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The *ispB* Gene Encoding Octaprenyl Diphosphate Synthase Is Essential for Growth of *Escherichia coli*

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The *Escherichia coli ispB* gene encoding octaprenyl diphosphate synthase is responsible for the synthesis of the side chain of isoprenoid quinones. We tried to construct an *E. coli ispB*-disrupted mutant but could not isolate the chromosomal *ispB* disrupted mutant unless the *ispB* gene or its homolog was supplied on a plasmid. The chromosomal *ispB* disruptants that harbored plasmids carrying the *ispB* homologs from *Haemophilus influenzae* and *Synechocystis* sp. strain PCC6803 produced mainly ubiquinone 7 and ubiquinone 9, respectively. Our results indicate that the function of the *ispB* gene is essential for normal growth and that this function can be substituted for by homologs of the *ispB* gene from other organisms that produce distinct forms of ubiquinone.

Escherichia coli, a member of the gram-negative and facultative anaerobic group of bacteria, usually obtains energy for growth through respiration under aerobic and anaerobic conditions, in addition to energy obtained from glycolysis (8, 10). In the respiratory chain of *E. coli*, two types of quinones, ubiquinone 8 (UQ-8) and menaquinone 8 (MK-8), are essential components (3, 5, 8, 10). UQ-8 is necessary for the transfer of electrons from NADH to succinate in the electron transfer system that has molecular oxygen as the final electron acceptor. MK-8 functions for the transfer of electrons from formate in the anaerobic electron transfer system which uses nitrate as the final electron acceptor. While *E. coli* has both UQ-8 and MK-8, some microorganisms contain only one type of quinone; e.g., *Bacillus* species produce only MK and *Acetobacter* species generally produces only UQ (3, 5). Why does *E. coli* synthesize two kinds of quinones when other bacteria can subsist on only one? To address this question, mutants defective in the synthesis of UQ, MK, or both have been isolated (19). *ubi* and *men* mutants are respiration defective under aerobic and anaerobic conditions, respectively (10, 19, 20). A strain defective in both *ubi* and *men* has been constructed and found to grow very slowly (19). However, it appears likely that the *ubi* mutation was leaky in this strain, as a small amount of UQ could still be detected (19). We have taken a different approach by isolating a mutant with a deletion of the *ispB* gene, which encodes octaprenyl diphosphate synthase (1). This enzyme is responsible for the synthesis of the side chain of both UQ and MK, and strains defective in this enzyme should not be able to synthesize active forms of UQ or MK (1). However, we found that it was impossible to obtain an *ispB* deletion mutant unless the *ispB* gene or its homolog was supplied on a plasmid. Thus, we suggest that the *ispB* gene is essential for the normal growth of *E. coli.*

Construction of an *ispB*-disrupted mutant. To investigate the function of the *ispB* gene, a plasmid (pTC2) used to disrupt this gene was constructed by inserting the chloramphenicol acetyltransferase (*cat*) gene into the *ispB* gene (Fig. 1). We attempted to obtain chloramphenicol-resistant strains by transforming strain FS1576 (*recD*) (15) with the linearized *Kpn*I-*Hin*dIII fragment from pTC2, but no Cm^r transformants were obtained. However, when FS1576 harboring a plasmid (pKA3) containing the *ispB* gene was used as a host cell, we obtained many Cm^r transformants. Several transformants were examined for proper replacement of the chromosomal *ispB* gene. One strain, designated KO229 (*ispB*::Cm^r), was confirmed to have the correct gene disruption by Southern blot analysis (data not shown). We attempted to cure strain KO229 of pKA3 to find out whether growth of KO229 is or is not dependent on pKA3. Strain KO229 harboring pKA3 (spectinomycin resistant) was subcultured five times on nonselective Luria (L) medium and then plated on L agar medium. When 1,000 colonies were replica plated on L agar medium containing of spectinomycin at 50 μg/ml, all of the colonies were spectinomycin resistant and a strain of KO229 that had lost pKA3 was never isolated. KO229 maintained pKA3, which contains the *ispB* gene, even under the nonselective conditions. KO229 harboring pKA3 showed growth characteristics similar to those of the wild-type strain, and the production of UQ-8 was normal (data not shown). To test further the importance of the *ispB* gene for the growth of *E. coli*, we recloned the *ispB* gene into plasmid pSI029, which has a temperature-sensitive (ts) replication origin (18), to yield plasmid pSI7 (Fig. 1). Strain KO229 harboring only pSI7 was obtained by swapping pKA3 for pSI7. KO229 harboring pSI7 could grow at 30°C (permissive temperature), while the same strain could not grow at 43°C (restriction temperature) (data not shown). This result indicates that the *ispB* gene is essential for the growth of *E. coli.*

Complementation of the *E. coli ispB* disruptant with homologs from *Haemophilus influenzae* and *Synechocystis* sp. strain PCC6803. To further investigate the significance of the *ispB* gene in *E. coli*, we used *ispB* homologs from *H. influenzae* (7) and *Synechocystis* sp. strain PCC6803 (9). Recently, the complete genomic sequences of *H. influenzae* and *Synechocystis* sp. strain PCC6803 were determined and IspB homologs with 64.9 and 34.5% identity to that of *E. coli* were reported for *H.*

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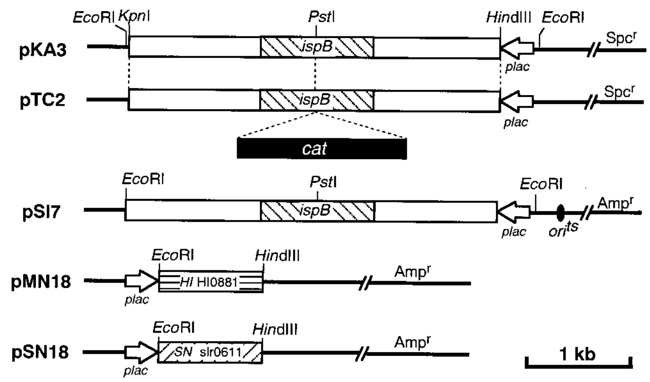

FIG. 1. Construction of the plasmids used in this study. pKA3 has the 3-kb fragment from the *E. coli* chromosome containing the *ispB* gene (1). The 1.5-kb *Hae*III fragment containing the *cat* gene from pACYC187 was blunt ended with T4 polymerase and inserted into the blunt-ended *Pst*I site of pKA3 (1), resulting in pTC2. The 4.5-kb *Kpn*I-*Hin*dIII *ispB*::Cm^r gene fragment was obtained from pTC2, and then *E. coli* FS1576 (*recD*) was transformed with this fragment. To express the *H. influenzae* and *Synechocystis* sp. strain PCC6803 *ispB* homologs in *E. coli*, oligonucleotide primers H1 (5′- GAATTCTATGAAGAAACAAGATCTT-3′ [sense]) and H2 (5′-AAGCTTCTAATAATTTCTATCTACAGACAA-3′ [antisense]) and S1 (5′- GAATTCTATGATCTCCACTACCTCCCTGTT-3′ [sense]) and S2 (5′- AAGCTTCTAATGGAGACGACCAAGCACATA-3′ [antisense]) were synthesized for amplification of both genes by PCR as described before (12). *H. influenzae* ATCC 51907 was obtained from the American Type Culture Collection, and *Synechocystis* sp. strain PCC6803 was obtained from S. Tabata (9). Chromosomal DNA was purified by the standard method as described before (13). pMN18 has the *H. influenzae ispB* homolog, and pSN18 has the *Synechocystis* sp. strain PCC6803 *ispB* homolog under control of the *lac* promoter of the expression vector pUC18. pSI7 has the *Eco*RI-*Eco*RI *ispB* gene fragment from pKA3 cloned into the *Eco*RI site of pSI029, which is temperature sensitive (ts) for DNA replication.

influenzae and *Synechocystis* sp. strain PCC6803, respectively (Fig. 2). The *H. influenzae ispB* homolog was suspected to encode an octaprenyl diphosphate synthase because of its high homology with *E. coli ispB*, but it is not known what type of demethyl-MK is synthesized in *H. influenzae* (3). The *Synechocystis* sp. strain PCC6803 *ispB* homolog was suspected to encode a solanesyl diphosphate synthase because *Synechocystis* sp. strain PCC6803 produces plastoquinone 9 (3). These homologs were obtained by PCR (12) as described in Fig. 1. PCR-amplified 1.0-kb fragments from *H. influenzae* and *Synechocystis* sp. strain PCC6803 were digested with *Eco*RI-*Hin*dIII and cloned into the same site of pUC18 to yield pMN18 and pSN18, respectively (Fig. 1). The authenticity of both cloned genes was confirmed by sequence analysis by the dideoxy chain termination method (14) on an ABI prism 377 sequencer. No discrepancies with respect to the reported sequences were found. KO229 harboring pKA3 was transformed with either pMN18 or pSN18, and the resulting transformants were both spectinomycin and ampicillin resistant. The transformants were subcultured five times in L medium containing ampicillin at 50 μg/ml and plated on L agar medium containing ampicillin. The resulting colonies were then replicated on L agar medium containing ampicillin or spectinomycin. Spectinomycin-sensitive and ampicillin-resistant strains which had only pMN18 or pSN18, but not pKA3, were selected. The exchange of pKA3 for pMN18 or pSN18 was confirmed by Southern blot analysis of the plasmid DNA (data not shown). No KO229 strain cured of both plasmids was obtained. These results indicate that the *H. influenzae* and *Synechocystis* sp. strain PCC6803 *ispB* homologs can complement a defect in the *E. coli ispB* gene and confirm that chromosomal *ispB* gene disruptants are not viable unless they carry a plasmid-borne copy of this gene.

Examination of the ubiquinone species in strain KO229 harboring pMN18 or pSN18. In strain KO229 harboring pMN18 or pSN18, the isoprenoid quinone side chain must be supplied by the product of the *ispB* gene on the plasmid. We examined species of UQs in such cells in accordance with a published procedure (4, 19). The purified UQs were analyzed by high-performance liquid chromatography with ethanol as the solvent (4). UQ-7 was detected mainly in KO229 harboring pMN18 (Fig. 3C), UQ-9 was detected mainly in KO229 harboring pSN18 (Fig. 3A), and UQ-8 was detected mainly in KO229 harboring pKA3 (Fig. 3B). These results indicate that the *H. influenzae ispB* homolog encodes heptaprenyl diphosphate synthase and that the *Synechocystis* sp. strain PCC6803 *ispB* homolog encodes solanesyl diphosphate synthase. We did not observe any significant differences in the growth properties of *E. coli* strains producing UQ-7, UQ-8, or UQ-9.

In summary, we conclude that *ispB* is a single-copy gene and essential for the normal growth of *E. coli* because a strain defective in this gene could not be isolated. It has been reported that an *E. coli* strain lacking both UQ-8 and MK-8 can grow very slowly on glucose-minimal medium supplemented with Casamino Acids (19). However, the strain still contained a small amount of UQ-8, indicating that it was not a completely quinone-free mutant. This report and our results indicate that quinones in *E. coli* are essential for growth. Quinones in *E. coli* might play roles other than as components of the electron transfer system, such as acting as antioxidants, as reported in eukaryotes (6).

So far, cells defective in polyprenyl diphosphate synthase have only been isolated from two yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. The *S. cerevisiae COQ1* gene encoding hexaprenyl diphosphate synthase is not essential for fermentative growth but is required for respiratory growth (2, 11). We have recently reported that the *S. pombe dps* gene encoding decaprenyl diphosphate synthase is not essential for growth in rich medium but is essential for growth on minimal medium (16). Interestingly, this *dps* disruptant can grow on minimal medium when supplemented with cysteine, glutathione, or α-tocopherol, indicating that UQ functions as an antioxidant in yeast (16). These results suggest that different organisms may have distinct requirements for isoprenoid quinones during growth. Our results suggest that the *ispB* gene is more important for growth in *E. coli* than in yeasts.

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EC   M------NLEKINELTAQDMAGVNAAILEQLNSDVQLINQLGYYIVSGGGKRIRPMIAVL   54
HI   MKKQDLMSIDEIQKLADPDMQKVNQNILAQLNSDVPLIGQLGFYIVQGGGKRIRPLIAVL   60
SN   M-----ISTTSLFAPVDQDLRLLTDNLKRLVGARHPILGAAAEHLFEAGGKRVRPAIVLL   55

EC   AARAVGYE---GNAHVTIAALIEFIHTATLLHDDVVDESDMRRGKATANAAFGNAASVLV  111
HI   AARSLGFE---GSNSITCATFVEFIHTASLLHDDVVDESDMRRGRATANAEFGNAASVLV  117
SN   VSRATLLDQELTARHRRLAEITEMIHTASLVHDDVVDEADLRRNVPTVNSLFDNRVAVLA  115

EC   GDFIYTRAFQMMTSLGSLKVLEVMSEAVNVIAEGEVLQLMNVNDPDITEENYMRVIYSKT  171
HI   GDFIYTRAFQLVAQLESLKILSIMADATNVLAEGEVQQLMNVNDPETSEANYMRVIYSKT  177
SN   GDFLFAQSSWYLANLDNLEVVKLLSEVIRDFAEGEILQSINRFDTDTDLETYLEKSYFKT  175

EC   ARLFEAAAQCSGILAGCTPEEEKGLQDYGRYLGTAFQLIDDLLDYNADGEQLGKNVGDDL  231
HI   ARLFEVAGQAAAIVAGGTEAQEKALQDYGRYLGTAFQLVDDVLDYSANTQALGKNVGDDL  238
SN   ASLIANSAKAAGVLSDAPRDVCDHLYEYGKHLGLAFQIVDDILDFTSPTEVLGKPAGSDL  235

EC   NEGKPTLPLLHAMHHGTPEQAQMIRTAIEQGNGRHLLEPVLEAMNACGSLEWTRQRAEEE  291
HI   AEGKPTLPLLHAMRHG-AQQAALIREAIEQGGKREAIDEVLAIMTEHKSLDYAMNRAKEE  298
SN   ISGNITAPALFAMEKY-PLLGKLIEREFAQAG---DLEQALELVEQGDGIRRSRELAANQ  291

EC   ADKAIAALQVLPDTPWREALIGLAHIAVQRDR*   323
HI   AQKAVDAIEILPESEYKQALISLAYLSVDRNY*   330
SN   AQLARQHLSVLEMSAPRESLLELVDYVLGRLH*   323
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FIG. 2. Alignment of the sequences of the *ispB* product from *E. coli* (EC) (GenBank accession no. U18997), its *H. influenzae* (HI) homolog HI0881 (GenBank accession no. U32770), and slr0611 from *Synechocystis* sp. strain PCC6803 (SN) (GenBank accession no. D90899). The conserved residues are shown as filled boxes. The DNA sequences were confirmed by us.

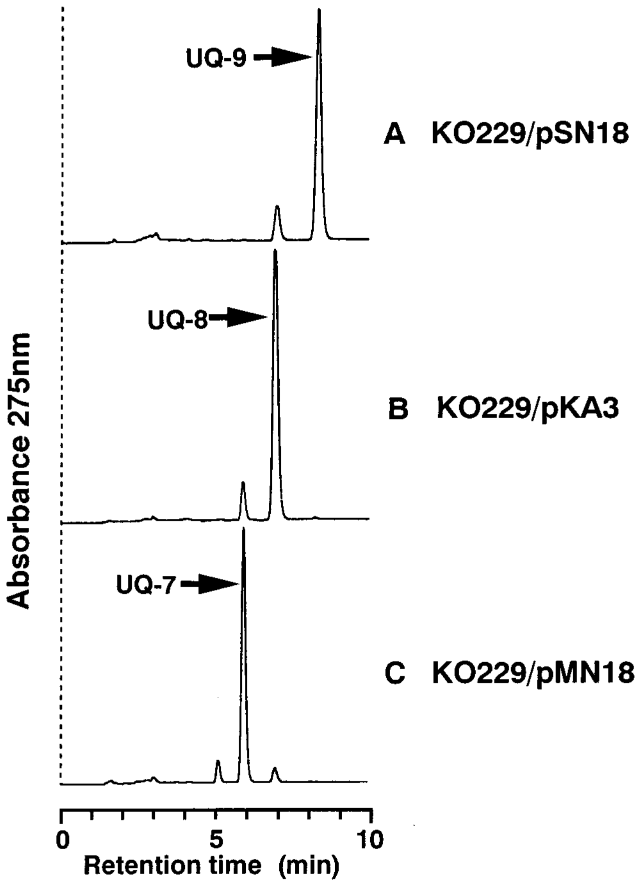

FIG. 3. Detection of UQ in strain KO229 harboring pMN18 or pSN18. The extracted crude ubiquinone was analyzed by normal-phase thin-layer chromatography with standard ubiquinone 10 carried out in a Kieselgel 60 F_{254} plate (Merck) with benzene-acetone (93:7, vol/vol). The UV-visualized band containing ubiquinone was collected from the thin-layer chromatography plate and extracted with chroloform-methanol (1:1, vol/vol). Samples were dried, and the precipitate was redissolved in ethanol. Samples were separated by high-performance liquid chromatography on a C_{18} reversed-phase column (YMC-Pack ODS-A; 150 by 60 mm [inside diameter]) with pure ethanol as the mobile phase, a flow rate of 1 ml/min, and detection at 275 nm. UQ was extracted from KO229 harboring pSN18 (A), KO229 harboring pKA3 (B), and KO229 harboring pMN18 (C). The peaks corresponding to UQ-7, UQ-8, and UQ-9 are indicated.

Furthermore, our results show that various kinds of UQs can be produced in *E. coli* by simply expressing the corresponding polyprenyl diphosphate synthase from different organisms without any apparent effect on its growth properties. Our results also support previous findings that *para*-hydroxybenzoate: octaprenyl diphosphate transferase (UbiA) has broad specificity with respect to its substrates (10, 17, 20). The UbiA protein could transfer not only the octaprenyl group but also the heptaprenyl and solanesyl groups to *para*-hydroxybenzoate.

In *E. coli*, the *H. influenzae ispB* homolog was found to produce UQ-7, although it was expected to encode octaprenyl diphosphate synthase on the basis of its high homology with this gene (64.9%). This indicates that homology does not always provide a notion of the precise function of a gene and that further experimental evidence is necessary to provide definite proof.

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