Construction and Characterization of *Escherichia coli* Disruptants Defective in the yaeM Gene

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*Escherichia coli* disruptants defective in the yaeM gene, which is located at 4.2 min on the chromosome map, were constructed and characterized. The disruptants showed auxotrophy for 2-C-methylyethritol, a free alcohol of 2-C-methyl-d-erythritol 4-phosphate that is a biosynthetic precursor in the nonmevalonate pathway. This result clearly shows that the yaeM gene is indeed involved in this pathway in *E. coli*.

**Key words:** biosynthesis; terpenoid; nonmevalonate pathway; 2-C-methyl-d-erythritol 4-phosphate; *Escherichia coli*

Since the initial discovery of the mevalonate pathway in the 1950s, it has been widely accepted that isopentenyl diphosphate (IPP), a fundamental unit in terpenoid biosynthesis, is synthesized in all living organisms only by the ubiquitous mevalonate pathway. On the other hand, it has recently been disclosed that several organisms, including many bacteria, green algae, and chloroplasts of higher plants, use an alternative mevalonate-independent pathway (nonmevalonate pathway) for the formation of IPP. Several recent experimental findings lend additional support to the operation of this novel pathway in terpenoid biosynthesis. The pathway, however, is not completely understood even in *Escherichia coli*. The first reaction in the biosynthesis of IPP in *E. coli* starts with the formation of 1-deoxy-d-xylulose 5-phosphate (DXP) from pyruvate and glyceraldehyde 3-phosphate catalyzed by 1-deoxy-d-xylulose 5-phosphate synthase (Fig. 1). The *dxs* gene encoding the enzyme has been already cloned. In the second step the intramolecular rearrangement of DXP to 2-C-methyl-d-erythritol 4-phosphate (MEP) was proposed to occur via a hypothetical intermediate, 2-C-methyl-d-erythrose 4-phosphate, followed by an unspecified reduction process of this intermediate. Precise details about the reductive mechanism of MEP formation, however, were neither disclosed nor made available from the earlier studies. In addition, the gene responsible for the formation of MEP had not been obtained.

By preparing mutants of *E. coli* requiring MEP for their growth, we have most recently succeeded in cloning and overexpression of a gene complementary to their blocked step. The encoded protein catalyzed transformation of DXP to MEP in a single step and was named DXP reductoisomerase. DNA sequencing of this gene showed that it is identical with the yaeM gene with unknown function located at 4.2 min on the chromosomal map of *E. coli*.

To confirm the in vivo role of this yaeM gene, yaeM disruptants were constructed by insertion of the aphII gene (a kanamycin-resistance gene) into the yaeM gene. pMEW41 and pMEW73 (Fig. 2) had been isolated as plasmids that complemented the IPP biosynthesis coding region of the *E. coli* mutants described above. In these plasmids, 2541 bp and 1501 bp *SalI* fragments containing the yaeM gene were each inserted into the *BamHII* site of a vector plasmid, pMW118 (Nippon Gene, Japan). *Tn5* was digested with *HindIII* and *SalI*, and treated with T4 DNA polymerase to provide a 1.3 kb DNA fragment carrying the aphII gene, which encoded aminoglycoside phosphoryltransferase. pMEW41 was digested with *BalI*, the recognition site of which was in the targeted yaeM gene, and then ligated with the 1.3-kb DNA fragment carrying the aphII gene to construct pMEW41BAR. A 3.5-kb linear *SacI*-*HindIII* fragment (Fig. 2) carrying yaeM and aphII genes was isolated from pMEW41BAR. *E. coli* FS1576, a *recD* mutant, was transformed with the *SacI*-*HindIII* fragment and then kanamycin-resistant transformants were selected on Luria-Bertani (LB) plates containing 15 μg/ml kanamycin and 0.1% 2-C-methylerythritol (ME), a free alcohol of MEP, because disruption of the yaeM gene could be lethal for *E. coli*.

To confirm the correct disruption by Southern hybridization using the yaeM and aphII genes as the probes, chromosomal DNAs were extracted from three transformants of 22 kanamycin-resistant transformants thus isolated. Southern hybridization was done with an ECL direct nucleic acid labeling and detection system (Amer sham Pharmacia) according to the protocol of the supplier (Fig. 3). When the yaeM gene was used as the probe, the parent strain, FS1576, showed a 4.6-kb *SalI* signal, while three putative disruptants, designated DYM1, DYM2, and DYM3, showed a 5.9-kb *SalI* signal. The aphII gene was present only in DYM1, DYM2, and DYM3, and not in the parent strain. The expected shift was also observed when *PstI* instead of *SalI* was used. These data clearly showed that all the three isolat-
ed colonies were yaeM disruptants generated by homologous recombination.

After construction of these disruptants, we next investigated the auxotrophy for DXP and MEP of the three disruptants carrying depletion of the yaeM gene. Since 1-deoxyxylulose and ME are used by *E. coli* to be incorporated into its ubiquinone, we used these free alcohols as supplements in the LB plates in the place of DXP and MEP. As shown in Fig. 4, these mutants were unable to grow on the LB plates. On the other hand, addition of ME, but not of 1-deoxyxylulose (data not shown), to the LB plates allowed good growth of these disruptants. In addition, the requirement of ME for the growth and survival of all the disruptants was suppressed by introducing a plasmid, pMEW73, containing the yaeM gene alone in its inserted DNA. These results show that the yaeM disruptants have a metabolic block between DXP and MEP, and strongly support the idea that the yaeM gene is involved in synthesizing MEP

Fig. 1. Alternative Nonmevalonate Pathway for the Isopentenyl Diphosphate Biosynthesis.

Fig. 2. Schematic Representation of a Linear DNA and Gene Disruption via Homologous Recombination.

The dashed lines represent the vector sequence. Introduction of the linear DNA into FS1576 and subsequent homologous recombination at the two sites resulted in the replacement of the chromosomal yaeM gene with the mutant yaeM gene. DNA fragments inserted in pMEW41 and pMEW73 are also shown.

Fig. 3. Southern Hybridization between *Pst* I- and *Sma* I-Digested Chromosomal DNAs and the Two Probes, the yaeM and *aphII* Genes.

Molecular sizes in kb are shown on the left. The banding patterns are in agreement with those expected from the replacement shown at the bottom of Fig. 2. DI-D3, yaeM disruptants DYM1-DYM3; P, the parent strain FS1576.

Fig. 4. Phenotypes of the yaeM Disruptants and the Parent Strain FS1576.

The three yaeM disruptants DYM1, DYM2 and DYM3 cannot grow on LB plates (LB), but can on LB plates supplemented with 0.1% ME (LB+ME). In addition, these disruptants transformed with pMEW73 (see Fig. 2) become able to grow on LB without ME (LB, Lower right). All plates are shown after 2 days at 37°C.
from DXP \textit{in vivo}.

We showed here that the \textit{yaeM} disruptants generated by homologous recombination were auxotrophic for ME, a free alcohol of MEP. The free alcohol incorporated into \textit{E. coli} cells could be converted to MEP by certain kinase(s) to be used as the precursor for the IPP formation, because MEP is an actual biosynthetic intermediate of IPP. The biosynthetic pathway from MEP to IPP, however, is not known at all. The \textit{yaeM} disruptant constructed in this study is a promising organism for searching for unknown precursors in the non-mevalonate pathway for the IPP biosynthesis, because such compounds could substitute for ME to meet the nutritional requirements of the disruptants.

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