Multicopy Suppressors of Prc Mutant *Escherichia coli* Include Two HtrA (DegP) Protease Homologs (HhoAB), DksA, and a Truncated RlpA

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We have isolated three multicopy suppressors of the conditional lethal phenotype of a *prc* (*tsp*) null strain of *Escherichia coli*. One of these suppressors included two novel putative protease genes in tandem that map to 3400 kb or 72.5 centisomes on the chromosome. We propose the names *hhoA* and *hhoB*, for *htrA* homolog, to denote that these genes encode proteins that are 58 and 35% identical, respectively, to the HtrA (DegP) serine protease and 36% identical to each other. The HhoA and HhoB proteins are predicted to be 455 and 355 amino acids, respectively, in length. The mature HhoA protein is periplasmic in location, and amino-terminal sequencing shows that it arises following cleavage of a 27-amino-acid signal peptide. Searches of the protein and DNA databases reveal a rapidly growing family of homologous genes in a variety of other bacteria, including several which are required for virulence in their host. Deletion of the *hhoAB* genes shows that they are not required for viability at high temperatures like the homologous *htrA* but grow more slowly than wild-type strains. A second multicopy *prc* suppressor is the *dksA* (*dnaK* suppressor) gene, which is also a multicopy suppressor of defects in the heat shock genes *dnaK*, *dnaJ*, and *grpE*. The *dksA* gene was independently isolated as a multicopy suppressor of a *mukB* mutation, which is required for chromosomal partitioning. A third dosage-dependent *prc* suppressor includes a truncated rare lipoprotein A (*rlpA*) gene.

The genes for least seven periplasmic proteases of *Escherichia coli* have been cloned, and there is evidence from in vivo and in vitro experiments that more exist. The function of many of these proteases in the cell is unknown, but most of them have been implicated in the cleavage of various recombinant proteins. The *ptr* gene encodes protease III or Pi, a zinc metalloprotease that is homologous to mammalian insulin-degrading enzymes and a variety of mitochondrial processing peptidases from yeasts to humans (16). The *htrA* (*degP*) gene encodes a trypsin-like serine protease that is required for growth at high temperatures (32). The *lep* and *lsp* genes encode proteases that cleave the signal peptides from exported proteins and lipoproteins, respectively (23, 62). Prc is a protease that processes the C terminus of FtsI (penicillin-binding protein 3) and is required for protection from combined thermal and osmotic stress (20). The *sppA* gene and probably the *sohB* gene encode homologous proteases that degrade signal peptides after their processing (4, 22). Additionally, the outer membrane-bound protease OmpT (protease VII) is specific for substrates with paired basic residues (55).

We have created a number of protease-deficient strains of *E. coli* to help examine their role in vivo and to help stabilize recombinant proteins produced in *E. coli*. We have also used several genetic selections to identify and clone additional protease genes. One of these selections involved isolating multicopy suppressors of the conditional lethal phenotype exhibited by a *prc* null strain grown at high temperature on hypotonic media.

The *prc* gene was isolated by complementation of a *prc*

mutant strain, and Prc was shown to cleave the C terminus of FtsI (PBP-3) in vivo (20). The 660-residue Prc (Tsp) protein was purified from *E. coli* by several groups, using its ability to cleave fragments of the λ *c*I repressor (47) and the B chain of human relaxin (43a) in vitro. Additionally, the *prc* gene was isolated as the target of a Tn*5* insertion that conferred increased susceptibility to a number of antibiotics (45). Prc homologs have been identified in a divergent group of prokaryotes including several cyanobacteria (9, 46), *Neisseria gonorrhoeae* (8), *Haemophilus influenzae* (17), and *Bartonella bacilliformis* (GenBank accession no. L37094). A domain in the Prc family of proteins is similar to a domain in the retinol-binding proteins, indicating a common folding domain that may form a binding pocket in these proteins for hydrophobic substrates (46, 47).

Strains with a *prc* null mutation exhibit a conditional lethal phenotype in that they filament and die at or above 40°C when grown in hypotonic media (20). Cell extracts from a *prc* null strain still contain proteolytic activity with a Prc-like substrate specificity (43b, 48). This suggests the presence of an additional protease(s) with a similar or overlapping substrate specificity. We reasoned that overproduction of this putative isofunctional protease may be able to suppress the *prc* null mutation. To this end, we isolated and characterized multicopy suppressors from a random plasmid library of *E. coli* chromosomal clones. Here, we present data for three multicopy Prc suppressors, one of which contains two novel putative serine protease genes, which we named *hhoA* and *hhoB* (for *htrA* homolog). These genes are identical to those subsequently named *degQ* and *degS* and presented in the accompanying article (60).

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains with their genotypes and sources are listed in Table 1. LB, 2YT, M9 glucose, and the hypotonic medium described

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TABLE 1. *E. coli* strains used in this study

Strain	Genotype	Source or reference
X90	*ara* Δ(*lac pro*) *gyrA argE*(Am) Rif^r *thi-1* F′ *lacI*^q *lac*^+ *pro*^+	48
KS1000	X90 Δ*prc*::Kan *eda-51*::Tn*10*	48
W3110	Wild type	ATCC 4474
WG1	Wild type	ATCC 5073
SB550	WG1 Δ*fhuA* Δ*hhoA*	This study
SB536	WG1 Δ*fhuA* Δ*hhoAB*	This study
SB600	WG1 Δ*fhuA* Δ*hhoAB* Δ*htrA*	This study
BW16824	F′ pOX38::Tn*10-11*/DE3 (*lac*)X74 *rep-71* Δ*phoA532 phn*(*Eco*B)	38
BW19333	F′ 128::Tn*10-11 lacI*^q *lac*ZM15/DE3(*lac*)X74 Δ*phoA532* Δ(*phnC?DEFGHIJKLMNOP*)-*33-30* Δ*uidA*::*pir-116*	B. Wanner

by Hara et al. (20) (5 g of yeast extract per liter, 2.5 g of tryptone per liter, 15 g of agar per liter) were used. Sucrose agar is LB agar with 5% sucrose and no NaCl. Low-phosphate medium for induction of the *phoA* promoter was described by Fuh et al. (19).

DNA manipulations. Enzymes for DNA manipulations were purchased from New England Biolabs, except for PCR reagents, which were purchased from Perkin-Elmer. DNA sequencing of both strands was performed in an ABI373 automated sequencer with dye-labeled terminators (Applied Biosystems Division of Perkin-Elmer) or Sequenase reagents (United States Biochemical) and [³³P]dATP (Dupont). Oligonucleotides were synthesized by the Genentech Organic Synthesis Department. ³³P-labeled nucleotides were obtained from Amersham.

Plasmids. A library of *E. coli* W3110 chromosomal DNA was prepared by ligating 3 to 6-kb fragments from a *Sau*3AI partial digest into the *Bam*HI site of dephosphorylated pBR322. The initial pHhoAB plasmid isolated from the library contained a 4-kb fragment including the sequence presented in Fig. 2, an additional 0.6 kb of 5′-flanking sequence, and an additional 0.3 kb at the 3′ end. The initial *rlpA* plasmid contained a 3.6-kb fragment of the *mrdAB-rlpA* locus (56) and a 3.2-kb fragment of the putative *yihKL* locus (42) that were artificially ligated during library construction. The suppression data presented in Table 2 were obtained with a deletion version of this plasmid (pRlpA′). The only intact open reading frame in the plasmid insert encodes a 123-residue fusion protein containing the first 102 residues of RlpA fused to residues 143 to 158 of YihK and the residues PRAFR encoded by pBR322. The initial *dksA* plasmid contained a 2.6-kb fragment with the *dksA* and *sfs1* genes (28, 29). Deletion of a 1.9-kb *Cla*I fragment from this plasmid leaves only the *dksA* coding sequence intact to form pDksA.

The *hhoAB* genes were both deleted by removing a 1.6-kb *Sac*II fragment (bases 1694 to 3286 in Fig. 2) from the original clone. Individual *hho* gene deletions were constructed by oligonucleotide-directed mutagenesis (31) of the cloned genes in an f1 ori plasmid derivative of phGHbp (19). The *hhoA* gene deletion includes all of the coding sequence and the intergenic region between *hhoA* and *hhoB*. It was created by using the oligonucleotide ACGAGAGCAG GAATA ATG TTTGTGAGCTCTTAC. This places the *hhoB* gene under control of the *hhoA* regulatory sequences. The *hhoB* gene deletion was created by using the oligonucleotide TGGTATGCTGCTGCCAATTAAGTCGTGCGC. The Δ*htrA* allele was constructed by PCR in vitro and recombined into the chromosome. The sequence of the deletion junction with an *Eco*RI restriction site is TCTTCGGTGAGAATTC GGGCACTCCG. These deletion alleles were subcloned into pS1080 for recombination into the chromosome as discussed below. Plasmid pS1080 (38) is a derivative of pWM7 (38) and pWM95 (60a). It has an R6Kγ *oriR* origin of replication, the multiple-cloning site and intergenic region of bacteriophage f1 from pBluescriptKS+ (Stratagene), the *bla* gene from pBR322, and the *sacB* gene from *Bacillus subtilis*.

Plasmids pHhoB and pHhoA are the *hhoB* and *hhoA* deletion constructs, respectively, in the phGHbp backbone. Oligonucleotide-directed mutagenesis was also used to fuse *hhoA* and *hhoB* to the *phoA* promoter in phGHbp for protein production in low-phosphate media (19). Plasmid pKS6-1w, which places the *prc* gene under control of the *lac* promoter, was obtained from Karen Silber (47). Details of any plasmid construction will be provided upon request.

Isolation of prc suppressors. The *E. coli* plasmid library was transformed into the *prc* null strain KS1000 (48). The transformants were grown at 37°C for at least 1 h and then plated onto hypotonic medium at 41°C. Single colonies were struck out onto hypotonic medium at 41°C, and plasmid DNA was isolated from those that grew well with single colonies. These plasmids were retransformed into KS1000 and plated on hypotonic medium at 41°C to confirm suppressor activity.

Efficiency of prc suppression. Cultures of KS1000 containing the suppressor plasmids were grown in Luria broth (LB) to log phase, diluted in phosphate-buffered saline, spread on LB or hypotonic agar, and grown at the appropriate temperature. Growth on LB was tested at 43°C, while growth on the hyptonic medium was tested at 40°C. Colonies were counted after 16 to 24 h of incubation, and the efficiency of plating was calculated as the ratio of CFU per milliliter obtained at high versus low temperatures. Plasmid pKS6-1w, which places the *prc* gene under control of the *lac* promoter, was used as a positive control. Cell morphologies were visualized in a Nikon microscope with dark-field and phase-contrast optics.

HhoA protein sequencing. Cultures of WG1 cells containing pBR322, *phoAp-hhoA*, or *phoAp-hhoB* plasmids were grown overnight in low-phosphate medium at 30°C. Periplasmic proteins were released from the cells by extraction into hypotonic buffer (10 mM Tris-HCl [pH 7.5], 2 mM EDTA) on ice. A large band migrating at the expected mass of HhoA (44 kDa) that is not present in the pBR322 control is visible by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. The HhoB protein was not observed in periplasmic or whole-cell extracts grown under similar conditions. Proteins separated by SDS-PAGE were electroblotted onto Millipore Immobilon-PSQ membranes for 1 h at 250 mA (constant current) in a Bio-Rad Trans-Blot apparatus (35). The polyvinylidene difluoride membrane was stained with 0.1% Coomassie blue R-250 in 50% methanol for 0.5 min and then destained for 2 to 3 min with 10% acetic acid in 50% methanol. The membrane was thoroughly washed with water and dried before storage at −20°C. Automated protein sequencing was performed on a model 470A Applied Biosystems sequencer. Electroblotted proteins were sequenced in the Blott cartridge. Peaks were integrated with Justice Innovation software and Nelson Analytical 760 interfaces. Sequence interpretation was performed on a VAX 8650 (21).

Gene deletions. The gene disruptions described above were subcloned into the R6Kγ ori plasmid pS1080 and recombined into the chromosome of BW16824 by M13 transduction and carbenicillin selection. Individual transductants were struck out on LB-carbenicillin plates and then blocked onto sucrose agar at room temperature for selection of plasmid resolvants. Chromosomal DNA from sucrose-resistant, carbenicillin-sensitive colonies was screened by PCR, using oligonucleotides flanking the deletion for those that carry the Δ*hhoAB* alleles in the chromosome. Details of this method will be published elsewhere.

Nucleotide sequence accession number. The 3,156-bp DNA sequence of the *hhoAB* genes shown in Fig. 2 was submitted to GenBank in October 1994 and given the accession number U15661.

RESULTS AND DISCUSSION

Isolation of multicopy prc suppressors. We reasoned that among the multicopy suppressors of a *prc* mutant should be genes encoding proteases with similar or overlapping substrate specificities. The increased gene dosage would lead to overproduction of the protease, which could at least partially overcome the loss of *prc* and allow *prc* mutant cells to grow under nonpermissive conditions. With this goal in mind, an *E. coli* chromosomal library was transformed into the *prc* null strain KS1000 and then plated on hypotonic medium at elevated temperatures. The number of colonies obtained was highly dependent on the plating temperature, which was adjusted to 41°C, at which the plating efficiency was ~10^{-6}. Transformants were then struck out under nonpermissive conditions and evaluated for stable growth of single colonies. At this point, the *prc* suppression could be due to a gene carried on the plasmid or from a spontaneous chromosomal mutation. To differentiate between these possibilities, plasmid DNA was isolated, transformed into fresh KS1000 cells, and plated under nonpermissive conditions. Of about 200 plasmids, 3 gave rise to a large number of transformants, indicating that the suppressor gene was located on the plasmid. These plasmids were further characterized by restriction mapping and deletion analysis to localize the suppressor activity to a smaller fragment. The DNA sequence of various clones was determined and screened against GenBank to see if these genes were previously known.

Isolation of hhoAB as a prc suppressor. One of the three multicopy *prc* suppressors that we characterized appeared to contain not one but two serine protease genes. It contained a 4-kb fragment that included a portion of the *mdh* gene. It had previously been proposed that this region encoded the 3′ portion an HtrA homolog if four frameshifts were included in the published sequence (6) (SwissProt entry HTRH_ECOLI by K. Rudd). We first determined the DNA sequence of both strands
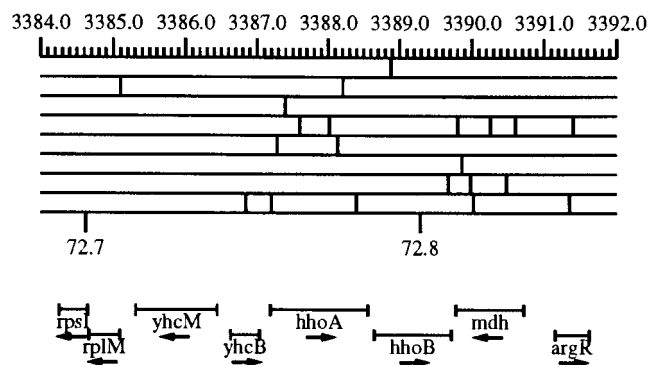
FIG. 1. Integrated map of the *hhoAB* gene locus of the *E. coli* chromosome (7). The upper scale shows the map position in kilobases, and the lower scale is in minutes (centisomes). The locations of the recognition sites for eight restriction enzymes are shown in each line and, from top to bottom, are *Bam*HI, *Hin*dIII, *Eco*RI, *Eco*RV, *Bgl*I, *Kpn*I, *Pst*I, and *Pvu*II. The open reading frames with gene names are shown below.

of a 1.9-kb fragment extending out from the *mdh* gene to a flanking *Hin*dIII site (Fig. 1), which should have encoded the 5′ region and the promoter. We found a complete open reading frame for an HtrA homolog, but to our surprise, this fragment also encoded the 3′ end of a second HtrA homolog. We then sequenced an additional 1.6-kb fragment to include the second gene and its promoter. We propose the names *hhoA* and *hhoB*, for *htrA* homolog, to denote these genes. HtrA is a serine protease that is required for growth at high temperatures (32, 33). It was independently isolated as a mutation (*degP4*::Tn*5*) that affected proteolysis in the periplasm (54). We are testing whether multicopy *htrA* will also suppress a *prc* mutation.

hhoAB DNA sequence analysis. The DNA sequence of a 3,156-bp fragment containing the *hhoAB* genes is shown in Fig. 2. The *hhoA* and *hhoB* genes have ATG start codons and are transcribed in the same direction. They are separated by an 89-bp spacer that has the potential to form a stable stem-loop structure, which may act as a regulatory element. Shine-Dalgarno sequences complementary to the 3′ end of the 16S rRNA are present at an appropriate spacing 5′ of the start codons for both genes. A potential bidirectional rho-independent transcriptional terminator is present between the convergent *hhoB* and *mdh* genes from bases 3069 to 3109 in Fig. 2. A potential σ^{70}-dependent promoter, containing 8 of 12 bases from the consensus −35 and −10 hexamers separated by the ideal-length 17-bp spacer, is found upstream of the *hhoA* gene. Transcription of the homologous *htrA* gene is induced at elevated temperatures by a σ^E-containing RNA polymerase, but nothing resembling a σ^E promoter sequence is apparent (15). The 3′ end of the sequence presented in Fig. 2 shows the proximity of the convergent *mdh* gene encoding malate dehydrogenase (59). An additional open reading frame that could encode a protein of 125 amino acids exists 5′ of the *hhoA* gene. Its putative TAA stop codon is boxed in Fig. 2. This protein has been given the name YHCB_ECOLI in the SwissProt database, and its function is unknown.

Protein sequences of HhoA and HhoB. The deduced protein sequences of HhoA and HhoB are 455 and 355 residues, respectively, and are shown in Fig. 2. The amino termini of both HhoA and HhoB have the characteristics of a standard signal peptide including basic N domains, hydrophobic cores, and signal peptidase cleavage sites (43). HhoA was overproduced by using a *phoA* promoter and was found in the periplasmic fraction of the cell. Amino-terminal sequencing of HhoA con-

firmed that the mature protein is derived from cleavage of a 27-residue signal peptide (Fig. 2). The cellular location of HhoB is unknown. The potential leader peptidase cleavage site of HhoB differs from a consensus Lep site in having a leucine residue at the −3 position, which is usually a small hydrophobic residue. HhoB may be exported into the periplasm and this signal may be cleaved or this hydrophobic domain may act as a membrane anchor.

Bazan and Fletterick (6) identified the HhoB open reading frame as a theoretical translation of the sequence flanking the *mdh* gene (59), which required four frameshifts. They aligned their putative EcORF (HhoB) with HtrA in a structurally based sequence alignment of cysteine and serine proteases. Their alignment dramatically shows how conserved the active-site catalytic triad of histidine, aspartate, and serine or cysteine residues is among a diverse group of proteases. The putative active-site catalytic triad residues for HhoA and HhoB are boxed in Fig. 2, and an alignment of these regions is presented in Fig. 3. Mutation of the putative active-site residues Ser-210 and His-105 of the *E. coli* HtrA protein was found to abolish its proteolytic activity, confirming the importance of these residues in catalysis (50). The open reading frame corresponding to HhoB was also identified by K. Rudd and labeled HtrH in SwissProt, but we show that this gene is not required for growth at high temperatures like the other *htr* genes.

The HtrA/DegP Family. A search of SwissProt, NBRF/PIR, GenBank, and GeneSeq (IntelliGenetics, Mountain View, Calif.) protein and DNA databases with the BLAST (1) and FASTA (41) programs revealed homologous proteins in a variety of bacteria including *E. coli* (32), *Salmonella typhimurium* (25), *Campylobacter jejuni*, (CJHTRA_1) (58), *Brucella abortus* (14, 44, 57), *Chlamydia trachomatis* (27), *Rochalimaea henselae* (3), *Rickettsia tsutsugamushi* (RIR47KDA_1), *Mycobacterium paratuberculosis* (11), *M. leprae* (MLU15180_31), *H. influenzae* (17) and *Pseudomonas aeruginosa* (8a). Multiple HtrA-like proteins have been found in *E. coli*, *B. abortus*, *H. influenzae*, and *P. aeruginosa*, suggesting that more family members remain to be identified in the other species.

These HtrA-like proteins are 27 to 59% identical to HhoA and 17 to 92% identical to each other overall. The bulk of the diversity is in the amino and carboxy termini, with a conserved core of about 200 residues including the putative catalytic triad. An alignment of the residues flanking the putative active-site catalytic triad of HhoA, HhoB, and their homologs is presented in Fig. 3. Interestingly, the *Rickettsia* protein (RIR47KDA_1) is clearly a family member, but lacks all three active-site residues. The putative protein listed as CHT59KD.1 was derived as an alternative translation with frameshifts of the CHT59KD GenBank entry from *C. trachomatis* (27).

Many of these species are facultative intracellular pathogens. A number of these HtrA homologs were isolated by using expression cloning systems with serum from infected patients as a probe for the antigen. This implies that the HtrA proteins are in the circulation of these patients. The HtrA proteins of *S. typhimurium* and *Brucella abortus* are required for virulence and survival in macrophages (5, 14, 25, 57). It is unclear whether HtrA proteolytic activity directly impedes host defenses or whether *htrA* mutants are less viable and more easily killed. The finding that *htrA* mutants are more susceptible to heat and oxidative stress suggests the latter is true (14, 25, 32). The *E. coli htrA* gene is required for growth above 42°C and is induced at high temperatures via a σ^E-containing RNA polymerase (32). The *Brucella abortus htrA* gene is also heat inducible and required for growth at 40°C (14).

The HtrA/DegP family of proteins are similar to other heat shock proteins in that they help cells adapt to environmental

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   1 TAATTGGGTT AGTCGTCGGC ATCATTATTG GTGCTGTGGC CATGCGTTTT GGTAATCGTA AACTACGCCA GCAACAGGCG TTGCAGTACG AACTGGAAAA

 101 GAATAAAGCT GAACTGGACG AGTATCGCGA AGAGCTGGTT AGCCACTTTG CCCGCAGCGC GGAATTACTG GATACCATGG CGCACGACTA TCGCCAGCTG

 201 TATCAGCACA TGGCAAAAAG CTCCAGCAGC CTGCTGCCGG AACTGTCTGC TGAAGCAAAC CCGTTCCGTA ATCGTCTGGC AGAGTCTGAA GCCAGCAACG

 301 ATCAGGCACC GGTGCAGATG CCTCGCGACT ATTCTGAAGG CGCATCCGGC CTGCTGCGTA CTGGCGCGAA GCGCGAC[TAA] TTTATTTTTC GGGCGCAGCC

 401 ATTGCGCCCT CCTCTTCTCT CCCTCCCCGA CTATCATTTA ATCTGGTGTC TCATTGTTAG CCGTCTGAAA ATTCAATAAC_ATCAAACTGT TTTGAATCTC
                                                              -35
 501 TTTTCTTATC ATTCAGGTAC GAGAGCAGGA ATAATGAAAA AACAAACCCA GCTGTTGAGT GCATTAGCGT TAAGTGTCGG GTTAACTCTC TCGGCGTCAT
 -27                        ____  ____  M   K   K   Q   T   Q   L   L   S   A   L   A   L   S   V   G   L   T   L   S   A   S   F
                                                                                               -10
 601 TTCAGGCCGT CGCGTCGATT CCAGGCCAGG TTGCCGATCA GGCCCCTCTC CCCAGTCTGG CTCCAATGCT GGAAAAAGTG CTTCCGGCAG TGGTGAGCGT
  -4   Q   A   V   A   S   I   P   G   Q   V   A   D   Q       A   P   L       P   S   L   A       P   M   L   E   K   V   L   P   A   V   V   S   V

 701 ACGGGTGGAA GGAACGGCCA GTCAGGGACA GAAAATCCCG GAAGAATTCA AAAAGTTTTT TGGTGATGAT TTACCGGATC AACCTGCACA ACCCTTCGAA
  30   R   V   E   G   T   A   S   Q   G   Q   K   I   P   E   E   F   K   F   F   G   D   D   L   P   D   Q   P   A   Q   P   F   E

 801 GGTTTAGGCT CCGGTGTCAT CATCAACGCC AGTAAAGGCT ATGTGCTGAC CAACAACCAT GTGATTAATC AGGCACAGAA AATCAGTATT CAGCTCAATG
  63   G   L   G   S   G   V   I   I   N   A   S   K   G   Y   V   L   T   N   N   [H]   V   I   N   Q   A   Q   K   I   S   I   Q   L   N   D

 901 ATGGGCGCGA GTTTGATGCA AAACTGATTG GTAGCGATGA CCAGAGCGAT ATCGCCCTGT TACAAATTCA AAACCCGAGC AAATTAACGC AAATCGCTAT
  97   M   G   R   E   F   D   A   K   L   I   G   S   D   D   Q   S   [D]   I   A   L   L   Q   I   Q   N   P   S   K   L   T   Q   I   A   I

1001 TGCCGACTCC GATAAATTGC GCGTCGGTGA TTTTGCCGTA GCGGTCGGTA ACCCATTTGG CCTTGGGCAA ACCGCCACCT CTGGCATTGT TTCCGCATTA
 130   A   D   S   D   K   L   R   V   G   D   F   A   V   A   V   G   N   P   F   G   L   G   Q   T   A   T   S   G   I   V   S   A   L

1101 GGCCGCAGCG GGTTGAATCT TGAAGGTCTG GAAAACTTTA TCCAGACAGA TGCTTCCATT AACGCGCGTA ACTCCGGCGG TGCACTATTA AACCTTAACG
 163   G   R   S   G   L   N   L   E   G   L   E   N   F   I   Q   T   D   A   S   I   N   R   G   N   [S]   G   G   A   L   L   N   L   N   G

1201 GTGAGTTAAT TGGCATCAAC ACTGCAATCC TTGCGCCTGG CGGCGGGAGC GTCGGGATTG GATTTGCCAT CCCCAGTAAT ATGGCGGCAA CACTGGCGCA
 197   E   L   I   G   I   N   T   A   I   L   A   P   G   G   G   S   V   G   I   G   F   A   I   P   S   N   M   A   R   T   L   A   Q

1301 GCAGCTTATC GACTTTGGTG AAATCAAACG CGGTTTGTTA GGCATCAAAG GCACCGAGAT GAGTGCCGAT ATCGCCAAAG CCTTCAACCT TGACGTGCAG
 230   Q   L   I   D   F   G   E   I   K   R   G   L   L   G   I   K   G   T   E   M   S   A   D   I   A   K   A   F   N   L   D   V   Q

1401 CGTGGCGCGT TTGTCAGCGA AGTGGTTGCCA GGTTCTGGCT CCGGCAAAAG CGGGCGTCAAA GCGGGCGATA TTATTACCAG CCTCAACGGC AAACCGCTGA
 263   R   G   A   F   V   S   E   V   L   P   G   S   G   S   A   K   A   G   V   K   A   G   D   I   I   T   S   L   N   G   K   F   L   N

1501 ATAGCTTTGC TGAGTTGCGC TCTCGTATCG CGACCACCGA GCCGGGCACG AAAGTGAAGC TTGGCCTGCT GCGTAACGGC AAACCACTGG AAGTAGAAGT
 297   S   F   A   E   L   R   S   R   I   A   T   T   E   P   G   T   K   V   K   L   G   L   L   R   N   G   K   P   L   E   V   E   V

1601 GACGCTCGAT ACCAGCACCT CTTCGTCGGC CAGCGCTGAA ATGATCACGC CAGCGCTGGA AGGTGCAACG TTGAGCGATG GTCAGCTAAA AGATGGCGGC
 330   T   L   D   T   S   T   S   S   S   A   S   A   E   M   I   T   P   A   L   E   G   A   T   L   S   D   G   Q   L   K   D   G   G

1701 AAAGGTATTA AAATCGATGA AGTTGTCAAA GGAAGCCCAG CTGCTCAGGC TGGCTTGCAA AAAGACGATG TGATCATTGG CGTCAACCGC GATCGGGTGA
 363   K   G   I   K   I   D   E   V   V   K   G   S   P   A   Q   A   G   L   Q   K   D   D   V   I   I   G   V   N   R   D   R   V   N

1801 ACTCGATTGC TGAAATGCGT AAAGTGCTGG CGGCAAAACC GGCCATCATC GCCCTGCAAA TTGTACGCGG CAATGAAAGC ATCTATCTGC TGATGCGTTA
 397   S   I   A   E   M   R   K   V   L   A   A   K   P   A   I   I   A   L   Q   I   V   R   G   N   E   S   I   Y   L   L   M   R

1901 ATGTCGTAAA CCGGGCATCA GGCTTACGTG TGATGTCCGG TTAACTCGTG GTATGCTGCT GCCGTTCCCT TTTTTAATGA CGCCTCCATC ATGTTTGTGA
   1                                                                                                                                   M   F   V   K

2001 AGCTCTTACG TTCCGTTGCG ATTGGATTAA TTGTCGGCGC TATTCTGCTG GTTGCCATGC CTTCGCTGCG CAGCCTTAAC CCGCTTTCCA CTCCGCAATT
   5   L   L   R   S   V   A   I   G   L   I   V   G   A   I   L   L   V   A   M   P   S   L   R   S   L   N   P   L   S   T   P   Q   F

2101 TGACAGTACC GATGAGACGC CTGCCAGCTA TAATCTGGCG GTTCCGCGCG CCGCGCCAGC GGTGGTTAAC GTTTACAACC GTGGTTTGAA CACCAACTCT
  38   D   S   T   D   E   T   P   A   S   Y   N   L   A   V   R   R   A   P   A   V   V   N   V   Y   N   R   G   L   N   T   N   S

2201 CACAACCAGC TTGAGATCCG CACCCTGGGA TCCGGTGTAA TCATGGATCA ACGCGGTTAT ATCATCACCA ATAAACACGT CATCAACGAC GCCGATCAGA
  71   H   N   Q   L   E   I   R   T   L   G   S   G   V   I   M   D   Q   R   G   Y   I   I   T   N   K   [H]   V   I   N   D   A   D   Q   I

2301 TCATCGTCGC CTTACAGGAT GGACGTGTAT TTGAAGCATT GCTGGTGGGA TCCGACTCTC TAACCGATCT GGCGGTACTT AAAATTAATG CCACTGGCGG
 105   I   V   A   L   Q   D   G   R   V   F   E   A   L   L   V   G   S   D   S   L   T   [D]   L   A   V   L   K   I   N   A   T   G   G

2401 TTTACCTACC ATTCCAATTA ATGCACGTGC CGTACCGCAC ATTGGCGACG TAGTACTGGC GATCGGTAAC CCGTACAACC TCGGGCAGAC CATTACCCAG
 138   L   P   T   I   P   I   N   A   R   R   V   P   H   I   G   D   V   V   L   A   I   G   N   P   Y   N   L   G   Q   T   I   T   Q

2501 GGGATTATTA GTGCCACGGG TCGAATCGGT CTGAACCCGA CCGGGCGGCA AAACTTCCTC CAAACCGATG CTTCCATTAA CCACGGTAAC TCTGGCGGCG
 171   G   I   I   S   A   T   G   R   I   G   L   N   P   T   G   R   Q   N   F   L   Q   T   D   A   S   I   N   H   G   N   [S]   G   G   A

2601 CGCTGGTGAA CTCGCTGGGC GAACTGATGG GCATTAATAC GCTGTCGTTT GATAAGAGTA ACGATGGCGA AACGCCGGAA GGTATCGGCT TTGCGATTCC
 205   L   V   N   S   L   G   E   L   M   G   I   N   T   L   S   F   D   K   S   N   D   G   E   T   P   E   G   I   G   F   A   I   P

2701 TTTCCAGTTA GCAACCAAAA TTATGGATAA GCTGATCCGC GATGGTCGCG TGATCCGCGG CTACATTGGT ATCGGCGGAC GTGAGATCGC ACCACTGCAC
 238   F   Q   L   A   T   K   I   M   D   K   L   I   R   D   G   R   V   I   R   G   Y   I   G   G   R   E   I   A   P   L   H

2801 GCGCAGGGCG GTGGTATAGA TCAACTGCAA GGGATCGTGG TTAATGAAGT GTCACCTGAC GGCCCGGCGG CGAATGCGGG TATTCAGGTC AACGATCTGA
 271   A   Q   G   G   G   I   D   Q   L   Q   G   I   V   V   N   E   V   S   P   D   G   P   A   A   N   A   G   I   Q   V   N   D   L   I

2901 TTATTTCGGT GGATAACAAA CCGGCCATCT CTGCTCTGGA GACGATGGAT CAGGTGGCGG AAATTCGCCC TGGTTCGGTG ATCCCTGTAG TAGTGATGCG
 305   I   S   V   D   N   K   P   A   I   S   A   L   E   T   M   D   Q   V   A   E   I   R   P   G   S   V   I   P   V   V   V   M   R

3001 TGATGATAAG CAGTTAACGC TGCAGGTCAC CATTCAGGAA TATCCGGCAA CCAATTAAGT CGTGCGCTCA AAACAAAAAA CCGGAGTCTG TGCTTCCGGTT
 338   D   D   K   Q   L   T   L   Q   V   T   I   Q   E   Y   P   A   T   N

3101 TTTTATTATC CGCTAATCAA TTACTTATTA ACGAACTCTT CGCCCAGGGC GATATC
 312                                     K   N   V   F   E   E   G   L   A   I   D
```

FIG. 2. DNA and deduced amino acid sequence of the *E. coli hhoAB* genes. A potential σ^{70} promoter, Shine-Dalgarno sequences, and the amino-terminal sequence of the mature HhoA protein are underlined. The putative active-site catalytic triad histidine, aspartate, and serine residues of HhoA and HhoB are boxed. The TAA stop codon of the upstream open reading frame (YhcB) is also boxed. The intergenic inverted repeats are arrowed. Note that the latter probably functions as a bidirectional transcription terminator. The sequence ends in the convergent *mdh* gene, and the Mdh carboxy-terminal sequence is shown.

stresses. The classic heat shock proteins (DnaJ, DnaK, GrpE, GroEL, and GroES in *E. coli*) act as chaperones to aid in the proper folding of proteins. They undergo cycles of binding and releasing denatured proteins and folding intermediates to prevent their nonproductive aggregation. The HtrA/DegP family of proteins may also act as chaperones by binding periplasmic proteins that have just been secreted or those that are denatured under stressful conditions. Either they could release the bound protein toward the proper folding pathway in a chaperone-like mode or the serine protease activity could degrade

it. The proteolytic efficiency of the complex would thus decide the fate of a protein. In support of this hypothesis, the *Rickettsia* HtrA homolog which lacks the active-site catalytic triad appears to act in a protease-independent manner. The precise role of the HtrA family members in cell growth and pathogenesis remains to be elucidated.

hhoAB gene deletions. The *hhoAB* genes were deleted in a two-step process involving homologous recombination of plasmid constructs into the chromosome. First, plasmids containing a gene deletion were integrated into the chromosomal gene
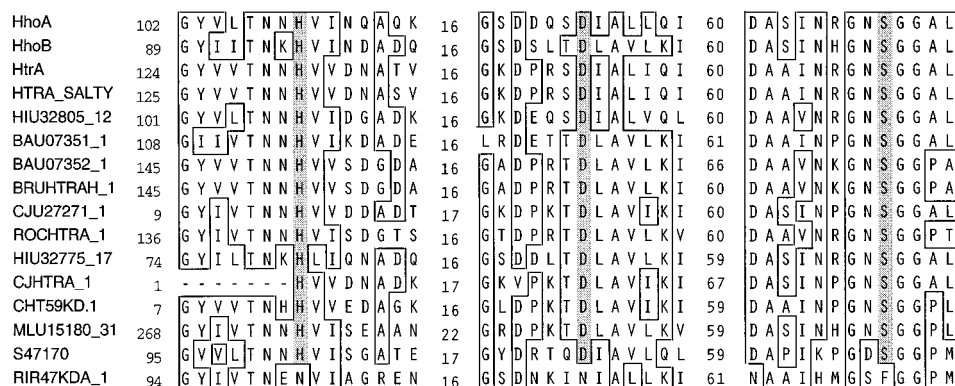
FIG. 3. Protein sequence alignment flanking the presumed active-site catalytic triad of histidine, aspartate, and serine residues (shaded) of the HtrA/DegP family of periplasmic serine proteases. The protein names are taken from the SwissProt, GenBank, and PIR databases. The numbers on the left side are those of the preproteins, while the other numbers represent sequence gaps. Note that the *Rickettsia* protein (RIR47KDA_1) lacks all three catalytic residues.

by homologous recombination, creating a direct repeat. A second homologous recombination event between repeats excises the plasmid and leads to replacement of the wild-type gene with the cloned deletion if the second event occurs on the opposite side of the deletion. The details of this method will be published elsewhere (4a).

In creating the *hhoAB* deletions, we found that they arose as very small colonies at room temperature on the sucrose plates after ~48 h whereas their wild-type counterparts appeared after ~24 h. Strains with the Δ*hhoA* and Δ*hhoAB* alleles formed smaller, flatter, more transparent colonies than wild-type cells but grew on LB, M9 glucose, and the hypotonic medium at all temperatures tested (25 to 42°C). Larger opaque colonies with normal growth characteristics appeared spontaneously in these cultures at a high frequency, suggesting the acquisition of a suppressor(s). Colonies arose at a reduced frequency at 42°C, and all had an opaque morphology, suggesting that they contained a second-site suppressor. We have termed these mutations *shh* (for suppressor of *hhoAB*) and are mapping their chromosomal locations. Initial attempts at the Δ*hhoB* allele replacement were unsuccessful.

A strain with a deletion of both the *hhoAB* and *htrA* genes was constructed. It was viable and similar to the Δ*hhoAB* strain in colony morphology at 37°C. This strain retained the temperature sensitivity of the *htrA* mutant strain in that it failed to grow on LB at 42°C. Larger opaque colonies were also seen arising in this strain, indicating that Shh activity is not due to a compensatory induction of *htrA*.

The accompanying report (60) describes a similar slow-growth phenotype with their *hhoB* and *hhoAB* deletion strains. However, their *hhoA* deletion strain, which retains the *hhoB* promoter, behaves like the wild type. This suggests that HhoB is either not expressed or inappropriately expressed from the *hhoA* promoter in our *hhoA* deletion strain.

***rlpA* as a suppressor of *prc*.** Another *prc* suppressor activity that we have characterized was localized to a 123-residue fusion protein containing the first 102 residues of the RlpA (rare lipoprotein A) protein. The mature RlpA protein is 345 residues in length and has a 17-amino-acid lipoprotein signal peptide to direct export to the periplasm. This signal is then cleaved, and the N-terminal cysteine residue of the resulting protein is modified to glycerylcysteine and then fatty acylated. This modification functions to anchor the protein in the cell membrane (56). This truncated RlpA retains its lipoprotein signal peptide and hence may be exported to the periplasm, modified, and anchored in the membrane. The role of RlpA in

vivo is unclear, but its gene is found in a cluster of genes (*mrdAB*) involved in cell shape determination and peptidoglycan synthesis (53).

The mechanism of Prc suppression by the truncated RlpA peptide is unclear. It is unlikely that deletion of 70% (residues 103 to 345) of the protein allows it to function normally. Recent findings suggest that it may indirectly lead to the induction of the HtrA protease. An increase in the concentration of certain outer membrane proteins leads to an increase in the concentration of the heat shock σ factor σ^E (σ^{24}) in the cell (37, 51). RNA polymerase with σ^E is known to transcribe the *htrA* protease and *rpoH* (σ^{32}) genes. The increased production of HtrA protease may then suppress the Prc defect by cleaving Prc substrates. Alternatively, other heat shock proteins induced by σ^E and/or σ^{32} may suppress the *prc* defect indirectly. These possibilities are currently being tested.

***dksA* is a multicopy suppressor of *prc*.** The third *prc* suppressor was localized to the *dksA* gene. The *dksA* gene was first characterized as a multicopy suppressor of the temperature-sensitive growth of a *dnaK* null strain and was also found to partially suppress the temperature sensitivity of *dnaJ* and *grpE* mutant strains (28). Null mutations in *dnaK* or *grpE* or over-expression of some mutant *dnaK* alleles also leads to formation of nonseptate filaments (10, 28, 36), which multicopy *dksA* is able to at least partially suppress. The *dksA* gene was also isolated as a multicopy suppressor of the temperature-sensitive phenotype of a *mukB* mutant strain (63). The MukB protein is required for chromosomal partitioning, because defects in it cause filamentation and anucleated cells.

The *dksA* gene is nonessential and encodes a protein of 151 amino acids, whose cellular location and function in vivo are unknown (28). The ability of DksA to suppress a number of heat shock proteins suggests its ability to function in this role. Alternatively, the DksA protein may act by stimulating the production of stress response factors. Along these lines, the DksA protein has been postulated to contain a zinc-binding motif ($Cx_2Cx_{17}Cx_2C$), similar to a number of eukaryotic (30) and prokaryotic (26, 61) regulatory proteins. DksA is similar to a number of *E. coli* proteins including the TraR transcriptional activator (13, 18), the YBII protein (40), the CP80 protein of bacteriophage 186 (49), and the YO80 protein of bacteriophage P2 (34). Homologous proteins have also been characterized in *H. influenzae* (12, 17) and *B. subtilis* (BSU20447). The role of DksA in the suppression of any of these defects remains to be elucidated. The finding that multicopy *dksA* is able to suppress

TABLE 2. Efficiency of plating of *prc* suppressors

Relevant genotype	EOP (43/37°C) on LB	EOP (40/37°C) on hypotonic medium
Wild type	1	1
prc/pBR322	1	10^{-5}
prc/pR1pA′	1	1
prc/pDksA	1	1
prc/pHhoA	1	1
prc/pHhoB	1	10^{-5}
prc/pHhoAB	1	1
prc/pKS6-1w	10^{-5}	1

temperature-sensitive filamentation caused by these different mechanisms is intriguing.

Characterization of *prc* suppressors. The efficiency of *prc* suppression was evaluated by comparing the plating efficiencies of *prc* suppressors under various conditions and is presented in Table 2. The efficiency of plating on LB agar at 30, 37, or 43°C was equivalent for all strains tested, except that overproduction of Prc by the control plasmid pKS6-1w was lethal at high temperatures (Table 2). Surprisingly, this toxicity was not observed on the hypotonic medium. Perhaps Prc is less active under hypotonic conditions. This helps explain a discrepancy between laboratories in that Hara et al. (20) reported that Prc overproduction is lethal whereas we and others (47) did not see this effect. Hara et al. overproduced Prc by using a λp_L promoter and thermosensitive *c*I857 repressor, so that their overproduction conditions were at 42°C whereas ours were at 37°C.

The efficiency of plating at 40°C on hypotonic medium is significantly different from that on LB, as previously shown (20). Colony formation by the *prc* mutant strain is at least 5 orders of magnitude lower, and these colonies probably contain spontaneous *prc* suppressors (Table 2). This difference in plating efficiency provided the selective pressure for the isolation of *prc* suppressors. As seen in Table 2, multiple copies of the truncated *rlpA* gene, the *dksA* gene, and the *hhoA* gene allowed efficient colony formation under these conditions. However, colony size is reduced from that of wild-type cells at the higher temperature. The multicopy *hhoB* allele had no apparent effect. Cells containing multiple copies of *dksA* formed smaller colonies than the others, even at 30 and 37°C.

Microscopic observation of individual cells confirmed that *prc* strains are filamented on hypotonic medium at elevated temperatures (20). The degree of filamentation between cells varied from slightly longer rods to very long filaments. Most filaments were nonseptate, but some had very thick, irregularly spaced septa. We also found that a fraction ($\sim 10^{-3}$) of wild-type cells behaved similarly under these conditions. This suggests that the mechanism controlling septation is perturbed under these conditions, especially in the *prc* mutant. The different suppressors varied in their ability to alleviate this filamentation. Multicopy *dksA* was able to completely suppress the conditional filamentation of a *prc* mutant at 40 but not 42°C. The *rlpA* and *hhoA* genes decreased the frequency of filamented cells in the population, while *hhoB* had no apparent effect. All strains listed in Table 2 appeared normal when grown on LB agar at high and low temperatures.

Possible role of Prc in vivo. Prc has been shown in vivo to be a protease that cleaves 11 residues from the C terminus of FtsI (penicillin-binding protein 3) (39). FtsI is an essential enzyme that is required for septum formation in the dividing cell (52). FtsI catalyzes the transglycosylation and transpeptidation reactions in the final steps of peptidoglycan synthesis (24). Sep-

tation is a process that requires careful temporal and spatial control. It is tempting to speculate that C-terminal processing of FtsI and possibly other enzymes by Prc is part of this mechanism. Along these lines, the homologous CtpA (carboxyl-terminal processing) protease in the divergent cyanobacterium *Synechocystis* sp. PCC6803 has a proteolytic activity similar to Prc. It cleaves the C terminus of the D1 protein of the photosystem II complex, which is required for its activation. This activation of an inactive D1 protein precursor by C-terminal processing is a common mechanism in other oxygenic photosynthetic organisms including plants (2). Prc has been demonstrated in vitro to be a protease that cleaves predominantly after small hydrophobic residues such as alanine and valine, especially in proteins with nonpolar C termini (43a, 47).

An alternative explanation is that Prc also functions as a chaperone involved in the folding and turnover of proteins in the periplasmic space. The high temperature and hypotonic conditions used in this selection can lead to the denaturation of proteins and exposure of hydrophobic core residues. Prc is thought to contain a hydrophobic binding pocket, which is a common feature among the chaperones. Prc may combine with the HtrA/DegP protein family to help (re)fold or degrade misfolded proteins in periplasm and allow normal growth. A general periplasmic chaperone in *E. coli* has not been demonstrated to date. Prc and HtrA (DegP) are both required for proper cell growth in response to environmental stresses like the other chaperones. The finding that multicopy suppressors of Prc include two new members of the HtrA/DegP family suggests that they have overlapping substrate specificities. Transcription of the *htrA* gene is known to be induced by heat, and multicopy *dksA* is able to suppress defects in a number of heat shock genes, lending further support for this model. Further characterization of the role of these and other proteases in the stability of recombinant proteins is under way.

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