Regulation of Diacylglycerol Kinase Biosynthesis in Escherichia coli

A TRANS-ACTING dgkR MUTATION INCREASES TRANSCRIPTION OF THE STRUCTURAL GENE

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The mechanism of a trans-acting mutation, dgkR1, which causes a 7-fold elevation of diacylglycerol kinase activity in membranes (Raetz, C. R. H., Kantor, G. D., Nishijima, M., and Jones, M. L. (1981) J. Biol. Chem. 256, 2109-2112) was investigated by direct measurement of diacylglycerol kinase polypeptide by high performance liquid chromatography and by construction of fusions of the dgkA promoter to β-galactosidase and galactokinase. The dgkR1 mutation was demonstrated to act by increasing the transcription of the structural gene for diacylglycerol kinase, dgkA. Additionally, sn-glycerol-3-phosphate acyltransferase activities were shown to be decreased 30-50% in membranes from dgkR1 mutant strains. Increased diacylglycerol levels occurred when cells were grown on low osmolarity media. This did not affect dgkA expression. In a dgkR1 background, enhanced expression of sn-1,2-diacylglycerol kinase activity in cells containing a high copy number plasmid bearing dgkA decreased sn-1,2-diacylglycerol levels. However, overproduction of diacylglycerol kinase in a dgkR1 genetic background did not affect diacylglycerol levels, suggesting that the dgkR1 mutation affects diacylglycerol metabolism by mechanisms additional to enhancement of dgkA transcription.

In Escherichia coli, diacylglycerol kinase functions to recycle sn-1,2-diacylglycerol generated from the turnover of membrane phospholipids back into the main pathway of phospholipid biosynthesis (1). During growth on media of low osmolarity, the major source of diacylglycerol is the transfer of phospholipid head groups to periplasmic membrane derived oligosaccharides (2-5). Some additional phospholipid turnover and diacylglycerol formation occurs which is not dependent on osmolarity (4, 5). This latter turnover may be related to the transfer of phosphorylethanolamine groups to outer membrane lipopolysaccharide (6). Phospholipase C (7, 8) and phosphatidic acid phosphatase (9, 10) activities have been measured in membranes from dgkR1 mutant strains. The dgkR1 mutation was shown to act by increasing the lipid biosynthetic enzyme, phosphatidylserine synthetase, in a trans-acting manner (12). In the case of the pssR mutation, it was possible to demonstrate that the elevated enzyme activity was associated with an increased level of the phosphatidylserine synthetase polypeptide in the cell membrane. The mechanism of the dgkR mutation, however, has not been elucidated.

Phospholipid biosynthesis in E. coli is clearly under regulatory controls. Phospholipids are maintained at a constant fraction of membrane mass (13) and pslB mutants, with a greatly reduced in vitro glycerol-3-phosphate acyltransferase activity, require the presence of a second mutation, pslX, to exhibit a growth phenotype (14). The pslX mutation presumably prevents the cellular regulatory machinery from adapting to the mutant pslB activity (14). Regulation is also exerted on the distribution of major phospholipid molecular species. The proportions of these species do not normally change except for a shift of the anionic lipid pool from phosphatidylglycerol to cardiolipin as cell cultures reach the stationary phase of growth (1). The enzymes at the cytidine diphosphate-diacylglycerol branchpoint of E. coli lipid biosynthesis have been overproduced both by recombinant DNA methods (15, 16) and, in the case of phosphatidylserine synthetase, by the presence of a trans-acting mutation (12). The changes in the cellular phospholipid composition induced by these manipulations have always been small in comparison to the changes in in vitro enzyme activity (12, 15, 16). Moreover, mutant strains of E. coli have been isolated in which the activity of one of the cytidine diphosphate-diacylglycerol branchpoint enzymes was markedly reduced, but the cellular phospholipid composition was unaltered (17, 18). These results indicate the functioning of enzymatic mechanisms in the regulation of phospholipid composition. The dgkR and pssR mutations indicate that genetic regulation of phospholipid metabolism is also occurring. Information on the molecular basis of these regulatory mechanisms and their integration in the overall control of phospholipid biogenesis is, however, lacking.

In this paper we report studies on the mechanism of action of the trans-acting dgkR1 mutation on diacylglycerol kinase activity and structural gene expression, the effect of elevated cellular diacylglycerol1 levels on dgkA expression, the effect of dgkA expression on diacylglycerol levels, and the effect of dgkR1 on diacylglycerol levels. The effect of the dgkR1 mutation on the activity of glycerol-P acyltransferase, which is transcribed divergently from diacylglycerol kinase off of a 170-base pair segment of DNA, was also investigated. The dgkR1 mutation was shown to act on dgkA expression at the transcriptional level. Increased cellular diacylglycerol production, induced by growth on a low osmolarity medium, did not affect dgkA expression. Increased diacylglycerol kinase activ-

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1 Throughout this paper, the term "diacylglycerol" refers exclusively to sn-1,2-diacylglycerol.

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The abbreviations used are: XGAL, 5-bromo-4-chloro-3-indolyl-
β-D-galactoside; EGTA, ethylene glycol tetraacetic acid; 
trahydrochloride, and ethidium bromide were from Sigma. 
Disodium indophenol, XGAL, were from Bio-Rad. Candida mycoderma glycerokinase and XGAL' 
were from Biochemicals. Nitrocefin was from BBL Microbiology Systems. Bo-
phospholipids were a gift of Dr. B. R. Ganong of our laboratory. All 
cating that the genes encoding this enzyme and diacylglycerol 
level. However, this effect was suppressed by the 
that the supernatants were discarded. The spermine pellets were resus-
ried in 100 µl of 10 mM Tris, 1 mM 
ethanol pellets were resuspended in 100 pl of 10 mM Tris, 1 mM 
press.

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**EXPERIMENTAL PROCEDURES**

**Materials**—Tryptone, yeast extract, vitamin assay casamino acids, 
peptone, and agar were from Difco. Antibiotics, lithium (d–)-lactate, 
(+)-galactose, diacylglycerol kinase, 2,6-dichlorophenyl-
indophenol, o-nitrophenyl-β-D-galactopyranoside, N-ethylmaleimide,
bovine pancreatic ribonuclease A was from Worthington. β-32P]ATP, 
3-[1-14C]galactose, and [1,2,3-3H]glycerol were from New England 
Nuclear. Restriction endonucleases, Tp, polynucleotide kinase, T4 
DNA ligase, and (GGAATTC) EcoRl linkers were from New 
England Biolaboratories or Bethesda Research Laboratories. Agarose 
and AG 1-X8 anion exchange resin (formate form, 200–400 mesh) 
were from Bio-Rad. *Candida mycoderma* glycerokinase and XGAL2 
were from Boehringer Mannheim. Triton X-100 was from Research 
Products International. 2-[1,2,3-3H]Glycerol-3-phosphate, prepared by 
glycerokinase catalyzed phosphorylation of [1,2,3-3H]glycerol (19), 
was a gift of W. O. Wilkinson of our laboratory. Purified total E. coli 
phospholipids were a gift of Dr. B. R. Ganong of our laboratory. 
All other materials were obtained as described previously (20, 21).

**Media, Growth Conditions, and Bacteriologic Techniques**—All bac-
terial strains used in this work were derivatives of E. coli K-12 and 
other materials were obtained as described previously (20, 21).

**Plasmid Markers/Description**

| Plasmid | Marker | Description | Source/Reference |
|---------|---------|-------------|-----------------|
| pBR322  | blac (cloning vector) | Laboratory collection (25) |
| pCV1    | blac (cloning vector) | Laboratory collection (20) |
| pMLB1034 | lacZ (lacZ fusion vector) | P. Bassford (26) |
| pKO1    | gal+ (TK) | Laboratory collection (27) |
| pJW1    | dgaK ble (ble fusion vector) | Laboratory collection (27) |
| pJW18   | dgaK ble (ble fusion vector) | This work |
| pJW20   | bleB dgaK ble tet | Laboratory collection |
| pJW27   | bleB dgaK lacZ | This work |
| pJW33   | bleB dgaK lacZ | This work |

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2 mM FeSO₄. Low osmolarity peptone medium contained 0.5% peptide, 0.04% tryptone, 0.02% yeast extract, and 0.2% glucose. High osmolarity peptone contained, additionally, 0.4 mM NaCl. Alternatively, for a low osmolarity medium, the NaCl was omitted from Luria broth. Unless otherwise indicated, media were supplemented with 0.2% glucose and 5 µg/ml tetracycline. For antibiotic selection, media were supplemented with either 5 µg/ml tetracycline or 25–50 µg/ml ampicillin. Transformation of plasmid DNA was performed as described by Kushner (29). Glycerol-3-phosphate prototrophic (pob+) transformants of strain VL3 were selected on M9 medium containing 0.2% glucose, 0.05% casamino acids, and appropriate antibiotics. β-Galactosidase fusions were scored on M9 plates containing 0.5% glucose, 0.2% casamino acids, and 40 µg/ml XGAL.

DNA—All plasmids used in this work are listed in Table I. Plasmid DNA was prepared by alkaline lysis of 1.5 ml overnight cultures as described by Maniatis et al. (28) through the ethanol precipitation step. Eppendorf microcentrifugation was for 10 min in all cases. The ethanol pellets were resuspended in 100 µl of 10 mM Tris, 1 mM EDTA, pH 8.0 (TE). Ammonium acetate (50 µl of 7.5 M) was then added and a precipitate allowed to form at room temperature for 5 min. After centrifugation at room temperature, the supernatants were transferred to fresh tubes, 0.3 ml of ethanol was added, and a precipitate allowed to form for 30 min at −20 °C. After centrifugation at 4 °C, the pellets were dried for a few minutes in vacuo. The dried pellets were resuspended in 50 µl of TE containing 50 µg/ml ribonuclease A and incubated at 37 °C for 1 h. Plasmid DNA was then precipitated by adding 50 µl of 20 mM spermine, 200 mM NaCl and keeping the tubes on ice for 15 min (30). After centrifugation at 4 °C, the supernatants were discarded. The supernatants were discarded after centrifugation at 4 °C and the pellets dried for a few minutes in vacuo. The dried pellets were resuspended in 50 µl of TE and stored at 4 °C until used. Restriction endonuclease digestions were performed as recommended by the suppliers. Other nucleic acid methods, including agarose-ethidium bromide electrophoresis, DNA ligations, and attachment of oligonucleotide linkers were according to standard pro-

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**Preparation of Membranes and Cell Free Supernatants**—Membranes were prepared from 100 ml cultures of cells grown in LB medium to an optical density at 600 nm of 0.6. Cells were harvested by centrifugation at 7500 × g for 10 min and resuspended in 25 ml of 0.1 M sodium phosphate, pH 7.0, 5 mM 2-mercaptoethanol, 1 mM diethylnterlaminepentethacetic acid. The cells were lysed by passage through a French pressure cell at 18,000 psi. Unbroken cells and large debris were removed by centrifugation at 5,000 × g for 10 min. Membranes were then pelleted by centrifugation at 160,000 × g for 90 min in a 30% glycerol cushion. Membrane phosphatidyl inositol, 2% glucose, 7.0, 5 mM 2-mercaptoethanol, 1 mM diethylnterlaminepentethacetic acid. These membrane suspensions were stored at −70 °C until used.

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**Cell free supernatants used in assays of soluble enzymes** were prepared from 12.5 ml cultures of cells grown to an optical density at 600 nm of 0.6. Cells were harvested by centrifugation at 7500 × g for 10 min and washed in 5 ml of the appropriate assay buffer. The washed cell pellets were resuspended in 2 ml of assay buffer and the pellets broken by two 15-s bursts from a Branson sonifier at a microtip setting of 3 (40 W) on ice. The broken cell suspensions were centrifuged for 15 min at 4 °C in an Eppendorf microcentrifuge and the supernatants saved for assay.

**Enzyme Assays**—Diacylglycerol kinase (EC 2.7.1.107) was assayed as described previously (21). From 0.1 to 4 µg of membrane protein was used, depending on the level of diacylglycerol kinase expression. Glycerol-3-phosphate acyltransferase (EC 2.3.1.15) was assayed by a modification of a previously described method (32). Membranes (1–2 mg of protein/ml) were first diluted to 0.35 mg protein/ml with an appropriate volume of 25 mM Tris, pH 8.4, 5 mM 2-mercaptoethanol. Triton X-100 was added to a final concentration of 0.5% and the membranes allowed to solubilize on ice for 10 min. Then 20 µl of this mixture was combined with 190 µl of reconstitution buffer (described below) and allowed to stand on ice for an additional 10 min. Assays were performed by combining 2–20 µl of reconstituted enzyme, 50 µl of 2X GPT buffer (0.2 M Tris, pH 8.4, 0.8 M NaCl, 10 mM MgCl₂, 10 mM 2-mercaptoethanol), 5 µl of 0.5 mM palmitoyl 

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*Wilkinson, W. O., Walah, J. P., Corless, J. M., and Bell, R. M. (1986) J. Biol. Chem. 261, 9951–9958*
coenzyme A, 5 μl of 20 mg/ml bovine albumin, and water to give a total of 80 μl. The reaction was initiated by addition of 20 μl of 10 mM sn-1,2-diacylglycerol-3-phosphate and terminated after 10 min at 25 °C by addition of 0.6 ml of 1% HClO4 and 3 ml of methanol/chloroform (2:1, v/v). The [3H]lysophosphatidic acid product was extracted into 1.8 ml of chloroform as described previously (21) and 1 ml of this chloroform phase was recovered by centrifugation and the supernatants transferred to fresh tubes. Phases were broken by addition of 2 ml of chloroform and 2 ml of 1.0 M NaCl and separated by centrifugation. The lower chloroform phase was washed twice with 4 ml of 1.0 M NaCl and saved for analysis. Diaslylglycerol content was determined by quantitative phosphorus determination with [3P]ATP using E. coli diacylglycerol kinase as described elsewhere (44). The radioactive product from every experiment was analyzed by thin layer chromatography and, in all cases, comigrated as a single spot with the phosphatic acid standard. No additional [3P] products were observed. Total lipid phosphorus was determined by the method of Ames and Dubin (45). If the insoluble material from the organic extraction was re-extracted with acidified solvent (4), no diacylglycerol and only 2% of the initial phosphorous were recovered.

Other Methods—Incorporation of [3H]lysophosphatidic acid into membrane-derived oligosaccharides and total cellular lipid was determined as described by Rutberg and Raetz (46). Protein concentrations were determined by the Peterson modification of the method of Lowry (46). Thin layer chromatography of [3P]-phosphorylated E. coli lipid extracts was performed on 0.25-mm silica gel 60 plates (Merck). The plates were developed in chloroform/methanol/acetic acid (65:25:5, v/v).

RESULTS AND DISCUSSION

Overproduction of the Diacylglycerol Kinase Polypeptide in dgkR+ Bearing E. coli Cells—The dgkR1 mutation results in a 7-fold elevation of diacylglycerol kinase activity in membranes (11). To determine whether this mutation caused an increase in the diacylglycerol kinase polypeptide or enhanced activity by some other mechanism, membranes from dgkR1 and dgkR+ E. coli strains were extracted with trifluoroacetic acid/heptane/2-propanol (0.025:1:4, v/v) and the extracted peptides subjected to high performance liquid chromatography analysis. Prior work demonstrated that the diacylglycerol kinase polypeptide was quantitatively extracted and migrated as a single peak (20). The strains used were transformed with pJW1, a high copy number plasmid which contains the diacylglycerol kinase structural gene (20), to increase the diacylglycerol kinase polypeptide signal. The high performance liquid chromatography profile of the dgkR1 strain, G1/KJW1, had a prominent diacylglycerol kinase polypeptide peak which was absent in the dgkR+ strain, R477/pJW1 (Fig. 1). This demonstrated that the dgkR1 mutation increased the level of diacylglycerol kinase polypeptide and ruled out a post-translational modification mechanism for the increased diacylglycerol kinase activity. However, this experiment did not establish the mechanism causing the elevated diacylglycerol kinase polypeptide, which could reflect changes in transcription, translation, or enzyme degradation. Hence, additional studies on the mechanism of the dgkR1 mutation were undertaken.

Construction of dgkA Transcriptional and Translational Fusion Plasmids—To ascertain whether the dgkR1 mutation affects diacylglycerol kinase transcription, translation, or both, fusion plasmids were constructed with the dgkA promoter controlling the synthesis of β-galactosidase and galactokinase. The structures of these plasmids are shown in Fig. 2, and the details of their construction are given in the figure legend. Plasmid pW27 encodes a translational fusion of the first two amino acids of diacylglycerol kinase to a functional β-galactosidase. Hence, additional studies on the mechanism of the dgkR1 mutation were undertaken.

Analysis of Total Cellular Diacylglycerol—Total E. coli lipids were extracted as described by Rotering and Raetz (4) except that the extracting solvent was not acidified in order to avoid acyl chain migration of sn-1,2-diacylglycerols (43). Cultures (100 ml) were grown to an optical density at 600 nm of 0.6 and harvested by centrifugation. The cells were washed in 0.2 M sodium phosphate, pH 7.0, 1 mM EDTA and resuspended in 1 ml of 1.0 M NaCl. Four ml of methanol and 2 ml of chloroform were added. After 1 h the cell debris was removed by centrifugation and the supernatants transferred to fresh tubes. Phases were broken by addition of 2 ml of chloroform and 2 ml of 1.0 M NaCl and separated by centrifugation. The lower chloroform phase was washed twice with 4 ml of 1.0 M NaCl and saved for analysis. Diaslylglycerol content was determined by quantitative phosphorus determination with [3P]ATP using E. coli diacylglycerol kinase as described elsewhere (44). The radioactive product from every experiment was analyzed by thin layer chromatography and, in all cases, comigrated as a single spot with the phosphatic acid standard. No additional [3P] products were observed. Total lipid phosphorous was determined by the method of Ames and Dubin (45). If the insoluble material from the organic extraction was re-extracted with acidified solvent (4), no diacylglycerol and only 2% of the initial phosphorous were recovered.
elsewhere (20). Tokinase synthesized by this mRNA is under the control of which begins with the first two amino acids of diacylglycerol protein, respectively. An arrow indicates the prominent diacylglycerol maxicells (20). Additional details of the methods used are given membrane preparations used were 0.39 and 4.64 pmol/min/mg of transcribed under control of the dgkA promoter. The galactosidase, and galactokinase activities were determined in E. coli strains R477 (dgkR+) and GK1 (dgkR1) as described above. Recombinants with the insert in the correct orientation were identified by restriction mapping. Plasmid pJW18 was derived from pK01 (27) by insertion of a d(GGAATTC) EcoRI linker into the Smal site and concomitant deletion of the small EcoRI-Smal fragment. The construction of plasmid pJW20, the source of the 2700-base pair, pBluescript DNA fragment used in these constructions, is detailed elsewhere (20).

Inasmuch as E. coli membrane preparations can be contaminated with a variable amount of outer membrane, the membrane bound D-lactate dehydrogenase activity was assayed as an inner membrane marker (34). The specific activities observed in cells bearing the fusion plasmids with the ratios observed in cells not bearing a plasmid indicates, however, that such an effect is not occurring with dgkA. When β-galactosidase and galactokinase activities were determined in these strains bearing the fusion plasmids, activity inductions of 9.1- and 8.3-fold, respectively, were obtained. When these numbers were corrected for differences in plasmid copy number, as reflected by the β-lactamase activities, the ratios became 7.0- and 7.5-fold, respectively. Background activities, observed in these strains without the fusion plasmids, were negligible.

The excellent agreement of the ratios for native diacylglycerol kinase activity with those for the fusion plasmid activities indicates that the effect of the dgkR1 mutation is exerted solely at the level of transcription.

Effect of the dgkR Mutation on plsB Expression—In E. coli the ploB and dgkA genes are transcribed divergently from a 170-base pair region of DNA (54). It is, therefore, possible

4 When the diacylglycerol kinase activities with plasmid pJW1 are corrected for both the marker lactate dehydrogenase and the difference in β-lactamase activities, the activities ratio becomes 5.8. An increased plasmid copy number in strain GKI was also observed with plasmid pCV1, the parent vector of pJW1 (20). β-Lactamase activities in strains R477/pCV1 and GKI/pCV1 were 11.8 and 19.6 μmol/min/mg of protein, respectively. This effect is therefore not due to the presence of cloned dgkA DNA.

5 Plasmids pMLB1034 and pK01, the parent vectors for pJW27 and pJW20, do not encode β-galactosidase or galactokinase activities (25, 26). In strain R477, the background β-galactosidase and galaktokinase activities were 0.003 and 0.01 μmol/min/mg of protein, respectively. Identical background activities were observed in strain GKI.
Regulation of dgkA Transcription in E. coli

TABLE II
Enzyme activities from strains R477 (dgk\(^+\)) and GK1 (dgkR1) bearing dgkA transcriptional and translational fusion plasmids

| Strain | Diacylglycerol kinase | Lactate dehydrogenase | \(\beta\)-Lactamase | \(\beta\)-Galactosidase | Galactokinase |
|--------|---------------------|----------------------|------------------|------------------|---------------|
| R477/No plasmid | 38 | 46 | 8.2 | 0.16 | 0.20 |
| GK1/No plasmid | 241 | 42 | 17.6 | 1.45 | 1.66 |
| R477/pJW1 | 265 | 39 | 8.3 | 1.45 | 1.66 |
| GK1/pJW1 | 4,010 | 48 | 10.8 | 1.45 | 1.66 |
| R477/pJW27 | 38 | 40 | 12.3 | 1.45 | 1.66 |
| GK1/pJW27 | 315 | 50 | 13.6 | 1.45 | 1.66 |
| R477/pJW33 | 35 | 39 | 13.6 | 1.45 | 1.66 |
| GK1/pJW33 | 217 | 33 | 13.6 | 1.45 | 1.66 |

Effect of Growth Medium Osmolarity on Diacylglycerol Kinase Gene Expression—During growth on media of low osmolarity, E. coli synthesizes large amounts of membrane-derived oligosaccharide, generating diacylglycerol as a byproduct. When maximally induced, MDO biosynthesis is the major source of cellular diacylglycerol (4, 5). Expression of dgkA may thus be regulated by growth medium osmolarity. To examine this possibility, diacylglycerol kinase activities were determined in membranes from strains GK1 (dgkR1) and R477 (dgkR*) grown on media of low osmolarity. The results are shown in Table IV. These activities, after correction for small differences in the marker lactate dehydrogenase (data not shown). This result suggests that the dgkA and plsB genes may be reciprocally regulated. Additional studies will be needed, however, before a firm conclusion can be drawn.

5 The small decreases in the fusion plasmid-encoded activities on low osmolarity media were accompanied by corresponding decreases in the \(\beta\)-lactamase activities, indicating a plasmid copy number effect. A similar decrease in \(\beta\)-lactamase activity was observed in strains bearing plasmid pBR322 grown on low osmolarity medium. \(\beta\)-Lactamase activities in strains R477/pBR322 and GK1/pBR322 were, respectively, 15.0 and 14.9 \(\mu\)mol/min/mg of protein on LB with 0.1 M NaCl and 12.4 and 12.8 \(\mu\)mol/min/mg of protein on LB without the NaCl. This effect is therefore not due to the presence of cloned dgkA DNA.

8-Lactamase activities in strains R477/pBR322 and GK1/pBR322 were, respectively, 15.0 and 14.9 \(\mu\)mol/min/mg of protein on LB with 0.1 M NaCl and 12.4 and 12.8 \(\mu\)mol/min/mg of protein on LB without the NaCl. This effect is therefore not due to the presence of cloned dgkA DNA.

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TABLE III
sn-Glycerol-3-phosphate acyltransferase activities in strains R477 (dgk\(^+\)) and GK1 (dgkR1) bearing various plasmids

| Strain | No plasmid | pJW1 | pJW27 | pJW33 |
|--------|------------|------|-------|-------|
| R477 (dgk\(^+\)) | 15.7 | 10.4 | 60 | 84 |
| GK1 (dgkR1) | 6.2 | 7.9 | 31 | 55 |

TABLE IV
Enzyme activities from strains R477 (dgk\(^+\)) and GK1 (dgkR1) bearing dgkA fusion plasmids and grown on a low osmolarity medium

| Strain | Diacylglycerol kinase | Lactate dehydrogenase | \(\beta\)-Galactosidase | Galactokinase |
|--------|----------------------|----------------------|------------------|---------------|
| R477 | 43 | 53 | 7.4 | 8.6 |
| GK1 | 288 | 52 | 9.2 | 9.5 |

Effects of Growth Medium Osmolarity and Diacylglycerol Kinase Overproduction on Cell Diacylglycerol Levels—The observations that diacylglycerol kinase expression and activity were unaffected by growth medium osmolarity suggested that cellular diacylglycerol levels may vary in response to osmolarity. Diacylglycerol levels were, therefore, determined in strains R477 and GK1 grown on media of high and low osmolarity (Table VI). Growth on a low osmolarity medium caused a 3- to 8-fold increase in cell diacylglycerol. The mole percent increase was similar for all strains on a given medium, about 0.3% for cells grown on LB and 0.5% on peptone. This suggested that removal of diacylglycerol generated during MDO biogenesis was not limited by diacylglycerol kinase activity.
the rate of diacylglycerol generation on these two media, and the structural gene on the high copy number plasmid, pJW1, was expressed in high and low osmolarity peptone media and analyzed for its significance. This resulted in a 2.5- to 4-fold reduction of the diacylglycerol levels. Enhanced expression of diacylglycerol kinase by amplification of its structural gene on the high copy number plasmid, pJW1, without effect on the diacylglycerol levels. The dgkR mutant strain was additionally altered in its diacylglycerol production. The present work demonstrates that the E. coli dgkR1 mutation acts by increasing the transcription of the dgkA gene. Additionally, the observation of decreased glycerol-3-phosphate acyltransferase activities in the dgkR mutant strain suggests that the structural genes for this enzyme and diacylglycerol kinase are reciprocally regulated. The biological significance of dgalR, however, remains unknown. The finding that diacylglycerol kinase overproduction only in the dgkR+ genetic background was unexpected. This indicated that the dgkR1 mutant strain was not altered in its diacylglycerol metabolism in some way not attributable to the enhanced diacylglycerol kinase activity. It is possible that the increased dgkA transcription in this strain is secondary to this other effect. Overall, these results indicate that diacylglycerol metabolism in E. coli is more complicated than previously thought. Given that diacylglycerols function as second messengers in eukaryotic cells (59, 60), the possibility that they function as signal molecules in E. coli as well is worthy of consideration. A recent report that transfer of phosphoglycerol transferase I, an MDO biosynthetic enzyme, was synthesized constitutively and not induced by low osmolarity (55). The increase in the steady state membrane diacylglycerol content caused by growth on low osmolarity media was not affected by the amount of diacylglycerol kinase present in the membrane. This may reflect compartmentalization of this diacylglycerol pool. Diacylglycerol generated during MDO biosynthesis is localized to the periplasmic space (56). Therefore, translocation of diacylglycerol generated during MDO synthesis from the outer to the inner leaflet of the cytoplasmic membrane must occur prior to its phosphorylation by diacylglycerol kinase. If this diacylglycerol translocation was rate limiting for its phosphorylation, then the diacylglycerol in the outer leaflet of the cytoplasmic membrane could be the low osmolarity pool inferred above. However, data from other membrane systems indicates that transmembrane movement of diacylglycerol is an extremely rapid process (57, 58) and other explanations for this result should be considered.

The activity in dgkR+ cells grown on high osmolarity media, enhanced expression of diacylglycerol kinase by amplification of its structural gene on the high copy number plasmid, pJW1, resulted in a 2.5- to 4-fold reduction of the diacylglycerol content. However, in the dgkR1 genetic background, overproduction of diacylglycerol kinase activity up to 100-fold was without effect on the diacylglycerol levels. The dgkR1 mutant strain, GK1, appears to have an alteration in its diacylglycerol metabolism which is additional to the enhanced dgkA transcription. Diacylglycerol levels in cells grown on the high osmolarity peptone medium were generally higher than those grown on the high osmolarity LB. This probably reflects a difference in the rate of diacylglycerol generation on these two media, inasmuch as a similar increase was observed in strain RZ6, which lacks diacylglycerol kinase activity.

### CONCLUDING DISCUSSION

The present work demonstrates that the E. coli dgkR1 mutation acts by increasing the transcription of the dgkA gene. Additionally, the observation of decreased glycerol-3-phosphate acyltransferase activities in the dgkR mutant strain suggests that the structural genes for this enzyme and diacylglycerol kinase are reciprocally regulated. The biological significance of dgkR, however, remains unknown.

Given that transcription of dgkA is regulated in E. coli, the question arises as to the mechanism operating on its promoter. The observation that expression is proportional with gene dosage in high copy number plasmid vectors argues against the involvement of a DNA-binding molecule present at only a few copies per cell, such as a repressor (51-53). This result is consistent with previous studies in which genes for lipid biosynthetic enzymes were shown to be regulated in a gene dose-dependent manner (15, 16). The availability of the dgkA-3lac'Z fusion plasmid should facilitate the application of E. coli lactose operon methodology (26) to further investigation of the dgkA regulatory mechanism.

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### TABLE V

Membrane derived oligosaccharide biosynthesis in dgkR1 and wild type strains grown on media of high and low osmolarity.

| Strain      | MDO synthesis  |
|-------------|----------------|
|             | −NaCl | +NaCl |
| R477 (dgkR*)| 0.42  | 0.058 |
| GK1 (dgkR1) | 0.41  | 0.043 |

### TABLE VI

Effects of diacylglycerol kinase overproduction on membrane diacylglycerol levels in cells grown on high and low osmolarity media.

| Strain | Relative diacylglycerol kinase activity | Total diacylglycerol* |
|--------|----------------------------------------|-----------------------|
|        | +NaCl | −NaCl | +NaCl | −NaCl | Peptone |
| R477   | 1**  | 0.13  | 0.40  | 0.19  | 0.76   |
| GK1    | 7     | 0.10  | 0.44  | 0.16  | 0.69   |
| R477/pJW1 | 9    | 0.05  | 0.30  | 0.05  | 0.46   |
| GK1/pJW1 | 100  | 0.10  | 0.39  | 0.22  | 0.75   |
| RZ6    | 1     | 0.25  | 0.25  | 0.25  | 0.25   |

*Expressed as percent of total lipid phosphorous (mol/mol).
**Specific activity of membranes from strain R477 was 40 nmol/min/mg of protein with all four media.
***Strain RZ6 does not grow on low osmolarity media.
