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Correlation of 3,4-Dihydroxybutyl 1-Phosphonate Resistance with a Defect in Cardiolipin Synthesis in *Escherichia coli*

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Escherichia coli treated for 1 h with 100 μM *rac*-3,4-dihydroxybutyl 1-phosphonate (DBP), a glycerol-3-phosphate analog, die when stored at 5°C, whereas the viability of untreated cells is relatively unaffected. This observation formed the basis of a selection procedure that was used to isolate mutants that are partially resistant to DBP. One such mutant, strain 6204, is constitutive for DBP transport, exhibits a particularly high degree of cold resistance, has the same doubling time as the parent, and is similar to the parent strain in terms of incorporation of DBP into the lipid fraction. Glycerol-3-phosphate and phosphatidylglycerol phosphate synthetases obtained from strain 6204 and its parent were identical in terms of DBP recognition. The parent strain is killed when incubated in the presence of a combination of 70 μM *rac*-DBP and 0.25% deoxycholate, whereas strain 6204 continues to grow, albeit more slowly, in the presence of this combination. Strain 6204 can be distinguished from the parent strain on agar plates (low phosphate minimal medium with glucuronate as the sole carbon source) containing 15 μM *rac*-DBP. The insertion of Tn*10* near the 6204 mutation has facilitated genetic manipulations. All phenotypic effects attributed to strain 6204 appear to be due to a single mutation. Genetic analysis indicates that Tn*10*, inserted near the gene responsible for DBP resistance, maps in the vicinity of 27 min. Three-factor crosses reveal a gene order of *hemA*-Dbp^r-Tn*10*(*zch*)-*trp*. The only gene for phosphoglyceride metabolism known to map in this region is the gene associated with cardiolipin synthetase, *cls*. Genetic results suggest that the mutation responsible for DBP resistance maps in or very near *cls*. Analysis of the lipids isolated from untreated strain 6204 (and from each of the transductants prepared by P1 *vir*-mediated transfer of DBP resistance of wild-type strains) reveals that cardiolipin synthesis is defective. These results strongly suggest that the mutation responsible for DBP resistance has its primary effect on cardiolipin synthesis. To further test this hypothesis, strains with an authentic *cls* mutation were constructed and examined for resistance to DBP. These strains had growth properties that were identical with those of strain 6204. Wild-type strains and mutants defective in cardiolipin synthesis were treated with DBP and 20 mM magnesium or calcium chloride. Simultaneous treatment of either cell type with DBP and divalent cation not only failed to stimulate growth but, quite the contrary, had a marked synergistic growth inhibitory effect.

The fluid mosaic model proposed by Singer and Nicolson pictures a biological membrane as a fluid lipid bilayer containing integral proteins embedded in an oriented fashion, as well as more loosely associated peripheral proteins (33). The phosphoglycerides are major components of the lipid bilayer of biological membranes, and in some cases they are the sole structural lipids. The phosphoglyceride composition of membranes isolated from different biological sources is complex and diverse. Although the physicochemical properties of the bulk lipid phase of membranes are known to reflect the lipid composition (19) and certain membrane proteins appear to require specific lipids to function (18), it is not clear why such a complex and diverse class of phosphoglycerides has evolved. The membranes of *Escherichia coli* contain only three structural phosphoglycerides: phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL). Although each of these phosphoglycerides is actually a molecular mixture containing different fatty acids, *E. coli* provides a relatively simple system for evaluating the regulation of phosphoglyceride metabolism and for examining phosphoglyceride function. Much of our current knowledge in this field has come from genetic and biochemical studies (5, 24). Despite their proven value in other biochemical areas, drugs have played a relatively minor role in the examination of phosphoglyceride metabolism and function.

Several years ago, we initiated a research program to determine whether 3,4-dihydroxybutyl 1-phosphonate ($CH_2OHCHOHCH_2CH_2PO_3H_2$; DBP), a structural analog of glycerol-3-phosphate (G3P), could be used to study phosphoglyceride metabolism and function. DBP is taken up by both the G3P and hexose phosphate transport systems of *E. coli* (8, 15). In vitro examination of enzymes obtained from this microorganism revealed that DBP is an inhibitor of G3P synthetase and is a competitive substrate of PG phosphate (PGP) synthetase but is not recognized by either G3P dehydrogenase or G3P acyltransferase (3). At concentrations greater than 0.3 mM, *rac*-DBP completely blocks the growth of *E. coli* 8, a strain that is constitutive for the *glp* regulon but defective in the catabolic G3P dehydrogenase (17, 30). At 30 μM, *rac*-DBP has only a slight growth inhibitory effect on strain 8 but causes an immediate 50% decrease in the rate of PG synthesis and a delayed but almost equally pronounced inhibition in the rate of PE synthesis (31). Analyses of the total PG, PE, and CL content of cells treated with 30 μM *rac*-DBP for 1 h revealed normal amounts of CL and slightly below normal amounts of PE (31). However, PG content is drastically reduced due to the continued turnover of preformed PG and to the inhibition of new synthesis (31). A new lipid, the phosphonic acid analog of PGP, is formed in cells treated with DBP (35). Neither the

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intracellular nucleoside triphosphate pool size nor the rate of macromolecular synthesis is markedly affected by the addition of 30 μM *rac*-DBP to a culture of strain 8 (31, 32, 34). In view of the marked change in phosphoglyceride composition of *E. coli* treated with 30 μM *rac*-DBP, the nearly normal doubling time is very puzzling. A priori, one might have expected such a profound change in the phosphoglyceride composition to be accompanied by serious growth problems. The present study demonstrates that DBP-treated cells do have difficulty surviving under certain growth conditions and describes the isolation of mutants that are no longer sensitive to DBP under these growth conditions. Furthermore, genetic and biochemical evidence is presented which suggests that DBP resistance is due to a single mutation in the *cls* gene.

MATERIALS AND METHODS

Chemicals. *rac*-3,4-Dihydroxy[3-^{3}H]butyl 1-phosphonate and the unlabeled material were synthesized as previously described (7). Ethyl methanesulfonate, *rac*-G3P, adenine, L-arginine, L-histidine, L-isoleucine, L-methionine, L-threonine, L-tryptophan, casein hydrolysate, sodium deoxycholate, sodium glucuronate, δ-aminolevulinic acid, lactose, tetracycline, fosfomycin (disodium salt), and streptomycin were purchased from Sigma Chemical Co., St. Louis, Mo. Tryptone, yeast extract, and agar were products of Difco Laboratories, Detroit, Mich. Sodium [1-^{14}C]acetate was purchased from New England Nuclear Corp., Boston, Mass. Membrane filters (type HAWP; pore size, 0.45 μm) were obtained from Millipore Corp., Bedford, Mass. All other chemicals were reagent grade.

Bacterial strains and culture conditions. The minimal medium (GL medium) used was that of Garen and Levinthal (6), and carbon sources were present at 0.4%. CH medium contained 1% casein hydrolysate (pH 7.4) and 0.5% sodium chloride. L medium contained 1% tryptone, 0.5% yeast extract, and 0.5% NaCl. Media in agar plates were solidified with 1.5% agar. All required amino acids were supplied at a concentration of 0.5 mM. When required, adenine and δ-aminolevulinic acid were added to a final concentration of 0.5 mM and 40 μg/ml, respectively. Unless otherwise stated, bacteria were cultured at 37°C, and cell growth and viability were monitored as previously described (30, 34). The bacterial and viral strains used are described in Table 1. P1 *vir* transduction was performed by the standard procedure of

TABLE 1. Bacterial and viral strains

Strain	Relevant properties	Source or reference
Bacterial		
7	HfrC *glpR*	E. C. C. Lin (17)
7-T	strain 7 *glpT*	This study
7-1	strain 7 *uhpR*	This laboratory (8)
8	strain 7 *glpD*	E. C. C. Lin (17)
6204	strain 7-1 Dbp^r	This study
CSH57	F$^-$ *leu purE lacY trp his argG ilv metA rpsL*	(20)
CSH62	HfrH	(20)
KL226	HfrC	CGSC[a]
KL208	HfrB7	KL226
R477	F$^-$ *thr1 leu6 his4 lac rpsL136*	C. R. H. Raetz (26)
S370	F$^-$ *purB51 hemA30 trp45 his68 tyrA2 pyr65 lacY1 rpsL125*	CGSC[a]
T1GP	F$^-$ *ilv met lacI cls*	P. Overath (22)
Hfr3000	HfrH λ$^-$	CGSC
HW10	HfrC *zhe*::Tn*10 glpR*	This study
HW11	HfrC *zhe*::Tn*10 glpR glpD*	This study
HW21	HfrH *zhe*::Tn*10 glpR glpD* λ$^-$	This study
HW22	HfrH *glpR* λ$^-$	This study
HW30	HfrC *zch*::Tn*10 glpR* Dbp^r	This study
HW31	HfrC *zch*::Tn*10 glpR* Dbp^r	This study
HW35	HfrC *zch*::Tn*10 glpR glpD*	This study
HW36	HfrC *zch*::Tn*10 glpR glpD* Dbp^r	This study
HW41	CSH62 *zch*::Tn*10*	This study
HW42	KL226 *zch*::Tn*10*	This study
HW43	KL208 *zch*::Tn*10*	This study
HW50	HfrC *glpR glpD trp45 zch*::Tn*10*	This study
HW51	HfrC *glpR glpD* Dbp^r	This study
HW53	HfrC *glpR* Dbp^r	This study
HW55	HfrC *glpR cls*	This study
HW56	HfrC *glpR*	This study
HW60	HfrC *glpR glpD hemA30 zch*::Tn*10*	This study
QC104	HfrC *glpR glpD*	This study
QC120	HfrC *glpR glpD cls*	This study
Viral		
P1 *vir*		Obtained from W. Maas, New York University Medical School, New York
Lambda NK370	*b221 c*I857 *c*I171::Tn*10 o*uga261	Botstein strain (13) obtained through W. D. Nunn, University of Calif., Irvine

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

Miller (20) except that the transduced cells were plated directly without using top agar. Conjugation mapping by gradient transmission was performed by the procedure of Miller (20), and counterselection against male strains was achieved by using plates containing 100 μg of streptomycin per ml.

DBP transport and enzyme assays. The DBP transport assay was a modification of that previously described (15). Cells cultured in CH medium were collected by centrifugation, washed, and suspended at a density of 100 Klett units in GL medium lacking a carbon source. Uptake was initiated by adding 50 μl of [^{3}H]DBP (specific activity, 30 μCi/μmol) to 1 ml of cell suspension at room temperature. Samples of 100 μl each were collected on membrane filters (pore diameter, 0.45 μm) after 1- and 5-min incubations. The filters were washed once with 1 ml of 0.85% sodium chloride and dried under a heating lamp. The radioactivity retained on the filter was measured in an Isocap 300 scintillation counter. Assays on G3P and PGP synthetases were performed as previously described (3).

Lipid analysis. Cultures (5 ml) in the late-log phase were incubated with 5 μCi of [1-^{14}C]acetate (500 μCi/μmol) for 1 h. Cells were collected by centrifugation and resuspended in 1 ml of distilled water, and the phosphoglycerides were then extracted as previously described (31). Cell debris was removed by passing the extract through glass wool. The lipids were dried under a stream of nitrogen and redissolved in a small volume of chloroform-methanol (1:1). Before spotting, the silica gel-impregnated paper (Whatman SG81) was heated for 40 min at 110°C. A chloroform-methanol-acetic acid (65:25:8) solvent system was used to develop the chromatogram. The radioactive lipids were detected with a Packard Chromatogram Scanner (model 7220/21). The order of lipid migration was the same as that obtained on silica gel thin-layer plates. The methods used to prepare and chromatograph the labeled lipids in experiments designed to evaluate the effect of DBP on phosphoglyceride metabolism were the same as those described above except that the cultures were in the early rather than late-log phase. The incorporation of DBP into the lipid fraction was followed as previously described (31).

Mutant isolation. *E. coli* 7-1 (*glpR uhpR*) was mutagenized with ethyl methanesulfonate by the procedure of Miller (20). After phenotypic segregation, the cells were diluted into fresh CH medium. When the turbidity reached 15 Klett units, *rac*-DBP was added to a final concentration of 100 μM, and the culture was incubated for an additional hour. The DBP-treated cells were stored at 5°C for ca. 6 weeks during which time they lost viability, whereas untreated cells did not. The surviving cells were cultured in fresh CH medium, and the DBP–cold-storage cycle was repeated. The surviving cells of the second-cycle selection were plated on CH medium. Colonies were tested for the ability to use G3P as the sole carbon source and for sensitivity to 15 μg of fosfomycin per ml. Cells that appeared to have retained the G3P transport system were assayed for the uptake of DBP. Individual survivors were also evaluated for cold sensitivity after incubation with 100 μM *rac*-DBP for 1 h at 37°C. As expected, survival rates for most were considerably higher than those for the parent strain. Strain 6204 was selected for further study.

Strain construction. To facilitate genetic manipulations, it was necessary to insert the Tn*10* transposon into a position near the mutation that enabled strain 6204 to survive cold storage. This was achieved by a modification of the procedure of Kleckner et al. (13). Before this could be accomplished it was necessary to construct a strain that was constitutive for the *glp* regulon and that was not lysogenic for lambda phage. Accordingly, strain HW22 which meets these requirements was constructed from strain Hfr3000 by taking advantage of the fact that *glpR* and *glpD* are adjacent (4). A Tn*10* random insertion pool was generated by infecting strain Hfr3000 with lambda NK370 (13). A P1 lysate of the pooled tetracycline-resistant cells was used to transduce strain 8. Cells were selected that were resistant to tetracycline and able to use G3P as the sole carbon source. Strain HW10, which meets these requirements and retains constitutivity for G3P transport, was infected with P1 *vir*, and the lysate was used to transduce strain 8. Strain HW11 was isolated as a tetracycline-resistant transductant that is unable to use G3P as the sole carbon source. A P1 *vir* lysate of HW11 was used to transduce strain Hfr3000 to obtain strain HW21, a tetracycline-resistant *glpD glpR* transductant. A P1 *vir* lysate of strain 7 was used to transduce strain HW21, and cells were selected for the ability to use G3P as the sole carbon source. Strain HW22 lost its tetracycline resistance in concomitance with gaining the ability to use G3P as the sole carbon source.

Strain HW22 was infected with phage lambda NK370 to generate a random Tn*10* insertion pool. Approximately 20,000 tetracycline-resistant cells were pooled (HW22 Tet^r pool). A P1 *vir* lysate, obtained from the HW22 Tet^r pool, was used to transduce strain 6204 to tetracycline resistance. Approximately 20,000 tetracycline-resistant colonies were pooled (6204 Tet^r pool). A P1 *vir* lysate obtained from the 6204 Tet^r pool was used to transduce strain 7-1. Again, 20,000 tetracycline-resistant colonies were pooled (strain 7-1 Tet^r pool). The strain 7-1 Tet^r pool was cultured in CH medium and treated with a combination of 70 μM *rac*-DBP and 0.25% deoxycholate. This combination is bactericidal to strain 7-1 but not to strain 6204 (see below). Survivors were tested for the ability to form colonies on plates containing GL medium with glucuronate as the sole carbon source and 15 μM *rac*-DBP. Although this selection technique is somewhat leaky, as a general rule we have observed that strain 6204 is able to form colonies on these plates, whereas strain 7-1 is not. Transductants that appeared to have the 6204 phenotype were tested for their ability to survive in the cold after a 1-h incubation with 100 μM *rac*-DBP and also for their ability to grow in the presence of 70 μM *rac*-DBP and 0.25% deoxycholate. Presumably, cells with the 6204 phenotype obtained in this fashion should have Tn*10* inserted near the Dbp^r mutation. The closeness of Tn*10* to the Dbp^r lesion was verified by P1 transduction with Dbp^r Tet^r cells as donors and strain 7-1 as the recipient. In such transductions, tetracycline resistance was always used for selection, and tetracycline-resistant transductants were then tested for DBP sensitivity as described above. Strain HW31, one such isolate, was selected for further study.

A P1 *vir* lysate of strain HW31 was used to transfer Tn*10* inserted near Dbp^r to different Hfr strains. Transduction of strains CSH62, KL226, and KL208 in this fashion yielded tetracycline-resistant strains HW41, HW42, and HW43, respectively. The HW31 lysate was also used to transfer Tn*10* to strain S370, and ca. 300 Tet^r transductants were pooled (S370 Tet^r pool). A P1 *vir* lysate of the S370 Tet^r pool was used to convert strain 8 to tetracycline resistance. Individual transductants of strain 8 were tested for a tryptophan requirement. Strain HW50 is a Tet^r, tryptophan-requiring derivative of strain 8. To construct strain HW60, a few of the tetracycline-resistant, δ-aminolevulinic acid-requiring transductants of S370 were pooled and infected with P1 *vir*.

The lysate thus obtained was used to convert strain 8 to strain HW60 (Tet^r *hemA*). P1 *vir*-mediated transduction was used to transfer Dbp^r from strain 6204 to strain HW50 to yield strain HW51. Initial selection, based on the ability of transductants to grow in the absence of tryptophan, was followed by selection on GL-succinate medium plates containing 30 μM *rac*-DBP. Strain HW50 was converted to strains QC104 (*cls*$^+$) and QC120 (*cls*) in a similar fashion. However, in this case, strain T1GP served as the donor, and the transductants were screened for the absence of CL by phosphoglyceride analysis. The *glpD*$^+$ allele of strain 7 was transferred to strains HW51, QC120, and QC104 by P1 *vir*-mediated transduction to yield strains HW53, HW55, and HW56, respectively. These transductants were isolated on the basis of their ability to use G3P as the sole carbon source.

RESULTS

Although strain 8 was used in most of our previous investigations concerning the effects of DBP on microbial physiology, strain 7-1 appeared to offer two major advantages for the present investigation. Strain 7-1 is constitutive for the hexose phosphate transport system and for G3P dehydrogenase but is otherwise isogenic with strain 8. At a concentration of 70 μM, *rac*-DBP inhibits the growth of strain 7-1 cultured in CH medium, whereas the growth of strain 8 is not affected under these conditions (data not shown). The constitutively produced catabolic G3P dehydrogenase of strain 7-1 probably keeps the intracellular G3P concentration much lower in this strain than in strain 8. Since the presence of G3P offsets the growth inhibitory effects of DBP (35), strain 7-1 would be expected to be more sensitive to DBP than is strain 8. The presence of the hexose phosphate transport system does not account for the difference in sensitivity (see below). The second reason for working with strain 7-1 is that it can use G3P as the sole carbon source, and this provides a simple method for eliminating transport mutants. The hexose phosphate transport system provides an alternate route for DBP uptake (8), and its presence was deemed to be desirable to help eliminate transport mutants. In the course of these studies it was discovered that although this rationale applies to high concentrations of DBP it does not apply to concentrations below 100 μM.

The direct approach to the isolation of DBP-resistant mutants, mutagenesis followed by the isolation of colonies that appear on nutrient agar plates containing high concentrations of DBP, produced only transport mutants. A new approach to the problem became evident after the discovery that cells treated with 100 μM *rac*-DBP for 1 h die when stored at 5°C, whereas untreated cells are relatively unaffected by storage in the cold (Fig. 1A). The details of this approach are discussed above. Briefly, strain 7-1 was mutagenized, incubated in CH medium in the presence of 100 μM DBP, and then stored in the cold. Survivors, demonstrated to have normal transport properties, were evaluated for cold sensitivity after incubation with 100 μM *rac*-DBP for 1 h at 37°C. As expected, survival rates for most mutants were considerably higher than for the parent. Strain 6204 was selected for further study because it is constitutive for DBP transport (Table 2), exhibits a particularly high degree of cold resistance (Fig. 1B), and has the same doubling time as the parent in the various media tested. Although the doubling time of strain 6204 is affected considerably less by 30 to 300 μM *rac*-DBP than is the parent, the growth of each strain is completely inhibited by 1 mM *rac*-DBP in CH

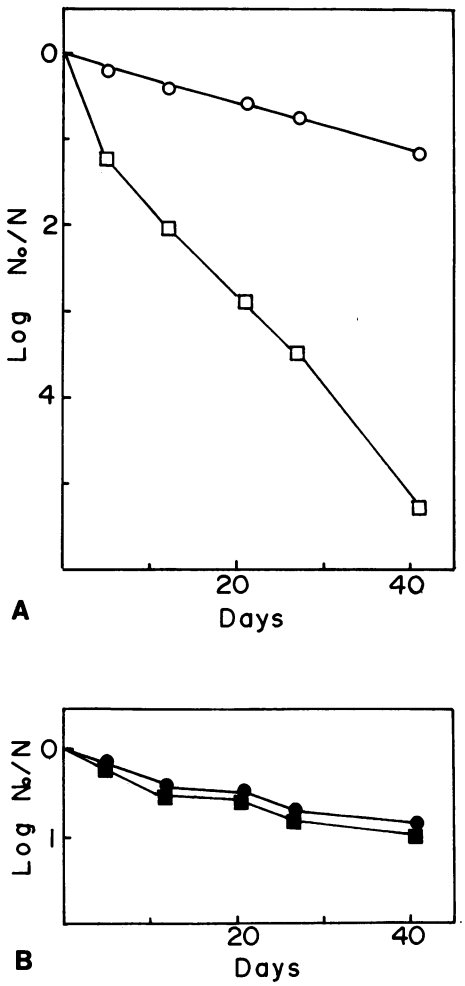

FIG. 1. Effect of DBP treatment on the ability of *E. coli* to survive in the cold. Early-log-phase cells (ca. 1.2×10^8 cells per ml) were treated with 100 μM *rac*-DBP for 1 h at 37°C. After exposure to DBP the cells were stored at 5°C for the indicated number of days. Viability results are expressed as the ratio of No (viability on day 0) to N (viability on a given day after exposure to DBP). (A) Strain 7-1 treated with DBP (□) and untreated (○); (B) Strain 6204 treated with DBP (■) and untreated (●).

medium (data not shown). The ability of DBP to inhibit lipid synthesis of strain 6204 and of the parent strain were compared as was its ability to be incorporated into the lipid fraction of each strain. No differences were detected (data not shown). In agreement with these results, the apparent K_m and K_i values for DBP and G3P of the PGP synthetase obtained from strain 6204 were almost identical to those obtained from strain 7-1 (Table 3). Furthermore, G3P synthetase isolated from each strain exhibited identical sensitivity to DBP (Table 3). Comparison of the V_{max} values also failed to reveal any significant differences (data not shown).

The cold-storage selection technique requires such a long period of time that it was not practical for genetic manipulations. Therefore, other selective pressures were tried. As might be expected of an enteric bacteria, *E. coli* grows quite well in the presence of the bile salt deoxycholate. However, concentrations of *rac*-DBP as low as 30 μM kill strain 8 cultured in GL-succinate medium containing 0.25% deoxy-

TABLE 2. DBP transport in various strains[a]

Strain	DBP transport (cpm) after	
	2 min	5 min
7-1	8,165	21,289
6204	8,088	20,584
Hfr3000	965	2,410
7-T	677	904

[a] The transport assay was performed with washed cells that had been cultured in CH medium. The cells were resuspended to a final density of 100 Klett units in GL medium lacking a carbon source. Uptake was initiated by the addition of *rac*-[^{3}H]DBP (specific activity, 30 μCi/μmol); final concentration, 30 μM. Cells (0.1 ml) were collected, and radioactivity was determined as described in the text. Strain 7-T was isolated as a spontaneous mutant resistant to 2 mM *rac*-DBP.

cholate (unpublished data in this laboratory). This synergistic effect was applied to strain 7-1 cultured in CH medium. Although *rac*-DBP at 70 μM is bacteriostatic to strain 7-1, simultaneous treatment of cells with 70 μM *rac*-DBP and 0.25% deoxycholate does cause a loss of viability (Fig. 2A). Although only a five- to sixfold decrease in viability is evident after 7 h of treatment, this is a sufficient selective pressure if the exposure is continued for a few more hours because strain 6204 continues to divide under these conditions (Fig. 2B).

The cold sensitivity and synergistic effects, evident in liquid medium, do not translate to agar plates, and it was therefore necessary to discover conditions that would permit a distinction to be made between strains 7-1 and 6204 on agar plates. Various media were tried in an attempt to achieve this goal. Strain 7-1 is considerably more sensitive to DBP than is strain 6204 when cultured in GL medium containing glucuronate as the sole carbon source. At 70 μM, DBP is bactericidal to strain 7-1 cultured in GL-glucuronate medium, whereas strain 6204 continues to grow (Fig. 3). Furthermore, the parent strain does not form colonies on agar plates containing GL-glucuronate medium plus 15 μM *rac*-DBP, whereas strain 6204 does.

Because the range of DBP concentrations that permit selection on GL-glucuronate medium plates is narrow, the carbon source is critical, and the procedure is somewhat leaky, it was necessary to insert Tn*10* near the gene responsible for DBP resistance to facilitate genetic studies. A random-insertion pool of Tn*10* in strain Hfr3000 was prepared. An attempt to simultaneously transfer Tet^r and Dbp^r into strain 7-1 failed because of a very high background of

TABLE 3. Apparent K_m and K_i values of PGP and G3P synthetase[a]

Synthetase	K_m (mM)			DBP K_i (mM)
	G3P	DBP	DHAP[b]	
PGP				
7-1	0.095	0.500		0.780
6204	0.090	0.500		0.800
G3P				
7-1			0.160	0.060
6204			0.160	0.060

[a] PGP synthetase and G3P synthetase were assayed as previously described (3).
[b] DHAP, Dihydroxyacetone phosphate.

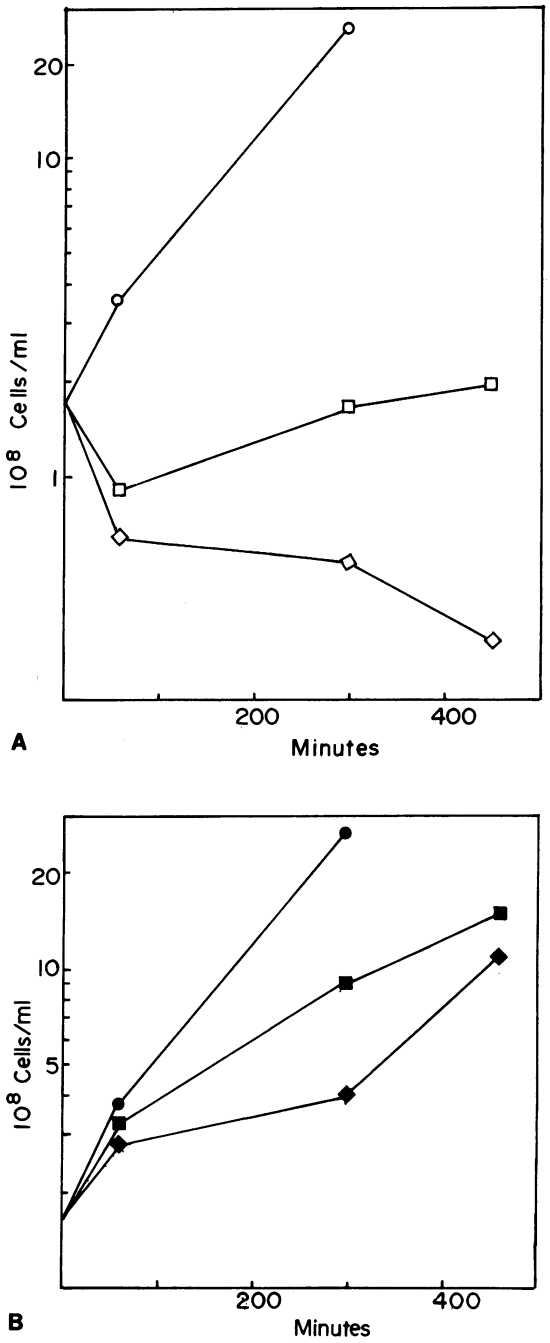

FIG. 2. Effect of DBP plus sodium deoxycholate on the viability of *E. coli* 7-1 (A) and 6204 (B). The bacteria were cultured in CH medium. At the time indicated as zero on the graph, 70 μM *rac*-DBP, 0.25% sodium deoxycholate, or both were added to growing bacterial cultures. Strain 7-1 treated with DBP (□); strain 7-1 treated with DBP plus deoxycholate (◇); strain 6204 treated with DBP (■); and strain 6204 treated with DBP plus deoxycholate (◆). In either strain, cultures treated with deoxycholate alone behaved identically to untreated cultures: Strain 7-1 (○); Strain 6204 (●).

transport-negative transductants that had received the wild-type control gene for the *glp* regulon from Hfr3000. The hexose phosphate transport system is not sufficiently active at the relatively low concentrations of DBP used in the

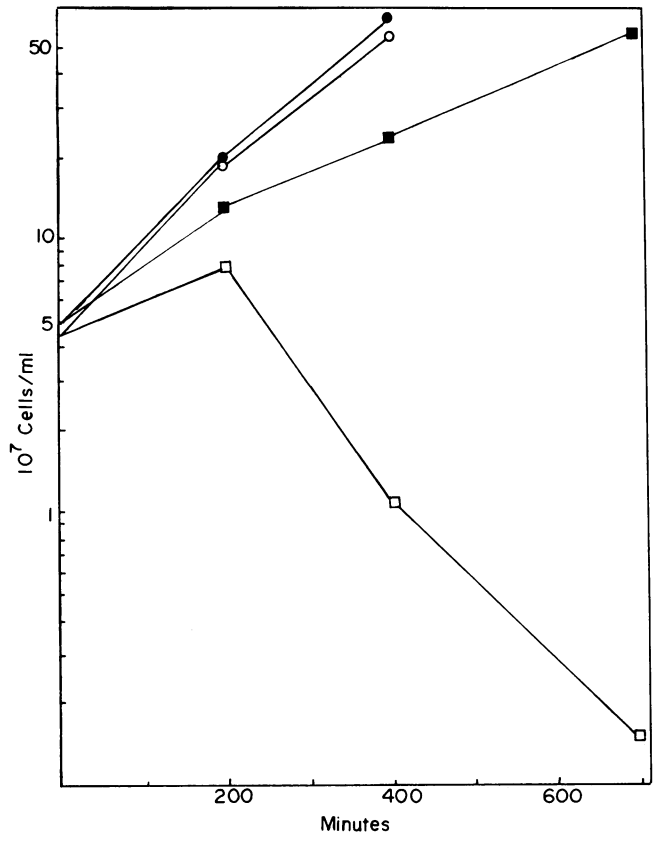

FIG. 3. Effect of 70 μM *rac*-DBP on the viability of *E. coli* 7-1 and 6204 (DBP-resistant strain) in GL medium containing glucuronate as the sole carbon source. DBP was added to growing cells at the time indicated as zero on the graph. Strain 7-1 treated with DBP (□) and untreated (○); strain 6204 treated with DBP (■) and untreated (●).

selection procedures. It was, therefore, necessary to construct strain HW22 to avoid this problem. A few strains that have Tn*10* inserted near the Dbp^r mutation were isolated by the procedures described above. One of these, strain HW31, was studied more carefully. P1 *vir* lysates prepared on strain HW31 were used to transduce strain 7-1. Approximately 60% of the transductants selected for tetracycline resistance were found to also have been converted to the 6204 phenotype, as judged by the ability to form colonies on agar plates containing GL-glucuronate medium plus 15 μM *rac*-DBP. Individual colonies were then tested for the ability to grow in the presence of DBP and deoxycholate and to survive storage in the cold after DBP treatment. These two properties are cotransduced with a frequency of 100%, suggesting that they are both caused by the same mutational event. To test whether the Dbp^r mutation is capable of being expressed in strain 8, a P1 *vir* transduction experiment was carried out with strain HW31 as the donor and strain 8 as the recipient, and tetracycline-resistant transductants were selected. Although strain 8 does not grow on plates containing GL-glucuronate or GL-succinate medium and 30 to 50 μM *rac*-DBP, ca. 60% of the transductants selected for tetracycline resistance were able to do so. Strain HW36, a Dbp^r derivative of strain 8, continues to grow in GL-succinate medium containing 0.3 mM *rac*-DBP. Strain HW35, lacking the mutation that confers DBP resistance but otherwise isogenic to strain HW36, is unable to grow under these conditions (data not shown).

To determine the map location of Tn*10* inserted near the mutation responsible for DBP resistance, the tetracycline resistance of strain HW31 was transferred to various Hfr strains by P1-mediated transduction. The resulting Tet^r Hfr strains, bearing a Tn*10* insertion at the same site as that in strain HW31, were mated with suitable F$^-$ strains. Recombination data, obtained from the conjugation of strains HW41 (HfrH) and HW42 (HfrC) with strain R477, suggest that Tn*10* might be located between the origin of HfrC and *his* (Table 4 and Fig. 4). Furthermore, when strain HW43 (HfrB7) was mated with strain CSH57, the transfer of Tn*10* was found to be closely associated with the *trp* operon (Table 4). The proximity of Tn*10* to the *trp* operon was confirmed by transduction. When strain HW31 (Tet^r *trp*$^+$) was used as the donor and strain CSH57 (*trp*) as the recipient, 63 of the 72 tetracycline-resistant transductants were found to be *trp*$^+$ also. In contrast, a similar experiment in which strain HW11 (*zhe*::Tn*10 trp*$^+$) was used as the donor revealed that none of the 45 Tet^r transductants tested were *trp* (data not shown). The location of Dbp^r and Tn*10* relative to two neighboring markers, *hemA* and *trp*, was determined by three-factor crosses. Because the DBP resistance phenotype can only be detected in a *glpR* background, it was first necessary to construct strains HW50 (*glpR glpD trp zch*::Tn*10*) and HW60 (*glpR glpD hemA zch*::Tn*10*) as described above. Strains HW50 and HW60 were used as recipients and strain 6204 (Dbp^r *trp*$^+$ *hemA*$^+$) was used as the donor for P1 *vir*-mediated transduction. Transductants of strain HW50 that no longer required tryptophan and of strain HW60 that no longer required δ-aminolevulinic acid were selected. The transductants were then scored for the ability to grow on GL-succinate medium plates containing 30 μM *rac*-DBP and on L medium plates containing 25 μg of tetracycline per ml (Table 5 and Fig. 5). The order of the markers is *hemA*-Dbp^r-Tn*10*-*trp*.

The *cls* gene is the only gene known to code for an enzyme

TABLE 4. Conjugational mapping of the location of Tn*10*[a]

Donor	Recipient	Selected marker	% of coinheritance of unselected markers						
			thr$^+$	*leu*$^+$	*lac*$^+$	*pur*$^+$	*gal*$^+$	*his*$^+$	Tet^r
HW41(HfrH)	R477	*leu*$^+$	–	–	32	–	–	1	5
HW42(HfrC)	R477	*leu*$^+$	64	–	–	–	–	0	0
HW43(HfrB7)	CSH57	*trp*$^+$	–	12	32	39	39	–	94

[a] Conjugational mapping was performed by the procedure of Miller (20). Hfr and F$^-$ strains at the mid-log phase were mixed in a 1:20 ratio for 60 min at 37°C. After dilution, the recombinants were selected on medium containing 100 μg of streptomycin per ml. Individual colonies with the selected phenotype were then tested for the unselected markers. A minus either means that the marker was not tested or is not applicable.

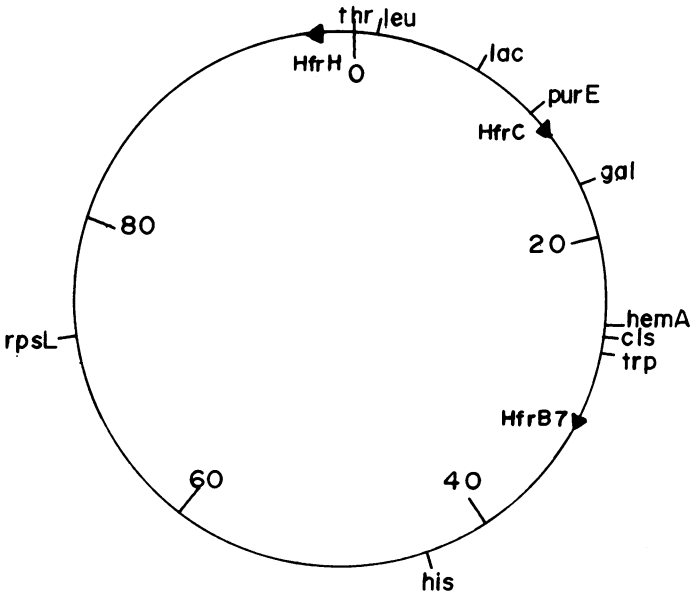

FIG. 4. Map locations on the *E. coli* chromosome of genes relevant to this study. The orders and locations of these genes are by the assignments of Bachmann (1). The origin and direction of chromosome transfer by each of the three Hfr strains used in the conjugational mapping of Tn*10* are indicated by arrowheads.

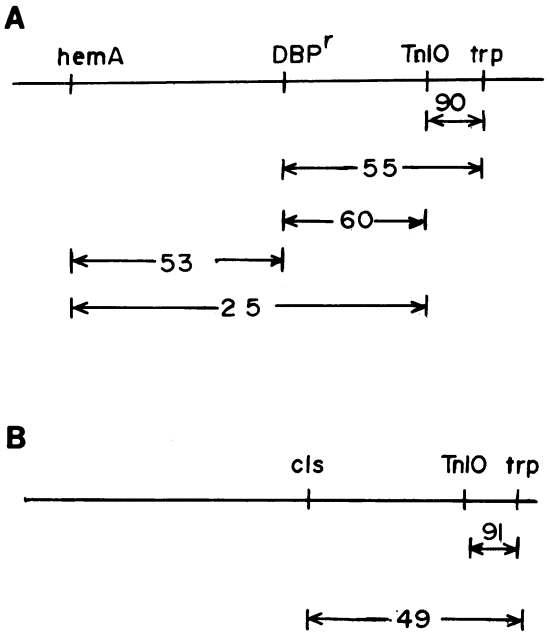

FIG. 5. Location and linkage of DBP resistance (A) and *cls* (B) in relation to other markers in the 26- to 27-min map region of the *E. coli* chromosome. This figure summarizes the data obtained from the transductional analysis presented in Table 3. Refer to Fig. 4 for the positions of *trp* and *hemA* relative to other genetic markers.

involved in phosphoglyceride metabolism that maps in the region between *hemA* and *trp* (1). We therefore examined whether strain 6204 synthesizes CL. Lipid extracted from [1-^{14}C]acetate-labeled strain 7-1 was compared with that extracted from strain 6204. The results of the lipid analysis clearly indicate that strain 6204 either does not contain CL or has so little of this phosphoglyceride that it cannot be detected by the method used (Fig. 6). Experiments were performed to determine whether resistance to DBP could be correlated with an absence of CL. P1 *vir*-mediated transduction was used to transfer DBP resistance to strain HW50. The phosphoglyceride composition of each of 10 randomly selected transductants that were able to grow on GL-succinate medium plates containing 30 μM *rac*-DBP was examined. In each case the DBP-resistant transductant was found to be unable to synthesize CL (data not shown).

Because the genetic and biochemical evidence suggested that DBP resistance is related to the gene responsible for CL synthetase it was important to determine whether a cell bearing the *cls* mutation isolated by Pluschke et al. (22) is resistant to DBP. To examine this possibility, strain QC120 was constructed by transferring the *cls* mutation of strain T1GP to strain HW50, a strain with a *glpR* background. Since the DBP-resistant phenotype was originally characterized in bacteria having an active catabolic G3P dehydrogenase, strains QC120 (*glpR glpD cls*) and QC104 (*glpR glpD*) were converted to strains HW55 (*glpR cls*) and HW56 (*glpR*), respectively (see above). Strain HW55 is indistinguishable from strain 6204 based on the observations that it (i) remains viable in the cold after incubation for 1 h in CH medium containing 100 μM *rac*-DBP, (ii) continues to grow in CH medium containing 70 μM *rac*-DBP and 0.25% deoxycholate, and (iii) is able to grow in GL-glucuronate medium containing 70 μM *rac*-DBP (data not shown). In contrast, strain HW56 exhibits the same sensitivity to DBP as that evident in strain 7-1 (data not shown).

The position of *cls* relative to Tn*10* (in the *zch* location) and *trp* was mapped by a three-factor cross. A P1 *vir* lysate of strain HW55 was used to convert strain HW50 to tryptophan independence. Two unselected traits, *cls* and Tet^r,

TABLE 5. Three-factor analyses[a]

Cross	Donor	Recipient	Selected markers	Unselected markers	No. of cotransductants
1	6204	HW50	*trp*$^+$	Tet^r Dbp^r	3
				Tet^s Dbp^r	60
				Tet^s Dbp^s	43
				Tet^r Dbp^s	8
2	6204	HW60	*hemA*$^+$	Tet^r Dbp^r	35
				Tet^s Dbp^r	23
				Tet^s Dbp^s	4
				Tet^r Dbp^s	48
3	HW55	HW50	*trp*$^+$	Tet^r Dbp^r	6
				Tet^s Dbp^r	55
				Tet^s Dbp^s	59
				Tet^r Dbp^s	5

[a] A P1 *vir* lysate of strain 6204 (Dbp^r Tet^s *trp*$^+$) was used to transduce strains HW50 (Dbp^s Tet^r *trp*) for cross 1 and HW60 (Dbp^s Tet^r *hemA*) for cross 2. Tryptophan-independent colonies selected from the first transduction and δ-aminolevulinic acid-independent colonies selected from the second were purified and scored for sensitivity to tetracycline and DBP. For cross 3, P1 *vir* lysates of strains HW55 (*cls* Tet^r *trp*$^+$) were used to transduce strain HW50. Tryptophan-independent transductants were selected, purified, and scored for their sensitivity to tetracyline and DBP. It should be noted that the *cls* mutation was scored on the basis of DBP resistance.

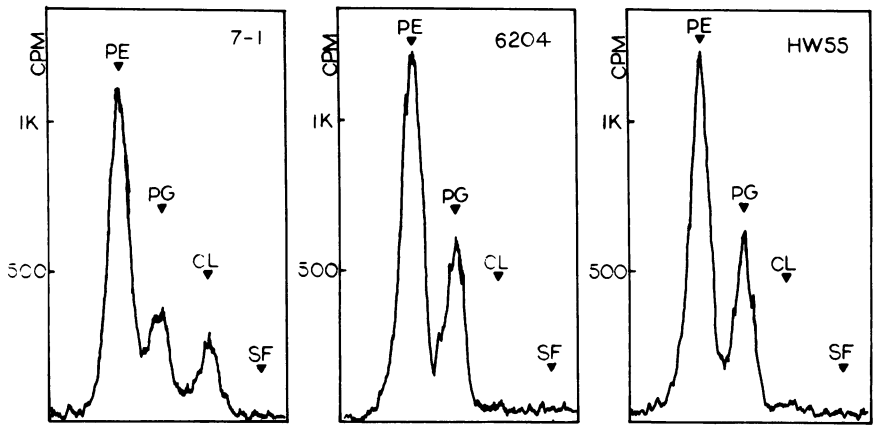

FIG. 6. Phospholipid profiles of strains 7-1 (*cls*$^+$), 6204 (Dbp^r), and HW55 (*cls*). Lipids were isolated from 5 ml of late-log-phase cultures and labeled with 1 μCi of [1-^{14}C]acetate (specific activity, 500 μCi/μmol) per ml for 1 h at 37°C as previously described (31). After the lipids were spotted on silica gel-loaded paper (Whatman SG81), the chromatogram was developed with a chloroform-methanol-acetic acid (65:25:8) solvent system. The radioactive lipids were detected with a Packard chromatogram scanner. SF, Indicates the solvent front.

were then scored. In this case, detection of the *cls* mutation was based upon the ability of mutant cells to form colonies on GL-succinate medium plates containing 30 μM *rac*-DBP. The results of this cross indicate that the gene order is *cls* (scored as DBP resistance)-Tn*10*(*zch*)-*trp* (Table 5 and Fig. 5). In a parallel experiment, when a P1 *vir* lysate of strain HW56 was used to convert HW50 to tryptophan independence none of the transductants were able to grow on GL-succinate medium plates containing 30 μM *rac*-DBP (data not shown). The frequency of cotransduction of *cls* and *trp* is ca. 50%. These results are very similar to those obtained from a cross between strains 6204 and HW50 (Table 5 and Fig. 5) and suggest that the mutation in strain 6204 responsible for DBP resistance either lies within the structural gene for CL synthetase or is at a position very close to it. The cotransduction frequency of *trp* and *cls* that we obtained (49%) is slightly higher than the one (35%) reported by Pluschke et al. (22).

The effect of DBP on phosphoglyceride synthesis in strains HW53, HW55, and HW56 cultured in CH medium was studied. Cells were pretreated with 30 μM *rac*-DBP for 20 min before the addition of labeled acetate. The relative concentrations of each of the phosphoglycerides were determined from the peak areas of the radioscan of the chromatogram (Table 6). It is evident that DBP exerts the same inhibitory effect on PG synthesis in each strain, despite the fact that DBP has much less effect on the growth of strains HW53 and HW55 than it does on the growth of strain HW56. Higher concentrations of DBP cause even greater inhibition of PG formation (data not shown).

The addition of magnesium cations to temperature-sensitive phosphatidylserine synthetase (*pss*) and phosphatidylserine decarboxylase (*psd*) mutants cultured at nonpermissive temperatures produces a partial phenotypic suppression that may be due to a stabilization of the membranes containing excess anionic lipids (9, 21, 25). Since DBP treatment leads to the accumulation of an abnormal anionic phosphoglyceride, the phosphonic acid analog of PGP, the possibility was considered that magnesium or calcium chloride would offset the effect of DBP. However, the addition of 20 mM magnesium chloride to DBP-treated cultures failed to offset the effect of DBP but instead had a synergistic growth inhibitory effect on both strains HW55 (*cls*) and HW56 (*cls*$^+$) (Fig. 7). Thus in this case, the presence or absence of CL synthetase does not seem to have any effect. The addition of 20 mM calcium chloride causes the same type of growth inhibitory effect as does the addition of magnesium chloride (data not shown).

DISCUSSION

E. coli 8, treated with low concentrations of DBP, exhibits major perturbations in phosphoglyceride metabolism but only slight changes in doubling time. Since *E. coli* did not evolve to survive in the medium used in the laboratory, our failure to detect an effect of DBP on growth in synthetic medium should not have been too surprising. Certain environmental conditions to which *E. coli* are ordinarily exposed are quite lethal to bacteria treated with low concentrations of DBP. *E. coli* normally grow quite well in the presence of 0.25% deoxycholate. However, this bile salt is quite deleterious to cells that are incubated in the presence of low concentrations of *rac*-DBP (Fig. 2A; unpublished data of this

TABLE 6. Phosphoglyceride analysis of strains HW53, HW55, and HW56 cultured in the presence or absence of DBP[a]

Strain	DBP treatment	%			Total lipids (cpm/ml of cells)
		PE	PG	CL[b]	
HW53 (Dbp^r)	−	75	25	ND	73,950
	+	89	11	ND	17,370
HW55 (*cls*)	−	74	26	ND	74,050
	+	89	11	ND	16,290
HW56 (*cls*$^+$)	−	73	20	7	71,170
	+	78	8	14	14,230

[a] Early-log-phase cultures (5 ml; 1.2 × 10^8 cells per ml) in CH medium were incubated with [1-^{14}C]acetate (500 μCi/μmol) for 1 h at 37°C. In experiments involving DBP treatment, cells were pretreated with 30 μM *rac*-DBP for 20 min before the addition of labeled acetate. The lipids were isolated and analyzed as indicated in the legend to Fig. 6. The concentrations of the individual phosphoglycerides were calculated from the areas in the radioscan of the chromatogram.

[b] ND, Not detectable by this method.

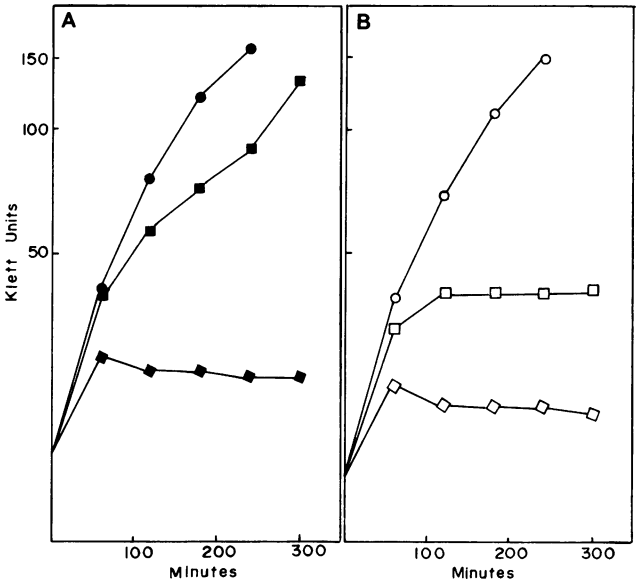

FIG. 7. Effect of DBP plus magnesium chloride on the growth of *E. coli* HW55 (*cls*) (A) and HW56 (*cls*$^+$) (B). The bacteria were cultured in CH medium. At the time indicated as zero on the graph, 70 μM *rac*-DBP, 20 mM magnesium chloride, or both were added to the growing bacterial cultures. Strain HW55 with DBP and magnesium chloride (◆), with DBP (■), and untreated (●); strain HW56 with DBP and magnesium chloride (◇), with DBP (□), and untreated (○).

laboratory). Furthermore, strain 7-1 incubated in the presence of 100 μM *rac*-DBP for 1 h die when stored at 5°C, whereas untreated cells remain viable (Fig. 1A). These effects suggest that DBP treatment is causing a major change in membrane structure. A single mutation found in strain 6204 permits it to resist the effects of DBP under these environmental stresses (Fig. 1B and 2B). The mutation does not affect the incorporation of DBP into the phosphoglyceride fraction or the ability of PGP or G3P synthetases to recognize DBP (Table 3). Furthermore, total lipid synthesis in strain 6204 is just as sensitive to the presence of DBP as is that of the parent strain. Tn*10* has been inserted near the mutation responsible for DBP resistance, and transduction experiments have revealed that the synergistic and cold-storage effects are caused by the same gene.

Experiments were performed to map the location of Tn*10*, inserted near the DBP resistance mutation, as well as the mutation itself. These experiments suggest that the mutation maps in or very near *cls*, a gene associated with CL formation. Consistent with this notion, strain 6204 does not form CL. Furthermore, DBP-resistant transductants of strain 7 are also unable to synthesize CL, and a strain constructed with an authentic *cls* lesion has the same resistance to DBP as does strain 6204.

It is not at all clear why the absence of CL should enable the cells to have enhanced resistance to DBP. Three, among the many possible, explanations will be briefly considered. First, it is possible that the accumulation of both CL and the PGP analog (35) might create a membrane lipid bilayer that is too anionic in character to permit normal function. In this case, the absence of CL might help to compensate for the appearance of the new anionic phosphoglyceride, the analog of PGP. In a related situation, the accumulation of anionic phospholipids in temperature-sensitive *pss* and *psd* mutants results in growth inhibition at nonpermissive temperatures (9, 21, 25). The growth phenotype at the restrictive temperature can be suppressed by increasing the concentration of magnesium and calcium cations (9, 21, 25). However, the addition of cations does not restore the cellular phosphoglyceride composition to normal (9, 21, 25). Therefore, the effect of magnesium and calcium cations on the growth of DBP-treated cells was examined. Contrary to expectation, the addition of either 20 mM magnesium or calcium chloride increased the sensitivity of the cells to DBP regardless of the *cls* genotype (Fig. 7). This seems to argue against the buildup of anionic lipids being solely responsible for the growth inhibitory effects observed. Second, it is possible that phosphoglyceride perturbations resulting from DBP treatment raise the phase transition temperature of the bacterial membranes. Cells do not grow at temperatures below the phase transition temperature of their membranes (19). Membranes isolated from *E. coli* lacking CL synthetase have been shown to have a lower phase transition temperature than do membranes isolated from wild-type cells (23). If DBP treatment raises the phase transition temperature of bacterial membranes then the absence of CL might offer some advantages. The fact that wild-type cells treated with DBP are killed during prolonged storage in the cold may be due to a DBP-induced change in membrane fluidity. In this regard, it should be noted that divalent cations increase the phase transition temperature of membranes made of synthetic PG (36). If they also do so for membranes containing the analog of PGP in place of PG then a change in membrane fluidity might also explain the synergistic effect observed when divalent ions are added to DBP-treated cells. Further work is required to establish a correlation between DBP-related growth phenomena and membrane fluidity. Third, it is possible that a certain amount of PG in the membrane is essential for normal cell function. In addition to its role as a membrane component, PG is also a precursor for CL (11), lipoprotein (39), and membrane-derived oligosaccharide (37). Therefore, in cells treated with DBP, a defect in CL synthetase may permit the cell to channel the scarce PG into other metabolic products. Cells with a *cls* mutation do indeed have a slower rate of PG turnover (22). However, it is difficult to see why channeling the PG to lipoprotein or membrane-derived oligosaccharide synthesis would cause a marked effect on cell growth. Mutants that either synthesize a lipoprotein devoid of covalently bound lipid or that are unable to synthesize lipoprotein appear to be able to grow (2, 10, 39). Membrane-derived oligosaccharide has recently been implicated in osmotic adaptation (12), but again, it does not appear to be essential for cell growth (28, 29). Numerous membrane-bound enzymes depend on the interaction of specific phospholipids for their activity (18). In particular, the phosphoenolpyruvate-sugar phosphotransferase system is reported to require PG (14). Perhaps a defect in CL synthetase enables the cells to retain a critical membrane concentration of PG to interact with enzymes and transport systems that require it. The possibilities discussed are not mutually exclusive or the only ones that might be put forward to explain why mutants with a defect in CL synthetase exhibit enhanced resistance to DBP.

The fact that deoxycholate acts synergistically with DBP suggests that DBP treatment alters the outer membrane of the bacteria. Studies of lipopolysaccharide of gram-negative bacteria indicate that certain mutants of the genus *Salmonella* which have defective lipopolysaccharide are quite sensitive to deoxycholate (27, 38). Lipopolysaccharide is general-

ly thought to serve on the outer membrane of gram-negative bacteria as a physical barrier that excludes hydrophobic compounds and detergents (16). DBP may affect the synthesis of lipopolysaccharide, its insertion into the outer membrane, or some other aspect of outer membrane structure. Any explanation of why deoxycholate acts synergistically with DBP must also account for the fact that cells with a lesion in *cls* do not exhibit this effect.

Although a great many questions have been raised by the observation that cells with a defect in CL synthetase exhibit enhanced resistance to DBP, one application of this knowledge is evident. DBP can be used to screen for the *cls* mutation. In addition, by raising the level of DBP it is possible to have cells continue to divide despite the fact that the membranes are devoid of CL and nearly devoid of PG. It should be noted that a mutation in *cls* does not confer complete resistance to DBP. At concentrations greater than 1 mM, strains HW53 and HW55 cultured in CH medium are still completely inhibited (data not shown). Attempts are currently being made to isolate second-step mutations that confer complete resistance to DBP and to determine why strains with defective CL synthetase have enhanced resistance to DBP.

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