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The *lytB* Gene of *Escherichia coli* Is Essential and Specifies a Product Needed for Isoprenoid Biosynthesis

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LytB and GcpE, because they are codistributed with other pathway enzymes, have been predicted to catalyze unknown steps in the nonmevalonate pathway for isoprenoid biosynthesis. We constructed a conditional *Escherichia coli lytB* mutant and found that LytB is essential for survival and that depletion of LytB results in cell lysis, which is consistent with a role for this protein in isoprenoid biosynthesis. Alcohols which can be converted to pathway intermediates beyond the hypothesized LytB step(s) support limited growth of *E. coli lytB* mutants. An informatic analysis of protein structure suggested that GcpE is a globular protein of the TIM barrel class and that LytB is also a globular protein. Possible biochemical roles for LytB and GcpE are suggested.

The *Escherichia coli* K-12 chromosome has about 4,300 genes, approximately one-third of which have unverified or unknown functions (4). In order to extend the annotation of the *E. coli* genome, we have deleted uncharacterized genes and analyzed the resultant phenotypes. Among our targets was the gene that was called *yaaD* in EcoMap 9 (3), was later designated *slpA* (12), and now is designated *fkpB* (22) following its functional characterization as a gene that encodes a peptidyl-prolyl *cis-trans* isomerase of the FKBP family. We show here that our inability to delete *fkpB* in haploid bacteria can be attributed to the polar effect of deletion of *fkpB* on expression of the downstream gene *lytB*.

lytB is highly conserved and has a pattern of distribution similar to that of genes in the nonmevalonate pathway for isoprenoid biosynthesis used by bacteria and plants (7). Isoprenoids are universally required metabolites that in bacteria have roles in processes as diverse as respiration (ubiquinones) and cell wall synthesis (bactoprenols) (23). Recent evidence indicates that the *Synechocystis* sp. strain PCC 6803 *lytB* homolog is required for isoprenoid production and is probably essential for survival (7). We found that the *E. coli lytB* gene is essential for viability and has a phenotype consistent with a role in isoprenoid synthesis.

FkpB is dispensable but LytB is essential for survival in rich medium. In order to delete *fkpB*, a crossover PCR product was constructed (15) (Fig 1) and cloned into the *Pac*I site of pNEB193 (New England Biolabs). (Crossover PCR produces a fragment with a central deletion.) A Cmp^r cassette was inserted into the central *Ecl*136II site to obtain pfkpB<>CAT. (In this paper we use the nomenclature suggested by Yu et al. [24], where a<>b means that gene a is replaced by gene b.) P1 phage transduction was used to recover MG1655 mutants in which *fkpB* had been replaced by CAT, as described previously (8). P1 prepared on CAG18442 (pfkpB<>CAT), in which *thr-39*::Tn*10* (Tet^r) is <1 min to the left of *fkpB*, was used to transduce MG1655 to Cmp^r with Tet^r as an external linked marker. Of several hundred Tet^r Cmp^r progeny screened, none was Amp^s (i.e., had resolved the duplication resulting from plasmid insertion to replace *fkpB*), suggesting that *fkpB* could not be deleted and might be essential.

We modified our procedure to use an external marker, *dapB*, very closely linked to *fkpB* (Fig. 1) and introduced compatible, complementing plasmids into both the donor and the recipient so that chromosomal deletants did not lack essential gene functions. The donor used was MG1655(pfkpB<>CAT, pGB-XY), and the *dapB* recipient was AT999(pGB-XY). pGB-XY contains the insert from pGM21 (17) (kindly provided by E. Ishiguro) which includes the contiguous *ileS*, *lsp*, *fkpB*, and *lytB* genes and part of the downstream *yaaF* gene recloned into pGB2 (6), a Spc^r pSC101 replicon. *fkpB* and *lytB* are thought to be transcribed from promoters located within *ileS* (18). Dap$^+$ Cmp^r Amp^s transductants were obtained from the cross, indicating that replacement is possible in the presence of pGB-XY. Replacement of *fkpB* was verified by Southern blotting, and the close linkage of *dapB* and Cmp^r was confirmed by further transduction (data not shown). The deletion/replacement was transduced to MG1655(pGB-XY), selecting for Cmp^r, but could not be transferred to plasmid-free recipients.

Because *fkpB* is cotranscribed with the downstream *lytB* gene, it was not clear whether *fkpB* or *lytB* or both are essential. To determine this, we transduced the *fkpB* replacement to MG1655 with extrachromosomal copies of both *fkpB* and *lytB* on pSP47, of only *fkpB* on SP47ΔlytB, or of only *lytB* on pBAD-L (pBAD18 [9] in which the *lytB* coding sequence was cloned under the control of the arabinose operon promoter, P_{BAD}). The chromosomal DNA in these plasmids is shown in Fig. 1. Table 1 shows that *fkpB* is dispensable but LytB is not. The growth rate of MG1655 *fkpB*<>CAT (pBAD-L) on Luria-Bertani (LB) medium containing arabinose was identical to that of MG1655 (data not shown). Microscopic observation showed that cells were normal in appearance. We concluded that *fkpB* is not needed for normal growth under these conditions.

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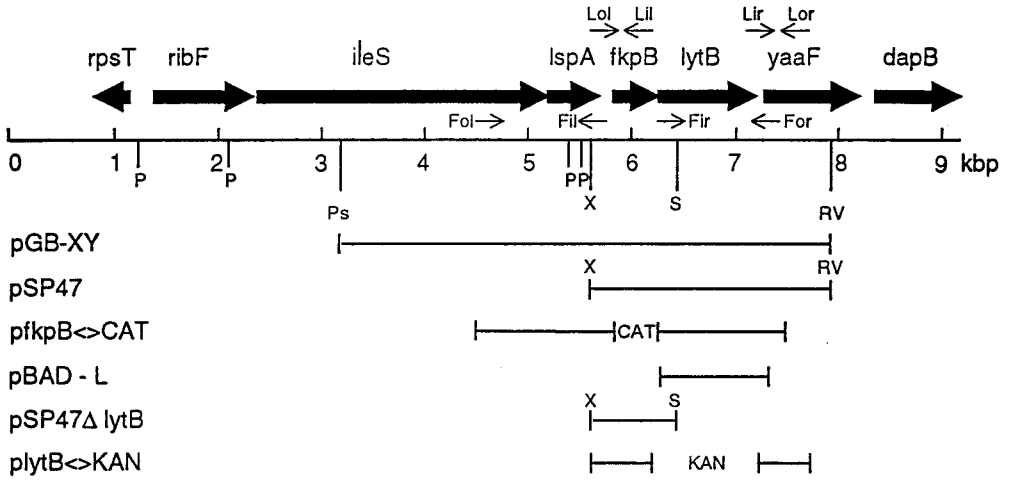

FIG. 1. The 0.5-min region of the *E. coli* chromosome. The genes referred to in this paper are drawn to scale, and the directions of transcription are indicated by the arrowheads. Promoters predicted from the sequence (3) are indicated by P. Chromosomal inserts in the plasmids are shown below the map as lines of the appropriate lengths. The chromosomal restriction sites used in plasmid construction are shown (Ps, *Pst*I; X, *Xba*I; RV, *Eco*RV; S, *Sna*BI). pSP47ΔlytB was constructed from pSP47 by deletion; the inserts for the remaining plasmids, other than pGB-XY, were constructed by using PCR amplification. The following primers were used to construct deletion plasmid pfkpB<>CAT: Fol (5′AATTCGCGT ATTAATTAAACGATTTCCACGAAGTG), For (5′AATTCTCCGCATTAATTAAATGCAGCAGTTGCAGG), Fil (5′CCGTGTACCCGGG AGCTCGATGCGTCTGTACAGATTCAGACATGCAGG), and Fir (5′CGCATCGAGCTCCCGGGTACACGGCGTAACATGCAGATCCT GTTGGCC). The following primers were used to construct deletion plasmid plytB<>KAN: Lol (5′ATTGCTGCGAAATCGTCGACCG), Lil (5′AACCGTGTAGCGGCCGCGTAGCGTGTTACGCCTCCAGTGCCGGATCG), Lor (5′ATCACCAGCCCGGGAATATACG), and Lir (5′ACGCTACGCGGCCGCTACACGGTTGTCATTAGCAGCCTAAGTTATGCG). The DNA between the primer pairs is absent from the chromosomes of deletion strains.

To study LytB function further, we constructed a conditionally expressing system. Crossover PCR was used to construct a 1-kb fragment in which the sequence flanking *lytB* was joined. The fragment was cloned into the *Sma*I/*Sal*I sites of pKO3 (15), and a Kan^r cassette was cloned into the central *Not*I site created during amplification. The resulting plasmid, plytB<>KAN, was transformed into MG1655(pBAD-L) grown with arabinose, and replacements were isolated as described previously (15). A representative construct, strain MGΔLy, was unable to form colonies under conditions in which P_{BAD} is inactive (arabinose absent, glucose present), confirming that LytB is an essential protein.

TABLE 1. Successful transduction of fkpB<>CAT requires *lytB* expression

Plasmid	Arabinose	No. of Cmp^r transductants
pSP47[a]	NA[c]	200
pSP47ΔL[b]	NA	2[d]
pBAD18	−	6[d]
pBAD18	+	3[d]
pBAD-L	−	1[d]
pBAD-L	+	223

[a] pSP47 (a gift from E. Ishiguro) is pET30-c in which an *Xba*I-*Eco*RV fragment of the pGM21 (17) insert was cloned. pSP47 lacks all DNA upstream of the final 20 bp of *lsp* (which contains a putative σ70 promoter).

[b] pSP47ΔL was made by deleting all DNA between the *Sna*BI site 240 bp downstream from the *lytB* start and the *Eco*RV site at the end of the region cloned in pSP47.

[c] NA, not applicable.

[d] The small number of Cmp^r transductants were Amp^r and most likely resulted from insertion and transduction of the complementing plasmid from donor to recipient.

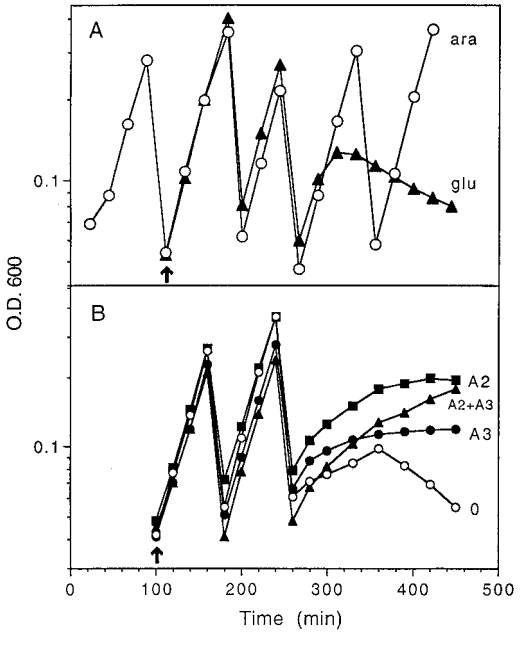

FIG. 2. Growth of MGΔLy after depletion of LytB. Cultures grown in LB medium containing 0.2% arabinose and 50 μg of ampicillin per ml were diluted and grown in the same medium for 3.5 generations. At the times indicated by the arrows cultures were diluted into medium with arabinose or glucose (0.2%). Cultures were maintained in the exponential phase at all times. Large decreases in optical density at 600 nm (O.D. 600) indicate times of dilution. (A) Growth with arabinose or glucose; (B) cultures contain glucose and each of the alcohols indicated at a concentration of 10 mM.

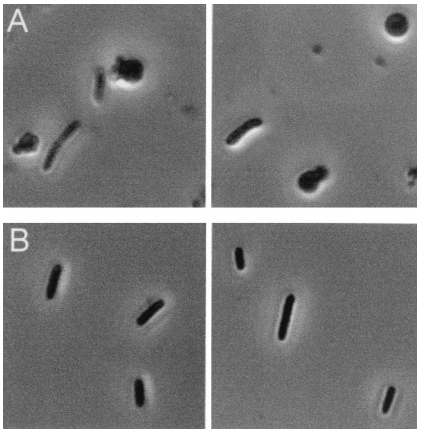

FIG. 3. Cell lysis in LytB-depleted cultures. Samples were taken from the cultures described in the legend to Fig. 2A at 360 min, fixed, and later photographed. (A) Spheroplasting in LytB-depleted cultures grown with glucose; (B) cells grown with arabinose.

Evidence that LytB is defective in isoprenoid synthesis. Because LytB synthesis in MGΔLy cultures could be inhibited by removing arabinose and adding glucose, it was possible to examine the effects of LytB depletion on cells. During depletion, growth continued normally for about 3 h and then slowed; lysis followed at about 4.6 h (Fig. 2A). Examination of the cultures showed that cells were converted to spheroplasts en route to lysis (Fig. 3). This phenotype can be explained since isoprenoids are required to make the bactoprenols which transport peptidoglycan precursors to the periplasm. Cunningham et al. (7) used 3-methyl-3-buten-1-ol (A3) and 3-methyl-2-buten-1-ol (A2), alcohol analogs of 3-methyl-3-buten-1-ol diphosphate (isopentenyl diphosphate [IPP]) and 3-methyl-2-buten-1-ol diphosphate (dimethyallyl diphosphate [DMAPP]) (see pathway in Fig. 4), to support the growth of *Synechocystis* cells deficient in LytB. We tested these alcohols to see if they were able to replace the requirement for LytB in *E. coli*. We found that they could not (presumably because they were not efficiently converted to the diphosphorylated derivatives that they would need to replace) during exponential growth in broth. However, they must have been successfully transported into the cell to some extent because they prevented lysis and allowed growth to continue for a period beyond the time when lysis would have occurred (Fig. 2B). The response to A2 was better than the response to A3 and was similar to the response to the two alcohols together. On LB medium plates containing glucose (to repress P_{BAD}) adding A2 or both alcohols resulted in slow colony formation, most likely because viability was sustained until changed intracellular conditions resulted in P_{BAD} induction. To show that the alcohols circumvent the *lytB* mutation rather than prevent lysis generally, we added the alcohols to *dapA* cells which had been deprived of diaminopimelic acid. The time and rate of lysis of the *dapA* mutant were not altered.

We also constructed a strain in which a single copy of *lytB* was present on the chromosome under the control of the P_{BAD} promoter by using the method and a plasmid kindly provided by Hans Loferer (2). This strain, MG1655 *araBCD<>lytB*, was not able to form colonies in the absence of arabinose on rich or minimal solid media with or without alcohols, showing that the alcohols could not replace LytB activity. Addition of alcohols to broth cultures of this strain delayed lysis (data not shown).

Nonmevalonate pathway and LytB function. The 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway (14) is used in green plants and many bacteria instead of the mevalonate pathway to

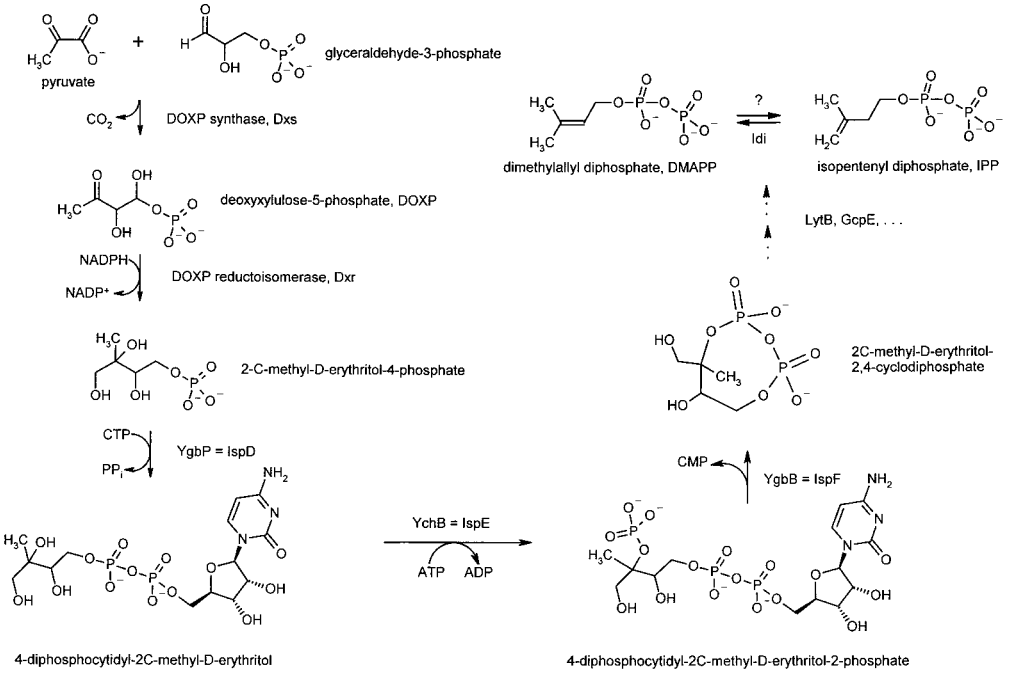

FIG. 4. Known and postulated reactions of the DOXP pathway.

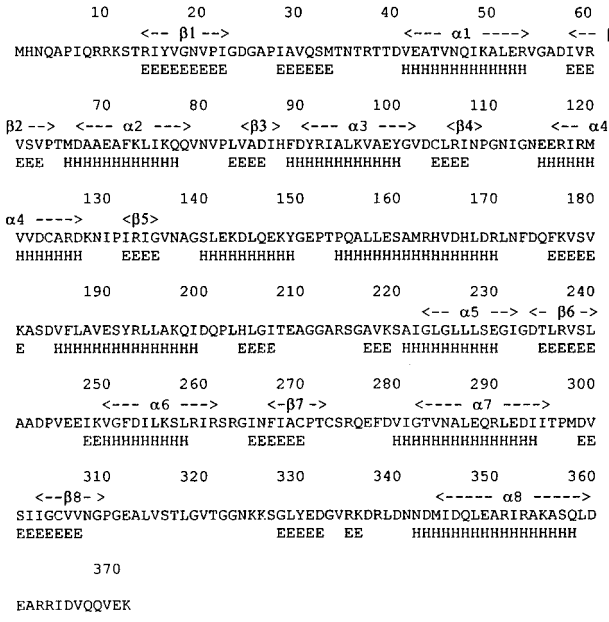

FIG. 5. Predicted secondary and tertiary structures for GcpE. H and E beneath the protein sequence indicate that the corresponding residues are predicted by PHD (21) to form parts of an α-helix and an extended structure (β-sheet), respectively. Information above the sequence indicates the predicted positions and extents of the backbone α- and β-units of the TIM barrel tertiary structure, inferred from a comparison of the sequences of homologues of GcpE and of the PDB structure 1THF.

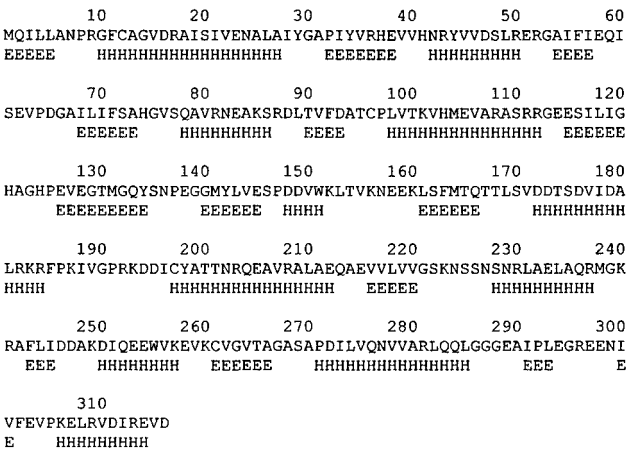

FIG. 6. Predicted secondary structure for LytB. For an explanation of the labels see the legend to Fig. 5.

generate the isoprenoid precursors IPP and DMAPP. This pathway (Fig. 4) originates with pyruvate and glyceraldehyde-3-phosphate. Almost all of the biochemical steps are now known.

The most recently identified reactions and genes are those that follow the formation of DOXP. The first reaction is simultaneous reduction and isomerization to 2-*C*-methyl-D-erythritol-4-phosphate, catalyzed by the *dxr* gene product. This enzyme has been isolated from *E. coli* and characterized (13). The chemistry of the three following steps has also been established: CTP-dependent cytidylation catalyzed by YgbP (20) (now renamed IspD; see EcoCyc at www.ecocyc.org/ for the *isp* pathway), ATP-dependent phosphorylation catalyzed by YchB (now IspE) (16), and cyclization with loss of CMP catalyzed by YgbB (now IspF) (11). Uncertainty remains about the final steps between 2*C*-methyl-D-erythritol-2,4-cyclodiphosphate and the isomers IPP and DMAPP. It has been shown (10) that although *idi*, the IPP isomerase gene of the mevalonate pathway, is present and active in *E. coli*, it is dispensable, suggesting that DMAPP and IPP are produced independently in the DOXP pathway; additional evidence that this is the case has been presented recently (19).

The remaining chemical steps in the pathway are two reductions (reduction of a primary alcohol and reduction of a secondary alcohol) and a ring-opening elimination reaction. Either IPP or DMAPP could be the product of this reaction, depending on which carbon contributes the hydrogen atom to the elimination. There are no known examples of reduction of alcohols in a single enzymatic step. Most commonly, these reactions occur in two steps: dehydration followed by enoyl reduction by NADPH. These considerations imply either that there are still a number of unrecognized genes in the DOXP pathway or that the alcohol reduction steps occur by non-pathway-specific mechanisms.

The two remaining genes with a pattern of homology that indicates that their functions may be specific to this pathway are *lytB* and *gcpE* (7). Our results and those of Cunningham et al. (7) show that *lytB* is an essential gene in the pathway. *gcpE* has recently also been shown to be essential (1, 5). Secondary-structure predictions (Fig. 5 and 6) indicate that both proteins are globular, α/β proteins with generally alternating α-helix and β-strand units. For GcpE this secondary structure was shown to be compatible with the eight-strand, αβ-TIM barrel tertiary structure (data not shown). For neither protein is there structural or sequence evidence for a binding site of a reduced coenzyme. If the alcohol level reductions are carried out by non-pathway-specific reductase systems, then it is possible that LytB and GcpE both catalyze elimination reactions and that the branching of the pathway is due to these enzymes acting in parallel.

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ADDENDUM IN PROOF

After this paper was submitted, Altincicek et al. (A. Altincicek, A.-K. Kollas, M. Eberl, J. Wiesner, S. Sanderbrand, M. Hintz, E. Beck, and H. Jomaa, FEBS Lett. **499:**37–40, 2001) also reported that the *E. coli lytB* gene is an essential gene of isoprenoid biosynthesis.

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