Identification of the Diacylglycerol Kinase Structural Gene of *Rhizobium meliloti* 1021

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The cyclic β-1,2-glucans of *Rhizobium* may function during legume nodulation. These molecules may become highly substituted with phosphoglycerol moieties from the head group of phosphatidylglycerol; diglyceride is a by-product of this reaction (K. J. Miller, R. S. Gore, and A. J. Benesi, J. Bacteriol. 170:4569-4575, 1988). We recently reported that *R. meliloti* 1021 produces a diacylglycerol kinase (EC 2.7.1.107) activity that shares several properties with the diacylglycerol kinase enzyme of *Escherichia coli* (W. P. Hunt, R. S. Gore, K. J. Miller, Appl. Environ. Microbiol. 57:3645-3647, 1991). A primary function of this rhizobial enzyme is to recycle diglyceride generated during cyclic β-1,2-glucan biosynthesis. In the present study, we report the cloning and initial characterization of a single-copy gene from *R. meliloti* 1021 that encodes a diacylglycerol kinase homolog; this homolog can complement a diacylglycerol kinase deficient strain of *E. coli*. The sequence of the rhizobial diacylglycerol kinase gene was predicted to encode a protein of 137 amino acids; this protein shares 32% identity with the *E. coli* enzyme. Analysis of hydropathy and the potential to form specific secondary structures indicated a common overall structure for the two enzymes. Because diglyceride metabolism and cyclic β-1,2-glucan biosynthesis are metabolically linked, future studies with diacylglycerol kinase mutants of *R. meliloti* 1021 should further elucidate the roles of the cyclic β-1,2-glucans in the *Rhizobium*-legume symbiosis.

Additional keywords: *Agrobacterium*, *Bradyrhizobium*.

Bacteria within the Rhizobiaceae family are distinguished by their ability to infect plants. Three well-known genera within this family are *Rhizobium*, *Bradyrhizobium*, and *Agrobacterium*. Plant infection by species of *Rhizobium* and *Bradyrhizobium* leads to the production of beneficial nitrogen-fixing nodules on the roots of legumes. In contrast, infection by species of *Agrobacterium* leads to tumor formation.

The cell surface carbohydrates of all three genera may function during plant infection processes. Indeed, several laboratories have recently provided definitive evidence that rhizobial glucosamine oligosaccharides act as signal molecules during plant infection (Lerouge *et al.* 1990; Roche *et al.* 1991a; Roche *et al.* 1991b; Spaink *et al.* 1991a; Spaink *et al.* 1991b; Truchet *et al.* 1991). Studies in our laboratory have focused on a different class of oligosaccharides produced by these bacteria, the cyclic β-1,2-glucans. These glucans are apparently synthesized by all species of *Rhizobium* and *Agrobacterium* (Dell *et al.* 1983; Hisamatsu *et al.* 1983; Koizumi *et al.* 1983; York *et al.* 1980; Zevenhuizen and Scholten-Koerselman 1979), and studies have indicated that these glucans may function during plant infection as well as during bacterial adaptation to hypo-osmotic stress (Cangelosi *et al.* 1990; Douglas *et al.* 1985; Dylan *et al.* 1990a; Dylan *et al.* 1986; Dylan *et al.* 1990b; Miller *et al.* 1986; Puvanesarajah *et al.* 1985; Zorreguieta *et al.* 1990).

Although the backbone structure of the cyclic β-1,2-glucans of species of *Rhizobium* and *Agrobacterium* is essentially identical, the glucans from different species contain varying amounts of substituents. For example, the cyclic β-1,2-glucans of *A. tumefaciens* (Smith and Townsend) Conn C58, *Rhizobium* sp. NGR234, and *R. meliloti* Dangeard strains 1021, K-24, A-145, A-148, SU-47, SU-231, SU-255, and SU-256 contain *sn*-1-phosphoglycerol as a major substituent (Batley *et al.* 1987; Miller *et al.* 1988; Miller *et al.* 1987; Zevenhuizen *et al.* 1990). In contrast, the cyclic β-1,2-glucans of *R. meliloti* 102F34 and *R. trifolii* Jordan strains 0403, TA-1, and In2 appear to be unsubstituted (Dylan *et al.* 1990a; Zevenhuizen *et al.* 1990), whereas those of *A. radiobacter* (Beijerinck and van Delden) Conn A1-5, IFO 12665b1, IFO 13127b, *R. phaseoli* Jordan AHU 1131, M7-5, and *R. trifolii* 4S contain methylmalonic and/or succinic acid substituents (Hisamatsu *et al.* 1987). The functions of the substituents on the cyclic β-1,2-glucans are unclear; however, anionic moieties, such as *o*-acetyl and sulphate substituents, on the glucosamine oligosaccharides of *Rhizobium* species appear to function in determining host specificity during legume nodulation (Roche *et al.* 1991a; Roche *et al.* 1991b; Spaink *et al.* 1991a; Spaink *et al.* 1991b; Truchet *et al.* 1991).

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Previously, we showed that the sn-1-phosphoglycerol substituent present on the cyclic β-1,2-glucans of R. meliloti 1021 is from the head group of phosphatidylglycerol (Miller et al. 1988). In fact, this substitution reaction may be directly analogous to the phosphoglycerol transfer reaction mediated by phosphoglycerol transferase I during membrane-derived oligosaccharide (MDO) biosynthesis by Escherichia coli (Migula) Castellani and Chalmers (Jackson and Kennedy 1983). This phosphoglycerol transfer reaction appears to represent the primary pathway of phospholipid turnover in E. coli and R. meliloti 1021. Specifically, Kennedy and co-workers (Schneider et al. 1979) estimated that approximately 90% of the phospholipid turnover in E. coli is attributed to this reaction. Likewise, we estimated that greater than 60% of the total phospholipid turnover in R. meliloti results from the transfer of phosphoglycerol head groups to the cyclic β-1,2-glucans (Miller et al. 1988).

sn-1,2-Diglyceride is a by-product of the phosphoglycerol transferase I reaction (Jackson and Kennedy 1983); however, diglyceride does not accumulate within the membranes of E. coli. Instead, the diglyceride product generated during MDO biosynthesis is recycled into the phospholipid pool as phosphatidic acid via the action of diacylglycerol kinase (Raetz 1982). This "diglyceride cycle" is advantageous to the cell because most of the ATP required for membrane phospholipid biosynthesis is expended during the formation of the fatty acid chains (Raetz and Newman 1979). Furthermore, diglyceride accumulation within E. coli may be detrimental. This is indicated in previous studies of diacylglycerol kinase mutants of E. coli. Specifically, these mutants grew poorly in media of low osmolarity, a condition that favors high levels of MDO biosynthesis (Raetz and Newman 1978). Furthermore, these strains were extremely sensitive to the β-glucoside, arbutin (Jackson et al. 1984). This sensitivity to arbutin arises because this glucoside serves as an excellent substrate for phosphoglycerol transfer as mediated by phosphoglycerol transferase I (Jackson and Kennedy 1983). Thus, high levels of diglyceride accumulate within diacylglycerol kinase mutants when they are grown in media containing arbutin.

On the basis of our previous demonstration that phosphoglycerol transfer to the cyclic β-1,2-glucans of R. meliloti 1021 appears to be directly analogous to the transfer reaction that occurs during MDO biosynthesis in E. coli, we predicted that a diglyceride cycle should also operate within R. meliloti (Hunt et al. 1991). Recently, we reported evidence for such a cycle through the identification of diacylglycerol kinase activity within cell extracts of R. meliloti 1021 (Hunt et al. 1991). Further, we have shown that the rhizobial diacylglycerol kinase shares many properties with the E. coli enzyme (Hunt et al. 1991). In the present study, we report the cloning, expression, and sequence of the diacylglycerol kinase structural gene from R. meliloti 1021.

MATERIALS AND METHODS

Bacterial strains, plasmids, phage vectors, and culture conditions. All bacterial strains, plasmids, and phage vectors used in this study are listed in Table 1. R. meliloti 1021 was cultured in YM medium (Miller et al. 1990) at

30° C. E. coli strains were cultured in LB medium (Miller 1972) at 37° C. LB medium was supplemented with ampicillin (50 μg/ml), chloramphenicol (20 μg/ml), kanamycin (50 μg/ml), spectinomycin (100 μg/ml), streptomycin (50 μg/ml), or tetracycline (12.5 μg/ml) as needed.

Construction of a diacylglycerol kinase mutant of Escherichia coli, strain WH1061, through disruption of the dgkA gene. A selectable marker for kanamycin resistance (1,270-bp kanamycin cassette, derived from Tn903 in pUC4K [Pharmacia, Piscataway, NJ]) was inserted at the unique MscI site of the E. coli diacylglycerol kinase structural gene dgkA. The dgkA gene was provided by Robert Bell (Duke University Medical Center, Durham, NC) within a 566-bp EcoRI fragment originally identified in plasmid pJW6 (Loomis et al. 1985). The entire 1,836-bp EcoRI fragment (dgkA-kan-dgkA) was treated with the DNA polymerase I Klenow fragment and ligated to plasmid pMAK705, which had previously been digested with HindIII and also treated with the DNA polymerase I Klenow fragment. We used the resulting temperature-sensitive plasmid, pWPH705, to introduce the disrupted dgkA gene into the chromosome of E. coli strain MC1061; we used the gene replacement method developed by Hamilton et al. (1989). Southern analysis confirmed that the resulting strain, WH1061, contained only one copy of the dgkA gene and that this copy contained the kanamycin cassette at the MscI site.

Table 1. Bacterial strains, plasmids, and phage vectors used in this study

Strain/plasmid	Description	Source[a]
Rhizobium meliloti 1021		F. Ausubel
Escherichia coli strains		
MC1061	ara leu lac gal hsdR rpsL	M. Casadaban
WH1061	dgkA-kan-dgkA derivative of MC1061	This study
RZ60	HfrH thi-1 mal⁺ dgk-6 relA1	E. P. Kennedy
RZ600	dgk⁺ (isogenic with RZ60)	E. P. Kennedy
Plasmids		
pUC18	Ap^r	Bethesda Research Laboratories (BRL)
pUC19	Ap^r	BRL
pUC4K	Kan^r::Tn903	Pharmacia
pMAK705	Cm^r, t.s. replicon[b]	S. Kushner
pWPH705	Cm^r, t.s. replicon dgkA-kan-dgkA	This study
pMM1	pUC19 containing a 2.6-kbp insert from R. meliloti 1021	This study
pMM2	pUC19 containing a 5.6-kbp insert from R. meliloti 1021	This study
Phage vectors		
M13mp18		BRL
M13mp19		BRL

[a] F. Ausubel, Massachusetts General Hospital, Boston, MA; M. Casadaban, University of Chicago, Chicago, IL; E. P. Kennedy, Harvard Medical School, Boston, MA; Bethesda Research Laboratories, Gaithersburg, MD; Pharmacia, Piscataway, NJ; S. Kushner, University of Georgia, Athens, GA.
[b] Temperature-sensitive replicon.

Genomic DNA and plasmid isolation. Genomic DNA was isolated from *R. meliloti* 1021 with the procedure described by Wilson (1987). Large-scale plasmid preparations from *E. coli* were isolated by the alkaline lysis method and purified by centrifugation through a CsCl gradient as described by Sambrook *et al.* (1989). We obtained small-scale plasmid preparations from 3-ml or 10-ml *E. coli* cultures by using the alkaline lysis method described by Sambrook *et al.* (1989). DNA preparations were analyzed by horizontal agarose gel electrophoresis; agarose concentrations ranging between 0.7 and 1.2% were used. Electrophoresis was routinely performed with a constant voltage between 25 and 100 V with TBE (45 mM Tris-borate, 1.0 mM EDTA, pH 8.0) or TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) (Sambrook *et al.* 1989).

Southern analysis. DNA preparations were electrophoresed within agarose gels and subsequently blotted onto nylon filters (Gene Screen Membranes; New England Nuclear, Boston, MA) according to instructions outlined by Devlin *et al.* (1988). Probes were labeled with [α-^{32}P]dATP with a random primer kit (Boehringer Mannheim, Indianapolis, IN). Hybridization and washing conditions were adapted from Devlin *et al.* (1988).

DNA sequencing. DNA sequencing was performed by the dideoxy nucleotide chain termination method (Sanger *et al.* 1977) with Sequenase (version 2, United States Biochemical Corporation, Cleveland, OH); [α-^{35}S]dATP was used as the radioactive label. A series of deletion fragments were generated by using *Bal*31 exonuclease, and these fragments were subcloned into M13mp18 and M13mp19. The sequences of both strands were determined from each series of deletion fragments.

Sequence analysis. Computer analysis of DNA and protein sequences was performed at The Pennsylvania State University Biocomputing Center with programs from Intelligenetics (Mountain View, CA) and the University of Wisconsin Genetics Computer Group (Devereux *et al.* 1984).

Diacylglycerol kinase assay. Diacylglycerol kinase activity was measured within cell extracts and membrane fractions with [γ-^{32}P]ATP and *sn*-1,2-dioleoylglycerol as previously described (Hunt *et al.* 1991).

RESULTS

***E. coli* strain WH1061 lacks diacylglycerol kinase activity.** Because the only copy of the diacylglycerol kinase

gene within strain WH1061 was disrupted with the kanamycin resistance cassette from Tn*903*, it was expected that no functional diacylglycerol kinase would be expressed by this strain and that this strain should represent a null mutant. Indeed, no diacylglycerol kinase activity was detected within cell extracts or within membrane preparations derived from strain WH1061 (Table 2). In contrast, cell extracts prepared from the parent strain, MC1061, contained diacylglycerol kinase activity at levels similar to those previously reported for other strains of *E. coli* (Table 2).

Consistent with this lack of detectable diacylglycerol kinase activity, strain WH1061 was impaired for growth in media of low osmolarity (Fig. 1A vs. 1B). Furthermore, the growth of this strain was extremely impaired in media supplemented with arbutin (Fig. 1C). The sensitivity of *E. coli* diacylglycerol kinase mutants to low osmolarity media and media containing arbutin has previously been noted by Raetz and Newman (1978) and Kennedy and co-workers (Jackson *et al.* 1984) as described above. However, these earlier studies examined diacylglycerol kinase mutants
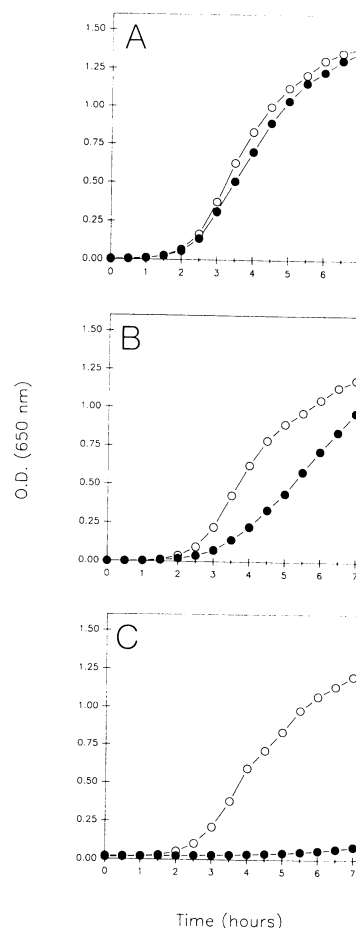

Fig. 1. Growth of *Escherichia coli* strains MC1061 and WH1061 at varying osmolarities and in media containing arbutin. Aliquots (0.1 ml) of precultures of MC1061 and WH1061 grown in LB medium were used to inoculate 10-ml cultures of **A**, LB medium; **B**, LB medium containing no added NaCl; or **C**, LB medium containing 90 mM arbutin. Cultures were incubated at 37° C on a gyratory shaker, and growth was monitored at 650 nm. O-O, MC1061; ●-●, WH1061.

Table 2. *Escherichia coli* strain WH1061 (*dgkA-kan-dgkA*) lacks detectable diacylglycerol kinase activity

Bacterial strain	Diacylglycerol kinase activity[a]	
	Crude sonicate	Membrane fraction
E. coli MC1061	10.2	23.0
E. coli WH1061 (*dgkA-kan-dgkA*)	ND	ND
E. coli RZ600 (*dgk*⁺)	11.7	26.2
E. coli RZ60 (*dgk-6*)	ND	ND

[a] Activity was measured within crude cell sonicates or membrane fractions as described by Hunt *et al.* (1991) and is expressed as nanomoles [γ-^{32}P]ATP converted to phosphatidic acid per minute per milligram of protein. ND, none detected.

obtained after chemical mutagenesis, and the precise nature of these mutations has never been defined.

Construction of a *R. meliloti* 1021 genomic library and subsequent screening for the rhizobial diacylglycerol kinase gene through complementation of strain WH1061. On the basis of the extreme sensitivity of strain WH1061 to growth media containing arbutin, we predicted that the diacylglycerol kinase structural gene from *R. meliloti* 1021 could be identified through complementation studies with a genomic DNA library. The feasibility of this approach was examined through preliminary studies with the *E. coli dgkA* gene. These studies revealed that the poor growth arbutin phenotype of strain WH1061 could be complemented by the *dgkA* gene present within the high copy number plasmid pUC18. Indeed, colonies containing the *dgkA* gene within pUC18 were readily detected within 16 hr of growth at 37° C on agar medium containing 90 mM arbutin. In contrast, strain WH1061 requires at least 24 hr to form visible colonies on this medium.

R. meliloti 1021 genomic DNA was partially digested with *Sau*3A, and 2- to 6-kbp fragments were retrieved from low melting point agarose gels. These genomic DNA fragments were then ligated with pUC19 plasmid DNA that had been previously digested with *Bam*HI and dephosphorylated with calf intestine alkaline phosphatase. Ligation products were used to transform *E. coli* WH1061. Transformants were plated onto LB medium containing kanamycin (50 μg/ml), ampicillin (50 μg/ml), and arbutin (90 mM). Plates were incubated for 16 hr at 37° C.

Out of 21,600 recombinant WH1061 transformants plated on 90 mM arbutin medium, two visible colonies were identified after 16 hr of incubation, indicating possible expression of the rhizobial diacylglycerol kinase gene. The plasmids present within these transformants were designated pMM1 and pMM2 and contained genomic inserts of 2.6 and 5.6 kbp, respectively. When cell extracts prepared from these transformants were assayed for diacylglycerol kinase activity, only transformants containing plasmid pMM1 expressed diacylglycerol kinase activity (Table 3). As shown in Table 3, the level of diacylglycerol kinase activity within transformants containing plasmid pMM1 was almost 10-fold greater than the level within the parent strain MC1061. This high level of activity is consistent with the presence of multiple copies of pMM1 within strain WH1061.

Localization of the *R. meliloti* 1021 diacylglycerol kinase gene within a 1.3-kpb *Pst*I fragment from pMM1. A

Table 3. Diacylglycerol kinase activity within transformants of *Escherichia coli* strain WH1061

E. coli strain	Plasmid	Diacylglycerol kinase activity[a]
MC1061	None	3.9
WH1061	None	ND
WH1061	pUC19	ND
WH1061	pMM1	38.2
WH1061	pMM2	ND

[a]Specific activity is expressed as nanomoles of $[\gamma^{-32}P]$ATP converted to phosphatidic acid per minute per milligram of protein. Crude cell extracts obtained after sonication were the source of the enzyme. ND, none detected.

diagram of plasmid pMM1 is presented in Figure 2. Initial restriction analyses revealed a unique *Pst*I site near the middle of the genomic DNA insert. Subsequent subcloning studies demonstrated that the complementing DNA was localized within the 1.3-kbp *Pst*I fragment isolated from pMM1.

On the basis of the above, the nucleotide sequence of the 1.3-kbp *Pst*I fragment from pMM1 was determined, and the sequence is presented in Figure 3. Three major open reading frames (ORF) preceded by potential ribosome-binding sites were identified: ORF1 (bp 90–716), ORF2 (bp 130–552), and ORF3 (bp 757–1,170). When the programs FASTP and FASTDB were used to search the PIR (release 29; 31,895 entries), SWISS-PROT (release 19; 21,795 entries), and GenBank (release 69; 55,631 loci) databases for sequences similar to each ORF, only one obviously significant match was detected. This similarity was between ORF3 and the *E. coli* diacylglycerol kinase enzyme (14.45 and 11.60 standard deviations above random with the FASTDB program from Intelligenetics and FASTP from the PIR, respectively). An optimal alignment containing two, single-residue gaps is presented in Figure 4. Not only did the predicted rhizobial protein have a size similar to the *E. coli* diacylglycerol kinase enzyme (137 and 122 amino acids, respectively), but it also shared 39 amino acid identities (32% identity). When considering conservative substitutions, defined as small hydrophilic (P,T,G,A,S), acidic (D,N,E,Q), basic (H,K,R), small hydrophobic (L,I,V,M), or aromatic (F,Y,W) residues, we
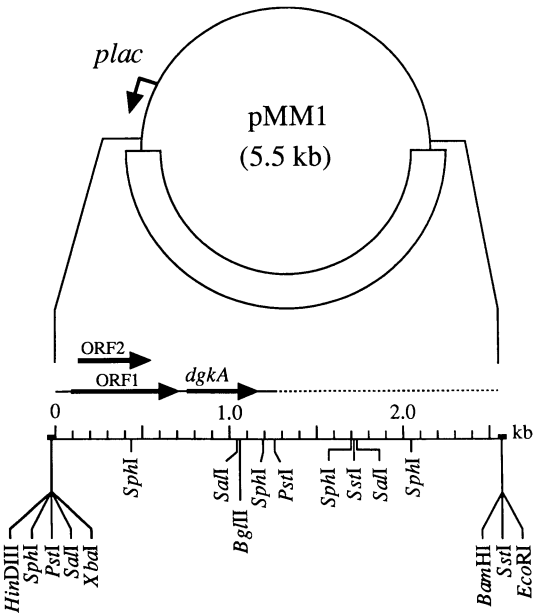

Fig. 2. Restriction map of the 2.6-kbp *Rhizobium meliloti* 1021 *Sau*3A genomic fragment within plasmid pMM1. The genomic fragment was inserted at the *Bam*HI site within the polylinker region of pUC19 as described in the text. Subcloning studies revealed that the *R. meliloti* diacylglycerol kinase structural gene (*dgkA*) was present on the 1.3-kbp *Pst*I fragment from this plasmid. Sequence analysis of this 1.3-kbp *Pst*I fragment revealed the presence of three open reading frames (see Figure 3). The relative positions of these three open reading frames (ORF1, ORF2, and ORF3 [*dgkA*]) within the original 2.6-kbp genomic *Sau*3A fragment are indicated.

found that the apparent homology between the two proteins increases to 55%. This is also apparent when one plots a moving average of the 250 PAMs Mutation Data Matrix (MDM) values (George et al. 1988) for the optimized alignment (because the related proteins from which this MDM was derived are largely soluble proteins, the similarity between the two diacylglycerol kinase enzymes may be somewhat underestimated).

When the 1.3-kbp PstI fragment from pMM1 was used as a probe in Southern hybridization analyses, it hybridized to a single 12.2-kbp EcoRI fragment from R. meliloti 1021 genomic DNA (data not shown). Thus, it is likely that the rhizobial diacylglycerol kinase gene is present as a single copy within the R. meliloti 1021 genome.

The predicted secondary structure for the rhizobial diacylglycerol kinase enzyme is similar to that predicted for the E. coli enzyme. Loomis et al. (1985) have proposed that the E. coli diacylglycerol kinase enzyme has three transmembrane α-helical segments, an amphipathic helix, and an α-helix. When the putative diacylglycerol kinase from R. meliloti was examined by the methods of Chou and Fasman (1978), secondary structure predictions generally matched those predicted for the E. coli enzyme (not shown). However, the rhizobial enzyme showed a greater tendency for α-helical structure, whereas the E. coli sequence favored β-sheets. Hydropathy plots obtained for both enzymes by the method of Kyte and Doolittle (1982), with a window of seven residues, are shown in Figure 4. In each enzyme, at the same relative positions, there are three regions predicted to be strongly hydrophobic. However, amino acid similarity, defined by the MDM 250 PAMs values, is only apparent in the latter portion of the second of these three hydrophobic regions (compare the similarity and hydropathy windowed averages in Figure 4). Immediately adjacent to the second hydrophobic segment is an apparently highly conserved region that may form an amphipathic helix. This helix is followed by

hydrophilic residues in both enzymes. A helical wheel projection for the putative amphipathic helix of each enzyme is presented in Figure 5.

DISCUSSION

Recently, we reported that the diacylglycerol kinase enzyme of R. meliloti 1021 shares many properties with the E. coli enzyme (Hunt et al. 1991). Consistent with this report are the results of the present study; these results reveal that the two enzymes share homology and are integral membrane proteins with the same predicted secondary structure.

Bell and co-workers have extensively characterized the diacylglycerol kinase enzyme from E. coli (Loomis et al. 1985; Walsh and Bell 1986a; Walsh and Bell 1986b; Walsh et al. 1990). Their studies have revealed a great deal of information about substrate specificity, lipid cofactor requirements, and divalent cation dependence. These researchers have also proposed a model for the tertiary structure of this enzyme (Loomis et al. 1985); the model predicts the presence of three transmembrane α-helical domains. An amphipathic α-helical domain occurs between the second and third hydrophobic transmembrane domains. This model is shown in Figure 6.

The results of the present study now permit a direct comparison of the sequences of two bacterial diacylglycerol kinase enzymes. This comparison reveals four regions of near identity that may be needed for efficient folding of the enzyme, the binding of MgATP and diglyceride substrates, or subsequent catalytic events. The comparison is perhaps best shown by modifying the tertiary model of E. coli diacylglycerol kinase proposed by Loomis et al. (1985) so that it indicates the residues that are identical in both enzymes (Fig. 6).

The greatest regions of identity occur in the domains predicted by Loomis et al. (1985) to be at or near the

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   1 GATCTGCTGTCATCGGCGAGATGGGCATCGGCAACACCACGATCGCGGCCGCAATCAATCTCGGCCTCTATGGCGGCACGGCAGAGGAATGGGTCGGCC
 101 CCGGTACCGGTTCGGAAGGCGAGGTGCTCATGCGCAAGATCGCTGCTGTCGAAAAGGCCGTTGCGCTGCACCGCGACCACTTGTCCGATCCGCTCGAAGT
 201 CATGCGGCGGCTTGGCGGCCGCGAGATCGCGGCGATGGCGGGCGCAATTCTCGCCGCGCGGATGCAGAAGGTGCCCGTCATCATCGACGGCTACGTCGCG
 301 ACCGCGGCCGCGGCGATCCTCAAGGCCGCCAACCCGGCGGCGCTCGACCATTGCCTGATCGGCCATGTTTCAAGCGAGCCCGGTCACATGCGGGCGATCG
 401 AGAAACTCGGCAAGACACCGCTCCTGGCTCTCGGCATGCGGCTTGGCGAAGGCACGGGTGCGGCTCTTGCAGCAGGCATCGTCAAGGCGGCGGCTGCCTG
 501 CCACAGCGGCATGGCGACCTTTGCGCAGGCCGGCGTAAGCAACAAGGAGTAGGGCACGCTCCGGCGCTTGCTGTCGATTGGCCGTCATGCGTGCTATGCT
 601 CGTGCCGTGCAGCTATTGATGCGGGGCGGCTTCTCGCATATCCGGGAAGCGCCGGGTCATCGGTTCGGGCGGCCTCGCAATGCGGCATTGGTGCCGCCTT

                                                 M  D  A  K  N  T  T  S  R  N  G  T  A  A  P
 701 TGATTCGCCTCAATAGGAACGTCGAAGCTGTGCCATCTCCCAACCCGCGAGACGAAATGGACGCCAAGAACACTACCTCCCGCAACGGCACCGCCGCGCC

       V  H  K  H  R  G  I  R  H  L  F  A  A  A  S  Y  S  F  G  G  A  K  R  L  I  G  E  A  A  F  R  H  E
 801 GGTCCACAAGCATCGTGGTATCCGGCATCTTTTCGCCGCGGCCAGCTATTCCTTCGGCGGTGCGAAACGCCTGATCGGCGAGGCGGCATTCAGGCACGAA

       L  I  A  F  A  V  A  M  V  A  F  M  I  V  G  A  N  L  F  Q  Y  V  A  M  A  I  L  F  L  L  M  M  A  F
 901 CTCATCGCCTTCGCGGTCGCGATGGTCGCGTTCATGATCGTCGGCGCGAACCTGTTCCAGTACGTCGCGATGGCAATCCTGTTTCTGCTGATGATGGCCT

       E  A  I  N  T  A  I  E  E  I  V  D  R  V  S  P  E  I  S  E  M  G  R  N  A  K  D  L  G  S  F  A  C
1001 TCGAGGCCGATCAACACCGCGATCGAGGAGATCGTCGACCGCGTCTCGCCGGAGATCTCCGAAATGGGCAGGAACGCAAAGGATCTCGGCTCCTTCGCGTG

       L  C  L  I  L  A  N  A  A  Y  A  L  Y  V  I  F  L  A  R  F  L  G  *
1101 CCTTTGCCTCATTCTCGCCAATGCGGCCTATGCGCTGTACGTGATATTTCTCGCTCGCTTCCTGGGCTGACCAGCAACGCGGCGCCGCATGCAGATGATC

1201 AGGCAAAGCTCCTTCGCCGGCGGGTGAGGGAAGGGACGTCCTCGACCGGGGGCAAGCT
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Fig. 3. Nucleotide sequence of the 1.3-kbp PstI fragment containing the Rhizobium meliloti 1021 diacylglycerol kinase gene. The predicted amino acid sequence of the diacylglycerol kinase enzyme is given above in single-letter code. The putative Shine-Dalgarno sequence is underlined.

membrane surface. With the exception of a helix-breaking, moderately hydrophobic proline near the middle of the putative amphipathic helix of the *R. meliloti* enzyme, a striking amount of identity is present in the polar face of the two putative amphipathic helices. Loomis *et al.* (1985) predicted an interaction between the three glutamyl residues present within this amphipathic helix and Arg10 and Lys13. Such an interaction may also occur between the conserved glutamyl residues and Lys18, Arg20, or Arg23 of the rhizobial enzyme.

Strong identity is also evident in a loop predicted to join the amphipathic helix with the third membrane-spanning helix (RAKDMGS in *E. coli* and NAKDLGS

ALIGNMENT OF *E. coli* AND *R. meliloti* DGK

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EcDGK    1 -------------MANNTTGFTRIIKAAGYSWKGLRAAWINEAAFRQEGVAVL
              ♦  ○○ ♦♦○♦♦○ ♦ ○   ♦ ♦♦♦♦♦ ♦ ○♦
RmDGK    1 MDAKNTTSRNGTAAPVHKHRGIRHLFAAASYSFGGAKRL-IGEAAFRHELIAFA

EcDGK   41 LAVVIACWLDVDAITRVLLISSVMLVMIVEILNSAIEAVVDRIGSEYHELSGRA
            ○♦○♦    ○  ○   ♦ ○   ○♦○♦  ♦ ○♦○♦♦♦ ○♦♦♦○○○♦ ♦○○ ♦
RmDGK   54 VAMVAFMIVGANLFQYVAMAILFLLMMAFEAINTAIEEIVDRVSPEISEMGRNA

EcDGK   95 KDMGSAAVLIAIIV-AVITWCILLWSHFG-
            ♦♦○♦♦ ♦ ♦ ♦○ ♦ ○ ○○ ○○♦
RmDGK  108 KDLGSFACLCLILANAAYALYVIFLARFLG
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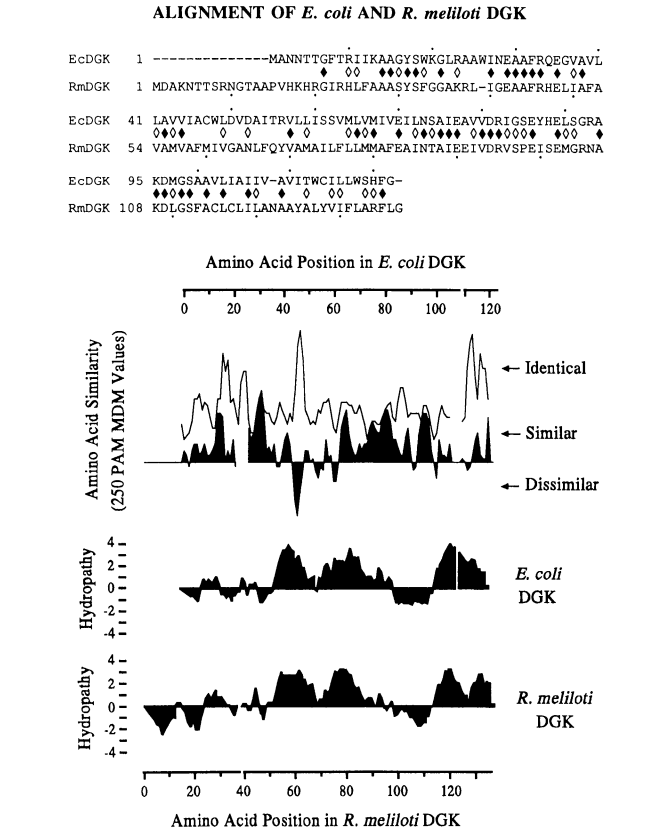Amino Acid Position in *E. coli* DGK

Fig. 4. Similarity between the *Rhizobium meliloti* 1021 and *Escherichia coli* diacylglycerol kinase enzymes. The ALIGN program of the PIR was used with a gap penalty of 16 and Mutation Data Matrix (MDM) values of 250 PAMs (George *et al.* 1988) to obtain an optimal alignment between the *R. meliloti* (Rm) and *E. coli* (Ec) diacylglycerol kinase sequences (11.6 SD units above the average randomized alignment score; 32% identity indicated by solid diamonds). Additional similarity is evident when conservative substitutions are permitted (open diamonds) with the amino acid groups: sulfhydryl (C); acid, acid amide, hydrophilic (DNEQ); basic (HKR); small hydrophilic (PTGAS); small hydrophobic (LIVM); and aromatic (FYW). This is also illustrated below the alignment by a plotting of a three-residue moving average of the 250 PAMs MDM values (shaded curve) with the values obtained for self-alignment of *E. coli* diacylglycerol kinase enzyme (DGK) to indicate 100% identity (unshaded curve). Three-residue windows containing gaps were omitted from each plot. Values above the baseline represent likely substitutions in conserved proteins, whereas those below it represent substitutions not likely to occur in conserved proteins. Hydropathy plots that used values of Kyte and Doolittle averaged over seven residue windows are aligned below the similarity plot (averages were calculated without gaps, and then gaps were introduced between appropriate windows to maintain the alignment).
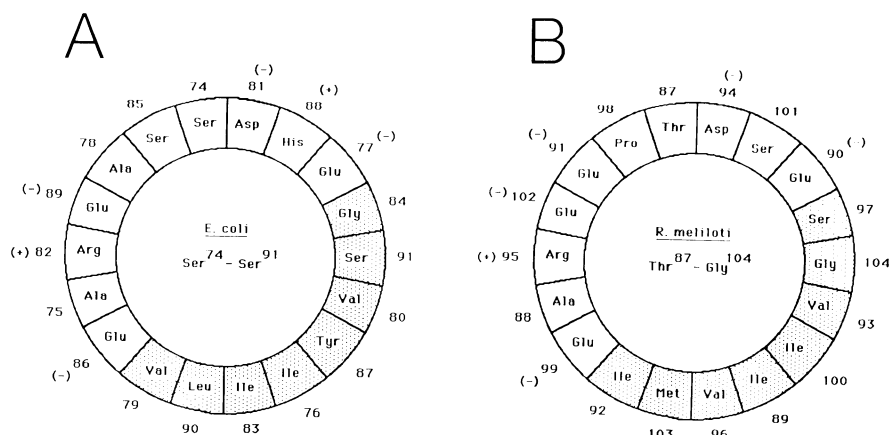
in *R. meliloti*), in another loop predicted to precede the first α-helix (AAGYS in *E. coli* and AASYS in *R. meliloti*), and in a rather highly charged region in the beginning of that helix (EAAFRQE in *E. coli* and EAAFRHE in *R. meliloti*). We note that the predicted hydrophilic nature of this region may not be compatible for forming part of a membrane-spanning helix, unless the charges interact with some other protein surface within the membrane. If this charged region is actually in the cytosolic environment, then the 36 residues joining this region with the proposed amphipathic helix may be too few to form two complete membrane-spanning helices. Experiments designed to explore the topology of these diacylglycerol kinase enzymes are clearly needed to test these tentative hypotheses.

Further comparison between the two enzymes reveals one notable difference: Unlike the *E. coli* enzyme, the rhizobial enzyme does not contain a cysteine residue within the first predicted transmembrane α-helix. Loomis *et al.* (1985) have suggested that a disulfide bond may form between Cys47 (putative transmembrane α-helix 1) and Cys114 (putative transmembrane α-helix 3) within the *E. coli* enzyme. However, a disulfide bond between putative transmembrane helices 1 and 3 would not be possible in the rhizobial enzyme.

The primary function of the diacylglycerol kinase enzyme of *E. coli* is to recycle diglyceride generated as a by-product of MDO biosynthesis. Indeed, it has been estimated that about two-thirds of the diglyceride produced by *E. coli* results from the transfer of phosphoglycerol head groups from phosphatidylglycerol to the MDO as catalyzed by phosphoglycerol transferase I (Rotering and Raetz 1983). Recently, we provided evidence that the primary source of diglyceride production in *R. meliloti* 1021 results from the transfer of phosphoglycerol head groups to the periplasmic cyclic β-1,2-glucans. Thus, we predict that the primary function of the diacylglycerol kinase enzyme of *R. meliloti* is to recycle diglyceride generated as a by-product of cyclic β-1,2-glucan biosynthesis. Furthermore, we predict that diacylglycerol kinase mutants of *R. meliloti* should be impaired for growth when exposed to conditions that favor high levels of cyclic β-1,2-glucan biosynthesis (e.g., low osmolarity; Miller *et al.* 1986). Thus, it is possible that diacylglycerol kinase mutants of *R. meliloti* 1021 could be utilized as background strains in future studies aimed at identifying genetic loci (e.g., through suppressor mutations) involved in various stages of cyclic β-1,2-glucan biosynthesis. In fact, this strategy should yield suppressor mutations for structural and regulatory genes involved with both backbone biosynthesis as well as phosphoglycerol substitution. Indeed, Kennedy and co-workers used this same strategy to identify *E. coli* mutants blocked for phosphoglycerol transfer to MDO (Jackson *et al.* 1984).

The isolation of a variety of rhizobial mutants defective for cyclic β-1,2-glucan biosynthesis is required for the further understanding of the roles of these unusual oligosaccharides during legume nodulation. To date, only two genes have been identified in *Rhizobium* that are involved in cyclic β-1,2-glucan biosynthesis: *ndvA* and *ndvB*. The *ndvA* gene product is involved in glucan export (Stanfield *et al.* 1988), whereas the *ndvB* gene encodes a 319-kDa protein involved in cyclic β-1,2-glucan biosynthesis (Ielpi

Fig. 5. Helical wheel projections for the predicted amphipathic helices of the **A**, *Escherichia coli* and **B**, *Rhizobium meliloti* diacylglycerol kinase enzymes. The helical wheel projection for residues 74-91 in the *E. coli* enzyme was previously presented by Loomis *et al.* (1985) with the graphical method described by Kaiser and Kezdy (1984). The same method was used, and a similar amphipathic helix is predicted for residues 87-104 of the rhizobial enzyme. The shaded amino acids in both diagrams represent amino acids that could interact with membrane lipids.
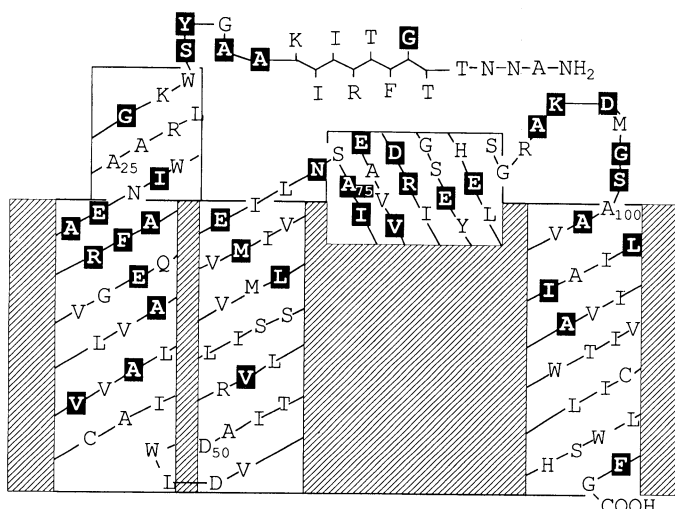

Fig. 6. Proposed model for the tertiary structure of the *Escherichia coli* diacylglycerol kinase enzyme. This tertiary structure was previously proposed by Loomis *et al.* (1985) for the *E. coli* enzyme. The predicted α-helical segments are outlined in boxes. The location of the three transmembrane-spanning helices and amphipathic helix is shown with respect to the phospholipid bilayer (diagonal shading). The amino acid residues present in both the *E. coli* and rhizobial enzymes are highlighted in shaded boxes.

et al. 1990). *R. meliloti* mutants at either locus are unable to invade alfalfa, and they elicit only ineffective, small white pseudonodules (Dylan *et al.* 1986; Dylan *et al.* 1990b). Although these studies indicate an important role for the cyclic β-1,2-glucans during legume nodulation, spontaneous symbiotic pseudorevertants of both classes of *ndv* mutants have recently been identified (Dylan *et al.* 1990b; Nagpal *et al.* 1992). Thus, although the cyclic β-1,2-glucans may provide important functions during legume nodulation, these molecules do not appear to be essential. However, these symbiotic pseudorevertants produce altered amounts of acidic exopolysaccharides (EPS), and Nagpal and co-workers have shown that *exo* genes are involved

in the suppression of the *ndv* mutant phenotype (Nagpal *et al.* 1992). On the basis of these results, Nagpal and co-workers suggested that cyclic β-1,2-glucans and EPS might interact during early nodule development. These researchers also proposed that the cyclic β-1,2-glucans are involved in the maintenance of turgor pressure within the elongating infection thread during nodulation (Nagpal *et al.* 1992). This possible function for the cyclic β-1,2-glucans is suggested from studies that revealed the *ndv* mutants to be impaired for growth at low osmolarity (Dylan *et al.* 1990b). Dylan and co-workers further suggested that the cyclic β-1,2-glucans act as signal molecules during legume nodulation (Dylan *et al.* 1990b). This signaling function is suggested from the finding that the addition of physiological levels of cyclic β-1,2-glucans to the alfalfa-*R. meliloti* nodulation assay system results in the enhancement of nodule number and nodulation kinetics (Dylan *et al.* 1990b).

Although the above studies indicate that the cyclic β-1,2-glucans are important for successful legume nodulation by *Rhizobium*, the functions of these unusual oligosaccharides and their substituents remain unclear. The identification of the diacylglycerol kinase gene of *R. meliloti* 1021 is the first step in a novel strategy for identifying additional genetic loci involved in cyclic β-1,2-glucan biosynthesis. Ultimately, these studies should lead to a better understanding of the roles of the cyclic β-1,2-glucans in the *Rhizobium*-legume symbiosis.

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