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Identification of Genes Affecting Lycopene Formation in *Escherichia coli* Transformed with Carotenoid Biosynthetic Genes: Candidates for Early Genes in Isoprenoid Biosynthesis

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Although isopentenyl diphosphate is a precursor of isoprenoids in *Escherichia coli*, the genes and enzymes involved in its biosynthesis have not been identified. Thus, we tried to isolate *E. coli* mutants deficient in the biosynthesis and their complementary genes by use of an artificial phenotypic screening system employing three carotenoid biosynthetic genes, *crtE*, *crtB*, and *crtI*. Cells were mutagenized with ethylmethanesulfonate, then transformed with a plasmid for expression of the carotenogenic genes. Mutants deficient in biosynthesis of isopentenyl diphosphate were expected to form white colonies, because they are unable to produce enough lycopene, whereas wild-type cells form red colonies. Among large numbers of red colonies, we identified 117 white colonies. Next, we transformed each mutant with an *E. coli* genomic library. Twenty-nine complementary genes that restore red color of host colonies were isolated. A homology search and further complementation study using subcloned genes revealed that the true complementary genes encode isopentenyl diphosphate isomerase, subunits of ATP synthase, enzymes of the Krebs cycle, some aldehyde dehydrogenases, phosphate acetyltransferase, and enzymes which relate to the biosynthesis of ubiquinones and menaquinones. Two unknown genes were also found, designated *elb1* and *2*, which may be involved in the early steps of isoprenoid biosynthesis.

Key words: carotenoid, complement, *Escherichia coli*, isoprenoid, mutant.

Isoprenoids are formed by condensation of branched five-carbon isoprene units and include a huge variety of biomolecules such as carotenoids, sterols, quinones, terpenes, dolichols, natural rubber, isopentenyladenosine, hemes, hopanoids, and prenylated proteins (*1-13*). Their common precursor is isopentenyl diphosphate (IPP) in all organisms, and the biosynthetic pathway of IPP in eukaryote and archaea has been confirmed to be the mevalonate pathway (*14, 15*). Condensation of three molecules of acetyl-CoA followed by reduction generates mevalonate *via* acetoacetyl-CoA and 3-hydroxy-3-methylglutaryl-CoA. Three subsequent steps of phosphorylation of mevalonate and one of decarboxylation give IPP. Although this pathway had been believed to be common to all organisms, several groups demonstrated that some eubacteria, *e.g.*, *Escherichia coli* (*16-18*), *Agrobacterium tumefaciens*, *Azotobacter vinelandii*, a *Pseudomonas* sp. (*16*), *Arthrobacter* (*19*), and *Zymomonas mobilis* (*18*), do not utilize mevalonate as the precursor of isoprenoids. In incorporation experiments with ^{13}C-labeled substrates such as glucose, Rohmer *et al.* showed that labeling patterns observed in bacteriohopane of *Rodopseudomonas palustris* and ubiquinone-8 (UQ-8) of *E. coli* differed from the patterns expected for the mevalonate pathway (*20, 21*). From these results and those of further experiments using triose phosphate metabolic mutants of *E. coli*, they postulated a novel pathway, which starts with condensation of pyruvate and glyceraldehyde-3-phosphate (*22*). Similar results has been observed in some plants, which suggests the existence of a novel pathway in plants, particularly in their plastids (*23-25*). However, most this pathway remains hypothetical, with neither the enzymes nor the genes that constitute the pathway having been discovered.

One of the best methods to elucidate an unknown biosynthetic pathway is to isolate mutants that have defects in the pathway. Detailed analyses of the mutants will provide important clues. For example, genes involved in the pathway can be isolated by their ability to complement the mutants, and their functions can be inferred from their homology to genes with known functions. Accumulation of an intermediate in mutant cells also provides clues about reactions in the pathway. Enzymatic assay using the intermediate often allows confirmation of the exact reactions. Many biosynthetic routes have been unraveled by this technique. In the case of isoprenoids, several trials have been made, some of which achieved remarkable success, mainly in *Saccharomyces cerevisiae*. Mutants of *S. cerevisiae*, each of which has a defect in the biosynthesis of isoprenoids such as sterol, ubiquinone, and dolichol, were selected based on auxotrophy (*26-28*). Their analyses helped the elucidation of the pathway and the cloning of

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Abbreviations: DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; IPP, isopentenyl diphosphate; MK, menaquinone; UQ, ubiquinone.

some genes of isoprenoid biosynthesis (*29-33*). Ergosterol-auxotrophic mutants assigned to almost all stages in the biosynthetic pathway of ergosterol were isolated, and some of the complementary genes, including phosphomevalonate kinase gene *erg8* (*34*), were cloned using the phenotypic screening system.

Similar attempts have been made to isolate mutants of *E. coli* deficient in the biosynthesis of isoprenoid. For example, Gibson isolated many mutants unable to synthesize ubiquinone, according to their nonviability on medium containing malate or succinate (*35*). Some genes were cloned by complementary screening of the mutants (*35-37*), but no genes of early isoprenoid biosynthesis, meaning the steps before the consecutive condensation by prenyltransferase, were isolated. Sherman *et al.* screened temperature-sensitive mutants of ubiquinone biosynthesis by use of aminoglycoside antibiotics, which require an energy transport system for their uptake into *E. coli* cells. They isolated several candidates, but failed to clone the early isoprenoid biosynthetic genes by complementary screening using those mutants (*38*). The difficulty in isolating mutants of the early stage of isoprenoid biosynthesis is thought to result from two factors: the complexity of the biosynthetic pathway of isoprenoids in *E. coli*, and the importance of the final products of the pathway. The pathway of isoprenoid biosynthesis has branches that diverge from the common intermediate, farnesyl diphosphate (FPP), and up- or down-regulation of one branch might complement a defect in another branch. Moreover, the final products of the branches are essential for normal growth of *E. coli* cells, and thus a defect in a step prior to the branching point causes pleiotrophic and frequently lethal effects on the mutant. If a defect in biosynthesis of a compound, such as ubiquinone, is accompanied by deficiency of other essential products, the formation of the compound cannot be used as a phenotypic marker for screening mutants deficient in the biosynthesis. Additionally, the ambiguousness of the biosynthetic pathway of IPP in *E. coli* makes the identification of mutants more difficult, in contrast with *S. cerevisiae*. Therefore, we needed to develop a new screening system that allows easy and efficient isolation of the mutants of early isoprenoid biosynthesis.

Previously we succeeded in cloning an archaeal geranylgeranyl diphosphate (GGPP) synthase gene by using an artificial phenotypic marker (*39*). In this work, we showed that the co-expression of three exogenous genes of GGPP synthase (*crtE*), phytoene synthase (*crtB*), and phytoene desaturase (*crtI*) in *E. coli* cells is sufficient for the conversion of IPP and FPP to a red-colored carotenoid, lycopene, and that formation of lycopene in *E. coli* cells can be detected by the color of colonies on agar plates. These observations suggest the possibility of establishing a novel system for the identification of mutants of early isoprenoid biosynthesis with the following features. (i) If the three genes of *crtE, crtB*, and *crtI* are introduced into a mutant that has a defect in the biosynthesis of IPP, the mutant may form white colonies on agar plates, which would be readily distinguishable from the red colonies of the wild-type. (ii) The artificial system is barely affected by the metabolism of the host cell, which is expected to have no system to control activities of exogenous enzymes and genes. Consequently the mutants and their complementary genes can be isolated efficiently. (iii) The system can detect the partial deficiency of IPP formation based on the depth of red coloration of the mutant cells. Such a system is very useful when lethal effects result from complete inactivation of an enzyme, such as those of early isoprenoid biosynthesis.

In this paper, we develop a unique system of mutant screening, which utilizes three exogenous carotenoid biosynthetic genes, to elucidate the early stage of isoprenoid biosynthesis. Using this system, we isolated many mutants deficient in artificial lycopene production and identified their complementary genes. Because IPP and FPP are converted to lycopene by the three enzymes of this artificial system, the mutations should have occurred in earlier steps of isoprenoid formation, and their complementary genes are candidates for the early genes of isoprenoid biosynthesis.

MATERIALS AND METHODS

Chemicals—UQ-10 was purchased from E. Merck. Other ubiquinones, as authentic samples, were donated by Dr. M. Tsujii. Antibiotics and ethylmethanesulfonate were purchased from Nacalai Tesque. Other chemicals were of analytic grade.

Bacterial Strains and Plasmids—*E. coli* DH5α strain was used for all manipulations. Plasmid pCAR25, which contained six carotenogenic genes, was donated by Dr. N. Misawa (*40*). A 1.3 kbp *Kpn*I-*Hpa*I fragment carrying *crtE* gene was isolated from pCAR25. The fragment was blunt-ended and inserted into the *Sca*I site of pACYC-IB (*39*). The resulting plasmid pACYC-IBE carries three *Erwinia uredovora* genes, *crtE, crtB*, and *crtI*, encoding GGPP synthase, phytoene synthase and phytoene desaturase, respectively (Fig. 1).

Growth Media—LB broth and LB agar were used. Ampicillin (60 μg/ml) and tetracycline (50 μg/ml) were added to media if necessary (*41*).

Isolation of Mutants and Their Complementary Plasmids—Cells of *E. coli* were mutagenized using ethylmethanesulfonate by the method of Miller (*42*). Competent cells derived from the mutagenized cells were transformed with pACYC-IBE and spread on LB agar plates containing tetracycline. The transformants were cultured at 37°C for 12 h, then the plates were placed at room temperature. About 3 to 5 days later, most of the colonies colored red, indicating the accumulation of lycopene. From 21,000 transformants, 117 colonies with reduced red coloration were isolated.

After making the competent cells from each selected mutant harboring pACYC-IBE, the cells were transformed with plasmids from an *E. coli* genomic library, which was prepared by partial *Sau*3AI digestion of *E. coli* genome and insertion of 3-6 kbp fragments into the *Bam*HI site of pUC119. The transformants were spread on LB agar plates containing ampicillin and tetracycline. Most of them formed white colonies. However, some colonies became red, which indicated the recovery of lycopene production, and we selected them from among 5,000-20,000 white colonies. In total, we isolated 29 red colonies from 25 mutants, and the plasmids derived from the genomic library were obtained from the colonies. Both ends of inserts of all plasmids and the full-length inserts of some plasmids were sequenced by the dideoxy method, and the genes included

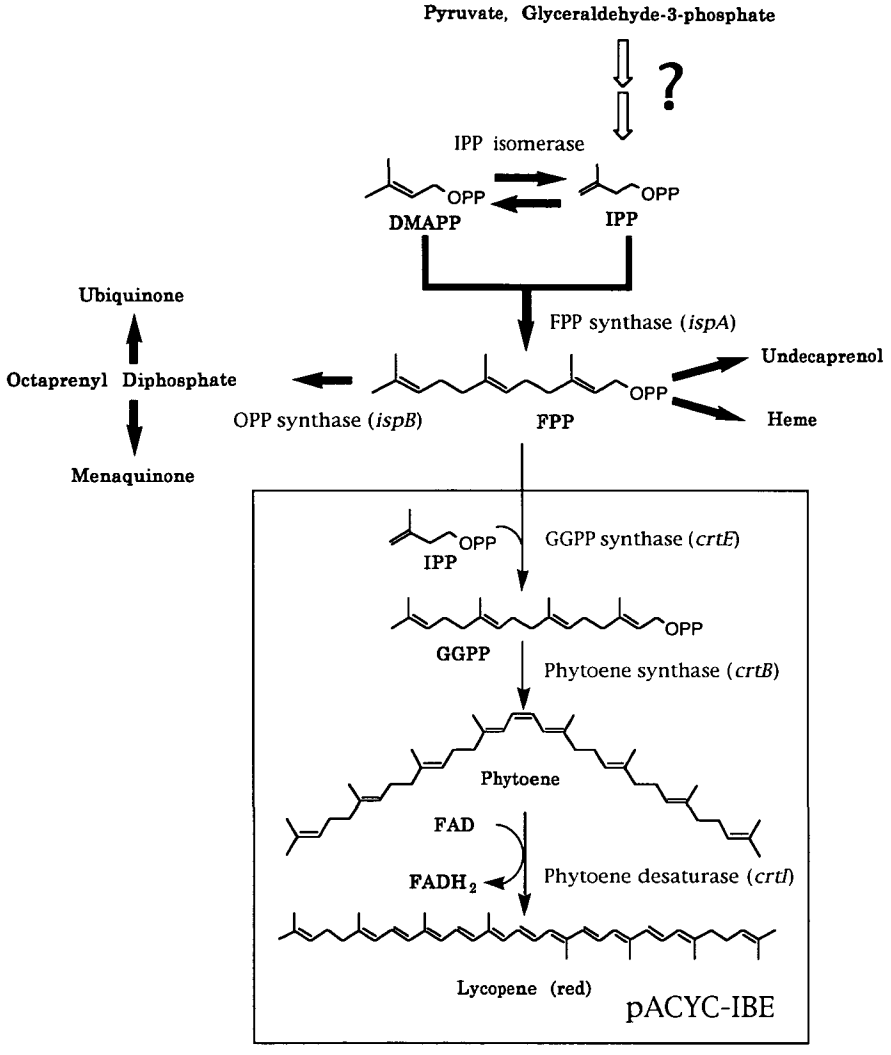

Fig. 1. **Isoprenoid biosynthesis in *E. coli* harboring the carotenogenic plasmid pACYC-IBE.** The enzymatic reactions encoded on the plasmid are shown inside the box.

in the inserts were confirmed by searching several databases (EMBL/GenBank/DDBJ) using the BLAST software at the National Center for Biotechnology Information. Almost all sequences and restriction maps that we determined were identical with those in the databases.

Determination of Complementary Genes—To select true complementary genes that can restore the color of mutant colonies from among the genes in the insert of each complementary plasmid, we subcloned some fragments of the insert and determined their ability to complement the deficiency in lycopene biosynthesis. An *Sma*I-*Stu*I fragment of about 1.4 kbp, containing only *sucB*, derived from pWL301-03 was subcloned into pUC118 *Sma*I vector, and the plasmid generated from the residual region contained only *sucC*. Mutant WL301 was shown to be complemented by the former. The plasmids derived from deletion of the inserts of p3-7-01 and p3-8-01 were utilized to determine the complementary genes for the corresponding mutants. Mutants 3-7 and 3-8 were complemented by the plasmid carrying only *f107* and only *ubiB*, respectively. From the insert of p3-24-01, which is almost identical to p3-50-05, an 1.8 kbp *Eco*RI fragment containing intact *f101* and *menF* whose product lacked part of its NH_2 terminus, was subcloned and proved to have complementary activity. We constructed a plasmid carrying only the large part of *menF* by deleting the *Eco*RI fragment, and the plasmid complemented both mutant 3-24 and 3-50. From the insert of p3-26-01, both a 2.7 kbp *Sal*I fragment and the residual 1.6 kbp region, including only *eutG* and only *eutE*, respectively, were subcloned, and the resultant plasmids were utilized to determine the complementary gene. The latter complemented mutants 3-26, 3-48, 3-81, and 3-102, but not mutant 3-69, whereas its complementary plasmid p3-69-02 contained *eutE*. Mutant 3-47 was complemented by p3-47-11⊿*Eco*RI, which includes 1.3 kbp region derived from *Eco*RI digestion of p3-47-11 and contains only *o182*. From the insert of p3-69-02, a 3.2 kbp *Sma*I fragment was removed, and the residual 1.1 kbp region carrying only *eutI* was subcloned. The resultant plasmid complemented mutant 3-69, whereas the former and other plasmids derived from p3-26-01 did not. To determine the complementary gene for mutant 3-123, we used plasmids containing deleted fragments of the insert of p3-123-01. The plasmid containing only *yhbL* was shown to complement the mutant.

Measurement of Lycopene and Ubiquinone—*E. coli* cells were cultured on LB agar plates or in LB broth. Plate cultures were incubated at 37°C for 24 h to form a lawn,

then cells were harvested by scratching. In liquid culture, cells were grown aerobically and harvested at stationary phase. The cells from plate and liquid cultures were lyophilized and weighed out, then 20 mg portion of the freeze-dried cells were suspended in 2 ml of methanol-0.3% aqueous NaCl (10:1, by volume). After addition 3 mg of UQ-10 or UQ-6 as the internal standard, the cells were sonicated ten times, each for 1 min interspersed with 30 s intervals of cooling in ice. From the suspensions, hydrophobic compounds were extracted twice with 2 ml of *n*-pentane, and the extracts were evaporated to dryness. The residues were dissolved with 500 μl of 2-propanol. All possible processes were manipulated in darkness (*43*).

TLC analyses confirmed that the major colored compound in the extracts was lycopene (Merck Kieselgel 60 F_{254}, developed with hexane: chloroform = 1:1, data not shown) (*40*). The amounts of lycopene in the extracts were determined from its absorption at 472 nm by use of a Hitachi U-2000 spectrophotometer.

Ubiquinone in the extracts was determined by high-performance liquid chromatography with a Hitachi 655A chromatograph by a modification of the method of Fujisaki *et al.* (*43*). An aliquot of each extract was loaded on 5 μm LiChrosorb RP-18 column (Cica-MERCK) and developed with 99.5% ethanol at a flow rate of 1 ml/min. The eluent was monitored at 275 nm. The amount of UQ-8 was determined from its absorption relative to those of UQ-10 and UQ-6 as the internal standards.

RESULTS

Construction of an Artificial Mutant Screening System—A new "red-white mutant screening system" was developed in order to isolate mutants deficient in the earlier steps of isoprenoid biosynthesis. First, we constructed a plasmid for expression of the three enzymes, GGPP synthase, phytoene synthase, and phytoene desaturase. A 1.3 kbp *Kpn*I-*Hpa*I fragment carrying *crtE* gene was isolated from pCAR25, a plasmid carrying *E. uredovora* carotenoid biosynthesis gene cluster (*40*). This fragment can express active GGPP synthase in *E. coli* by itself. The fragment was blunt-ended and inserted into the *Sca*I site of pACYC-IB (*39*), making plasmid pACYC-IBE, which has the three carotenogenic genes. Because this plasmid is derived from pACYC-184, it can co-exist with pUC vectors and contains the tetracycline resistance gene. Wild-type *E. coli* can form red colonies on agar plates when it is transformed with the plasmid, and the transformant produces about 10 μg of lycopene per g of dry cells when it is grown in liquid medium.

Isolation of Mutants with the Red-White Mutant Screening System—*E. coli* cells mutagenized with ethylmethanesulfonate were transformed with pACYC-IBE and cultured at 37°C on LB agar plates containing tetracycline to form colonies, then the plates were placed at 20°C. After 3-5 days, the majority of colonies became red. This coloration indicates the production of lycopene in the cells. However, some mutants delayed their accumulation of carotenoid and formed whiter colonies than others. Through three screenings, 117 white colonies were isolated from *ca.* 21,000 colonies. These mutants should have some defect in the biosynthetic pathway of lycopene, including formation of IPP. They might also include mutants in which the pACYC-type plasmid has become unstable. To exclude mutants of this type, all mutants were transformed with pCAR25, a plasmid encoding the six enzymes sufficient for production of zeaxanthin-β-diglucoside and carrying *Col*E1 origin (*40*), and then their colors were checked. As a result, 9 mutants were excluded whose colonies strongly colored yellow, indicating that their mutations lie not in genes responsible for carotenoid biosynthesis, but in those that affect pACYC-IBE.

To analyze effects of the mutations, we collected cells of the mutants, for which complementary genes were isolated later, by scraping confluent cultures on the agar plates, then measured the amounts of lycopene in the cells, as shown in Table I. All mutants were then transformed with pUC119 so that they could be correctly compared with complemented ones later. Most of the mutants showed reduced production of lycopene in comparison with wild-type cells harboring both pACYC-IBE and pUC119. Because [all-*E*]-octaprenyl side chain of ubiquinone, a cofactor of the electron transfer system, is derived from IPP, we also determined the amounts of UQ-8 in the cells of mutants. Wild-type cells harboring pACYC-IBE and pUC119 produced a considerably smaller amount of UQ-8 than wild-type cells which were transformed with pACYC-184 and pUC119. The levels of UQ-8 formation in most of the mutants were slightly higher than the former, but still lower than the latter (Table I).

Isolation of Complementary Plasmids of the Mutants—Plasmids derived from *E. coli* genomic library were introduced into each of the isolated mutants harboring pACYC-IBE. The library was constructed using pUC119, which can co-exist with pACYC-IBE because of the difference in their

TABLE I. **Levels of lycopene and UQ-8 in mutant cells.** The mutants were grown on LB agar plates at 37°C, and after 24 h, confluent cultures on the plates were harvested by scraping. The amounts of lycopene and UQ-8 in the cells were measured as described under "MATERIALS AND METHODS."

Host mutant/plasmid	Lycopene (μg/g of dried cells)	UQ-8 (μg/g of dried cells)
Wild type/pACYC184, pUC119	—	1,050±132
Wild type/pACYC-IBE, pUC119	76.9± 7.2	513± 39
WM301/pACYC-IBE, pUC119	36.8± 8.3	650± 8
WL301/pACYC-IBE, pUC119	15.8± 5.5	588± 36
WM302/pACYC-IBE, pUC119	14.7± 2.6	531± 50
3-7/pACYC-IBE, pUC119	55.4±23.6	525± 72
3-8/pACYC-IBE, pUC119	64.9± 3.0	526± 59
3-12/pACYC-IBE, pUC119	23.1± 9.5	677± 57
3-20/pACYC-IBE, pUC119	10.1± 1.3	646±122
3-21/pACYC-IBE, pUC119	14.1±14.0	681± 51
3-23/pACYC-IBE, pUC119	29.3± 1.4	631± 45
3-24/pACYC-IBE, pUC119	20.5± 3.9	677± 67
3-26/pACYC-IBE, pUC119	37.9±18.7	524± 24
3-42/pACYC-IBE, pUC119	22.7± 5.8	654± 29
3-47/pACYC-IBE, pUC119	24.4± 7.0	628± 42
3-48/pACYC-IBE, pUC119	41.0± 6.0	651±153
3-50/pACYC-IBE, pUC119	24.1± 2.8	642± 50
3-52/pACYC-IBE, pUC119	54.9± 3.7	589± 26
3-53/pACYC-IBE, pUC119	29.4±17.8	913±151
3-68/pACYC-IBE, pUC119	69.7± 8.3	397± 64
3-69/pACYC-IBE, pUC119	41.9± 7.4	456± 12
3-81/pACYC-IBE, pUC119	25.4± 4.0	798± 23
3-90/pACYC-IBE, pUC119	23.0± 4.8	725± 80
3-93/pACYC-IBE, pUC119	69.6±17.4	573± 53
3-94/pACYC-IBE, pUC119	70.2±12.2	516± 80
3-102/pACYC-IBE, pUC119	44.2± 3.6	570± 36
3-123/pACYC-IBE, pUC119	60.6±16.0	656± 85

origins. If a transformant recovers its carotenoid production, it will form a redder colony than those other transformants form. This recovery indicates that the introduced plasmid might encode the same enzyme actually damaged in the mutant, or another enzyme that has the same function. As a result of screening of the plasmids that caused the formation of red colonies, 29 complementary plasmids were isolated from 25 out of 117 mutants (Table II).

To confirm whether each complementary plasmid actually increases formation of lycopene in the mutant harboring pACYC-IBE, the amount of lycopene in the cells harboring both pACYC-IBE and the complementary plasmids was determined and compared with that in the mutant cells harboring both pACYC-IBE and pUC119 (Table III). Increases in the accumulation of lycopene occurred in many mutants as the result of complementation, and the complementary plasmids can be classified into four groups according to the extent of the increase. The first group of plasmids, containing pWL301-03, p3-20-01, p3-21-02, p3-23-04, p3-24-01, p3-42-01, p3-47-13, p3-50-05, p3-53-02, and p3-53-04, markedly increases the production of lycopene in the corresponding mutants. The second group, including p3-8-01, p3-47-02, p3-47-11, p3-69-02, p3-81-02, p3-90-02, and p3-123-01, increases the lycopene accumulation to some degree. The third group, which contains pWM301-01, pWM302-01, pWM302-03, p3-7-01, p3-48-02, p3-68-01, p3-94-02, and p3-102-01, does not change the amount of lycopene in the mutants, though some of them increase the production of lycopene when the cells are grown in liquid medium (data not shown). The last group, which includes p3-12-01, p3-26-01, p3-52-01, and p3-93-03, reduces formation of lycopene. The results obtained with the latter two groups might arise from the difference in growth conditions, because confluent cultures were used in the measurement of lycopene.

We also determined effects of the complementation on production of UQ-8 in the mutants. HPLC analyses revealed slight decreases in UQ-8 production caused by the complementation in most mutants, while some mutants showed increased production (Table III).

Assignment of Complementary Genes—To determine the genes that were contained in the complementary plasmids, both ends of the inserted fragment of each plasmid and the full-length inserts of some plasmids were sequenced, then homologous sequences in DNA databases (EMBL/GenBank/DDBJ) were searched by using BLAST software. Because the whole genome sequence of *E. coli* has now been determined (*44*), we could get information about all fragments, *i.e.*, map positions, full DNA sequences, included

TABLE II. **Complementary genes and map positions.** All genes included in isolated plasmids are indicated, and the complementary genes, decided by subcloning or deletion of the plasmids excepting seven clones which contained several *unc* genes as described under "MATERIALS AND METHODS," are shown in boldface and underlined. The complementary genes in p3-7-01 and p3-123-01 have been designated as *elb1* and *2*, respectively, and their names on the databases are indicated in following parentheses.

Host mutant	Plasmid	Complementary genes	Map position (min)
WM301	pWM301-01	*uncG, uncD, uncC*	84.3-84.5
WL301	pWL301-03	***sucB***, *sucC*	16.2-16.5
WM302	pWM302-01	*uncG, uncD, uncC, glmU*	84.3-84.5
WM302	pWM302-03	*uncG, uncD, uncC, glmU*	84.3-84.5
3-7	p3-7-01	*ycfK, o137, ycfA, f179, pin, mcrA,* ***elb1*** *(f107)*	26.0
3-8	p3-8-01	***ubiB***, *yigC, rfaH, yigW*	86.8
3-12	p3-12-01	*yiaW*, ***aldB***	80.9-81.0
3-20	p3-20-01	***gltA***	16.2-16.5
3-21	p3-21-02	***sucA***	16.2-16.5
3-23	p3-23-04	***gltA***	16.2-16.5
3-24	p3-24-01	*f101*, ***menF***	51.3
3-26	p3-26-01	***eutE***, *eutJ, eutG*	55.3-55.4
3-42	p3-42-01	*uncG, uncD, uncC, glmU*	84.3-84.5
3-47	p3-47-02	*uncG, uncD, uncC, glmU*	84.3-84.5
3-47	p3-47-11	*o276, f163, f644, o505,* ***o182***	65.0-65.1
3-47	p3-47-13	***o182***, *o505*	65.0-65.1
3-48	p3-48-02	***eutE***, *eutJ, eutG*	55.3-55.4
3-50	p3-50-05	*f101*, ***menF***	51.3
3-52	p3-52-01	***uncD***	84.3-84.5
3-53	p3-53-02	*uncE, uncF, uncH*	84.3-84.5
3-53	p3-53-04	*uncE, uncF, uncH, uncA, uncG, uncD, uncC*	84.3-84.5
3-68	p3-68-01	***aldH***	29.3
3-69	p3-69-02	*eutJ, eutE, cchB, cchA,* ***eutI***	55.3-55.4
3-81	p3-81-02	*eutG, eutJ,* ***eutE***, *cchB*	55.3-55.4
3-90	p3-90-02	*selB, yiaY,* ***aldB***	80.9-81.0
3-93	p3-93-03	*yiaW*, ***aldB***	80.9-81.0
3-94	p3-94-02	*yiaW*, ***aldB***	80.9-81.0
3-102	p3-102-01	***eutE***, *eutJ, eutG*	55.3-55.4
3-123	p3-123-01	***elb2*** *(yhbL), yrbM, o210*	72.3

TABLE III. **Effects of complementation on levels of lycopene and UQ-8 in mutant cells.** Complementary plasmids were introduced in corresponding mutants that harbored pACYC-IBE, and the transformants were grown on LB agar plates as described under "MATERIALS AND METHODS." The amounts of lycopene and UQ-8 in the cells were measured and compared with those in the cells of corresponding mutants harboring both pACYC-IBE and pUC119 to determine their relative amounts.

Complementary plasmid	Lycopene (μg/g of dried cells)	Relative amount of lycopene (%)	UQ-8 (μg/g of dried cells)	Relative amount of UQ-8 (%)
pWM301-01	28.5± 5.6	77	672± 51	103
pWL301-03	50.8±12.7	322	560± 60	95
pWM302-01	13.6± 4.1	93	620± 29	117
pWM302-03	12.7± 1.4	86	625± 76	118
p3-7-01	55.0±16.3	99	543± 56	103
p3-8-01	84.3± 8.2	130	319± 6	61
p3-12-01	11.9± 1.9	52	724± 20	107
p3-20-01	49.8± 2.7	493	408± 28	63
p3-21-02	33.8± 3.7	240	610± 7	90
p3-23-04	83.2± 4.2	284	424± 37	67
p3-24-01	77.0± 0.6	376	366± 24	54
p3-26-01	24.8± 7.0	65	547± 83	104
p3-42-01	53.5± 2.6	236	564± 67	86
p3-47-02	39.3±16.6	161	558± 32	89
p3-47-11	43.1± 6.9	177	790± 15	126
p3-47-13	55.8±19.3	229	772± 71	123
p3-48-02	51.1± 7.3	125	397± 14	61
p3-50-05	56.5±11.0	234	570± 43	89
p3-52-01	37.9±11.0	69	548± 85	93
p3-53-02	61.7±15.1	210	672±116	74
p3-53-04	65.3±14.3	222	701± 49	77
p3-68-01	59.2±12.6	85	445± 48	112
p3-69-02	56.1±10.5	134	356± 21	78
p3-81-02	38.2± 8.1	150	620±177	78
p3-90-02	40.1± 4.3	174	669± 62	92
p3-93-03	50.8± 1.4	73	577± 76	101
p3-94-02	67.2± 6.5	96	687± 40	133
p3-102-01	40.0± 4.7	90	507± 32	89
p3-123-01	98.7±15.5	163	408± 55	62

ORFs and encoded enzymes. Average length of the inserted fragments is *ca.* 4.3 kbp. Most of the fragments have several ORFs (Table II). The sequences derived from the databases are almost identical to those that we determined, and almost all restriction sites are conserved. Thus, using appropriate restriction enzymes, we subcloned digested parts of the fragments into pUC118 or pUC119, considering the direction of *lac* promoter, to determine which genes actually function in the complementary plasmids. Deletion of the inserted fragments was also performed for the same purpose. If the subcloned DNA fragment contains the complementary gene, the resultant plasmid must have the same ability as the parental plasmid to restore the color of the corresponding mutant cells.

As shown in Table II, we determined almost all complementary genes for the mutants. Mutant 3-47 is complemented by p3-47-11 and p3-47-13, both of which contained *o182*, a gene of *E. coli* IPP isomerase (*45*). The subcloned plasmid p3-47-11⊿*Eco*RI, carrying only *o182*, can complement mutant 3-47. Plasmid p3-47-02, which does not contain *o182*, also complements the mutant, though it has only weak ability to restore the color of mutant cells.

Some clones are mapped in the same region, though they were isolated by screening for different mutants. The inserted DNA fragments of pWM301-01, pWM302-01, pWM301-03, p3-42-01, p3-47-02, p3-52-01, p3-53-02, and p3-53-04 are mapped at 84.3-84.5 min, a region that includes *uncE, uncF, uncH, uncA, uncG, uncD*, and *uncC*, which encode the subunits of ATP synthase (*46*), and *glmU*, which encodes *N*-acetylglucosamine-1-phosphate uridyltransferase (*47, 48*). This result clearly indicates the requirement for ATP for the production of lycopene, therefore we did not determine the subunit that actually complements the mutant.

Five clones, p3-26-01, p3-48-02, p3-69-02, p3-81-02, and p3-102-01, are mapped at the same region within 55.3-55.4 min, which includes parts of the ethanolamine utilization operon (*49*). Mutants 3-26, 3-48, 3-81, and 3-102 are complemented by *eutE*, which encodes an aldehyde dehydrogenase, whereas mutant 3-69 is complemented by *eutI*, the gene for phosphate acetyltransferase. The DNA fragment of pWL301-03, p3-20-01, p3-21-02, and p3-23-04, are mapped in the same region of 16.2-16.5 min, which contains an operon related to the Krebs cycle (*50-52*). The operon consists of the genes for citrate synthase (*gltA*), succinate dehydrogenase (*sdhC, sdhD, sdhA*, and *sdhB*), 2-oxoglutarate dehydrogenase (*sucA*), dihydrolipoamide succinyl transferase (*sucB*), and succinyl-CoA synthase (*sucC* and *sucD*); and the mutants WL301, 3-20, 3-21, and 3-23 are complemented by different genes of *sucB, gltA, sucA*, and *gltA*, respectively. Four clones, p3-12-01, p3-90-02, p3-93-03, and p3-94-02, are mapped at 80.9-81.0 min and contain only *aldB*, the aldehyde dehydrogenase gene (*53*). Each clone can complement all of the mutants 3-12, 3-90, 3-93, and 3-94, and this strongly suggests *aldB* is the complementary gene. Besides, mutant 3-68 is complemented by a distinct aldehyde dehydrogenase gene (*aldH*) that is included in p3-68-01 and mapped at 29.3 min (*54*). Lycopene formation of mutants 3-24 and 3-50 is complemented by *menF* gene, which is included in p3-24-01 and p3-50-05 and encodes isochorismate synthase (*55*). The complementary gene in p3-8-01 is *ubiB*. It encodes an enzyme of ubiquinone biosynthesis, which catalyzes the hydroxylation of 2-octaprenylphenol to 2-octaprenyl-6-hydroxyphenol (*56*). However, the production of UQ-8 in mutant 3-8 is not increased by the transformation with p3-8-01 (Table III). Mutants 3-7 and 3-123 are complemented by genes mapped at 26.0 min (*f107*) and 72.3 min (*yhbL*), respectively, but their functions are unknown. We designated these genes as *elb* (enhancing lycopene biosynthesis) *1* and *2*.

TABLE IV. **Effect of complementary plasmids on levels of lycopene and UQ-8 in wild-type cells.** Wild-type cells transformed with pACYC-IBE and the complementary plasmids were grown in LB medium and harvested at stationary phase. The amounts of lycopene and UQ-8 in the cells were measured as described under "MATERIALS AND METHODS."

Plasmid	Lycopene (μg/g of dried cells)	UQ-8 (μg/g of dried cells)
pUC119	16.9± 1.4	457±144
pWL301-03	47.1±10.8	460±137
p3-7-01	49.1±19.5	487±186
p3-8-01	79.4±20.2	411±128
p3-20-01	53.4±21.8	405±132
p3-21-02	64.6±10.2	523± 13
p3-23-04	46.2±21.8	401±148
p3-24-01	47.1±13.7	477±122
p3-47-11	28.9± 4.0	524±196
p3-47-13	37.3± 6.3	578±179
p3-50-05	75.2±20.4	586± 88
p3-53-02	31.6±10.1	452±106
p3-53-04	27.0± 1.7	541±177
p3-69-02	87.9±12.4	362±119
p3-123-01	27.5± 2.7	456±166

Effects of Complementary Genes on Lycopene Production in Wild-Type E. coli—The isolated genes were examined for their abilities to incerase the accumulation of lycopene in wild-type cells. Wild-type *E. coli* cells harboring pACYC-IBE were transformed with each of the complementary plasmids, pWL301-03, p3-7-01, p3-8-01, p3-20-01, p3-21-02, p3-23-04, p3-24-01, p3-47-11, p3-47-13, p3-50-05, p3-53-02, p3-53-04, p3-69-02, and p3-123-01. The transformants were cultured in liquid medium, and the amounts of lycopene and ubiquinone-8 in the cells were measured (Table IV). Most of the transformants produced larger amounts of lycopene than the wild-type cells harboring both pACYC-IBE and pUC119.

Because an enlarged peak was detected near that of UQ-10, the regular internal standard, in HPLC analysis for ubiquinone in wild-type cells that harbored p3-24-01 or p3-50-05, we exceptionally used UQ-6 as an internal standard to observe the peak. When the cells were transformed with p3-24-01 or p3-50-05, the peak became three or seven times larger than that detected in the analysis of wild-type cells harboring pUC119, respectively. The peak is expected to be identical to MK-8 from its elution pattern previously reported (*43*).

DISCUSSION

In this work, we selected 117 mutants of *E. coli* whose colonies showed reduced coloration derived from artificial formation of lycopene. Since most of them showed a real decrease in lycopene production and are expected to have a defect in an early step of isoprenoid biosynthesis, we screened an *E. coli* genomic library for their complemen-

tary genes and succeeded in isolating 29 genes from 25 mutant lines. No complementary genes have yet been isolated from the rest of the mutants. This difficulty might result from multiple mutations of the mutants. Most of the complementary plasmids can increase the lycopene production of the corresponding mutants and even of wild-type *E. coli*. We investigated the genes included in the plasmids because of the strong possibilities that they encode the early steps of isoprenoid biosynthesis.

We succeeded in isolating a known gene that encodes an early step of isoprenoid biosynthesis. The complementary gene, which is included in p3-47-11 and p3-47-13, encodes IPP isomerase. This enzyme catalyzed the conversion between IPP and DMAPP. This reaction precedes the carotenogenic steps encoded in pACYC-IBE (Fig. 1). *E. coli* IPP isomerase gene has recently been identified by database searches based on the homology with IPP isomerase of *Rhodobacter capsulatus* (*45*), and it has been reported that overexpression of this enzyme in *E. coli* cells harboring carotenoid biosynthetic genes increases the production of carotenoids in the cells (*57*). The acquisition of the gene in this work clearly demonstrates the advantage of this screening system in isolating the early genes of isoprenoid biosynthesis.

Some of the complementary plasmids include several genes of subunits of ATP synthase and enzymes of the Krebs cycle. This might simply indicate the requirement for high energy compounds in some step of carotenogenesis, because no compound in the Krebs cycle seems to be directly involved in the hypothetical pathway proposed by Rohmer *et al.* (*22*). Since at least two phosphorylation processes should be involved in the biosynthesis of IPP, it is reasonable that mutation of these enzymes causes the decrease of lycopene formation.

We selected a gene of biosynthesis of UQ-8, *ubiB*, as an effectual gene on carotenoid biosynthesis in *E. coli*. It was expected that the product of *ubiB* gene should produce a positive effect on the ubiquinone synthesis, since it catalyses the hydroxylation of 2-octaprenylphenol under aerobic growth conditions (*58*). However, no significant changes were observed in ubiquinone production in either mutant 3-8 or wild-type cells when they were transformed with *ubiB*. This might suggest that a later step is rate-limiting in ubiquinone biosynthesis, and indicate that the effect of *ubiB* on lycopene formation does not derive from an increase of UQ-8. If so, the enzyme encoded in *ubiB* or its product (or derivatives of it) should activate the biosynthesis of lycopene at the stage of IPP formation or later in an unknown way.

On the other hand, mutants 3-24 and 3-50 are complemented by *menF*, which encodes an enzyme of menaquinone (MK) biosynthesis, isochorismate synthase. When wild-type cells harboring p3-24-01 or p3-50-05 were grown in liquid medium, a peak that was thought to be identical to MK-8 was increased. Consequently, it is conceivable that an increased amount of MK-8 affects the lycopene production. Even under these aerobic growth conditions, an increase of MK-8 might have a positive effect on the energy generation as a hydrogen carrier for anaerobic oxidation (*59*), if the formation of lycopene produced reductive conditions and caused oxygen deficiencies in the cells. This hypothesis is supported by the fact that some genes of aldehyde dehydrogenase, *aldB*, *aldH*, and *eutE*, were isolated as complementary genes. They might restore the color of mutant colonies by resolving the accumulation of toxic aldehydes caused by the reductive conditions.

However, other possibilities remain, such as the acceleration of some step in the early isoprenoid biosynthetic pathway by MK-8. Alternatively, the increase of isochorismate synthase or intermediates of menaquinone biosynthesis may stimulate IPP formation by regulating expression of enzymes of IPP biosynthesis. Whatever the case, the finding that the biosynthetic genes of ubiquinone and menaquinone affect the production of lycopene might indicate the existence of an unknown mechanism regulating the biosynthesis of isoprenoids in *E. coli*.

The plasmids including the ethanolamine utilization operon complement five mutants. Four of the mutants are complemented by *eutE*, and one by *eutI*. Because the radioactivity of [^{14}C]ethanolamine was found to be incorporated not into neutral lipids, which contain some isoprenoid compounds, but into fatty acids in *E. coli* (*60*), it is hard to imagine that all genes in the operon are directly involved in the biosynthetic pathway of lycopene. However, phosphate acetyltransferase, encoded in *eutI*, produces acetylphosphate from acetyl-CoA, and this product might act as a donor of an acetyl group or a phosphate group in the biosynthesis of IPP. Alternatively, acetylphosphate might facilitate carotenogenesis in *E. coli* cells by influencing other metabolic pathways.

Genes with unknown functions were also isolated. The complementary gene included in p3-7-01 was *f107*, designated as *elb1*. The gene *elb1* encodes a small hypothetical protein, whose predicted molecular mass is 12 kDa. No protein homologous to the product of *elb1* could be found by a search of databases. Mutant 3-123 was complemented by *yhbL*, designated as *elb2*. This gene encodes sigma cross-reacting protein 27A, which might affect the lycopene production as the transcriptional regulator of other genes. It is conceivable that the product of *elb2* regulates the biosynthetic pathway of ubiquinone, including early steps of isoprenoid biosynthesis, because *elb2* is located beside *arcB*, which encodes aerobic respiration control sensor protein, and they seem to be contained in the same operon. The fact that the wild-type *E. coli* harboring pACYC-IBE also shows increased production of lycopene on transformation with p3-7-01 or p3-123-01 strongly suggests the involvement of the two isolated genes, *elb1* and *2*, in the early steps of isoprenoid biosynthesis.

In this study, we developed a new screening system to isolate mutants of the early stage of isoprenoid biosynthesis and complementary genes for the mutants. These complementary genes are candidates for the genes that relate directly or indirectly to the formation of isoprenoid. They might be applied to industrial use because they can increase the production of lycopene even in wild-type cells. This system is simple to use and efficient, and it will allow selection of new genes from newly selected mutants or mutants whose complementary genes have not been isolated yet. However, the complementation might not be very strict, because mutant 3-47 harboring pACYC-IBE is complemented by different genes. This indicates that some mutants might be complemented not only by the gene in which the mutant has a defect, but also by other genes, which at least affect lycopene production. Therefore, further investigation into the isolated genes is needed. Never-

theless, this system will provide a clue to the elucidation of early isoprenoid biosynthesis.

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