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De Novo Synthesis of Thymidylate via Deoxycytidine in *dcd* (dCTP Deaminase) Mutants of *Escherichia coli*

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***dcd* (dCTP deaminase) mutants of *Escherichia coli* were reported not to require thymidine for growth even though most of the thymidylate that is synthesized de novo arises from cytosine nucleotides through a pathway involving dCTP deaminase. We found, however, that the fresh introduction of *dcd* mutations into many strains of *E. coli* produced a requirement for thymidine for optimum aerobic growth, but the mutants readily reverted to prototrophy via mutations in other genes. One such mutation was in *deoA*, the gene for deoxyuridine phosphorylase. However, a *dcd deo* mutant became thymidine dependent once again if a *cdd* mutation (affecting deoxycytidine deaminase) were introduced. The results indicate that *dcd* mutants utilize an alternative pathway of TMP synthesis in which deoxycytidine and deoxyuridine are intermediates. A *cdd* mutation blocks the pathway by preventing the conversion of deoxycytidine to deoxyuridine, whereas a *deoA* mutation enhances it by sparing deoxyuridine from catabolism. The deoxycytidine must arise from dCTP or dCDP via unknown steps. It is not known to what extent this pathway is utilized in wild-type cells, which, unlike the *dcd* mutants, do not accumulate dCTP.**

In *Escherichia coli*, dUTP is an obligatory precursor for the biosynthesis of thymidylate (Fig. 1) (14, 24). Previous studies in *E. coli* and *Salmonella typhimurium* suggested that under aerobic conditions, only about 25% of the dUTP is synthesized in a manner analogous to that of the other deoxynucleoside triphosphates, i.e., by the phosphorylation of dUDP that is generated by the reduction of UDP. The remaining 75% of dUTP is produced by dCTP deaminase (15), the product of the *dcd* gene (27). Accordingly, *dcd* mutants were noted to have a reduced TTP pool (16) and an increased sensitivity to 5-bromodeoxyuridine (BrdUrd) (7). This sensitivity is consistent with a defect in the synthesis de novo of thymidylate, which would compete with the analog for incorporation into DNA. Nevertheless, *dcd* mutants have been described as prototrophs (5, 14, 16, 17); they do not require thymidine for growth. Although some *dcd* mutants with extensive deletions grew slowly and manifested some filamentation, these effects were thymidine independent (16). These results might lead us to the unexpected conclusion that the pathway by which most TMP is synthesized is dispensable. In this study, we examine alternative pathways that may be used by *dcd* mutants.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. Additional *dcd* and *deoA* derivatives were constructed from these strains in the following way. *dcd-12*::Tn*10*d*kan* was introduced by P1-mediated transduction from BW1040 with selection for kanamycin resistance. *deoA22* was introduced by cotransduction with *thr-34*::Tn*10* from BW933 with selection for tetracycline resistance. *deoA22* transductants were detected by their inability to use thymidine as the sole carbon source. The *thr*::Tn*10* marker was removed either by transduction of *thr*$^+$ from KL16 or by spontaneous precise excision of Tn*10*.

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Media. TY (tryptone-yeast extract) medium, used for routine growth of all organisms, contained (per liter) 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl and was adjusted to pH 7.2 with NaOH. VB minimal medium was medium E of Vogel and Bonner (26) containing thiamine (1 μg/ml); glucose (at 0.4%) was used routinely as the carbon source. Amino acids were added, as required, at 100 μg/ml. Nucleosides and nucleobases were added to minimal medium at 0.5 mM when required as nutrients or at 5 mM when replacing glucose as a carbon source. To reduce the outgrowth of phenotypic revertants, *dcd* mutants were routinely propagated in TY medium supplemented with thymidine at 125 μg/ml, and reference cultures were stored frozen in 7% dimethyl sulfoxide (12). Norit-treated Casamino Acids were prepared by filtering a hot 10% solution of vitamin assay Casamino Acids (Difco) through Norit until there was no further change in color. Ampicillin was used at 100 μg/ml, tetracycline was used at 15 μg/ml, and kanamycin was used at 25 μg/ml.

Sensitivity to BrdUrd. Cultures were grown to saturation in microwell plates containing TY broth plus thymidine. With the aid of a multipronged inoculator (29), the cultures were diluted into 0.1 ml of 10 mM $MgSO_4$ and patched onto the surface of VB-glucose agar supplemented with 0.1% Norit-treated Casamino Acids and BrdUrd at 100 μg/ml. The plates were incubated in the dark for 18 h at 42°C. For complementation tests, ampicillin was added to the medium.

Other microbiological methods. Phage P1 transductions (20) and plasmid transformations (4) were performed as previously described. *cdd* was scored by growth on 0.4% cytidine as the carbon source. Colony sizes, expressed as area, were calculated from diameter measurements performed with a ×6 magnifier that was equipped with a reticle containing a linear scale graduated in tenths of a millimeter (Edmund Scientific Corp.). Dilutions of cultures were spread on duplicate plates to yield about 150 to 250 colonies per plate. For each strain, 50 random colonies (25 from each plate) were measured. For anaerobic growth, the cells were suspended in an agar underlay containing a membrane fraction of *E. coli*

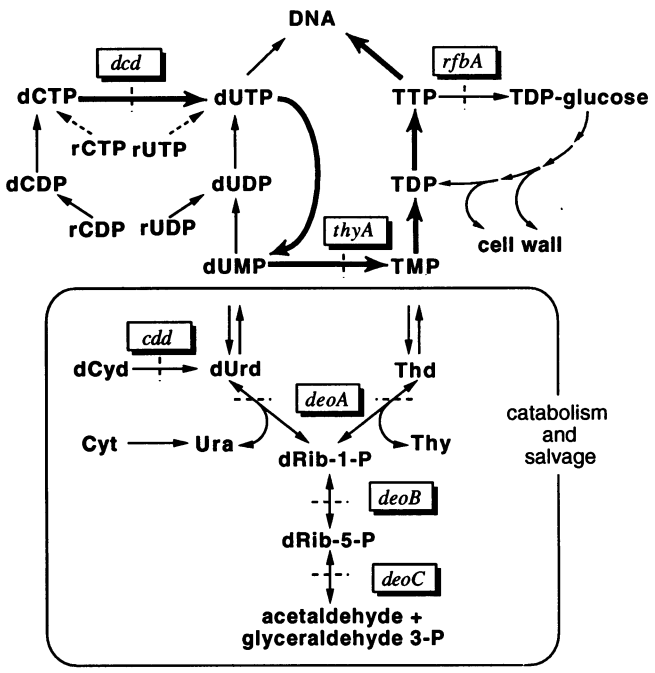

FIG. 1. Metabolism of deoxyribonucleotides in *E. coli*. Heavy arrows mark the major pathway (dCTP pathway) for the synthesis de novo of thymidylate in DNA. Broken arrows indicate the anaerobic pathway of nucleotide reduction. Rectangles denote the mutant genes mentioned in this work.

(Oxyrase, Inc., Ashland, Ohio) and lactate as a reducing substrate (1).

Other methods. Thymidine phosphorylase activity was determined by the spectrophotometric measurement of the phosphate-dependent cleavage of thymidine (13), and enzyme units were as thus defined. Assays were performed on sonicates of log-phase cells grown either under inducing conditions (TY broth containing 8 mM thymidine) or under noninducing conditions (VB minimal medium without thymidine), as stated. Protein was measured by the bicinchoninic acid method (21).

RESULTS

Thymidine auxotrophy and BrdUrd sensitivity. Because most thymidylate is made via dCTP deaminase in *E. coli*, it is surprising that strains carrying *dcd-1* or *dcd* deletion mutations were not noted to require thymidine for growth, a finding that we confirmed with the following strains that were descendants or derivatives of the early mutants: strains HD1038 (*dcd-1*), LD175 (*dcd-1 cdd-50*), CS3840 (Δ*dcd-86*), and QE35 (Δ*dcd-87*). On the other hand, we observed that our *dcd* insertion mutations, such as *dcd-12*::Tn*10*d*kan*, did specify a partial requirement for thymidine (27). However, these insertions were studied in a different parental strain, KL16, and the mutants were propagated in rich, thymidine-supplemented media to reduce secondary mutation to thymidine independence. When the *dcd-1* and *dcd* deletion mutations were transduced into strain KL16, they now specified a thymidine requirement just as the insertion mutants did. All the mutants required thymidine for optimum growth (Fig. 2), although thymidine was unable to fully restore the growth rate of the deletion mutants, perhaps because they lack many genes in addition to *dcd*. The mutants were also found to be sensitive to BrdUrd when tested as described under Materials and Methods.

It should be noted that the *his-dcd* deletions that we tested (Fig. 2) encompass the *rfbABD* gene cluster, which is required for the synthesis of TDP sugars. TDP is used merely as a carrier for the insertion of some sugars into the cell wall and is consequently recycled. Nevertheless, it was reported that mutations in the *rfb* genes can reduce the effect of partial thymine starvation on the TTP pool and on the rate of DNA synthesis (18). However, in our experiments, the loss of the *rfb* genes did not alleviate the thymidine requirement specified by the *dcd* mutation.

Complementation tests. Insertions as well as deletions might affect the expression of multiple genes. To verify that the traits of the mutants are due specifically to the loss of *dcd*, we performed complementation tests with the recombinant plasmid pET11-dus67 (27), a plasmid in which the only cloned coding sequence is that of *dcd*. The plasmid restored the thymidine independence and BrdUrd resistance that were lacking in the *dcd* insertion and deletion mutants (strains BW1040, BW1095, and BW1099), whereas a control plasmid, pBR322 (23), did not. Complementation was also demonstrated in the original *dcd-1* mutant, strain HD1038, in which the *dcd* mutation specified sensitivity to BrdUrd at 42°C. Therefore, in these mutants, thymidine dependence and sensitivity to BrdUrd are due specifically to the loss of dCTP deaminase.

The results also indicated that *dcd-1* belongs to the same complementation group as *dcd-12*::Tn*10*d*kan* and five other mutations that were previously studied (27). This fact had been in doubt because, unlike the insertion mutations, *dcd-1* was reported to be nontransducible with *his* (7), a finding that we could not confirm; when we transduced strain HD1038 (*dcd-1 his-1*) to histidine independence with a phage P1 lysate of strain KL16 (*his*$^+$), 24 of the 94 selected recombinants were resistant to BrdUrd (*dcd*$^+$).

Thymidine dependence and the genetic background of *dcd* mutants. Is there something unusual about strain KL16 that enables a *dcd* mutation to specify thymidine auxotrophy? We transduced the *dcd-12*::Tn*10*d*kan* mutation into other *E. coli* strains. Most of the strains tested showed some enhancement of growth by thymidine, and two of the K-12 derivatives (strains KL16 and Y-Mel) showed strong dependence (Fig. 3A). Although thymidine did not appear to affect the average colony size of the *E. coli* B *dcd* mutant, in the absence of thymidine the colonies were flatter and crenulated.

The thymidine requirement became more pronounced during the faster growth of the cells on an enriched medium, i.e., one containing a casein hydrolysate that had been treated with Norit to remove nucleotide precursors (Fig. 3C). On this medium, *dcd* mutants of MG1655 and of *E. coli* B displayed a difference in colony size in the presence of thymidine, whereas they had not on unsupplemented minimal medium (Fig. 3A). In all of these growth experiments (Fig. 2 and 3), even those strains that produced microcolonies in the absence of thymidine demonstrated no reduction in plating efficiency that would indicate thymineless death. This observation even applied to the *dcd* mutant of wild-type K-12, which bears an inducible λ prophage.

Requirements during anaerobic growth. Under anaerobic conditions, the colony size of strain BW881 (KL16 *dcd*) was no longer affected by the presence of thymidine. However, the bacteria grew more slowly in the absence of air, and the growth rate or medium might affect the thymidine requirement (Fig. 3C versus A). Therefore, we do not know whether the loss of thymidine stimulation can be attributed to an anaerobically

TABLE 1. *E. coli* strains used

Strain	Description[a]	Reference and/or source[b]
BL21	Strain B *hsdS gal*	22
BW865	KL16 *dcd-12*::Tn*10*dkan[c] *dut-22 zic-4901*::Tn*10 deoA22*	27
BW874	KL16 *dcd-12*::Tn*10*dkan *deoA22*	P1(BW865) × BW934
BW881	KL16 *dcd-12*::Tn*10*dkan *hisG4*	27
BW933	KL16 *deoA22 thr-34*::Tn*10*	6
BW934	KL16 *deoA22*	6
BW1040	KL16 *dcd-12*::Tn*10*dkan	P1(BW865) × KL16
BW1094	QE35 *zed-977*::Tn*10*	P1(BW6163) × QE35
BW1095	KL16 Δ(*attP2H-dcd*)*87*[d] *zed-977*::Tn*10*	P1(BW1094) × BW1040
BW1099	KL16 Δ(*attP2H-udk*)*86 rcsA*::(mini)Tn*10*	P1(CS3840) × BW1040
BW1124	KL16 *dcd-1*	P1(BW1136) × BW881
BW1136	As HD1038 but *his*$^+$	P1(KL16) × HD1038
BW1142	LD175 *cir-52*::Tn*10*	P1(LA5523) × LD175
BW6163	KL16 *zed-977*::Tn*10*	28; CGSC
C	Wild-type strain C; prototroph	ATCC 13706
CR63	F$^+$ *supD60 lamB63*	2; CGSC
CS3840	W1485 Δ(*attP2H-udk*)*86 rcsA*::(mini)Tn*10*	C. Schnaitman
HD1038	*dcd-1 leuB6 tonA2 lacY1 supE44 gal-6 galP63? hisG1 rfbD1? argG6 malT1 xyl-7 mtlA2 metB1*	17; J. A. Fuchs
Hfr 3000	HfrH PO1 *thi-1 relA1 spoT1*	2; CGSC
K-12	Wild-type strain K-12; F$^+$ (λ); prototroph	2; CGSC4401
KL16	Hfr PO45 *thi-1 relA1 spoT1*	2
LA5523	HfrC PO2A *cis-52*::Tn*10 fpk-1 metB1 uhp-1 pyrE41 relA1 tonA22 ompF627 spoT1*	11
LD175	HD1038 *cdd-50*	7
MG1655	Prototroph	2
QE35	HfrH PO1 Δ(*attP2H-dcd*)*87 thi-1 relA1 spoT1*	5; CGSC
W1485	F$^+$	2; CGSC
W3110	IN(*rrnD-rrnE*)*1*	2; ATCC 27325
Y-Mel	*supF58 mel-1*	2; laboratory strain

[a] All strains were derivatives of *E. coli* K-12, except for strain BL21 (an *E. coli* B derivative) and strain C. Unless otherwise noted, the strains were λ$^-$ and F$^-$.

[b] Phage P1 transductional crosses are described as follows: P1(donor) × recipient. Abbreviations: CGSC, *E. coli* Genetic Stock Center, Yale University School of Medicine, New Haven, Conn.; ATCC, American Type Culture Collection, Rockville, Md.

[c] Tn*10*dkan designates the transposed portion of a mini-Tn*10* element (27).

[d] Although the Δ*87* deletion specifies a defect in methyl-galactoside transport (5), it cannot extend as far as the *mglBACD* gene cluster because we find that the strain is *cdd*$^+$ *gat*$^+$ *metG*$^+$. The deleted *mgl* locus may be one that was mapped by transduction (5) to a site 1.5 to 1.9 min counterclockwise of where current maps (3, 19) place the *mglBACD* cluster.

induced metabolic shift in nucleotide metabolism or whether it is merely the result of a slowing of protein synthesis that enables more balanced growth.

***deoA22* suppresses thymidine requirement.** For each strain tested, introduction of a *deoA* mutation increased the colony size of a *dcd* mutant on thymidineless medium (Fig. 3B and D) and reduced its degree of dependence on thymidine for optimum growth. In control experiments (data not shown), neither thymidine supplementation nor *deoA* mutations affected the colony sizes of *dcd*$^+$ strains. *deoA* is the gene for thymidine (deoxyuridine) phosphorylase, an enzyme that participates in nucleoside catabolism. Apparently, this block can cause sufficient salvage of deoxyuridine or thymidine to offset the need for exogenous thymidine in *dcd* mutants.

Spontaneous mutations that suppress *dcd-12*. Strain BW881 (*dcd-12*) produced microcolonies on minimal medium lacking thymidine (Fig. 2). After 72 h of growth, there emerged from about 700 microcolonies 15 larger colonies of thymidine-independent revertants. They retained the *dcd-12*::Tn*10*dkan mutation. From their patterns of nutrient utilization (Table 2), six were identified as *deoA* mutants. Three of these were tested under inducing conditions and found to have 3 to 5% of the thymidine phosphorylase activity of the parent strain. None were mutants for *udp*, which encodes uridine phosphorylase, an enzyme that weakly catalyzes the same reactions. The results confirm that it was the *deoA22* allele that produced the

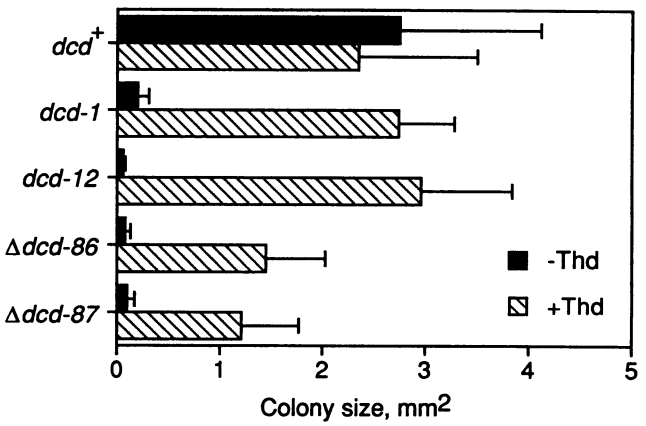

FIG. 2. Thymidine requirement specified by *dcd* mutations in strain KL16. Cells were grown for 48 h at 37°C on minimal media supplemented with histidine and either with or without thymidine. Each filled bar represents the mean of 50 measurements. Error bars (1 standard deviation) are presented not for statistical analysis but only as a conventional representation of the range of variability; the values would not be expected to follow a Gaussian distribution. The strains used were KL16 (*dcd*$^+$) and the following *dcd* mutant derivatives: BW1124 (*dcd-1*), BW881 (*dcd-12*::Tn*10*dkan), BW1099 [Δ(*attP2H-udk*)*86*], and BW1095 [Δ(*attP2H-dcd*)*87*].

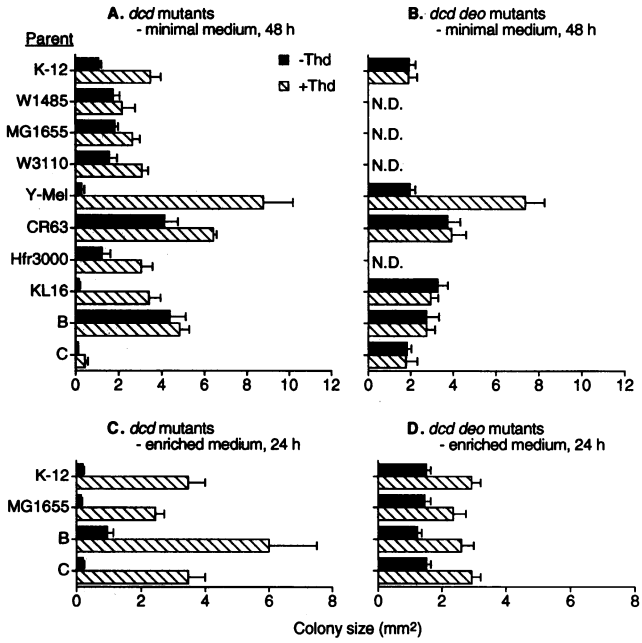

FIG. 3. Thymidine dependence of *dcd* and *dcd deo* mutants. For each of the indicated parental strains, derivatives containing either *dcd-12*::Tn*10dkan* (A and C) or both *dcd-12*::Tn*10dkan* and *deoA22* mutations (B and D) were constructed. The parental *E. coli* B strain used was BL21. The *E. coli* C strain was the wild type (ATCC 13706). Others were derivatives of *E. coli* K-12 (Table 1). The colonies were grown at 37°C for 48 h on VB minimal medium (A and B) or for 24 h on VB medium that was enriched with 0.2% Norit-treated Casamino Acids (C and D). The colony sizes were measured and recorded as for Fig. 2. N.D., not determined.

phenotypic suppression of *dcd* mutants (Fig. 3) and not a hidden mutation in a cotransduced gene.

Thymidine phosphorylase levels in *dcd*$^+$ strains. Of all the strains that were tested, the greatest thymidine dependence was displayed by *dcd* mutants of KL16 and Y-Mel (Fig. 3A). We asked whether this property were due to unusually high levels of thymidine phosphorylase in the parental (*dcd*$^+$) strains under our growth conditions. Cells grown in minimal medium without thymidine had the following levels of thymidine phosphorylase (units per milligram of protein): K-12 (control), 42; Y-Mel, 66; and KL16, 26. Although strain Y-Mel had a 57% higher level than K-12, the significance of this difference is questionable because the enzyme is normally capable of being induced at least 60-fold by thymidine (8). Therefore, KL16 and Y-Mel may have an elevation of another catabolic enzyme, or they may have a relatively slower rate of TMP synthesis via an alternative pathway.

TABLE 2. Thymidine-independent phenotypic revertants of a *dcd* mutant

Growth on carbon source:			Presumed genotype[a]	No. found
Thymidine	Deoxyadenosine	Uridine		
−	+	+	*deoA*	6
−	−	−	*deoB*	0
−	−	+	*deoC*	0
+	+	−	*udp*	0
+	+	+	?	9

[a] Based on reference 8. ?, genotype unknown.

TABLE 3. Growth requirements of a *dcd* mutant[a]

Supplement[b]	Colony size[c] (*mm*^{2})
None	0.09 ± 0.02
Thymidine	2.81 ± 0.27
Thymine	2.11 ± 0.43
Deoxyuridine	1.74 ± 0.37
Uridine	1.70 ± 0.36
Uracil	1.81 ± 0.35
Cytosine	1.54 ± 0.28
Adenine	0.11 ± 0.08
Deoxyadenosine	<0.01
Adenosine	<0.01

[a] Strain BW881 (KL16 *dcd-12 his*).
[b] Supplements were at a final concentration of 0.5 mM in a glucose minimal agar medium containing histidine.
[c] Mean ± 1 standard deviation for 50 colonies after 48 h of growth at 37°C.

Compounds that can substitute for thymidine. A *dcd* mutant of strain KL16 was grown on a minimal agar medium supplemented with various nucleosides or nucleobases (Table 3). Although thymidine could best satisfy the nutritional requirements, any of the other pyrimidine compounds tested could substitute for thymidine. The adenine derivatives were ineffective, including deoxyadenosine, which is a potential source for the deoxyribose in salvaged TMP. The effective compounds, namely, thymidine, thymine, deoxyuridine, uridine, uracil, and cytosine, are all precursors for TMP via salvage pathways (Fig. 1). In addition, exogenous uridine is a competitive inhibitor of thymidine phosphorylase (8) and may thus produce an effect like that of a *deoA* mutation.

The deoxycytidine pathway. The effect of a *deoA* mutation suggested that salvage of deoxyuridine is required for efficient synthesis of thymidylate in a *dcd* mutant. What is the source of this deoxyuridine? Although it could arise from dUMP, an obligatory precursor of TMP, previous evidence suggests that there is little deoxyuridine formed except in thymidylate synthase mutants, which accumulate dUMP (9). An alternative possibility is that deoxyuridine may arise from the breakdown of dCTP, the pool of which may be 20-fold elevated in *dcd* mutants (16). In these mutants, the deamination of cytosine compounds to uracil analogs would have to occur at the deoxynucleoside level via cytidine (deoxycytidine) deaminase, the product of the *cdd* gene (Fig. 1). To test this hypothesis, the *cdd-50* mutation of strain BW1142 was transduced into strain BW874 (KL16 *dcd-12 deoA*) via linkage with *cir*::Tn*10*, and the recombinants were tested for their thymidine requirements (Fig. 4). The *dcd deoA* parental strain was a prototroph; the thymidine dependence specified by *dcd-12* was suppressed by the *deoA* mutation. However, a *cdd-50* transductant of this strain was a thymidine auxotroph. Of 42 tested tetracycline-resistant (*cir*::Tn*10*) transductants, 17 were *cdd* mutants, and there was a one-to-one correspondence between the *cdd* defect and thymidine auxotrophy. This finding suggested that deoxycytidine and deoxyuridine are intermediates in an important alternative pathway for TMP synthesis in *dcd* mutants. However, the *cdd* mutation had no apparent effect on the growth of wild-type cells, which were tested in the same manner (Fig. 4). Therefore, the deoxycytidine pathway appears to be essential only in *dcd* mutants.

Phenotypic revertants of *dcd deo cdd* mutants. When plated on thymidineless medium, a *dcd-12 deoA cdd* strain yielded thymidine-independent mutant derivatives with a frequency of

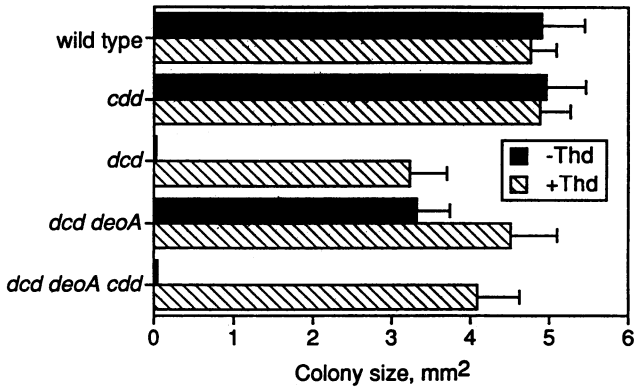

FIG. 4. Requirement for the *cdd* gene for thymidine independence of *dcd deoA* mutants. Colony size was recorded after 24 h of growth at 37°C on VB minimal medium containing 0.2% Norit-treated Casamino Acids, with or without added thymidine. All strains were derivatives of KL16. With the exception of the *dcd* mutant (strain BW1040), they contained *cir-52*::Tn*10* in addition to the other listed markers. The alleles used were *cdd-50*, *dcd-12*::Tn*10*d*kan*, and *deoA22*.

about 10^{-5}. These phenotypic revertants retained the original mutations. It is not known whether the new mutations are in the same genes as those affected in the unidentified revertants in Table 2. Thus, even when two pathways of thymidylate synthesis are blocked, mutation may enhance a third to the point where it can suffice.

DISCUSSION

The findings suggest that *dcd* mutants depend on the following alternative pathway for the biosynthesis of thymidylate: dCTP (or dCDP)→→dCyd→dUrd→dUMP→TMP. The enzymatic steps by which dCTP or dCDP is degraded to deoxycytidine are unknown and beg further investigation. Unlike the other two pathways, which operate through the deamination of dCTP or the phosphorylation of dUDP, respectively, the deoxycytidine pathway does not require dUTPase. Thus, it may be the chief pathway operating in a *dut* (dUTPase) mutant, a possibility that we are now exploring.

Our observations suggest several reasons why previous investigators had not detected a thymidine requirement for *dcd* mutants. First, the effect is dependent on the parental strain used (Fig. 3). Second, because all the strains we tested showed at least some growth without thymidine, auxotrophy may have been easily undetected. Third, *dcd* mutants readily revert to greater thymidine independence when grown on limiting media. Adaptation of the cells to growth in thymidineless medium is further facilitated by the existence of alternative biosynthetic pathways. In addition to the deoxycytidine pathway described here, *E. coli* should also be able to compensate somewhat for a loss of the dCTP pathway by an increased flux of nucleotides through the dUDP pathway; the loss of dCTP deaminase produces a decrease in the TTP pool (16), which should lead to an increased production of dUDP by both derepression and allosteric activation of ribonucleoside diphosphate reductase (14, 25).

The *dcd* mutant strains that we examined varied greatly in their degree of dependence on thymidine and in the degree to which they responded to exogenous thymidine. These properties do not appear to be related to lineage. When we compare the pedigree of these strains (2) with the thymidine requirements of their *dcd* mutants (Fig. 3A), we can see no consistent pattern. For example; (a) the most defective strains are *dcd* mutants of Y-Mel and KL16, but their only common ancestor, K-12, is less defective; and (b) strain W1485 is not as deficient as its parent, strain K-12, nor its descendant, strain W3110. It is unlikely that so many independent mutations affecting thymidine synthesis arose coincidentally during construction of the parental (*dcd*$^+$) strains. Instead, they probably arose during the years of propagation, serial passage, and storage in agar media, which preceded their storage in freezers. Media rich in nucleotide precursors would select for the relative enhancement of catabolic (salvage) pathways, while poorer media would select for increased de novo synthesis. In this work, we have seen examples of the ease with which such changes can evolve: (a) when a *dcd* mutant was grown on thymidineless media, as many as a few percent of the colonies gave rise to faster-growing phenotypic revertants that were mutated in other genes; and (b) *dcd deo cdd* mutants yielded thymidine-independent revertants at a frequency equivalent to that of forward mutations.

Under conditions in which dCTP does not accumulate, the deoxycytidine pathway may be minor. However, it is difficult to assess the relative contribution of the three pathways to thymidylate synthesis in a wild-type cell because almost all studies of this type are based on the use of mutants. For example, experiments suggesting that most of the thymidylate in *E. coli* and *S. typhimurium* is made from deoxycytidine compounds were performed with pyrimidine auxotrophs that had *cdd* mutations and that were fed pyrimidines (10, 15). The balances in nucleotide metabolism are so intricate that interruption of a pathway or nutritional supplementation affects alternate pathways via allosteric and genetic regulation (14). Thus, we may not be able to extrapolate experimental results to the physiology of the unaltered cell. However, the relative roles of these pathways are probably of less importance than their existence and their diversity. This diversity provides a form of insurance for the cell. When one pathway is blocked, the others enable limited cell growth, thereby facilitating the outgrowth of phenotypic revertants, as we have seen in these studies.

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