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Cloning and Characterization of *cutE*, a Gene Involved in Copper Transport in *Escherichia coli*

SUZANNE D. ROGERS,[1]* MRINAL R. BHAVE,[2] JULIAN F. B. MERCER,[2] JAMES CAMAKARIS,[1] AND BARRY T. O. LEE[1]

Department of Genetics, The University of Melbourne,[1] and The Murdoch Institute, Royal Children's Hospital,[2] Parkville, 3052, Australia

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The copper-sensitive/temperature-sensitive phenotype of the *Escherichia coli cutE* mutant has been complemented by cloning wild-type genomic DNA into the plasmid vector pACYC184 and selecting transformants on medium containing 4 mM copper sulfate and chloramphenicol. One of these complementing clones, designated pCUT1, contained a 5.6-kb *Bam*HI fragment. This recombinant plasmid transformed *cutE*, allowing wild-type growth of transformants on medium containing copper sulfate. Complementation of copper sensitivity was assessed by comparing both cell survival at increased copper levels and the results of ^{64}Cu accumulation assays. An *Eco*RI subclone, 2.3 kb in size, was also shown to complement *cutE* when cloned in both medium- and high-copy-number vectors and was completely sequenced. This clone was mapped on the *E. coli* physical map at 705.70 to 707.80 kb. A series of subclones was constructed from pCUT1 and used to show that the large open reading frame of the translated sequence was essential for complementation. This open reading frame has a potential upstream promoter region, ribosome-binding site, and transcriptional terminator and encodes a putative protein of 512 amino acids that contains a region showing some homology to a putative copper-binding site.

Copper is essential for the function of a number of cellular enzymes, yet its inherent properties also result in toxicity. For example, copper is required by some enzymes which transfer electrons from substrates to oxygen. Cytochrome oxidase accepts electrons in the respiratory chain and transfers them to dioxygen, with the redox center of this enzyme functioning through the ability of the two associated copper ions to transfer electrons (16). However, copper-dependent protein degradation has been demonstrated elsewhere (11), and membranes have been shown to be inactivated because of the radical peroxidation of lipids following exposure to copper (38). Therefore, copper may indirectly cause severe damage in biological systems by virtue of its role in the production of free radicals (11, 36). Of necessity, therefore, the level of free copper within a cell must be limited, and the transport of copper into cells and its transfer to copper-requiring enzymes is expected to be tightly regulated. The mechanisms by which this process occurs are as yet poorly understood, but copper is likely to be transported within cells in a bound, presumably nontoxic form. In eukaryotic cells, the way in which metallothioneins bind heavy metals, including copper, has been studied extensively, although the exact role of these metalloproteins in response to levels of metal within the cell is unclear (13). A prokaryotic metallothionein in a *Synechococcus* sp. has been described, but its physiological importance is unknown (24).

Most investigations of the way in which bacterial cells handle heavy-metal ions have centered on compounds which exist in the environment as pollutants and are toxic but are not essential for cell survival. Bacterial resistance to mercury is the best studied of these systems (35, 37). The transposon Tn*501*, which confers mercury resistance in bacteria, is one system in which the genes and regulation of resistance have been studied in detail (20, 22, 35). Bacterial resistance to copper has also been reported where copper sprays have been used to control plant pathogens, and the genetics of this system is being elucidated (7). The use of copper as a growth enhancer for pigs has resulted in the emergence of copper resistance determinants such as the plasmid pRJ1004 (39), which confers copper-inducible copper resistance in *Escherichia coli* (31).

As part of an investigation into the ways in which cells are able to handle this toxic yet essential metal, we are studying chromosomally encoded mechanisms for copper transport and utilization in wild-type *E. coli* and therefore tolerance as differentiated from resistance to copper. Six chromosomal genes which may be involved in copper transport have previously been identified by the isolation and characterization of copper-sensitive, copper-dependent mutants (29). These mutants were designated *cutA* through *cutF*. One copper-sensitive mutant, *cutE*, is not altered in uptake (29) and is dependent on copper, as it will not grow on medium which has been depleted of copper but will grow when 0.05 mM copper sulfate is added to copper-depleted medium (28). It was proposed that copper, which is normally bound to a specific storage and/or transport protein encoded by *cutE*, is toxic when associated with other cellular components and is not transported to copper-dependent enzymes (29).

The availability of strains which are sensitive to copper and the ability to measure uptake, export, and accumulation of ^{64}Cu provided a strategy for cloning genes involved in copper transport. The present work describes the isolation and characterization of a gene which complements the *cutE* mutation. DNA sequence analysis of the complementing clone has allowed some further insight into the function of this gene.

MATERIALS AND METHODS

Media. All strains were cultured in Luria broth (LB) or Luria agar (LA) with selective agents when appropriate.

* Corresponding author.

Chloramphenicol at a final concentration of 20 μg/ml, carbenicillin at 250 μg/ml, and ampicillin at 100 μg/ml were used. Copper-containing medium was prepared by adding appropriate amounts of 1.0 M $CuSO_4$ to LB or LA and adjusting the pH to 7.5 with NaOH before autoclaving (39).

Bacterial strains, culture conditions, and plasmids. *E. coli* ED8739 (*hsdR hsdM metB gal lac supE supF*) (2) was used as wild type for copper sensitivity and was also used for construction of the genomic library. GME135 is the temperature-sensitive, copper-sensitive mutant (*cutE355*) derived from ED8739 (28, 29). The strain TG2 (*hsdR recA*::Tn*10*) (4) was used for maintenance of recombinant plasmids. Phage DNA isolation and plate lysates were performed with *E. coli* LE392 (2).

pACYC184, a medium-copy-number cloning vector (5), was used for construction of the genomic library. pUC19 (21) and Bluescript SK+ (Stratagene Cloning Systems) (34) were used for subcloning DNA fragments of interest. The plasmid pCUT1 is a pACYC184 recombinant containing a 5.6-kb *Bam*HI genomic insert which complements the copper sensitivity of GME135 (this study).

Recombinant DNA techniques. Restriction endonucleases and DNA ligase were purchased from Boehringer Mannheim Ltd. and used according to the manufacturer's specifications. Isolation of genomic and phage DNAs, purification of DNA on CsCl gradients, plasmid preparations, ligations, transformations, and phage growth and purification were performed as described by Sambrook et al. (32).

Construction of genomic library and isolation of clones that complement the *cutE* mutation. The genomic library from ED8739 was constructed by cloning a complete *Bam*HI digest of genomic DNA into the unique *Bam*HI site of pACYC184. The cloning vector had been treated with calf intestinal phosphatase (32). Competent cells of the copper-sensitive strain GME135 were prepared by using the protocol described by Brown and Lund (4), transformed with the recombinant pACYC184 plasmids (approximately 20 ng of plasmid), and plated onto LA plates containing 4 mM $CuSO_4$ and chloramphenicol. Colonies which grew on these selection plates were considered to complement the copper-sensitive phenotype of GME135 and were tested for copper survival and accumulation as described below. Plasmid DNA was prepared from complementing isolates, and the inserts were mapped with restriction enzymes and subsequently used for further analysis.

Spot recombination test. Overnight cultures of strain GME135 were grown in LB containing 0.2% maltose. Copper sulfate plates (4 mM) were spread with 2×10^8 cells and spotted with serial dilutions of phage. After incubation at 37°C, colonies which were tolerant of this level of copper sulfate could be counted.

Assay of copper sensitivity and ^{64}Cu accumulation. Copper sensitivity was assayed by using overnight LB cultures of the strains to be tested; 10-μl aliquots of serial 10-fold dilutions of each culture were plated on LA supplemented with various amounts of copper. Colonies were counted after 24 h at 37°C. For ^{64}Cu accumulation studies, radioactive copper (as cupric chloride) was obtained from the Australian Nuclear Science and Technology Organization, Lucas Heights, Australia, at a specific activity of 5,180 to 7,400 MBq/mg of Cu. Overnight cultures of the strains to be assayed were diluted 10-fold into LB and grown with shaking to an A_{620} of 0.7. Duplicate 3-ml samples were sedimented by centrifugation, washed twice in 3 ml of HBS (15 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.0], 0.9 mM $CaCl_2$, 2.65 mM KCl, 0.49 mM $MgCl_2$, 134.7 mM NaCl, 5.55 mM glucose, 100 μg of chloramphenicol per ml), and suspended in 1.5 ml of the same solution. Samples were stored on ice for 60 min and then warmed to 37°C (for 5 min) before the addition of 1.5 ml of ^{64}Cu solution (74 MBq/ml) in HBS (prewarmed to 37°C) to each suspension to initiate accumulation. The addition of ^{64}Cu represented time zero. The suspensions were briefly vortexed and then incubated with shaking at 37°C. Samples (0.5 ml) were taken at 0, 5, 10, 20, and 30 min and added to 0.7 ml of cold 0.85% NaCl. Samples were stored on ice (for approximately 30 min) to facilitate bulk processing of samples and then sedimented by centrifugation, washed twice in 0.8 ml of 0.85% NaCl–1 mM EDTA, and finally suspended in the same volume of saline. Centrifugation and washing rather than filtration were necessary, as significant nonspecific binding of copper to all types of membrane filters tested has been found (30). The radioactivity was counted in an LKB 1282 universal gamma counter. The amount of radioactivity at time zero (less than 10% of counts after 30 min of incubation) was subtracted from the amounts at other readings to eliminate the effect of nonspecific binding of ^{64}Cu to cell components. Counts were not converted to molar concentrations, since the specific activity of ^{64}Cu batches varied and atomic absorption facilities are required to obtain accurate copper concentrations. After counting, samples were stored at 4°C for 5 days (to allow radioactive decay over 10 half-lives) before their protein contents were determined by using the Bio-Rad protein assay reagent and the manufacturer's directions for microassay.

DNA sequencing. Subclones of pCUT1 in the Bluescript vector were produced by digestion with appropriate restriction endonucleases. Double-stranded sequencing was performed by the method of Sanger et al. (33) per the protocol supplied by the manufacturers of the Sequenase kit (United States Biochemical Corp.). The universal and T3 primers were used for sequencing initially, and then oligonucleotides synthesized on the basis of our sequence data were used. [α-^{35}S]dATP was purchased from Amersham International. The DNA was sequenced in both directions, and sequence data were compiled and analyzed by using the sequence analysis software and data bases of the Australian National Sequencing Analysis Facility.

Nucleotide sequence accession number. The nucleotide sequence presented here has been assigned the accession number X58070 and described as *E. coli cutE* gene by the EMBL Data Library.

RESULTS

Complementation of *cutE*. The medium-copy-number plasmid pACYC184(5) was initially chosen because it had been found previously (28) that copper resistance was not conferred in *E. coli* when the copper resistance determinant of pRJ1004 was subcloned into pBR322, which is a high-copy-number vector (1). In addition, we found that the activity of ampicillin but not of chloramphenicol was reduced by the levels of copper sulfate used in our experiments. Therefore, we were able to select directly for pACYC184 recombinant plasmids, which complemented *cutE*, in the presence of copper sulfate and chloramphenicol. It was subsequently found that complementing clones could be transferred into the high-copy-number vectors needed for template preparation for DNA sequencing and that these recombinant plasmids also complemented copper sensitivity (see below).

Transformation of *cutE* competent cells with the genomic library described above provided three chloramphenicol-

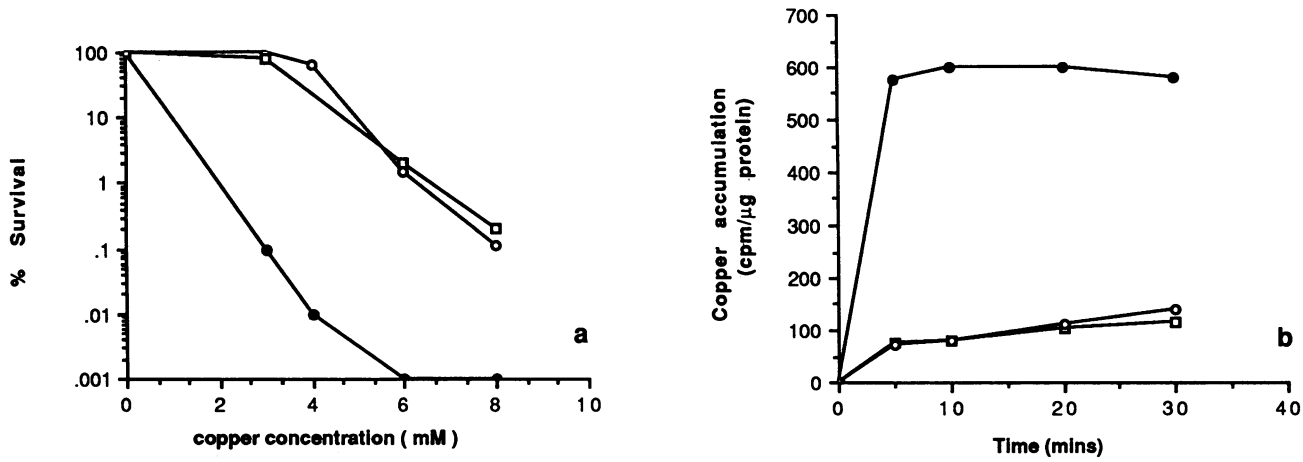

FIG. 1. Survival of CFU on medium containing copper sulfate (a) and accumulation of ^{64}Cu (b) by wild-type (○), *cutE* (●), and complemented *cutE* (□) strains. Survival and accumulation data shown for complemented *cutE* strains were obtained by using GME135 transformed with pCUT1. Since the specific activities of ^{64}Cu batches varied (see text), accumulation data are representative results of experiments performed with one batch of ^{64}Cu.

resistant, tetracycline-sensitive isolates which also showed the parental copper phenotype when grown on medium containing 4 mM copper sulfate. These contained pACYC 184 with a 5.6-kb *Bam*HI insert, and plasmid DNAs from these three isolates transformed *cutE*, giving approximately 10^5 transformants per μg of DNA when selected on medium containing 4 mM copper sulfate. No colonies were obtained on 4 mM copper sulfate selective medium when *cutE* was transformed with pACYC184 alone. Restriction endonuclease digestion of the plasmids indicated the presence of *Eco*RI, *Sal*I, and *Eco*RV sites within the inserts. Restriction patterns of the three clones with the enzymes named above were identical, and therefore one clone was chosen for further analysis and designated pCUT1. In order to test the specificity of pCUT1, this plasmid was transformed into copper-sensitive strains which have been previously described (29). The number of copper-tolerant colonies obtained on copper sulfate selection plates was not above the reversion frequency for any other known *cut* mutants (data not shown).

Results of measuring the toxic effect of copper sulfate on wild type, *cutE*, and *cutE* transformed with pCUT1 are given in Fig. 1a. It can be seen that no significant differences were found between the sensitivities of wild-type and complemented strains compared with the sensitivity of *cutE* strains. As it had been postulated that mutations in *cutE* result in defective storage and/or transport of copper (29), comparisons of ^{64}Cu accumulation were also made. Figure 1b demonstrates that, again, no differences between wild-type and complemented strains were observed but that under the experimental conditions used, strains containing a mutation in *cutE* accumulated up to five times more copper than did the wild-type and complemented strains. When *cutE* was transformed with pACYC184 alone, its sensitivity to and accumulation of copper were not altered (data not shown).

When subcloned into pACYC184, a 2.3-kb *Eco*RI fragment from the 5.6-kb *Bam*HI insert of pCUT1 also complemented *cutE*. By measuring the ability of transformed strains to grow on medium containing up to 8 mM copper sulfate and by using copper accumulation assays (data not shown), it was found that the recombinant plasmid containing this *Eco*RI fragment complemented *cutE* to the same extent as pCUT1. When the 2.3-kb *Eco*RI fragment was used to probe a Southern blot of *E. coli* wild-type genomic DNA, a single signal at 2.3 kb was obtained (data not shown), thus confirming the origin of the complementing DNA.

DNA sequence analysis of the complementing clone. The 2.3-kb *Eco*RI fragment was subcloned into pUC19 and the Bluescript sequencing vector, and these constructs also completely complemented *cutE*, as described for pCUT1. Nucleotide sequencing of the complementing clone gave an actual size of 2,333 bp for the *Eco*RI insert. Analysis of the translated nucleotide sequence revealed a single open reading frame (ORF) contained within this fragment, with good potential prokaryotic transcription signals corresponding to the consensus for the *E. coli* promoter (12). The ORF extended from positions 394 to 1925 in the nucleotide sequence (Fig. 2), giving a predicted protein of 512 amino acid residues. No large hydrophobic stretches of amino acids or evidence of a leader sequence was found by the hydropathy method of Kyte and Doolittle (15). The predicted protein gave a nonsignificant score when it was tested (by the method of Dodd and Egan [10]) for the presence of the helix-turn-helix DNA-binding motifs expected in regulatory proteins. Searches of the NBRF provisional proteins and EMBL data bases extracted no sequences significantly homologous to that presented here. However, one further feature noted (Fig. 2) is a short span of amino acids (His-X-X-Met-X-X-Met) which shows homology to a putative copper-binding motif (19).

Potential −10 and −35 sequences have been identified upstream of the ORF (Fig. 2) on the basis of consensus sequences derived by Harley and Reynolds (12). A potential ribosome-binding site and a proposed transcription start point that fit the criteria of those often found in *E. coli* (17, 27) were also present (Fig. 2). Alternative promoter sequences may be identified in this region, but those which were considered to best fit the consensus are shown in Fig. 2. We have noted several sequences following the stop codon of the predicted translation product that fit the requirements for an RNA transcriptional terminator (27), and the region which we consider to best fit these criteria is also underlined in Fig. 2. Regions of perfect dyad symmetry exist

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GAATTCGGTGGGGTTTCCGGTCTGGTGACCATTGAAGACATCCTGGAACTGATTGTTGGTGAGATTGAAGACGAGTATGACGAAGAAGATGATATCGACTTCCTGCAGCTGAGTCGTCAT  120

ACCTGGACCGTACGCGCACTGGCTTCCATTGAAGACTTCAACGAAGCGTTCGGCACCCACTTTAGCGATGAAGAAGTCGACACTATCGGTGGTCTGGTGATGCAGGCATTTGGGCATCTT  240

CCGGCGCGTGGCGAAACTATCGACATCGACGGTTACCAGTTCAAAGTGGCGATGGCCGACAGTCGGCGTATTATTCAGGTTCATGTCAAAATCCCGGATGACTCACCCCAGCCGAAGCTG  360
                                     = =  =         ======                  ======
                                                     <------    ------>                                   <----  ---->

                      RBS             M  A  F  A  S  L  I  E  R  Q  R  I  R  L  L  L  A  L  L  F  G  A  C  G  T  L  A  F  S
GATGAATAAAACCGAAACTGGATAGATAACTACATGGCTTTTGCCTCATTAATTGAACGCCAGCGCATTCGCCTGCTGCTGGCGTTATTATTCGGTGCCTGCGGAACGCTGGCCTTCTCT  480

 P  Y  D  V  W  P  A  A  I  I  S  L  M  G  L  Q  A  L  T  F  N  R  R  P  L  Q  S  A  A  I  G  F  C  W  G  F  G  L  F  G
CCTTACGACGTCTGGCCTGCGGCGATTATTTCGCTGATGGGGCTTCAGGCGTTGACCTTTAACCGCCGTCCACTCCAGTCTGCCGCTATTGGCTTTTGCTGGGGATTTGGCCTCTTTGGC  600

 S  G  I  N  W  V  Y  V  S  I  A  T  F  G  G  M  P  G  P  V  N  I  F  L  V  V  L  L  A  A  Y  L  S  L  Y  T  G  L  F  A
AGCGGTATTAACTGGGTCTATGTCAGCATCGCGACCTTTGGCGGAATGCCTGGCCCGGTTAACATCTTCCTGGTGGTGCTGCTGGCGGCGTATTTGTCGCTGTATACCGGACTGTTTGCT  720

 G  V  L  S  R  L  W  P  K  T  T  W  L  R  V  A  I  A  A  P  A  L  W  Q  V  T  E  F  L  R  G  W  V  L  T  G  F  P  W  L
GGCGTGCTGTCGCGTCTGTGGCCGAAAACCACCTGGCTGCGCGTAGCGATTGCCGCCCCTGCCCTCTGGCAAGTGACCGAGTTTCTGCGCGGTTGGGTACTGACCGGCTTCCCGTGGTTA  840

 Q  F  G  Y  S  Q  I  D  G  P  L  K  G  L  A  P  I  M  G  V  E  A  I  N  F  L  L  M  M  V  S  G  L  L  A  L  A  L  V  K
CAGTTCGGCTATAGCCAGATTGATGGCCCGTTAAAAGGGCTGGCACCGATAATGGGCGTGGAAGCCATTAACTTCCTGCTGATGATGGTTAGTGGCCTGCTGGCACTGGCGTTGGTCAAA  960

 R  N  W  R  P  L  V  V  A  V  V  L  F  A  L  P  F  P  L  R  Y  I  Q  W  F  T  P  Q  P  E  K  T  I  Q  V  S  M  V  Q  G
CGCAACTGGCGTCCGCTGGTGGTGGCCGTCGTGCTGTTTGCCCTTCCCTTCCCGCTGCGTTACATCCAGTGGTTTACCCCACAACCGGAGAAAACCATTCAGGTTTCGATGGTTCAGGGC  1080

 D  I  P  Q  S  L  K  W  D  E  G  Q  L  L  N  T  L  K  I  Y  Y  N  A  T  A  P  L  M  G  K  S  S  L  I  I  W  P  E  S  A
GATATTCCGCAATCGCTGAAATGGGACGAAGGCCAGCTTCTTAATACGCTGAAGATTTACTACAACGCAACGGCACCGCTGATGGGCAAATCATCGTTGATTATCTGGCCGGAGTCGGCG  1200

 I  T  D  L  E  I  N  Q  Q  P  F  L  K  A  L  D  G  E  L  R  D  K  G  S  S  L  V  T  G  I  V  D  A  R  L  N  K  Q  N  R
ATAACCGATCTGGAAATTAATCAGCAACCGTTCCTCAAAGCACTGGACGGTGAGTTGCGTGATAAAGGTAGCTCGCTGGTAACCGGGATTGTCGACGCGCGTCTCAATAAGCAGAACCGC  1320

 Y  D  T  Y  N  T  I  I  T  L  G  K  G  A  P  Y  S  Y  E  S  A  D  R  Y  N  K  N  H  L  V  P  F  G  E  F  V  P  L  E  S
TACGATACCTACAACACCATCATCACGCTGGGTAAAGGTGCGCCGTACAGCTACGAATCAGCCGATCGCTATAACAAAAACCATCTGGTGCCGTTTGGCGAGTTTGTCCCGCTGGAGTCG  1440

 I  L  R  P  L  A  P  F  F  D  L  P  M  S  S  F  S  R  G  P  Y  I  Q  P  P  L  S  A  N  G  I  E  L  T  A  A  I  C  Y  E
ATTCTGCGTCCGTTAGCACCGTTCTTTGATCTGCCGATGTCGTCGTTCAGCCGTGGGCCATATATCCAGCCGCCGCTGTCGGCAAATGGTATTGAGCTTACTGCGGCTATTTGCTACGAG  1560

 I  I  L  G  E  Q  V  R  D  N  F  R  P  D  T  D  Y  L  L  T  I  S  N  D  A  W  F  G  K  S  I  G  P  W  Q  H  F  Q  M  A
ATCATTCTCGGCGAGCAAGTGCGCGATAACTTCCGCCCGGATACCGACTATCTGCTGACTATCTCCAACGATGCGTGGTTTGGTAAATCTATTGGTCCATGGCAACACTTCCAGATGGCG  1680

 R  M  R  A  L  E  L  A  R  P  L  L  R  S  T  N  N  G  I  T  A  V  I  G  P  Q  G  E  I  Q  A  M  I  P  Q  F  T  R  E  V
CGAATGCGTGCGCTGGAGCTGGCGCGCCCACTGTTGCGCAGCACCAACAACGGCATTACGGCGGTGATTGGCCCGCAGGGTGAGATTCAGGCGATGATCCCGCAGTTCACCCGCGAGGTG  1800

 L  T  T  N  V  T  P  T  T  G  L  T  P  Y  A  R  T  G  N  W  P  L  W  V  L  T  A  L  F  G  F  A  A  V  L  M  S  L  R  Q
TTAACCACTAACGTGACGCCGACCACCGGACTCACACCATACGCACGTACCGGCAACTGGCCGCTGTGGGTGCTGACGGCATTGTTTGGTTTTGCTGCTGTGTTGATGAGTCTGCGTCAG  1920

 R  R  K  *
CGACGTAAATAATCCCTTCTTATCGTGCCGGAATACATCCCATATTTCGGCACGCTCACCGGATGCGGCATCAGTGCATTATCCGGATAACAATATTCTCCTATCCAACTTTGGCACATC  2040
                     ==============        ==============

TATTGCTTTGTTATACAAGGCAAACCCTGAAACAGCATCAGTACAACCTAGTTGCACCACAGTAATCCAGCGGTAGCACTAAAAGAGTGCATTTTAAGAATTTTGCTTCGGAATGGTGCG  2160

GCGCATCTCGCAAAAACAAACAATAAAACAGCAATATCTTTACATTTAGCCAAACTAAATGTTACCTAAGGAAGGTGCGAACAAGTCCCTGATATGAGATCATGTTTGTCATCTGGAGCC  2280

ATAGAACAGGGTTCATCATGAGTCATCAACTTACCTTCGCCGACAGTGAATTC  2333
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FIG. 2. Nucleotide sequence of the 2.3-kb *Eco*RI DNA fragment which complements *cutE* and sequence of the predicted translation product. Symbols: = potential promoter and transcriptional terminator stem-loop; *CAT*, potential transcription start point; *, stop codon; RBS, possible ribosome-binding site; <----- ----->, dyad symmetry. Amino acids which are italicized and underlined form a putative copper-binding site.

between the proposed −10 and −35 consensus sequences and downstream of the −10 sequence (Fig. 2).

Identification of complementing DNA. A series of subclones from the 2.3-kb *Eco*RI insert were prepared in the Bluescript vector in order to determine which region within the clone complements *cutE*. Each subclone was tested for complementation by transforming *cutE* and selecting on medium containing 4 mM copper sulfate. Where complementation occurred, at least 10^5 transformants per μg of DNA were obtained. The results shown in Fig. 3 reveal that only the complete 2.3-kb *Eco*RI fragment and the 2.1-kb *Sal*I-*Eco*RI fragment complemented copper sensitivity. These results, along with the DNA and amino acid sequence data (Fig. 2), suggest that the complete large ORF and proposed upstream promoter region are required for complementation.

Location of *cutE* on the *E. coli* physical map. After comparison of the restriction map of the 5.6-kb insert in pCUT1 with the physical map of the *E. coli* chromosome compiled by Kohara et al. (14), we located this fragment to the 702-kb region and more precisely at the 702.80- to 708.35-kb region on the scale of Medigue et al. (18). The *Eco*RI subfragment mapped at 705.70 to 707.80 kb (18); however, we had sized this fragment as 2.3 rather than 2.1 kb. Table 1 gives the map positions and some additional restriction sites of the 2.3-kb *Eco*RI fragment. All the appropriate restriction sites in the corresponding regions showed overall matches, with the exception of the *Pvu*II site at 707.90 kb (14). Sequencing of the 2.3-kb *Eco*RI fragment cloned from pCUT1 revealed this site to be 108 bp upstream rather than 100 bp downstream of the *Eco*RI site at position 707.80 kb (18). This discrepancy is likely to be due to the inaccuracies of restriction mapping such small fragments.

To confirm that this region of the *E. coli* chromosome does contain *cutE*, bacteriophages 3A2 and 16A8 (minisets 170 and 171, respectively), which were expected to carry the

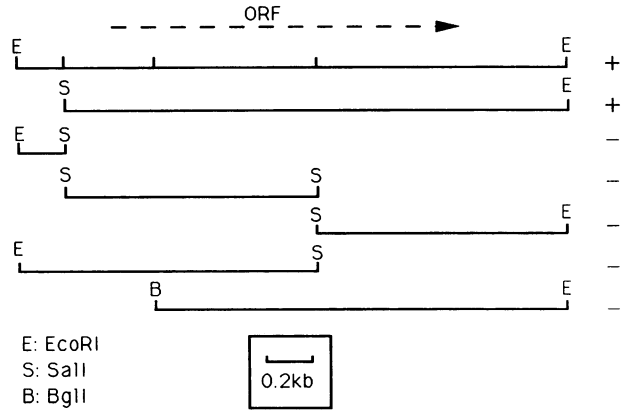

FIG. 3. Subclones of the 2.3-kb *Eco*RI fragment and their abilities to complement *cutE*. Clones which complemented the copper sensitivity of *cutE* as described in the text (+) and those which did not (−) are indicated. The position of the ORF described in the text is also indicated.

cutE gene, were obtained from Y. Kohara. Phage DNA was isolated, and the 2.3-kb *Eco*RI fragment from each stock was subcloned into Bluescript SK vectors. Plasmids carrying the 2.3-kb *Eco*RI fragment from miniset 170 or 171 were able to complement the copper sensitivity of *cutE* mutant cells as described above.

Spot recombination tests were also performed. When phage from miniset 170 or 171 was spotted on a lawn (2×10^8) of *cutE* mutant cells, 60 to 80 copper-tolerant colonies per 10^8 PFU could be counted on 4 mM copper sulfate plates. Control spot recombination tests using phage from minisets 320 and 463, which are from a different region of the genome, did not result in copper-tolerant colonies arising at more than the reversion frequency of 1 in 10^8 cells.

DISCUSSION

The copper sensitivity of cells carrying a mutation in *cutE* was complemented by a wild-type DNA clone (pCUT1), and we interpret this result as indicating that functional copies of the gene have been introduced. We are not able to state unequivocally that the clone which phenotypically complements the mutation is the *cutE* gene. Although pACYC184 is a relatively low-copy-number plasmid (5), it is possible that we have cloned a gene of similar function which is able to complement copper sensitivity if it is present in more than one copy but which is not actually *cutE*. However, the data indicate that cell survival of complemented cells in the presence of added copper is equivalent to that of wild type, which is in contrast to the greatly reduced survival of *cutE* (Fig. 1a), and that copper accumulation is also restored to normal in complemented cells (Fig. 1b). These results would probably not be obtained except in the unlikely event that more than one gene with very similar functions existed. A 2.3-kb *Eco*RI fragment isolated from pCUT1 also complement *cutE* when subcloned into pACYC184, pUC19, and Bluescript SK+. If our results were due to multicopy supression, we would expect some difference in the ability of lower (pACYC184)- and high (pUC19 and Bluescript)-copy-number constructs to complement *cutE*, but no difference was seen. In addition, we originally obtained three identical recombinant plasmids which complemented the copper sensitivity of GME135, and one of these, pCUT1, did not complement the copper sensitivity of strains carrying mutations in any of the other known *cut* loci, implying that complementation by pCUT1 is specific to the *cutE* locus.

By comparison of restriction sites, the *cutE* locus has been mapped on the published physical maps of *E. coli* (14, 18). The function of this region of the chromosome was indicated by the finding that plasmid vectors containing the 2.3-kb *Eco*RI fragment isolated from the appropriate phage miniset also complemented the copper sensitivity of *cutE* mutant cells. Spotting phage containing DNA from this region onto *cutE* mutant cells also resulted in copper-tolerant colonies, which we assume to result from double crossover events between the cloned and host DNA. This finding indicates that we have cloned the *cutE* locus and not a locus which suppresses the copper sensitivity of a cell when the locus is present in multiple copies. Therefore, *cutE* is located between *leuS* and *nagBE* on the map of sequenced *E. coli* genes (18).

By combining the sequence data seen in Fig. 2 with the transformation results for the various subclones (Fig. 3), we have been able to define the region within the 2.3-kb clone that is required for complementation. Of the subclones constructed, only the largest *Sal*I-*Eco*RI fragment also complemented copper sensitivity. Since the 1.9-kb *Bgl*I-*Eco*RI subclone will not transform *cutE* to wild-type levels of survival in the presence of copper sulfate and since a reasonably good ribosome-binding site (27) is situated upstream of the ORF start, we deduce that the methionine at position 394 is the start point for translation of the protein which provides complementation. Potential sequences necessary for initiation of transcription were identified upstream of this ORF (Fig. 2). The sequence TATTAT fits the −10 promoter consensus, TATAAT (12), well, but the proposed −35 sequence, TGGCGA, shows less homology to the consensus TTGACA for the sequences of known *E. coli* promoters (12). However, further indirect evidence that we have identified the promoter region is supplied by the presence of nucleotides upstream of the putative −35 sequence (Fig. 2) which are also often conserved in *E. coli* promoters (12). In addition, the proposed −10 and −35 sequences are separated by 16 bp, the spacing being 16 to 18 bp in 90% of known *E. coli* promoters (12). Previous work (29) has shown by P1 transduction analysis that the six known *cut* genes are not closely linked on the genome, suggesting that the copper genes do not exist as an operon. The sequence data presented here suggest that the identified ORF has good potential promoter consensus and transcriptional termination sequences (Fig. 2), which would support

TABLE 1. Restriction map of 2.3-kb *Eco*RI fragment that complements the *cutE* mutation

Restriction site	Physical map location	
	Published (17)	Present study
*Eco*RI	705.70	705.700
*Ssp*I	NT[a]	706.019
*Nco*I	NT	706.372
*Pvu*I	NT	706.649
*Sal*I	NT	706.738
*Bgl*I	707.05	707.440
*Sal*I	NT	707.833
*Pvu*II	707.90	707.925
*Eco*RV	707.50	707.940
*Eco*RI	707.80	708.033

[a] NT, not tested.

the conclusion that the known *cut* genes do not constitute an operon. We have not, however, excluded the possibility that copper transport genes are scattered throughout the genome yet coordinately controlled as a regulon, as is the case for *E. coli* genes which are regulated in response to iron (8).

There is a 6-bp region of perfect dyad symmetry (indicated in Fig. 2) in the 16-bp sequence separating the proposed −10 and −35 sequences. Mutations in a 7-bp symmetrical dyad between the −10 and −35 promoter elements of the mercury resistance operon of transposon Tn*501* prevent induction by the Mer-Hg(II) complex (23, 26). Regions of dyad symmetry are also the site for Fur (ferric uptake regulation) protein binding at the promoter of the aerobactin operon carried on pColV plasmids (9). Examination of the sequence shown in Fig. 2 revealed another region with dyad symmetry lying between the proposed −10 sequence and the translation start site. A potential promoter-operator site with dyad symmetry has also been noted between the −10 promoter sequence and the translation start site of a chromosomal mercury resistance determinant from *Bacillus* sp. (40). Although the significance of dyad symmetry upstream of the proposed *cutE* gene is as yet unknown, this symmetry may have a role in regulation.

If we assume that the clone described here encodes the CutE protein, we may speculate as to the function of the protein. As described above, strains carrying mutations in *cutE* are sensitive to but dependent on copper and show normal uptake of copper into the cell (29), suggesting that this gene is involved in storage and/or transport of copper. It has been shown previously that wild-type *E. coli* cells are able to control intracellular copper levels over a range of external copper concentrations (28). We found that a mutation in *cutE* also caused increased accumulation of copper within the cell, and although we cannot exclude the possibility that this is due to an export defect, the lack of hydrophobic stretches of amino acids in the protein predicted from the sequence of *cutE* indicates that it is most likely to be intracellular and therefore not a membrane-associated protein directly involved with uptake or export of copper.

Studies of the three-dimensional structures of copper-containing metalloproteins such as plastocyanin (6, 25) have shown that the amino acids cysteine, histidine, and methionine are involved in copper binding. The predicted translation product from the nucleotide sequence of copper resistance genes from *Pseudomonas syringae* pv. *tomato* showed the presence of a repeated motif (Asp-His-Ser-Gln/Lys-Met-Gln-Gly-Met) thought to be involved with the copper resistance role of ORF B (19). The general structure Asp-His-X-X-Met-X-X-Met is conserved in a similar repetitive region of a second ORF which is also essential for resistance (19). The sequence His-X-X-Met-X-X-Met appears once in the predicted translation product of *cutE* (Fig. 2) and has also been found in a repeated form in the deduced amino acid sequence of the copper resistance determinant from the *E. coli* plasmid pRJ1004 (3). We suggest that this sequence represents a possible copper-binding site in CutE. The CutE protein does not appear to be related to the metallothioneins, which are described as small, cysteine-rich proteins (13). Since the translation product predicted from the *cutE* sequence does not contain any likely helix-turn-helix motifs, it seems also that CutE is not a regulatory protein analogous to MerR. The data presented here are consistent with the hypothesis that CutE is involved with storage of copper within the cell and/or with transfer of copper to copper-requiring enzymes. Mutations in this gene could result in excess intracellular free copper which then becomes abnormally associated with cellular components, resulting in toxicity and copper sensitivity. The additional copper dependency suggests that *cutE* is involved in supplying copper to copper-dependent enzymes. We believe, therefore, that we have identified a specific copper transport gene which may play a role in the control of levels of this toxic yet essential heavy metal in *E. coli*.

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