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GcpE Is Involved in the 2-*C*-Methyl-D-Erythritol 4-Phosphate Pathway of Isoprenoid Biosynthesis in *Escherichia coli*

BORAN ALTINCICEK,* ANN-KRISTIN KOLLAS, SILKE SANDERBRAND, JOCHEN WIESNER, MARTIN HINTZ, EWALD BECK, AND HASSAN JOMAA

Institute of Biochemistry, Academic Hospital Centre, Justus Liebig University, D-35392 Giessen, Germany

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In a variety of organisms, including plants and several eubacteria, isoprenoids are synthesized by the mevalonate-independent 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway. Although different enzymes of this pathway have been described, the terminal biosynthetic steps of the MEP pathway have not been fully elucidated. In this work, we demonstrate that the *gcpE* gene of *Escherichia coli* is involved in this pathway. *E. coli* cells were genetically engineered to utilize exogenously provided mevalonate for isoprenoid biosynthesis by the mevalonate pathway. These cells were then deleted for the essential *gcpE* gene and were viable only if the medium was supplemented with mevalonate or the cells were complemented with an episomal copy of *gcpE*.

In all organisms studied so far, isoprenoids derive from the common isoprene units, isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). In mammals and in fungi, IPP and DMAPP are formed exclusively by the mevalonate pathway (11). In contrast, many eubacteria (including *Escherichia coli*), algae, and the plastids of higher plants synthesize IPP and DMAPP by the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (9, 34). The MEP pathway was also identified in a plastid-like organelle of malaria parasites (15). Since the MEP pathway is absent in humans, it has been validated as a drug target for the treatment of both bacterial and parasitic infections (15, 29).

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) (1, 6, 20, 22, 24, 25, 35, 38). DOXP is then converted by the DOXP reductoisomerase (Dxr) into MEP (Fig. 1) (1, 12, 21, 28, 30, 36, 40). According to recent findings, the enzymes encoded by the genes *ygbP, ychB*, and *ygbB* are able to catalyze the formation of 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate, with 4-diphosphocytidyl-2-*C*-methyl-D-erythritol as an intermediate (14, 18, 19, 26, 33, 39). The subsequent biochemical steps of the MEP pathway are still unknown.

Recent evidence (2, 7, 27, 32) indicates that the MEP pathway produces IPP and DMAPP separately after a branching point downstream from MEP. In addition, IPP and DMAPP can be interconverted in *E. coli* by the IPP isomerase (Ipi); however, this enzyme is not essential for survival and consequently absent in various other bacteria using the MEP pathway, as shown for *Synechocystis* (10, 13).

In a search for other genes involved in the MEP pathway, it was demonstrated that an enzyme encoded by the *lytB* gene catalyzes an essential step at, or subsequent to, the point at which the MEP pathway branches to form IPP and DMAPP (8). Using genomic databases, a pattern of occurrence identical to that of the described genes of the MEP pathway was identified for the genes *lytB* and *gcpE* (8). Therefore, *gcpE* must be considered a candidate for another gene of the MEP pathway. In former work, *gcpE* was shown to be essential for the growth of bacteria, but no clear function could be attributed to it (4).

In this work, we demonstrate that *gcpE* is essentially involved in the MEP pathway. In a first step, *E. coli* cells were genetically engineered to utilize exogenously provided mevalonate for isoprenoid biosynthesis by introduction of three genes of the yeast mevalonate pathway (Fig. 1). In a second step, the chromosomal *gcpE* gene of the engineered cells was deleted. The resulting mutants were viable only when the culture medium was supplemented with mevalonate, similar to *dxr*-deficient bacteria serving as controls. The ability to grow in the absence of mevalonate could be restored by transformation with a plasmid containing the *gcpE* gene.

MATERIALS AND METHODS

Strains and media. All plasmids were constructed in *E. coli* TOP10 (Invitrogen). For gene replacement experiments, the recombination-proficient wild-type *E. coli* K-12 strain DSM 498 (ATCC 23716) was used. Bacteria were grown in Standard 1 medium (Merck) at 37°C with aeration. *Saccharomyces cerevisiae* strain BJ1991 (16) was grown in YPD medium (3) at 30°C with aeration. For solid medium, agar (Difco Bacto Agar) was added to 1.5% (wt/vol). Media were supplemented with 150 μg of ampicillin/ml, 25 μg of chloramphenicol/ml, or 100 μM mevalonate, where appropriate. Mevalonate was prepared as described elsewhere (32). For selection against *sacB*, salt-free Luria-Bertani medium (5) was supplemented with sucrose to a final concentration of 6% (wt/vol).

Recombinant DNA techniques. Plasmid isolation, agarose gel electrophoresis, ligation, and transformation of plasmid DNA were carried out according to standard protocols (3). For analytical plasmid preparation, a GFX Micro Plasmid Prep kit (Amersham Pharmacia) was used. DNA fragments were gel purified using an Easy Pure kit (Biozym Diagnostik). Restriction endonuclease digestions were carried out as specified by the manufacturer (Promega). Genomic DNA from *S. cerevisiae* was prepared as described elsewhere (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 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.

* Corresponding author. Mailing address: Institute of Biochemistry, Academic Hospital Centre, Justus Liebig University, Friedrichstr. 24, D-35392 Giessen, Germany. Phone: 49-6419947528. Fax: 49-6419947529. E-mail: Boran.Altincicek@biochemie.med.uni-giessen.de.

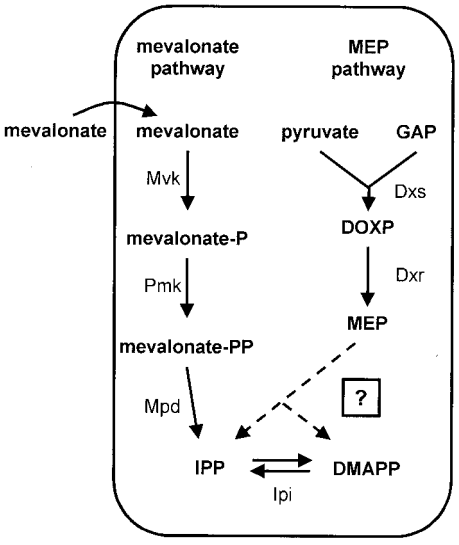

FIG. 1. The MEP pathway of IPP and DMAPP biosynthesis in *E. coli* and genetically engineered synthesis of IPP from exogenously supplied mevalonate. Interrupted lines indicate not fully elucidated steps. Mvk, mevalonate kinase; Pmk, phosphomevalonate kinase; Mpd, mevalonate pyrophosphate decarboxylase; Dxs, DOXP synthase; Dxr, DOXP reductoisomerase; Ipi, IPP isomerase; GAP, D-glyceraldehyde 3-phosphate; P, phosphate; PP, pyrophosphate.

Construction of the synthetic mevalonate operon pSC-MVA. To generate an *E. coli* strain capable of using exogenously provided mevalonate to synthesize IPP, a synthetic operon was constructed by a PCR-based method (Fig. 2). In the first step, genomic DNA of *S. cerevisiae* was used as template to amplify the genes for mevalonate kinase (Mvk; EC 2.7.1.36), phosphomevalonate kinase (Pmk; EC 2.7.4.2), and mevalonate pyrophosphate decarboxylase (Mpd; EC 4.1.1.33) in three asymmetric PCRs, using primer pairs in a 10:1 molar ratio (500 and 50 nM). In the second step, the three fragments were annealed at their overlapping regions including synthetic ribosome binding sites (5′-AGGAGG-3′) eight nucleotides upstream of the start codon of the relevant genes and amplified to a single fragment, using 500 nM outer primers. The final fragment was cloned into a pBAD vector using the pBAD-TOPO-TA cloning kit (Invitrogen) and verified by restriction analysis and sequencing.

The following set of oligonucleotide primers was used: Mev-kin-Sc-for, 5′-T*AGGAGG*AATTAACCATGTCATTACCGTTCTTAACT-3′; Mev-kin-Sc-rev, 5′-**TTGATCTG***CCTCCT***ATGAAGT**CCATGGTAAATT-3′; Pmev-kin-Sc-for, 5′-**ACTTCAT***AGGAGG***CAGATCA**AATGTCAGAGTTGAGAGCCTTC-3′; Pmev-kin-Sc-rev, 5′-**GAGTATTA***CCTCCT***ATTTATC**AAGATAAGTTTC-3′; Decarb-Sc-for, 5′-**GATAAAT***AGGAGG***TAATACTC**ATGACCGTTTACACAGC ATCC-3′; and Decarb-Sc-rev, 5′-TTATTCCTTTGGTAGACCAGT-3′. Overlapping sequences are in boldface, and sequences defining ribosome binding sites are in italics. To test the functionality of the synthetic operon, bacteria transformed with pSC-MVA were tested for fosmidomycin resistance in a diffusion assay. The bacteria were spread on plates with and without mevalonate, and filter paper disks soaked with 2 μl of 100 mM fosmidomycin in water were placed in the middle of the plates.

Construction of the gene replacement plasmids pKO3-Δ*dxr* and pKO3-Δ*gcpE*. For generation of precise in-frame deletion mutants of *E. coli*, the pKO3 vector was used (23). Crossover PCR deletion products were constructed basically as described previously (23). First, two different asymmetric PCRs were used to generate fragments upstream (525 bp) and downstream (558 bp) of the sequences targeted for deletion. 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 outer primers. 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.

To construct the gene replacement plasmid pKO3-Δ*dxr* for deletion of *dxr*, the following set of oligonucleotide primers was used for crossover PCR: Dxr-N-out, 5′-TAGGATCCCATTGTCGTGGAATATTACGG-3′; Dxr-N-in, 5′-**CCCATC CACTAAACTTAAACA**CTTCATGAAACATCCAGAGTT-3′; Dxr-C-in, 5′-**TG TTTAAGTTTAGTGGATGGG**GAAGTCGCCAGAAAAGAGGT-3′; and Dxr-C-out, 5′-TAGTCGACCCCACACAAACAGTTCCATTA-3′; To construct the gene replacement plasmid pKO3-Δ*gcpE* for deletion of *gcpE*, the following set of oligonucleotide primers was used for crossover PCR: Gcpe-N-out, 5′-TAGGA TCCCCAGCGTCTGTGGATACTAC-3′; Gcpe-N-in, 5′-**CCCATCCACTAAAC TTAAACA**TTGAATTGGAGCCTGGTTATG-3′; Gcpe-C-in, 5′-**TGTTTAAG TTTAGTGGATGGG**TAATAACGTGATGGGAAGCGC-3′; and Gcpe-C-out, 5′-TAGTCGACAGTGAGCATAATCAGTTCAGC-3′. The restriction sites for *Bam*HI and *Sal*I are underlined; overlapping sequences defining the 21-bp in-frame insertion are in boldface.

Construction of the deletion mutant strains wtΔ*dxr* and wtΔ*gcpE*. Gene replacement experiments were carried out as described previously except for supplementing the plates with 100 μM mevalonate (23). The gene replacement plasmids pKO3-Δ*dxr* and pKO3-Δ*gcpE* were transformed into wild-type *E. coli* cells harboring pSC-MVA 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 *dxr* deletion was confirmed using two different primer pairs: Dxr-con-N (5′-TTCTCAGGACGATGTACAGAA-3′) plus Dxr-con-C (5′-AGCAGACAACATCACGCGTTT-3′) and ecolyaemfor (5′-GCGGATCC ATGAAGCAACTCACCATTCTG-3′) plus ecolyaemrev (5′-CCGGAAGCTTT CAGCTTGCGAGACGCATCA-3′). The *gcpE* deletion was confirmed using two primer pairs: Gcpe-con-N (5′-CTGGAGGTCACTGATGCTAC-3′) plus Gcpe-con-C (5′-ATTTCACTGTAACCGTAGCTG-3′) and ecolgcpefor (5′-GG ATCCATGCATAACCAGGCTCCAATTCAA-3′) plus ecolgcpcrev (5′-AAGC TTTTTTTCAACCTGCTGAACGTCAAT-3′). Bacteria with the desired deletion as verified by PCR were tested for growth with and without mevalonate.

Complementation experiments. The mutant strains wtΔ*dxr* and wtΔ*gcpE* were complemented by transformation with plasmids pQE-dxr and pQE-gcpE, respectively. Plasmid pQE-dxr was constructed as described above but using the primers ecolyaemfor and ecolyaemrev (40). In a similar way, pQE-gcpe was constructed using the primers ecolgcpefor and ecolgcperev.

RESULTS

gcpE represents a highly conserved gene identified in a variety of organisms including eubacteria, plants, and the malaria parasite *Plasmodium falciparum*, all of them known to possess the MEP pathway (Fig. 3). In organisms using the mevalonate pathway, including animals, fungi, archaebacteria and some

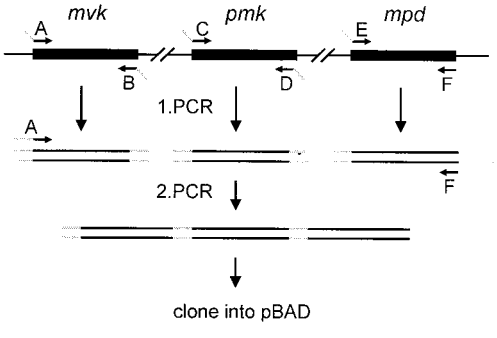

FIG. 2. Construction of the synthetic operon conferring the ability to utilize mevalonate for IPP synthesis. The genes coding for Mvk, Pmk, and Mpd were amplified from genomic yeast DNA, thereby introducing ribosome binding sites (indicated by gray lines) with the various primers (A, Mev-kin-Sc-for; B, Mev-kin-Sc-rev; C, Pmev-kin-Sc-for; D, Pmev-kin-Sc-rev; E, Decarb-Sc-for; F, Decarb-Sc-rev). The three PCR products were annealed at their overlapping regions defined by the specific primers and assembled in a second round of amplification using the outer primers. The synthetic operon was cloned into the pBAD vector.

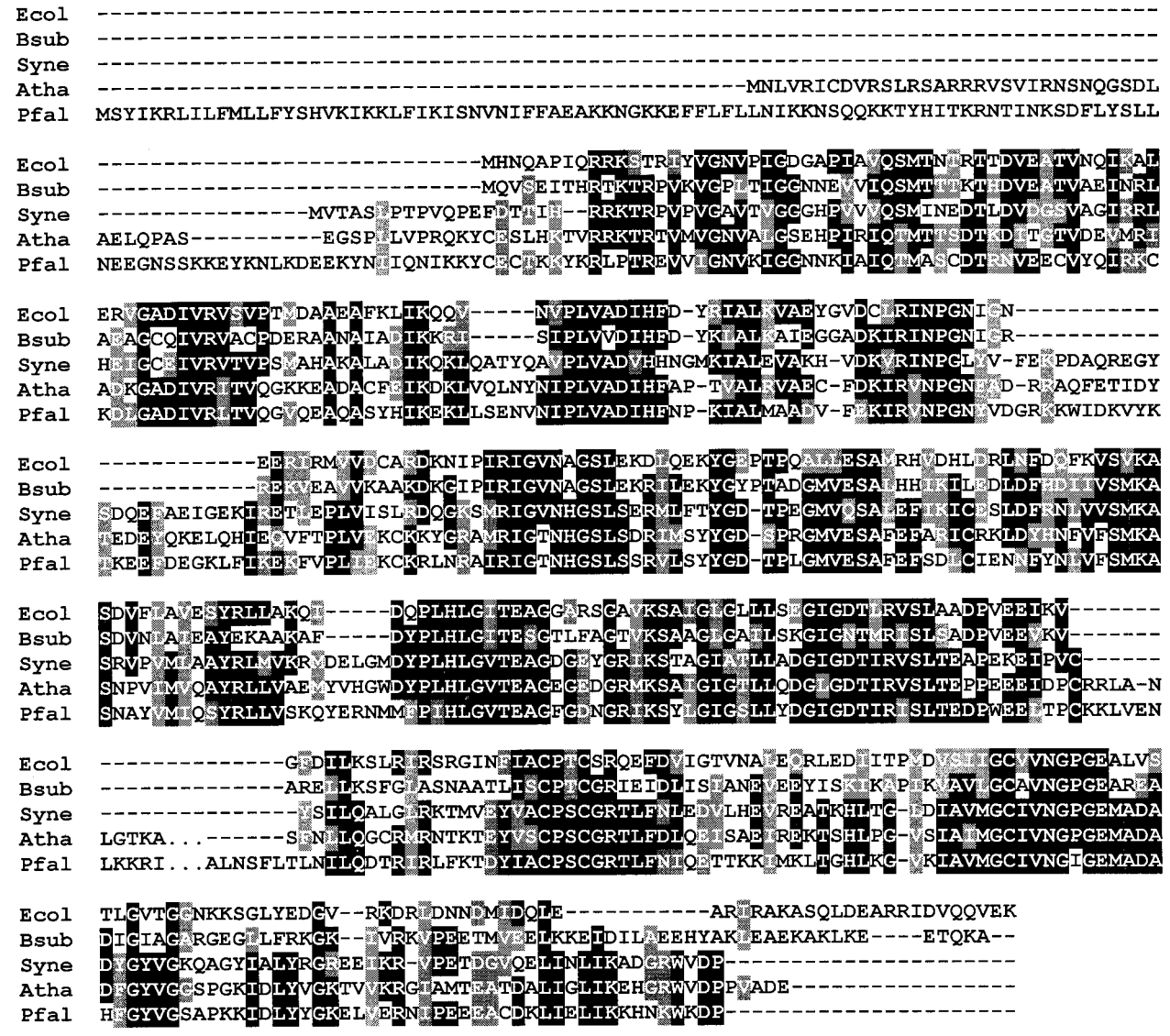

FIG. 3. Alignment of the deduced amino acid sequence of *gcpE* from *E. coli* and other organisms using the MEP pathway. Ecol, *E. coli* (Swiss-Prot accession no. P27433); Bsub, *Bacillus subtilis* (Swiss-Prot accession no. P54482); Pfal, *Plasmodium falciparum* (assembled from different sequences from The Institute for Genomic Research and Sanger databases and deposited in GenBank with accession no. AF323928); Syne, *Synechocystis* sp. strain PCC6803 (Protein Identification Resource accession no. S77159); Atha, *Arabidopsis thaliana* (GenBank accession no. BAB09833). Three dots indicate sequence insertion of 258 amino acids in the sequence of *A. thaliana* and of 304 amino acids in the sequence of *P. falciparum* with weak similarities to each other. Black and gray outlines indicate identical and similar amino acid residues, respectively.

eubacteria (41), no homologues of GcpE can be found in genome databases. An overview of the occurrence of GcpE homologues is displayed in Table 1.

To demonstrate a role for *gcpE* in the MEP pathway, in a genetic approach *E. coli* cells with a disrupted *gcpE* gene were constructed and analyzed for loss of the ability to synthesize isoprenoids via the MEP pathway. Since *E. coli* mutants blocked in isoprenoid biosynthesis are not viable under normal growth conditions (7, 40), *E. coli* transformants capable of utilizing mevalonate for IPP synthesis were generated. For this purpose, a synthetic operon containing the yeast genes for Mvk, Pmk, and Mpd was constructed (Fig. 2). The single genes were obtained by PCR amplification, thereby introducing a ribosome binding site in the 5′ region of each gene. The three genes were assembled in a second round of amplification and cloned into the pBAD expression vector.

To demonstrate functionality of the artificial mevalonate operon, the sensitivity to fosmidomycin of *E. coli* cells harboring this construct was tested. Fosmidomycin is a strong and specific inhibitor of the DOXP reductoisomerase and known to inhibit the growth of wild-type *E. coli* (17). As expected, bacteria containing the synthetic operon survived in the presence of fosmidomycin when the medium was supplemented with mevalonate. Optimal growth rates were observed in the presence of 100 to 200 μM mevalonate (data not shown). Without mevalonate, the bacteria could not grow in the presence of fosmidomycin.

To inactivate the *gcpE* gene, the coding sequence was com-

TABLE 1. Accession numbers of GcpE homologues in various organisms

Organism	GcpE accession no.
Eubacteria	
Aquifex aeolicus	sp O67496
Bacillus subtilis	sp P54482
Chlamydia muridarum	tr Q9PKY3
C. pneumoniae	tr Q9Z8H0
C. trachomatis	sp O84060
Escherichia coli	sp P27433
Haemophilus influenzae	sp P44667
Helicobacter pylori	tr Q9ZLL0
Mycobacterium tuberculosis	sp O33350
Synechocystis strain PCC6803	pir S77159
Thermotoga maritima	tr Q9WZZ3
Treponema pallidum	sp O83460
Neisseria meningitidis	tr Q9JZ40
Campylobacter jejuni	tr Q9PPMI
Deinococcus radiodurans	tr Q9RXC9
Pseudomonas aeruginosa	tr AAG07190
Vibrio cholerae	tr Q9KTX1
Staphylococcus aureus	
Streptococcus pyogenes	
S. pneumoniae	
Borrelia burgdorferi	
Mycoplasma genitalium	
M. pneumoniae	
Rickettsia prowazekii	
Archaebacteria	
Archaeoglobus fulgidus	
Methanobacterium thermoautotrophicum	
Aeropyrum pernix K1	
Methanococcus jannaschii	
Pyrococcus horikoshii	
Halobacterium sp. strain NRC-1	
Pyrococcus abyssi	
Eucaryota	
Plasmodium falciparum	gb AF323928
Arabidopsis thaliana	gb BAB09833
Saccharomyces cerevisiae	
Drosophila melanogaster	
Caenorhabditis elegans	
Homo sapiens	

pletely removed from the bacterial genome by homologous recombination and replaced by a synthetic 21-bp sequence (Fig. 4A). This was accomplished by using the pKO3 gene replacement vector that allows the generation of precise in-frame deletion mutants in *E. coli* wild-type strains (23). The gene replacement procedure was performed in a wild-type *E. coli* K-12 strain harboring the synthetic mevalonate operon, using mevalonate-supplemented medium. Bacteria containing the desired *gcpE* deletion were identified by PCR analysis (Fig. 4B). Finally, it was demonstrated that *gcpE* deletion mutants depend on exogenously provided mevalonate (Fig. 5). In a control experiment, the *dxr* gene was deleted in *E. coli* by the same technique. The resulting Δ*dxr* strain was dependent on mevalonate in the same way as the *gcpE* deletion mutant (Fig. 5). These data provide clear evidence that *gcpE* is functionally involved in the MEP pathway.

To further confirm this result, the generated *E. coli* Δ*gcpE* strain was complemented by transformation with a plasmid containing an intact *gcpE* gene under the control of the *tac* promoter. The complemented cells regained the ability to grow on medium without mevalonate (Fig. 5C). Similarly, Δ*dxr* bacteria could be successfully complemented with the respective episomal copy of the intact *dxr* gene (Fig. 5C).

DISCUSSION

The genomic distribution of GcpE homologues is a strong indication that this gene is involved in the MEP pathway. Sequence extensions at the NH_2 terminus of the GcpE homologues 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. This provides further evidence for a role of GcpE in the MEP pathway as all enzymes of this pathway described so far in plants are localized in the plastids.

In addition, the *gcpE* gene of *Streptomyces coelicolor* A3(2) is located directly upstream of the *dxs* gene for the DOXP synthase, indicating that both genes may be transcribed as one cistron, thus implying a functional relationship between GcpE and the MEP pathway (EMBL accession no. AL049485). Interestingly, *S. coelicolor* A3(2) possesses an additional copy of the *gcpE* gene with 94.8% identity of the predicted proteins

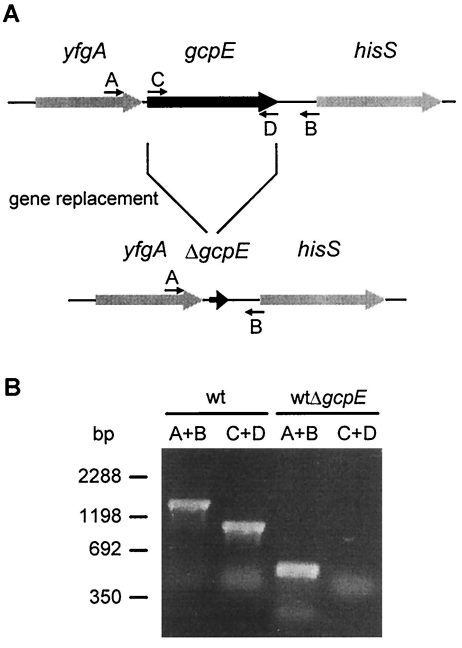

FIG. 4. Replacement of the *gcpE* gene with a precisely engineered deletion. (A) Diagram of the *gcpE* region of the wild-type strain and the *gcpE* deletion mutant. Small arrows indicate the primer sites used for PCR analysis. Primers: A, Gcpe-con-N; B, Gcpe-con-C; C, ecolgcpefor; D, ecolgcperev. (B) Verification of the deletion of the *gcpE* gene by PCR. After selection for integrates of the gene replacement vector pKO3-Δ*gcpE* 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 530 bp obtained using the primer pair A plus B of the *gcpE* mutant strain is the expected 1,070 bp smaller than the wild-type product of 1,600 bp. Using the primer pair C plus D, the *gcpE* gene (1,116 bp) was amplified in the wild-type strain, and no product was obtained in the *gcpE* mutant strain.

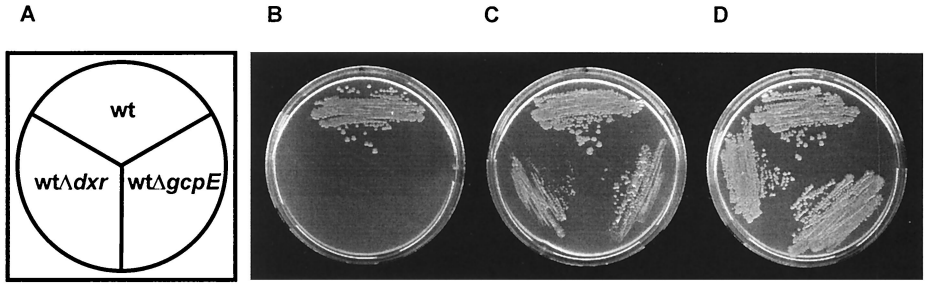

FIG. 5. Growth of the *E. coli* strains indicated in panel A (wt, wild type; wtΔ*dxr,* *dxr* deletion mutant; wtΔ*gcpE,* *gcpE* deletion mutant) on medium without (B) and with (C) mevalonate and after complementation of the mutant strains with episomal *dxr* and *gcpE* genes, respectively, without mevalonate (D).

located downstream of the *dxr* gene for the DOXP reductoisomerase separated by a gene for a putative metalloprotease with similarity to the YaeL protein of *E. coli* (Swiss-Prot accession no. P37764; EMBL accession no. AL355913). In *E. coli*, a yet uncharacterized open reading frame, *yfgA*, may be cotranscribed with *gcpE*. YfgA (Swiss-Prot accession no. P27434) is supposed to be a transcriptional regulator in *E. coli* because a helix-turn-helix motif can be found.

In earlier work, the *gcpE* homologue of *Providencia stuartii* was described as *aarC* and identified as a negative regulator of the 2′-*N*-acetyltransferase [Aac(2′)-Ia] involved in the acetylation of peptidoglycan and certain aminoglycosides in *P. stuartii* (31). However, as *gcpE* homologues are highly conserved in bacteria lacking *aac(2′)-Ia* such as *E. coli* and *Haemophilus influenzae*, the authors concluded that GcpE must additionally carry out essential housekeeping functions. A single point mutation in the *aarC* gene of *P. stuartii* resulted in a slow-growth phenotype and altered cell morphology, with the formation of very short rods, many of which were spherical (31). This observation is consistent with the fact that inhibition of the MEP pathway impaires cell wall biosynthesis (37).

The gene disruption experiments performed in the present study demonstrate unambiguously an essential role of GcpE in the MEP pathway. Similar approaches introducing the partial mevalonate pathway for IPP biosynthesis from mevalonate in *E. coli* have been successfully applied in previous work to demonstrate the involvement of YgbP, YchB, and YgbB in the MEP pathway (18, 19, 39) and to provide evidence for its branching to form IPP and DMAPP (32).

The amino acid sequence predicted from the *gcpE* gene provides no obvious evidence for the function of the polypeptide since no significant sequence motifs or similarities to polypeptides of known function were identified. Consequently, the exact function of GcpE within the MEP pathway requires further investigation.

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