Isolation and Characterization of *Escherichia coli* Strains Defective in CDP-diglyceride Hydrolase*

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CDP-diglyceride, an obligatory intermediate in the biosynthesis of the glycerophospholipids in *Escherichia coli*, is cleaved *in vitro* to phosphaacid and CMP by a membrane-bound hydrolase. Previous work from our laboratory (Bulawa, C. E., Hermes, J. D., and Raetz, C. R. H. (1983) *J. Biol. Chem.* 258, 14974-14980) has demonstrated that this enzyme also catalyzes the transfer of CMP from CDP-diglyceride to phosphate and numerous phosphomonoesters. We now report the isolation of *E. coli* mutants which are defective in CDP-diglyceride hydrolase. These mutations, designated *cdh*, map at minute 88 between *phkA* and *tpi*. This information permitted the identification of a ColEl hybrid plasmid, pLC16-4, which causes the overproduction of hydrolase activity. The isolation of deletion and Tn10 insertion mutants at *cdh* suggests that the hydrolase is nonessential for cell growth. Hydrolase mutants are defective in both CDP-diglyceride hydrolysis and CDP-diglyceride-dependent cytidylylation, indicating that both activities are encoded by the *cdh* gene. Although previously described as a ribospecific enzyme, we have found that incubation of the partially purified hydrolase with [α-32P]dCDP-diglyceride and phosphate yields two products, [32P]dCMP and [α-32P]dCDP. That a single enzyme utilizes both CDP- and dCDP-diglyceride is demonstrated by the following. (i) The hydrolysis of [α-32P]dCDP-diglyceride is inhibited by nonradioactive dCDP-diglyceride and vice versa. (ii) Utilization of both liponucleotides is inhibited by AMP. (iii) Mutants in the *cdh* gene are defective in both CDP- and dCDP-diglyceride hydrolysis, while *cdh* clones overproduce both activities. (iv) Hydrolase mutants accumulate both CDP- and dCDP-diglyceride.

CDP-diglyceride is the precursor of all the glycerophospholipids in *Escherichia coli* and related Gram-negative bacteria. The enzymatic synthesis of this chloroform-soluble nucleotide was originally reported by Carter (1), and, subsequently, Raetz and Kennedy showed that both the ribo- and deoxy-forms of the liponucleotide exist in living cells (2). The utilization of CDP-diglyceride in the synthesis of phosphatidylserine and phosphatidylglycerophosphate was demonstrated in *E. coli* extracts by Kanfer and Kennedy (3). In more recent studies, Ganong and Raetz have shown that conditionally lethal CDP-diglyceride synthetase mutants are defective in the formation of both phosphatidyethanolamine and phosphatidylglycerol under the nonpermissive conditions (4). Thus, a substantial body of biochemical and genetic evidence has established that CDP-diglyceride is the sole phosphatidyl donor for glycerolipid biosynthesis in *E. coli*.

The first indication that CDP-diglyceride had an additional metabolic role came from the work of Raetz et al. (5). These investigators found that CDP-diglyceride was cleaved *in vitro* to phosphatidic acid and CMP by a membrane-bound hydrolase. Partial purification of this activity (6) showed that it was distinct from the lipid biosynthetic enzymes. Although several ribonucleotides, such as CDP-diglyceride, UDP-diglyceride, and CDP-serine, were rapidly cleaved by the hydrolase, dCDP-diglyceride was not utilized at an appreciable rate (6). In addition, AMP- and ADP-diglyceride were identified as potent inhibitors of CDP-diglyceride hydrolysis (5, 6). Recently, Rittenhouse et al. have reported a similar AMP-sensitive pyrophosphatase in guinea pig brain (7). In the latter case, both CDP- and dCDP-diglyceride were substrates for the enzyme.

Recent studies from our laboratory have provided new insights into the function of the CDP-diglyceride hydrolase. We have demonstrated that the partially purified hydrolase catalyzes the transfer of CMP from CDP-diglyceride to various phosphomonoester acceptors (8). To further explore the phenomenon of CDP-diglyceride-dependent cytidylylation, we have isolated *E. coli* mutants defective in CDP-diglyceride hydrolyase (9). In the present study, we show that the hydrolase and cytidylyltransferase activities are encoded by a single locus, designated *cdh*. Contrary to previous reports, we found that dCDP-diglyceride was efficiently hydrolyzed *in vitro* and, furthermore, that it could serve as a dCMP donor. Analysis of hydrolase mutants revealed the accumulation of CDP- and dCDP-diglyceride, indicating that both liponucleotides are substrates for the hydrolase *in vitro*.

**EXPERIMENTAL PROCEDURES**

*Materials*—Chemicals were purchased from the following companies: ribo- and deoxy-α-[32P]CTP, Amersham Corp.; [5-3H]CTP and 3P, New England Nuclear; tetrabutylammonium bromide, Aldrich. Other fine chemicals were from Sigma. Purified phosphatidylserine synthase (10), used for the synthesis of radiolabeled CDP- and dCDP-diglyceride, was the gift of Dr. William Dowhan, Silica Gel 60 thin layer plates (0.25 mm) and PEI-cellulose F thin layer plates (0.1 mm) were the products of E. Merck, Darmstadt, Germany. Aquasol was purchased from New England Nuclear.

*Bacterial Strains, Plasmids, and Growth Media*—The strains of *E. coli* K12 used in this work are listed in Table I. Three different media were employed for the growth of bacteria: LB (16), G56 (17), and minimal A (16). Carbon sources were included at a final concentration of 1. The abbreviations used are: PEI, polyethyleneimine; Mes, 2-(N-morpholino)ethanesulfonic acid; HPLC, high pressure liquid chromatography.
of 0.2%. All other nutrients were present at the levels recommended by Miller (16). For strains harboring Tn10, tetracycline was added to the culture at 15 μg/ml. Plates contained 15 g of agar/liter of medium.

Preparation of Crude Extracts—Extracts were prepared from fresh overnight cultures unless otherwise indicated. Cells were grown at 37 °C in LB medium and then harvested at 4 °C by centrifugation. After washing once with cold 10 mM potassium phosphate, pH 7.5, the cells were resuspended in the same buffer at a final concentration of about 5 mg/ml of protein. The cells were broken by passage through an Aminco pressure cell at 18,000 p.s.i. After removing intact cells by centrifugation, extracts were assayed as described below.

Purification of CDP-diglyceride Hydrolase—CDP-diglyceride hydrolase was partially purified from the overproducing strain JA200/pLC16-4 through the DEAE-cellulose step (6). The final preparation was purified 300-fold relative to a crude extract from a wild-type strain and was free of phosphatidylglycerophosphate activities.

Synthesis of CDP-diglyceride.—CDP-diglyceride was prepared from egg phosphatidic acid and CMP-morpholinate as reported previously (2, 18). dCDP-diglyceride was synthesized by the same procedure except that E. coli phosphatidic acid and dCMP-morpholinate were used. Ribo- and deoxy-[α-32P]CDP-diglyceride and [5-3H]CDP-diglyceride were generated by the CMP:CDP-diglyceride exchange reaction (6, 19) of phosphatidylserine synthase. Radioactive cytidine monophosphates were prepared by acid hydrolysis of the corresponding triphosphates (8).

Enzyme Assays—CDP-diglyceride hydrolase activity was determined at 37 °C as follows, unless otherwise indicated. The substrate was either [α-32P]CDP-diglyceride or [α-32P]dCDP-diglyceride (1000 cpm/nmol) at a final concentration of 0.33 mM. In addition, the assay mixture contained 100 mM potassium phosphate, pH 7.5, 0.1% Triton X-100, and 0.7 mg/ml of bovine serum albumin in a total volume of 60 μl. Reactions were terminated by Bligh-Dyer extraction at pH 2 (solvents and volumes are given in the legend to Fig. 5), and 2 ml of the upper phase were transferred to a scintillation vial. After the addition of 10 ml of Aquasol, the radioactivity was quantitated by liquid scintillation spectrometry. We recently discovered that when phosphate is used as the buffer, the hydrolase catalyzes the formation of two water-soluble products, [32P]CMP and [cY-32P]CDP (6). Therefore, using this assay, CDP-diglyceride hydrolase activity is measured as the nanomoles of CMP plus CDP produced/min/mg of protein.

| Strain    | Relevant genotype                        | Source (Reference) |
|-----------|------------------------------------------|--------------------|
| RA5       | F- leu, proC, his, trp, ilv, argH, parE, pss-5, gal, xyl, mtl, malA, ara, lac, thiA, rpsL | C. R. H. Raetz (11) |
| RA5-1     | cdh-1 derivative of RA5                  | This work          |
| RA5-2     | cdh-2 derivative of RA5                  | This work          |
| RA5-3     | cdh-3 derivative of RA5                  | This work          |
| HfrH      | Hfr thi-1, relA1, spoT1, X-, supQ80       | CGSC               |
| KL25      | Hfr supE42, X-                          | CGSC               |
| R477      | F- thr-1, leu-6, his-4, ada, rpsL136, tonA136 | J. Adler (12)     |
| M2508     | Hfr metB1, relA1, melA7, spoT1           | CGSC               |
| RES4300   | Hfr malB29, dnaB107, thi*                | C. R. H. Raetz (13) |
| AM1       | Hfr pfsA1, relA1, tonA22, T2a, pit10, spoT1 | CGSC               |
| FRAG-1    | F- thi-1, rha-4, lac22s, gal-33           | CGSC               |
| EJ482     | argH* metB1 cdh-1 transductant of RA5-1, M2508 as donor | This work |
| MA1000    | metB* pfsA1 transductant of M2508, AM1 as donor | This work |
| W3110     | Wild type strain of E. coli K-12, X-, F- | CGSC               |
| MW1104    | pfsA* cdh-4::Tn10 transductant of MA1000 | This work          |
| MW1204    | pfsA* rha-4::Tn10 transductant of MA1000 | This work          |
| JF1010    | Hfr ΔpfsA, recA56, relA1, spoT1, Δ(rha-pfsA), tonA22, T2a | CGSC               |
| ET2036    | F- Δ(rha-tpi), Δ(gal-uraB), 808h          | D. Fraenkel (14)   |
| JC411     | F- leuB6, hisG1, argG6, metB1, lacY1, gal-6, xyl-7, met-2, malA1, rpsL104, tonA2, tss-1, supE44, X- | CGSC               |
| JB1104    | metB* cdh-4::Tn10 transductant of JC411, MW1104 as donor | This work          |
| JB1204    | metB* rha-4::Tn10 transductant of JC411, MW1204 as donor | This work          |
| RA2021    | pss-21                                   | C. R. H. Raetz (15) |
| RB421     | cdh-4::Tn10 pss-21 transductant of RA2021, MW1104 as donor | This work          |
| RB400     | pss* cdh-4::Tn10 transductant of RB421, MW1104 as donor | This work          |

*CGSC, E. coli Genetic Stock Center, Yale University, New Haven, CT.
stopped by acidic Bligh-Dyer extraction, and a portion of the upper phase was analyzed by PEI-cellulose thin layer chromatography as described previously (8). Two different solvent systems were utilized. Solvent A (0.55 M NaCl in 0.2% formic acid) separated nucleotides of different net charge (20), while solvent B (2 M LiCl, 2% boric acid (1:2, v/v)) resolved ribo- and deoxyribonucleotides (21). After chromatography, the positions of the radioactive products were determined by autoradiography. The spots were scraped from the plate and counted in 10 ml of Patterson and Green scintillation fluid (22). Cytidylyltransferase activity is defined as nanomoles of [cY-32P]CDP formed/min/mg of protein.

Isolation of a Tn10 Insertion in cdh—For mutagenesis of W3110 with Tn10, cells were grown in λ m broth (1% Tryptone, 0.25% NaCl, 0.2% maltose, and 0.01% yeast extract) to early stationary phase, harvested, and resuspended in 0.05 volume of fresh medium. After addition of ANK370 (221 cl871 Tn10 Oga2821) (N. Kleckner, Biological Laboratories, Harvard University, Cambridge, MA) at a multiplicity of infection of 0.2, the suspension was incubated at 37 °C for 45 min. Portions (1.5 x 10^9 cells/plate) were then spread on LB agar containing 15 μg/ml of tetracycline and 0.025 M sodium pyrophosphate. The plates were incubated at 42 °C, and approximately 8500 tet' colonies were obtained. To enrich for cdh::Tn10 mutants, the colonies were harvested from the plates, infected with Pluir, and the resulting lysate was used to transduce MA10000 to p/hA+tet'. Recombinants were selected at 37 °C on minimal A plates containing 15 μg/ml of tetracycline and 0.2% mannitol as the sole carbon source. CDP-diglyceride hydrolase activity was scored by colony autoradiography. Transductants were also scored on McConkey plates containing 1% rhamnose. A rha/Tn10 mutant was isolated and used as a cdh+ter' control in experiments studying the cdh-4::Tn10 strain. Genetic manipulations using Pluir were performed as described by Miller (16).

Miscellaneous—Mutagenesis with N-methyl-N'-nitro-N-nitrosoguanidine, preparation of Pluir lysates, genetic crosses, and transductions were carried out as described by Miller (16). Protein concentration was determined by the method of Lowry et al. (23) using bovine serum albumin as the standard. For autoradiography of 32P-containing compounds, Kodak XAR-5 film was used in conjunction with an intensifying screen (Dupont Lightning Plus). HFLC was performed with a Waters M6000 solvent delivery system, a model 720 system controller, a U6K universal liquid chromatograph injector, and a λmax 480 LC spectrophotometer.

RESULTS

Isolation of Mutants Defective in CDP-diglyceride Hydro-lase—Cells of strain RA5 (Table I) were treated with N-methyl-N'-nitro-N-nitrosoguanidine and plated on LB agar. After incubation at 30 °C for 24 h, the colonies were transferred to filter paper, lysed in situ, the assayed for CDP-diglyceride hydrolase as reported previously (9). In a screening of approximately 16,000 colonies, five putative mutants were identified. Cell-free extracts were prepared from these strains and assayed by quantitative methods. Three strains had reduced levels of CDP-diglyceride hydrolase (data not shown); the most defective mutant, RA5-2, retained approximately 15% of the wild type activity. The isolation of these mutants was reported in a previous communication (9).

Mapping of the CDP-diglyceride Hydrolase (cdh) Defect—Preliminary mapping of cdh-1 was conducted by conjugation of RA5-1 with various Hfr strains. These studies located cdh-1 between the points of origin of KL25 and HfrH (Fig. 1A) based on the following observations. (i) In a mating of HfrH with RA5-1, no cdh* streptomycin-resistant exconjugants were obtained when leu', trp', or his' was the selected marker. (ii) However, approximately 64% of the argH' streptomycin-resistant recombinants were cdh* in a cross of KL25 and RA6-1 (data not shown).

The position of cdh-1 was more precisely determined by Pluir mapping of the minute 85 to 95 interval. Linkage to argH and p/kA (Table II and Fig. 1B) placed cdh-1 near minute 88, and this conclusion was substantiated by a 3-factor cross. A Pluir lysate of Frag-1 (rha'' cdh'' metB') was used to transduce EJ482 (rha'' cdh'' metB') to methionine prototrophy. Of the 100 transductants scored for the unselected genes, none were simultaneously cdh-1 and rha'' (data not shown). Thus, cdh-1 lies between the rha and metB loci (Fig. 1B). Like cdh-1, cdh-2 and cdh-3 were co-transducible with metB (data not shown), suggesting that these three mutations were allelic. Finally, these data demonstrate that cdh is distinct from the other enzymes of CDP-diglyceride metabolism, since these genes map far away from minute 88 (Fig. 1).

Overproduction of CDP-diglyceride Hydrolase in a Hybrid Plasmid-bearing Strain—The mapping of cdh-1 facilitated the
identification of a ColEl hybrid plasmid carrying the cdh gene. Clones of rha and pfkA, two genes in close proximity to cdh, had been previously isolated from the Clarke and Carbon hybrid plasmid bank (14, 25). Since these plasmids contain as much as 0.5 min of chromosomal DNA (26), it was possible that cdh was also carried on one of these rha or pfkA clones. Because ColEl plasmids are retained in multiple copies per cell (26), the presence of cdh was expected to produce an elevation in enzyme activity. Cell-free extracts were prepared from a pfkA tpi clone (JA200/pLC16-4), a rha clone (JA200/pLC5-5), and a control (JA200/pLC44-14), and assayed for CDP-diglyceride hydrolase activity. As shown in Table III, pLC16-4 caused a 6-fold overproduction of the hydrolase, indicating that the cdh gene was present on this plasmid. In a complementation analysis, pLC16-4 corrected cdh-2 but not gplK (data not shown). Several conclusions can be drawn from these data. First, the overproduction of hydrolase activity by pLC16-4 suggests that cdh is the structural gene for the enzyme, although the possibility that it encodes a regulatory element cannot be excluded. Second, complementation of cdh-2 shows that this mutation is recessive to the wild type allele. Finally, cdh must be counterclockwise from gplK since the latter is not carried on pLC16-4.

Isolation of Deletion and Tn10 Insertion Mutants at cdh—The most defective strain isolated in our initial screening still possessed 15% of the activity present in wild type strains. We attempted to obtain mutants completely defective in the hydrolase in two ways: 1) by insertion of Tn10 within cdh and 2) by deletion of the cdh gene. 1) To accomplish the former, cells of W3110 were infected with λ::Tn10, and random transposition into the chromosome produced a collection of tet' mutants. To enrich for insertions near minute 88, a Pluir MA1000 element cannot be excluded. Second, complementation of cdh-2 shows that this mutation is recessive to the wild type allele. Phosphatidylserine Synthase—As discussed above, we obtained mutants defective in CDP-diglyceride hydrolase by three methods: chemical mutagenesis, transposon insertion, and deletion. In each case, approximately 15% of the wild type activity remained. This constant amount of residual activity suggested the existence of one or more additional enzyme(s) capable of cleaving CDP-diglyceride in vitro. Of particular relevance was the previous observation that phosphatidylserine synthase will catalyze CDP-diglyceride hydrolysis in the absence of serine (19). To assess the contribution of this enzyme to the residual activity, a cdh-4::Tn10 pss-21 double mutant was constructed. Assay of a crude extract prepared from this strain indicated that phosphatidylserine synthase accounted for about 80% of the activity in the cdh-4::Tn10 mutant (Table IV, experiment C).

The activity remaining in RB421 (cdh-4::Tn10 pss21), which is only 5% of the wild type value, may represent the minimal level of hydrolase able to support cell growth. Alternatively, the hydrolase may be nonessential, and nonspecific pyrophosphatases may account for the 5% residuum. Current mapping data support the latter. Since cdh is counterclockwise from tpi, the rha-tpi deletion should remove the entire cdh gene. Recently, Southern blot analysis has confirmed the absence of cdh DNA in ET2036. Thus, CDP-diglyceride hydrolase appears to be a nonessential enzyme in E. coli.

Characterization of CDP-diglyceride Hydrolase Mutants—To determine whether or not there was a phenotype associated with the cdh lesion, we examined the growth of hydrolase mutants under a variety of conditions. None of the cdh mutants were temperature- or osmotically sensitive. Furthermore, exposure to numerous detergents, colicins, and antibiotics failed to reveal any difference between cdh- and cdh+ strains.

Next, we analyzed the phospholipid composition of JB1105, JB1204, and JB1204/pLC16-4; these strains possess reduced, normal, and elevated levels of CDP-diglyceride hydrolase.

### Table III

Identification of a hybrid plasmid carrying cdh

Cultures were grown in LB broth containing colicin E1. Extracts were prepared and assayed for CDP-diglyceride hydrolase activity.

| Strain*  | Cloned gene(s)* | Hydrolase activity nmol/min/mg protein |
|----------|-----------------|---------------------------------------|
| JA200/pLC44-14 | uvrA dnaB lexA | 5.0                                   |
| JA200/pLC5-5 | rha             | 4.4                                   |
| JA200/pLC16-4 | tpi, pfkA       | 29.5                                  |

* The construction of these plasmids appears in Ref. 26.

* Taken from Table I in Ref. 25.

### Table IV

Comparison of the residual activity in various cdh mutants

| Experiment | Strain | Genotype | Hydrolase activity nmol/min/mg |
|------------|--------|----------|--------------------------------|
| A          | MW1104 | cdh-4::Tn10 | 0.83                           |
|            | MW1204 | rha::Tn10  | 5.33                           |
| B          | JP1010 | ∆rha-pfk   | 4.78                           |
|            | ET2036 | ∆rha-tpi   | 0.64                           |
| C          | RB460  | pss+ cdh::Tn10 | 0.71                         |
|            | RB421  | pss-21 cdh::Tn10 | 0.15                         |

* Taken from Table I in Ref. 25.

### Table V

Phosphatidylserine Synthase activity

| Strain | Genotype | Activity nmol/min/mg |
|--------|----------|---------------------|
| JA200/pLC5-5 | rha-tpi  | 2.1                 |

* Taken from Table I in Ref. 25.
respectively. After uniformly labeling cells with $^{32}$P, the phospholipids were extracted by the method of Bligh and Dyer (28) and separated by thin-layer chromatography on silicic acid. The results, presented in Table V, A, showed that neither the hydrolase mutant nor the overproducer had any alteration in the levels of the major phospholipids.

Since CDP-diglyceride is a substrate for the hydrolase in vitro, we looked for a perturbation in the cellular levels of this metabolite in hydrolase mutants and overproducers. To measure CDP- and dCDP-diglyceride pools, cells were grown in medium containing $^{32}$P, and then Bligh-Dyer extracted (28) under acidic conditions (see legend to Table VII). After the addition of [5-$^3$H]CDP-diglyceride as an internal standard, the chloroform-soluble material was incubated with purified phosphatidylserine synthase and serine which generated the reaction,

$$\text{CDP-diglyceride + dCDP-diglyceride + serine} \rightarrow \text{phosphatidylserine + CMP + dCMP}.$$  

The water-soluble reaction products were isolated and analyzed by high pressure liquid chromatography. A typical elution profile is depicted in Fig. 2, and the quantitation of the results is given in Table V, B. Comparison of cdh+ and cdh- strains revealed a 5-fold elevation in the level of CDP-diglyceride in the mutant, indicating that this compound is a physiologically relevant substrate for the hydrolase. Surprisingly, dCDP-diglyceride also accumulated, a finding which was not expected since earlier studies indicated that this liponucleotide was not utilized in vitro (6). Overproduction of the hydrolase had no effect on either ribo- or deoxy-CDP-diglyceride pools, suggesting that the excess enzyme is not active in vivo.

dCDP-diglyceride Is a Substrate for the Hydrolase—In order to explain the elevation of dCDP-diglyceride in vivo, we re-examined the substrate specificity of the hydrolase. Contrary to previous studies, we found that the partially purified hydrolase converted [a-$^3$P]CDP-diglyceride to a water-soluble radioactive product which was identified as dCMP by PEI-cellulose thin-layer chromatography (Fig. 3, lane 5). While this reaction was linear with both time and protein (data not shown), the rate was 5- to 6-fold lower than the hydrolysis of the ribo-substrate (Table VI, A). Identical results were obtained using crude cell extracts (data not shown).

Importantly, utilization of dCDP-diglyceride by the partially purified hydrolase showed three characteristics previously observed with the ribo-substrate. 1) Analogous to CDP-diglyceride hydrolysis, cleavage of dCDP-diglyceride was optimal at an acidic pH. As shown in Table VI, A, changing the pH from 7.5 to 6.0 produced a 5-fold stimulation in dCDP-diglyceride hydrolysis. Since CDP-diglyceride hydrolysis increased by only 70%, the discrepancy between ribo- and deoxy-activities was less than 2-fold at pH 6.0. 2) AMP, a potent inhibitor of CDP-diglyceride hydrolysis (6), also re-

### Table V

**Comparison of the phospholipid composition and the CDP-diglyceride levels in cdh mutants and overproducers**

| Strain          | PE% | PG% | CL% | PA% | Other% |
|-----------------|-----|-----|-----|-----|--------|
| JB1104 cdh-     | 67.1| 15.5| 4.6 | 9.15| 12.8   |
| JB1204 cdh+     | 67.4| 14.1| 4.8 | 6.14| 13.6   |
| JB1204/pLC16-4  | 64.1| 14.7| 3.7 | 0.18| 17.6   |

**A**

| Strain          | % of total CHCl_3-soluble $^{32}$P |
|-----------------|-----------------------------------|
| JB1104 cdh-     | 0.228                             |
| JB1204 cdh+     | 0.084                             |
| JB1204/pLC16-4  | 0.066                             |

**B**

| Strain          | % of total CHCl_3-soluble $^{32}$P |
|-----------------|-----------------------------------|
| JB1104 cdh-     | 0.228                             |
| JB1204 cdh+     | 0.084                             |
| JB1204/pLC16-4  | 0.066                             |

* Other represents the remainder of the lane, including the origin. The amount of radioactivity at the origin is variable and is frequently 5-10% of the total under these conditions.

Values are corrected for the recovery of [5-$^3$H]CDP-diglyceride, which was between 75-78% for the three determinations shown above. It was assumed that CDP- and dCDP-diglyceride were recovered with equal efficiency.  

![Fig. 2. Separation of liponucleotide-derived CMP and dCMP by HPLC.](image-url)
duced dCDP-diglyceride utilization (Table VI, B). 3) Both CDP- and dCDP-diglyceride functioned as nucleotidyl donors (discussed further below). As shown in Fig. 3, lane 6, the hydrolase catalyzed the formation of dCDP in a reaction dependent on inorganic phosphate. In addition, nonradioactive CDP-diglyceride inhibited the hydrolysis of [α-³²P] dCDP-diglyceride (Table VI, B), suggesting that a single enzyme was responsible for the cleavage of both liponucleotides. When the converse experiment was performed, the expected inhibition of [α-³²P] dCDP-diglyceride hydrolysis by dCDP-diglyceride was observed (Table VI, B). However, higher levels of dCDP-diglyceride were required, perhaps indicating a lower affinity of the enzyme for the deoxy-compound.

Further evidence supporting dCDP-diglyceride as a hydro-

![FIG. 3. Products generated by the hydrolase from ribo- and deoxy-α-[³²P] dCDP-diglyceride in the presence or absence of inorganic phosphate. Assays were performed in 100 mM Mes, pH 6.0, and 1.3% octyl-β-D-glucoside. The substrate was either α-[³²P] dCDP-diglyceride (lanes 1–3) or [α-³²P] dCDP-diglyceride (lanes 4–6) at a final concentration of 0.33 mM (1 × 10⁶ cpm/nmol). In lanes 3 and 6, 16.6 mM potassium phosphate was included as the cytidylyl acceptor. After addition of a buffer control (10 mM Mes, pH 6, lanes 1 and 4) or 98 pg of DEAE-purified hydrolase (lanes 2, 3, 5, and 6), the tubes were incubated for 30 min at 37°C. The water-soluble reaction products were isolated by extraction and analyzed by PEI-cellulose thin layer chromatography in solvent B. The autoradiogram of the plate is shown above. The arrows indicate the position of authentic chemical standards.

Table VI

| A. Substrate | pH | Hydrolase activity (nmol/min/mg protein) |
|--------------|----|-----------------------------------------|
| CDP-diglyceride | 7.5 | 800 |
|              | 6.0 | 1360 |
| dCDP-diglyceride | 7.5 | 145 |
|              | 6.0 | 889 |

| B. Substrate | Inhibitor | Hydrolase activity (nmol/min/mg protein) |
|--------------|-----------|-----------------------------------------|
| CDPDG*       | None      | 1680 |
|              | 0.33 mM dCDPDG | 1180 |
|              | 0.99 mM dCDPDG | 498 |
| dCDPDG None  | 911 |
|              | 0.11 mM CDPDG | 217 |
|              | 0.33 mM CDPDG | 31 |
|              | 0.33 mM AMP | 24 |

*CDPDG, CDP-diglyceride; dCDPDG, dCDP-diglyceride.

TABLE VII

| Strain         | Hydrolyase activity |
|----------------|---------------------|
|                | CDPDG*  | CDPDG |
| JB1104         | 0.082   | 0.053 |
| JB1204         | 7.36    | 4.38  |
| JB1104/pLC16-4 | 66.1    | 39.4  |

*CDPDG, CDP-diglyceride; dCDPDG, dCDP-diglyceride.

Hydrolase Mutants Are Defective in Cytidylylation—We recently reported that a partially purified preparation of CDP-diglyceride hydrolase catalyzed the formation of CDP from CDP-diglyceride and inorganic phosphate (8). Since homogeneous CDP-diglyceride hydrolase was not employed, it was possible that the cytidylyltransferase activity was due to a different enzyme. To demonstrate that both the hydrolase and the transferase activities are encoded by the cdh locus,
we assayed CDP synthesis in extracts of hydrolase mutants and clones. Analogous to CDP-diglyceride hydrolysis, the formation of CDP was absent in the mutant and elevated in the clone (Table VIII). Similar results were obtained when dCDP-diglyceride was used as the nucleotidyl donor (data not shown). Thus, the cdh gene product catalyzes the transfer of CMP or dCMP from the lipopolysaccharide to water or inorganic phosphate.

**DISCUSSION**

We have isolated E. coli mutants defective in CDP-diglyceride hydrolase. This mutation, designated cdh, maps at minute 88 and is carried on the ColE1 hybrid plasmid (26, 27), pLC16-4, as judged by the 5-fold overproduction of hydrolase activity. Using recombinant DNA techniques, further overproduction of the hydrolase should be possible, thereby simplifying its purification to homogeneity.

The CDP-diglyceride hydrolase from E. coli was originally reported to specifically hydrolyze ribolipid monomethylglucoside (6). Subsequently, Rittenhouse et al. discovered a similar pyrophosphatase in brain, except both CDP- and dCDP-diglyceride served as substrates (7). The present finding that dCDP-diglyceride is utilized (though less effectively) by the E. coli enzyme eliminates this discrepancy. In addition to the similarity in substrate specificity, both enzymes have an acidic pH optimum and both are inhibited by AMP- and ADP-diglyceride (6, 7). In view of these common characteristics, the possibility that the prokaryotic and eukaryotic hydrolases perform the same cellular function deserves consideration.

The revision of the substrate specificity has important implications for considerations of hydrolase function. The original notion that ribospecific hydrolysis controls the intracellular ratio of CDP- to dCDP-diglyceride (6) is eliminated by the present work. Currently, no E. coli enzyme has been identified which distinguishes between CDP- and dCDP-diglyceride, and the physiological significance of the two lipoid monomethylglucoside forms, if any, remains obscure.

Interestingly, extracts prepared from cdh- Tn10 pss-21 double mutants can catalyze CDP-diglyceride hydrolysis at a slow rate. One intriguing possibility is the existence of a second form of the hydrolase, analogous to the two E. coli isozymes of phosphofructokinase (29, 30). Further characterization of CDP-diglyceride hydrolysis in cdh+ pss- extracts may reveal yet another lipoid monomethylglucoside-specific hydrolase.

The recent discovery of CDP-diglyceride-dependent cytidylation (8) suggests a biosynthetic role for the hydrolase. Previous enzymological studies from our laboratory showed that the partially purified hydrolase catalyzes the transfer of CMP from CDP-diglyceride to phosphatase as well as to a variety of phosphomonooesters (8). In the present study, we have demonstrated that the hydrolase and cytidyltransferase activities reside in a single enzyme, the product of the cdh gene.

In some respects, the hydrolase is similar to the phospholipases of E. coli, particularly the detergent-resistant phospholipase A of the outer membrane. Both enzymes appear to be nonessential for cell survival (31). In addition, Nishijima et al. have obtained evidence suggesting that the phospholipase A is involved in the formation of acylyphosphatidylglycerol (32). Thus, it is possible that these apparently catalytic enzymes actually catalyze the synthesis of cell envelope components.

The accumulation of CDP- and dCDP-diglyceride in cdh mutants establishes that these two lipoid monomethylglucoses are physiological substrates for the hydrolase. The question of whether they are indeed cytidyl donors in vivo can only be resolved by the elucidation of the "true" CMP acceptor. Currently we are analyzing cdh- and cdh+ strains by radiochemical and chromatographic methods. The results of these studies should provide new insight into the nature of the physiological CMP acceptor.

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