Escherichia coli engineered to synthesize isopentenyl diphosphate and dimethylallyl diphosphate from mevalonate: a novel system for the genetic analysis of the 2-*C*-methyl-D-erythritol 4-phosphate pathway for isoprenoid biosynthesis

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Isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) constitute the basic building block of isoprenoids, a family of compounds that is extraordinarily diverse in structure and function. IPP and DMAPP can be synthesized by two independent pathways: the mevalonate pathway and the recently discovered 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway. Although the MEP pathway is essential in most eubacteria, algae and plants and has enormous biotechnological interest, only some of its steps have been determined. We devised a system suitable for the genetic analysis of the MEP pathway in *Escherichia coli*. A synthetic operon coding for yeast 5-diphosphomevalonate decarboxylase, human 5-phosphomevalonate kinase, yeast mevalonate kinase and *E. coli* isopentenyl diphosphate isomerase was incorporated in the chromosome of this bacterium. The expression of this operon allowed the synthesis of IPP and DMAPP from mevalonate added exogenously and complementation of lethal mutants of the MEP pathway. We used this system to show that the *ygbP*, *ychB* and *ygbB* genes are essential in *E. coli* and that the steps catalysed by the products of these genes belong to the trunk line of the MEP pathway.

Key words: metabolic engineering, *ychB*, *ygbB*, *ygbP*.

INTRODUCTION

The isoprenoids are a large family of compounds with more than 30000 members known to date [1]. Some isoprenoids have essential functions in the cell. For instance, sterols are required as structural components of membranes, dolichol as a sugar carrier in the glycosylation of proteins, ubiquinone and plastoquinone as electron carriers in photosynthesis and respiration, and abscisic acid, cytokinins, gibberellic acid and steroids as hormones that control physiological processes and development. In spite of the structural and functional diversity of isoprenoids, all derive from isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), which represent the five-carbon basic building block of isoprenoids. Both IPP and DMAPP are essential intermediates for the synthesis of isoprenoid compounds.

There are two pathways for the synthesis of IPP and DMAPP: the well-known mevalonate (MVA) pathway and the newly discovered 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway, first reported by Rohmer et al. [2]. Three enzymic reactions are necessary to convert MVA to IPP (Scheme 1). In the first, 5-phosphomevalonate is synthesized from MVA by the action of mevalonate kinase. A second phosphorylation reaction catalysed by 5-phosphomevalonate kinase affords 5-diphosphomevalonate, which is subsequently tranformed to IPP by 5-diphosphomevalonate decarboxylase. Isopentenyl diphosphate isomerase (IDI) mediates the interconversion between IPP and DMAPP. Condensation of one or several molecules of IPP to a priming molecule of DMAPP allows the synthesis of prenyl chains of increasing size, which are the starting points for the ramifications of the pathway that lead to the final isoprenoid products.

The MEP pathway for isoprenoid biosynthesis is essential in most eubacteria, like *Escherichia coli*, algae and plants [3–6]. Although this pathway was shown by labelling experiments in many systems, it has not been fully elucidated yet. The initial step involves the formation of 1-deoxy-D-xylulose 5-phosphate from pyruvate and glyceraldehyde 3-phosphate (Scheme 1). This reaction is catalysed by 1-deoxy-D-xylulose 5-phosphate synthase, which is encoded by the *dxs* gene in *E. coli* [7,8]. 1-Deoxy-D-xylulose 5-phosphate is converted to isoprenoids but is also a precursor of thiamin and pyridoxol in plants and microorganisms [9,10]. In the second step MEP is synthesized by 1-deoxy-D-xylulose 5-phosphate reductoisomerase, which is encoded by the *dxr* gene [11].

The results of *in vitro* enzymic assays led to the proposal that, in *E. coli*, MEP is converted to 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (ME-2,4cPP) in three enzymic steps catalysed,

Abbreviations used: CDP-ME, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol; CDP-ME2P, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate; DMAPP, dimethylallyl diphosphate; DX, 1-deoxy-D-xylulose; IDI, isopentenyl diphosphate isomerase; ecIDI, *Escherichia coli* IDI; hPMK, human 5-phosphomevalonate kinase; IPP, isopentenyl diphosphate; ME-2,4cPP, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; MVA, mevalonate; ORF, open reading frame; yMVK, yeast mevalonate kinase; yPMD, yeast 5-diphosphomevalonate decarboxylase; CAT, chloramphenicol acetyltransferase.

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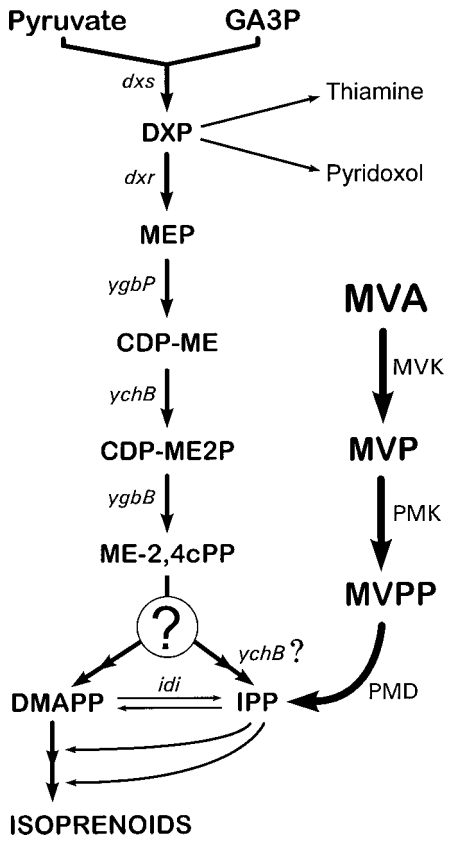

Scheme 1 The MEP pathway and the partial MVA pathway engineered in *E. coli*

The intermediates of the MEP pathway (on the left) are as follows: GA3P, glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; CDP-ME, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol; CDP-ME2P, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate; ME-2,4cPP, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate. The genes encoding the enzymes of the MEP pathway are indicated. The intermediates of the MVA pathway (on the right) are as follows: MVP, 5-phosphomevalonate; MVPP, 5-diphosphomevalonate. The enzymes of the MVA pathway are as follows: MVK, mevalonate kinase; PMK, 5-phosphomevalonate kinase; PMD, 5-diphosphomevalonate decarboxylase.

respectively, by the products of the *ygbP*, *ychB* and *ygbB* genes (Scheme 1) [12–14]. The *ygbP* gene product mediates the condensation of MEP with CTP to form 4-diphosphocytidyl-2-*C*-methyl-D-erythritol (CDP-ME) [12]. CDP-ME is then phosphorylated by the *ychB* gene product to 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate (CDP-ME2P) [13]. The last step of the proposed sequence, catalysed by the *ygbB* gene product, is the elimination of the CMP moiety from CDP-ME2P to yield ME-2,4cPP [14]. The participation of CDP-ME, CDP-ME2P and ME-2,4cPP in the MEP pathway is supported by experiments on the incorporation of ^{14}C-radiolabelled specimens of these compounds into carotenoids by isolated chromoplasts from *Capsicum annuum* [12–14]. However, genetic evidence is required to confirm that the results of the incorporation experiments are not due to reversibility of one or several steps of the proposed sequence.

The analysis of the function of the *ychB* gene *in vivo* is particularly relevant. In contrast to the above-mentioned results, the product of this gene was proposed to catalyse the terminal step in the synthesis of IPP [15]. This proposal is based on the results of *in vitro* assays performed with crude *E. coli* extracts enriched with the *ychB* gene product, which suggested that this product catalyses the phosphorylation of isopentenyl monophosphate to IPP. The occurrence of this reaction *in vivo* is supported by experiments on the incorporation of [4-^{14}C]-isopentenyl monophosphate into total terpenoids of isolated peppermint-oil gland secretory cells [15].

Several labelling studies have suggested that, in *E. coli*, IPP and DMAPP are synthesized from MEP by routes that separate at some point [16–18]. The operation of separate routes for the synthesis of IPP and DMAPP in *E. coli* has been confirmed genetically [19]. In contrast, a single route leading in the first instance to IPP seems to be present in the bacterium *Zymomonas mobilis* [20] and *Catharanthus roseus* cells [21]. Although interesting, these differences might complicate the elucidation of the MEP pathway. The branching point in *E. coli* might be located immediately after MEP or further downstream in the pathway. An open question is whether the reactions catalysed by the *ygbP*, *ychB* and *ygbB* gene products belong to the common segment of the pathway or to one of the branches.

We addressed the above questions with a novel system suitable for the genetic analysis of the MEP pathway. *E. coli* was engineered to synthesize IPP and DMAPP from MVA. The presence of an alternative pathway for the production of isoprenoid units in the bacterium allowed the rescue of otherwise lethal mutants of the MEP pathway. We used this approach to show that the *ygbP*, *ychB* and *ygbB* genes are essential in *E. coli* and that the steps catalysed by the products of these genes belong to the trunk line of the MEP pathway.

EXPERIMENTAL

Oligonucleotides, *E. coli* strains and supplements for growth media

The oligonucleotides used in this study are listed in Table 1. A brief description of the relevant *E. coli* strains generated is given in Table 2. *E. coli* cells were grown in 2 × TY medium [22]. The antibiotics chloramphenicol (17 μg/ml), kanamycin (25 μg/ml), tetracycline (6 μg/ml) and ampicillin (100 μg/ml) were used as selective agents. Other supplements were added to the medium at the following concentrations: 0.5 mM 1-deoxy-D-xylulose (DX), 0.2% (w/v) D-glucose, 1 mM MVA and 0.02 or 0.2% (w/v) L-arabinose, as indicated. DX was synthesized enzymically, as described in [8]. MVA was prepared from mevanolactone (Sigma) by hydrolysis of 1 vol. of 1 M mevanolactone with 1.05 vol. of 1 M KOH at 37 °C for 30 min. The final pH of the MVA stock solution was approx. 7.5.

Isolation and mutagenesis of yeast 5-diphosphomevalonate decarboxylase (yPMD), human 5-phosphomevalonate kinase (hPMK), yeast mevalonate kinase (yMVK) and *E. coli* IDI (ecIDI) coding sequences

The coding sequence of yPMD [open reading frame (ORF) YNR043w, *ERG19*] was amplified by PCR using genomic DNA from *Saccharomyces cerevisiae* strain FY1679 as a template and the oligonucleotides INI-5 and INI-6 as primers. The resulting PCR product was reamplified using oligonucleotides PMD-Nde5′ and PMD-Eco3′ as primers (see Table 1). The final PCR product contained the yPMD coding sequence flanked by *Nde*I and *Eco*RI restriction sites at the 5′ and 3′ ends, respectively. The clone ym0505.r1 (IMAGE clone 46897; GenBank accession number H09914) coding for hPMK was from Research Genetics. The hPMK coding sequence was amplified by PCR using oligonucleotides hPMK1 and hPMK4 as primers. The final PCR

Table 1 Oligonucleotides used in this study

hPMK, human 5-phosphomevalonate kinase; MVK, mevalonate kinase; CAT, chloramphenicol acetyltransferase.

Name	Sequence
CAT1	5′-GAGTCCGAATAAATACCTGTG-3′
CAT4	5′-CCGAATTTCTGCCATTCATCC-3′
DX1	5′-AAAGCCCGGGATCTGATCGAC-3′
DX2	5′-CCCGTCAGTCGCTGAAACAAC-3′
DX3	5′-AAGTCGGGAAACGCGAAGGTC-3′
DX4	5′-CTGGCTCGCCAAAGGTCATAC-3′
hPMK1	5′-TGGTTAACATATGGCCCCGCTGGGAGGCGC-3′
hPMK4	5′-AGGTTAACTCAATTAAAGTCTGGAGCGGATAAATTCTATC-3′
idi-3X	5′-GGCTCGAGTTATTTAAGCTGGGTAAATGCAG-3′
idi-5X	5′-CCCCTCGAGATTATGCAAACGGAACACGTC-3′
INI-1	5′-CGGGCCTCGTTTGGCTGTCGCACTG-3′
INI-2	5′-CGCGGGTGGAAGGACCTTGTGGAGG-3′
INI-5	5′-CGCTGCCCAGAATGGACCTCCCTAG-3′
INI-6	5′-CAGCCGCGTTTTGACTTGAAACGTGC-3′
PMD-Eco3′	5′-TCGAATTCTCATTATTCCTTTGGTAGACCAGTCTT-3′
PMD-Nde5′	5′-GCCATATGACCGTTTACACAGCATCCG-3′
MVK-Hpa3′	5′-CGGTTAACTCATTATGAAGTCCATGGTAAATTCG-3′
MVK-Hpa5′	5′-AAGTTAACATATGTCATTACCGTTCTTAACTTC-3′
pBAD-D2	5′-TCATACTCCCGCCATTCAGAG-3′
pBAD-Link1	5′-AATTCTAAGGAGGTTTAAACTAAGGAGGTACGTAAGGAGG-3′
pBAD-Link2	5′-TCGACCTCCTTACGTACCTCCTTAGTTTAAACCTCCTTAG-3′
pBAD-mut1	5′-CTGAGAGTGCACCATCTGCGGTGTGAAATACC-3′
pBAD-U3	5′-CCGCCAAAACAGCCAAGCTTG-3′
pRS-L1	5′-GATCCGTTTAAACGCCCGGGCGGCCGCG-3′
pRS-L2	5′-AATTCGCGGCCGCCCGGGCGTTTAAACG-3′
ychB-1	5′-TCCGTGCGGTTGCACGTCGAC-3′
ychB-2	5′-CACCATCGGCTGCGCAAGCAG-3′
ychB-5	5′-GCAGGTGAAGGAGGCATAGGTC-3′
ychB-6	5′-GCGCGGAAAGGCATCACATGTG-3′
ygb-F	5′-AATTAGCGGCCGCTGGCGATTTGCGAAG-3′
ygb-DR	5′-AACGGATCTCTACGGTGACGTC-3′
ygb-R	5′-ATTATCTCGAGTGCCAGCGCTTCACG-3′
ygb-UF	5′-GCGCCGAACGCCAGATGGTTC-3′

Table 2 *E. coli* strains used in this study

Strain	Description
MC4100	*araD139*, Δ(*lac*)*U169*, *strA*, *thi* [37]
MC4100(pAB-M2)	MC4100 containing plasmid pAB-M2
MC4100(pAB-M3)	MC4100 containing plasmid pAB-M3
EcAB1-1	MC4100 *dxs*::*CAT* [16]
EcAB1-1(pAB-M2)	EcAB1-1 containing plasmid pAB-M2
EcAB1-1(pAB-M3)	EcAB1-1 containing plasmid pAB-M3
EcAB1-5	EcAB1-1 carrying the MVA$^+$ operon in the chromosome
TE2680	F$^-$, λ^-, IN(*rrnD-rrnE*)*1*, Δ(*lac*)*X74*, *rpsL*, *galK2*, *recD1903*::Tn*10d*-Tet, *trpDC700*::*putPA1303*::[Kan^s-Cam^r-*lac*] [27]
EcAB3-1	TE2680 carrying the MVA$^+$ operon in the chromosome
EcAB3-2	EcAB3-1 *dxs*::*CAT*
EcAB3-4	EcAB3-1 *ygbP*::*CAT*
EcAB3-5	EcAB3-1 *ychB*::*CAT*
EcAB3-6	EcAB3-1 *ygbB*::*CAT*

product contained the hPMK coding sequence flanked by an *Hpa*I restriction site at both ends. The coding sequence of yMVK (ORF YMR208w, *ERG12*) was amplified by PCR using genomic DNA from *S. cerevisiae* strain FY1679 as a template and the oligonucleotides INI-1 and INI-2 as primers. The resulting PCR product was reamplified using oligonucleotides MVK-Hpa5′ and MVK-Hpa3′ as primers. The final PCR product contained the yMVK coding sequence flanked by an *Hpa*I restriction site at both ends. The coding sequence of the ecIDI was amplified by PCR using genomic DNA from strain W3110 as a template and oligonucleotides idi-5X and idi-3X as primers. In this PCR, an *Xho*I restriction site was introduced at both ends of the coding sequence. The final PCR products containing the ORFs coding for yPMD, hPMK, yMVK and ecIDI were cloned, separately, into the *Sma*I restriction site of plasmid pBluescript SK+. Sequencing was performed to ensure that no mutation had been introduced during amplification.

Assembly of the synthetic operons

Synthetic operons (Figures 1A–1C) were assembled in a derivative of the high-copy plasmid pBAD-GFPuv (Clontech; GenBank accession number U62637). In this vector, expression is under control of the P_{BAD} promoter, which can be induced in the presence of L-arabinose and repressed in the absence of L-arabinose and presence of D-glucose [23,24]. First, the *Nde*I restriction site located between pBR322*ori* and the *ara*C coding region of plasmid pBAD-GFPuv (position 4926–4931) was eliminated by site-directed mutagenesis as described in [25], using the oligonucleotide pBAD-mut1 as a mutagenic primer. The mutation was confirmed by restriction analysis and sequencing. The *Nde*I–*Eco*RI restriction fragment of the mutated plasmid, containing the green fluorescent protein (GFP) coding sequence (positions 1341–2064), was replaced by an *Nde*I–*Eco*RI restriction fragment containing the yPMD coding sequence, taking advantage of the modifications introduced at the ends of the yPMD sequence (see above). This cloning step positioned the yPMD ORF just dowstream of the P_{BAD} promoter. To clone the following coding sequences, a polylinker was introduced between the *Eco*RI and *Sal*I restriction sites of the plasmid. This polylinker, generated by annealing the oligonucleotides pBAD-Link1 and pBAD-Link2, contained the restriction sites *Pme*I and *Sna*BI, flanked by cohesive ends of *Eco*RI and *Sal*I sites. Restriction sites *Pme*I, *Sna*BI and *Sal*I were preceeded by the Shine–Dalgarno consensus sequence TAAGGAGG [26]. The modified inserts coding for hPMK and yMVK (see above) were digested with *Hpa*I and blunt-end ligated, respectively, into the *Pme*I and *Sna*BI restriction sites. The modified insert coding for ecIDI (see above) was digested with *Xho*I and ligated into the *Sal*I restriction site of the plasmid. Insert orientation was confirmed, after each step, by PCR and sequencing. The plasmid containing the ORFs coding for yPMD, hPMK and yMVK was named pAB-M2 (Figure 1B). The plasmid containing, in addition, the ORF coding for ecIDI was termed pAB-M3 (Figure 1C). The operon containing ORFs coding for yPMD, hPMK, yMVK and ecIDI was designated MVA$^+$ (Figure 1A).

Stable integration of the MVA$^+$ operon into the *E. coli* chromosome

The MVA$^+$ operon was transferred to the *E. coli* chromosome by a genetic system based on two elements: the *E. coli* strain TE2680 [27] and a pRS550-derived plasmid [28]. Sequences to be introduced in the genome of strain TE2680 can be cloned in plasmid pRS550, between a kanamycin-resistance gene and a promoterless version of the *lac* operon [28]. A double crossing-over event involving these two flanking sequences allows the integration of a single copy of the cloned sequence in the TE2680 chromosome. The incorporated sequence is perpetuated through cell division.

A polylinker containing the restriction sites *Pme*I, *Sma*I and *Not*I was introduced between the *Eco*RI and *Bam*HI sites of vector pRS550 to clone the MVA$^+$ operon. The polylinker was generated by annealing the oligonucleotides pRS-L1 and pRS-L2. The MVA$^+$ operon was amplified by PCR, using the pAB-

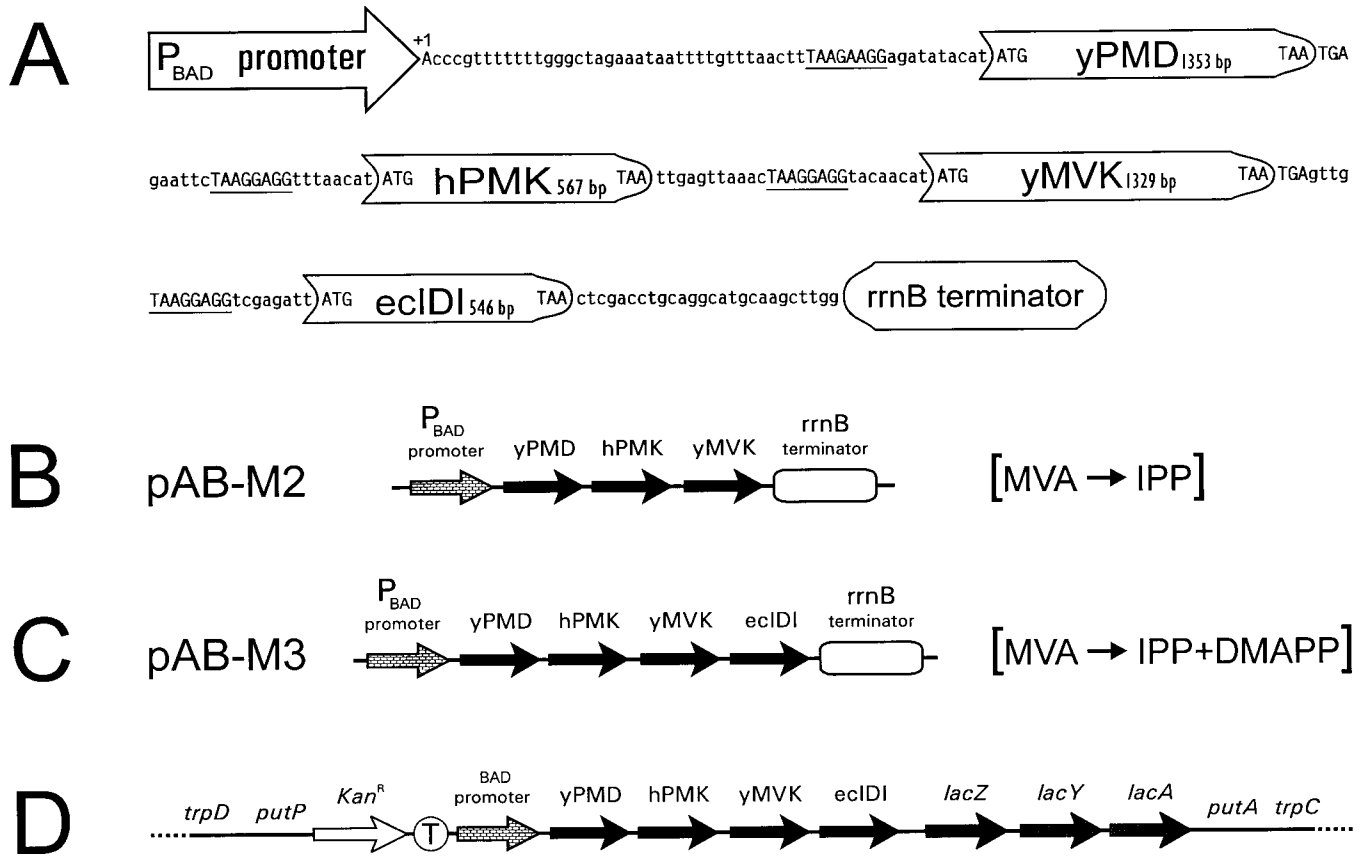

Figure 1 Synthetic operons for MVA utilization in *E. coli*

(**A**) The MVA$^+$ operon. Boxes representing the P_{BAD} promoter, the ORFs coding for yPMD, hPMK, yMVK and ecIDI and the rrnB transcription terminator are combined with the sequence of the transcribed untranslated regions (coding strand). The sequences of the ORFs can be accessed at the NCBI database with the following accession numbers: yPMD, U49261; hPMK, NM-006556; yMVK, X55875 and ecIDI, AF119715. The transcription start site (position +1), the Shine–Dalgarno sequences (underlined), and the translation start and stop codons are shown in capital letters. (**B**) Plasmid pAB-M2 contains an incomplete version of the MVA$^+$ operon in which the ORF coding for ecIDI is missing. The gene products encoded in the synthetic operon of plasmid pAB-M2 allow synthesis of IPP from MVA. (**C**) Plasmid pAB-M3 contains the complete MVA$^+$ operon. The gene products encoded in this operon allow synthesis of IPP and DMAPP from MVA. (**D**) Map of the genomic region containing the MVA$^+$ operon in the chromosome of *E. coli* strain EcAB3-1 and derivatives. This synthetic operon is located between the transcription terminator (T) of a chimaeric kanamycin-resistance gene (*Kan*^{R}) and a promoterless *lac* operon containing ORFs *lacZ*, *lacY* and *lacA*. The construct interrupts the tryptophan operon of the *E. coli* chromosome. It is located between ORFs *trpC* and *trpD*. The genes *putA* and *putP* are derived from the *Salmonella typhimurium put* operon.

M3 plasmid as a template and oligonucleotides pBAD-D2 and pBAD-U3 as primers. The PCR product was cloned in the *Pme*I restriction site of the pRS550 derivative in the same orientation as the promoterless *lac* operon, to restore the transcription of this operon. The derivative of plasmid pRS550 that carried the MVA$^+$ operon was named pRS-MVA$^+$. Digestion of plasmid pRS-MVA$^+$ with *Sal*I and *Sca*I restriction enzymes rendered a 3196 bp fragment containing the ampicillin-resistance gene and the origin of replication, and a 13406 bp fragment containing the kanamycin-resistance gene, the MVA$^+$ operon and the *lac* operon sequences. The linear plasmid DNA was used to transform competent TE2680 cells. Transformants were grown in the presence of kanamycin and tetracycline to select recombinants carrying the MVA$^+$ operon and the *recD* mutation. Sensitivity to chloramphenicol and ampicillin was confirmed by replica plating. The presence of the MVA$^+$ operon in the chromosome of the novel strain was shown by PCR. The transcriptional activity of this operon was confirmed by the appearance of blue colonies in plates containing 40 μg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (Xgal). The TE2680 derivative containing a functional copy of the MVA$^+$ operon in the chromosome was named EcAB3-1. The MVA$^+$ operon was transferred to strain EcAB1-1, defective in the *dxs* gene, by phage P1 transduction. The EcAB1-1 derivative carrying a copy of the MVA$^+$ operon in the chromosome was named EcAB1-5.

Deletion of *E. coli ygbP*, *ychB* and *ygbB* and *dxs* genes in strain EcAB3-1

As mentioned above, *E. coli* strain EcAB3-1 was derived from the *recD* mutant host TE2680 by incorporation of the MVA$^+$ operon into the chromosome. Since strain EcAB3-1 carries the *recD* mutation, gene disruption in this strain can be accomplished by homologous recombination between appropriate linear DNA constructs and the genome. To delete *ygbP*, *ychB* and *ygbB* genes in the genome of strain EcAB3-1 (Figure 2), we prepared deletion constructs in which the *CAT* gene (encoding chloramphenicol acetyltransferase) was surrounded by the flanking regions of the target genes. First, a 960 bp fragment containing the *CAT* gene was obtained by PCR, using plasmid pCAT19 [29] as a template and oligonucleotides CAT1 and CAT4 as primers. The amplified

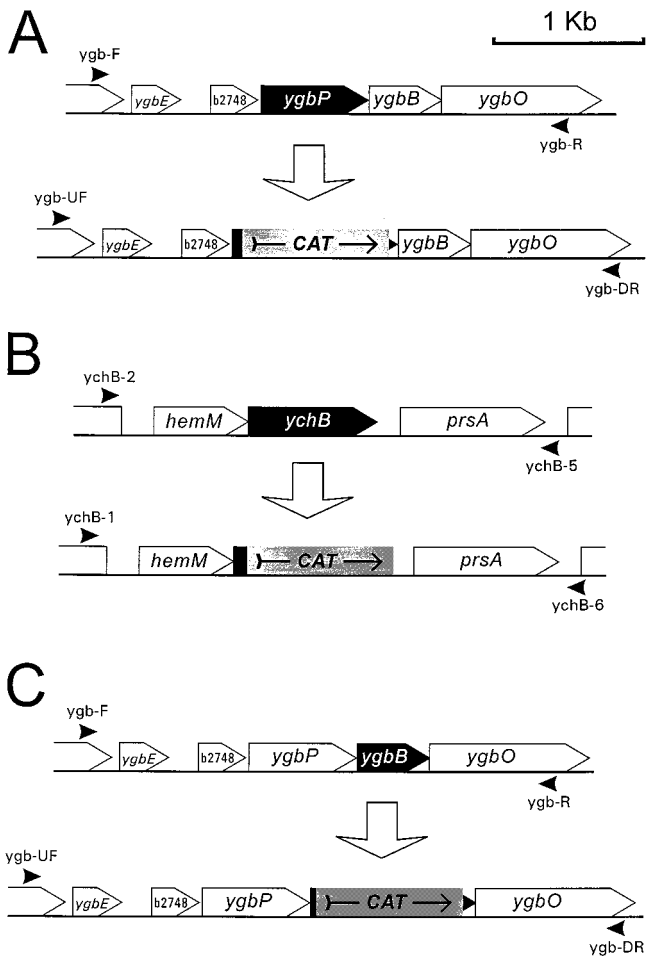

Figure 2 Deletion of *E. coli ygbP*, *ychB* and *ygbB* genes

Maps of the *E. coli* genomic regions containing *ygbP* (**A**), *ychB* (**B**) or *ygbB* (**C**) loci before and after substitution of *ygbP*, *ychB* or *ygbB* coding sequences by the *CAT* gene. Maps are drawn to scale. The direction of transcription is indicated for each ORF. Arrowheads indicate the positions of the oligonucleotides used to prepare the deletion constructs (primers ychB-2, ychB-5, ygb-F and ygb-R) and to analyse the mutated loci (primers ychB-1, ychB-6, ygb-UF and ygb-DR).

sequence contained the complete ORF coding for CAT, together with the corresponding promoter elements, but did not contain any transcription-termination signal. Therefore, no polar effect dowstream of the gene disrupted by the *CAT* gene is expected, as long as these two genes have the same transcriptional orientation.

E. coli genomic regions containing the *ygbP*, *ychB* and *ygbB* genes were amplified by PCR, using genomic DNA from strain EcAB3-1 as a template. The chromosomal region containing the *ychB* gene was amplified with oligonucleotides ychB-2 and ychB-5 as primers. This region corresponds to positions 7530–4540 of section 109 of the *E. coli* complete genome sequence (accession number AE000219). The PCR product was cloned and a fragment of this product containing most of the *ychB* coding sequence (positions 6480–5715 of the section 109 sequence) was replaced by the 960 bp fragment containing the *CAT* gene. Similarly, the genomic region containing *ygbP* and *ygbB* genes was amplified using oligonucleotides ygb-F and ygb-R as primers. This region corresponds to positions 8565–5455 of section 248 of the *E. coli* complete genome sequence (accession number AE000358). To disrupt the *ygbP* gene, the region corresponding to positions 7392–6816 of the section-248 sequence was replaced by the 960 bp fragment containing the *CAT* gene, and to disrupt the *ybgB* gene, the region corresponding to 6670–6315 of section 248 was replaced by the same 960 bp fragment. Details of the cloning procedures are available on request. To obtain the deletion construct corresponding to the *dxs* gene, genomic DNA from strain MC4100 *dxs*::*CAT* [16] was amplified by PCR using oligonucleotides DX2 and DX3 as primers.

Linear DNA obtained by PCR amplification of the deletion constructs was used to transform competent cells of strain EcAB3-1. Transformants were plated in media containing 0.2 % (w/v) L-arabinose, 1 mM MVA and the antibiotics chloramphenicol, kanamycin and tetracycline to select, respectively, the *CAT* gene, the MVA$^+$ operon and the *recD* mutation. Substitution of the *ygbP*, *ychB*, *ygbB* and *dxs* genes by the *CAT* gene in the chromosome was confirmed by PCR using oligonucleotides complementary to genomic sequences located outside the regions manipulated in the deletion constructs. Oligonucleotides ychB-1 and ychB-6 were used to confirm the deletion of the ychB coding sequence. Oligonucleotides ygb-UF and ygb-DR were used to confirm the deletion of the ygbP and ygbB coding sequences. Oligonucleotides DX1 and DX4 were used to confirm deletion of the *dxs* gene. The identity of the PCR products was confirmed by restriction analysis. The genomic regions containing the *ygbP*, *ychB* and *ygbB* disrupted genes are shown in Figure 2. A scheme of the genomic region containing the *dxs* disrupted gene has been reported elsewere [16].

RESULTS AND DISCUSSION

Engineering *E. coli* for the synthesis of IPP from MVA

To metabolically engineer *E. coli* for the production of IPP from MVA, we constructed a synthetic operon coding for yPMD, hPMK and yMVK (Figures 1A and 1B). These enzymes were chosen because they have been shown to be active after expression in *E. coli* [30–32]. The three coding sequences were cloned in the high-copy plasmid pBAD-GFPuv, between the P_{BAD} promoter and the rrnB transcriptional terminator. Expression of the P_{BAD} promoter can be activated with L-arabinose and repressed with D-glucose [23,24]. The first ORF of the operon codes for yPMD and is preceded by the leader region of the T7 gene 10 (Figure 1A). The ORFs coding for hPMK and yMVK are preceded by ribosomal binding sites consisting of a Shine–Dalgarno sequence and an AT-rich translation spacer of eight bases, reported as the optimal distance to the ATG start codon [26]. The plasmid containing the synthetic operon coding for yPMD, hPMK and yMVK was named pAB-M2 (Figure 1B).

To analyse the functionality of the synthetic operon, plasmid pAB-M2 was introduced into *E. coli* strain EcAB1-1, in which the gene coding for 1-deoxy-D-xylulose 5-phosphate synthase (*dxs*) had been disrupted [16] (Table 2). As shown in Figure 3(A), strain EcAB1-1(pAB-M2) could grow only when DX or MVA were added to the medium. After 18 h at 37 °C, tiny colonies of this strain were visible in the plate containing MVA and D-glucose, while they were still too small to be distinguished in the plate containing MVA and L-arabinose. Approx. 2–3 days of growth were required for the apperance of well-grown colonies of strain EcAB1-1(pAB-M2) in the medium containing MVA and L-arabinose (results not shown). In spite of the slow growth, it can be concluded that the engineered bacteria can synthesize IPP from MVA. This implies that MVA can be transported into the *E. coli* cell and that conversion of this compound to IPP is compatible with the *E. coli* metabolism. In the *dxs* mutant background, IPP derived from MVA was the only source of the five-carbon prenyl unit. Since DMAPP is essential for isoprenoid

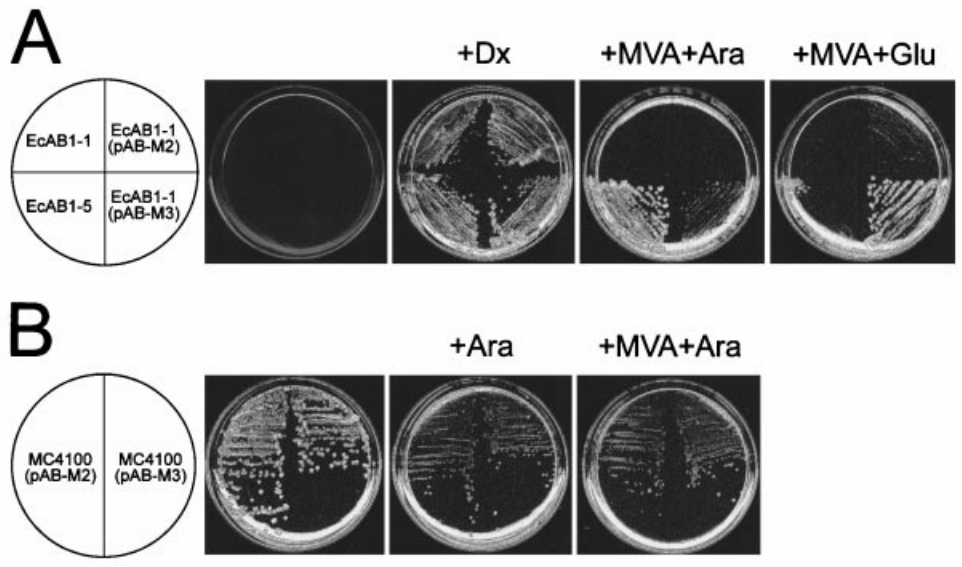

Figure 3 Complementation of *E. coli dxs* mutant strains with MVA

(**A**) *E. coli* cells of the mutant strains EcAB1-1, EcAB1-1(pABM2), EcAB1-1(pAB-M3) and EcAB1-5 were plated on 2 × TY medium containing 17 μg/ml chloramphenicol to select the *dxs*::*CAT* disruption. The medium was supplemented with 0.5 mM DX, 0.02% L-arabinose (Ara), 0.2% (w/v) D-glucose (Glu) and 1 mM MVA, as indicated. Plates were incubated at 37 °C for 18 h. (**B**) *E. coli* cells from the *dxs*$^+$ control strains MC4100(pAB-M2) and MC4100(pAB-M3) were plated on 2 × TY medium containing 100 μg/ml ampicillin to prevent plasmid loss. The medium was supplemented with 0.02% L-arabinose and 1 mM MVA, as indicated. Plates were grown at 37 °C for 14 h.

biosynthesis, it can be inferred that IPP was converted to DMAPP by endogenous IDI activity. This conclusion is relevant because either very low [33] or no IDI activity [34,35] was detected in various analyses of crude extracts from wild-type *E. coli* cells.

Incorporation of the ecIDI coding sequence in the synthetic operon

We aimed to determine whether the low endogenous IDI activity was limiting for the growth of strain EcAB1-1(pAB-M2) when MVA was used to complement the *dxs* mutant. We thus included the ORF coding for ecIDI in the synthetic operon. The operon containing ORFs coding for yPMD, hPMK, yMVK and ecIDI was named MVA$^+$ (Figure 1A). Plasmid pAB-M3 containing this synthetic operon (Figure 1C) was introduced into strain EcAB1-1. As shown in Figure 3(A), the resulting strain EcAB1-1(pAB-M3) grew in the presence of MVA at a higher rate than strain EcAB1-1(pAB-M2). Therefore, the incorporation of the ORF coding for ecIDI in the synthetic operon improved the complementation system. However, strains EcAB1-1(pAB-M2) and EcAB1-1(pAB-M3) grew better in the presence of D-glucose, the repressor of the P_{BAD} promoter, than in the presence of L-arabinose, the activator of the promoter (Figure 3A). In addition, the growth of both strains, as estimated by colony size, was inversely correlated with L-arabinose concentration in the medium (results not shown). These observations suggested that, although the expression of the synthetic operon present in plasmids EcAB1-1(pAB-M2) and EcAB1-1(pAB-M3) was required for complementation of the *dxs* mutant, it inhibited growth. To confirm this interpretation further, we examined whether plasmids pAB-M2 and pAB-M3 affected the growth of *E. coli* strain MC4100, from which mutant strain EcAB1-1 had been generated. As shown in Figure 3(B), when expression of the P_{BAD} promoter was induced with L-arabinose, the growth of *E. coli* MC4100 cells carrying plasmids pAB-M2 or pAB-M3 was significantly inhibited. No further inhibition of growth was observed in the plate containing L-arabinose and MVA (Figure 3B). It can be concluded that the expression of the synthetic operon of plasmids pAB-M2 and pAB-M3 inhibited growth of MC4100 cells, probably because of side effects caused by the encoded products, whereas the metabolization of MVA in these cells did not affect growth significantly.

Integration of the MVA$^+$ operon in the *E. coli* chromosome

Since pAB-M2 and pAB-M3 are high-copy plasmids, we reasoned that the high number of copies of the MVA$^+$ operon present in the engineered *E. coli* cells might be responsible, at least in part, for the inhibitory effect described above. To improve growth of the complemented mutant, we integrated a single copy of the MVA$^+$ operon into the *E. coli* chromosome. The MVA$^+$ operon was amplified by PCR using plasmid pAB-M3 as a template. The amplified sequence contained the complete promoter, including the regulatory sequences that respond to L-arabinose and D-glucose, and the four ORFs that allow conversion of MVA to IPP and DMAPP, but lacked the transcription-termination signals that were originally present in the expression cassette. The PCR product was integrated in the chromosome of *E. coli* strain TE2680, a *recD* mutant host that allows efficient recombination of linear DNA with homologous sequences in the chromosome. The resulting strain was named EcAB3-1 (Table 2). The MVA$^+$ operon was incorporated upstream of a promoterless *lac* operon containing *lacZ*, *lacY* and *lacA* coding sequences (Figure 1D). In the parent strain TE2680 this defective operon is not transcribed [27,28]. Integration of the MVA$^+$ operon in the appropriate orientation restored the expression of the *lacZ* gene that codes for β-galactosidase, thus confirming that the incorporated operon was expressed. β-Galactosidase activity was detected in strain EcAB3-1 by the appearance of

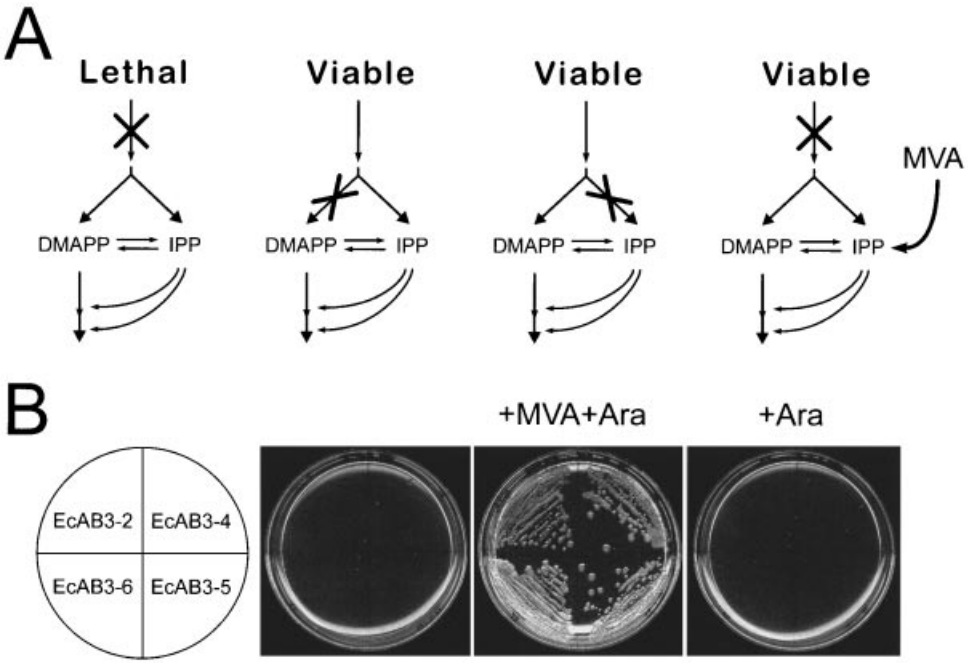

Figure 4 Phenotypic analysis of *E. coli ygbP*, *ychB* and *ygbB* deletion mutants

(**A**) Expected phenotypic effect of mutations blocking the common segment or the branches of the MEP pathway. Lethal mutants of the pathway can be rescued by synthesis of IPP and DMAPP from MVA. (**B**) *E. coli* cells from the mutant strains EcAB3-2, EcAB3-4, EcAB3-5 and EcAB3-6 were plated on medium containing 17 μg/ml chloramphenicol, 25 μg/ml kanamycin and 6 μg/ml tetracycline to select, respectively, the corresponding disrupted gene, the MVA$^+$ operon and the *recD* mutation. The medium was supplemented with 0.2% L-arabinose (Ara) and 1 mM MVA, as indicated. Plates were incubated at 37 °C for 16 h.

blue colonies after plating in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (Xgal; results not shown). Interestingly, the colour intensity of the colonies increased in the presence of L-arabinose and was very weak in the presence of D-glucose.

Strain EcAB1-5 was obtained from strain EcAB1-1, defective in the *dxs* gene, by transduction of the MVA$^+$ operon, using phage P1. As expected, DX added to the medium restores growth of the mutant strain EcAB1-5 (Figure 3A). This mutant can also be complemented with MVA. When MVA was added, the growth of strain EcAB1-5 was much higher in the presence of L-arabinose than in the presence of D-glucose (Figure 3A). Growth in the presence of MVA and D-glucose was not evident until approx. 2 days at 37 °C. Growth of strain EcAB1-5 was correlated directly with the L-arabinose concentration up to 0.2% (w/v; results not shown), which is 10 times higher than the L-arabinose concentration used in the experiment of Figure 3. In conclusion, a single copy of the MVA$^+$ operon is enough for complementation of the *dxs* mutant and expression of this operon can be modulated finely with L-arabinose and D-glucose. Growth of *E. coli* strains containing a single copy of the MVA$^+$ operon in the chromosome is not inhibited by L-arabinose (results not shown).

Strain EcAB3-1 as a tool for genetic analysis of the MEP pathway

As mentioned above, the MEP pathway for IPP and DMAPP biosynthesis is branched. Since IPP and DMAPP are essential in *E. coli*, any mutation blocking the trunk line of the pathway is expected to be lethal (Figure 4A). However, mutations blocking one of the branches alone are not expected to be lethal because the block would be circumvented by the IDI function. As shown by our results, the disruption of the *dxs* gene, which blocks the first step of the trunk line of the MEP pathway, results in a lethal phenotype. Neither IPP nor DMAPP can be synthesized in a non-supplemented *dxs* mutant. However, the synthesis of IPP from MVA, mediated by the synthetic operon of plasmid pAB-M2, suffices for the complementation of this mutant, because IPP can be converted to DMAPP by endogenous IDI activity. This activity should also mediate conversion of IPP to DMAPP, or vice versa, when a single branch of the MEP pathway is blocked.

E. coli strain EcAB3-1, carrying a single copy of the MVA$^+$ operon in the chromosome, is a suitable system for genetic analysis of the MEP pathway. As mentioned above, this strain is a *recD* mutant host which allows efficient recombination of linear DNA with homologous sequences in the chromosome. Candidate genes can be disrupted or deleted in strain EcAB3-1 upon transformation with linear DNA of appropriate constructs. Lethal mutants affecting the trunk line of the MEP pathway are rescued in this strain in the presence of MVA and L-arabinose.

Biochemical evidence was provided recently indicating that the products of the *ygbP*, *ychB* and *ygbB* genes from *E. coli* are involved in the MEP pathway [12–14]. To confirm that these genes are required for the synthesis of IPP and DMAPP, we deleted the ORFs of these genes in the EcAB3-1 genome (see the Experimental section for details). *E. coli* strain EcAB3-4 was obtained by deletion of the *ygbP* gene, EcAB3-5 by deletion of the *ychB* gene and EcAB3-6 by deletion of the *ygbB* gene (Table 2). In addition, strain EcAB3-2 was obtained from strain EcAB3-1 by disruption of the *dxs* gene. As shown in Figure 4(B), none of these strains grew in the absence of MVA and L-arabinose. None of the *ygbP*, *ychB* and *ygbB* mutants nor the control *dxs* mutant were complemented with L-arabinose alone, which was added to promote expression of ecIDI encoded in the MVA operon. Rescue of these mutants required the availability of

MVA that was converted to IPP and DMAPP. Thus, *ygbP*, *ychB* and *ygbB* are essential genes of the MEP pathway. No other *E. coli* gene can substitute for them under the growth conditions tested. If the *ygbP*, *ychB* and *ygbB* genes are involved in other biochemical processes in *E. coli*, these are not essential. Moreover, our results indicate that the enzymic reactions catalysed by the *ygbP*, *ychB* and *ygbB* gene products belong to the trunk line of the pathway. The branching of the pathway leading to IPP and DMAPP should thus occur after ME-2,4cPP (Scheme 1).

Our results are particularly relevant in what concerns the *ychB* gene. The results of *in vitro* assays and incorporation experiments led to the proposal of two functions for the *ychB* gene product in the MEP pathway: the conversion of CDP-ME to CDP-ME2P [13] and the conversion of isopentenyl monophosphate to IPP [15] (Scheme 1). The kinase encoded by the *ychB* gene is unlikely to have both functions *in vivo* because of the differences between the substrates and reactions proposed. The second proposal implies that the *ychB* gene product catalyses the last step of the branch that leads to IPP. This is not consistent with our genetic data, which show that the *ychB* gene is required for the trunk line of the MEP pathway. This argument is reinforced by our finding that the *ygbP* and *ygbB* genes, which code for enzymes catalysing the previous and following steps, are also required for the trunk line of the pathway. However, the genetic data presented do not allow us to rule out the involvement of the *ychB* gene product in the branch of the MEP pathway that leads to IPP.

Our system is compatible with strategies intended to identify novel genes of the MEP pathway. Preliminary experiments have shown that mutants of this pathway can be obtained by transposon tagging in an *E. coli* strain containing the MVA^+ operon in the chromosome (results not shown). The feasibility of an IPP-producing system for the isolation of mutants of the MEP pathway has been reported recently [36], although no precise description of the system used has been provided.

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