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Isolation and Characterization of the *Escherichia coli htrD* Gene, Whose Product Is Required for Growth at High Temperatures

JOHN M. DELANEY,* DEBBIE ANG, AND COSTA GEORGOPOULOS

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

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Those genes in *Escherichia coli* defined by mutations which result in an inability to grow at high temperatures are designated *htr*, indicating a high temperature requirement. A new *htr* mutant of *E. coli* was isolated and characterized and is designated *htrD*. The *htrD* gene has been mapped to 19.3 min on the *E. coli* chromosome. Insertional inactivation of *htrD* with a mini-Tn*10* element resulted in a pleiotropic phenotype characterized by a severe inhibition of growth at 42°C and decreased survival at 50°C in rich media. Furthermore, *htrD* cells were sensitive to H_2O_2. Growth rate analysis revealed that *htrD* cells grow very slowly in minimal media supplemented with amino acids. This inhibitory effect has been traced to the presence of cysteine in the growth medium. Further studies indicated that the rate of cysteine transport is higher in *htrD* cells relative to the wild type. All of these results, taken together, indicate that the *htrD* gene product may be required for proper regulation of intracellular cysteine levels and that an increased rate of cysteine transport greatly affects the growth characteristics of *E. coli*.

The means by which bacteria survive and adapt to changing environmental conditions is a fundamental question. Fluctuation in growth temperature is one condition with which bacteria must cope. *Escherichia coli* is able to survive over a wide range of temperatures, from 10°C to 49°C (16). Many physiological changes occur within the cell to allow adaptation to these varying growth temperatures (16). Among those changes which occur in response to increased temperature are altered membrane composition (16), lipid metabolism (25), growth rate (16), and marked changes in the overall pattern of gene expression (14). In response to increasing temperature, perhaps one of the most striking changes in gene expression is the induction of the heat shock response.

The heat shock response is a highly conserved cellular response to environmental stress, especially rapid changes in temperature (for a review, see reference 30). In *E. coli*, about 20 genes are transiently expressed at increased levels following a shift up in growth temperature. These genes form a regulon under the coordinate control of the positive transcriptional regulator σ^{32}. At least some of the heat shock genes are essential for *E. coli* growth at all temperatures, indicating that these genes, and indeed the heat shock response itself, play a fundamental role in bacterial physiology.

In order to better understand how *E. coli* survives and adapts to exposure to high temperatures, a study has been initiated to define new genes whose products are required for growth at high temperatures. Libraries of *E. coli* cells harboring transposon insertions were created at 30°C. These libraries were subsequently screened at 42°C for the inability to grow well at this higher temperature. The genes defined by these insertions are designated *htr* (for high temperature requirement). Some of the genes isolated by this method have been described and fall into two broad classes based upon regulation of expression. The first class of *htr* genes is composed of previously unidentifi d heat shock genes and includes the *htrA* and *htrC* genes (22, 32). The *htrA* gene exemplifies a new class of heat shock genes, being under the exclusive control of the newly discovered σ^E protein (11). The HtrA protein has been shown to be a periplasmic endopeptidase (23). The *htrC* gene is a typical heat shock gene, being transcribed with the help of the σ^{32} polypeptide (32). The precise function of the HtrC protein is unknown, but it appears to be involved in global regulation of gene expression (32). The second class of *htr* genes defines a unique class of previously unidentified genes whose products are required at high temperatures but whose expression is not increased at the higher temperatures (17). This class includes the *htrB* and *htrP* genes (17). The precise function of the *htrB* and *htrP* gene products is unknown.

In this paper we describe the isolation and initial characterization of a new *htr* mutant, *htrD*. The *htrD* gene product is required for efficient cell growth at 42°C and for cell survival at 50°C. Mapping data indicate that the *htrD* gene is located at 19.3 min on the *E. coli* chromosome. In addition to sensitivity to high temperature, inactivation of the *htrD* gene leads to H_2O_2 sensitivity and to growth inhibition in the presence of cysteine. Further studies have shown that the *htrD* gene product may be involved in the regulation of cysteine transport.

MATERIALS AND METHODS

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

Media and bacterial growth. Bacteria were routinely cultured in either L broth or M9 minimal medium (27). When required, antibiotics were added to the following final concentrations: kanamycin, 100 μg/ml; ampicillin, 50 μg/ml; tetracycline, 15 μg/ml. Glucose and glycerol were added to a final concentration of 0.4% each. Sodium acetate was added to a concentration of 0.2%. Sucrose, when used for suppression of the HtrD temperature sensitivity (Ts^-) phenotype, was added to L agar plates at a concentration of 3 M. Amino acids were made up as 200× stock solutions and added as

* Corresponding author.

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

Strain, bacteriophage, or plasmid	Relevant characteristics[a]	Reference or source
Strains		
A326	*trxB*::Kan^r	M. Russel (35)
CF1652	Δ*relA*	M. Cashel
CF1693	Δ*relA* Δ*spoT*	M. Cashel
CG1071	*htrD*::mini-Tn*10* (Ts$^-$)	This paper
MC4100	*relA1* (AT^s)	6
JD158	MC4100 *htrD*::mini-Tn*10*	This paper
B178	W3110 *galE sup*$^+$	Our collection
JD214	B178 Δ*relA* (AT^s)	This paper
JD215	B178 *htrD*::mini-Tn*10*	This paper
JD216	B178 Δ*relA htrD*::mini-Tn*10*	This paper
JD283	MC4100 (λ*cIind*)	This paper
Bacteriophages		
T4gt7	Generalized transduction	G. G. Wilson (41)
P1 L4	Generalized transduction	L. Caro
λ1098	λ mini-Tn*10* transposon	J. C. Way (40)
1H1	λ *htrD*$^+$	18
1F10	λ *htrD*$^+$	18
λ*cIind*	Forms stable lysogens	Our collection
Plasmids		
pREG153	Low-copy-number cosmid vector, Amp^r	D. Low (31)
DA379	pREG153 *htrD*$^+$ cosmid	This paper
DA381	pREG153 *htrD*::mini-Tn*10* cosmid	This paper
pPMR20	M13mp18 *trxB*$^+$	M. Russel (35)

[a] AT^s, 3-amino-1,2,4-triazole sensitive.

described by Davis et al. (9). For growth inhibition, cysteine and cystine were added as described previously (9). For restoration of growth of cysteine-inhibited cells, the minimal media were supplemented with methionine and threonine to a final concentration of 0.5 mM (8), and homoserine was added to a final concentration of 6 mM. Modified low-phosphate MOPS (morpholine propanesulfonic acid) minimal medium (29), supplemented with glucose as described by Bochner and Ames (3), was used for labelling of cells for guanosine 3',5'-bispyrophosphate (ppGpp) assays (3, 34). Medium E (39), supplemented with 0.5% glucose, was used for amino acid transport assays (1). Bacterial sensitivity to 3-amino-1,2,4-triazole and to 1,2,4-triazole-3-alanine was determined as described by Rudd et al. (34). Bacterial growth was determined by monitoring the optical density at 595 nm.

Chemicals. Antibiotics, carbon sources, MOPS, amino acids, 3-amino-1,2,4-triazole, 1,2,4-triazole-3-alanine, H_2O_2, benzoate, and salicylate were obtained from Sigma Chemical Co. $^{32}P_i$ (500 μCi/mmol) was purchased from NEN/Dupont. L-[^{35}S]cysteine (1,042 Ci/mmol) was from ICN Biomedicals, Inc. L-[4,5-^{3}H]leucine (72 Ci/mmol) was obtained from Amersham.

Genetic techniques. Transduction crosses were performed by using either P1 or T4gt7 as described by Miller (27) and Wilson et al. (41), respectively. Inheritance of the *relA* allele was determined by screening for 3-amino-1,2,4-triazole sensitivity (34). Insertional mutagenesis utilizing the λ1098 (mini-Tn*10*) system was done as previously described (40).

Recombination of the *htrD*::mini-Tn*10* insertion onto Kohara bacteriophages 1H1 and 1F10 (18) was accomplished by first growing these bacteriophages on *htrD*::mini-Tn*10* bacteria, using the resulting lysates to infect MC4100 (λ) bacteria (strain JD283), and selecting for Tet^r transductants.

The *E. coli* genomic cosmid library was constructed by O'Connor et al. (31) in pREG153, a low-copy-number cosmid vector (two to four copies per cell). The original library was grown on a *supE* host. Helper bacteriophage λ *c*I857 *O*am29 was grown on this library, and the lysate was used to infect *htrD* (*sup*$^+$) bacteria in order to select for cosmids able to complement the *htrD* Ts$^-$ phenotype at 42°C on L agar plates containing ampicillin. Plasmid DNA was extracted for further characterization from those isolates that formed colonies at 42°C.

Recombination of the *htrD*::mini-Tn*10* insertion was accomplished by transducing the *htrD*-complementing cosmid DA378 into *htrD*::Tn*10* mutant bacteria by using transducing bacteriophage T4gt7 and selecting for Amp^r at 30°C. Amp^r transductants were then grown with selection in L broth and infected with T4gt7. The resulting lysates were used to infect wild-type B178. Bacteria carrying recombinant (Amp^r Tet^r) cosmids were selected on L agar plates supplemented with the appropriate antibiotics.

DNA manipulations. Cosmid DNA was extracted from bacteria and purified by the alkaline lysis procedure as described by Maniatis et al. (24). Cosmid DNA was labelled with [α-^{32}P]dATP (NEN/Dupont) by using the Bethesda Research Laboratories, Inc., nick translation kit. Hybridization of nick-translated DNA to the Kohara library was conducted as described previously (36).

Heat and H_2O_2 treatments. Bacterial cultures, grown overnight in L broth at 30°C with selection, were diluted 1:100 into L broth and incubated with shaking at 30°C to a cell concentration of approximately 2×10^8/ml. For measuring the cellular sensitivity to heat, 1 ml of cells either was taken from this culture and directly placed at 50°C or, for thermotolerance assays, was first placed at 42°C for 15 min prior to the 50°C treatment. Culture samples were withdrawn at specific intervals, serially diluted, and spotted onto L agar plates. Plates were incubated at 30°C for 36 to 48 h. For measuring H_2O_2 sensitivity, cultures were grown as described above for heat treatment. For single-H_2O_2-concentration experiments, 1 ml of cells was placed in a microcentrifuge tube at room temperature (23°C). $CuSO_4$ was added to a final concentration of 100 μM to enhance hydroxyl radical formation (5). H_2O_2 was then added to a final concentration of 8.8 mM. Cell viability was measured as described above. For experiments with various H_2O_2 concentrations, 10 ml of cells was centrifuged at 1,500 × *g* for 10 min, washed once in M9 salts solution, and resuspended in an equal volume of M9 salts solution. One milliliter of this cell suspension was then pipetted into each of seven microcentrifuge tubes (one tube for each dosage of H_2O_2). $CuSO_4$ was added to a final concentration of 100 μM. In some cases, cysteine (0.3 mM), benzoate (10 mM), or salicylate (10 mM) was also added. Cell viability was determined as described above. Alternatively, amino acids, benzoate, or salicylate (at concentrations described above) was added to M9 minimal agar plates. Plates were then overlaid with cells in M9 minimal soft agar. H_2O_2 (5 μl of an 8.8 M solution) was then spotted onto the center of the plate. Plates were incubated at 30°C overnight. Relative zones of killing were then measured and compared.

Measurement of ppGpp. $^{32}P_i$ labelling of nucleotides, formic acid extraction, and precipitation of excess $^{32}P_i$ were performed as described by Bochner and Ames (3). Basal ppGpp levels were determined by the method of Rudd et al. (34). Twenty-five microliters of extracted samples was ap-

plied to polyethyleneimine cellulose sheets (Brinkman Instruments, Inc.). Chromatography was conducted in solvents Tb and Sb as described by Bochner and Ames (3). The radioactive spots were visualized by autoradiography after overnight exposures. To measure changes in ppGpp levels in the presence of cysteine, cells were grown in MOPS supplemented with all amino acids except cysteine to an optical density of 0.5 at 595 nm. Cysteine was added, and the culture was incubated at 37°C. Culture samples were taken prior to and 15 min after cysteine addition. The ppGpp levels were determined as described above.

Amino acid transport. Growth and preparation of bacteria and measurement of amino acid transport were performed by the procedure described by Baptist and Kredich (1). L-[^{35}S]cysteine and L-[4,5-^{3}H]leucine were diluted in nonradiolabelled solutions of either amino acid prior to use. Osmotic shock was conducted by the method of Berger and Heppel (2) as described by Baptist and Kredich (1). Cell protein concentrations were determined by the method of Bradford (4) by using bovine serum albumin as a standard.

RESULTS

Isolation and mapping of the *htrD* gene. In an attempt to isolate genes required for cell growth only at high temperatures, a library of mini-Tn*10* (Tet^r) transposon insertions was created in the wild-type *E. coli* strain B178 at 30°C. This library was then screened for mutants which were unable to grow at 42°C on rich media. One of these temperature-sensitive mutants was designated *htrD*. The Ts$^-$ phenotype and the mini-Tn*10* insertion defining *htrD* were shown by P1 transduction analysis to be 100% linked. Furthermore, when transduced into other wild-type strains, the *htrD*::mini-Tn*10* insertion was found to breed true for temperature-sensitive growth (data not shown).

To localize the position of the *htrD* gene on the *E. coli* chromosome, an *E. coli* genomic library, constructed with a pREG153 (Amp^r) low-copy-number cosmid vector, was screened for complementation of the HtrD Ts$^-$ phenotype. Isolates which enabled *htrD* bacteria to grow at the nonpermissive temperature were selected for further study. Cosmid DNA, extracted from these isolates and transformed back into *htrD*::mini-Tn*10* cells, was shown to fully complement the Ts$^-$ phenotype. In addition, it was shown that these complementing cosmids were able to recombine with the *htrD* mini-Tn*10* insertion. Cosmids isolated in this manner were then labelled with ^{32}P by nick translation and used to probe the *E. coli* genomic library of Kohara et al. (18). Specific hybridization was observed with Kohara bacteriophages λ213 (1H1) and λ214 (1F10), which carry DNA from the *E. coli* 19.3 min region. To ensure that the *htrD* gene mapped to this region, bacteriophages λ213 and λ214 were grown on *htrD*::mini-Tn*10* cells and the resulting lysates were used to transduce the mini-Tn*10* Tet^r marker into wild-type bacteria. By using this procedure, it was shown that only bacteriophages λ213 and λ214 were capable of recombining with the *htrD*::mini-Tn*10* insertion. Since there is approximately 9 kb of overlap between bacteriophages λ213 and λ214, the *htrD* gene must be located in that region (18). A thioredoxin reductase null mutation (*trxB*::Kan^r) was also found to recombine with Kohara bacteriophages λ213 and λ214. However, the *htrD* gene is not allelic to *trxB* because a *trxB* minimal clone (pPMR20) (35) did not complement the HtrD Ts$^-$ phenotype. Furthermore, restriction analysis has revealed that the *htrD*::mini-Tn*10* insertion lies within approximately 200 bp of the 3′ end of the *trxB* open reading frame (data not shown).

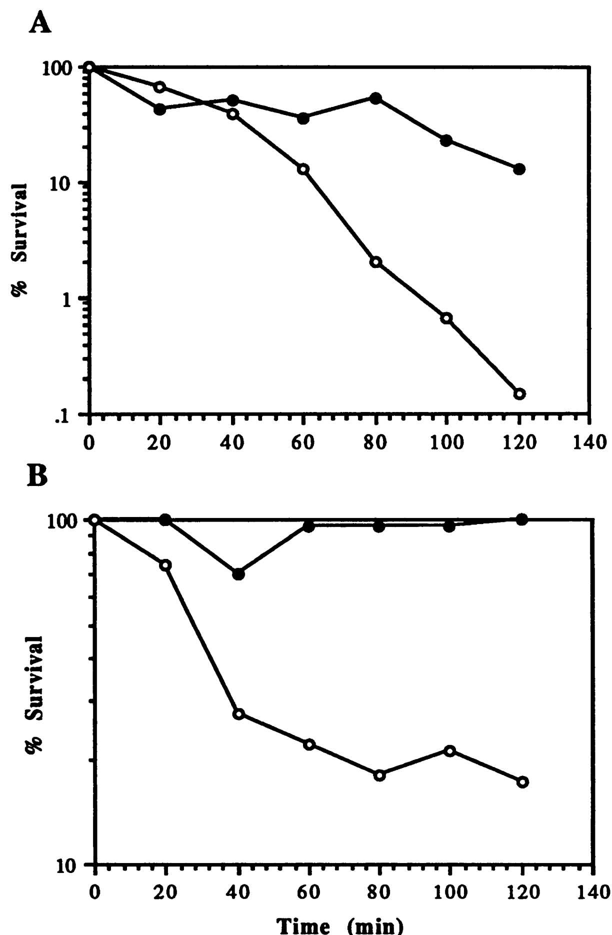

FIG. 1. Sensitivity of *htrD* cells to heat. Cells were grown at 30°C in L broth and either placed immediately at 50°C (A) or pretreated for 15 min at 42°C prior to placement at 50°C (B). Symbols: ●, wild-type (MC4100); ○, *htrD*::mini-Tn*10* mutant (JD158).

Phenotypic characterization of the *htrD* mutant. An interesting aspect of the Ts$^-$ phenotype of *htrD* cells is that at 42°C cell survival was not noticeably affected. Rather, cell growth is severely inhibited. When *htrD* cells are incubated at 42°C on L agar plates for 24 h, colonies eventually appear at 100% plating efficiency but colony growth is very slow. When such colonies are returned to 30°C, normal growth is restored. Cellular morphology appears to be unaffected at any temperature by the *htrD*::mini-Tn*10* insertion mutation.

To determine whether the *htrD* gene product is required for cell survival at higher temperatures, wild-type and *htrD* cells were exposed to 50°C (Fig. 1). As can be seen, the *htrD*::mini-Tn*10* mutant was much more sensitive to the lethal effects of heat than was its wild-type, isogenic parent. This result indicates that *htrD* is essential for survival at 50°C. When *E. coli* cells are exposed to a brief, sublethal dose of heat, they become resistant to a subsequent, potentially lethal heat exposure. This phenomenon is known as thermotolerance (30). Isogenic wild-type and *htrD* cells were treated at 42°C for 15 min and then exposed to 50°C to determine whether the *htrD* mutation affects the ability of *E.*

coli to develop thermotolerance. By comparing the relative rates of killing for heat-pretreated and nontreated cultures, it can be seen that the survival of both wild-type and *htrD* cells was enhanced when a 15-min heat shock was administered (Fig. 1B). However, *htrD* cells did not achieve the same degree of heat resistance as wild-type cells. These results indicate that *htrD* cells are inherently more sensitive to potentially lethal heat exposures and that the *htrD* gene product, while not required for growth at lower temperatures, is essential for survival at extreme temperatures.

Since the modalities of action of heat are similar to those of H_2O_2 (12, 26, 33) and since expression of some heat shock genes is induced by H_2O_2 (28, 38), the sensitivity of *htrD* cells to H_2O_2 was investigated. The *htrD* insertion mutant was more sensitive to H_2O_2 than the wild-type parent (Fig. 2A), such that after a 60-min exposure to 8.8 mM H_2O_2 the survival of *htrD* cells was decreased approximately 10-fold relative to that of the wild type. Toxicity of *E. coli* to increasing concentrations of H_2O_2 is not a linear function but instead is characterized by a bimodal curve, showing two regions of killing separated by a region of relative resistance (5). Mode I killing occurs at low H_2O_2 concentrations, whereas higher concentrations result in mode II killing (15). When wild-type and *htrD* cells were exposed to increasing H_2O_2 concentrations, the *htrD*::mini-Tn*10* mutant again exhibited an increased sensitivity to the chemical (Fig. 2B). The *htrD* mode II survival curve is shifted to the left relative to that of the wild type. This may indicate either a greater resistance to mode I killing combined with an increase in sensitivity to mode II killing or a general overall increase in H_2O_2 sensitivity.

Growth characteristics of *htrD* cells. To study the effect of the *htrD* mutation on cell growth, the *htrD*::mini-Tn*10* insertion mutation was freshly transduced into wild-type bacteria and, after single-colony isolation to remove contaminating bacteriophage, the resulting fresh transductants were immediately inoculated into rich media (L broth) for growth rate analysis. It was found that growth of *htrD* mutant cells was inhibited for several hours relative to that of the wild type (Fig. 3). After this initial lag period, growth resumed and continued at a wild-type rate until stationary phase was reached. Both wild-type and *htrD* cells eventually achieved the same cell density. Interestingly, the length of the observed lag period was dependent upon the relative age or experience of the cells used as the inoculum. As these cells were serially recultured in either liquid or solid media, the lag phase exhibited decreased until the *htrD* growth curve was indistinguishable from that of the wild type (data not shown). Presumably, the cause of this decrease in the growth lag could be the acquisition of extragenic suppressors in the culture. Since serial propagation in either liquid or solid media resulted in a decrease in the length of the lag phase, it does not seem likely that some contaminant in the agar-based solid media resulted in a dehabituation of cells to growth in broth. In addition, it was found that neither the length of the lag phase nor the relative rate of logarithmic growth in rich media was influenced by the growth temperature (data not shown). The observed lag does not seem to be due to the accumulation of dead cells contributing to the optical density, since the *htrD*::mini-Tn*10* mutant did not lose viability during stationary phase compared with the wild type, even after several days of incubation at 30°C (data not shown).

When *htrD* cells were grown on rich media at the nonpermissive temperature, some large colonies appeared that were subsequently shown to retain the ability to grow at high

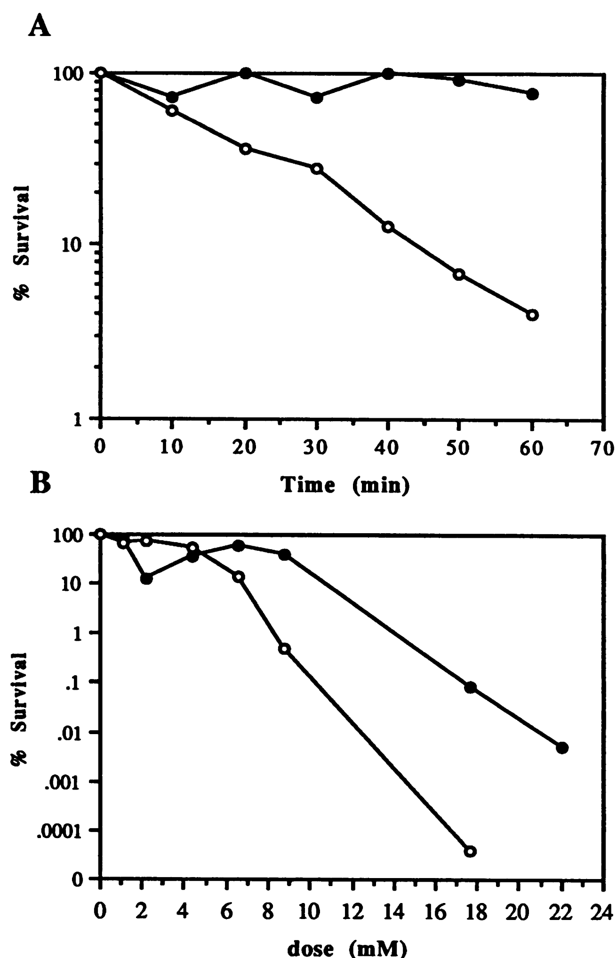

FIG. 2. Sensitivity of *htrD* cells to H_2O_2. (A) Exposure to 8.8 mM H_2O_2. (B) Exposure to increasing concentrations of H_2O_2. Symbols: ●, wild-type (MC4100); ○, *htrD*::mini-Tn*10* mutant (JD158).

temperature. Several of these revertants have been analyzed genetically and were found to be the result of spontaneous extragenic suppressor mutations. That is, no *htrD*-suppressing mutations were found to be linked to the *htrD*::mini-Tn*10* (Tet^r) insertion through cotransduction analysis using bacteriophage P1. This result may indicate that the *htrD*::mini-Tn*10* insertion lies within the *htrD* gene itself and is not exerting a polar effect on a downstream gene. The rate at which these suppressors were formed was quite high in *htrD* cells, being approximately 5×10^{-3} on rich media. Interestingly, on rich media, the rate of suppressor formation decreased by an order of magnitude in relaxed (*relA*) bacterial backgrounds. Furthermore, an *htrD relA spoT* triple mutant had an even lower rate of suppression, being about 100-fold less than in the *htrD* strain. The exact cause of this decrease in the observed rate of suppression is unknown but may be due to an ineffectiveness of some suppressors in a relaxed background.

Analysis of basal ppGpp levels. In *E. coli* during periods of amino acid limitation, the nucleotide ppGpp rapidly accumulates within the cell (7). This accumulation is the hallmark of the stringent response and is dependent upon both the *relA* and *spoT* genes (7, 10). Induction of the stringent response has a global effect on gene expression (7). To determine

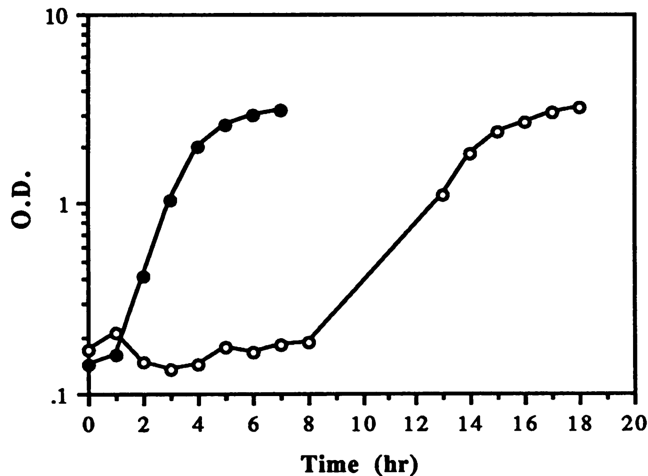

FIG. 3. Growth lag of *htrD* cells grown at 30°C in L broth. The optical density (O.D.) of the culture was determined at 595 nm. A representative experiment is shown. Symbols: ●, wild-type (MC4100); ○, *htrD*::mini-Tn*10* mutant (JD158).

whether *htrD* has any effect on the stringent response, basal levels of ppGpp were analyzed in both isogenic wild-type and *htrD* strains (Table 2). First, when cells were grown in unsupplemented minimal media, basal ppGpp levels were approximately twofold lower in the *htrD* mutant than in the isogenic, wild-type parent. As expected, a *relA* mutation resulted in lower ppGpp levels in both wild-type and *htrD*::mini-Tn*10* strains. Second, in accordance with a normal stringent response, when wild-type cells were grown in minimal media supplemented with a mixture of amino acids, ppGpp levels were reduced relative to the levels seen in unsupplemented media. Interestingly, the opposite result was seen for the *htrD* mutant. In this case, ppGpp levels were actually higher in the presence of amino acids than in unsupplemented media. Indeed, when grown in amino acid-supplemented media, basal ppGpp levels in the *htrD* mutant were about fivefold higher than in wild-type cells grown in the same medium. These data imply that, in *htrD*::mini-Tn*10* cells, the stringent response was induced in the presence of amino acids and furthermore that this induction could be relieved by omitting amino acids from the growth media. This unusual increase in ppGpp levels in the presence of amino acids occurred even in a *relA htrD* double mutant, indicating that the *htrD* mutation affects the *E. coli* stringent response at a fundamental level and does not simply reflect a regulatory interaction between HtrD and RelA.

At least one prediction can be made on the basis of the finding of relatively lower basal ppGpp levels in *htrD* cells in minimal media without amino acids. That is, on unsupplemented minimal media, *htrD* cells should be sensitive to the drugs 3-amino-1,2,4-triazole and 1,2,4-triazole-3-alanine (34). These drugs are both histidine antimetabolites. Since histidine biosynthesis is dependent upon ppGpp, cells exhibiting reduced ppGpp levels are more sensitive to both 3-amino-1,2,4-triazole and 1,2,4-triazole-3-alanine than are cells with normal ppGpp levels (7). Indeed, when plated on minimal media containing either 3-amino-1,2,4-triazole or 1,2,4-triazole-3-alanine, *htrD* growth was much more inhibited compared with the wild type, consistent with the lower basal ppGpp levels in these cells (data not shown).

TABLE 2. Effect of *htrD*::mini-Tn*10* on ppGpp levels

Strain	ppGpp level (pmol/OD_{600})[a] in:	
	Minimal medium (no amino acids)	Minimal medium plus all amino acids
B178	21.7	4.5
JD215	12.1	24.1
JD214	14.0	7.4
JD216	5.0	15.0

[a] Residual ppGpp levels in the *relA* strains are probably due to the presence of a wild-type *spoT* gene (10). OD_{600}, optical density at 600 nm.

Effect of amino acids on *htrD* growth. Since the addition of amino acids had an obvious effect on *htrD*::mini-Tn*10* cells, the growth rate of *htrD* cells in the presence or absence or specific amino acids was examined more precisely. It should be noted that the *htrD* mutation did not result in auxotrophy for any amino acid (data not shown). Instead, growth of *htrD* cells was severely inhibited in media supplemented with all amino acids (Fig. 4A). This growth inhibition was found to occur independently of the *relA* gene (Fig. 4A) and is in agreement with the *relA*-independent accumulation of ppGpp found in *htrD* cells grown in amino acid-supplemented media (Table 2).

To determine whether the growth inhibition is due to amino acid supplementation in general or is instead caused by the presence of a specific amino acid, individual amino acids were sequentially removed from or added to the growth medium. The results of these experiments revealed that the presence of cysteine alone was sufficient to inhibit the growth of *htrD* cells (Fig. 4B). When cysteine was removed from the amino acid supplement, *htrD* cell growth was indistinguishable from that of the wild type. It should be noted that the *htrD*::mini-Tn*10* insertion mutant was also sensitive to cystine (data not shown). Equivalent sensitivities were obtained when equal molar concentrations of either cysteine or cystine were used. Furthermore, no *htrD* suppressors were found to be cysteine auxotrophs.

Cysteine is known to inhibit the activity of several enzymes (8, 13, 21). One such enzyme is homoserine dehydrogenase, which reduces aspartate semialdehyde to form homoserine (8). Homoserine is a common intermediate in the biosynthesis of methionine and threonine, and inhibition of homoserine dehydrogenase activity, therefore, leads to a growth inhibition resulting from a blockage of methionine and threonine biosynthesis (8). This inhibition can be overcome by the addition of these two amino acids to the growth medium (8). Consistent with this prediction, the addition of methionine and threonine or of homoserine to minimal media containing only cysteine overcame the growth inhibition of *htrD* cells (Fig. 4C). Growth was not completely restored by these supplements, but this may be due to the inhibition of other biosynthetic pathways by cysteine. Temperature did not seem to have an effect on any of these basic findings, except that *htrD* cells grew slightly more slowly than wild-type cells in M9 minimal media at 42°C (data not shown). Furthermore, no differences in any *htrD* phenotypes were observed when the growth medium sulfur concentration or sulfur compound (i.e., sulfite or sulfate) was altered. In addition, there was no observable effect of adding *O*-acetylserine to the growth media. *O*-Acetylserine is the immediate precursor of the carbon moiety of cysteine in *E. coli* and is also an internal inducer of cysteine biosynthesis in this organism (20). Therefore, the observed phenotypes of the *htrD*::mini-Tn*10* mutant are likely due to the effects of cysteine alone.

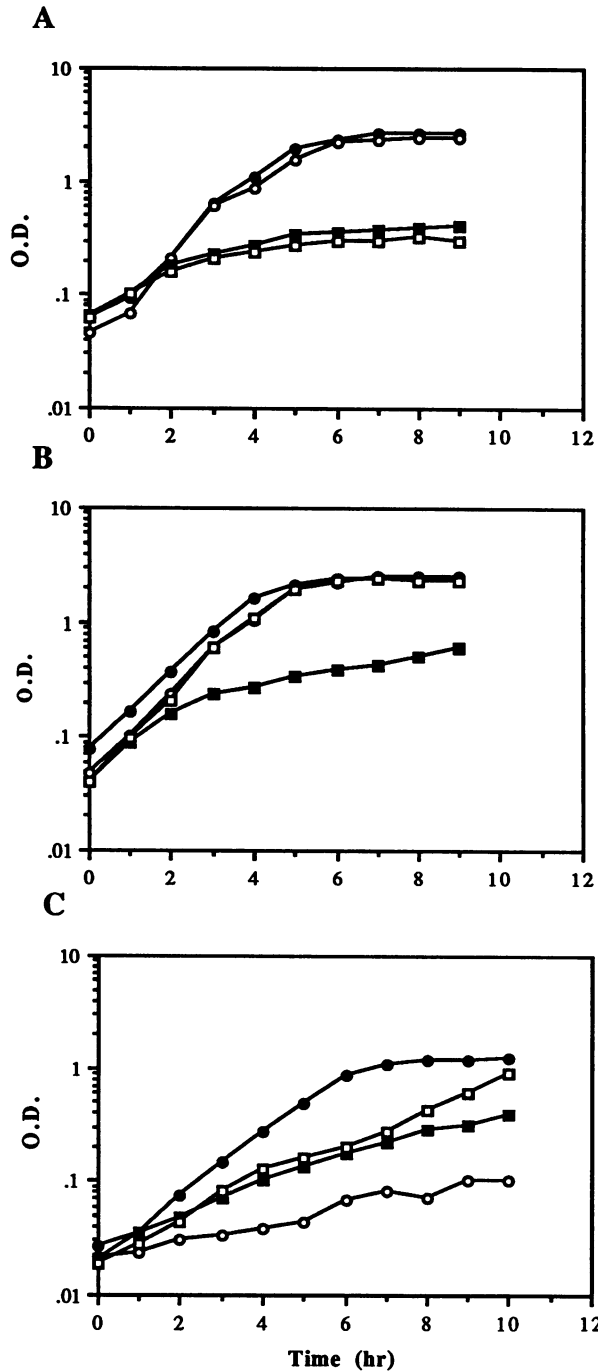

FIG. 4. Effect of amino acid supplementation on *htrD*::mini-Tn*10* mutant growth. Cells were grown at 30°C in M9 minimal (cysteine-free) medium overnight. These cultures were then diluted 1:100 into M9 minimal medium with or without supplements and incubated with shaking at 30°C. The optical density (O.D.) of the cultures was determined at 595 nm. (A) M9 minimal medium supplemented with a mixture of all 20 amino acids. ●, wild type (B178); ○, Δ*relA* mutant (JD214); ■, *htrD*::mini-Tn*10* mutant (JD215); □, Δ*relA htrD*::mini-Tn*10* mutant (JD216). (B) M9 minimal medium supplemented with a mixture of all 20 amino acids (filled symbols) or all amino acids except cysteine (open symbols). ● and ■, wild type (B178) and *htrD*::mini-Tn*10* mutant (JD215), respectively. (C) *htrD*::mini-Tn*10* mutant (JD215) growth in M9 minimal medium supplemented with no amino acids (●), cysteine only (○), cysteine and homoserine (■), or cysteine, methionine, and threonine (□).

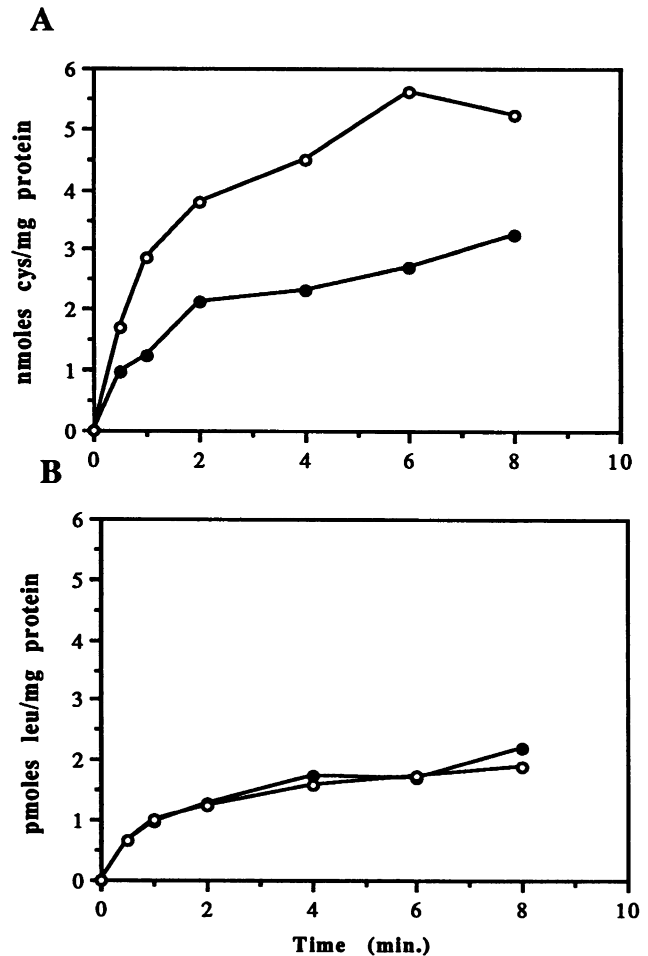

FIG. 5. Rate of amino acid transport in wild-type (B178) (●) and *htrD*::mini-Tn*10* mutant (JD215) cells (○) at 25°C. (A) Rate of cysteine transport. (B) Rate of leucine transport.

Since cysteine addition results in methionine and threonine limitation, we suspected that ppGpp levels may be influenced by the presence of cysteine. In fact, addition of cysteine to the growth medium did affect ppGpp accumulation. In wild-type cells, ppGpp levels decreased 50% within 15 min of cysteine addition. In contrast, ppGpp levels in the *htrD*::mini-Tn*10* mutant increased almost twofold under the same conditions (data not shown).

Cysteine transport. Given that certain enzymatic activities, particularly homoserine dehydrogenase, are inhibited by such a common amino acid as cysteine, the intracellular concentration of cysteine must be tightly regulated. One way to control intracellular levels of a substance is to limit its entry into the cell. Since the *htrD* mutant was affected by normal extracellular levels of cysteine, the transport of this amino acid may be altered in *htrD* cells, resulting in higher intracellular cysteine levels.

In order to investigate this possibility, the rate of cysteine transport in wild-type and *htrD* mutant cells was examined. The results of these experiments indicate not only that the initial rate of cysteine transport was higher in the *htrD*::mini-Tn*10* mutant but also that the overall steady-state rate was higher (Fig. 5A). At higher temperatures (i.e., 42°C), the rate of cysteine transport in both wild-type and

htrD mutant cells increased proportionally to the rates observed at the lower temperature, such that the rate of transport was still dramatically higher in the *htrD* mutant as compared with wild-type bacteria (data not shown). To establish that amino acid transport is not generally defective in *htrD* cells, the rate of leucine uptake was studied. Leucine transport in the *htrD*::mini-Tn*10* mutant was indistinguishable from that in the isogenic, wild-type parent (Fig. 5B). It has been established that a periplasmic protein facilitates cystine transport in *E. coli*, rendering this transport system sensitive to osmotic shock (1, 2, 20). The rate of cysteine transport in osmotically shocked wild-type and *htrD* mutant cells was dramatically reduced (data not shown), the observed basal levels of transport being the same in both cell types. These results indicate that cysteine transport in the *htrD*::mini-Tn*10* mutant was sensitive to osmotic shock and, more importantly, that the regulation of this transport system was affected by the *htrD* mutation.

DISCUSSION

Cysteine biosynthesis plays an important role both in cellular metabolism and in the sulfur cycle itself since it involves the critical process of covalent binding (fixation) of reduced, inorganic sulfur to carbon (19). The pathway of cysteine biosynthesis has been studied extensively and is well characterized for both *Salmonella typhimurium* and *E. coli* (20). Most of the genes involved in cysteine metabolism, including those responsible for sulfur uptake and reduction, have been identified and studied in both of these organisms (1, 20). These genes make up a regulon, being under the common control of the *cysB* gene product (19, 20). The *cysB* gene product appears to be autoregulated (20).

The transport of cystine into the cell has been relatively well characterized (2, 19, 20). In *S. typhimurium*, cystine enters the cell by three different systems (CTS-1, CTS-2, and CTS-3), each characterized by unique reaction kinetics (20). Only CTS-1 appears to be part of the cysteine regulon and is also unique among these systems in its sensitivity to osmotic shock, implying the existence of a periplasmic cystine-binding protein (20). Regulatory mutations resulting in high expression of CTS-1 lead to cystine sensitivity (1). In *E. coli*, two cystine transport systems have been identified (20). Both of these systems are sensitive to osmotic shock, and in fact a cystine-binding protein has been purified from this organism (2). Only one of these systems is associated with the cysteine regulon (20). Unfortunately, the genes responsible for cystine or cysteine transport have not been identified in either *S. typhimurium* or *E. coli* (20).

Cellular sensitivity to cysteine seems to result from the inhibition of homoserine dehydrogenase activity. This leads to a decreased intracellular level of homoserine, which should effectively block methionine and threonine biosynthesis (8). This conclusion is supported by the finding that addition of either homoserine or methionine and of threonine can partially restore growth to cysteine-inhibited *htrD*::mini-Tn*10* cells (Fig. 4C). Starvation for methionine and threonine, caused by an increase in intracellular cysteine, may also explain the high basal ppGpp levels found in *htrD* cells in minimal media supplemented with amino acids. Amino acid limitation induces the *E. coli* stringent response (7), and it has recently been shown that addition of cysteine to the growth medium results in a transient amino acid starvation in *E. coli* (37). As indicated by the accumulation of ppGpp levels, cysteine induces the stringent response in *htrD* cells at cysteine levels that do not induce the stringent response in wild-type cells. It was found that *htrD* cells actually begin to accumulate ppGpp after cysteine is added to the growth medium, even though ppGpp levels decline in wild-type cells treated in a similar manner. This indicates that cysteine addition results in an amino acid limitation in the *htrD*::mini-Tn*10* mutant.

Wild-type *E. coli* growing on minimal medium plus amino acids is more sensitive to H_2O_2 than when growing on unsupplemented medium. Removal of cysteine from the medium greatly decreases sensitivity to H_2O_2. Likewise, wild-type cells growing on minimal medium supplemented with cysteine alone are more sensitive to H_2O_2 than when cysteine is not present (unpublished observations). Other amino acids also contribute to this enhancement of H_2O_2 sensitivity; however, cysteine seems to play a more prominent role. Therefore, the H_2O_2 sensitivity of the *htrD*::mini-Tn*10* mutant may be the result of an abnormally high intracellular cysteine concentration as well. A high concentration of cysteine may change the reducing potential of the cytoplasm enough to effectively increase the relative rate of hydroxyl radical formation, thus rendering the cell more susceptible to H_2O_2. Alternatively, cysteine may act as a weak acid within the cell, effectively enhancing the lethal effects of oxidants such as H_2O_2. In support of this idea, addition of benzoate or salicylate to the growth medium increases the H_2O_2 sensitivity of wild-type cells in an additive fashion (data not shown). Furthermore, the addition of cysteine had a similarly additive effect, increasing the effect of benzoate and salicylate on the H_2O_2 sensitivity of wild-type cells. By effectively increasing the oxidizing potential of H_2O_2, increased intracellular cysteine levels may increase the sensitivity of the *htrD*::mini-Tn*10* mutant to this chemical.

The *E. coli htrD* gene was isolated in a study aimed at defining new genes whose products are required for growth at high temperature. Insertional inactivation of this gene leads to a significantly increased rate of cysteine transport as compared to the wild type. The specific cysteine transport system affected by this mutation is unknown. However, osmotic shock reduces cysteine transport activity in *htrD* cells, indicating the involvement of a periplasmic protein in the affected system. Interestingly, the temperature sensitivity of *htrD* cells on rich medium seems to be suppressed by the addition of large amounts of sucrose to the medium (data not shown). Addition of sucrose is known to interfere with transport processes, suggesting that inhibition of cysteine transport in *htrD* cells may restore normal growth. The immediate result of this higher rate of cysteine transport is a cellular sensitivity to cysteine.

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