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pH-Sensitive CDP-Diglyceride Synthetase Mutants of *Escherichia coli*: Phenotypic Suppression by Mutations at a Second Site

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In *Escherichia coli*, mutations which lower the level of CDP-diglyceride synthetase are designated *cds* and map at min 4. The *cds-8* mutation resulted in strikingly defective enzyme activity and also rendered cells pH sensitive for growth. Both the inhibition of growth and the massive accumulation of phosphatidic acid which occur in a *cds-8* mutant at pH 8 were suppressed by mutations at a second locus, designated *cdsS*, which mapped between *argG* and *gltB* near min 68. The *cdsS3* mutation by itself did not affect CDP-diglyceride synthetase activity in wild-type cells, but it caused a twofold stimulation of the residual activity present in strains harboring *cds-8*. Both the insensitivity to pH and the twofold stimulation of residual activity were lost by introduction of an F′ strain carrying $cdsS^+$ into a *recA1 cds-8 cdsS3* host. When a culture of a *cds-8* $cdsS^+$ strain was shifted to pH 8, the residual specific activity of synthetase dropped by 75% within 100 min. In a *cds-8 cdsS3* double mutant under the same conditions, the activity declined appreciably less, about to the level found in the *cds-8* $cdsS^+$ strain under permissive conditions (pH 6). Thus, it appears that mutations in the *cdsS* gene suppress the pH sensitivity of *cds* mutants by inhibiting the decay of residual CDP-diglyceride synthetase activity at the nonpermissive pH. The *cdsS* locus appears to be distinct from any known nonsense or missense suppressor.

Escherichia coli must possess about 100 structural genes for the biogenesis of membrane lipids and related envelope components (13). Despite much progress in the last 5 years (13), many of these genes remain uncharacterized, and very little is known about control mechanisms, either at the genetic or at the metabolic level.

Since many of the existing genetic lesions in the lipid system give rise to conditional lethalities (13), an analysis of second-site bypass mutants represents a powerful (and unexploited) approach to the elucidation gene interactions and regulatory mechanisms. The only studied example in the *Escherichia coli* phospholipid system is the phenotypic suppression of *plsB* lesions. The *plsB* mutation causes a 10-fold elevation in the K_m of glycerol-3-phosphate acyltransferase for its substrate glycerol-3-phosphate, and consequently $plsB^-$ strains are glycerol-3-phosphate auxotrophs (2, 3). In these mutants the altered acyltransferase is unable to use the endogenous glycerol-3-phosphate pool because the concentration is kept low by feedback regulation of the biosynthetic glycerol-3-phosphate dehydrogenase. This was shown by the isolation of second-site revertants of *plsB*, in which the glycerol-3-phosphate dehydrogenase (*gpsA*) had become feedback insensitive, resulting in a higher steady-state level of glycerol-3-phosphate (4, 6).

In the present work, we have isolated and characterized mutations which bypass the conditional lethality associated with defects in CDP-diglyceride synthetase. We previously showed that *E. coli* mutants defective in CDP-diglyceride synthetase (*cds*) are pH sensitive for growth (9). For instance, strains carrying the *cds-8* allele are unable to grow at or above pH 8 and accumulate large amounts of phosphatidic acid. In a stationary culture of a *cds-8* strain, the incidence of revertants able to grow at pH 8.5 is about 10^{-7}. Most of the faster growing of such revertant strains possess nearly normal levels of CDP-diglyceride synthetase activity. Others do not recover measurable activity, but nevertheless map at the *cds* locus and represent partial intragenic suppressors. In a small fraction of the revertants, however, the *cds-8* mutation is unchanged, and its continued presence can be demonstrated genetically. These strains carry extragenic suppressors of the pH sensitivity caused by *cds-8*. In the present work the charac-

TABLE 1. Strains of *E. coli* K-12

Strain	Relevant genotype	Source/reference
W3110	$F^- \lambda^-$ wild type	CGSC[a]
GN80	*thr-1 his-4 cds-8*	9
Hfr3000	Hfr *cds*$^+$	CGSC
SJ16	*panD2 zad-220*::Tn*10 metB1 gyrA216*	This work
Hfr3000-8	*cds-8* derivative of Hfr3000, GN80 donor	This work
PK3	Hfr *thr-1 leuB6 cds*$^+$	CGSC
PK3-8	*cds-8* derivative of PK3, GN80 donor	This work
KL16	Hfr *cds*$^+$	CGSC
KL16-8	*cds-8* derivative of KL16, GN80 donor	This work
KL16-99	Hfr *recA1 his*$^+$	CGSC
KLF2/JC1553	*leuB6 hisG1 argG6 metB1 rpsL104 recA1* F'102	CGSC
AB2497	*argE3 thr-1 leuB6 proA2 his-4 thyA12 rpsL31 cds*$^+$ *cdsS*$^+$ F^-	CGSC
AB2497A	*argE*$^+$ transductant of AB2497, W3110 donor	This work
AB2497-3	*cdsS3* derivative of AB2497A, GR8-3 donor	This work
GL99	*dapD4 cds*$^+$	9
GL85	*dapD*$^+$ *cds*$^+$ transductant of GL99, GN80 donor	This work
GL85-3	*cdsS3* derivative of GL85, GR8-3 donor	This work
GR8	*cds-8* derivative of AB2497, GN80 donor	This work
GR8-1	*cdsS1* derivative of GR8	This work
GR8-2	*cdsS2* derivative of GR8	This work
GR8-3	*cdsS3* derivative of GR8	This work
GR8-3A	*argE*$^+$ transductant of GR8-3, W3110 donor	This work
GR8A	*argE*$^+$ transductant of GR8, W3110 donor	This work
GR8-30	*cdsS3* derivative of GR8A, GR8-3 donor	This work
GR8AZ	*zgi-203*::Tn*10 argG6* transductant of GR8A, SK2262 donor	This work
GR8AZ-4	*his*$^+$ *recA1* recombinant of KL16-99 × GR8AZ	This work
GR8-388	*argG6 cdsS3 zgi-203*::Tn*10 his*$^+$ *recA1* construct of GR8A	This work
PC3	*leuB6 thyA47 dnaG3 rpsL153* F^-	CGSC
PC3-8	*leuB*$^+$ *cds-8* recombinant of Hfr3000-8 × PC3	This work
JC411	*leuB6 hisG1 argG6 metB1 rpsL104* F^-	CGSC
JC411-8	*leuB*$^+$ *cds-8* recombinant of Hfr3000-8 × JC411	This work
SK2262	*leuB6 hisG1 metB1 argG6 zgi-203*::Tn*10*	CGSC
PA340	*thr-1 leuB6 hisG1 argH1 gdh-1 gltB31*	CGSC
PA340AZ	*argH*$^+$ *argG6 zgi-203*::Tn*10* construct of PA340	This work

[a] CGSC, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

terization of such bypass revertants has led to the discovery of the *cdsS* locus, a novel suppressor which is far removed from the *cds* structural gene.

MATERIALS AND METHODS

Chemicals. Phosphatidic acid (derived from egg phosphatidylcholine) was purchased from Sigma Chemical Co., St. Louis, Mo. $^{32}P_i$ (carrier-free) was the product of New England Nuclear, Boston, Mass., and [α-^{32}P]dCTP was obtained from Amersham Corp., Arlington Heights, Ill. *N*-Decyl-*N*,*N*-dimethyl-3-ammoniopropane-1-sulfonic acid (DAPS) was purchased from calbiochem, La Jolla, Calif.

Bacterial strains and growth conditions. All bacterial strains used in this work are derivatives of *E. coli* K-12 (Table 1). Strains carrying *dapD4* were convenient recipients for all *cds* alleles by P1 *vir* transduction, since *dapD*$^+$ transductants can be selected on LB medium (12), which contains negligible levels of diaminopimelic acid. The availability of a Tn*10* insertion close to *dapD*, *zad-288*::Tn*10*, in turn facilitated the introduction of *dapD4* into any tetracycline-sensitive strain. Alternatively, *cds* alleles could be introduced by transduction into *pan*$^-$ strains, which could be constructed by transduction with *zad-220*::Tn*10* linked to *panD2*. This method was used to construct all Hfr strains carrying *cds-8* (Table 1). In a few cases, *cds-8* strains were obtained by conjugation with Hfr3000-8. Similarly, *cdsS* alleles are highly cotransducible with *argG*. The transposon insertion *zgi-203*::Tn*10* was closely linked to *argG6*, permitting the introduction of *cdsS* mutations in two steps.

Cultures were generally grown on LB medium (12) containing 10 g of NaCl, 10 g of Tryptone (Difco Laboratories, Detroit, Mich.), and 5 g of yeast extract (Difco) per liter. For routine growth of pH-sensitive *cds* mutants, LBM broth was used, which was prepared by buffering LB broth at pH 6 with 50 mM morpholinoethanesulfonic acid. To test for pH sensitivity, LB medium was titrated to pH 8.5 with 1 M NaOH. For radiochemical labeling experiments we used H56 salts (9) supplemented with 0.2% glucose, 0.4% vitamin-free Casamino Acids (Difco), and 5 μg of thiamine per ml.

Recombinants from genetic crosses and transductions were selected on plates containing 1.5% agar in minimal A salts (12), supplemented with 0.2% glucose and 5 μg of thiamine per ml. In crosses and transduc-

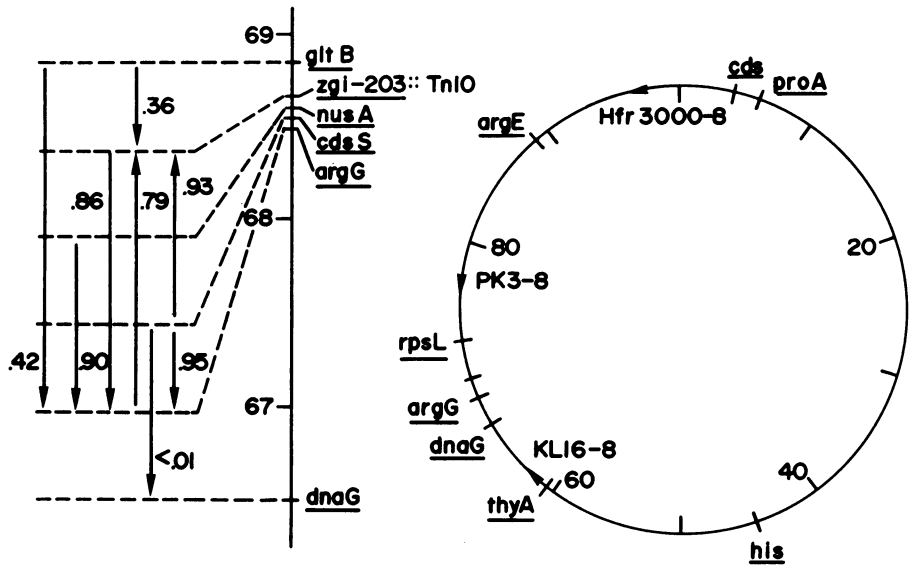

FIG. 1. Mapping of the *cdsS* locus. Cotransduction data are from this work and reference 7.

tions in which pH-sensitive *cds* alleles were involved, we used minimal A6.5 salts (final pH 6.5), which contain 9.1 g of KH_2PO_4, 4.6 g of K_2HPO_4, 1 g of $(NH_4)_2SO_4$, 0.5 g of sodium citrate, and 1 ml of 20% $MgSO_4$ per liter, supplemented with 0.2% glucose and 5 μg of thiamine per ml.

Required amino acids and thymine were added to minimal media at 50 μg/ml, pantothenic acid was supplied at 1 μg/ml, and tetracycline was added at 30 μg/ml. Bacterial cultures were incubated at 37°C.

CDP-diglyceride synthetase assays. Cell-free extracts were prepared by French pressure cell lysis at 18,000 lb/in^2, and CDP-diglyceride synthetase activity was determined at 37°C as described previously (9). Assays were carried out in triplicate. To measure residual activity in *cds-8* strains, 0.2% DAPS was used as detergent instead of Triton X-100, and assays were performed at pH 7 for 20 min. The assay of CDP-diglyceride synthetase by colony autoradiography was carried out as described in a previous report (8).

Miscellaneous procedures. Phospholipids were extracted by the method of Ames (1) and analyzed by thin-layer chromatography on Silica Gel 60 plates (250 μm; E. Merck AG, Darmstadt, West Germany) in the solvent system chloroform-pyridine-formic acid (50:30:7, vol/vol). CDP-diglyceride levels were measured as previously described (9). Protein in cell extracts was measured by the procedure of Lowry et al. (11), with bovine serum albumin as the standard. Mutagenesis with ethyl methane sulfonate, preparation of P1 *vir* lysates, genetic crosses, and transductions were performed as described by Miller (12).

RESULTS

Isolation of extragenic suppressors of *cds-8*. A culture of GR8 (*cds-8*) growing exponentially in minimal A6.5 medium was treated with ethyl methane sulfonate and divided into several independent cultures. These were grown with shaking to stationary phase, and a portion of each was plated on H56 (pH 8.5). After 24 h of growth at 37°C, two large colonies were chosen from each plate. A P1 *vir* lysate was grown on each and used to transduce GL99 to *dapD*$^+$, a locus which is situated within 0.5 min of *cds* (8). Transductants were scored for pH sensitivity on LB medium at pH 8.5. The pH sensitivity was recoverable from 3 of 46 independent pH-resistant clones. These three presumably carry suppressors of *cds-8*. One of them, GR8-3, which showed the strongest suppression as judged by growth on plates at pH 8.5, was selected for further study.

Conjugational mapping of the suppressor locus (*cdsS*). To detect the suppressor in recombinants, it was necessary to use Hfr donors carrying *cds-8*. The locations of the origins of transfer in these strains, as well as of other pertinent chromosomal markers, are shown in Fig. 1.

PK3-8 was allowed to mate with GR8-3 for 90 min, and prototrophic *rpsL31* recombinants were selected on A6.5 medium and scored for pH sensitivity (Table 2). Only in the case of selection for *thyA*$^+$ were pH-sensitive recombinants found, at a frequency of about 10%. When Hfr3000-8 was the donor, the same result was obtained: only *thyA*$^+$ recombinants recovered pH sensitivity. Finally, from a mating with KL16-8, *his*$^+$ recombinants were selected after 30 min, and all recombinants were pH resistant. These results indicate that *cdsS* is between *thyA* and *rpsL* and probably quite close to the latter.

Linkage of *cdsS* to markers near min 68. The linkage of *cdsS* to a number of markers between *thyA* and *rpsL* was tested by P1 *vir* transduction.

TABLE 2. Conjugation of Hfr strains with GR8-3[a]

Cross	Recombinants selected	Recombinants per ml	% pH sensitive (no./total tested)
PK3-8 × GR8-3	*argE*$^+$ *rpsL31*	3.8×10^6	0 (0/50)
	proA$^+$ *rpsL31*	1.6×10^6	0 (0/50)
	his$^+$ *rpsL31*	8.4×10^4	0 (0/50)
	thyA$^+$ *rpsL31*	2.0×10^4	10 (5/50)
Hfr3000-8 × GR8-3	*his*$^+$ *rpsL31*	8.0×10^4	0 (0/50)
	thyA$^+$ *rpsL31*	5.0×10^3	10 (2/20)
	argE$^+$ *rpsL31*	6.7×10^3	0 (0/50)
KL16-8 × GR8-3[b]	*his*$^+$ *rpsL31*	4.2×10^5	0 (0/50)

[a] Cells growing on LBM broth were allowed to mate for 90 min at 37°C. Recombinants selected on minimal A6.5 agar were repurified and scored for pH sensitivity on LB medium at pH 8.5. All Hfr strains are *argE*$^+$ *proA*$^+$ *his*$^+$ *thyA*$^+$ *cds-8 cdsS*$^+$ and streptomycin sensitive; GR8-3 carries *argE3 proA2 his-4 thyA12 cds-8 cdsS3 rpsL31*.

[b] Mating was for 30 min.

The suppressor was closely linked to *argG* and to *zgi-203*::Tn*10*, an unidentified Tn*10* insertion close to *argG* (Table 3). The suppressors in the two other bypass mutants, GR8-1 and GR8-2, were also found to be close to *zgi-203*::Tn*10*. Furthermore, from a separate collection of 11 independent spontaneous revertants of GR8 carrying second-site suppressors, two were linked to *argG* (not shown). These observations imply that *cdsS*, at min 68, is an important, but not the only, locus at which mutations can bypass the pH sensitivity of a *cds-8* strain.

To determine the location of *cdsS3* with respect to *argG*, *zgi-203*::Tn*10*, and *gltB*, two three-factor crosses were performed (Table 4). In the first cross, a P1 *vir* lysate on SK2262 (*argG6 cdsS*$^+$ *zgi-203*::Tn*10*) was used to transduce GR8-3A (*argG*$^+$ *cdsS3* Tet^s) to tetracycline resistance on LBM plates. Two hundred transductants were scored for pH sensitivity and requirement for arginine. None of the pH-resistant transductants was auxotrophic for arginine, indicating that *cdsS* is between *argG* and *zgi-203*::Tn*10*.

A second cross was carried out to determine on which side of *argG* the Tn*10* insertion lies. A P1 *vir* lysate prepared on W3110 (*argG*$^+$ Tet^s *gltB*$^+$) was used to transduce PA340AZ (*argG6 zgi-203*::Tn*10 gltB1*), and 100 *argG*$^+$ transductants were scored for tetracycline sensitivity and aspartate requirement. The data show that *zgi-203*::Tn*10* and, therefore, *cdsS* are clockwise from *argG*.

Suppression of other *cds* mutations. Five other *cds* alleles which confer pH sensitivity were introduced into AB2497A and AB2497-3, and *cds*$^-$ transductants, identified by colony autoradiography, were tested for pH sensitivity by scoring for growth on LB medium at pH 8.5. All five mutations were suppressed by *cdsS3* (data not shown).

Effects of *cdsS3* in a *cds*$^+$ background. The isogenic transductants GL85 (*cds*$^+$ *cdsS*$^+$) and GL85-3 (*cds*$^+$ *cdsS3*) were examined for antibiotic sensitivity with a number of drugs, at four different pH values between 6 and 8.5, by a disk clearing assay (14). No significant differences were found, in contrast to *cds-8*, which gives rise to low-level erythromycin resistance (9).

When AB2497A (*cds*$^+$ *cdsS*$^+$) and AB2497-3 (*cds*$^+$ *cdsS3*) were tested for pH sensitivity by determining their plating efficiency on minimal media from pH 8.5 to 10, both strains formed colonies equally well at pH 8.5 and 9, and neither grew above pH 9. The liponucleotide pools of these strains were also measured in G56 medium. In AB2497A, the CDP-diglyceride content was 20 pmol/mg of protein, and that of

TABLE 3. Linkage between *cdsS* mutations and markers near min 68

P1 donor	Recipient	Selected marker	Unselected marker	Cotransduction frequency	No. unselected/ no. selected
GR8-3 (*cdsS3*)	PC3-8 (*cdsS*$^+$)	*dnaG*$^+$	*cdsS3*	<0.1	0/100
SK2262 (*cdsS*$^+$)	GR8-3 (*cdsS3*)	*zgi-203*::Tn*10*	*cdsS*$^+$	0.93	93/100
GR8-3 (*cdsS3*)	JC411-8 (*cdsS*$^+$)	*argG*$^+$	*cdsS3*	0.96	45/47
SK2262 (*cdsS*$^+$)	GR8-1 (*cdsS1*)	*zgi-203*::Tn*10*	*cdsS*$^+$	0.90	45/50
SK2262 (*cdsS*$^+$)	GR8-2 (*cdsS2*)	*zgi-203*::Tn*10*	*cdsS*$^+$	0.88	44/50

TABLE 4. Three-factor analysis of *argG6 cdsS3* and *gltB31*

P1 donor × recipient	Genotype		Total no. of markers
	Selected marker	Unselected markers	
SK2262 (*zgi-203*::Tn*10 cdsS*$^+$ *argG6*) × GR8-3A (Tet^s *cdsS3 argG*$^+$)	*zgi-203*::Tn*10*	*cdsS*$^+$ *argG6*	181
	zgi-203::Tn*10*	*cdsS3 argG*$^+$	10
	zgi-203::Tn*10*	*cdsS*$^+$ *argG*$^+$	9
	zgi-203::Tn*10*	*cdsS3 argG6*	0
W3110 (*argG*$^+$ Tet^s *gltB*$^+$) × PA340AZ (*argG6 zgi-203*::Tn*10 gltB31*)	*argG*$^+$	Tet^s *gltB31*	44
	argG$^+$	Tet^s *gltB*$^+$	42
	argG$^+$	Tet^r *gltB31*	14
	argG$^+$	Tet^r *gltB*$^+$	0

dCDP-diglyceride was 6.6 pmol/mg of protein. In AB2497-3 the values were 18 and 8.6 pmol, respectively.

No differences between *cds*$^+$ strains carrying *cdsS*$^+$ and *cdsS3* have been observed, and the only way to demonstrate the presence of the latter is to show that when a P1 *vir* lysate grown on a putative *cdsS3* mutant is used to generate *argG*$^+$ transductants of GR8AZ (*cds-8 argG6 cdsS*$^+$), the majority become pH resistant.

Suppression of phosphatidic acid accumulation. To cultures of AB2497A (*cds*$^+$ *cdsS*$^+$), AB2497-3 (*cds*$^+$ *cdsS3*), GR8A (*cds-8 cdsS*$^+$), and GR8-30 (*cds-8 cdsS3*) growing exponentially in H56 (pH 6) containing 5 μCi of $^{32}P_i$ per ml, 5 mM KOH was added to shift the pH to 8.0 (Fig. 2). Samples of the cultures were removed at various times for analysis of phospholipids (Table 5).

In the *cds*$^+$ strains there was no effect of *cdsS3* upon either the growth rate or the lipid composition at the elevated pH. The effects of *cdsS3* in the *cds-8* strains were striking. The growth rate of GR8-30 (*cds-8 cdsS3*) after the pH shift was almost the same as that of AB2497A (*cds*$^+$ *cdsS*$^+$), whereas the turbidity of the GR8A (*cds-8 cdsS*$^+$) culture increased about threefold and stopped. The phosphatidic acid content of GR8A increased from the steady-state level of about 4% to over 30%, whereas in GR8-30 the phosphatidic acid pool never rose above 5% of the lipid phosphorus (Table 5). Thus, *cdsS3* suppresses both the growth inhibition and the phosphatidic acid accumulation caused by a pH upshift.

Effect of *cdsS3* on CDP-diglyceride synthetase activity in vitro. CDP-diglyceride synthetase ac-

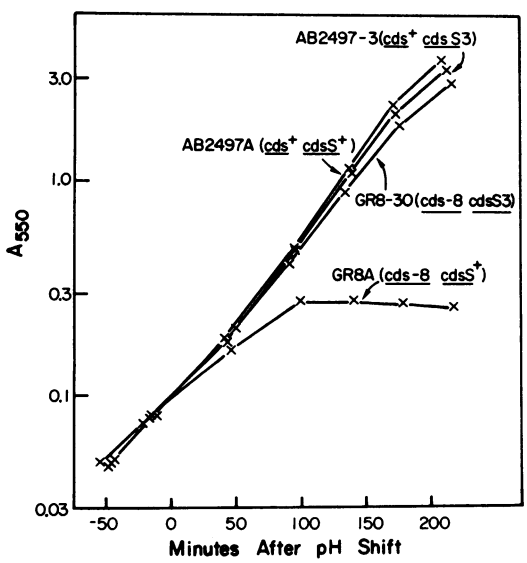

FIG. 2. Suppression of the pH sensitivity of growth in a *cds-8* mutant by *cdsS3*. Growth conditions are described in the text. A_{550}, Absorbance at 550 nm.

TABLE 5. Suppression of phosphatidic acid accumulation in a *cds-8* mutant at pH 8 by *cdsS3*[a]

Strain	pH	% of total phospholipid			
		PE	PG + CL	PA	Others
AB2497A (*cds*$^+$ *cdsS*$^+$)	6	75.1	22.3	0.3	2.3
	8	73.4	25.2	0.3	1.1
AB2497-3 (*cds*$^+$ *cdsS-3*)	6	74.2	22.7	0.3	2.8
	8	73.4	25.4	0.2	1.0
GR8A (*cds-8 cdsS*$^+$)	6	74.6	19.6	2.9	2.9
	8	50.1	12.1	35.4	2.5
GR8-30 (*cds-8 cdsS3*)	6	74.7	21.3	1.5	2.5
	8	77.2	18.0	3.3	1.5

[a] Growth conditions are described in the text. Phospholipid compositions were determined from samples taken immediately before and about 3 h after the pH shift. PE, Phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; PA, phosphatidic acid. Other compounds include monoacyl phospholipids and phospholipid precursors. The results shown are from a single representative experiment.

TABLE 6. Residual CDP-diglyceride synthetase activity in a *cds-8* mutant: stimulation by *cdsS3* and a zwitterionic detergent[a]

Strain	Sp act (nmol/min per mg)	
	Triton X-100	DAPS
AB2497A (*cds*$^+$ *cdsS*$^+$)	10.9	4.4
AB2497-3 (*cds*$^+$ *cdsS3*)	11.7	4.5
GR8A (*cds-8 cdsS*$^+$)	0.029	0.96
GR8-30 (*cds-8 cdsS3*)	0.041	1.53

[a] Cell-free extracts were prepared from cultures growing exponentially in LB medium. CDP-diglyceride synthetase activity was determined at 37°C and pH 7.5 (AB2497A and AB2497-3), or pH 7 (GR8A and GR8-30), in the presence of either 0.2% Triton X-100 or 0.2% DAPS. The results shown are from a single representative experiment.

tivity was assayed in strains carrying *cds*$^+$, or *cds-8*, and the effect of the *cdsS3* allele was examined (Table 6). Activity of the enzyme from the wild-type (*cds*$^+$) strains was not significantly different when the *cdsS3* allele was present. However, marked stimulation was observed in a *cds-8* background. Residual CDP-diglyceride synthetase activity was reproducibly stimulated from 1.6- to 2.2-fold by *cdsS3* (cf. Tables 7 and 8).

Enzyme activity was also determined in these strains when DAPS was used in place of Triton X-100 as detergent (Table 6). DAPS inhibits wild-type activity by 60%, but stimulates *cds-8* activity 10- to 30-fold. Neither *cdsS3* nor DAPS alters the characteristic pH profile of CDP-diglyceride synthetase activity in *cds-8* strains (data not shown; see reference 9). In the presence of DAPS, the same stimulations of residual activity by *cdsS3* are observed, but the residual activity is much easier to detect.

When cell-free extracts of GR8A (*cds-8 cdsS*$^+$) and GR8-30 (*cds-8 cdsS3*) are mixed, resultant activity is additive (Table 7). Thus, the *cdsS3* mutation does not seem to affect a soluble activator or inhibitor of the mutant enzyme.

Dominance of *cdsS*$^+$. F'102, which carries the *E. coli* chromosomal region from min 68 to 70, was introduced into GR8-388 (*cds-8 cdsS3 argG6 recA1*) by conjugation with KLF2/JC1553 and selection of *argG*$^+$ *metB*$^+$ exconjugants. The resulting *cdsS*$^+$/*cdsS3* merodiploid was tested for growth on LB medium at pH 8.5 and was found to be pH sensitive.

Residual CDP-diglyceride synthetase activity was measured in GR8-388, GR8-388/F'102, and GR8AZ-4, which is isogenic with GR8-388 except *cdsS*$^+$. Specific activities were 1.10, 0.42, and 0.50 nmol/min per mg, respectively. GR8-388 has twice the activity of GR8AZ-4, but this twofold stimulation is lost in the merodiploid. As determined both in vivo and in vitro, *cdsS*$^+$ is dominant to *cdsS3*.

Loss of residual activity due to a pH shift. Two cultures each of AB2497A, AB2497-3, GR8A, and GR8-30 were prepared in 100 ml of H56 (pH 6). When the absorbance at 550 nm reached 0.4, one culture of each strain was shifted to pH 8 by adding 5 M KOH, and incubation was continued for 100 min before harvesting. The remaining cultures (pH 6) were harvested at an absorbance at 550 nm of 0.8. Wild-type and residual activities in cell-free extracts were determined under appropriate conditions (Table 8). In the wild type (*cds*$^+$), specific activities increased somewhat as a result of a pH shift. The significance of this is not clear, but *cdsS3* had no effect.

The residual activity in GR8A (*cds-8 cdsS*$^+$) decreased by 75% as a result of the pH shift, whereas GR8-30 (*cds-8 cdsS3*) retained over 50% of its CDP-diglyceride synthetase activity. After 100 min at pH 8, GR8-30 possessed the same level of activity as GR8A had at pH 6.

DISCUSSION

Strains of *E. coli* which carry the *cds-8* allele are deficient in CDP-diglyceride synthetase activity and are unable to grow above pH 8. When a culture of such a strain growing exponentially at pH 6 is shifted to pH 8.5, growth ceases within one generation and CDP-diglyceride synthesis is markedly inhibited, as indicated by a sudden rise in the phosphatidic acid level and a decrease in the incorporation of $^{32}P_i$ into phosphatidylethanolamine and phosphatidylglycerol (9). All of the phenotypes associated with this mutation revert simultaneously in the majority of pH-resistant revertants.

However, in a small fraction of revertants the *cds-8* allele is not changed, and the mutation conferring pH resistance is located at a second site on the chromosome. Three strong suppressors of pH sensitivity have been isolated by chemical mutagenesis of strain GR8 (*cds-8*) fol-

TABLE 7. Residual CDP-diglyceride synthetase activity in a *cds-8* mutant: effect of *cdsS3* on activity in mixed extracts[a]

Sample	nmol of dCDP-diglyceride formed[b]
GR8A (*cds-8 cdsS*$^+$)	0.304 ± 0.001
GR8-30 (*cds-8 cdsS3*)	0.538 ± 0.018
GR8A + GR8-30	0.809 ± 0.007

[a] Assays were carried out with 10 μl of each crude extract, containing about 40 μg of protein, at pH 7 in the presence of 0.2% DAPS. Incubation was at 37°C for 20 min.

[b] Average of triplicate determinations ± standard deviation.

TABLE 8. Residual CDP-diglyceride synthetase activity in *cds-8* mutants: loss of activity after a pH shift[a]

Strain	Sp act (nmol/min per mg) at pH of growth medium:	
	6	8
AB2497A (*cds*$^+$ *cdsS*$^+$)	9.6	10.8
AB2497-3 (*cds*$^+$ *cdsS3*)	9.7	10.3
GR8A (*cds-8 cdsS*$^+$)	0.74	0.18
GR8-30 (*cds-8 cdsS3*)	1.29	0.69

[a] Cultures in H56 medium were harvested during exponential growth at pH 6, or 100 min after a shift to pH 8, and CDP-diglyceride synthetase activity was determined in cell-free extracts. Assays were carried out at pH 7.5 for 10 min with 0.2% Triton X-100 (AB2497-3) or at pH 7 for 20 min with 0.2% DAPS (GR8A, GR8-3). The results shown are from a single representative experiment.

lowed by screening for the presence of an unaltered *cds-8* allele. These three suppressors were found to map at a single site between *argG* and *gltB* near min 68.

Weak suppressors may be isolated spontaneously with 10 to 20 times the frequency of true revertants. From a collection of 11 independent spontaneous second-site revertants, 2 were found also to be linked to *argG*. Thus, although more than one locus may be involved in suppression of the *cds-8* lesion, the strongest suppression is exerted by mutations at a site, designated *cdsS*, near min 68. The suppression of other pH-sensitive *cds* alleles by a *cdsS* mutation indicates that the phenotypes associated with these other alleles probably have a common cause.

The mechanism of suppression by second-site revertants depends upon the molecular basis of pH sensitivity, which is not entirely clear. The data depicted in Fig. 2 and Table 5 show that the *cdsS3* mutation in strain GR8-30 suppresses both the inhibition of growth and the accumulation of phosphatidic acid which occur at pH 8 in the isogenic *cdsS*$^+$ strain GR8A. Since phosphatidic acid does not accumulate and net phosphatidylethanolamine and phosphatidylglycerol increase normally in GR8-30 (not shown), *cdsS3* must either stabilize or stimulate the CDP-diglyceride synthetase-catalyzed reaction in vivo under otherwise nonpermissive conditions.

The effect of *cdsS3* on residual CDP-diglyceride synthetase activity supports this conclusion (Table 6). Residual activity in cell-free extracts is consistently elevated from 1.6- to 2.2-fold in strains carrying *cdsS3* (Tables 6 to 8). The absence of any obvious effect of *cdsS3* in the wild type (*cds*$^+$) indicates a specific interaction between the *cdsS* gene product and the mutant *cds-8* enzyme. The striking stimulation of residual activity in GR8A and GR8-30 by the zwitterionic detergent DAPS permits a more reliable analysis of effects of *cdsS3* on *cds-8* activity in vitro.

The mixing experiment of Table 7 argues against the elevation of residual activity in a *cds-8 cdsS3* double mutant by loss of a soluble inhibitor or by a noncovalent protein-protein interaction. Rather, the results suggest that this elevation is due to a change in the structure or amount of the mutant enzyme itself.

The *cdsS*$^+$ allele is dominant to *cdsS3*. A *cdsS*$^+$/*cdsS3* diploid is pH sensitive and does not exhibit the twofold elevation of residual activity that is observed in a *cds-8 cdsS3* double mutant. The dominance of *cdsS*$^+$ implies that the function of the *cdsS*$^+$ gene product is at least partly responsible for the loss of activity of the mutant CDP-diglyceride synthetase polypeptide at pH 8. Suppression of *cdsS3* presumably results from the mutational removal of this function.

Residual CDP-diglyceride synthetase activity in GR8A is unstable at elevated pH. When a culture of GR8A is shifted from pH 6 to 8 and cell-free extracts are made after 100 min, only 25% of the activity present at pH 6 remains (Table 8). The mutant polypeptide appears to be irreversibly inactivated or degraded by incubation in the more alkaline medium. In contrast, GR8-30 loses less than half of its activity under the same conditions and at pH 8 possesses the same level of activity as GR8A has at pH 6. This decrease in activity as measured in vitro correlates well with the in vivo results (Table 5). During growth at pH 8, the phosphatidic acid content of GR8-30 rises to about the same level seen in GR8A at pH 6.

Suppression by *cdsS3* appears to result from the stabilization of the mutant CDP-diglyceride synthetase polypeptide in vivo when a *cds-8* mutant is shifted to the nonpermissive pH. *cdsS3* probably does not cause an increase in the amount of mutant enzyme by affecting expression of *cds*, since it has no effect on activity in the wild-type strain. The same argument holds for models of suppression involving elevation of the liponucleotide pool or a more nonspecific protection from elevated pH.

The most straightforward interpretation of the above results is that some nonessential function of the *cdsS* gene product causes the irreversible loss of activity of the mutant enzyme at elevated culture pH by modification of the defective polypeptide itself. In *cdsS*$^-$ mutants this loss of activity does not occur or occurs slowly enough to permit cell growth.

One possibility that is consistent with these data is that in *cdsS*$^+$ strains there is a nonessential post-translational modification of CDP-di-

glyceride synthetase which renders the mutant enzyme unstable, and in *cdsS*$^-$ mutants this modification does not take place. Another possible model is that the mutant enzyme coded for by *cds-8* is still sufficiently active to permit growth at the nonpermissive pH, but a minor conformation change makes it a substrate for a protease which is the product of the *cdsS* gene. This model is appealing because there is precedent for phenotypic suppression by protease mutants. Mutations in the *lon* locus are defective in degradation of proteins and lack one of the ATP-dependent proteases (5), and several temperature-sensitive mutants of bacteriophages T5 and λ are suppressed by *lon* mutations (10).

Because the CDP-diglyceride synthetase has not been purified to homogeneity, or even identified on a two-dimensional gel, it would be difficult to distinguish among these or other plausible models of suppression by *cdsS3*. Nevertheless, the present work demonstrates that suppression of conditionally lethal *cds* lesions can result from mutations at more than one (perhaps several) other locus. Additional study of other suppressors of *cds-8*, as well as suppressors of other conditionally lethal phospholipid mutants, should contribute significantly to our knowledge about the interactions of phospholipid enzymes with other cellular components.

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