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# Identification of *cutC* and *cutF* (*nlpE*) Genes Involved in Copper Tolerance in *Escherichia coli*

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**It has been suggested previously that copper transport in *Escherichia coli* is mediated by the products of at least six genes, *cutA*, *cutB*, *cutC*, *cutD*, *cutE*, and *cutF*. A mutation in one or more of these genes results in an increased copper sensitivity (D. Rouch, J. Camakaris, and B. T. O. Lee, p. 469–477, *in* D. H. Hamer and D. R. Winge, ed., *Metal Ion Homeostasis: Molecular Biology and Chemistry*, 1989). Copper-sensitive *cutC* and *cutF* mutants were transformed with a genomic library of *E. coli*, and copper-tolerant transformants were selected. Two distinct clones were identified, each of which partially restores copper tolerance in both the *cutC* and *cutF* mutants of *E. coli*. Subcloning, physical mapping, and sequence analysis have revealed that the *cutC* gene is located at 42.15 min on the *E. coli* genome and encodes a cytoplasmic protein of 146 amino acids and that the *cutF* gene is located at 4.77 min on the *E. coli* genome and is allelic to the *nlpE* gene independently identified by Silhavy and coworkers (W. B. Snyder, L. J. B. Davis, P. N. Danese, C. L. Cosma, and T. J. Silhavy, J. Bacteriol. 177:4216–4223, 1995). Results from the genetic mapping of the copper-sensitive mutations in the *cutF* mutant and sequencing of the *cutC* and *cutF* (*nlpE*) alleles from both *cutC* and *cutF* mutants indicate that both the *cutC* and *cutF* mutants are in fact double mutants altered in these two genes, and mutations in both the genes appear to be required for the copper-sensitive phenotype in each mutant.**

Copper is an essential heavy metal trace element, which plays a vital role in the growth and physiology of aerobic organisms; however, excess of this metal results in cell death. Organisms have evolved to possess effective means of achieving the fine balance between copper requirement and copper toxicity. Genetic alterations in the mechanisms involved in copper metabolism result in copper deficiency and/or copper sensitivity, as exemplified by Wilson's disease and Menkes' disease in humans (4) and *ctr1* mutants of *Saccharomyces cerevisiae* (9, 10).

The mechanism of copper transport and homeostasis in *E. coli* is not well understood. Plasmid-borne copper resistance determinants have been identified in *E. coli* and *Pseudomonas syringae* (2, 3, 22, 29). On the basis of a preliminary characterization of copper-sensitive mutants, it was proposed that six genes (*cutA*, *cutB*, *cutC*, *cutD*, *cutE*, and *cutF*) are involved in the uptake, intracellular storage and delivery, and efflux of copper in *E. coli* (30). Of these six structural genes, the *cutA* locus and the *cutE* gene have been cloned and sequenced (15, 28). The *cutA* locus and the *cutE* gene are located at 94 and 15 min on the *E. coli* chromosome, respectively (15, 28). The *cutA* locus consists of two operons, one containing a single open reading frame (ORF) encoding a cytoplasmic protein of 13 kDa (CutA1) and the other consisting of two genes encoding 50-kDa (CutA2) and 24-kDa (CutA3) inner membrane proteins. The *cutA2* gene is allelic to *dipZ* (15), which encodes a protein disulfide isomerase required for the biogenesis of *c*-type cytochrome (8). The functions of CutA1 and CutA3 are not clear (15). The *cutE* gene is allelic to *lnt*, the gene encoding apolipoprotein *N*-acyltransferase (17), and the CutE protein, composed of 512 amino acid residues, contains a sequence, H-F-Q-M-A-R-M, which is homologous to a putative copper-binding motif in plasmid-encoded copper resistance proteins from *P. syringae* and *E. coli* (2, 22, 28).

The *cutC* mutant is copper sensitive ($Cu^s$), and the *cutF* mutant is $Cu^s$, temperature sensitive (ts), and conditionally copper dependent. Both mutants accumulate copper but have apparently normal kinetics of copper uptake (30). To further characterize the genetic determinants of copper metabolism in *E. coli*, we have identified two clones from an *E. coli* genomic library which complement the $Cu^s$ phenotype of the *cutC* and *cutF* mutants. Sequence analysis of the cloned *cutC* gene suggests that CutC is a cytoplasmic copper-binding protein. Subcloning and complementation tests indicate that the *cutF* gene is allelic to the *nlpE* gene identified independently by Snyder et al. (35). This paper provides evidence that the $Cu^s$ phenotype of the *cutC* and *cutF* mutants results from at least two mutations, one in the *cutC* gene located at 42.15 min and the other in the *cutF* gene located at 4.77 min of the *E. coli* chromosome.

## MATERIALS AND METHODS

**Chemicals and enzymes.** [$\alpha$-$^{35}$S]dATP and [$\alpha$-$^{35}$S]dCTP were purchased from Amersham Corp., Arlington Heights, Ill. Restriction enzymes, T4 DNA polymerase, and T4 DNA ligase were obtained from New England Biolabs Inc., Beverly, Mass. The random primer kit (Prime-It 11) for labeling DNA probes was obtained from Stratagene, La Jolla, Calif. The PCR kit was obtained from Perkin-Elmer Cetus, Norwalk, Conn. The TA cloning kit for direct cloning of PCR products was obtained from Invitrogen Corp., San Diego, Calif. 5-Bromo-4-chloro-3-indolyl-$\beta$-D-galactoside (X-Gal) was purchased from Bachem, Torrance, Calif. The Qiagen kit and Wizard minipreps DNA purification system used for the preparation of plasmid DNA were obtained from Qiagen Inc., Chatsworth, Calif., and Promega Corp., Madison, Wis., respectively. Oligonucleotides were synthesized with an Applied Biosystems Synthesizer. DNA sequencing was carried out with a Perkin-Elmer Cetus model 480 DNA thermal cycler, a Taq Dye Deoxy Terminator Cycle sequencing kit, and a Model 373 A, version 1.2.0 DNA sequenator from Applied Biosystems.

**Bacterial strains.** The strains used in the present study are listed in Table 1. DH5$\alpha$ cells were used as the host for transformations. Plasmid vectors pBR322 and pK184 (18) were used for subcloning.

**Media and growth conditions.** Media used include L-broth (LB) or LB agar, which was supplemented with 4 mM $CuSO_4$ for the assay of the copper-sensitive

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TABLE 1. Bacterial strains and relevant genotypes

| Strain | Genotype | Source or reference |
|---|---|---|
| ED8739 (wild type) | *hsdR hsdM metB gal lac supE supF* | 30 |
| GME111 | ED8739 *cutC* | 30 |
| GME137 | ED8739 *cutF* | 30 |
| CAG18436 | *zae-502*::Tn*10* | 34 |
| CAG18580 | *zae-502*::Tn*10kan* | 34 |
| RK4375 | *fhuB478*::Tn*10* (3.7 min) | 19 |
| JW353 | *zae-502*::Tn*10* (4.7 min)[a] | B. J. Bachmann |
| NK5525 | *pro-81*::Tn*10* (5.7–5.8 min) | B. J. Bachmann |
| CAG12156 | *uvrC279*::Tn*10* (42.25 min) | 34 |
| CAG12126 | *zeb-3199*::Tn*10* | 34 |
| WBS262 | MC4100 *nlpE*::*spc* | 35 |
| PNDC2000 | MC4100 *cpxR*::*spc* | 6 |
| WBS1141 | MC4100 *degP*::Tn*10* | 35 |

[a] Cotransduces 55% with *dnaE* (*polC*) at 4.7 min.

($Cu^s$) phenotype. Antibiotics used for selecting transformants or recombinants were ampicillin (50 μg/ml), kanamycin (50 μg/ml), tetracycline (10 μg/ml), and spectinomycin (50 μg/ml). The *E. coli* genomic library, LBST, which contained 0.5- to 15-kb partial *Sau*3AI-*Taq*I digests of MC4100 chromosomal DNA in *Cla*I- and *Bam*HI-cleaved pBR322, was a gift from D. Oliver, Wesleyan University, Middletown, Conn., via J. Lutkenhaus, Kansas University Medical Center, Kansas City, Kans. To determine the copper tolerance of the *cutC* and *cutF* mutants harboring the complementing clones (pCUTC2 or pCUTF1), 500 to 10,000 cells were spread on LB-ampicillin (LB-Amp) plates containing different concentrations of copper sulfate. Copper-containing media were prepared by adding appropriate amounts of 1 M $CuSO_4$ $5H_2O$ and adjusting the pH to 7.5 with NaOH before autoclaving (38). After 24 to 48 h of incubation at 30°C, colony numbers were determined and the percent survival was calculated.

**Recombinant DNA techniques.** Plasmid preparations, ligations, preparation of competent cells, transformations, and agarose gel electrophoresis were performed by standard procedures (32). DNA fragments were recovered from agarose by the freeze-thaw method of Benson and Zagursky (33).

**Isolation of clones complementing *E. coli cutC* and *cutF* mutants.** Competent cells of $Cu^s$ *cutC* mutant strain GME111 and those of $Cu^s$ and ts *cutF* mutant strain GME137 were transformed with LBST genomic library DNA (approximately 20 ng of plasmid DNA), plated on 10 LB-Amp agar plates, and incubated overnight at 30°C. $Amp^r$ transformants of *cutC* were replica-plated on LB-Amp agar plates containing 4 mM $CuSO_4$. $Amp^r$ *cutF* transformants were replica plated on LB-Amp agar with or without 4 mM $CuSO_4$; the LB-Amp agar plates were incubated at 42°C for 24 h, and the LB-Amp plates containing 4 mM $CuSO_4$ were incubated at 30°C for 24 to 48 h. Plasmid DNA was prepared from copper-tolerant or temperature-resistant (tr) clones and retransformed into the *cutC* or *cutF* mutant, and the transformants were checked for the copper-tolerant or temperature-resistant phenotype without prior selection. Plasmid DNA was purified after treatment with proteinase K and digested with different restriction enzymes, and the restriction maps were constructed. On the basis of the presence or absence of common DNA fragments, these clones were classified and used subsequently for physical mapping on the *E. coli* chromosome. Dot blot hybridization was also performed to further confirm the presence of common fragments in different clones. Two distinct clones, pCUTC1 and pCUTF1, based on their complementation of the $Cu^s$ phenotype of the *cutC* or *cutF* mutant, were identified.

**Mapping of *cutC*- and *cutF*-complementing clones.** pCUTC1 was digested with *Pst*I, and pCUTC2 and pCUTC5 were digested with *Eco*RI and *Sal*I; 1.6- and 1.7-kb *Pst*I fragments from pCUTC1 and 0.8- and 3.2-kb *Eco*RI-*Sal*I fragments from pCUTC2 and pCUTC5, respectively, were eluted from the gel and labeled with [α-$^{35}$S]dCTP. Likewise, pCUTF1 was digested with *Hin*dIII to generate a 4.5-kb *Hin*dIII fragment, which was labeled with [α-$^{35}$S]dCTP. The labeled probes were purified in spun columns (5 prime—3 prime Inc., Boulder, Colo.) and were used to hybridize with the *E. coli* lambda phage library of Kohara et al. (20) by using an *E. coli* gene-mapping membrane according to the protocol provided by the vendor (PanVera Corp., Madison, Wis.). After being washed, the membrane was air dried, covered with plastic wrap, and scanned for the positive hybridization signals with a Phosphor-Imager series 400 (Molecular Dynamics).

**Dot blot analysis of *cutC* and *cutF* clones.** For the preparation of the dot blot, plasmid DNA was heated to 95°C for 5 min and chilled on ice for 2 min. Samples (2 μl) containing 50 ng of DNA were spotted on Hybond-N membranes. After drying, the DNA was denatured in 1.5 M NaCl–0.5 N NaOH for 1 min, neutralized, and fixed (32). Hybridization with the labeled probe was carried out as described above for the *E. coli* gene-mapping membrane.

**Genetic mapping of $Cu^s$ mutations in the GME137 (*cutF*) mutant.** Transduction of mutant strain GME137 was carried out (33) with P1 lysates from strains carrying Tn*10* or Tn*10kan* linked to 42 or 4.7 min on the *E. coli* chromosome (listed in Table 1). The transductants were purified on LB-tetracycline (LB-Tet) or LB-kanamycin (LB-Kan) plates containing 10 mM citrate and scored for $Cu^s$/copper-tolerant and ts/tr phenotypes.

**Subcloning and complementation.** On the basis of the restriction map of the pCUTF1 clone, different DNA fragments were cloned in pBR322 or pK184 vectors and checked for complementation of $Cu^s$ or ts phenotypes of the *cutF* mutant. Clone pCUTF11 (~5.6 kb) was constructed by deleting the *Bam*HI-*Bss*HII fragment (~4.7 kb) in pCUTF1 and religating the remaining DNA fragment including the vector after creating blunt ends with T4 DNA polymerase. The *Hin*dIII fragment (4.5 kb) was cloned at the *Hin*dIII site of pBR322 to generate pCUTF12. The *Ssp*I-*Hin*dIII fragments were cloned at the *Eco*RV and *Hin*dIII sites of pBR322 to generate pCUTF13 and pCUTF14. The *Bss*HII-*Bss*HII (1.4-kb) fragment was deleted from pCUTC5 to obtain pCUTF15, and pCUTF18 was obtained by cloning the *Bss*HII-*Bss*HII (1.4-kb) fragment after making blunt ends at the *Eco*RV site of pBR322. The *Pst*I-*Pst*I (1.8-kb) fragment and *Pst*I-*Hin*dIII (2-kb) fragment were cloned in pK184 at *Pst*I and *Pst*I-*Hin*dIII sites to obtain pCUTF16 and pCUTF17, respectively.

To define a single ORF responsible for conferring the copper-tolerant phenotype to *cutF*, the DNA fragments 1.338, 1.184, and 0.877 kb in size were amplified by PCR with pCUTF1 as the template and 5′ primer CutF1 (5′ATCT GGACGATGAACACTG3′) from upstream of ORF140 and 3′ primers CutF3 (5′ATATAAGCTTCTGGCCTG3′), CutF4 (5′AGTAAGCTTCTACTGACA G3′), and CutF5 (5′CAAGCTTGTCAGCGGTTCG3′) from the C-terminal end of ORF236 to produce plasmids pF3, pF4, and pF5, respectively (see Fig. 2). *Hin*dIII sites were introduced in primers CutF3, CutF4, and CutF5 to facilitate subsequent cloning at the *Pst*I and *Hin*dIII sites in vector pK184. PCR products were first cloned in TA cloning vector pCR11 and checked for complementation. pF6 was constructed by amplifying the *nlpE* gene with primers CutF113F (5′AC CCACGGATCCCACCCGTGCATCGAAA3′) and CutF33F (5′ATGTTGAA GCTTATCCTTCTGGCCTGTT3′) and cloned in TA cloning vector pCR11. The *nlpE* alleles in *cutC* and *cutF* mutants were cloned by PCR with CutF113F and CutF33F primers; clones containing upstream of ORF140 and *nlpE* from *cutC* and *cutF* mutants were obtained by PCR with CutF1 and CutF33F primers. The *cutC* alleles from the *cutC* (strain GME111) and *cutF* (strain GME137) mutants were cloned by PCR with CutC7 (5′GATCACGGTTATCGTTCGT3′) and CutC8 (5′TGTCGATATGCCACGAATG3′) primers and chromosomal DNA as the template in TA cloning vector pCR11 for sequencing.

**Nucleotide sequence determination of the *cutC* and *cutF*/*nlpE* alleles from the wild-type (MC4100), *cutC* mutant (GME111), and *cutF* mutant (GME137) strains.** The 600-bp insert in pCUTC2 was sequenced in both directions with an automated sequenator. Primers adjacent to the *Eco*RI and *Bam*HI sites in pBR322 were used first, followed by additional primers based on the sequence data thus generated. Additional sequence upstream of the *cutC* gene was determined with pCUTC1 as the template. Even though the *cutF* region of the *E. coli* genome has been sequenced previously and independently by two groups (H. Mori, Institute for Virus Research, Kyoto, Japan, and T. Silhavy, Princeton University, Princeton, N.J.), this region was sequenced in both directions with primers from the known sequence and pCUTF1 and pCUTF11 insert DNA as the templates. The *nlpE* and *cutC* alleles in the *cutC* and *cutF* mutants were also sequenced in both directions. Sequence data were analyzed with the University of Wisconsin Genetics Computer Group sequence analysis software package.

**Nucleotide sequence accession number.** The sequence of the *cutC* gene has been submitted to the NCGR (accession number L38618). The sequence of the *cutF* region has been submitted to the NCGR (accession number L38619).

## RESULTS

**Isolation of clones complementing $Cu^s$ mutations in the *cutC* and *cutF* mutants.** The $Cu^s$ *cutC* and *cutF* mutants of *E. coli* were transformed with the LBST genomic library, and the transformants were replica plated under nonpermissive conditions. Seven copper-tolerant clones (pCUTC1 to pCUTC7) of the *cutC* mutant and three tr and copper-tolerant clones (pCUTF1 to pCUTF3) of the *cutF* mutant were obtained.

On the basis of restriction enzyme analysis of the DNA inserts, clones 2, 3, 6, and 7 among the *cutC*-complementing clones were identical; they all had an insert of about 600 bp and lacked commonly used restriction enzyme sites (Fig. 1). pCUTC1 and pCUTC4 had 5.5- and 8-kb DNA inserts, respectively, and they both contained common *Pst*I (1.6- and 1.7-kb) fragments, indicating that these two clones had overlapping sequences. pCUTC5 had an insert of ~3 kb, and its restriction map was different from those of the pCUTC1 and pCUTC4 clones (Fig. 1 and 2). [α-$^{35}$S]dCTP-labeled pCUTC2 insert was found to hybridize with pCUTC1 and pCUTC4 but not with pCUTC5 or any of the pCUTF clones. *Pst*I fragments of the pCUTC1 clone hybridized with pCUTC4 and not with

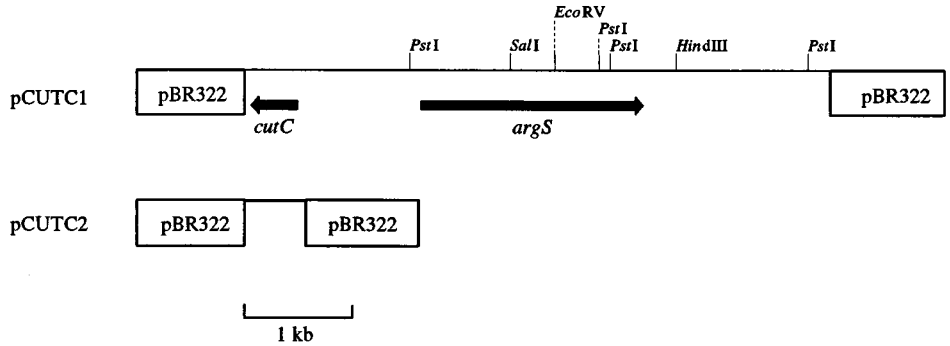


FIG. 1. Restriction map of plasmid pCUTC1 and determination of the physical map position of the *cutC* gene on the *E. coli* chromosome. The position of pCUTC2 and the orientation of *cutC* were determined by nucleotide sequence determination of the DNA insert in pCUTC2 and partial sequencing of the DNA insert in pCUTC1 near the *Bam*HI site in pBR322 and 5′ upstream of the *argS* gene. Boxes indicate pBR322 sequences, and arrows designate the directions of transcription of the *cutC* and *argS* genes.

pCUTC2, pCUTC5, or any of the pCUTF clones (data not shown). These results support the conclusion that pCUTC1, pCUTC2, and pCUTC4 are overlapping clones and that pCUTC5 is different from all the other pCUTC clones. The smallest clone, pCUTC2 (about 600 bp), conferred copper tolerance to the *cutC* mutant. Two clones (pCUTC1 and pCUTC2) were chosen for further studies.

Restriction enzyme analysis of the *cutF*-complementing clones indicated that clones 1, 2, and 3 were identical. pCUTF1 contained an insert of 6.3 kb and was chosen for further studies (Fig. 2). Furthermore, pCUTC5, isolated as a *cutC*-complementing clone, had DNA fragments common to pCUTF1 (Fig. 2). The [α-$^{35}$S]dCTP-labeled *Hin*dIII fragment of the pCUTF1 clone hybridized with the pCUTF2, pCUTF3, and pCUTC5 clones but not with any of the other pCUTC clones (data not shown). These data suggest that pCUTF1 and pCUTC5 are also overlapping clones.

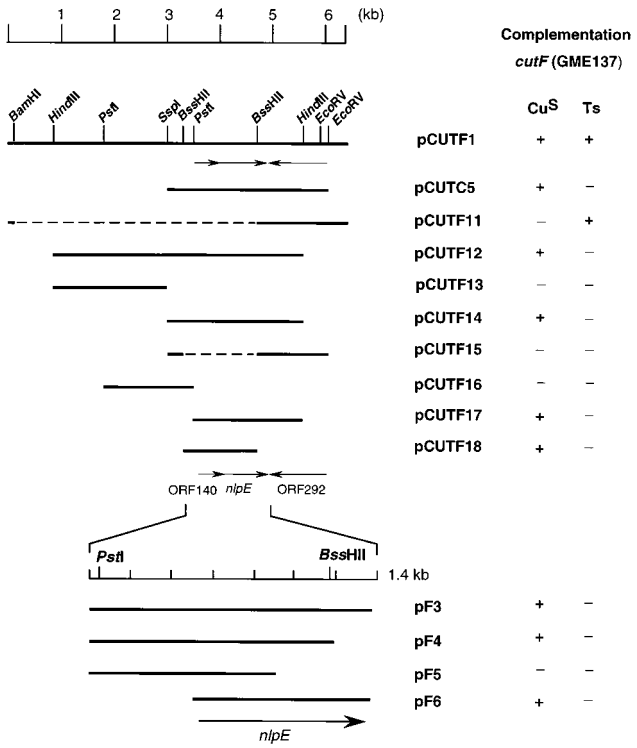


FIG. 2. Restriction map of the pCUTF1 clone and identification of the *cutF* gene by subcloning and complementation. A simplified physical map of the *E. coli* pCUTF1 clone and the inserts in the subclones are shown at the top. Dashed lines indicate deletions, and arrows denote the direction of transcription of ORF140, ORF236 (*cutF*/*nlpE*), and ORF292. The ability of the subclones to complement Cu$^s$ and ts phenotypes of the *cutF* mutant or its lack thereof is expressed as + or − and is indicated on the right. Plasmids pF3, pF4, pF5, and pF6 indicate subclones constructed by PCR.

**Mapping of the pCUTC1, pCUTC2, and pCUTF1 clones on the *E. coli* chromosome.** [α-$^{35}$S]dCTP-labeled pCUTC1 and pCUTC2 DNA were found to hybridize with Kohara lambda phage clones 337 and 338 (20), which map to 42 min on the *E. coli* chromosome (31). pCUTC1 covered the region from kb 1969 to 1974.5 on the *E. coli* chromosome (31) (Fig. 1). Since the *Pst*I fragments of pCUTC1 did not hybridize with pCUTC2 DNA and the *Pst*I-*Hin*dIII fragment of pCUTC1 contains the gene encoding arginyl-tRNA synthetase (*argS*) (14), we conclude that the pCUTC2 insert lies to the left of *argS*, as shown in Fig. 1.

[α-$^{35}$S]dCTP-labeled pCUTF1 or pCUTC5 DNA was found to hybridize with Kohara phage clones 122 and 123 (20), which map at 4.7 min on the *E. coli* chromosome (31). Comparison of the restriction map of pCUTF1 with those of the Kohara phage clones 122 and 123 indicated that the insert in pCUTF1 covered the region from kb 218.8 to 225.1 on the *E. coli* chromosome (31) (Fig. 2).

**Sequence determination of the *cutC* gene.** The DNA sequence of the 583-bp insert in plasmid pCUTC2 was determined (Fig. 3). Computer analysis of the DNA sequence revealed a single ORF composed of 146 codons. To define the exact location and orientation of the *cutC* gene relative to that of *argS* on the *E. coli* chromosome, the insert in pCUTC1 was partially sequenced with a primer adjacent to the *Bam*HI site in pBR322 and a primer 5′ upstream of the *argS* gene. The sequence obtained with the primer near the *Bam*HI site was identical to that obtained with the same primer and pCUTC2 as the template DNA, indicating that the *cutC* gene is located about 1 kb from the 5′ end of the *argS* gene and is transcribed in the opposite direction to *argS* (Fig. 1).

**Subcloning of pCUTF1 and pCUTC5.** pCUTF1 was found to confer both copper-tolerant and tr phenotypes on the *cutF* mutant, while the overlapping clone, pCUTC5, selected as a complementing clone of the *cutC* mutant, conferred copper tolerance but not the tr phenotype to the *cutF* mutant. Subcloning and complementation studies revealed that the 4.5-kb *Hin*dIII fragment cloned in pBR322 (plasmid pCUTF12), the 2.4-kb *Ssp*I-*Hin*dIII fragment cloned in pBR322 (plasmid pCUTF14), the 2-kb *Pst*I-*Hin*dIII fragment cloned in pK184

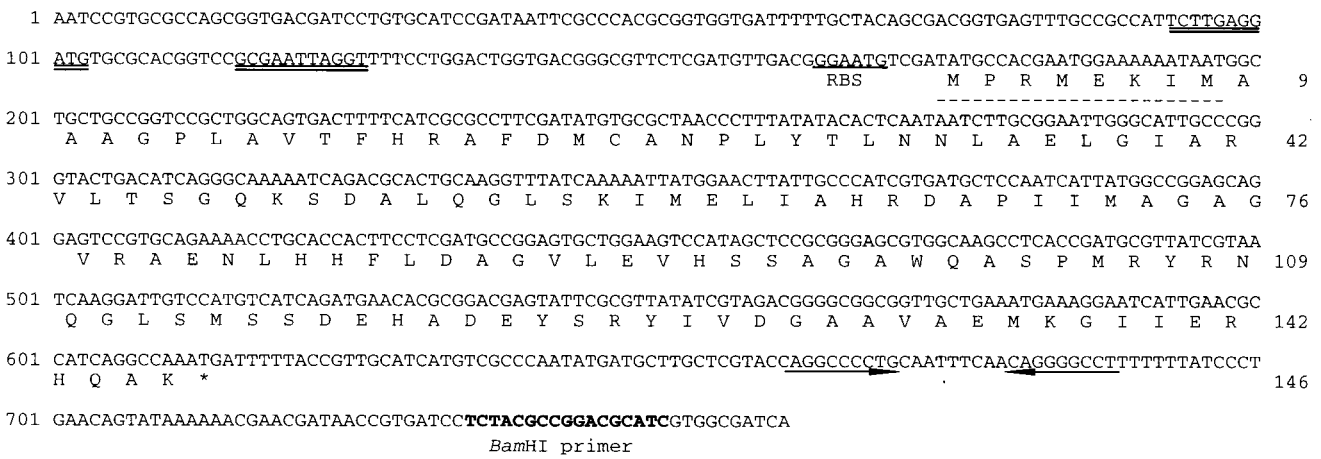


FIG. 3. Nucleotide sequence of the *cutC* gene and deduced amino acid sequence of its gene product. The putative ribosome-binding site (RBS), potential promoter, and inverted repeat (the putative transcription termination stem-loop structure) are underlined, double underlined, and marked with arrows, respectively. The nucleotides are numbered on the left, and the deduced amino acid residues for the CutC protein (numbered on the right) are given below the DNA sequence in the single-letter amino acid code. The putative copper-binding motif at the N terminus is marked with a dashed line. The sequences near the *Bam*HI primer in pBR322 are in boldface type. *, stop codon.

(plasmid pCUTF17), and the 1.4-kb *Bss*HII-*Bss*HII fragment cloned in pBR322 (pCUTF18) all conferred the copper-tolerant but not the tr phenotype to the *cutF* mutant (Fig. 2). Plasmid pCUTF11 conferred only tr to the *cutF* mutant. pCUTF13 (containing the 2.1-kb *Hin*dIII-*Ssp*I fragment in pBR322), and pCUTF15, and pCUTF16 (containing the 1.8-kb *Pst*I fragment in pK184) did not complement either the $Cu^s$ or the ts phenotype. These results clearly showed that the ts and $Cu^s$ phenotypes of the *cutF* mutant were due to separate, albeit closely linked, mutations.

The nucleotide sequence of the genes near the 4.7-min region on the *E. coli* chromosome has been previously determined by H. Mori and his team (22a) while sequencing the *E. coli* genome and by T. Silhavy and his team (35). We sequenced the region of interest in pCUTF1 and pCUTF11 to resolve some discrepancies in the sequences obtained from Mori's and Silhavy's groups. The sequence data indicate that the *Pst*I-*Hin*dIII fragment (Fig. 4, bp 201 to 2083), which conferred only copper tolerance to the *cutF* mutant, contains two ORFs, ORF140 and ORF236, and a part of ORF292 (Fig. 4). Plasmid pCUTF11 containing only ORF292 conferred tr, suggesting that ORF292 immediately 3′ to ORF236 but in the opposite orientation was responsible for conferring the tr phenotype to the *cutF* mutant.

Complementation analysis of the *cutF* mutant with PCR-generated subclones revealed that clone pF6 containing only ORF236 conferred copper tolerance to the *cutF* mutant (Fig. 2). A clone with a deletion of the C-terminal 30 to 35 amino acids (pF4) still complemented the $Cu^s$ phenotype of the *cutF* mutant, while a larger C-terminal deletion (about 130 amino acids) (pF5) abolished the complementation (Fig. 2). ORF236 has been identified as a multicopy suppressor of the toxicity caused by the periplasmic LamB-LacZ-PhoA tripartite fusion protein (36) and has been shown to encode a new outer membrane lipoprotein, NlpE (35). This suppression is presumably mediated by the induction of the periplasmic DegP protease as a result of the overexpression of the outer membrane NlpE protein (11, 35), and the expression of the *degP* gene is partially epistatic to the multicopy expression of the NlpE protein (35). We have examined the role of DegP protein in the function of NlpE protein in partially restoring copper tolerance to the *cutF* mutant. As shown in Table 2, the cloned *cutF* (*nlpE*) gene conferred the copper-tolerant phenotype to a *degP*::Tn*10* derivative of the *cutF* mutant, suggesting that the complementation of the $Cu^s$ mutation in the *cutF* mutant does not require the induction of the DegP protease by overexpression of the NlpE protein. Strain WBS262 (MC4100 *nlpE*::*spc*), a null *nlpE* mutant, is slightly $Cu^s$ compared with its wild-type parental strain MC4100, and this copper sensitivity is complemented by the plasmid harboring the *cutF* (*nlpE*) gene but not suppressed by overproduction of the CutC protein (Table 2).

**Complementation of *cutC* and *cutF* mutants with pCUTC and pCUTF.** Transformation of the *cutC* mutant with pCUTF1 and pF6 conferred the copper-tolerant phenotype, and, reciprocally, pCUTC1 and pCUTC2 conferred copper tolerance but not the tr phenotype to the *cutF* mutant (Table 2). These results suggested two possibilities: (i) pCUTC1/pCUTC2 complemented the *cutC* mutation and multicopy suppressed the $Cu^s$ phenotype of the *cutF* mutant, while pCUTF1/pCUTC5 complemented the *cutF* mutation and multicopy suppressed the $Cu^s$ phenotype of the *cutC* mutant; or (ii) both the *cutC* and the *cutF* mutants isolated as $Cu^s$ mutants were in fact double mutants, altered in both the *cutC* and *cutF* genes identified above. Genetic mapping by P1 transduction as well as cloning and sequence determination of the *cutC*/*cutF* (*nlpE*) alleles from both the *cutC* and the *cutF* mutants were carried out to distinguish between these two possibilities.

**Copper tolerance of the *cutC* and *cutF* mutants harboring pCUTC or pCUTF clones.** The copper tolerance of the *cutC* and *cutF* mutants with or without the complementing clones was determined by the colony-forming assay on LB plates containing different concentrations of copper. The wild-type strain ED8739 formed normal-sized colonies after 24 h at 30°C on LB agar plates containing up to 4 mM copper with 100% survival, whereas the *cutC* and *cutF* mutants grew more slowly and formed colonies after 48 h at 30°C with 70 and 4% survival, respectively. After 48 h at 30°C, the wild-type cells formed colonies on LB plates containing 6 mM copper sulfate with 0.9% survival but the *cutC* and *cutF* mutants did not. The *cutC* and *cutF* mutants containing the complementing clones (pCUTC2, pCUTF1, or *nlpE* (*cutF*) cloned in pBR322) still grew more slowly on LB plates containing ≥2 mM copper sulfate; after 48 h at 30°C, the *cutC* mutant containing either the cloned *cutC* or the *cutF* gene formed colonies with 100%

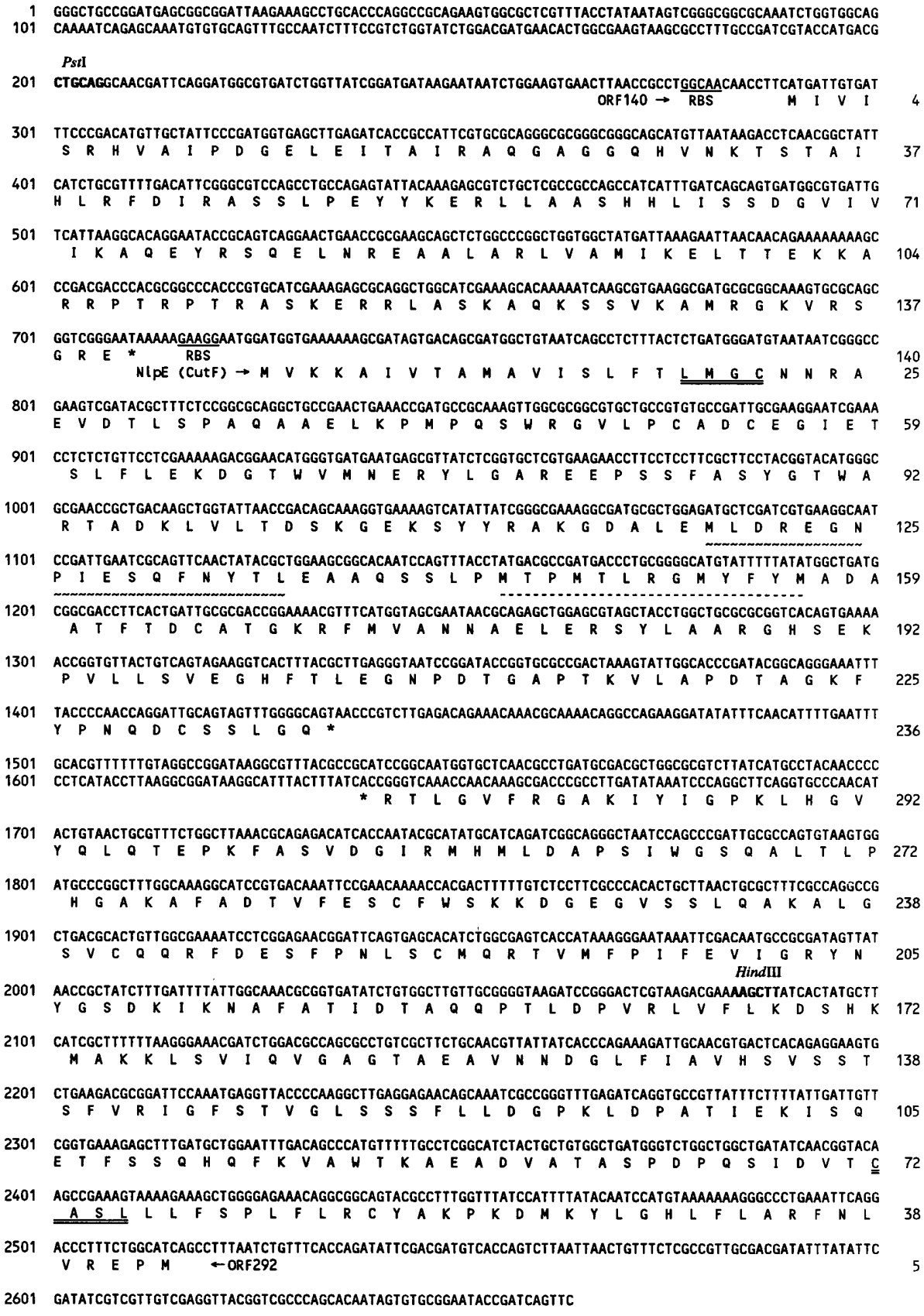


FIG. 4. Nucleotide sequence of the 2.7-kb fragment from pCUTF1 containing the *cutF* gene. Primers were designed from the known sequence, and DNA from pCUTF1 or pCUTF11 was sequenced in both directions. The putative ribosome-binding sites (RBS) are underlined. The nucleotides are numbered on the left, and the deduced amino acid sequences for ORF140, CutF (NlpE) protein, and ORF292 (numbered on the right) are given below the DNA sequence in the single-letter amino acid code. The lipobox in CutF and ORF292, the putative copper-binding sites, and the signature sequence of the soybean trypsin inhibitor in CutF (NlpE) are double underlined and indicated by – – – – and ~ ~ ~ ~ ~ ~, respectively. *, stop codon. The *Pst*I and *Hin*dIII restriction sites are in boldface type.

TABLE 2. Complementation of the $Cu^s$ and ts phenotypes of mutants with pCUTC or pCUTF plasmids and the plasmids harboring the mutant alleles of the *cutC* and *cutF* genes[a]

| Plasmid[b] | Complementation with[c]: | | | | |
|---|---|---|---|---|---|
| | *cutC* (GME111) ($Cu^s$) | *cutF* (GME137) | | *cutF degP*::Tn*10* ($Cu^s$) | MC4100 *nlpE*::*spc* ($Cu^s$) |
| | | $Cu^s$ | ts | | |
| pCUTC1/C2 | + | + | − | + | − |
| pCUTF1 | + | + | + | + | + |
| pF6 | + | + | − | + | + |
| pSDG1 | − | − | − | ND | ND |
| pSDG2 | − | − | − | ND | ND |
| pSDG3 | − | − | − | ND | ND |
| pSDG4 | + | − | − | ND | ND |

[a] Competent cells of the *cutC* (GME111), *cutF* (GME137), *cutF degP*::Tn*10*, and MC4100 *nlpE*::*spc* mutant strains were transformed with the plasmids, and the transformants were checked for copper-tolerant or tr phenotype by patching on LB-Amp plates with or without 4 mM copper and incubating at 30 or 42°C.

[b] Plasmids pSDG1 and pSDG2 contain the *cutC* alleles from the $Cu^s$ *cutC* and *cutF* mutants, respectively, and plasmids pSDG3 and pSDG4 carry the *cutF* alleles from the *cutC* and *cutF* mutants, respectively.

[c] +, ability to complement; −, lack of complementation; ND, not determined.

TABLE 3. Mapping of the $Cu^s$ and ts mutations in the *cutF* mutant by P1 transduction[a]

| Donor strain | Selected marker | Unselected markers | No. of transductants |
|---|---|---|---|
| RK4375 (*fhuB478*::Tn*10*) | $Tet^r$ | tr Cu tolerant | 9 |
| | | tr $Cu^s$ | 13 |
| | | ts Cu tolerant | 43 |
| | | ts $Cu^s$ | 35 |
| JW353 (*zae-502*::Tn*10*) | $Tet^r$ | tr Cu tolerant | 21 |
| | | tr $Cu^s$ | 49 |
| | | ts Cu tolerant | 9 |
| | | ts $Cu^s$ | 21 |
| NK5525 (*pro-81*::Tn*10*) | $Tet^r$ | tr Cu tolerant | 0 |
| | | tr $Cu^s$ | 3 |
| | | ts Cu tolerant | 0 |
| | | ts $Cu^s$ | 97 |
| CAG12156 (*uvrC279*::Tn*10*) | $Tet^r$ | tr Cu tolerant | 0 |
| | | tr $Cu^s$ | 0 |
| | | ts Cu tolerant | 30 |
| | | ts $Cu^s$ | 70 |
| CAG12126 (*zeb-3199*::Tn*10kan*) | $Kan^r$ | tr Cu tolerant | 0 |
| | | tr $Cu^s$ | 0 |
| | | ts Cu tolerant | 52 |
| | | ts $Cu^s$ | 48 |

[a] P1 lysates of different donor strains carrying Tn*10* or Tn*10kan* linked to 4.7 min or 42 min on the *E. coli* chromosome were used in transduction experiments with the *cutF* mutant (strain GME137) as the recipient. $Tet^r$ or $Kan^r$ transductants were purified on LB-Tet or LB-Kan plates, respectively, containing 10 mM citrate and were then scored for the tr phenotype by incubating the plates at 42°C and for copper tolerance by patching on LB agar plates containing 4 mM $CuSO_4$ and incubating at 30°C.

survival on LB plates containing 4 mM $CuSO_4$. The *cutF* mutant containing either pCUTF1 or the cloned *cutC* or the *cutF* gene formed colonies with 50 to 100% survival on LB plates containing 4 mM $CuSO_4$. Like the wild-type cells, the *cutC* mutant harboring the complementing clones formed colonies with 1 to 1.5% survival on LB plates containing 6 mM $CuSO_4$. These results suggest that the *cutC* or the *cutF* clone partially restores the copper-tolerant phenotype of both the *cutC* and the *cutF* mutants; the growth rate of mutant cells with the complementing clone is still lower than that of the wild-type parental strain.

**Mapping of $Cu^s$ mutations in *cutF*.** Cloning and subcloning data indicated that the mutations responsible for the $Cu^s$ phenotype in both the *cutC* and the *cutF* mutants were located near 4.7 and 42 min on the *E. coli* chromosome. The *cutC* mutant was P1 resistant, rendering the mapping of the $Cu^s$ mutation(s) by P1 transduction a difficult task. With the *cutF* strain as the recipient strain and RK4375 (*fhuB478*::Tn*10*), JW353 (*zae-502*::Tn*10*), and NK5525 (*pro-81*::Tn*10*) as the donor strains, we showed the existence of a $Cu^s$ mutation and a closely linked ts mutation in the *cutF* mutant located at 4.2 and 4.5 min, respectively (Table 3; Fig. 5A). In addition, copper-tolerant transductants of the *cutF* mutant (GME137) were obtained by Tn*10*- and Tn*10kan*-linked transduction with *uvrC279*::Tn*10* (30%) and *zeb-3199*::Tn*10kan* (52%) as the donors (Table 3), indicating the existence of a second mutation responsible for the $Cu^s$ phenotype located at 41.6 min on the *E. coli* chromosome (Fig. 5B). These results strongly suggest that the *cutF* mutant contained two mutations, one in the *cutF* (*nlpE*) gene and the other in the *cutC* gene, and that mutations in both genes are required for the $Cu^s$ phenotype of the *cutF* mutant. This conclusion is supported by the finding that Tn*10*-linked transduction of the wild-type strain with a *zae-502*::Tn*10* $Cu^s$ ts derivative of the *cutF* mutant as the donor yielded ts transductants, as expected, but no $Cu^s$ transductants (data not shown).

**Cloning and sequence determination of the *cutC* and *cutF* (*nlpE*) alleles from the *cutC* and *cutF* mutants.** The *cutC* and *cutF* (*nlpE*) alleles in the *cutC* mutant (strain GME111) were cloned by PCR as described in Materials and Methods. The complete sequences of both the *cutC* gene (about 600 bp) and the *cutF* (*nlpE*) gene (about 877 bp) were determined in both directions. The *cutC* allele in the *cutC* mutant was found to contain a mutation at bp 146 (from A to G), resulting in an amino acid substitution of Lys-49 by Arg-49. Analysis of the *cutF* (*nlpE*) allele from the *cutC* mutant showed that it contained two mutations, one in the signal peptide (bp 32 from C to T), resulting in the substitution of Ala-−10 by Val-−10 and the other in the mature protein (bp 301 from A to T), resulting in the replacement of Thr-81 by Ser-81. These results indicated that indeed the *cutC* mutant contained mutations in both the *cutC* gene and the *cutF* (*nlpE*) gene and that the restoration of the copper-tolerant phenotype of the *cutC* mutant by the cloned *cutC* or *cutF* gene is by complementation and not by multicopy suppression.

The *cutF* (*nlpE*) and *cutC* alleles were also cloned from the *cutF* mutant (strain GME137). The *cutF* (*nlpE*) gene from the *cutF* mutant was found to contain a mutation at bp 683 (A to C), resulting in the replacement of Asn-208 by Thr-208, and a second mutation upstream of ORF140 at position −91 bp (C to T); this second mutation may affect the promoter of ORF140 and *nlpE*. The *cutC* allele in the *cutF* mutant was found to contain a mutation at 197 bp (from G to A), resulting in an amino acid change from Arg-66 to His-66. The mutant *cutC* alleles from the *cutC* and *cutF* mutants cloned in pBR322 (plasmids pSDG1 and pSDG2, respectively) failed to complement the $Cu^s$ phenotype of both *cutC* and *cutF* mutants. The mutant *cutF* alleles from both the *cutC* and *cutF* mutants cloned in pBR322 (plasmid pSDG3 and pSDG4, respectively) also did not complement the $Cu^s$ phenotype of the *cutF* mutant. Plasmid pSDG3 (containing the *cutF* allele from the *cutC* mutant) did not complement the $Cu^s$ phenotype of the *cutC*

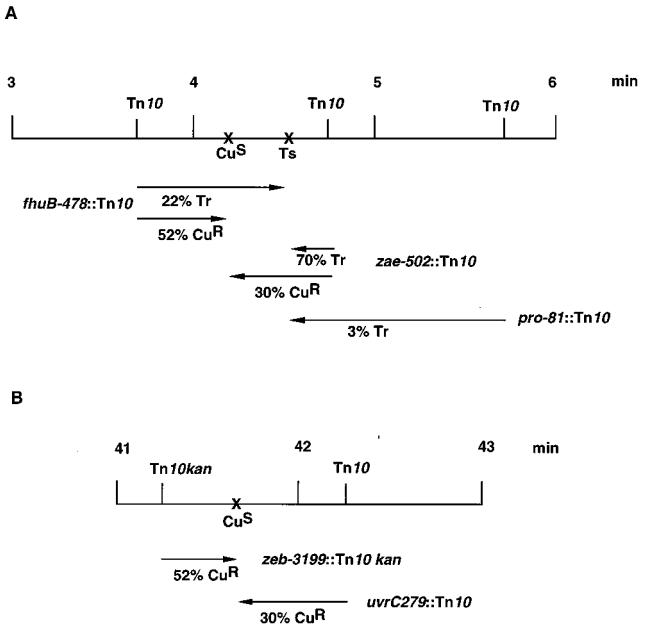


FIG. 5. Mapping of the $Cu^s$ and ts mutations in the *cutF* mutant by P1 transduction. (A) Genetic mapping of $Cu^s$ and ts mutations in the *cutF* mutant near 4.75 min on the *E. coli* chromosome. The *cutF* mutant cells were transduced with P1 phage grown on *fhuB478*::Tn*10*, *zae-502*::Tn*10* or *zae-502*::Tn*10kan*, and *pro-81*::Tn*10* strains as donors at 30°C. The linkages of the $Cu^s$ or ts phenotype to these transposons were determined by scoring the colonies on LB plates with or without 4 mM copper at 30 or 42°C, respectively. (B) Location of the $Cu^s$ mutation in the *cutF* mutant near 42 min on the *E. coli* chromosome. Tn*10kan* or Tn*10* from strains carrying *zeb-3199*::Tn*10kan* and *uvrC279*::Tn*10* were transduced into the *cutF* mutant. The $Tet^r$ or $Kan^r$ transductants were scored for copper tolerance, and the linkages to the transposons were determined.

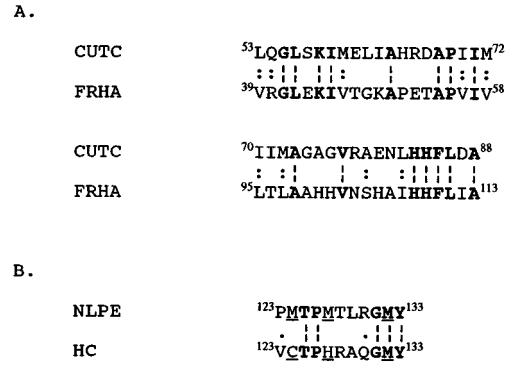


FIG. 6. Sequence homology of *E. coli* CutC and CutF proteins with the protein data bank. (A) Sequence homology between *E. coli* CutC and flavin-reducing hydrogenase α from *M. thermoautotrophicum*. Deduced amino acid sequences of CutC were compared with the gene bank by the BLAST program (37). (B) Sequence homology between *E. coli* NlpE and *N. pharaonis* halocyanin (HC).

mutant, but plasmid pSDG4 (containing the *cutF* allele from the *cutF* mutant) complemented the $Cu^s$ phenotype of the *cutC* mutant (Table 2). These results support the conclusion that both the *cutC* and *cutF* mutants were double mutants with mutations in the *cutC* and the *cutF* (*nlpE*) genes and that mutations in both genes are required for the $Cu^s$ phenotypes of these two mutants. The apparent complementation of the $Cu^s$ phenotype of the *cutC* mutant by pSDG4 might result from the leakiness of the *cutF* allele in the *cutF* mutant.

## DISCUSSION

The *cutC* and *cutF* mutants were both isolated as $Cu^s$ mutants which accumulated copper and showed a decreased efflux of copper (30). In addition to being $Cu^s$, the *cutF* mutant is ts and copper dependent. These differences in the phenotypes of the *cutC* and *cutF* mutants, along with the data on the kinetics of copper transport and accumulation in both mutants, led to the postulation that *cutC* and *cutF* mutants belonged to two distinct classes of $Cu^s$ mutants of *E. coli* (30). The *cutC* gene was postulated to code for an efflux protein which removes excess copper from the cytoplasm, and the CutF protein was proposed to be responsible for protecting the cell from copper toxicity and for delivering copper to the sites of assembly of copper proteins (30).

In the present study, we have identified two genes, *cutC* and *cutF* (*nlpE*), each of which confers copper tolerance to both *cutC* and *cutF* mutants of *E. coli*. We have located the *cutC* gene at 42.15 min and the *cutF* gene at 4.77 min of the *E. coli* chromosome. Analysis of the deduced amino acid sequence of the CutC protein suggests that it is a hydrophilic protein with multiple charged residues lacking an N-terminal signal sequence. Comparison of the predicted CutC sequence with the protein data bank by using the BLAST program (37) has revealed regions of significant homology with 8-hydroxy-5-deazaflavin-reducing hydrogenase α from *Methanobacterium thermoautotrophicum* (1) (Fig. 6A). Amino acid residues 53 to 72 and 70 to 88 of the CutC protein exhibit 40 and 36% identities and 65 and 57% similarities to amino acid residues 39 to 58 and 95 to 113, respectively, of hydrogenase α from *M. thermoautotrophicum*, a Ni-containing enzyme with homology to large subunits of other Ni-containing hydrogenases (1). In addition, the N terminal of CutC contains the M-1–PRMEKIM-8 sequence, a pattern (**M**-X-X-**M**-X-X-X-**M**) similar to a putative copper-binding motif (**M**-X-X-X-X-**M**-X-X-**M**) present in the CopB ATPase from *Enterococcus hirae* (23). The deduced amino acid sequence of the CutC protein suggests that it is a cytoplasmic copper-binding protein.

The *cutF* (*nlpE*) gene is flanked by ORF140 and ORF292. ORF140 and the *nlpE* gene are separated by 13 bp and probably share a promoter. ORF140 has 75% similarity and 60% identity to an ORF of unknown function adjacent to the gene encoding β-keto adipate succinyl coenzyme A transferase in *P. putida* (24). Using the BLAST program, we have identified two different regions (amino acids 8 to 39 and 70 to 103) in ORF140 showing significant homology to peptide chain release factors in *E. coli*, *Salmonella typhimurium*, *Bacillus subtilis*, and *S. cerevisiae* mitochondria (7, 12, 25, 26). ORF292, which is immediately 3′ to the *nlpE* gene and responsible for conferring tr to the *cutF* mutant, is separated by 199 bp and is transcribed in the direction opposite that of *nlpE*. Examination of the NlpE sequence reveals several interesting features. It contains a typical lipobox (LGMC) in its N-terminal signal sequence, and the predicted lipoprotein nature of NlpE protein has been verified (35). The LMGC sequence is followed by NNRAE, and its localization in the outer membrane has been demonstrated experimentally (35). Odermatt et al. (23) recently identified two P-type copper efflux ATPase genes, *copA* and *copB*, in a gram-positive bacterium, *E. hirae*, and showed that a disruption of either *copA* or *copB* results in copper sensitivity. NlpE contains a sequence **M**-124–TP**M**TLRG**M**YFY**M**-136 which is similar to the putative copper-binding motif **M**-X-X-X-X-**M**-X-X-**M** present three times at the N terminal of CopB ATPase from *E. hirae* (23). Halocyanin, a membrane-associated copper-binding lipoprotein isolated from the archaeal bacterium

*Natronobacterium pharaonis*, contains seven potential copper-binding domains with conserved sequences based on comparison with other cyanins, pseudoazurin, and azurin proteins (21). One of the H.8 antigens of *Neisseria gonorrhoeae* is also an outer membrane lipoprotein, and its C-terminal 127 amino acids are homologous to copper-containing proteins known as azurins (16). Comparison of the NlpE sequence with that of halocyanin has revealed a motif of P-123–MTPMTLRGMY-133 with 45% identity and 64% similarity to one of the conserved and potential metal-binding domains in these small blue copper proteins (Fig. 6B). The presence of this potential metal-binding motif **M**-124–X-X-**M**-X-X-X-X-**M**-X-X-X-**M**-136 in CutF (NlpE) suggests that NlpE is an outer membrane lipoprotein with metal-binding capacity, a function similar to those of CopA, CopB, and CopC encoded by plasmid-borne *cop* genes in *P. syringae* (2, 5).

NlpE also contains a sequence of M-99–LDREGN-PIESQFNYSL-115, similar to the signature sequence of serine protease inhibitors (27). This motif is present at the N terminus of serine protease inhibitors (27), while in NlpE it is present in the middle of the protein. According to Silhavy and his colleagues, an overexpression of NlpE suppresses the toxic effects of periplasmic LamB-LacZ-PhoA fusion protein by inducing the synthesis of DegP (a serine protease) via a two-component system, *cpxR* and *cpxA* (6, 11, 35). Thus, the multicopy suppression of the maltose sensitivity caused by the expression of periplasmic LamB-LacZ-PhoA fusion protein is dependent on a functional DegP protein. However, CutF (NlpE) protein partially restores copper tolerance in the *cutF* mutant in the absence of a functional DegP. Preliminary studies show that the *cpxR* null mutants are also more copper sensitive than their wild-type parental strains, and this $Cu^s$ phenotype of the *cpxR* mutant is suppressed by a plasmid carrying the *cutC* gene and not by a plasmid containing the *nlpE* gene. Whether the CpxA-CpxR system is involved in the function of CutF remains to be ascertained. The possible role of the putative signature sequence of the soybean trypsin inhibitor proteins in NlpE in inducing DegP synthesis (11) and in maintaining copper tolerance, if any, also remains to be elucidated.

A comparison of the sequence of ORF292 (complementing the ts phenotype of the *cutF* mutant) with the gene bank has revealed a 100% identity with the partial ORF present at the 3′ end of *drpA*/*proRS* genes (13, 39). The *drpA* gene product is involved in global RNA synthesis (39), and *proRS* codes for prolyl-tRNA synthetase (ProRS) (13). The predicted *drpA*/*proRS* gene products differ in size because of the discrepancies in their reported sequences (13, 39). A motif search of ORF292 by the Prosite program has revealed that the sequence LSAC at amino acid residues 36 to 39 may be a lipobox. However, a closer inspection of the N-terminal sequence suggests that the putative signal sequence containing this lipobox is unusual both in the lengths of its *n* segment (26 residues) and *h* plus *c* segment (9 residues) and in the overall length of the signal sequence. The lipoprotein nature of this ORF remains to be ascertained.

The ts allele in the *cutF* mutant could be transduced into a wild-type background. On the other hand, no $Cu^s$ transductant of the wild-type strain was obtained when the *cutF* mutant carrying Tn*10* at either 4.7 or 42 min was used as the donor. These results suggest that ts and $Cu^s$ mutations (between 4 and 5 min) are distinct and that more than one mutation is required for the $Cu^s$ phenotype. Consistent with the result is the finding that a null mutant of *nlpE* is slightly $Cu^s$ and that this copper sensitivity is suppressed by the plasmids harboring the *cutF* (*nlpE*) gene but not the *cutC* gene. The exact functions of CutC and CutF proteins are not clear, but each protein is required for copper tolerance in *E. coli*. CutC has been previously suggested to be involved in the efflux of copper (30). It remains possible that CutC is a cytosolic component of an efflux pathway for copper. While the CutF protein has been suggested to be involved in both copper efflux and the delivery of copper to copper-dependent enzymes (30), our results suggest that CutF is an outer membrane protein with potential copper-binding sites.

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