JOURNAL OF BACTERIOLOGY, Sept. 1992, p. 5647–5653
0021-9193/92/175647-07$02.00/0

Vol. 174, No. 17

dcd (dCTP Deaminase) Gene of *Escherichia coli*: Mapping, Cloning, Sequencing, and Identification as a Locus of Suppressors of Lethal *dut* (dUTPase) Mutations

LINGHUA WANG AND BERNARD WEISS*

Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0602

Received 16 April 1992/Accepted 27 June 1992

In *Escherichia coli*, most of the dUMP that is used as a substrate for thymidylate synthetase is generated from dCTP through the sequential action of dCTP deaminase and dUTPase. Some mutations of the *dut* (dUTPase) gene are lethal even when the cells are grown in the presence of thymidine, but their lethality can be suppressed by extragenic mutations that can be produced by transposon insertion. Six suppressor mutations were tested, and all were found to belong to the same complementation group. The affected gene was cloned, it was mapped by hybridization with a library of recombinant DNA, and its nucleotide sequence was determined. The gene is at 2,149 kb on the physical map. Its product, a 21.2-kDa polypeptide, was overproduced 1,000-fold via an expression vector and identified as dCTP deaminase, the enzyme affected in previously described *dcd* mutants. Null mutations in *dcd* probably suppress the lethality of *dut* mutations by reducing the accumulation of dUTP, which would otherwise lead to the excessive incorporation of uracil into DNA.

In *Escherichia coli* and *Salmonella typhimurium*, dCTP deaminase forms most of the dUTP (22), an obligatory precursor for the de novo synthesis of thymidylate (30). dUTP is degraded by dUTPase, the product of the *dut* gene, yielding PP_i and dUMP, a substrate for thymidylate synthetase. This breakdown of dUTP also prevents its utilization by DNA polymerases in place of dTTP. If uracil is incorporated into DNA, it is removed via an excision repair process initiated by uracil-DNA glycosylase (8), an enzyme that probably evolved to remove mutagenic deaminated cytosine residues from DNA. If large amounts of uracil are incorporated, its removal may produce lethal double-strand breaks.

Insertions into the *dut* gene are lethal, and the lethality could not be suppressed by known mutations affecting the synthesis of dUTP or its removal from DNA (10). We produced a conditionally lethal *dut*(Ts) allele (9) so that we could look for phenotypic suppressor mutations in new genes that, when identified, would reveal the mechanism of the lethality. We described one such mutation (9) that occurred spontaneously and that also suppressed the lethality of a *dut* insertion mutation. The gene was tentatively designated *dus*, a mnemonic for *dut* suppressor. In this study, we set out to identify the *dus* gene by first creating additional mutations via transposon insertion. The presence of an antibiotic marker in the gene facilitated its transfer, mapping, and isolation. We present evidence that the *dus* mutations are actually *dcd* alleles and that they are altered in the structural gene for dCTP deaminase.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains used are listed in Table 1. Plasmid vectors were as follows. pUC18 (35), a high-copy-number plasmid, was used for initial cloning; pTZ19 (18), a high-copy-number expression vector with a phage T7 promoter, was used as an intermediate in subcloning and for the initial characterization of gene products; M13mp18 and M13mp19 phages (35) were used in DNA sequence analysis; and pET-11 (29), an expression vector with a T7 promoter, was used to overproduce and characterize the *dcd* gene product. Figure 1 shows the structures of most of the recombinant plasmids prepared in this study. Plasmid pUC18-dusH1 contains a 3.4-kb *Hin*dIII fragment of λ 355 (Fig. 2). pTZ19-dus2 contains a *Hin*dIII-*Eco*RV fragment of pUC18-dusH1 replacing a *Hin*dIII-*Hin*cII segment of the vector's polylinker. The fragment was excised from the polylinker region with *Hin*dIII and *Bam*HI and subcloned in similarly digested vectors to form M13mp18-dus and M13mp19-dus. pET11-dus55 and pET11-dus67 consist, respectively, of *Hin*cII and *Hin*cII-*Xmn*I fragments of pUC18-dusH1 ligated into the *Bam*HI site of pET-11.

Bacteriological methods. The following were as previously described: media and antibiotic supplements (26), phage P1 transductions, transformations with plasmids (6), and random transposition of mini-Tn*10* elements (34). *dut* mutants were routinely grown in media supplemented with thymidine at 125 μg/ml (10).

DNA hybridization. Preparation of lysates of a λ phage library of *E. coli* genomic DNA was as described previously (17). Droplets were applied to nitrocellulose membranes, and hybridization was performed (1) with a probe that was generated by the random primer method (11, 12) and labeled with deoxyadenosine 5′-[α-^{35}S]thiotriphosphate.

Cloning and DNA sequence analysis. General techniques for producing recombinant DNA and for restriction enzyme mapping were as described elsewhere (25). DNA sequencing was performed with Sequenase version 2.0 (U.S. Biochemical Corp.); ambiguities were resolved by the use of Ssb protein and dITP according to the manufacturer's directions. Both strands were completely sequenced. Oligonucleotides used were complementary either to adjacent vector sequences or to portions of the cloned DNA that had just been sequenced. To analyze regions bordering a Tn*10*d*kan* insertion, we used the primer GTATCCACCTTAACTTAATG, which is complementary to sequences 32 nucleotides from

* Corresponding author.

TABLE 1. Bacterial strains

Strain	Genotype[a]	Reference or source
AB1157	*hisG4*[b]	2
BL21(DE3)	*E. coli* B; *hsdS gal* (λ *imm*21 *int*::Φ[*lacUV5*-T7 gene *1*])	29
BW863	KL16[c] *dut-22*(Ts) *zic-4901*::Tn*10 deoA22*	P1(BW952) × BW934
BW865	BW863 *dcd-12*::Tn*10dkan*[d]	This study
BW870	AB1157 *dcd-12*::Tn*10dkan*	P1(BW865) × AB1157
BW881	KL16 *dcd-12*::Tn*10dkan hisG4*	P1(BW870) × KL16
BW901	KL16 *dut-22*(Ts) *ttk-1*::*kan*[e]	As BW741 (9)
BW934	KL16 *deoA22*	9
BW940	KL16 *dcd-11*[f] *dut-22*(Ts) *ung-1 ttk-1*::*kan*	9
BW952	KL16 *dut-22*(Ts) *zic-4901*::Tn*10*	P1(CAG8492) × BW901
CAG18492	*zic-4901*::Tn*10*	27
KL16	Hfr PO-45 *thi-1 relA1 spoT1*	2
N100	*recA galK2* IN(*rrnD-rrnE*)	R. N. Rao

[a] Unless otherwise noted, all strains were derivatives of *E. coli* K-12 F⁻ λ⁻.
[b] AB1157 is a multiple auxotroph. For complete genotype, see reference 2.
[c] Derivatives of KL16 that bear *dut* or *zic*::Tn*10* mutations may have lost the closely linked *spoT1* marker of KL16 during transduction.
[d] Tn*10dkan* denotes the transposed portion of a mini-Tn*10* element.
[e] *ttk-1*::*kan* is downstream of *dut*, in the second gene of the *dut* operon (10).
[f] *dcd-11* was previously called *dus-1* (9).

both ends of the transposon (15). Computer software for general sequence analysis (7, 24) was obtained from the Genetics Computer Group, Inc.

Protein analysis. Cell extracts were prepared as described below. Samples of the supernatants were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a discontinuous system, and the gels were stained with Coomassie blue dye G250 or R250 (25). Markers were bovine erythrocyte carbonic anhydrase (29 kDa), bovine chymotrypsinogen (25 kDa), soybean trypsin inhibitor (20 kDa), and bovine β-lactoglobulin (18 kDa). Sequencing of the amino terminus of dCTP deaminase was performed with an Applied Biosystems model 470 sequencer.

Enzyme and protein assays. dCTP deaminase activity was determined on sonic extracts of growing cells by a spectrophotometric method (4). When the enzyme was not overproduced via an expression vector, its assay required prior partial purification of the cell extracts by streptomycin precipitation (21). β-Galactosidase activity (19) and protein (28) were determined as described previously.

Induction of *dcd* in expression vectors. Strain BL21(DE3) carrying a recombinant pET-11 plasmid was grown at 37°C in tryptone-yeast broth containing ampicillin at 100 μg/ml. At a cell density of about 2 × 10^8/ml, IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a concentration of 0.4 mM. After 30 to 40 min, rifampin was added to a concentration of 150 μg/ml, and incubation was continued for 2 h.

Purification of dCTP deaminase. Strain BL21(DE3, pET11-dus55) was induced with IPTG and treated with rifampin 30 min later as described above. After 2 h of further incubation, the cells were harvested by centrifugation at 4°C and stored at −20°C. Subsequent steps were at 4°C. The cells were thawed and suspended in 2 volumes of 50 mM potassium

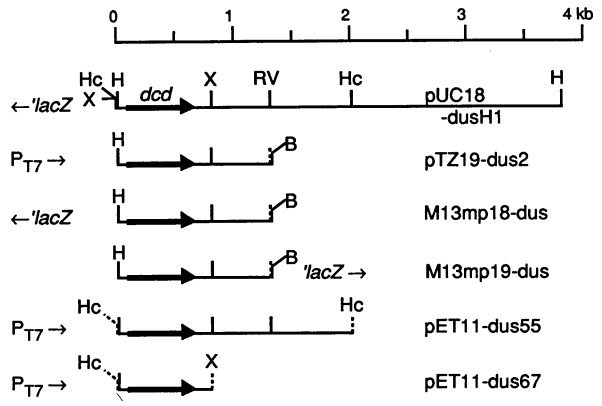


FIG. 1. Structures of *dcd* plasmids. Restriction maps are of the cloned inserts and include some sites from the adjacent polylinker regions (bent lines). Arrows and the symbols *'lacZ* (a 5′-truncated gene) and P_{T7} (T7 phage promoter) indicate the orientations of the vector DNA. Constructions are described in Materials and Methods. Dashed lines denote former cleavage sites that were obliterated by blunt-end ligations. Abbreviations: B, *Bam*HI; H, *Hin*dIII; Hc, *Hin*cII; RV, *Eco*RV; X, *Xmn*I.

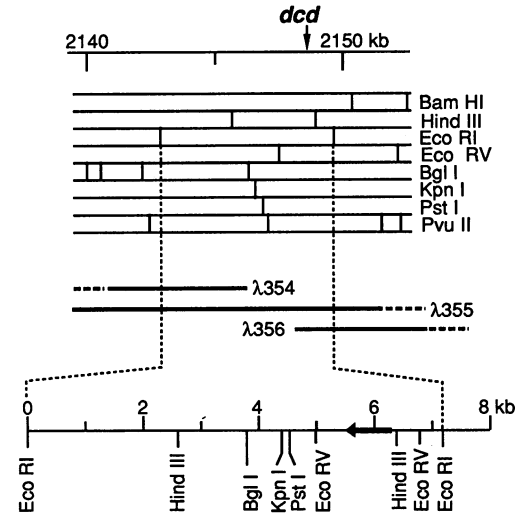


FIG. 2. Location of *dcd* on the physical map of *E. coli*. A restriction map of the cloned 7.2-kb *Eco*RI fragment that complements the suppressor mutations is aligned with a corresponding portion of the physical map of *E. coli* (17). Also shown are the portions of this region contained in λ phages 354, 355, and 356 of the genomic library (17).

phosphate (pH 6.8)–2 mM Na_3EDTA (buffer A). A sonic extract was prepared, and cell debris was removed by centrifugation for 15 min at 27,000 × g. To the supernatant (fraction I), 0.25 volume of a 15% solution of streptomycin sulfate was added slowly with stirring. After 30 min, the suspension was centrifuged for 30 min at 27,000 × g. The supernatant was dialyzed against 100 volumes of buffer A containing 1 mM dithiothreitol and 10% glycerol (buffer B), yielding fraction II. A column of DEAE-Sephadex A50 (1.5 by 12 cm) was equilibrated with buffer B. One milliliter of fraction II was diluted to 10 ml in buffer B and applied to the column at a rate of 10 ml/h. The column was washed with 40 ml of buffer B, and the protein was eluted with 200 ml of buffer B containing a linear gradient of 0 to 0.6 M NaCl. Fractions of 3.5 ml were collected at a rate of 15 ml/h. A peak of protein at 0.4 M NaCl corresponded to that of dCTP deaminase activity (see Results).

Nucleotide sequence accession number. The DNA sequence of the *dcd* region (Fig. 3) has been submitted to GenBank under accession number M90069.

RESULTS

Isolation of *dus* insertion mutants. A *dut-22*(Ts) mutant is inviable at high temperatures, but it readily yielded temperature-resistant pseudorevertants that acquired extragenic mutations designated by the symbol *dus* (9). Our rationale for attempting to isolate *dus* insertion mutants was as follows. Extragenic suppressors often turn out to be translational (e.g., missense) suppressors or mutations in another polypeptide that complexes with the mutated one. Such mutations usually appear with low frequency because they are limited to no more than a few sites in a gene, and their suppressor activity is allele specific. However, spontaneous *dus* mutations occurred with high frequency ($>10^{-5}$), and the one which we studied (*dus-1*) was not allele specific; it suppressed both *dut-22*(Ts) and the insertion mutation *dut-21*::*cat* (9). Therefore, we reasoned that *dus* mutants may be null mutants and that we should be able to obtain them by transposon insertion.

Strain BW863 [*dut-22*(Ts)] was infected with λ 1105. The phage contains a mini-Tn*10* element specifying kanamycin resistance, which we refer to as Tn*10*d*kan*. To ensure independent mutations, the infected cells were grown in five separate flasks at ambient temperature before kanamycin-resistant derivatives were selected at 42°C. The kanamycin selected for random insertions of the transposon into the chromosome, whereas the high temperature selected for loss of the λ *c*I857(Ts) phages as well as for reversion or suppression of *dut-22*(Ts). Our object was to obtain *dus*::Tn*10*d*kan* mutants. However, because of the high frequencies of random transposition and spontaneous reversion, a large fraction of the survivors were expected to have Tn*10* insertions unlinked to their *dus* mutations. Therefore, they were tested by a transductional backcross. Phage P1 lysates were prepared from 15 colonies and used to infect strain BW863 [*dut-22*(Ts)]. Each culture was then grown overnight at 32°C, and diluted portions were plated at both 32 and 42°C on a tryptone-yeast extract agar containing thymidine and kanamycin. Nine of the fifteen transductions yielded temperature-resistant recombinants; the others produced fewer than 1% survivors at 42°C. Five temperature-resistant mutants of independent origin (one from each original flask) were selected for further study. The new *dus* alleles are currently designated *dcd-12*::Tn*10*d*kan* to *dcd-16*::Tn*10*d*kan*.

Cloning and mapping of *dcd-12*::Tn*10*d*kan*. *Eco*RI digests of DNA from the *dcd-12*::Tn*10*d*kan* mutant and of plasmid pUC18 were mixed, treated with DNA ligase, and used to transform strain N100 (*recA*) to kanamycin resistance. Recombinant plasmids had 9-kb inserts, of which 2 kb was Tn*10*d*kan* DNA. Because Tn*10*d*kan* contains no *Eco*RI sites, the cloned fragments contained *dus* (*dcd*) sequences adjacent to both ends of the transposon. The plasmid contained three *Sma*I sites, one of which was in Tn*10*d*kan*. Therefore, *dus* sequences adjacent to each end of the transposon were contained in separate *Sma*I fragments. For DNA sequencing, the fragments were denatured and annealed to an oligonucleotide primer that was complementary to the inverted repeat sequences found at each end of the transposon, and the polymerizations were directed outward into the bacterial sequences.

A sequence of 260 nucleotides was determined. It contained an open reading frame of at least 140 nucleotides into which the transposon was inserted 33 nucleotides from the start. A *Hin*dIII site was located 93 nucleotides upstream of the gene. The nucleotide sequence and predicted peptide sequence resembled those of no known gene or protein.

The cloned segment was then used to locate similar sequences in an ordered library of recombinant λ phage clones containing the *E. coli* genome. A hybridization probe was prepared from the 3.2-kb *Sma*I fragment of the plasmid, which contained *dcd* sequences downstream of the insertion. It hybridized to λ phages 354, 355, and 356 of the miniset of Kohara et al. (17). According to the physical map (17), the phages have bacterial DNA overlapping a 7-kb *Eco*RI piece and a 3.6-kb *Hin*dIII piece (Fig. 2). Therefore, these segments were isolated from λ 355 and subcloned in plasmid pUC18. The recombinant plasmids complemented the *dcd-12*::Tn*10*d*kan* mutation; i.e., they restored temperature sensitivity to strain BW865 [*dut-22*(Ts) *dcd-12*::Tn*10*d*kan*]. Through further restriction mapping of these new plasmids and the *dcd-12*::Tn*10*d*kan* plasmid, we located the *dcd* gene at kb 2149 on the physical map (Fig. 2), corresponding to about 45.0 min on the linkage map (3), or 1 min clockwise of *his*.

One locus for all the suppressor mutations. P1 lysates of the five *dus* (*dcd*) insertion mutants were used to transduce a *hisG* mutant (strain AB1157 [2]) to kanamycin resistance. Ninety-five colonies were scored from each transduction; 4 to 17% of the recombinants were His^+. Plasmid pUC18-dusH1 (Fig. 1) was introduced (by transformation at 30°C) into the five *dut*(Ts) *dcd*::Tn*10*d*kan* mutants and into the previously isolated spontaneous *dus* mutant BW940 [*dut*(Ts) *dcd-11*]. Each strain became temperature sensitive again. The same results were obtained when the experiment was later repeated with pET11-dus67, which we shall show contains only one intact gene. In a control experiment, when the mutants were transformed with just the plasmid vectors, they remained temperature resistant. Therefore, all of the suppressor *dcd* mutations belong to the same complementation group, and they are recessive to the wild-type allele.

DNA sequence. A 1.3-kb *Hin*dIII-*Eco*RV DNA fragment covering the *dcd* region was subcloned in M13mp18 and M13mp19 (Fig. 1), and its sequence was determined (Fig. 3). The site of *dcd-12*::Tn*10*d*kan* insertion was found to be located in an open reading frame of 579 nucleotides, encoding a polypeptide with a predicted molecular mass of 21.2 kDa and a pI of 5.7. It is preceded by a putative ribosome binding site. A possible promoter sequence, indicated in Fig. 3, was predicted by the algorithm of Mulligan and McClure (20) at a confidence level of 99.94%. Twenty-five nucleotides

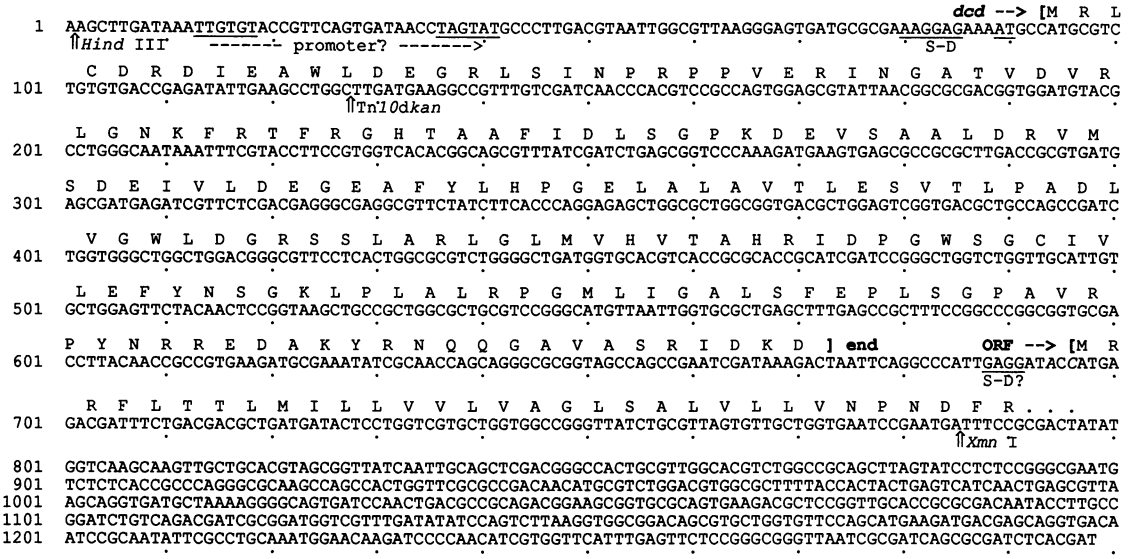


FIG. 3. DNA sequence of the *dcd* region. The sequence is that of the *Hin*dIII-*Eco*RV cloned segment of M13mp18-dus and M13mp19-dus (Fig. 1). Homologies with the Shine-Dalgarno (S-D) consensus sequence and putative −35 and −10 promoter hexamers are underlined. A translation is also shown for the initial portion of an open reading frame (ORF) downstream of *dcd*, for which no product has yet been demonstrated.

downstream of the *dcd* gene is a partially sequenced open reading frame with a coding capacity of >200 amino acids. It has an average codon preference score (14) of 0.6678 (randomized sequence score = 0.4691), which is consistent with that for a bona fide but weakly expressed gene. Its apparent lack of an independent putative promoter sequence and its proximity to *dcd* suggest that it may belong to the same operon. The putative product of this second open reading frame contains a hydrophobic region of at least 20 amino acids near its amino terminus, which is preceded by two positively charged amino acids (arginines). This motif is associated with secreted proteins (23). The genes and their predicted products contained no significant homology to any known genes or proteins. There were no other known dCTP deaminase sequences available for comparison.

Detection of the gene product. A 785-bp *Hin*dIII-*Xmn*I segment of DNA, which contained only the *dcd* gene (Fig. 3), was cloned in the expression vector plasmid pET-11. The product was pET11-dus67 (Fig. 1), in which the *dcd* gene was aligned with a phage T7 promoter and placed upstream of a transcriptional terminator. The host strain, BL21(DE3), contained an IPTG-inducible T7 RNA polymerase gene. After induction, the cells overproduced a polypeptide that appeared by SDS-PAGE to have a molecular mass of about 22 to 23 kDa (Fig. 4). Within the limits of accuracy of the method, this value is consistent with that inferred from the DNA sequence (21.2 kDa).

A *dus* (*dcd*) mutant lacks dCTP deaminase. A *dus* mutant was grown in a medium containing $^{32}P_i$, and its radiolabeled nucleotides were extracted and separated by two-dimensional thin-layer chromatography (Fig. 5). The mutant accumulated dCTP, the immediate precursor for most of the cellular dUTP. This result suggested a deficiency of dCTP deaminase. The mutant was tested and found to have ≤10% of wild-type dCTP deaminase activity.

The cloned *dcd* gene encodes dCTP deaminase. In strain BL21(DE3, pET11-dus67), IPTG caused an induction of dCTP deaminase activity to a level of 1,400 U/mg of protein, whereas a strain carrying the vector (pET-11) produced only 1.3 U/mg. Is the cloned *dcd* gene merely a regulatory gene, or is it the structural gene for dCTP deaminase? Once the T7 RNA polymerase is induced, transcription directed by *E. coli* RNA polymerase should not be able to compete with the T7 polymerase-promoter system. Therefore, any overproduced protein (apart from the T7 polymerase) is bound to be the product of the cloned DNA (29), which, in pET11-dus67, contains only one complete open reading frame (Fig. 1 and 3). The addition of rifampin, an inhibitor of *E. coli* RNA polymerase, should further block chromosomal transcription. The time course of dCTP deaminase induction is shown in Fig. 6. As a control, we also measured β-galactosidase,

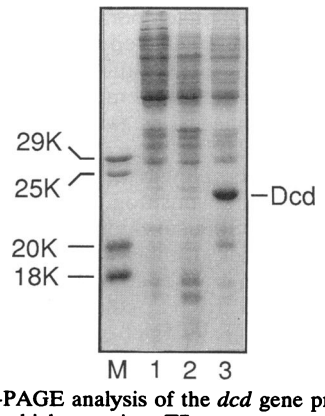


FIG. 4. SDS-PAGE analysis of the *dcd* gene product. The indicated plasmids, which contain a T7 promoter, were propagated in strain BL21(DE3), and some of the growing cultures were treated with IPTG to induce T7 RNA polymerase. Lanes: M, markers; 1, vector (pET-11) plus IPTG; 2, plasmid pET11-dus67, uninduced; 3, plasmid pET11-dus67 plus IPTG. An equal mass of cells was analyzed in each lane. The separating gel contained 15% polyacrylamide.

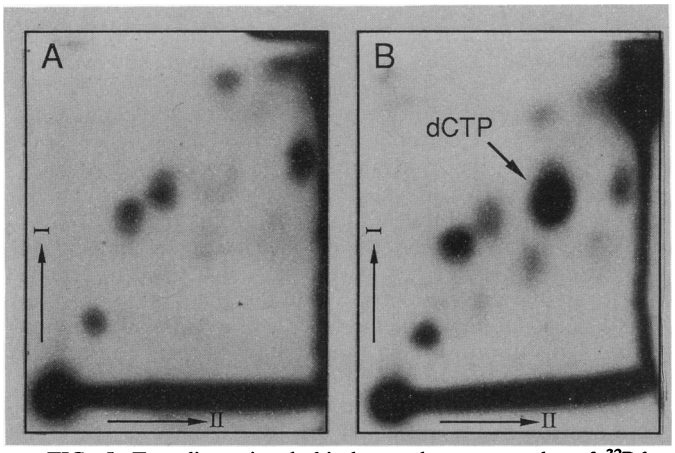


FIG. 5. Two-dimensional thin-layer chromatography of ^{32}P-labeled cellular nucleotides. (A) Strain KL16 (*dcd*$^+$); (B) strain BW881 (*dcd-12*::Tn*10dkan*). The cells were labeled for 2 h in minimal medium lacking thymidine and containing $^{32}P_i$ (100 μCi/ml). Procedures were those of Bochner and Ames (5). Their solvent Fa was used for the first dimension (I), and solvent Pb was used for the second dimension (II) (see Fig. 2C of reference 5). A major spot on the autoradiograph (B, short arrow) corresponded to a dCTP marker that was detected by UV absorbance.

the product of the chromosomal *lacZ* gene, which is transcribed by *E. coli* RNA polymerase and is also induced by IPTG. Although rifampin was added 30 min after IPTG, it failed to inhibit the induction of dCTP deaminase, which continued after the synthesis of β-galactosidase stopped. The results indicate that dCTP deaminase is expressed from the cloned segment, which contains only the *dcd* gene.

Enzyme purification. We purified the overexpressed enzyme by using a procedure modified from that for the dCTP deaminase of *S. typhimurium* (4). The enzyme constituted about 40% of the soluble protein in the crude extract; a 2.2-fold purification brought it to >90% purity, with an overall yield of 27% in the purest fraction (Table 2 and Fig.

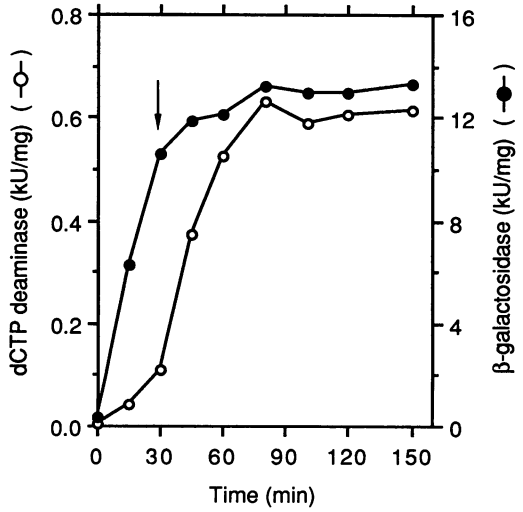


FIG. 6. Induction of the plasmid *dcd* and chromosomal *lacZ* genes. A growing culture of strain BL21(DE3, pET11-dus67), which carries an IPTG-inducible T7 RNA polymerase on a λ phophage (DE3) and a T7 promoter on a recombinant plasmid, was treated at zero time with IPTG. Rifampin was added at 30 min (arrow).

TABLE 2. Purification of overexpressed dCTP deaminase

Fraction	Total activity (10^4 U)[a]	Total protein (mg)	Sp act (10^3 U/mg) of protein)	Yield (%)
I (sonic extract)	4.14	31.6	1.3	100
II (streptomycin)	4.11	25.5	1.6	99
III (DEAE-Sephadex)[b]	1.13	4.1	2.9	27

[a] Values are based on the purification of enzyme from 0.35 g of cell paste. Procedures were as described in Materials and Methods.
[b] Tubes 36 to 38 (Fig. 7) were pooled to give fraction III.

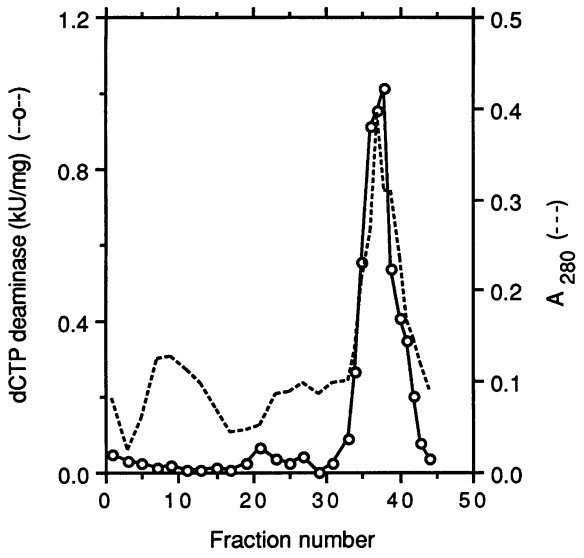
7). The entire eluate from a DEAE-Sephadex column contained 56% of the activity originally present in the crude extract. The elution profile (Fig. 7A) revealed a correspondence between a major protein peak, dCTP deaminase activity, and a polypeptide of about 21 kDa (Fig. 7B). Another protein of about 30 kDa was prominent in the crude extract and in a separate absorption peak (fraction 10) of the DEAE-Sephadex elution profile. Whereas its size corresponds to that of the vector-encoded β-lactamase, it might instead be the product of the second open reading frame in the cloned DNA, the size of which is unknown.

N-terminal sequence. The purified protein was analyzed by limited Edman degradation. The sequence of the first 10 amino acids matched that predicted from the DNA sequence, except for a cysteine, which could not be determined by this procedure. The results confirm that our cloned *dcd* gene is the structural gene for dCTP deaminase and not merely a regulatory gene.

DISCUSSION

A major function of dUTPase is to keep uracil out of DNA. This function is especially important in *E. coli*, in which most thymidylate is made via the following circuitous pathway in which dUTP is an intermediate: dCTP → dUTP → dUMP → dTMP. Therefore, *dut* mutants, which should accumulate dUTP, incorporate large amounts of uracil into DNA. Uracil-containing DNA is repaired by a pathway requiring uracil-DNA glycosylase (*ung* gene), the endonucleolytic activity of exonuclease III (*xth*), DNA polymerase I (*polA*), and DNA ligase (*lig*) (30). When a nonlethal (leaky) *dut* mutation is combined with a nonlethal *xth*, *polA*, or *lig* mutation, the result may be conditionally lethal (temperature sensitive). The double mutants die because they cannot complete the excision repair of uracil-containing DNA. However, at high temperatures, they yield phenotypic revertants, many of which have acquired *dcd* mutations (30, 32). The cells survive because they lack dCTP deaminase, the major source of dUTP. Consequently, in a previous study from our laboratory (10), when we produced a *dut* insertion mutation, we expected that its lethality might be suppressed by a *dcd* mutation. We examined three *dcd* mutant alleles, both alone and in combination with *ung* mutations, but none were suppressors of *dut* lethality. Therefore, in the current study, we were not prepared to find that our *dus* mutations were in *dcd*.

An obvious explanation for the failure of previously tested *dcd* mutations to suppress *dut* lethality is that those mutations may have been leaky, whereas our insertion mutations are tight. One of the previously tested *dcd* mutations arose spontaneously as a phenotypic revertant of a temperature-sensitive *dut-1 xth* mutant. It had simultaneously acquired defects in both *dcd* and *his*, a common occurrence in *dut xth*



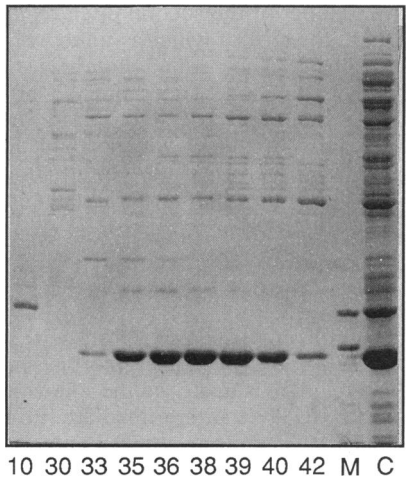


FIG. 7. Copurification of the dCTP deaminase and the 21-kDa polypeptide. (A) Elution profile of dCTP deaminase and protein (A_{280}) during DEAE-Sephadex chromatography (see Materials and Methods). (B) SDS-PAGE of DEAE-Sephadex column fractions. Numbers are those of the fractions represented in panel A; samples of equal volume were analyzed. Lane M, markers (29 and 25 kDa); lane C, crude extract (fraction I). The separating gel contained 10% polyacrylamide.

(30) and in *dut lig* (32) double mutants. It was therefore presumed to contain a *his-dcd* deletion, which should, of course, have no residual dCTP deaminase activity. However, we have recently found that its histidine requirement is revertable, suggesting that it is not a deletion mutant. Unfortunately, the possible leakiness of these *dcd* alleles cannot be tested by enzymatic assay. All of the mutants had ≤10% of wild-type activity, which was the lower limit of detection in partially purified extracts in which there were interfering enzymes.

The original *dcd* mutation (*dcd-1*) had been crudely mapped in the vicinity of our suppressor mutations (13), and deletions encompassing both *his* and *dcd* had been obtained (21). However, unlike our insertion mutations, *dcd-1* was not cotransducible with *his* (13) and did not suppress *dut* lethality (10), raising the possibility that it is in a different gene, perhaps that for a regulatory protein. We are now investigating this matter. The *dcd-1* mutation cannot, however, be in a gene for another subunit of the enzyme because the deaminase is made up of only one type of polypeptide, as indicated by gel electrophoresis and protein sequence analysis. However, the 21-kDa polypeptide probably exists as a tetramer; the native enzyme from *S. typhimurium* is about 82 kDa, as determined by gel filtration chromatography (4).

Of the six mutations studied, five arose by transposon insertion. Although we cannot assume that these insertions are located at different sites within the gene, it is nevertheless remarkable that in six independent events, suppressors of *dut* lethality arose only by mutation of *dcd*. The results further suggest that the mutations cannot be in another gene upstream of *dcd* in the same operon. The mutations were complemented by plasmids pET11-dus67 and pUC18-dusH1. The cloned segment in plasmid pET11-dus67 bore only one open reading frame, that of *dcd*. It was inserted near a T7 promoter that should not function in the mutants because they lack T7 RNA polymerase (29). pUC18-dusH1 contained no vector-derived promoter aligned with *dcd*. Therefore, the cloned segments must carry the *dcd* promoter. Moreover, they possess no open reading frame upstream of *dut*. Therefore, if *dcd* is within an operon, it must be the first gene of that operon. Thus, the complementation experiments indicate that all of the mutations tested must be within the *dcd* gene or its promoter region.

El-Hajj et al. (10) speculated that the lethality of *dut* mutations might be unrelated to the synthesis of uracil-containing DNA. This hypothesis was based on their findings that the lethality was not suppressed by mutations in genes, including *dcd*, that affect the synthesis of dUTP or the removal of uracil from DNA. The results of the present study repudiate the basis for that hypothesis. Lethality appears to be directly related to the accumulation of dUTP; it results from mutations in *dut*, which should enhance dUTP accumulation, and it is alleviated by mutations in *dcd*, which should reduce it. Consistent with these results is an earlier finding that even a viable *dut* mutant is killed by a high level of exogenous uracil (16).

Why is the accumulation of dUTP or the incorporation of uracil into DNA lethal? The stable replacement of at least 10% of DNA thymine with uracil does not significantly affect viability (33). However, the cell might not tolerate the higher levels of replacement that might occur in our tight *dut* mutants, for any of the following reasons: (i) even in an *ung* mutant, uracil-containing DNA may be subject to degradation by other repair enzymes; (ii) uracil-containing DNA may not be recognized by essential DNA-binding enzymes or regulatory proteins; or (iii) a control mechanism may turn off protein synthesis in response to the accumulation of dUTP or to its incorporation into DNA (9). We should note that a *dut* insertion mutant is inviable even in the presence of exogenous thymidine and even when containing a *deoA* (thymidine phosphorylase) mutation, which should enhance thymidine utilization (10). Therefore, either dUTP accumulation is lethal by itself or the dTTP synthesized from exogenous thymidine is unable to compete adequately with endogenous dUTP unless dUTP synthesis is reduced by a tight mutation in *dcd*.

ACKNOWLEDGMENTS

We gratefully acknowledge the capable technical assistance of Fred Kung.

This work was supported by research grant NP-770T from the

American Cancer Society. Computer services were supported by NIH grant MO1 RR 00042.

REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1989. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
2. **Bachmann, B. J.** 1987. Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12, p. 1190–1219. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
3. **Bachmann, B. J.** 1990. Linkage map of *Escherichia coli* K-12, edition 8. Microbiol. Rev. **54:**130–197.
4. **Beck, C. F., A. R. Eisenhardt, and J. Neuhard.** 1975. Deoxycytidine triphosphate deaminase of *Salmonella typhimurium*. J. Biol. Chem. **250:**609–616.
5. **Bochner, B. R., and B. N. Ames.** 1982. Complete analysis of cellular nucleotides by two-dimensional thin-layer chromatography. J. Biol. Chem. **257:**9759–9769.
6. **Chung, C. T., S. L. Niemela, and R. H. Miller.** 1989. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. USA **86:**2172–2175.
7. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
8. **Duncan, B. K.** 1981. DNA glycosylases, p. 565–586. *In* P. D. Boyer (ed.), The enzymes, vol. 14. Academic Press, New York.
9. **El-Hajj, H. H., L. Wang, and B. Weiss.** 1992. Multiple mutant of *Escherichia coli* synthesizing virtually thymineless DNA during limited growth. J. Bacteriol. **174:**4450–4456.
10. **El-Hajj, H. H., H. Zhang, and B. Weiss.** 1988. Lethality of a *dut* (deoxyuridine triphosphatase) mutation in *Escherichia coli*. J. Bacteriol. **170:**1069–1075.
11. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
12. **Feinberg, A. P., and B. Vogelstein.** 1984. Addendum: a technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **137:**266–267.
13. **Fuchs, J. A., and H. O. Karlström.** 1976. Mapping of *nrdA* and *nrdB* in *Escherichia coli* K-12. J. Bacteriol. **128:**810–814.
14. **Gribskov, M., J. Devereux, and R. R. Burgess.** 1984. The codon preference plot: graphic analysis of protein coding sequences and prediction of gene expression. Nucleic Acids Res. **12:**539–549.
15. **Halling, S. M., R. W. Simons, J. C. Way, R. B. Walsh, and N. Kleckner.** 1982. DNA sequence organization of IS*10*-right of Tn*10* and comparison with IS*10*-left. Proc. Natl. Acad. Sci. USA **79:**2608–2612.
16. **Hochhauser, S. J., and B. Weiss.** 1978. *Escherichia coli* mutants deficient in deoxyuridine triphosphatase. J. Bacteriol. **134:**157–166.
17. **Kohara, Y., K. Akiyama, and K. Isono.** 1987. The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. Cell **50:**495–508.
18. **Mead, D. A., E. Szczesna-Skorupa, and B. Kemper.** 1986. Single-stranded DNA 'blue' T7 promoter plasmids: a versatile tandem promoter system for cloning and protein engineering. Protein Eng. **1:**67–74.
19. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Mulligan, M. E., and W. R. McClure.** 1986. Analysis of the occurrence of promoter-sites in DNA. Nucleic Acids Res. **14:**109–126.
21. **Neuhard, J., and E. Thomassen.** 1976. Altered deoxyribonucleotide pools in P2 eductants of *Escherichia coli* K-12 due to deletion of the *dcd* gene. J. Bacteriol. **126:**999–1001.
22. **O'Donovan, G. A.** 1977. Thymidine metabolism in bacteria, p. 219–253. *In* I. Molineux and M. Kohiyama (ed.), DNA synthesis, present and future. Plenum Publishing Corp., New York.
23. **Oliver, D. B.** 1987. Periplasm and protein secretion, p. 56–69. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
24. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444–2448.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
26. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **Singer, M., T. A. Baker, G. Schnitzler, S. M. Deischel, M. Goel, W. Dove, K. J. Jaacks, A. D. Grossman, J. W. Erickson, and C. A. Gross.** 1989. A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. Microbiol. Rev. **53:**1–24.
28. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150:**76–85.
29. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.
30. **Taylor, A. F., and B. Weiss.** 1982. Role of exonuclease III in the base-excision repair of uracil-containing DNA. J. Bacteriol. **151:**351–357.
31. **Tsaneva, I. R., and B. Weiss.** 1990. *soxR*, a locus governing a superoxide response regulon in *Escherichia coli* K-12. J. Bacteriol. **172:**4197–4205.
32. **Tye, B.-K., and I. R. Lehman.** 1977. Excision repair of uracil incorporated in DNA as a result of a defect in dUTPase. J. Mol. Biol. **117:**293–306.
33. **Warner, H. R., B. K. Duncan, C. Garrett, and J. Neuhard.** 1981. Synthesis and metabolism of uracil-containing deoxyribonucleic acid in *Escherichia coli*. J. Bacteriol. **145:**687–695.
34. **Way, J. C., M. A. Davis, D. Morisato, D. E. Roberts, and N. Kleckner.** 1984. New Tn*10* derivatives for transposon mutagenesis and for constructions of *lacZ* operon fusions by transposition. Gene **32:**369–379.
35. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.