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The Deoxycytidine Pathway for Thymidylate Synthesis in *Escherichia coli*[▽]

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When thymidylate production is diminished by a mutation affecting dCTP deaminase, *Escherichia coli* is known to use an alternate pathway involving deoxycytidine as an intermediate. The pathway requires the gene for any of three nucleoside diphosphate kinases (*ndk*, *pykA*, or *pykF*) and the gene for a 5′-nucleotidase (*yfbR*).

Escherichia coli has three known routes for the aerobic de novo synthesis of dUMP, the substrate for thymidylate synthetase. They are referred to as the dCTP, dUDP, and deoxycytidine (dCyd) (17, 29) pathways after their characteristic intermediates. In *E. coli* and in *Salmonella enterica* serovar Typhimurium, dCTP is a precursor for 75% to 80% of thymidylate that is synthesized de novo (17). In the first step of this dCTP pathway, dCTP is converted to dUTP by dCTP deaminase, which is encoded by the *dcd* gene (Fig. 1). Then, dUTPase (*dut* gene) catalyzes the hydrolysis of a pyrophosphate from dUTP, yielding dUMP. Most of the remaining synthesis of dUMP (and hence TMP) is presumed to occur through the dUDP pathway, in which UDP is reduced enzymatically to dUDP. Some of the dUDP may produce dUMP through the reversible thymidylate kinase reaction, but most of it is phosphorylated to dUTP, which is then degraded to dUMP by dUTPase (Fig. 1). The dCyd pathway (Fig. 2) comes into play when dCTP deaminase is inactivated by a *dcd* mutation, thereby disrupting the major (dCTP) pathway for dUMP synthesis (29). *dcd* mutants grow poorly in the absence of thymidine (Thd), indicating that the dUDP pathway is inadequate to supply the needs of the cell. However, *dcd* mutants readily acquire Thd independence through spontaneous mutations in the *deoA* gene, which encodes a phosphorylase for both Thd and deoxyuridine (dUrd) (29). This finding suggested that dUrd could be an endogenous precursor for TMP. It was surprising because de novo synthesis of nucleic acid precursors occurs through nucleotide rather than nucleoside intermediates. Therefore, the dCyd pathway must rely on salvage enzymes. The blockage of dCTP deaminase in *dcd* mutants was postulated to lead to a metabolic detour. The massively accumulating dCTP is degraded to dCyd, which is then deaminated to dUrd. dUrd is converted to dUMP by Thd kinase, which, like other enzymes that act on Thd, acts on dUrd as well. The *deoA* mutation enables this pathway by blocking the breakdown of a critical intermediate, dUrd, and thereby renders a *dcd* mutant Thd independent. The major evidence for this pathway was that a mutation in *cdd*, the gene for cytidine (and dCyd) deaminase, caused a *dcd deoA* mutant to again require Thd (29), indicating that dCyd and dUrd are intermediates. In this paper, a similar approach is used to identify the additional enzymes that are critical for this pathway, those involved in each step of the conversion of dCTP to dCyd. The disruption of the corresponding gene should cause a *dcd deoA* mutant to require Thd.

Strain construction. A series of mutations that might affect the dCyd pathway (Table 1) were transduced into BW1929 (*dcd deoA*) for testing. The *pykF* mutation is a transposon insertion at nucleotide 812 of the 1,412-nucleotide open reading frame, as determined by inverse PCR (26). Each of the other mutations, with the exception of *cdd-50*, was produced by the replacement of 80% to 97% of the gene by transformation with a PCR product containing an antibiotic resistance marker (31). To alleviate the selective pressure for hidden mutations to Thd independence, all newly constructed strains were isolated and propagated using Luria-Bertani medium (16) supplemented with Thd at 125 μg/ml, which has been shown to be necessary and sufficient for the preservation of the Thd requirement of *dcd* mutants (29). The following antibiotics were

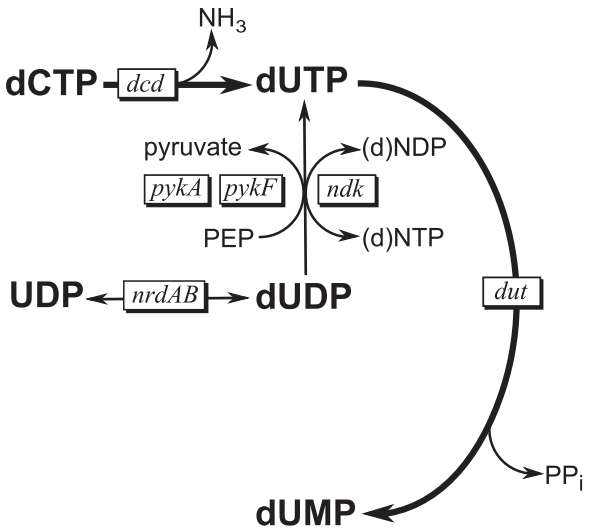

FIG. 1. dCTP and dUDP pathways for de novo synthesis of dUMP. The major (dCTP) pathway is shown with bold arrows. The genes and their products are as follows: *dcd*, dCTP deaminase; *dut*, dUTPase (dUTP pyrophosphohydrolase); *ndk*, nucleoside diphosphate kinase; *nrdAB*, ribonucleoside diphosphate reductase; *pykA*, pyruvate kinase I; and *pykF*, pyruvate kinase II. Abbreviations: (d)NDP and (d)NTP, any ribo- or deoxyribonucleoside diphosphate and triphosphate, respectively; PEP, phosphoenolpyruvate.

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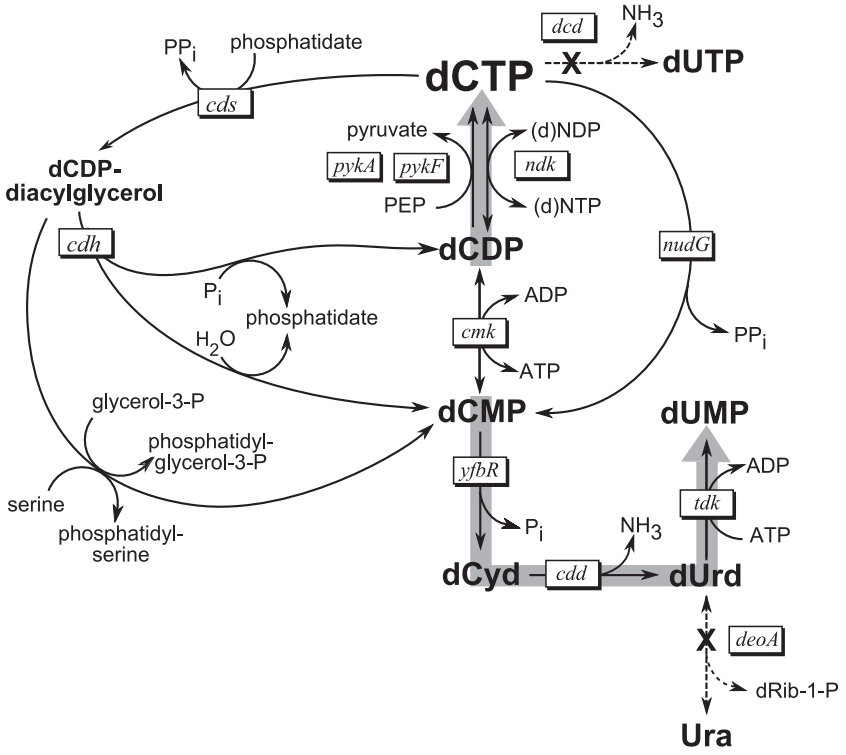

FIG. 2. The dCyd pathway, an alternative, salvage pathway for the synthesis of dUMP in a *dcd deoA* mutant. The broad shaded arrows cover identified steps, including those established in this work. The genes and their corresponding products are as follows: *cdd*, cytidine (or dCyd) deaminase; *cdh*, (d)CTP-diglyceride hydrolase; *cds*, (d)CDP-diglyceride synthetase; *cmk*, (d)CMP kinase; *deoA*, Thd (dUrd) phosphorylase; *nudG*, Nudix (d)CTP pyrophosphohydrolase; *tmk*, Thd (dUrd) kinase; and *yfbR*, a cytoplasmic 5′-nucleotidase. Other symbols and abbreviations are as described in the legend to Fig. 1.

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant genotype[a]	Source or reference[b]
Strains		
BW934	KL16 *deoA22*	8
BW1146	KL16 *dcd-12*::Tn*10*d *kan deoA22 cdd-50 cir-52*::Tn*10*	8
BW1670	λ*c*I857 Δ(*cro-bioA*) *hsdR2 mdoB202*::Tn*10*	28
BW1670 *dcd*	BW1670 Δ*dcd*::(FRT-Sp-FRT)	PCR(pUC18::Sp) × BW1670
BW1670 *nudG*	BW1670 Δ*nudG*::(FRT-Tp-FRT)	PCR(BW1915) × BW1670
BW1679	*pykF*::Tn*10*d*cat*	This lab; random insertion of element 105 (14)
BW1697	BW1670 Δ*yggV*::(FRT-*kan*-FRT)	PCR(pKD4) × BW1670
BW1914	BW1670 Δ*pykA*::(FRT-Gm-FRT)	PCR(pKD4::Gm) × BW1670
BW1915	BW1670 Δ*yggV*::(FRT-Tp-FRT)	PCR(EZ-Tn<DHFR-1>Tnp[c]) × BW1697
BW1929	KL16 Δ*dcd*::(FRT-Sp-FRT) *deoA22*	P1(BW1670 *dcd*) × BW934
JW0893_11	Δ*cmk*::(FRT-*kan*-FRT)	Keio collection (1)
JW2288_11	Δ*yfbR*::(FRT-*kan*-FRT)	Keio collection (1)
JW2502_11	Δ*ndk*::(FRT-*kan*-FRT)	Keio collection (1)
JW2714_11	Δ*surE*::(FRT-*kan*-FRT)	Keio collection (1)
JW3889-1	Δ*cdh*::(FRT-*kan*-FRT)	Keio collection (1)
KL16	Hfr KL16 (PO-45) *thi-1 relA1 spoT1*	2
Plasmids		
pKD4	*ori*γ *bla* FRT-*kan*-FRT	6
pKD4::Gm	*ori*γ *bla* FRT-Gm-FRT	This lab[d]
pUCGm	pUC18::Gm *bla*; Gm cassette in multiple cloning site	24
pUC18::Sp	pUC18::(FRT-Sp-FRT) *bla*	This lab[e]

[a] All strains are derivatives of *E. coli* K-12 and are F^- λ^- unless indicated otherwise. *cat*, *kan*, Sp, Gm, and Tp indicate resistance to chloramphenicol, kanamycin, spectinomycin, gentamicin, and trimethoprim, respectively. FRT (FLP recombinase target) sequences enable excision of the bounded resistance gene by site-specific recombination (6).

[b] Transductions with phage P1 *dam rev6* (25) are described as follows: P1(donor) × recipient. Deletion/substitution mutations were produced by allelic replacement with PCR-amplified DNA and are described as follows: PCR(donor template) × recipient. Details are available on request.

[c] Transposon DNA from Epicentre Biotechnologies, Madison, WI.

[d] An 855-bp SmaI Gm cassette of pUCGm (24) was cloned into pKD4, replacing a 360-bp PvuII segment containing the *kan* gene.

[e] A HindIII segment of pKD3 (6) that contains an FRT-*cat*-FRT cassette was cloned in pUC18 (30), and then the *cat* gene was excised by digestion with AscI and replaced by a PCR product of the Sp determinant of pKRP13 (22).

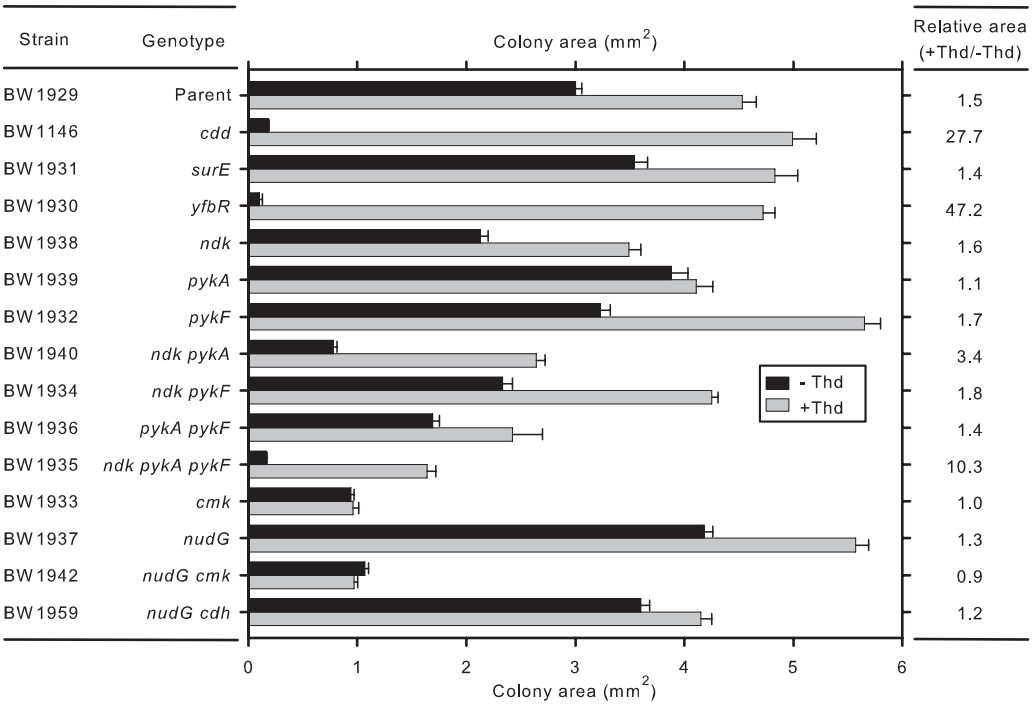

FIG. 3. Thd requirement of strain BW1929 (*deoA dcd*) and mutant derivatives. The strains were constructed by phage P1-mediated transduction (25) of mutations from the strains listed in Table 1. Saturated cultures were diluted in 10 mM $MgSO_4$ and plated on a minimal agar containing 0.4% glucose and 1% Norit-treated Difco Casamino Acids (28), both with and without Thd at 125 μg/ml. After 24 h of growth at 37°C, from 25 to 50 uncrowded colonies were measured. Each bar represents the mean colony area ± standard error of the mean. The relative area (+Thd/−Thd) is the ratio of the lengths of the corresponding bars.

used at the indicated concentrations (in μg/ml): kanamycin, 35; chloramphenicol, 20; spectinomycin, 50; gentamicin, 20; and trimethoprim, 10. The absence of a wild-type copy of each newly introduced mutant gene was verified by PCR.

From dCMP to dCyd. The mutant derivatives of the *dcd deoA* strain BW1929 were grown in the presence or absence of Thd, and their colony sizes were measured (Fig. 3). The parental strain, BW1929, should rely mainly on the dCyd pathway for the synthesis of TMP. Any mutation blocking this route should result in a growth requirement for Thd. A *cdd* mutation, which is known to interrupt the dCyd pathway (29), provided a positive control. Whereas the growth of the parental strain was enhanced only 50% by exogenous Thd, that of the *cdd* derivative was enhanced about 28-fold (Fig. 3). The small growth enhancement of the parental *dcd deoA* strain, which was previously observed and not seen in the wild type (29), suggests that the dCyd pathway is not as robust as the dCTP pathway.

The previous step, dCMP→dCyd, was then examined. Most of the known enzymes with 5′-nucleotidase activities are located in the periplasm and are therefore not likely to be accessible to cytoplasmic nucleotides (27). Two newly discovered cytoplasmic nucleotidases, YfbR and SurE (21), were considered. They each have a broad specificity and can hydrolyze dCMP. The *yfbR* derivative of the *dcd deoA* strain became dependent on Thd for optimum growth, whereas the *surE* mutant did not (Fig. 3). The *yfbR* mutation is unlikely to affect any genes other than *yfbR*; the deletion is confined to the gene (1), there are no overlapping genes, and the next gene downstream is in the opposite orientation (23). Therefore, YfbR is a dCMP phosphohydrolase that is essential for the dCyd pathway. This is the first physiological role found for this enzyme. The conversion of dCMP to dCyd is likely to be unidirectional because there is no dCyd kinase in *E. coli*.

From dCDP to dCMP. During aerobic growth, the reduction of ribonucleotides to deoxyribonucleotides occurs at the diphosphate level. Therefore, dCDP is the first dCyd nucleotide synthesized and could be the direct precursor for dCMP. There are three known theoretical pathways from dCDP to dCMP (Fig. 2). The first is that of a direct reaction catalyzed by dCMP kinase, encoded by *cmk*. This freely reversible reaction may be driven toward dCDP by the high concentration of cellular ATP, or it may be driven toward dCMP by the subsequent hydrolysis of dCMP by YfbR. The other two pathways involve dCTP as intermediates. dCTP may be hydrolyzed directly to dCMP in a reaction catalyzed by NudG, or it may be converted to dCMP indirectly during membrane phosholipid biosynthesis.

The CMP kinase pathway was tested first. CMP kinase, encoded by *cmk*, catalyzes the reversible interconversion of (d)CMP and (d)CDP. It is responsible for 94% of the ATP-dependent CMP kinase activity in a cell extract of *E. coli* (9). Because the cytosine residue is synthesized in the cell from uracil at the triphosphate level, dCMP is not an intermediate in the biosynthesis of dCTP. Therefore, CMP kinase is a salvage enzyme. The slow growth produced by a *cmk* mutation (Fig. 3) was previously attributed (3, 9) to the kinase's role in helping to regenerate the (d)CTP that is consumed during membrane

TABLE 2. Doubling times in liquid culture of some mutant derivatives of BW1929 (*dcd deoA*) grown with and without Thd

Strain	Additional mutation(s)	Doubling time (min)[a]	
		+Thd	−Thd
BW1929	None	38	44
BW1930	*yfbR*	42	340
BW1935	*ndk*, *pykA*, *pykF*	60	148

[a] The liquid medium was similar to the agar medium used for colony size measurements. Cells at an initial concentration of about 2×10^7/ml were aerated by shaking at 37°C in flasks with sidearm cuvettes, and growth was monitored by determining the optical density at 560 nm by using a Klett colorimeter. Readings were taken periodically at cell densities between 1×10^8 and 4×10^8/ml.

phospholipid biosynthesis (Fig. 2). However, the growth defect persists despite a *dcd* mutation, which produces a greatly expanded pool of dCTP, suggesting the possibility that an important additional role for the enzyme exists, such as the salvage of CMP generated by mRNA turnover. Although the *dcd deoA cmk* mutant grew slowly, it did not display a Thd requirement. Therefore, dCMP kinase is not essential for the dCyd pathway. dCTP-dependent routes were investigated next.

Role of nucleoside diphosphate kinases. dCTP is a likely precursor for dCyd in this pathway. In *dcd* mutants grown without Thd, dCTP has been measured at levels ranging from 40% (19) to 150% (18) those of ATP and constituted from 17% to 40% of the total nucleoside triphosphate pool. There are multiple nucleoside diphosphate kinases that may catalyze the conversion of dCDP to dCTP (15), only three of which are shown in Fig. 2. *ndk* encodes an enzyme that is not specific with respect to its nucleoside di- and triphosphate substrates (12, 13). *pykA* and *pykF* produce pyruvate kinases that catalyze the phosphorylation of any (deoxy)nucleoside diphosphate, using phosphoenolpyruvate as a phosphoryl donor (11). The reactions catalyzed by Ndk should be freely reversible because the reactants and the products both consist of a diphosphate and a triphosphate. However, the pyruvate kinase reaction, in which pyruvate is the phosphate acceptor, has an extremely unfavorable equilibrium. Its equilibrium constant (as measured with the rabbit muscle enzyme) is about 4×10^4 for production of the nucleoside triphosphate (7). All six permutations of *ndk*, *pykA*, and *pykF* mutations were tested (Fig. 3). It should be noted that although nucleoside diphosphate kinase activity is essential for polynucleotide biosynthesis, a mutant lacking these three enzymes is still viable because it retains similar activities associated with succinyl-CoA synthetase and adenylate kinase (15). The loss of all three genes was required for almost complete Thd dependence: whereas the parental *dcd deoA* strain displayed only a 50% enhancement of growth by Thd, the *pykA pykF ndk* derivative displayed a 10-fold enhancement (Fig. 3). The Thd dependence of this mutant, as well as that of the *yfbR* mutant, was confirmed during growth in liquid medium (Table 2).

Because of a general defect in nucleoside biosynthesis, the *pykA pykF ndk* strain grows slowly even in the presence of Thd. It could be argued, therefore, that the Thd dependence of this mutant is an artifact attributable to this slow growth, which might magnify the small effect that Thd has on the parental strain. However, the slow-growing *cmk* mutant (Fig. 3) displays no response to Thd, indicating that not just any growth defect in a *dcd deoA* mutant background will amplify an apparent Thd auxotrophy; in fact, as we might expect, slower growth may even reduce a nutritional requirement.

The growth requirement of the *pykA pykF ndk* derivative is specific for Thd or its precursors. In separate experiments (data not shown), neither deoxyadenosine nor deoxyguanosine could measurably stimulate the growth of the mutant. However, dCyd and dUrd, which are intermediates in the dCyd pathway, were as effective as Thd. Another possibility is that the *pykA pykF ndk* strain's need for Thd was created by a deficiency in the conversion of TDP to TTP, which was overcome by overloading the pathway with a very high concentration of exogenous Thd (125 μg/ml). This concentration is about 25 times that needed by a thymineless strain that has a *deoA* mutation. However, when the *ndk pykA pykF* strain was grown with Thd at 5 μg/ml, its average colony size (1.08 ± 0.03 mm^2) was 35 times greater than that of the strain grown in the absence of Thd (data not shown).

From dCTP to dCMP. The requirement for three nucleoside diphosphate kinases strongly suggests that dCTP is an intermediate in the dCyd pathway. There are two known alternate routes from dCTP to dCMP (Fig. 2). A direct reaction is catalyzed by NudG, a Nudix triphosphatase that releases pyrophosphate from 5-methyl-dCTP, dCTP, and CTP (20). A *nudG* mutation was tested (Fig. 3) but did not produce Thd auxotrophy even in combination with a *cmk* mutation. A second possible route is provided by membrane phospholipid biosynthesis, in which (d)CMP is produced indirectly from (d)CTP (5). Tight mutations of *cds*, which block the formation of dCDP-diglyceride, are lethal (10) and could not be tested. The pathway may be short-circuited by (d)CDP-diglyceride hydrolase (encoded by *cdh*), which can transfer the deoxycytidylyl group to phosphate or to water to generate dCDP or dCMP, respectively (4). However, a *cdh* mutation, even when combined with a *nudG* mutation, failed to produce a Thd requirement (Fig. 3).

Concluding remarks. The data clearly define a new component of the dCyd pathway: YfbR, which catalyzes the hydrolysis of dCMP to dCyd. Other steps are less clear. The *cmk*, *cdh*, and *nudG* genes are not essential, which would appear to suggest that they each have no significant role. However, it is just as likely that they participate in parallel redundant routes from dCDP to dCMP. The need for nucleoside diphosphate kinases combined with the dispensability of (d)CMP kinase suggests that (i) dCTP is an important intermediate in the dCyd pathway, (ii) the direct conversion of dCDP to dCMP is a much less significant route, and (iii) phospholipid metabolism may be relatively important for the production of dCMP from dCTP.

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