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Isolation and Characterization of the *Escherichia coli htrB* Gene, Whose Product Is Essential for Bacterial Viability above 33°C in Rich Media

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We have identified and studied the *htrB* gene of *Escherichia coli*. Insertional inactivation of the *htrB* gene leads to bacterial death at temperatures above 33°C. The mutant bacterial phenotype at nonpermissive temperatures includes an arrest of cell division followed by the formation of bulges or filaments. The *htrB*$^+$ gene has been cloned by complementation and shown to reside at 23.4 min on the *E. coli* genetic map, the relative order of the neighboring loci being *mboA-htrB-pyrC*. The *htrB* gene is transcribed in a counterclockwise fashion, relative to the *E. coli* genetic map, and its product has been identified as a membrane-associated protein of 35,000 Da. Growth experiments in minimal media indicate that the HtrB function becomes dispensable at low growth rates.

Escherichia coli is able to survive and grow over a wide temperature range, from 10 to 49°C, primarily because of its ability to adapt by altering its cellular composition (21). From 21 to 37°C, there are few adaptive changes that are required for efficient growth (19, 48). In contrast, when the temperature rises above 42°C, the composition of the cell is markedly changed. One of the most striking changes to occur is the expression pattern of proteins. For most cell proteins, a sudden increase in temperature causes a decrease in their production. However, a group of proteins called the heat shock proteins are transiently induced to high levels of synthesis at temperatures above 42°C (19, 37).

We have been interested in the role of the heat shock response in the maintenance of growth and viability of *E. coli*. There are approximately 20 heat shock genes that have been identified, all of which are under the positive control of the σ^{32} transcription factor. This heat shock regulon includes genes whose products are proteases (e.g., *lon*) and chaperonins, which assist the proper assembly and transport of proteins (e.g., *groEL* and *dnaK*), as well as the housekeeping transcription factor σ^{70} itself (i.e., *rpoD*) (15, 18, 37).

We recently initiated a set of experiments aimed at identifying additional *E. coli* genes whose products are required for cell growth and survival at high temperatures. The approach has been to create libraries of *E. coli* cells at 30°C carrying various transposon insertions. Such libraries are then screened for those insertions which result in the inability to form colonies at 42°C. This method has proven effective, and several new genes whose products are required only at a high temperature have been isolated. The genes that have been defined by such mutations have been named *htr* (for high temperature requirement). Two of the newly identified genes, *htrA* and *htrC*, are previously unidentified heat shock-inducible genes (32, 45). The HtrA protein is a periplasmic endopeptidase that may be required to remove polypeptides that become toxic to the cell when the temperature is raised above 42°C (31). The *htrA* gene is exclusively under the positive regulation of the newly identified σ^{24} (σ^{E}) transcription factor (9, 57). The *htrC* gene is under the positive regulation of σ^{32}, and its gene product may itself be a transcription factor or be capable of indirectly affecting the expression of a wide variety of genes involved in the growth and viability of *E. coli* (45).

In this work, we present genetic and physiological studies defining the *htrB* gene and its product. Insertional inactivation of the *htrB* gene leads to an exquisitely temperature-sensitive phenotype (Ts$^-$), in that *htrB* mutants can survive at temperatures up to 32.5°C but not above 33°C.

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MATERIALS AND METHODS

Bacteria, bacteriophages, and plasmids. The bacteria, bacteriophages, and plasmids used during the course of this work are described in Table 1.

Media. L broth is composed of 10 g of NZ-amine, 5 g of yeast extract, and 5 g of sodium chloride per liter. M9 minimal medium was made as previously described (31). Glucose and glycerol were added to a final concentration of 0.4%, and sodium acetate was added to a final concentration of 0.2%. Supplementing amino acids were a defined mixture of amino acids without methionine (16). Antibiotics were added when needed at a final concentration of 50 μg/ml for ampicillin (Amp) and kanamycin (Kan) and 15 μg/ml for tetracycline (Tet) and chloramphenicol (Cam).

Cell growth analysis. Culture growth was monitored by optical density measurement at 595 nm (OD_{595}). Cell viability was determined by plating various dilutions on L-agar plates at 30°C, as described by Miller (36). The number of cells was determined by counting with a Pettrof-Hausser counting chamber. Photographs of cells were taken after the cultures were first treated with 1.0% glutaraldehyde for 15 min at room temperature and then adhered to microscope slides by using poly-L-lysine. All cultures, except the B178 culture grown at 42°C, were concentrated 10-fold by centrif-

TABLE 1. *E. coli* strains, bacteriophages, and plasmids

Strain, bacteriophage, or plasmids	Relevant characteristics	Reference or source
Strains		
B178	W3101 *galE sup*$^+$	14
CG97	B178 *recA1* Tet^r	31
DH5α	*recA1*	Bethesda Research Laboratories
MLK217	B178 *htrB1*::mini-Tn*10*	This paper
MLK48	B178 *htrB3*::mini-Tn*10*	This paper
MLK79	B178(λ *c*I *ind*)	This paper
MLK50	MLK217(Mu Amp^r)	This paper
NFB216	JM83 *mdoA*::Tn*10 pyrC*	J.-P. Bohin (30)
RP501	*pyrC46*	J. S. Parkinson
MLK633	RP501 *htrB1*::mini-Tn*10*	This paper
TOE13	*ftsA*(Ts)	W. Donachie (56)
TOE1	*ftsQ*(Ts)	W. Donachie (2)
Ken90	*ftsZ*(Ts)	W. Donachie (33)
SP4500	*pbpA*(Ts)	W. Donachie (52)
SP5211	*rodA*(Ts)	W. Donachie (52)
D22	*envA1*	W. Donachie (40)
AX655	*pbpB2158*	B. Bachmann (11)
Bacteriophages		
1H7	λ *htrB*$^+$	27
E4H10S	λ *htrB*$^+$	27
λ *c*I *ind*	Forms stable lysogens	Our collection
Mu Amp^r	*c*$^+$ Amp^r	A. Toussaint
P1L4	Generalized transduction	L. Caro
λ1098	λ mini-Tn*10*	J. C. Way (58)
Plasmids		
KS$^+$ and SK$^+$	Bluescript, pUC derivative, plasmids with T7 and T3 promoters	Stratagene
pREG153	Low-copy-number cosmid vector, Amp^r	D. Low (28)
pBR322	Medium-copy-number vector, Amp^r Tet^r	5
Mu d5166	Mini-Mu vector, Cam^r	M. Casadaban (17)
Mu d5005	Mini-Mu vector, Kan^r	M. Casadaban (17)
pTSI	*htrB* on a 4.7-kbp *Sau*3A partially digested fragment in pBR322	This paper
pKS12	*htrB*$^+$ on the *Pvu*II-*Sal*I fragment from pTS1 in pBluescript KS$^+$	This paper
pSKS1	*htrB*$^+$ on the *Pvu*II-*Sal*I fragment from pTS1 in pBluescript SK$^+$	This paper
pBP-KS	*Bam*HI-*Pst*I fragment carrying *htrB*$^+$ and *mdoA*$^+$	This paper
CG1259	pREG153 *htrB*$^+$ cosmid	This paper
CG1262	pREG153 *htrB*$^+$ cosmid	This paper

ugation for 5 min at 6,000 × *g* before they were adhered to the microscope slide.

Genetic techniques. The procedure employed in mini-Tn*10* mutagenesis has been previously described (31, 58). P1 transductions were performed as described by Miller (36). Bacteriophage P1L4 was obtained from Lucien Caro.

The mini-Tn*10* insertions were recombined onto the cosmid clones CG1259 and CG1262 by first transducing the cosmid into *htrB1*::mini-Tn*10* or *htrB3*::mini-Tn*10* bacteria by T4GT7-mediated transduction (59). Mutant cells carrying either cosmid were grown overnight in L broth at 30°C, and the culture was diluted 1:10 in fresh broth and infected with T4GT7 bacteriophage at 37°C. The resulting lysate was used to infect B178 bacteria. Bacteria carrying cosmids that conferred both Amp^r and Tet^r were isolated on the appropriately supplemented L-agar plates and further characterized.

Recombination of the mini-Tn*10* insertions onto the bacteriophages 1H7 and E4H10S described by Kohara et al. (27) was achieved by first infecting *htrB1*::mini-Tn*10* and *htrB3*::mini-Tn*10* with these bacteriophages, using the resulting lysate to infect B178(λ) cells (to prevent killing by the infecting λ bacteriophage), and selecting for Tet^r colonies.

***E. coli* genomic libraries.** The cosmid library was made by O'Connor et al. from *Sau*3A partial fragments cloned into pREG153 (41), a low-copy-number vector (2 to 4 copies per cell) (28). The original cosmid library was prepared on a *supE* host. Helper bacteriophage λ *c*I857 *O*am29 was grown on this library and used to infect *htrB sup*$^+$ mutant bacteria. Following a 30-min incubation at 30°C to allow for Amp^r expression, the infected cultures were plated at 42°C on L-Amp plates. DNA was extracted from those colonies that grew at 42°C and was further characterized.

Mini-Mu libraries, derived from vectors Mu d5166 and d5005, were made as described by Groisman and Casadaban (17). Such lysates were used to infect *htrB*(Mu) lysogens (to prevent killing by the Mu helper bacteriophage), and the cells were plated at 42°C on either L-Cam, in the case of Mu d5166, or L-Kan, in the case of Mu d5005. Plasmid DNA from rescued colonies was purified and further characterized.

DNA manipulations. Plasmid DNA was purified from bacteria by the alkaline lysis method of Birnboim and Doly (4) as described by Maniatis et al. (34). Plasmid DNA was labeled with [α-^{32}P]dATP (NEN/Dupont) by using the nick translation kit sold by Bethesda Research Laboratories. Hybridizations were performed as described elsewhere (48a). All restriction endonucleases and T4 DNA ligase were used according to manufacturers' specifications.

Labeling of plasmid-encoded proteins. T7 RNA polymerase-promoter-directed expression and labeling of plasmid-encoded proteins were done by the methods of Tabor and Richardson (51) in the cell line DH5α.

Maxicell labeling of plasmid-encoded proteins was performed by the method of Sancar et al. (47) as described by Lipinska et al. (31).

RESULTS

Isolation and characterization of *htrB* insertion mutations. Two independently isolated alleles of *htrB* were identified in a search for mutations in genes that are required for growth only at high temperatures. These mutants were isolated from a library that was created in the wild-type strain B178 at 30°C by random insertion of a mini-Tn*10* transposon carrying the Tet^r gene (58). Subsequently, the library was screened for mutants that were unable to form colonies on L-agar plates at 42°C. The two *htrB* mutants, *htrB1*::mini-Tn*10* and *htrB3*::mini-Tn*10*, were found to belong to the same complementation group. By using P1 transduction, we were able to show that Tet^r and the inability to propagate at 42°C were 100% cotransducible. In all respects tested, *htrB1*::mini-Tn*10* and *htrB3*::mini-Tn*10* mutant bacteria behave similarly. In most studies reported below, the *htrB1*::mini-Tn*10* allele was used.

To determine the requirement for the *htrB* gene product, cells were plated at a variety of temperatures and on a

TABLE 2. Colony-forming ability of *htrB1*::mini-Tn*10* bacteria on M9 media at 42°C

Supplement(s)	Mass doubling time (min)[a]	*htrB* colony formation
Glucose	70	+
Glucose + amino acids	45	−
Glycerol	100	+
Glycerol + amino acids	55	−
Acetate	235	+
Acetate + amino acids	75	+

[a] Mass doubling times shown are those of the isogenic $htrB^+$ strain B178 grown at 42°C.

variety of media. On L-agar plates, *htrB* bacteria will form colonies at temperatures below 32.5°C but will not form colonies at temperatures above 33°C. This temperature effect is bactericidal; that is, *htrB* bacteria irreversibly lose their colony-forming ability. However, *htrB* mutant bacteria will form colonies at 42°C when grown on M9 plus glucose. If these M9-glucose plates are supplemented with a defined mixture of amino acids, *htrB* mutant bacteria will form colonies at 37 but not at 42°C. Colony formation does not appear to be a function of catabolite repression. If glucose is replaced with glycerol, the phenotypes remain the same, i.e., in the absence of amino acids, *htrB* bacteria grow at 42°C, whereas with amino acids, they do not. The addition of the amino acids per se does not inhibit growth at 42°C; when *htrB* bacteria are plated on M9 with acetate and amino acids, they form colonies at 42°C.

The ability of *htrB* mutants to propagate at 42°C on some media but not on others suggested that one requirement for HtrB may be a function of mass doubling time of the culture. For example, by supplementing minimal medium with amino acids and relatively good carbon sources such as glucose and glycerol, the cells grow at a rate that requires the function of the HtrB protein. However, when minimal medium is supplemented with amino acids and a poor carbon source, like acetate, the cells grow slower than the rate at which the HtrB protein is required. The mass doubling times of the isogenic parent, B178, in these different media at 42°C support such a hypothesis (Table 2). The *htrB* cells grow at 42°C when the mass doubling time is 70 min or higher but do not grow when the mass doubling time is 55 min or less.

We do not believe that the temperature sensitivity and growth rate dependence of the *htrB* mutants are identical phenomena. The mass doubling time of $htrB^+$ and *htrB* cells at 30°C in L broth is 55 min, as fast as cells growing at 42°C in M9 supplemented with glycerol and amino acids, a nonpermissive condition for *htrB*. Also, as mentioned above, *htrB* cells will grow in M9 supplemented with glucose and amino acids at 37°C, but the mass doubling time of wild-type cells at 37°C is faster than it is at 42°C (data not shown). The slower growth rate at the higher temperature may be caused in part by a starvation for methionine at temperatures above 40°C (46). If the rate of cell growth was the only determining factor for the requirement of the *htrB* gene product, one would expect that *htrB* cells would not grow in M9 supplemented with glucose and amino acids at 37°C and the maximal temperature the mutant cells could grow on L agar would be lower than 30°C. The HtrB protein must be required for a growth rate-dependent process that also has a unique temperature dependence.

The patterns of growth, division, and viability of *htrB* cells grown in L broth at 30 and 42°C were compared with those of B178, the isogenic wild-type parent (Fig. 1). The growth characteristics of *htrB* cells are very similar to those of the B178 isogenic parent at 30°C. However, at 42°C, all three of these properties are altered. When an *htrB* culture is shifted to 42°C, it continues to grow at the 30°C rate for 3 h (Fig. 1A). After 3.5 to 4 h, the cells begin to lyse with a concomitant decrease in the OD of the culture. Cell viability, as determined by colony formation at 30°C, is lost quickly; over 95% of the cells have lost viability in the first 2 h (Fig. 1B). The cell growth that occurs during the first 3 h at 42°C is not accompanied by cell division, since the number of cells in the culture remains constant after the first 30 min at 42°C (Fig. 1C). Within the first 30 min, the number of cells does not quite double, but often increases by approximately 50%. This increase may simply reflect the completion of already initiated cell divisions without the initiation of any new rounds of cell division. The divisions that occur in the first 30 min are not required for the HtrB phenotype to manifest itself, i.e., cells that are treated with the cell division inhibitor nalidixic acid, either before shifting to 42°C or at the time of the shift, still show the altered morphology (see below) that accompanies the death of *htrB* cells (data not shown).

Altered morphology of *htrB* cells. Figure 2 contains photographs of wild-type B178 and *htrB* cells grown at 30 or 42°C for 2.75 h. Figure 2D shows *htrB* cells that were shifted to 42°C when the culture was at an OD_{595} equal to 0.05. The *htrB* cells have formed large bulges at one of their ends or at their centers. If the culture is shifted when the OD is higher than 0.05, the cells gradually shift from a phenotype of forming bulges to a predominantly filamenting phenotype. Figure 2E and F show cells that were shifted at an OD_{595} equal to 0.4 and 1.0, respectively. Regardless of the initial OD of the culture when it is shifted, the cells cease dividing and eventually lose viability.

Double mutants of *htrB1*::mini-Tn*10* and mutations affecting cell division and cell wall formation. The filamentation and formation of bulges of *htrB* strains may indicate that HtrB plays a role in cell division and/or cell wall synthesis. To determine if this is the case, we constructed double mutants of *htrB* with mutations in the *ftsZ*, *ftsQ*, *ftsA*, *pbpA*, *pbpB*, or *rodA* genes. All of the double mutants behave similarly. When shifted to 42°C, the morphology of the double mutants is more normal than either of the single mutants alone, i.e., the cells do not form bulges or filaments nor do they become spherical. However, they rapidly cease dividing, stop growing, and eventually lyse.

A double *envA1 htrB* mutant was also constructed. Unlike the other double mutants, these cells grow at 42°C with a relatively normal morphology, except that the cells are slightly fatter and often connected (data not shown). The *envA1* allele is a nonconditional mutation that slows the final formation of cell wall during septation (39). This tardiness leads to an overabundance of connected cells in the culture. In other respects, however, individual cells have wild-type morphologies. Accompanying the effect on septation, the *envA1* mutation also leads to an extreme sensitivity to a wide variety of antibiotics such as rifampicin (38, 40). The double mutant is still sensitive to rifampicin (Rif^s), hence the *htrB* mutation does not show a reciprocal suppression of the *envA1* mutation.

To confirm that the suppression of the HtrB phenotypes comes from the *envA1* allele, we transduced *envA1* into wild-type strain B178. To do this, we first marked the *envA1* mutation with a mini-Tn*10* carrying Kan^r, called kan1029, that is approximately 20% linked to the *envA* gene. Because

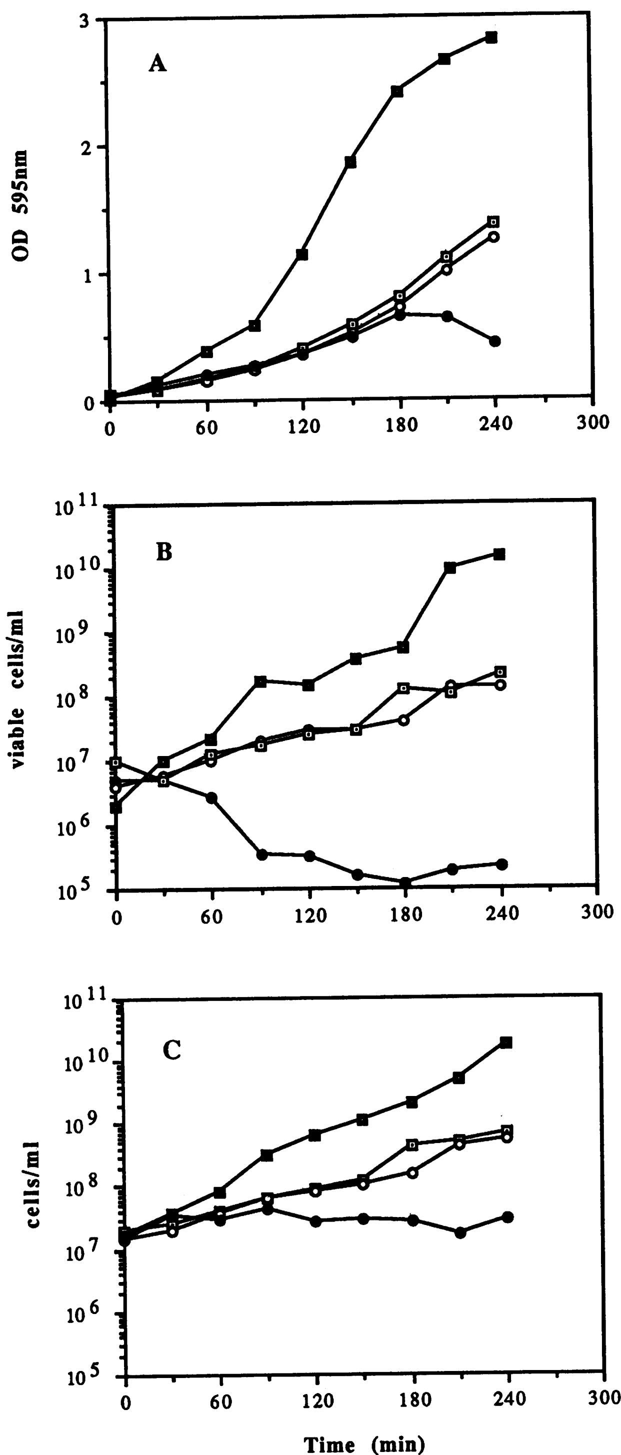

FIG. 1. Growth properties of *htrB* bacteria on L broth. Bacterial cultures of *htrB*$^+$ (B178) and *htrB1*::mini-Tn*10* were grown at 30°C to log phase, diluted to an OD_{595} equal to 0.05, and placed at 30 and 42°C at time zero. Aliquots were removed every 30 min for analysis. (A) Increase in culture mass as determined by measurement of OD_{595}. (B) Viable cells per ml of culture. Dilutions of each culture were plated on L agar at 30°C, and colonies were counted. (C) Cells per ml of culture. Cells were counted under a microscope with a Pettrof-Hausser counting chamber. ⊡, *htrB*$^+$ grown at 30°C; ○, *htrB1*:mini-Tn*10* grown at 30°C; ■, *htrB*$^+$ shifted to 42°C; ●, *htrB1*::mini-Tn*10* shifted to 42°C.

of the relatively weak effect of the *envA1* mutation on cell morphology, we used the Rif^s phenotype of *envA1* bacteria as the criterion of the mutation's presence or absence. This Kan^r marker was transduced into D22, the *envA1* strain, and both Kan^r *envA*$^+$ and *envA1* strains were isolated. A P1 transducing lysate was made on the Kan^r *envA1* strain and used to transduce B178 bacteria to Kan^r; these colonies were then screened for Rif^s. Subsequently, the *htrB1*::mini-Tn*10* allele was introduced into the D22 Kan^r *envA1* and *envA*$^+$ strains, as well as the B178 *envA1* strain. The Ts$^-$ phenotype of *htrB* was suppressed in the D22 background regardless of the presence or absence of the *envA1* allele. In addition, the *envA1* allele that was transduced into B178 was not able to suppress the Ts$^-$ phenotype of *htrB*. Thus, it appears that suppression is not mediated by the *envA1* mutation, but through another unlinked and unidentified mutation(s) in the D22 genetic background.

Cloning of the *htrB* gene. The *htrB* gene was identified and cloned from several different *E. coli* genomic libraries by complementation of the Ts$^-$ phenotype of the *htrB* mutations at 42°C. Five clones were isolated from two mini-Mu libraries in the Mu vectors d5166 and d5005 (17), and four clones were isolated from a genomic library made in the low-copy-number cosmid vector pREG153 (28, 41). Restriction mapping of these plasmid DNAs showed that they fall into two different classes of complementing clones. Clones from both classes were labeled and used to probe the overlapping λ clones of the *E. coli* genomic library made by Kohara et al. (27). One class of clones hybridized to bacteriophages 1H7 and E4H10S. This corresponds to position 23.4 min on the *E. coli* genetic map (Fig. 3). Two cosmids and four mini-Mu clones fell into this class. The remaining two cosmids and one mini-Mu clone form a second class and map to a different part of the *E. coli* genome. They appear to represent a class of multicopy suppressors of the HtrB phenotype (unpublished data). By a variety of criteria discussed below, it was determined that the first class of complementing clones carries the bona fide *htrB*$^+$ gene.

DNA from one of the mini-Mu clones from the first class was partially digested with *Sau*3A and cloned into the *Bam*HI site of pBR322. This subclone, pTS1, is capable of correcting all of the phenotypes of *htrB* mutants. pTS1 was labeled by nick translation and used to probe a Southern blot of wild-type and *htrB1*::mini-Tn*10* genomic DNA digested with either *Cla*I or *Ava*II (Fig. 4). The differences observed in restriction patterns between wild-type and *htrB* DNAs are consistent with the insertion of a 3.7-kbp piece of DNA, the size of the mini-Tn*10* used for the insertion mutagenesis.

The two cosmid clones from the first class, CG1259 and CG1262, as well as the two λ transducing phage described by Kohara et al., 1H7 and E4H10S, that hybridize to the first class of clones, can recombine with the insertionally inactivated *htrB* alleles, thus acquiring the mini-Tn*10* cassette. Restriction mapping of those cosmids that acquired the *htrB1*::mini-Tn*10* and *htrB3*::mini-Tn*10* alleles showed that the mini-Tn*10* insertions were approximately 300 bp apart from one another and located within the *Bgl*I-*Sac*II fragment at 6.25 to 7.25 kbp, as marked on the restriction map in Fig. 3 (data not shown).

Both the *pyrC* and *mdoA* genes have also been mapped to the 23.5-min region of the *E. coli* genome (30, 60). P1 transductions were done to determine the extent of the linkage of *htrB* to *pyrC*. It was found that the two genes are 95% cotransducible. Comparisons of the restriction map of the *pyrC* region, the restriction map of Kohara et al., and the restriction map of *htrB* place *pyrC* approximately 6 kbp

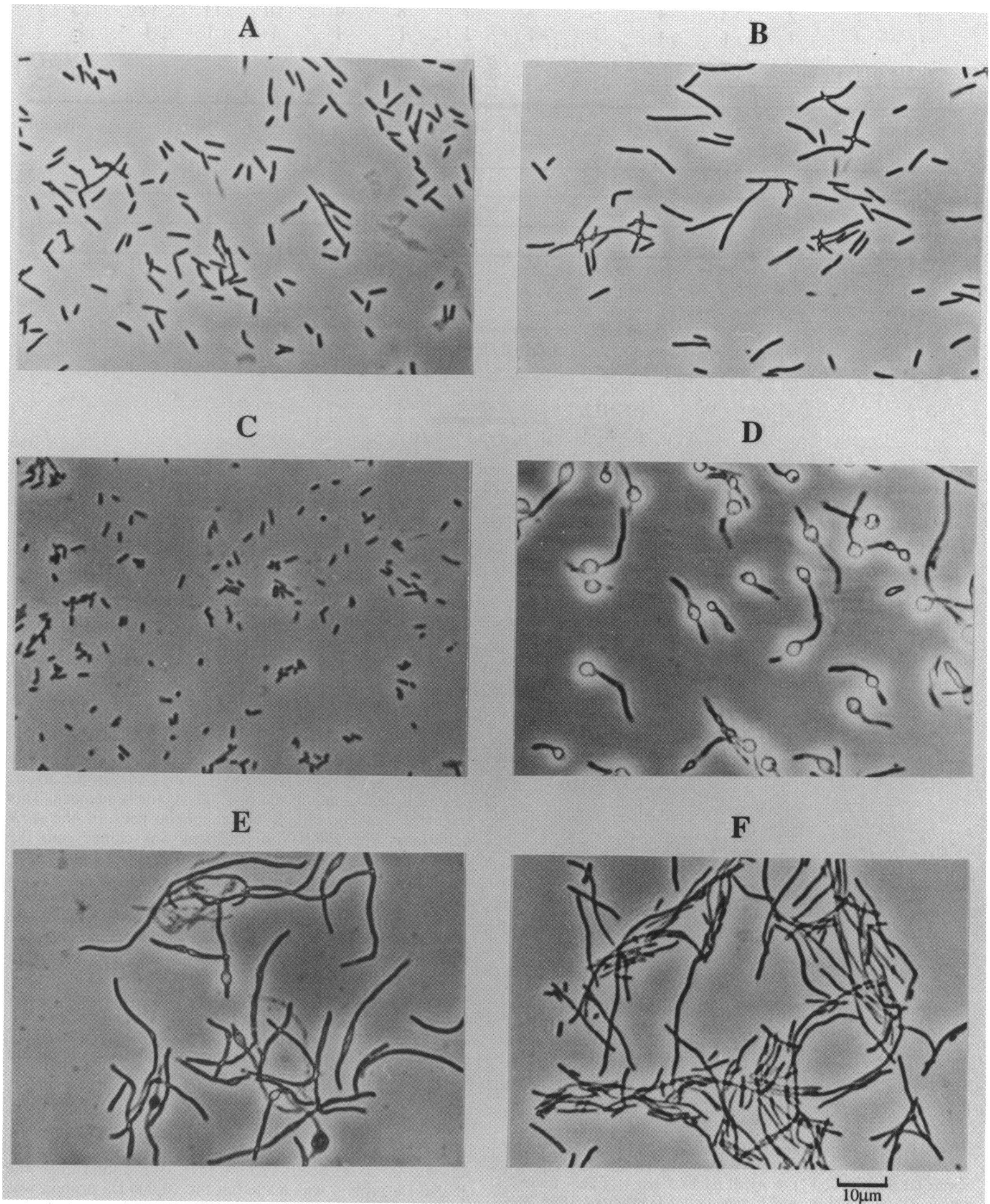

FIG. 2. Morphological changes in *htrB1*::mini-Tn*10* cells after a shift from 30 to 42°C. (A and B) *htrB*$^+$ (B178) (A) and *htrB1*::mini-Tn*10* (B) cultures grown to log phase in L broth at 30°C, diluted to an OD_{595} equal to 0.05, and grown for 2.75 h at 30°C. (C and D) Same cultures and conditions as in panels A and B, respectively, but shifted to 42°C. (E) Log phase culture of *htrB1*::mini-Tn*10* diluted to an OD_{595} of 0.4, shifted to 42°C, and grown for 2.75 h. (F) Culture of *htrB1*::mini-Tn*10* grown to an OD_{595} of 1.0 at 30°C, shifted to 42°C, and grown for 2.75 h.

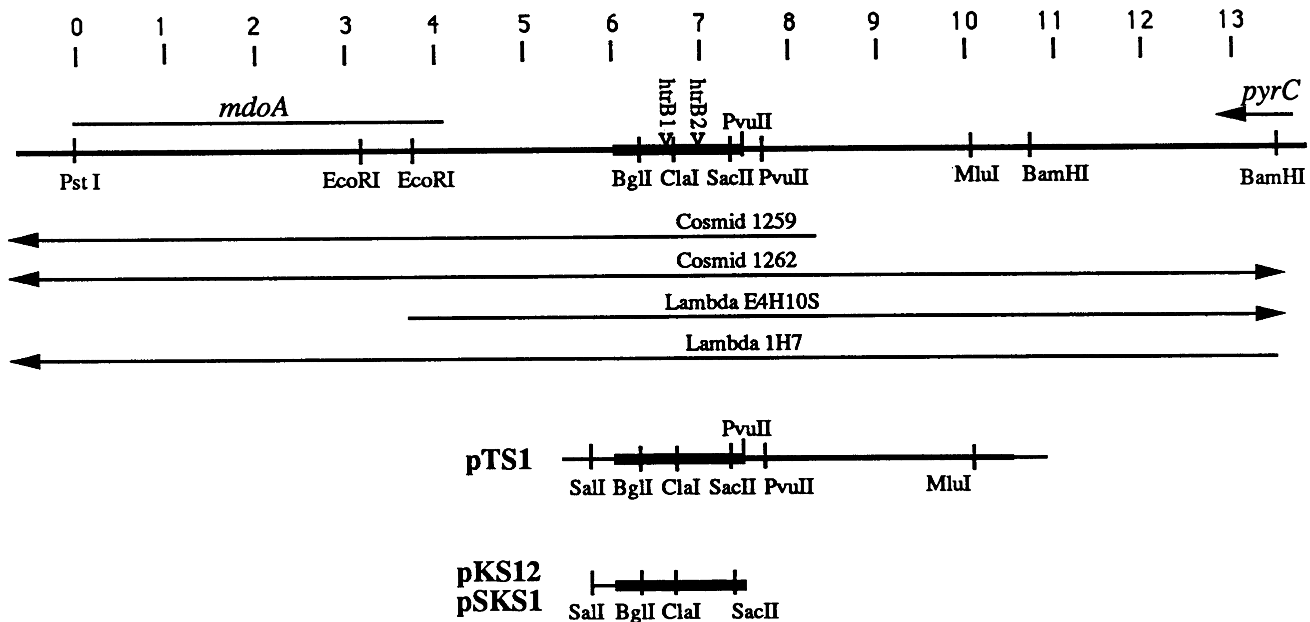

FIG. 3. Partial restriction maps of the genomic DNA, λ clones, and plasmids containing the *htrB* gene and the surrounding region at 23.4 min on the *E. coli* chromosome. All clones shown correct the HtrB phenotype. The location of the *htrB* gene is indicated by the bold line. The locations of *htrB1*::mini-Tn*10* and *htrB3*::mini-Tn*10* insertions are marked on the top genomic map. The locations of *pyrC* and *mdoA* were determined by restriction map comparisons (27, 30, 60). The thin lines in the pTS1, pKS12, and pSKS1 restriction maps indicate pBR322 sequences. Numbers at the top are kilobase pairs.

downstream of *htrB*, clockwise on the genetic map. This distance is consistent with a cotransduction frequency of 95%, by using Wu's formula (61). To confirm the close linkage of *htrB* and *mdoA*, we cloned the 10-kbp *Bam*HI-*Pst*I fragment from this region into the pBluescript KS$^+$ vector

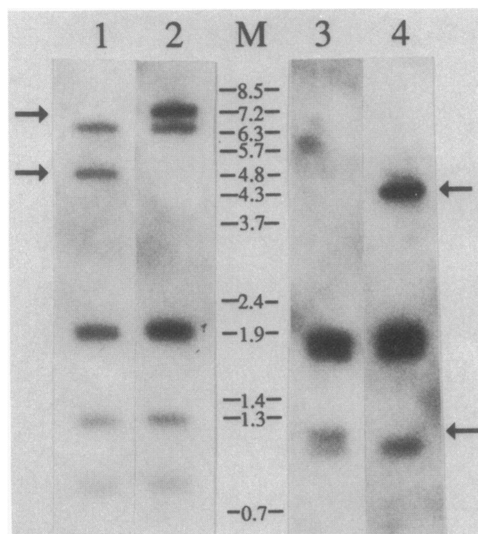

FIG. 4. Autoradiograph of a Southern blot of *htrB*$^+$ and *htrB1*::mini-Tn*10* genomic DNAs. Genomic DNAs were digested with *Cla*I (lanes 1 and 2) or *Ava*II (lanes 3 and 4). Lanes 1 and 3, *htrB*$^+$; lanes 2 and 4, *htrB1*::mini-Tn*10*. Transferred DNA was probed with nick-translated pTS1 plasmid DNA. DNA size standards (lane M) are a *Bst*EII digest of λ DNA probed with nick-translated λ DNA. Arrows indicate restriction fragments affected by the insertion of the mini-Tn*10*.

and showed that this fragment was able to recombine with the *htrB1*::mini-Tn*10* and the *mdoA*::Tn*10* mutant alleles. By comparing the restriction maps of the *mdoA* locus and pTS1, we deduce that *mdoA* must lie 1.5 to 2.0 kbp upstream of *htrB*. From the comparison of all of the restriction maps in this area and the P1 transductions, we deduce the order of the three genes on the *E. coli* chromosome to be *mdoA-htrB-pyrC*.

Identification of the *htrB* gene product. A subclone of pTS1 was made by digestion with *Pvu*II, which cuts in the insert, and *Sal*I, which cuts in the pBR322 vector sequences. This fragment can correct all of the phenotypes of the *htrB* mutations. The *Pvu*II-*Sal*I fragment was cloned into the *Eco*RV and *Sal*I sites of the pBluescript KS$^+$ and SK$^+$ vectors to give rise to subclones pKS12 and pSKS1, respectively. The pBluescript vectors are pUC derivatives with promoters for the T7 and T3 RNA polymerases flanking the polylinker region (Stratagene). The proteins that are encoded in the inserted DNA were labeled with [^{35}S]methionine by using the T7 expression system of Tabor and Richardson (51). When the T7 promoter is located at the *Pvu*II end of the fragment, as is the case in plasmid pKS12, a 35,000-Da protein is made (Fig. 5A, lane 2). When the T7 promoter is located at the *Sal*I end, as is the case in plasmid pSKS1, a 10,000-Da protein is made (lane 3).

Because the above system does not require a functional endogenous promoter, it is possible that some of these mRNAs are not expressed in vivo. To determine if this is the case, the proteins encoded by these same plasmids were labeled in a maxicell system (47). We found that the 35,000-Da protein was made but the 10,000-Da protein was not (Fig. 5B, lanes 3 and 4). We conclude that the 35,000-Da protein is the *htrB* gene product and that the 10,000-Da protein could be a fortuitous open reading frame that may not be expressed extensively in vivo.

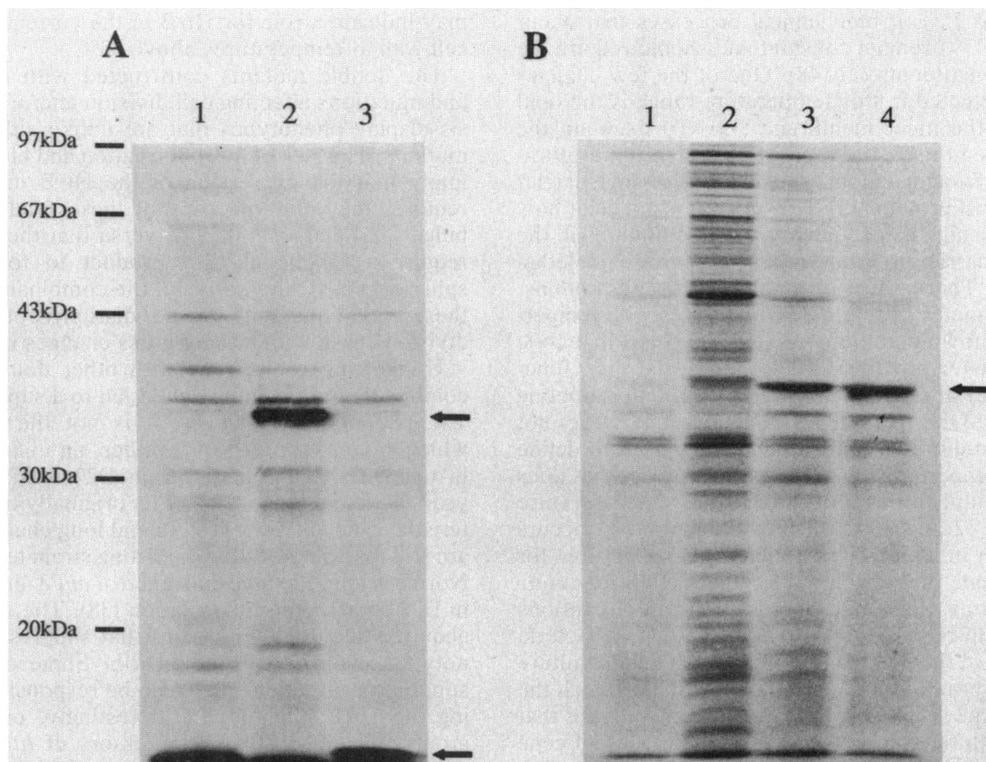

FIG. 5. Autoradiograph of [^{35}S]methionine-labeled proteins encoded by plasmids pKS12 and pSKS1. (A) Plasmid-encoded proteins labeled by using the T7 RNA polymerase-promoter expression system (51). Lane 1, pBluescript KS vector; lane 2, pKS12; lane 3, pSKS1. (B) Maxicell labeling of plasmid-encoded proteins (47). Lane 1, pBluescript KS vector; lane 2, pBluescript SK; lane 3, pKS12; lane 4, pSKS1. Arrows indicate labeled products from pKS12 and pSKS1.

In order to conclusively demonstrate that the inactivation of the 35,000-Da open reading frame is responsible for the HtrB phenotype, we constructed DNA deletions of plasmid pKS12 by using the DNase I method (20). The analysis of these deletions shows that there is a correlation between the presence of the 35,000-Da protein and the suppression of the HtrB phenotypes. No such correlation was found with the 10,000-Da protein (data not shown). We conclude that absence of synthesis of the 35,000-Da protein leads to the mutant HtrB phenotype.

Attempts to focus the HtrB protein on the standard O'Farrell two-dimensional gel system (42) have been unsuccessful. The HtrB protein may be membrane bound or have a charge that is outside of the pH 3.5 to 10 range of the gels. Nonequilibrium gels (43) have also been unsuccessful. In both cases, the protein remains at the origin of the isoelectric focusing gel as a smear and only enters the second dimension gel if the sample has been treated with sodium dodecyl sulfate (SDS). This type of behavior may indicate that the HtrB protein is associated with one of the *E. coli* membranes. Preliminary cell fractionations using the methods of Billmire and Duckworth (3) and Ito et al. (24) show that the HtrB protein, labeled in the T7 promoter-polymerase expression system, partitions almost exclusively with the membrane fractions (data not shown).

DISCUSSION

In an attempt to define previously unidentified genes whose products are required for survival at high temperatures, we have isolated two mutations in the *htrB* gene. Both mutant alleles were created by the insertion of a mini-Tn*10* transposon carrying the Tet^r gene. This method of mutagenesis was chosen in order to bias against mutations that merely lead to temperature-sensitive proteins. This appears to be the case for the two *htrB* mutations that we have studied. Although both alleles exhibit identical mutant phenotypes, they are not identical mutations, that is, the mapping of the insertions showed that they are approximately 300 bp apart. The *htrB* alleles appear to be null mutations that do not code for temperature-sensitive proteins.

The wild-type *htrB* gene has been cloned and localized to a 1.55-kbp fragment of DNA that corrects all of the HtrB phenotypes. This fragment encodes a 35,000- and possibly a 10,000-Da protein. We conclude that the 35,000-Da protein is the HtrB protein, since it is synthesized in both the T7 RNA polymerase-promoter and in the maxicell systems, while the 10,000-Da protein is only synthesized under T7 promoter control. In addition, a mutant plasmid that has lost the ability to direct the synthesis of the 35,000- but not the 10,000-Da protein loses its ability to suppress any of the HtrB mutant phenotypes. The 35,000-Da protein behaves like a membrane-associated protein, since (i) it does not focus well in either equilibrium or nonequilibrium two-dimensional gel electrophoresis, (ii) it does not enter the gel unless it is treated with SDS first, and (iii) it sediments with the membrane fraction during cell fractionation experiments. The sequencing of this gene should determine whether there are potential membrane-spanning regions within the HtrB protein, as would be expected of a membrane protein.

The *htrB* mutants show a unique temperature sensitivity, growing only at temperatures at or below 32.5°C. In general,

it is thought that *E. coli* biochemical processes that occur between 21 and 37°C remain constant and should require the same cellular constituents (21, 48). One of the few changes that has been detected in this temperature range is the lipid composition of the inner membrane (35). To maintain the fluidity of the membrane, the ratio of saturated to unsaturated fatty acids is adjusted by the 3-ketoacyl-acyl carrier protein synthase II protein (12). However, it is not clear how important this change is for viability, since mutations in the 3-ketoacyl-acyl carrier protein synthase II gene are not lethal to the cell (13). The expression pattern of a few proteins, termed "thermometer" proteins, also changes with temperature (19). No known mutations exist in the genes that code for the thermometer proteins to determine what their functions are or if they are essential. The HtrB protein is not one of the four defined thermometer proteins because it does not focus on the two-dimensional gels that were used to define these proteins. Apparently, HtrB affects a process that does not remain constant throughout the 21-to-37°C temperature range. Between 32 and 33°C, a transition probably occurs from a condition in which HtrB function is dispensable for cell viability to one that absolutely requires HtrB function.

Like temperature, there are also growth rate conditions that modulate the requirement for *htrB*, i.e., *htrB* bacteria will grow provided that the mass doubling time of the culture is 70 min or longer but will not grow on media for which the mass doubling time is 55 min or less. Mutant phenotypes that depend on growth rate are associated with a variety of gene mutants, for example, *ftsE* (53), *ftsM* (8), *ftsI* (54), *secB* (29), and *dnaK* (our unpublished data). For the most part, the phenotypes of slower-growing mutant bacteria are less extreme than the phenotypes at higher growth rates. It could be that such functions are involved in accelerating certain cellular processes to keep up with the faster growth rate. Alternatively, the processes for which these functions are needed could be required only at high growth rates. In contrast to the lack of cellular alterations seen with temperature changes, there is a large number of changes associated with growth rate. Cell mass, rate of protein expression, DNA concentration, rRNA and mRNA expression, and cell wall and membrane composition have all been shown to change with growth rate (6, 35, 44, 48, 55).

The morphology of *htrB* mutants grown at 42°C in L broth is quite striking. The large bulges that form may be indicative of alterations in the cell wall, and the filamentation of the cells implies that the cells continue to grow but are unable to divide. (It is not clear why there is a difference in the morphology depending on the density of the culture when it is shifted to 42°C.) Bulges that are less pronounced than those seen in *htrB* cells can be formed by treating wild-type cells with low concentrations of certain β-lactam antibiotics (7, 49, 50). These antibiotic-induced bulges are formed by the inactivation of penicillin-binding proteins PBP 2 and PBP 3 (50). Cells carrying double mutations in the *pbpA* and *pbpB* genes, coding for the PBP 2 and PBP 3 proteins, respectively, also produce bulges that are very similar to those produced by *htrB* cells (1). Both of these proteins are transpeptidases involved in shaping the peptidoglycan layer (22, 23). PBP 2 activity is responsible for forming the cylindrical portion of the cell wall, since in its absence spherical cells are produced (50). PBP 3 activity is required for the formation of the peptidoglycan needed for septum formation, i.e., in its absence, bacteria filament without septum formation (50). The similarity of *htrB* bulges with those formed by the *pbpA pbpB* double mutants and the similarity of its filamentation with that of the *pbpB* mutant may indicate a role for HtrB in the correct assembly of the cell wall at temperatures above 33°C.

The double mutants constructed with *htrB1*::mini-Tn*10* and mutations affecting cell division and/or cell wall synthesis display phenotypes that are unlike either of the single mutants. The lack of bulge formation and filamentation could imply that the expression of the HtrB mutant phenotype requires the wild-type copy of these products to form the bulges and filaments or vice versa that the other mutations require a functional *htrB* product to form filaments or spherical cells. Alternatively, the combinations of *htrB* with these mutations may be so disruptive that growth and division cease without any gross changes in morphology.

Unlike the results seen with other double mutants, the combination of *htrB* and *envA1* led to a suppression of HtrB Ts$^-$ phenotype. However, it is not the *envA1* mutation which suppresses *htrB* but, rather, an unlinked mutation(s) in the D22 background. Strain D22 was isolated over 20 years ago, and only a few of its originally described characteristics, such as slow growth and long chain formation (40), are still associated with the existing strain today. In addition, Normark identified the existence of *envA*-linked suppressors in D22 shortly after its isolation (38). The strain could have acquired additional mutations that suppress the EnvA phenotype, leading to faster growth. Some of these putative suppressor mutations may also be responsible for suppressing the HtrB phenotype. Interestingly, one member of a group of cold-sensitive suppressors of *htrB* that we have recently isolated maps to the transducing phage 15B8 described by Kohara et al., the bacteriophage that carries the *envA* gene (unpublished observation). The isolation and characterization of this suppressor mutation may illuminate a functional linkage to *htrB*.

P1 transduction and restriction map analyses have localized the *htrB* gene approximately 6 kbp counterclockwise from *pyrC* and approximately 1.5 to 2.0 kbp from the *mdoA* gene(s). Approximately 10 to 15 enzymes, including the acyl carrier protein and the *mdoA* gene product(s), are involved in the production of membrane-derived oligosaccharides (MDO) (26). The synthesis of these compounds has been shown to be osmotically regulated (25), and *mdoA* mutations affect outer membrane composition and chemotaxis (10). The close linkage to *mdoA* could indicate that *htrB* affects MDO synthesis; although we have not determined if MDO synthesis is affected in *htrB* cells, this seems unlikely in light of the differences between the phenotypes of MDO-deficient mutants and the severity of the HtrB mutant phenotype.

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