LytB, a novel gene of the 2-*C*-methyl-D-erythritol 4-phosphate pathway of isoprenoid biosynthesis in *Escherichia coli*

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Abstract The mevalonate-independent 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway for isoprenoid biosynthesis is essential in many eubacteria, plants, and the malaria parasite. Using genetically engineered *Escherichia coli* cells able to utilize exogenously provided mevalonate for isoprenoid biosynthesis by the mevalonate pathway we demonstrate that the *lytB* gene is involved in the trunk line of the MEP pathway. Cells deleted for the essential *lytB* gene were viable only if the medium was supplemented with mevalonate or the cells were complemented with an episomal copy of *lytB*.

Key words: Isoprenoid biosynthesis; 2-*C*-Methyl-D-erythritol 4-phosphate pathway; *lytB*

1. Introduction

Isoprenoids play essential roles in all organisms, as in electron transfer, photosynthesis, membrane stability, and cell signalling. In animals, fungi, archaebacteria, and certain bacteria the biosynthesis of isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP), the precursors of all isoprenoids, proceeds exclusively by the mevalonate (MVA) pathway. However, in many eubacteria, including *Escherichia coli*, algae, plastids of plants, and in the apicomplexan protozoa *Plasmodium falciparum*, the alternative MVA-independent 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway is operative for isoprenoid biosynthesis [1–4].

Although the first five enzymatic steps of the MEP pathway have been described, the terminal steps are still unknown. The pathway initiates with the formation of 1-deoxy-D-xylulose 5-phosphate (DOXP) by condensation of pyruvate and D-glyceraldehyde 3-phosphate catalyzed by the DOXP synthase (Dxs). DOXP is then converted into MEP by the DOXP reductoisomerase (Dxr) (Fig. 1). The enzymes encoded by the genes *ygbP* (*ispD*), *ychB* (*ispE*) and *ygbB* (*ispF*) mediate the formation of 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate, with 4-diphosphocytidyl-2-*C*-methyl-D-erythritol and 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate as intermediates [1–4].

By constructing deletion mutants of *E. coli* it was demonstrated that all known enzymes are involved in the trunk line of the MEP pathway in vivo [5,6] and that the pathway branches to synthesize IPP and DMAPP [7] (Fig. 1). These experiments were carried out with *E. coli* cells genetically engineered to be able to utilize exogenously provided MVA for isoprenoid biosynthesis by the classical MVA pathway. Using these cells it is possible to identify novel enzymes involved in the trunk line of this pathway as recently shown for GcpE [8,9].

Recently, evidence was given that an enzyme encoded by the *lytB* gene in the cyanobacterium *Synechocystis* catalyzes an essential step at, or subsequent to, the point at which the MEP pathway branches to form IPP and DMAPP [10]. *LytB* was originally described in *E. coli* as a gene involved in mediating penicillin tolerance [11], and subsequently identified in various other Gram-negative bacteria [12]. Using a genetic approach we now demonstrate that LytB is essentially involved in the trunk line of the MEP pathway in *E. coli*.

2. Materials and methods

2.1. Strains and media

All plasmids were constructed in *E. coli* TOP10 (Invitrogen). For gene replacement experiments, the recombination proficient wild-type K-12 strain *E. coli* DSM No. 498 (ATCC 23716) was used. Bacteria were grown in Standard 1 medium (Merck) at 37°C with aeration. For solid medium, agar (Difco Bacto agar) was added to 1.5% (w/v). Media were supplemented with 150 μg/ml ampicillin, 25 μg/ml chloramphenicol or 100 μM MVA, where appropriate. MVA was prepared as described [7]. For selection against *sacB*, salt-free LB medium [13] was supplemented with sucrose to a final concentration of 6% (w/v).

2.2. Recombinant DNA techniques

Plasmid isolation, agarose gel electrophoresis, ligation and transformation of plasmid DNA were carried out according to standard protocols [14]. For analytical plasmid preparation, the GFX™ Micro Plasmid Prep kit (Amersham Pharmacia) was used. DNA fragments were gel purified using the Easy Pure kit (Biozym Diagnostik). Restriction endonuclease digestions were carried out according to the specified recommendations of the manufacturer (Promega).

2.3. PCR

All PCRs were performed in a total volume of 20 μl using a Stratagene robocycler with a heated lid and the expand high fidelity PCR system (Roche Diagnostics). An initial denaturation at 94°C for 1 min was followed by 30 cycles of denaturation at 94°C for 30 s, annealing

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Abbreviations: DMAPP, dimethylallyl pyrophosphate; DOXP, 1-deoxy-D-xylulose 5-phosphate; IPP, isopentenyl pyrophosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; MVA, mevalonate

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Table 1
Oligonucleotides used in this study

Name	Sequence
LytB-N-out	5′-TAGGATCCCCGGCCTACAGATTGCTGCG-3′
LytB-N-in	5′-CCCATCCACTAAACTTAAACACAACAGGATCTGCA-TGTTACG-3′
LytB-C-in	5′-TGTTTAAGTTTAGTGGATGGGCGTGAAGTCGATT-AAGTCAT-3′
LytB-C-out	5′-TAGTCGACAGAACCACCCATGATCACC-3′
LytB-con-N	5′-CGATAAAACCACCTTCTCGT-3′
LytB-con-C	5′-ATTGCGGGTAGTTTTCTCAA-3′
Ec-LytB-for	5′-GGATCCATGCAGATCCTGTTGGCCAAC-3′
Ec-LytB-rev	5′-AAGCTTTTAATCGACTTCACGAATATCG-3′

at 50°C for 30 s and extension at 72°C for 30 s to 90 s, dependent on the expected size of the products. A final 7-min 72°C step was added to allow complete extension of the products.

2.4. Construction of the gene replacement plasmids pKO3-ΔlytB

For generation of precise in-frame deletion mutants of *E. coli*, the pKO3 vector was used [15]. Crossover PCR deletion products were constructed basically as described previously [15]. First, two different asymmetric PCRs were used with the primer pair LytB-N-out and LytB-N-in to generate a fragment upstream (559 bp) and with the primer pair LytB-C-in and LytB-C-out to generate a fragment downstream (537 bp) of the sequence targeted for deletion (Table 1). The primer pairs were in a 10:1 molar ratio (500 nM outer primer and 50 nM inner primer). Then, both fragments were annealed at their overlapping region and amplified to a single fragment, using 500 nM of the outer primers, LytB-N-out and LytB-C-out. The resulting fragment was cloned using the pCR-TOPO-TA cloning kit (Invitrogen) and verified by restriction analysis and sequencing. The fragment was released from the pCR-TA vector by *Bam*HI and *Sal*I digestion, gel-purified, ligated into the *Bam*HI- and *Sal*I-digested pKO3 vector, and transformed into wild-type *E. coli*. Colonies growing on chloramphenicol plates at 30°C were screened for inserts by analytical plasmid preparation and restriction analysis.

2.5. Construction of the mutant strain wtΔlytB

Gene replacement experiments were carried out as described previously except for supplementing the plates with 100 μM MVA [15]. The gene replacement plasmid pKO3-Δ*lytB* was transformed into wild-type *E. coli* cells harboring the pSC-MVA plasmid [8] and allowed to recover for 1 h at 30°C. Bacteria with the plasmid integrated into the chromosome were selected by a temperature shift to 43°C. By screening for sucrose resistance and chloramphenicol sensitivity, bacteria with lost vector sequences were selected and tested for the desired genotype by PCR. The *lytB* deletion was confirmed using the primer pair LytB-con-N and LytB-con-C. Bacteria with the desired deletion as verified by PCR were tested for growth with and without MVA.

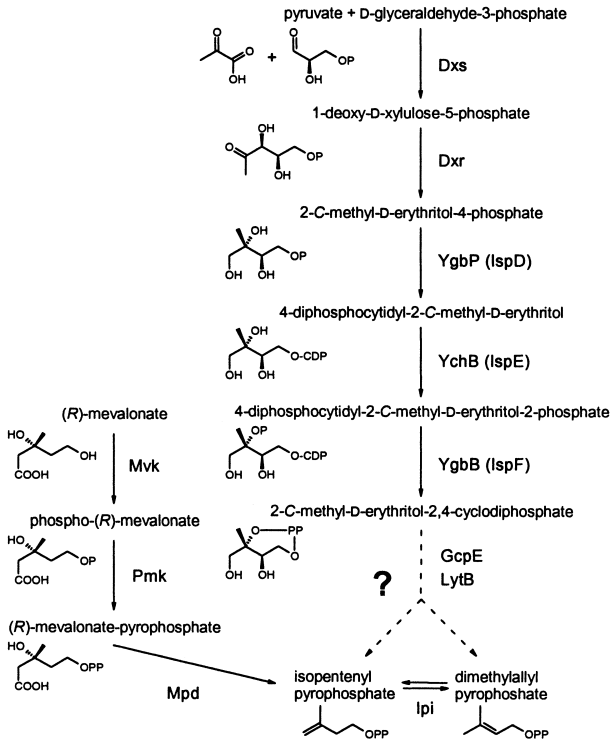

Fig. 1. The MEP pathway of IPP and DMAPP biosynthesis in *E. coli*, and genetically engineered synthesis of IPP from exogenously supplied MVA. Interrupted lines indicate not fully elucidated steps. Mvk, MVA kinase; Pmk, phosphomevalonate kinase; Mpd, MVA pyrophosphate decarboxylase; Dxs, DOXP synthase; Dxr, DOXP reductoisomerase; Ipi, IPP isomerase.

2.6. Complementation experiment

The mutant strain wtΔ*lytB* was complemented by transformation

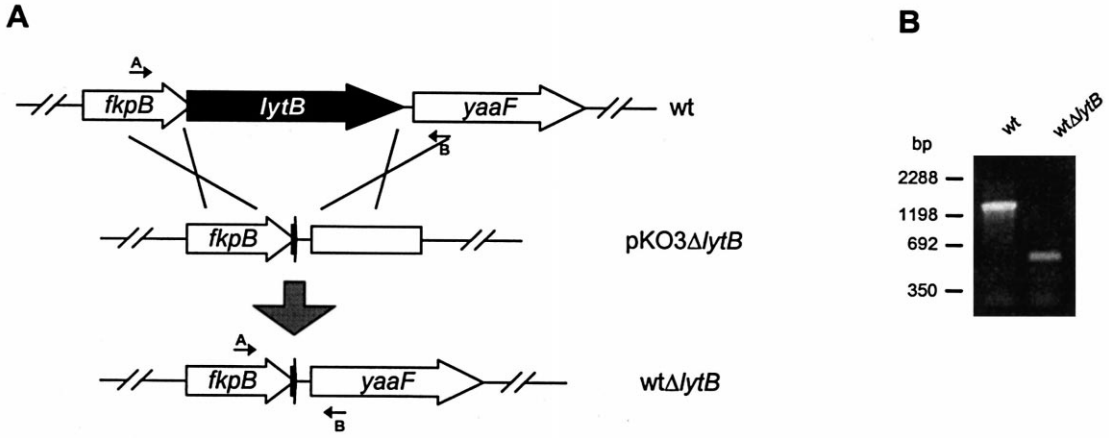

Fig. 2. Gene replacement of *lytB* with a precisely engineered deletion. A: Diagram of the *lytB* region of the wild-type strain and the *lytB* deletion mutant. Small arrows indicate the primer sites used for PCR analysis. The primers were: A, LytB-con-N; B, LytB-con-C. B: Verification of the deletion of the *lytB* gene by PCR. After selection for integrates of the gene replacement vector pKO3-Δ*lytB* into the chromosome at 43°C, bacteria were plated at 30°C on sucrose medium and replica plated onto chloramphenicol plates. The chloramphenicol-sensitive, sucrose-resistant colonies were screened by PCR. The PCR product of 560 bp using the primer pair A plus B of the *lytB* mutant strain is the expected 880 bp smaller than the wild-type product of 1440 bp.

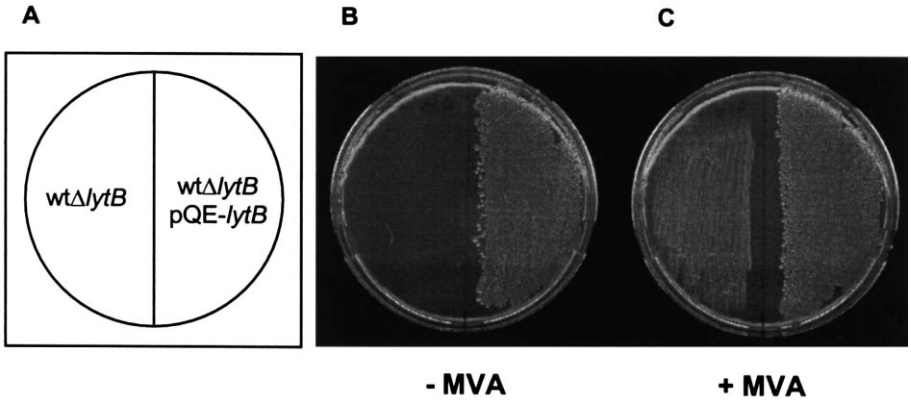

Fig. 3. Growth of the *E. coli* strains indicated in (A) (wtΔ*lytB*, *lytB* deletion mutant; wtΔ*lytB* pQE-*lytB*, *lytB* deletion mutant complemented with an episomal copy of the *lytB* gene) on medium without (B) and with (C) MVA.

with the plasmid pQE-*lytB*. This plasmid was constructed by inserting the PCR fragment generated using the primers Ec-LytB-for and Ec-LytB-rev into the *Bam*HI and *Hin*dIII sites of the vector pQE30 (Qiagen).

3. Results and discussion

LytB homologs exists in many eubacteria, plants, and in *P. falciparum* but are not detectable in animals, archaebacteria or in *S. cerevisiae*. The distribution pattern throughout the species is identical to Dxr (Table 2). N-terminal sequence extensions of the LytB homologs of the plant *Arabidopsis thaliana* and the parasite *P. falciparum* are likely to represent signal sequences targeting the polypeptides into the plastids of plants and the apicoplast (a plastid-like organelle) of malaria parasites, respectively (data not shown). Importantly, it needs to be stressed that the *E. coli lytB* gene characterized in the present study [11] is not homologous to the pneumococcal and streptococcal genes encoding murein hydrolases [16,17] that incidentally carry the same name.

In order to demonstrate a role for *lytB* in the MEP pathway, *E. coli* cells with a disrupted *lytB* gene were constructed and analyzed for loss of their ability to synthesize isoprenoids by the MEP pathway. Since *E. coli* mutants blocked in isoprenoid biosynthesis are not viable under normal growth conditions [18], *E. coli* transformants capable of utilizing MVA for IPP synthesis were used. This strain carries an artificial operon (pSC-MVA) encoding MVA kinase (Mvk), phosphomevalonate kinase (Pmk), and MVA pyrophosphate decarboxylase (Mpd) from yeast (Fig. 1) [8]. To inactivate the *lytB* gene, its coding sequence was completely removed from the bacterial genome by homologous recombination and replaced by a synthetic 21 bp sequence (Fig. 2A). This was accomplished by using the pKO3-Δ*lytB* gene replacement vector that allows the generation of a precise in-frame deletion mutant in *E. coli* wild-type strain. Bacteria containing the desired *lytB* deletion were identified by PCR analysis (Fig. 2B). Cells deleted for the essential *lytB* gene were viable only if the medium was supplemented with MVA or the cells were complemented with an episomal copy of *lytB* (Fig. 3). These data demonstrate that LytB acts in the trunk line of the MEP pathway upstream of the branching point and most probably after the formation of 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (Fig. 1).

Since in the present study LytB could be identified as another enzyme essential for the MEP pathway that is present in some important pathogenic agents but absent in mammalians, it represents an attractive drug target for a new class of antibiotics and antimalarials [19–22].

Table 2
Accession numbers of LytB and Dxr homologs in various organisms

Organism	LytB	Dxr
Eubacteria		
Aquifex aeolicus	sp O67625	sp P45568
Bacillus subtilis	sp P54473	sp O31753
Chlamydia pneumoniae	sp Q9Z6P2	sp Q9Z8J8
Chlamydia trachomatis	sp O84867	sp O84074
E. coli	sp P22565	sp P45568
Haemophilus influenzae	sp P44976	sp P44055
Helicobacter pylori	sp O25160	sp P56139
Mycobacterium tuberculosis	sp O53458	sp Q10798
Synechocystis PCC6803	sp Q55643	sp Q55663
Thermotoga maritima	sp Q9X1F7	sp Q9WZZ1
Treponema pallidum	sp O83558	sp O83610
Neisseria meningitidis	tr Q9JR39	sp Q9JX33
Campylobacter jejuni	sp P94644	sp Q9PMV3
Deinococcus radiodurans	tr Q9RSG0	sp Q9RU84
Pseudomonas aeruginosa	tr Q9HVM7	sp Q9KGU6
Vibrio cholerae	pir G82293	sp Q9KPV8
Staphylococcus aureus	–	–
Streptococcus pyogenes	–	–
Streptococcus pneumoniae	–	–
Borrelia burgdorferi	–	–
Mycoplasma genitalium	–	–
Mycoplasma pneumoniae	–	–
Rickettsia prowazekii	–	–
Archaebacteria		
Archaeoglobus fulgidus	–	–
Methanobacterium thermoautotrophicum	–	–
Aeropyrum pernix K1	–	–
Methanococcus jannaschii	–	–
Pyrococcus horikoshii	–	–
Halobacterium sp. *NRC-1*	–	–
Pyrococcus abyssi	–	–
Eucaryota		
P. falciparum	gb AAK12102	tr O96693
A. thaliana	pir T04781	sp Q9XFS9
Saccharomyces cerevisiae	–	–
Drosophila melanogaster	–	–
Caenorhabditis elegans	–	–
Homo sapiens	–	–

– means not detectable.

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References

[1] Rohmer, M. (1999) Nat. Prod. Rep. 16, 565–574.
[2] Boucher, Y. and Doolittle, W.F. (2000) Mol. Microbiol. 37, 703–716.
[3] Lange, B.M., Rujan, T., Martin, W. and Croteau, R. (2000) Proc. Natl. Acad. Sci. USA 97, 13172–13177.
[4] Eisenreich, W., Rohdich, F. and Bacher, A. (2001) Trends Plant. Sci. 6, 78–84.
[5] Takagi, M., Kuzuyama, T., Takahashi, S. and Seto, H. (2000) J. Bacteriol. 182, 4153–4157.
[6] Campos, N., Rodríguez-Concepción, M., Sauret-Gueto, S., Gallego, F., Lois, L.M. and Boronat, A. (2001) Biochem. J. 353, 59–67.
[7] Rodríguez-Concepción, M., Campos, N., Lois, L.M., Maldonado, C., Hoeffler, J.F., Grosdemange-Billiard, C., Rohmer, M. and Boronat, A. (2000) FEBS Lett. 473, 328–332.
[8] Altincicek, B., Kollas, A.K., Sanderbrand, S., Wiesner, J., Hintz, M., Beck, E. and Jomaa, H. (2001) J. Bacteriol. 183, 2411–2416.
[9] Campos, N., Rodríguez-Concepción, M., Seemann, M., Rohmer, M. and Boronat, A. (2001) FEBS Lett. 488, 170–173.
[10] Cunningham, F.X., Lafond, T.P. and Gantt, E. (2000) J. Bacteriol. 182, 5841–5848.
[11] Gustafson, C.E., Kaul, S. and Ishiguro, E.E. (1993) J. Bacteriol. 175, 1203–1205.
[12] Potter, S., Yang, X., Boulanger, M.J. and Ishiguro, E.E. (1998) J. Bacteriol. 180, 1959–1961.
[13] Blomfield, I.C., Vaughn, V., Rest, R.F. and Eisenstein, B.I. (1991) Mol. Microbiol. 5, 1447–1457.
[14] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1987) Current Protocols in Molecular Biology, John Wiley and Sons, New York.
[15] Link, A.J., Phillips, D. and Church, G.M. (1997) J. Bacteriol. 179, 6228–6237.
[16] García, P., González, M.P., García, E., López, R. and García, J.L. (1999) Mol. Microbiol. 31, 1275–1277.
[17] López, R., González, M.P., García, E., García, J.L. and García, P. (2000) Res. Microbiol. 151, 437–443.
[18] Takahashi, S., Kuzuyama, T., Watanabe, H. and Seto, H. (1998) Proc. Natl. Acad. Sci. USA 95, 9879–9884.
[19] Jomaa, H., Wiesner, J., Sanderbrand, S., Altincicek, B., Weidemeyer, C., Hintz, M., Turbachova, I., Eberl, M., Zeidler, J., Lichtenthaler, H.K., Soldati, D. and Beck, E. (1999) Science 285, 1573–1576.
[20] Altincicek, B., Hintz, M., Sanderbrand, S., Wiesner, J., Beck, E. and Jomaa, H. (2000) FEMS Microbiol. Lett. 190, 329–333.
[21] Wiesner, J., Hintz, M., Altincicek, B., Sanderbrand, S., Weidemeyer, C., Beck, E. and Jomaa, H. (2000) Exp. Parasitol. 96, 182–186.
[22] Reichenberg, A., Wiesner, J., Weidemeyer, C., Dreiseidler, E., Sanderbrand, S., Altincicek, B., Beck, E., Schlitzer, M. and Jomaa, H. (2001) Bioorg. Med. Chem. Lett. 11, 833–835.