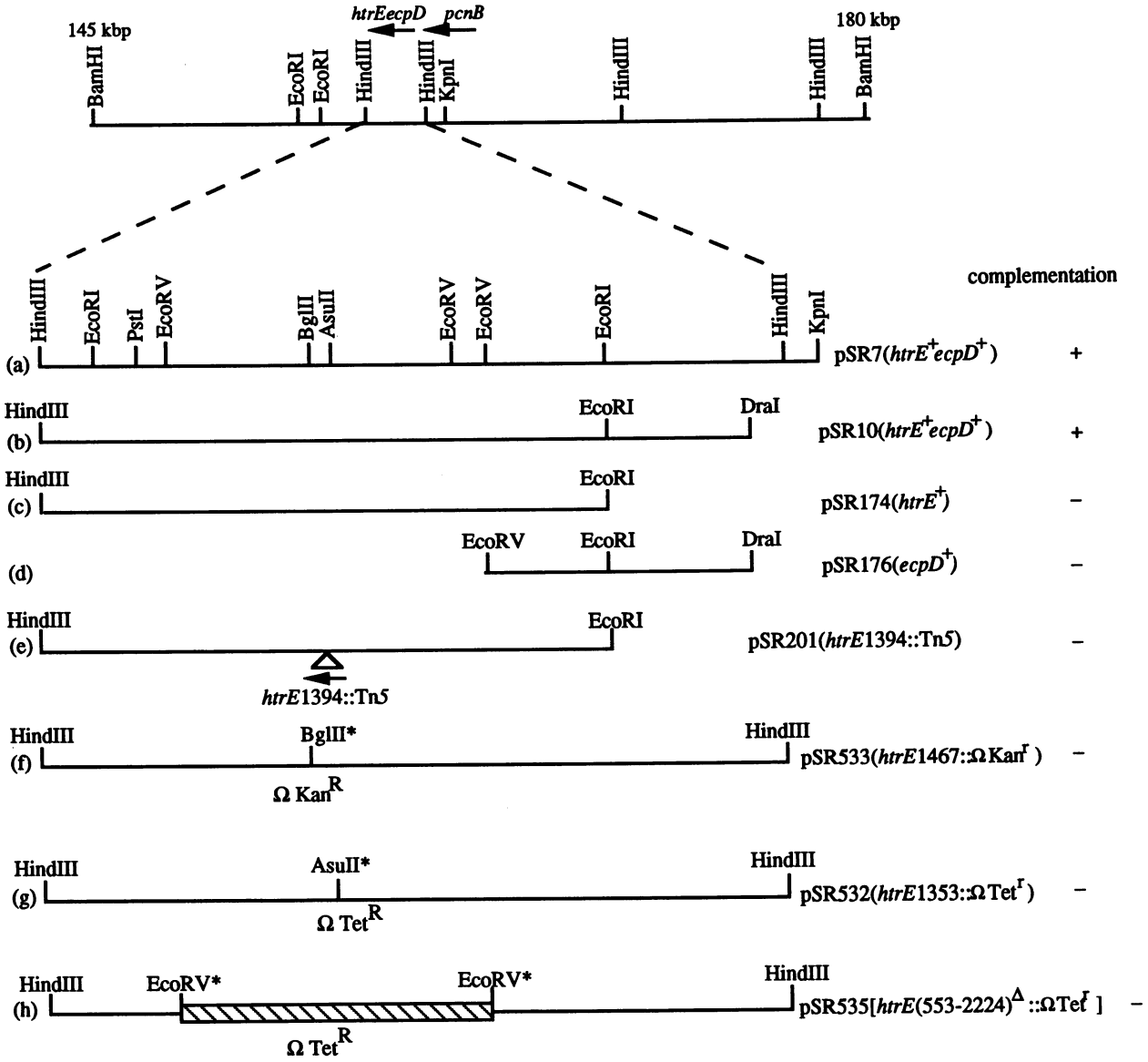

FIG. 1. Restriction map of the *htrE* operon and construction of *htrE* insertion and deletion derivatives. The right column indicates the complementation of the *htrE1394*::Tn*5* mutant bacteria. (a and b) Restriction maps of pSR7 and pSR10, both carrying the *htrE* and *ecpD* genes; (c) pSR174 carrying only the *htrE* gene; (d) pSR176 carrying the 1.5-kbp *Dra*I-*Eco*RV fragment which contains the *ecpD* gene along with its promoters and 500 bp of the coding region of *htrE* gene; (e) the location and orientation of the Tn*5* insertion in the *htrE* gene; (f, g, and h) in vitro insertions of Ω-Kan^r at the *Bgl*II site and Ω-Tet^r at the *Asu*II site and substitution of a 1.7-kb *Eco*RV fragment of the *htrE* gene by the Ω-Tet^r cassette.

cellular RNA to a uniformly radiolabeled probe containing the 5′ end *ecpD*-specific sequences followed by digestion of RNA-DNA hybrids with mung bean nuclease. First the promoter-containing region along with the flanking sequences was subcloned from pSR176(*ecpD*$^+$) as a 750-bp *Hin*cII-*Hin*dIII insert into the *Sma*I-*Hin*dIII sites of phagemid vector pBluescript KS, resulting into pDM655. To label the antisense strand, 1 μg of single-stranded DNA prepared from pDM655 with the help of helper phage M13KO7 was extended from the SK primer in the presence of [α-^{32}P] dCTP. From the replicated, double-stranded DNA a 315-bp *Hin*cII-*Dra*I promoter-containing probe was isolated by digestion with *Bam*HI and *Hin*dIII and then hybridized to approximately 20 μg of RNA under the conditions described previously (30). The RNA-DNA hybrids were digested with 300 U of mung bean nuclease. The protected fragment was sized and compared with pBR322 *Msp*I-digested DNA as well as results of a dideoxy sequencing reaction carried out with SK primer by using pDM655 as a template.

Genetic manipulations. Isogenic sets of various *E. coli* mutant derivatives were constructed by standard P1 transduction experiments (32). For most of the comparative studies dealing with the regulation of the *htrE* operon, MC4100 and its isogenic derivatives were used, except in certain cases as outlined in Table 1. The Δ*himA82* mutation was transduced from K1299 (12) into MC4100 by using the

Tet^r insertion in the *himA* gene. The *himD7* (*hip*) mutation was transduced from K1926 (12) by using a nearby Tn*10* Kan^r marker. This was achieved by using the Tn*10* Kan^r insertion from CAG18528, with Tn*10* Kan^r *zbj-3110* located at 20 min (45). Similarly, the *katF*::Tn*10* insertion mutation from AMS150 (31) was transduced into MC4100 by standard genetic techniques. The isogenic *rpoH*$^+$ and *rpoH* strains have previously been described (39).

Sequencing. Nucleotide sequence analysis of the 3.9-kb *Dra*I-*Hin*dIII chromosomal insert in pSR10 was performed by either subcloning various restriction fragments or using nested sets of exonuclease III-generated subclones. Both of the DNA strands were sequenced by the dideoxyribonucleotide chain termination reaction technique. The sequencing reactions were carried out with Sequenase kit (U.S. Biochemicals, Cleveland, Ohio).

Nucleotide sequence accession number. The GenBank accession number for the sequences described here is L00680. This includes both the *ecpD* and the *htrE* genes along with the DNA sequences from the flanking regions.

RESULTS

A pool of approximately 5×10^4 Tn*5* insertion events was obtained in CA8000 bacteria (39) and screened for isolates with a TS phenotype, i.e., inability to form colonies at 43.5°C. One such TS isolate, subsequently designated *htrE1394*::Tn*5*, was chosen for the present study. The *htrE* mutation was first shown by P1 transduction to confer a TS phenotype in various genetic backgrounds. In all such genetic backgrounds tested, the *htrE* mutation conferred the following phenotypes: (i) inability to form colonies above 43.5°C in either rich (Luria-Bertani) or minimal (M9) medium, (ii) reduced colony-forming ability on Luria-Bertani medium in the presence of a high salt concentration, e.g., 0.7 M NaCl, and (iii) lethal hypersensitivity to a normally sublethal heat shock at 50°C. All three phenotypes cotransduced 100% with the Tn*5*-encoded kanamycin resistance marker, demonstrating that all three phenotypes are the consequence of a single mutational event.

Cloning of the *htrE* gene. To isolate the wild-type *htrE* gene, an *E. coli* genomic library cloned in the low-copy-number cosmid vector pREG153 (36) was used to select for those clones which allow *htrE1394*::Tn*5* bacteria to form wild-type-like colonies at 43.5°C. In subsequent experiments, we also used the λ115 transducing bacteriophage, from Kohara's *E. coli* library (22), to clone the wild-type *htrE* gene (see below). Further subclones were constructed in either the low-copy-number vector pGB2 (6) or the pUC-derived Bluescript vector, pSK$^+$ (Stratagene). A common 7.0-kbp *Kpn*I fragment, obtained from either the *htrE*$^+$ cosmids or λ115, was shown to rescue all the mutant phenotypes exhibited by the *htrE1394*::Tn*5* mutation. Further subcloning experiments resulted in the identification of a 3.9-kbp *Dra*I-*Hin*dIII fragment (pSR10) which complemented the TS phenotype of *htrE* bacteria (Fig. 1).

Mapping of the *htrE* gene. To map the *htrE* gene, we used the ^{32}P-labeled 7.0-kbp *Kpn*I fragment derived from plasmid pSR7 (*htrE*$^+$) or the larger *htrE*$^+$ cosmid clones to hybridize to the λ *E. coli* DNA library (22). Hybridization with the *htrE*$^+$ cosmid clones was observed with λ clones 113 to 116. The smaller 7.0-kbp *Kpn*I probe hybridized only to bacteriophage λ115, which corresponds to kbp 150 to 160 of the *E. coli* physical map (22). To further confirm the map position assignment, the *htrE* mutants were lysogenized with different λ transducing bacteriophages spanning the region from kbp 140 to 170. Only recombinant bacteriophage λ115 was found capable of (i) complementing the TS phenotype and (ii) recombining with the kanamycin resistance marker of the *htrE1394*::Tn*5* mutation. By comparing the restriction pattern of pSR7(*htrE*$^+$) or a smaller clone, pSR10(*htrE*$^+$), with the *E. coli* restriction map, the *htrE* gene was localized more precisely within the region from kbp 153 to 157. These results were further verified by carrying out P1 transductions with drug resistance markers known to be located in this area. Results of such transduction experiments placed the *htrE* gene counterclockwise to the *pcnB* gene, the two genes being approximately 90% linked by P1 transduction. The *pcnB* gene product has been previously shown to be involved in copy number control of various plasmids (29). By using another nearby gene, *ponB*, which encodes penicillin-binding protein 1B and is located clockwise to *pcnB*, a cotransduction frequency of 60% was observed between a Tn*10* insertion in *ponB* from SR126 and the Kan^r marker from *htrE*. All of the data obtained are consistent with the mapping results presented above.

Construction of Ω insertion or substitution mutations in the *htrE* gene. Since in the original screen we isolated only a single *htrE* allele, *htrE1394*::Tn*5*, we also constructed three additional null alleles. All three alleles were constructed on plasmid pSR7 or pSR10 and transferred onto the chromosome as described in Materials and Methods. The three new null alleles were then transduced by P1 into various wild-type *E. coli* strains. In all genetic backgrounds tested, similar phenotypes were observed, including TS bacterial growth and hypersensitivity to a 50°C incubation. Thus, it is concluded that the *htrE* gene is dispensable for *E. coli* bacterial growth at temperatures up to 43°C but is required for *E. coli* bacterial viability at temperatures above 43.5°C (Fig. 2). In all cases tested, it was observed that bacterial cell growth stopped at the nonpermissive temperature, but neither a loss in colony-forming ability nor cell lysis occurred when a culture of *htrE* mutant bacteria was shifted to a high temperature (43.5°C). However, incubation at 50°C resulted in rapid bacterial cell lysis (Fig. 2), as compared with isogenic wild-type bacteria.

Sequencing of the *htrE* operon. The *htrE* gene, along with flanking sequences, was sequenced from the minimal subclone pSR10. The sequence analysis of a 3.8-kbp fragment of DNA revealed the existence of two open reading frames (ORFs). The first ORF, designated *ecpD* (*E. coli* PapD), starts with an ATG as the initiator codon at position 186 and terminates with a TAA codon at position 924 (Fig. 3). The second ORF defines the *htrE* gene. The ORF starts with a GTG as the initiator codon at position 961 and terminates with a TAG at position 3550. The sequence upstream of the *ecpD* gene shows certain interesting features, such as the presence of a 13-bp inverted repeat located between nucleotide positions 120 and 158 as presented in Fig. 3. Also observed was a potential secondary structure which could form a stem-loop-like structure, between nucleotide positions 179 and 212.

The exact position of the Tn*5* insertion event was determined by the cloning and sequencing strategy described earlier (39). Tn*5* was shown to be inserted between nucleotides 2354 and 2355, which should disrupt the *htrE* ORF at amino acid position 465 (Fig. 3).

The predicted molecular masses of the proteins encoded by the two ORFs are approximately 27 and 95 kDa, respectively. The 27-kDa protein is expected to be highly hydrophobic, with a predicted pI of 10.2. However, the 95-kDa protein is predicted to be mostly hydrophilic, with a pI of

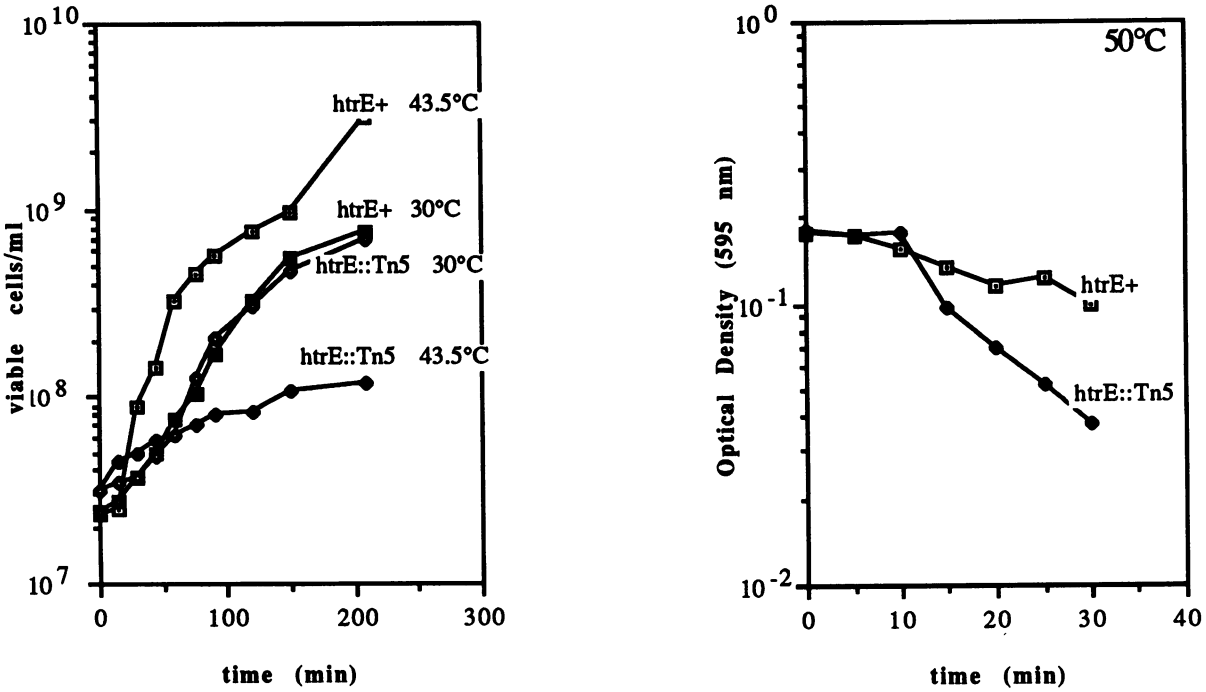

FIG. 2. Growth and viability curves of *htrE*$^+$ and *htrE1394*::Tn*5* isogenic bacteria. Exponentially growing cultures at 30°C were diluted 1:10 into prewarmed Luria-Bertani medium and incubated at 30, 43.5, or 50°C. Bacterial growth was monitored by measuring CFU per milliliter at various times following the temperature shift by plating serial dilutions and by measuring the optical density at 595 nm for experiments with incubation at 50°C.

4.6. These predictions were verified by labeling the proteins encoded by plasmids carrying *ecpD* and *htrE* under the T7 promoter expression system and by then performing two-dimensional gel electrophoresis (see below).

Identification of the *htrE* and *ecpD* gene products. Bacterial cultures of BL21(DE3) carrying pSR10, pSR174, or pSR176 were labeled with [^{35}S]methionine as described in the legend to Fig. 4. The presence of plasmid pSR10 resulted in the large overproduction of 95- and 27-kDa proteins (Fig. 4). In the presence of pSR174 (Fig. 5A), the synthesis of the 95-kDa species corresponding to the HtrE protein was induced. Plasmid pSR176 led only to overproduction of the 27-kDa polypeptide corresponding to the EcpD protein. Both of these polypeptides appeared to be processed. To verify that the 95-kDa protein was indeed processed, the bacterial cultures carrying pSR176 were pulse-labeled with [^{35}S]methionine for 1 or 3 min and then chased with a 1,000-fold excess mixture of cold methionine and cysteine for 5. The resolution of such labeled extracts following separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% acrylamide) showed that the 95-kDa protein is processed to a 92-kDa form. Both of these protein species were separated on isoelectric focusing gels and shown to migrate at the expected isoelectric points. The processing of the putative signal sequence of the 95-kDa protein resulted in a protein of a more acidic nature (Fig. 5B). These data correspond well with those expected from the DNA sequence analysis, although more-direct evidence would be from N-terminal sequencing of these products. Similarly, we showed by pulse-chase experiments that the 27-kDa polypeptide corresponding to the EcpD protein was also processed to an approximately 26-kDa mature product (Fig. 6).

Homology to other known proteins. Sequence similarity searches against the Swiss-Prot and GenPept data bases revealed strong homology of the HtrE and EcpD proteins to a family of prokaryotic proteins involved in pilin transport and assembly. This family includes proteins from at least five distinct *E. coli* serotypes and homologous proteins from *Haemophilus influenzae* and *Klebsiella pneumoniae* (18, 19). Of these, the HtrE protein is homologous to the PapC-like family of proteins, whereas the EcpD protein resembles the PapD-like family of proteins. In all cases analyzed so far, the PapD protein has been localized to the periplasm, while PapC is anchored onto the membrane and has been recently shown to act like a molecular usher (8). The 27-kDa protein encoded by the *ecpD* ORF shows an overall identity of 34 to 40% to PapD-like proteins. The overall similarity, taking into account conservative substitutions, rises to about 60% (Fig. 7). Similar scores were observed for homology of the HtrE protein at the amino acid level to the PapC-like family of proteins. PapD is the specific periplasmic chaperone required for the assembly of P pili (24, 27). The crystal structure of PapD has revealed that it has the overall topology of an immunoglobulin-like fold (17). Alignment of the EcpD protein sequence to that of the seven previously sequenced pilus chaperone sequences from *E. coli*, *H. influenzae*, and *K. pneumoniae* shows identity in 22 of the 23 conserved amino acid residues (Fig. 7). An additional 58 amino acid residues have been shown to be identical in at least four of the seven proteins. Our EcpD protein sequence shows identity in 53 of these 58 amino acid residues, with the remaining 5 reflecting only conservative substitutions. Most of these conserved amino acid residues found in PapD have been shown to participate in maintaining the overall structure of the various domains.

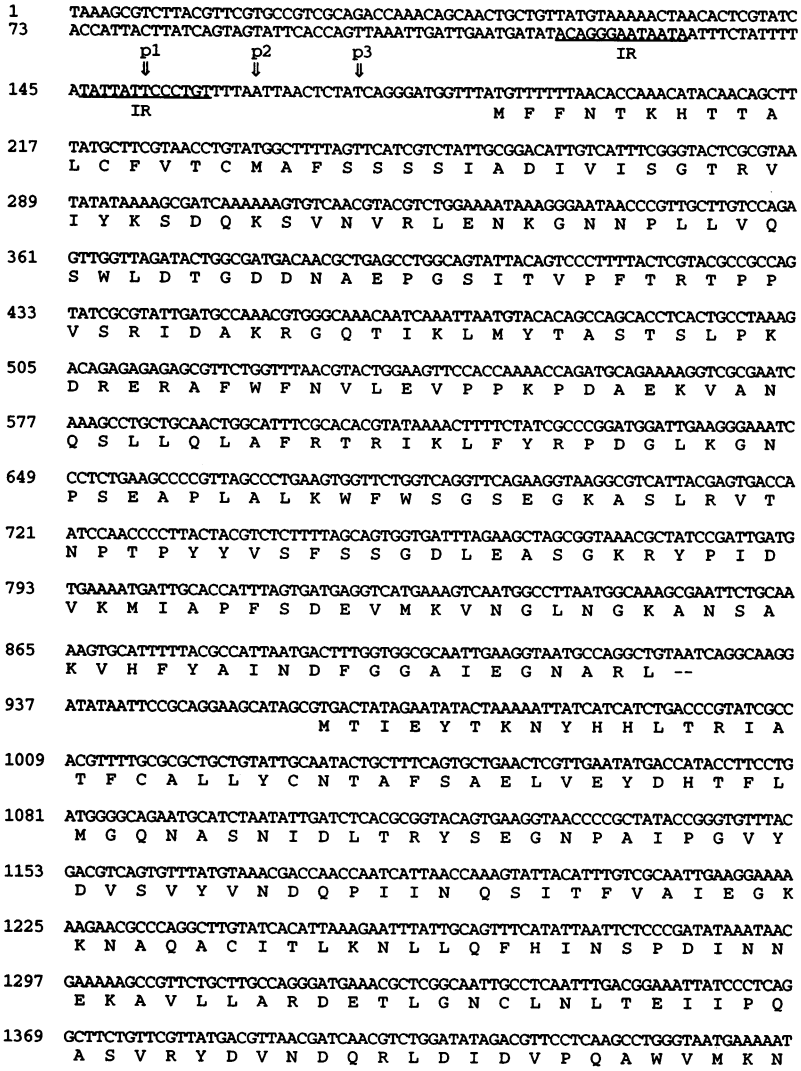

FIG. 3. Nucleotide sequence of the *htrE* and *ecpD* genes and the flanking regions. The three transcriptional start sites P1, P2, and P3 are shown by arrows. The inverted repeats present near the promoter region (IR) are underlined. The insertional site of Tn*5* is indicated by a triangle.

***ecpD* and *htrE* form an operon.** Our in vivo complementation experiments showed that the 3.0-kbp *Eco*RI-*Hin*dIII fragment cloned in either a low- or high-copy-number vector was unable to complement any of the known *htrE1394*::Tn*5* phenotypes (Fig. 1). Although a protein of 95 kDa was expressed in vivo from an inducible promoter (such as the T7 expression system), such a synthesis was not detected in the absence of such a system. These results suggested that a key regulatory element was missing from this clone, which includes 260 bp of sequence upstream of the GTG initiation codon. This idea is further supported by the fact that clones carrying complete copies of both *ecpD* and *htrE* complemented *htrE1394*::Tn*5* mutant bacteria, suggesting that all the necessary regulatory elements required for transcription of *ecpD* and *htrE* are located at the 5′ end of the *ecpD* gene. To identify the regulatory regions and the transcriptional start site(s), we carried out primer extension experiments, using RNA isolated from a variety of genetic backgrounds and under different growth conditions.

Mapping of *htrE* transcripts. The *ecpD* and *htrE* transcriptional start sites determined by the primer extension experiments and mung bean nuclease protection assay were shown to be located at nucleotide positions 152 (P1), 163 (P2), and 174 (P3). The sequences located upstream of the putative P1 and P3 transcriptional start sites have good homology to the −10 and −35 Eσ^{70} consensus sequences (Fig. 8 to 11). In the absence of mRNA capping experiments, we cannot rule out the possibility that one or more of these sites represent either transcriptional termination or mRNA processing sites. Among the three transcriptional start sites, only P2 varied in the relative amounts of transcripts made, depending upon the growth conditions as well as the strain background used.

Since *htrE* was essential for growth at high temperatures, we looked at the relative transcript levels under a wide range of temperatures. Interestingly, the level of transcripts observed at temperatures ranging from 37 to 42°C was significantly higher than that at the lower temperatures (Fig. 8). Judging from the amount of primer extension products made from RNA isolated from wild-type bacterial cultures, it is

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1441 TACCAAAACTATGTTGATCCATCGTTATGGGAAAACGGCATTAATGCGGCCATGTTGTCATACAACCTCAAC
       Y  Q  N  Y  V  D  P  S  L  W  E  N  G  I  N  A  A  M  L  S  Y  N  L  N

1513 GGATATCATAGTGAAACCCCTGGTCGAAAAAATGAAAGCATTTATGCTGCATTTAACGGTGGGATGAATTTA
       G  Y  H  S  E  T  P  G  R  K  N  E  S  I  Y  A  A  F  N  G  G  M  N  L

1585 GGTGCATGGCGACTGCGTGCCTCGGGCAACTACAACTGGATGACCGATTCTGGCAGTAATTATGATTTTAAG
       G  A  W  R  L  R  A  S  G  N  Y  N  W  M  T  D  S  G  S  N  Y  D  F  K

1657 AATCGGTATGTTCAGCGTGATATCGCCTCGCTGCGTTCTCAACTCATTCTTGGTGAGTCTTATACGACGGGC
       N  R  Y  V  Q  R  D  I  A  S  L  R  S  Q  L  I  L  G  E  S  Y  T  T  G

1729 GAAACCTTTGATTCCGTCAGTATCCGAGGCATTCGTTTATACAGTGACAGCCGCATGTTGCCTCCGACTTTA
       E  T  F  D  S  V  S  I  R  G  I  R  L  Y  S  D  S  R  M  L  P  P  T  L

1801 GCCAGCTTTGCGCCTATCATTCATGGCGTTGCCAATACCAACGCCAAAGTCACTATTACGCAAGGTGGCTAT
       A  S  F  A  P  I  I  H  G  V  A  N  T  N  A  K  V  T  I  T  Q  G  G  Y

1873 AAGATTTATGAAACGACGGTGCCGCCAGGCGCTTTCGTCATTGATGATCTGAGTCCGTCAGGGTACGGCAGC
       K  I  Y  E  T  T  V  P  P  G  A  F  V  I  D  D  L  S  P  S  G  Y  G  S

1945 GATCTTATTGTTACCATCGAAGAATCCGATGGCTCAAAGCGGACATTCTCGCAACCTTTCTCATCCGTTGTT
       D  L  I  V  T  I  E  E  S  D  G  S  K  R  T  F  S  Q  P  F  S  S  V  V

2017 CAAATGTTACGCCCTGGCGTTGGACGTTGGGATATTAGCGGCGGTCAGGTCTTAAAAGATGATATTCAGGAT
       Q  M  L  R  P  G  V  G  R  W  D  I  S  G  G  Q  V  L  K  D  D  I  Q  D

2089 GAGCCTAATTTATTTCAAGCAAGCTACTACTACGGCCTGAATAACTATCTGACGGGTTATACCGGTATTCAG
       E  P  N  L  F  Q  A  S  Y  Y  Y  G  L  N  N  Y  L  T  G  Y  T  G  I  Q

2161 ATAACCGATAATAACTATACCGCTGGTTTGTTAGGTCTTGGTCTGAATACTTCAGTTGGTGCATTTTCTTTC
       I  T  D  N  N  Y  T  A  G  L  L  G  L  G  L  N  T  S  V  G  A  F  S  F

2233 GATGTGACTCATTCCAATGTTCGTATCCCGGATGATAAAACATACCAGGGGCAAAGTTATCGTGTTTCCTGG
       D  V  T  H  S  N  V  R  I  P  D  D  K  T  Y  Q  G  Q  S  Y  R  V  S  W
                                                    ▽
2305 AACAAGTTATTCGAAGAAACAAGTACTTCACTGAATATCGCGGCCTATCGCTATTCGACACAGAATTACCTT
       N  K  L  F  E  E  T  S  T  S  L  N  I  A  A  Y  R  Y  S  T  Q  N  Y  L

2377 GGTCTTAATGATGCACTAACTCTAATTGATGAAGTGAAACATCCCGAACAAGATCTTGAACCGAAATCCATG
       G  L  N  D  A  L  T  L  I  D  E  V  K  H  P  E  Q  D  L  E  P  K  S  M

2449 CGTAATTACTCACGCATGAAAAATCAGGTTACGGTCAGTATTAACCAACCGTTGAAATTTGAGAAAAAAGAT
       R  N  Y  S  R  M  K  N  Q  V  T  V  S  I  N  Q  P  L  K  F  E  K  K  D

2521 TACGGTTCATTTTATCTTTCCGGAAGTTGGTCCGATTACTGGGCTTCCGGACAAAATCGTAGCAATTACTCT
       Y  G  S  F  Y  L  S  G  S  W  S  D  Y  W  A  S  G  Q  N  R  S  N  Y  S

2593 ATTGGCTACAGTAACAGTACATCCTGGGGCAGCTACAGTGTCAGTGCCCAGCGTTCATGGAATGAAGACGGC
       I  G  Y  S  N  S  T  S  W  G  S  Y  S  V  S  A  Q  R  S  W  N  E  D  G

2665 GACACTGACGATAGCGTTTATCTTAGTTTCACCATTCCAATTGAAAAATTACTTGGCACTGAACAACGTACT
       D  T  D  D  S  V  Y  L  S  F  T  I  P  I  E  K  L  L  G  T  E  Q  R  T

2737 TCAGGTTTCCAGAGTATTGATACTCAAATAAGCAGTGACTTTAAGGGTAATAACCAACTCAACGTTAGCAGC
       S  G  F  Q  S  I  D  T  Q  I  S  S  D  F  K  G  N  N  Q  L  N  V  S  S

2809 AGTGGCTATAGCGATAACGCTCGCGTCAGTTATAGCGTGAATACTGGCTATACGATGAATAAAGCCAGCAAA
       S  G  Y  S  D  N  A  R  V  S  Y  S  V  N  T  G  Y  T  M  N  K  A  S  K

2881 GATTTGAGTTATGTTGGGGGTTATGCCAGCTATGAGTCACCATGGGGAACGCTGGCAGGTTCAATTTCTGCA
       D  L  S  Y  V  G  G  Y  A  S  Y  E  S  P  W  G  T  L  A  G  S  I  S  A

2953 AATAGCGATAACAGCCGTCAAGTTTCTCTCAGCACCGACGGTGGTTTTGTATTGCATAGCGGTGGACTGACT
       N  S  D  N  S  R  Q  V  S  L  S  T  D  G  G  F  V  L  H  S  G  G  L  T

3025 TTCAGTAATGATAGTTTTAGCGACTCCGATACACTGGCGGTAGTTCAGGCTCCAGGTGCTCAAGGAGCGCGA
       F  S  N  D  S  F  S  D  S  D  T  L  A  V  V  Q  A  P  G  A  Q  G  A  R

3097 ATAAATTATGGCAACAGTACTATCGATCGATGGGGTTATGGTGTCACCAGCGCTCTTTCTCCTTATCATGAA
       I  N  Y  G  N  S  T  I  D  R  W  G  Y  G  V  T  S  A  L  S  P  Y  H  E

3169 AACCGTATCGCGCTGGATATCAACGATCTTGAGAACGATGTTGAATTAAAAAGTACCAGTGCAGTAGCTGTA
       N  R  I  A  L  D  I  N  D  L  E  N  D  V  E  L  K  S  T  S  A  V  A  V

3241 CCGCGTCAGGGTTCAGTCGTCTTTGCTGATTTTGAAACCGTGCAAGGGCAATCAGCCATTATGAACATCACA
       P  R  Q  G  S  V  V  F  A  D  F  E  T  V  Q  G  Q  S  A  I  M  N  I  T

3313 CGAAGTGATGGTAAAAATATTCCATTTGCTGCAGATATTTATGATGAGCAAGGCAATGTCATTGGTAATGTT
       R  S  D  G  K  N  I  P  F  A  A  D  I  Y  D  E  Q  G  N  V  I  G  N  V

3385 GGACACCGTGGACAAGCATTTGTTCGTGGTATTGAGCAGCAGGGAAATATCAGCATTAAATGGCTCGAACAA
       G  H  R  G  Q  A  F  V  R  G  I  E  Q  Q  G  N  I  S  I  K  W  L  E  Q

3457 AGTAAACCCGTAAGTTGTCTTGCGCATTATCAACAAAGCCCAGAAGCACCAAAAATAGCACAATCTATTATT
       S  K  P  V  S  C  L  A  H  Y  Q  Q  S  P  E  A  P  K  I  A  Q  S  I  I

3529 CTGAATGGAATTCTGTTTATATAGATTATGAAACCGAAGATGACACCTCCGATGGGGTTTACCTAATCAAGG
       L  N  G  I  L  F  I ---
3601 TAACGGCACATCACAGCCTCTTCATTTCCAGGCAACATTAAAGCAAGACGGGAATATTGCTATCGAACCCGG
3673 CGAATTTAAAGCCACCAGTACTTTCCAGGTAACCTATCCCTGATAACGTAGCAGCACTGTCACCTTCTGGCA
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FIG. 3—*Continued.*

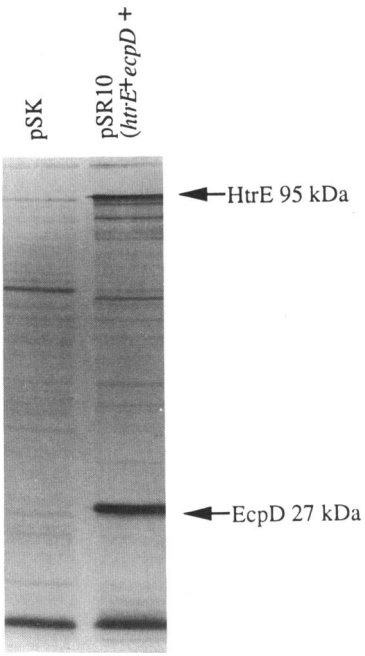

FIG. 4. Identification of the *htrE* and *ecpD* gene products. Cultures of the BL21(DE3) strain transformed with plasmid pBluescript SK$^+$ alone or pSR10 containing both the *htrE* and *ecpD* genes cloned under the T7 RNA polymerase promoter. Expression of T7 RNA polymerase was induced by addition of IPTG (isopropyl-β-D-thiogalactopyranoside) (0.5 mM). Following a 30-min incubation, cells were treated with rifampin (200 μg/ml) for another 15 min and then labeled with [^{35}S]methionine (50 μCi/ml) for 10 min. The proteins were resolved by SDS–12.5% PAGE.

clear that very little *ecpD*-specific mRNA accumulated at low temperatures, e.g., 22°C. The amount of transcript accumulation gradually increased following a temperature upshift. This type of temperature-induced transcriptional regulation is reminiscent of Eσ^{32} regulation of classical heat shock genes (34). However, unlike most of the heat shock genes, the transcript levels were markedly reduced at 50°C (Fig. 8).

Since it is known that σ^{32} (the *rpoH* gene product) is absolutely required for the transcription of the classical heat shock genes, we compared the mRNA levels from isogenic *rpoH*Δ and *rpoH*$^+$ strains. The results of the transcriptional analysis clearly showed that transcription of the *htrE* operon is not dependent on σ^{32}, since the levels of transcripts as well as the usage of the putative transcription initiation sites were identical in all genetic backgrounds studied, irrespective of the presence or absence of *rpoH*. In addition, it is known that σ^{32}-promoted transcription depends on the presence of canonical −10 and −35 consensus sequences unique to this class of genes (34). Such Eσ^{32}-dependent promoter consensus sequences were not found in the *ecpD* promoters, as shown in Fig. 8 and 9.

IHF-mediated positive regulation of the *htrE* operon. It is known that pilin operons are in either an on or off mode. The phase variation of the type I pilin-like *fim* operon of *E. coli* K-12 is known to be mediated by the DNA-binding protein IHF, known to bend DNA (1, 9). In contrast, type II pilins, exemplified by the Pap pilins, are regulated by PapI and PapB. PapI has been shown to be regulated positively by the global regulatory protein Lrp through methylation (5).

Since the *ecpD* and *htrE* gene products show a high level of sequence similarity to the pilin chaperone PapD and the pilin porin protein PapC, respectively, we analyzed the levels of transcripts in *lrp*$^+$ and *lrp*::Tn*10* strains, using the primer extension method (Fig. 9). In both strains and under all conditions studied, both the actual level of transcripts and

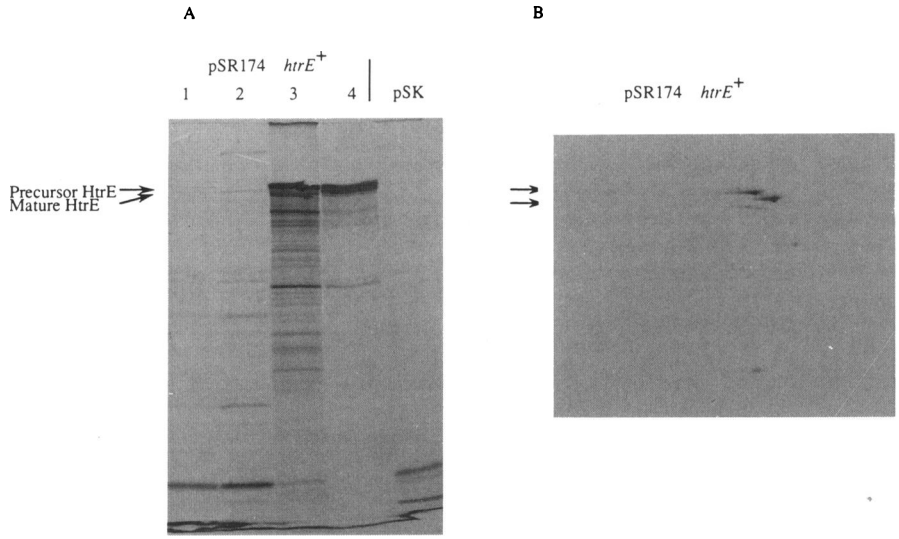

FIG. 5. In vivo processing of the HtrE protein. (A) Cultures of the BL21(DE3) strain transformed with vector pBluescript SK$^+$ alone or pSR174 containing the *htrE* gene under the T7 RNA polymerase promoter. Expression of T7 RNA polymerase was induced as described in the legend to Fig. 4, and cultures were labeled for 1 and 3 min (lanes 1 and 3) with [^{35}S]methionine (50 mCi/ml) and then chased for 5 min with a 1,000-fold excess of cold methionine (lanes 2 and 4). Proteins were precipitated with trichloroacetic acid (10%), washed with cold acetone, and then resolved by SDS–7% PAGE. (B) Two-dimensional PAGE of the same [^{35}S]methionine-labeled cell extracts as used in panel A prepared from bacteria carrying *htrE* on plasmid pSR174. In the first dimension, 2% ampholines (pH 3.5 to 10.5; Pharmacia) were used. The arrows indicate the positions of the precursor and mature HtrE species.

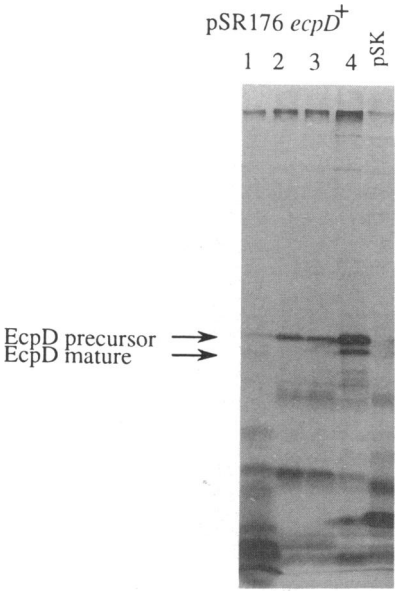

FIG. 6. Processing of the EcpD protein. Pulse-chased samples were prepared from cultures carrying the *ecpD* gene cloned under T7 RNA polymerase promoter (pSR176) or vector pSK alone. Cultures were labeled for 30 s (lane 1) or 1 min (lane 2) and then chased with cold methionine for either 1 min (lane 3) or 5 min (lane 4). The proteins were resolved by SDS–15% PAGE.

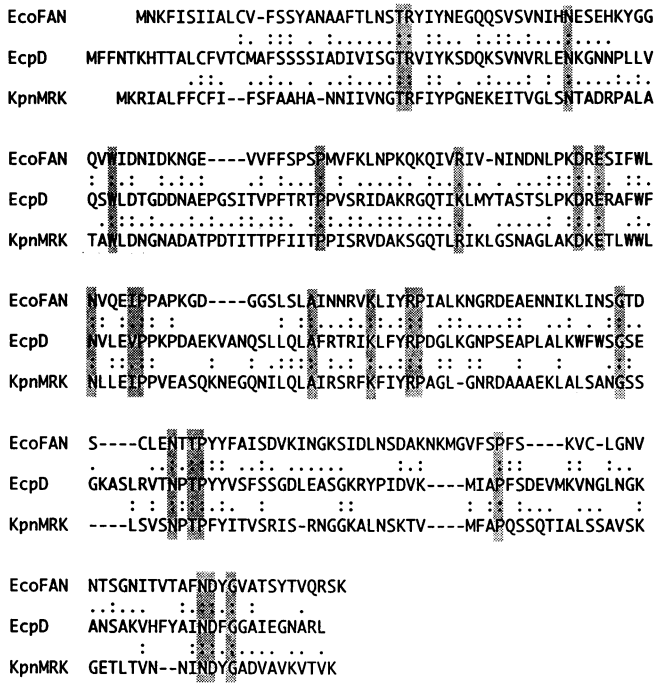

FIG. 7. Comparison of amino acid sequences of *E. coli* EcpD and two of the representative PapD-like proteins, MrkB and FanE. The identity retained for the consensus sequence for PapD family consisting of 23 invariant residues is shown by shaded bars. Identical residues are indicated by double dots; conserved residues are indicated by single dots.

the position of transcription initiation remained identical, demonstrating no direct participation of the *lrp* gene product in the regulation of the *ecpD* operon. This is consistent with the lack of Dam methylase recognition sites upstream of the *ecpD* promoter or near its leader sequence. This result is quite surprising since both *ecpD* and *htrE* structural genes are highly conserved with their corresponding *papD*-like and *papC*-like gene families.

Next, we isolated and studied mRNA from isogenic *himA*$^+$ and *himA*::Tn*10* (SR69 and SR1021) strains or from *himD* mutant bacteria (SR1022). The primer extension results showed comparatively low levels of mRNA transcripts initiating from all three putative promoters. Thus, it is clear that functional IHF is needed for efficient transcription of the *ecpD-htrE* operon. However, since some *ecpD-htrE* operon-specific transcripts were seen in strains lacking IHF, the requirement for the IHF protein is not absolute. The densitometry scanning of the autoradiograms showed a five- to sixfold decline in the levels of transcripts initiated from either of the three transcriptional start sites in strains carrying mutations in either *himA* or *himD*, which encode the two different subunits of the IHF protein.

***katF*-mediated positive regulation of the *htrE* operon.** Since the *htrE* gene is essential for *E. coli* growth at high temperatures, particularly under more severe stress conditions such as growth at 50°C, we used the Northern (RNA) technique to see whether transcriptional regulation is mediated by the alternative sigma factor σ^s (coded by the *katF* [=*rpoS*] gene product). Interestingly, the level of *ecpD-htrE* operon transcripts was substantially reduced in the *katF*::Tn*10* mutant bacteria. To determine which of the three transcriptional start sites in particular was used by Eσ^s, a primer extension analysis was done. Transcriptional initiation was reduced primarily from the P2 transcriptional start site (Fig. 10). The −10 region of the putative P2 promoter does in fact have similarity to −10 regions found in *katF*-dependent promoters. However, it may be noted that P2 promoter is still used in *katF*::Tn*10* mutant bacteria, although at significantly reduced rates. A similar dependence on the *rpoS* gene product has also been observed in case of curli, encoded by the curlin subunit gene, *csgA* (37).

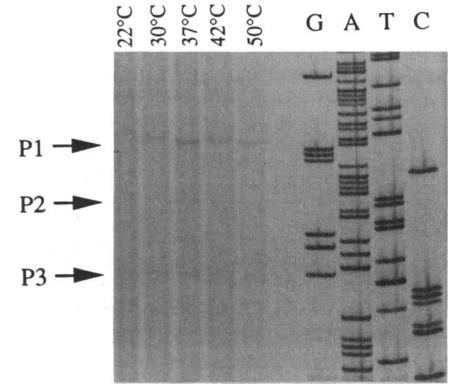

FIG. 8. Mapping of 5′ termini of *htrE ecpD* transcripts. Primer extension reactions of total cellular RNA hybridized to ^{32}P-end-labeled DNA oligonucleotide probe, complementary to nucleotides 83 to 106 in the *ecpD* sense strand. RNA was extracted from wild-type bacteria grown at 22°C or after a 10-min shift to 30, 37, 42, or 50°C. Lanes G, A, T, and C correspond to the dideoxy sequencing reactions carried out with the same oligonucleotide as the primer.

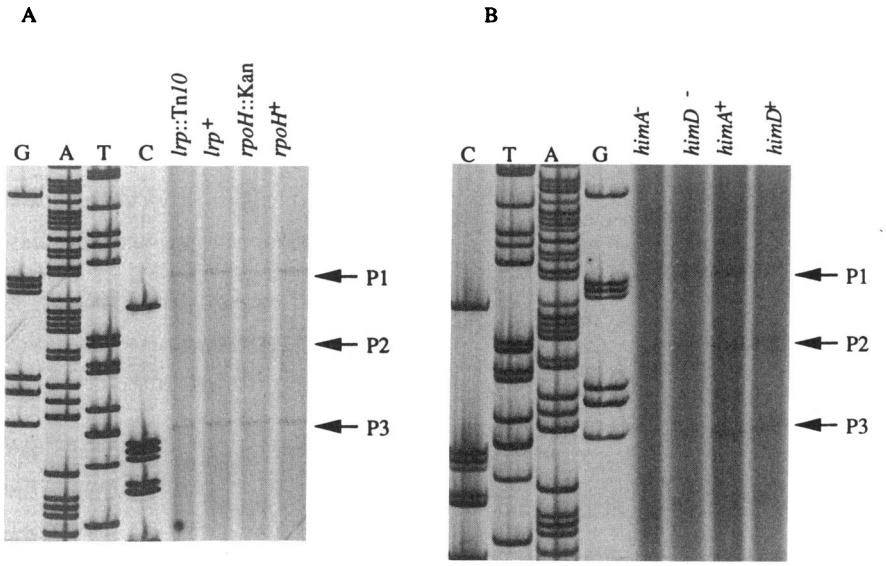

FIG. 9. IHF-mediated transcriptional regulation of the *htrE ecpD* operon. (A) RNA was extracted from isogenic rpoHΔ (KY1621) and *rpoH*$^+$ (SR407) strains at 30°C or from isogenic *lrp*::Tn*10* and *lrp*$^+$ strains. (B) Primer extension reactions were carried out with RNA extracted from isogenic *himA*::Tn*10* and *himD* mutant bacteria. The positions of the three transcriptional start sites are indicated by arrows.

DISCUSSION

From a library of random Tn*5* insertions in the *E. coli* chromosome, we characterized an insertion that results in a TS phenotype for bacterial growth at temperatures above 43.5°C. This Tn*5* insertion defined a locus, *htrE*, constituting an operon of at least two new genes, designated *ecpD* and *htrE*. The *htrE* locus was mapped to 3.3 min, corresponding to the region from kbp 153 to 157 of the physical map of the *E. coli* chromosome. The *ecpD* gene is the first gene of the operon and is located approximately 2.5 kbp counterclockwise of the *pcnB* gene, involved in copy number control of various plasmids (29). The direction of transcription of the *ecpD-htrE* operon was shown to be counterclockwise relative to the *E. coli* genetic map.

Sequence analysis of a 3.9-kbp minimal clone which complemented all phenotypes of *htrE*::Tn*5* insertion mutant bacteria revealed the existence of two ORFs. The first ORF identified encodes a protein with a predicted molecular mass of 27-kDa and a predicted pI of 10.2. The second ORF identified was found to encode a protein with a predicted molecular mass of 95 kDa and a predicted pI of 4.58. The predicted isoelectric point of the HtrE and EcpD proteins is in close agreement with their observed migration on two-dimensional SDS-polyacrylamide gels. In addition, both HtrE and EcpD proteins are synthesized as precursors and are processed to 92- and 26-kDa species, respectively. These results are also in agreement with a predicted transport signal sequence for both proteins. By sequencing from the Tn*5* insertion site in the *htrE* gene, we demonstrated that the *htrE* gene is encoded by the second ORF.

The EcpD and HtrE proteins were found to be highly homologous to the class of proteins involved in postsecretional assembly of pili, PapD and PapC, respectively, in *E. coli* or the related class of proteins from *K. pneumoniae*, MrkB and MrkC, as well as nonfimbrial adhesins, including AfaC. It has been proposed that PapC (or its homologs) plays an active role in the dissociation of pilin from the PapD chaperone (8). The 95-kDa HtrE protein and the 27-kDa EcpD protein show an overall identity of 34 to 40% over their entire polypeptide with their homolog PapC and PapD class of proteins (2, 7, 20, 33, 35, 42, 44). In the PapC family of proteins, one of the conserved features is the presence of two highly conserved cysteine residues, spaced approximately 27 amino acids apart. In the HtrE protein, these two cysteine residues are located at amino acid positions 94 and

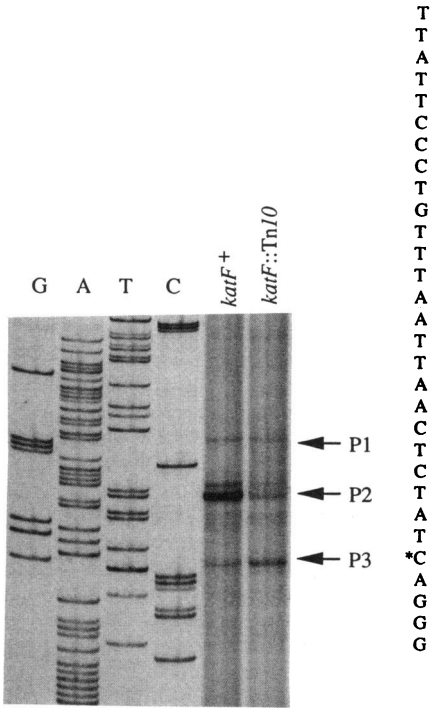

FIG. 10. Positive regulation of the P2 promoter by KatF. Primer extension reactions were carried out as described in the legend to Fig. 8, using template RNA from isogenic *katF*::Tn*10* and *katF*$^+$ bacteria harvested after the entry into stationary phase.

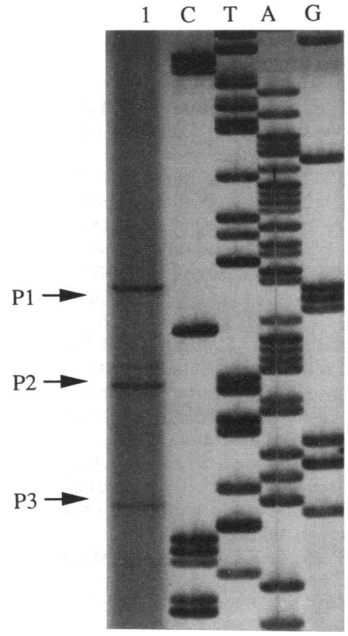

FIG. 11. Mapping of the 5′ termini by mung bean nuclease protection assay. Mung bean nuclease mapping used a uniformly labeled, single-stranded, 315-nucleotide *Hin*cII-*Dra*I DNA fragment as a probe. The probe (10^6 cpm) was hybridized to RNA extracted from MC4100 bacteria grown at 37°C, digested with 300 U of mung bean nuclease, and electrophoresed (lane 1). Lanes G, A, T, and C correspond to the dideoxy sequencing reactions carried out using pDM655 (*Hin*II-*Hin*dIII) as the template, initiated by the SK primer (Stratagene).

127. In the HtrE protein homologs, the first cysteine is approximately 100 amino acid residues from each amino terminus in all cases studied so far. A previous comparison of seven members of the PapD family has shown that 23 amino acid residues are identical in all cases (18). As can be seen in Fig. 7, this conservation has been retained in EcpD. This result strongly suggests a role for the EcpD protein similar to that of the other members of the PapD family.

It may be noteworthy to point out here that in certain *E. coli* strains a novel class of pilins, called curlins, has been detected (38). Although the genes encoding the structural subunit of curlin have been shown to map at 8 min on the *E. coli* chromosome, nothing is known about their assembly or transport. It is possible that the *htrE* and *ecpD* genes are involved in this pathway, since both the curlin operon and *htrE* operons seem to be incomplete by themselves. If the *htrE* operon is indeed used to transport pilin-like substrates, then certainly the corresponding structural genes are located elsewhere. However, the transcription of the *csgA* gene, which encodes the curlin subunit, is under the transcriptional regulation of a cytoplasmic protein, Crl. The transcriptional activation of the *csgA* gene by the Crl protein occurs only at lower temperatures, such as 26°C, but not at 37°C (3). Such a temperature-dependent transcriptional regulation is exactly the opposite of that found for the *ecpD-htrE* operon. However, one feature common to the *ecpD-htrE* operon and the curli-encoding *csgA* gene is the positive regulation by the *rpoS* gene product (37).

The main observations of the transcriptional regulation of the *ecpD-htrE* operon were that (i) transcription was induced with a temperature upshift from 22 to 37°C but was not controlled by the $E\sigma^{32}$ regulon, (ii) transcription declined at temperatures above 42°C and was much lower at 50°C than at 37°C, a situation reminiscent of most housekeeping genes, and (iii) transcription was severely reduced at low temperatures, such as 22°C. Similar results have also been obtained for *pap* transcription (14). However, unlike *pap* transcription, *ecpD-htrE* operon transcription is not regulated by Lrp (the product of the *lrp* [= *mbf*] gene). Instead, the main regulation seems to be through the DNA-binding protein IHF and the product of *katF* gene, which is known to function as an alternative sigma factor, judging from the fact that the levels of the *htrE* transcripts were reduced in both *himA* and *himD* mutant bacteria, as well as in bacteria carrying *katF*::Tn*10* mutations.

One of the main phenotypes associated with *htrE* null mutations is hypersensitivity to a brief exposure to 50°C, a condition that is normally sublethal for $htrE^+$ bacteria. The mechanism of thermotolerance at 50°C is not understood at present. We have previously shown that *htrC* mutant bacteria have a similar phenotype (39). Clearly the transcriptional regulation of the *htrC* gene, which is exclusively transcribed by $E\sigma^{32}$, is different from that of the *ecpD-htrE* operon. Interestingly, it has been shown that mutations in the RNA polymerase sigma subunit σ^s, encoded by *rpoS* (*katF*), are deficient in stationary-phase thermotolerance (25). Furthermore, the trehalose synthesis gene *otsA* (*osmA*), an osmotically inducible gene which is also transcribed by $E\sigma^s$, has been also implicated in such a thermotolerance at high temperatures (16). The P2 promoter of the *htrE* operon appears to be under positive regulation by $E\sigma^s$. Also, *htrE* null mutant bacteria do exhibit sensitivity to high osmolarity. Thus, some of the *rpoS*-regulated genes might be important to survival both at high temperatures and under other stress conditions. However, further studies are needed to see whether the *ecpD-htrE* operon is also subject to growth phase regulation and osmotic induction. Finally, it is worth mentioning here that some of the defects observed in *htrE* null mutant bacteria may be due to the possibility that the *htrE*-encoded PapC-like protein functions as a porin, perhaps involved in the transport of certain essential metabolites. Such a possibility is not unlikely since the PapC homolog of *Salmonella typhymurium* pathogenic bacteria has been shown to transport molecules such as vitamin B_{12} (41). The fact that *htrE* null mutants exhibit an osmosensitive phenotype is consistent with such a hypothesis.

ACKNOWLEDGMENTS

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