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# Analysis of the Decaprenyl Diphosphate Synthase (*dps*) Gene in Fission Yeast Suggests a Role of Ubiquinone as an Antioxidant[1]

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***Schizosaccharomyces pombe*** **produces ubiquinone-10 whose side chain is thought to be provided by the product generated by decaprenyl diphosphate synthase. To understand the mechanism of ubiquinone biosynthesis in *S. pombe*, we have cloned the gene encoding decaprenyl diphosphate synthase by the combination of PCR amplification of the fragment and subsequent library screening. The determined DNA sequence of the cloned gene, called *dps*, revealed that the *dps* gene encodes a 378-amino-acid protein that has the typical conserved regions observed in many polyprenyl diphosphate synthases. Computer-assisted homology search indicated that Dps is 45 and 33% identical with hexaprenyl diphosphate synthase from *Saccharomyces cerevisiae* and octaprenyl diphosphate synthase from *Escherichia coli*, respectively. An *S. pombe dps*-deficient strain was constructed. This disruptant was not able to synthesize ubiquinone and had no detectable decaprenyl diphosphate synthase activity, indicating that the *dps* gene is unique and responsible for ubiquinone biosynthesis. The *S. pombe dps*-deficient strain could not grow on either rich medium supplemented with glycerol or on minimal medium supplemented with glucose. The *dps*-deficient strain required cysteine or glutathione for full growth on the minimal medium. In addition, the *dps*-deficient strain is more sensitive to $H_2O_2$ and $Cu^{2+}$ than the wild type. These results suggests a role of ubiquinone as an antioxidant in fission yeast cells.**

**Key words: antioxidant, decaprenyl diphosphate synthase, glutathione, isoprenoid, ubiquinone.**

Isoprenoids of various lengths exist in nature. Relatively short-chain isoprenoids such as farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP) are utilized for the modification of proteins and for the synthesis of sterols and dolichols (*1*), while long-chain isoprenoids (6 to 10 units of isoprene) are mainly used to form the side chain of ubiquinones (*2-4*). Isoprenoids are synthesized by a 1′-4 condensation reaction between IPP and allylic diphosphates. Farnesyl diphosphate is the common substrate for the so-called branch-point enzymes, *i.e.*, the enzymes catalyzing the first committed steps in the biosynthesis of cholesterols, dolichols, ubiquinones, isoprenylated hemes, and proteins. Various isoprenoids are synthesized from farnesyl diphosphate by various prenyl diphosphate synthases. However, our knowledge of the biosynthesis of the long-chain isoprenoids is limited compared to that concerning the biosynthesis of the short-chain isoprenoids.

Ubiquinone (UQ) functions as an electron transporter between lipoprotein complexes of the respiratory chain. Various organisms produce different type of UQ; for example, *Saccharomyces cerevisiae* mainly produces ubiquinone-6 (*2*), *Escherichia coli* mainly produces ubiquinone-8 (*3*), and *Schizosaccharomyces pombe* and humans mainly produce ubiquinone-10 (*1*). We have recently proved that the species of ubiquinone is determined by polyprenyl diphosphate synthase, not by the specificity of PHB (*p*-hydroxybenzoate)-polyprenyl transferase (*5, 6*). Expression of the *ispB* gene in an *S. cerevisiae* COQ1-deficient strain enabled the cells to produce UQ-8 instead of original UQ-6. The finding that the UQ species is determined by the enzymatic specificity of polyprenyl diphosphate synthase raised the question of what structure is important for the product specificity of polyprenyl diphosphate synthase. During the past few years, the structural genes for several farnesyl diphosphate synthases (*7-9*), geranylgeranyl diphosphate synthases (*10-12*), a hexaprenyl diphosphate synthase (*13*), a heptaprenyl diphosphate synthase (*14*), and an octaprenyl diphosphate synthase (*15*) have been identified and characterized. Se-

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Abbreviations: DPS, decaprenyl diphosphate synthase; IPP, isopentenyl diphosphate; FPP, (*E,E*)-farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; S, solanesyl diphosphate; TLC, thin layer chromatography; PCR, polymerase chain reaction; kb, kilo base pair(s); leu, leucine; ura, uracil; ade, adenine; cys, cysteine; HPLC, high-performance liquid chromatography; UQ, ubiquinone; glut, glutathione.



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quence comparisons of those prenyltransferase revealed the existence of two conserved DDXX(XX)D aspartate-rich domains, which are thought to be the binding sites for the diphosphate moieties in IPP and the allylic substrate (*16, 17*). Recently two amino acids (AS) in domain II of geranylgeranyl diphosphate synthase have been defined as the most important determinant of the product length (*18*). However, this idea is not applicable to long-chain-length-producing polyprenyl diphosphate synthase, because the corresponding region does not differ among these enzymes. One of the ways to study the specificity of the enzymes is to accumulate information on the genes encoding different types of prenyl diphosphate synthase. The gene encoding the polyprenyl diphosphate synthase that produces prenyl diphosphate with more than 9 units of isoprene has not yet been cloned.

To increase our knowledge of prenyltransferase and its role in ubiquinone biosynthesis, we cloned the gene encoding decaprenyl diphosphate synthase that is responsible for forming the 10-isoprene-unit side chain of ubiquinone. The analysis of decaprenyl diphosphate synthase is important not only to elucidate how the enzyme recognizes the length of the chain, but also for medical purposes, because ubiquinone-10 is used for treating several types of heart disease (*4*). We also discuss the possible role of ubiquinone as an antioxidant in fission yeast.

## EXPERIMENTAL PROCEDURES

*Materials*—Restriction enzymes and other DNA-modifying enzymes were purchased from Takara Shuzo and New England Biolabs. [1-$^{14}$C]IPP (1.96 TBq/mol) was from Amersham. IPP, all-*E*-farnesyl diphosphate (FPP), geranylgeraniol (GGOH), solanesol (all-*E*-nonaprenol), and polyprenols ($C_{40}$-$C_{60}$) from *Ailanthus altissima* were purchased from Sigma Chemical. Kiesel gel 60 $F_{254}$ thin-layer plates were purchased from Merck. Reversed-phase LKC-18 thin-layer plates were purchased from Whatman Chemical Separation.

*Strains and Plasmids*—*E. coli* strains DH10B, DH5$\alpha$, and XL1-Blue were used for the general construction of plasmids (*19*). Plasmids pBluescript II KS+/−, pREP1 (*20*), pWH5 (*21*), and YEp13M4 (*22*) were used as the vectors. The *S. pombe* homothallic wild-type strain SP870 (h$^{90}$ *leu1-32, ade6-M210, ura4-D18*) and SP826 (h$^+$, *leu1-32, ade6-M210, ura4-D18*/h$^+$, *leu1-32, ade6-M216, ura4-D18*) diploid cells were used to produce a *Δdps::ura4* strain by homologous recombination. Yeast cells were grown in YE (0.5% yeast extract, 3% glucose) or PM minimal medium with appropriate supplements as described by Moreno *et al.* (*23*). YEA and PMA contains 75 $\mu$g/ml adenine in YE and PM, respectively. The concentration of supplemented amino acids was 100 $\mu$g/ml. The plasmid libraries of *S. pombe* genomic DNA cloned into the shuttle vector pWH5 containing the *LEU2* marker gene were a gift from Dr. David Beach. Construction of the *S. pombe* cDNA library was described before (*24*). Yeast transformation was performed essentially according to the method described by Rose *et al.* (*25*).

*DNA Manipulations and Sequencing*—Cloning, restriction enzyme analysis and preparation of plasmid DNAs were performed essentially as described by Maniatis *et al.* (*19*). PCR was done according to the procedure described before (*26*). The PCR product was used as a probe for screening the cDNA libraries constructed in λZAPII. An insert fragment from a positive clone was subcloned into the *Not*I site of pBluescript II SK− by *in vivo* excision (Stratagene). Deletion clones for sequencing were constructed using exoIII nuclease (TaKaRa Shuzo). Both directions of double-stranded DNA were sequenced by the chain-termination method of Sanger *et al.* (*27*).

To generate a *dps* disruptant, the plasmid pKS1100XU was constructed. The 4 kb *Sma*I fragment from pKS100 (from the genome library) was cloned in the *Eco*RV site of pBluescript II KS+. The resulting plasmid, named pKS-1100, was digested by *Xba*I and self-ligated to remove the *Eco*RI site of the multiple cloning site to yield pKS1100X. The *ura4* cassette was excised from pIRT5 (*24*) by digesting with *Hin*dIII, and cloned into the pBluescript II KS+ *Hin*dIII site; the resulting plasmid was named pBSURA4. The 1.8 kb *Eco*RI-*Hin*cII fragment from pBSURA4 was inserted into *Eco*RI-*Eco*RV-digested pKS1100X to yield pKS1100XU. To construct pREP1DPS, the oligonucleotides TGATTCTTCCGTTCTTTGAAA (118 bp upstream from the ATG translation start codon) and CGTTGGCAG-TTGTCGTTCAC (205 bp downstream from the TAG stop codon) were used to amplify the *dps* gene. The amplified 1.5 kb fragment was cloned into the *Eco*RV site of pBluescript II KS+, and the resulting plasmid was named pBSDPS. pBSDPS was digested with *Sal*I and *Sma*I and cloned into the same site of pREP1 to yield pREP1DPS.

*Gene Disruption*—The one-step gene disruption technique was used according to Rothstein (*28*). Plasmid pKS1100XU was linearized by appropriate restriction enzymes, and the linearized plasmid was used to transform SP870 and SP826 to uracil prototrophy.

*Prenyl Diphosphate Synthase Assay and Product Analysis*—Prenyl diphosphate synthase activity was measured by the slightly modified method described before (*5*). Yeasts were grown to the mid-to-late log phase in the PMA medium containing glucose with Leu or Leu and Ura. All subsequent steps were carried out at 4°C. The cells were first pelleted by centrifugation at 2,500×*g* for 1 min and washed once with $H_2O$. Cells were resedimented at 2,500×*g* for 1 min and resuspended in a buffer containing 100 mM potassium phosphate pH 7.4, 1 mM EDTA, 10% ethylene glycol, 10 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 1 $\mu$g/ml pepstatin A. The washed cells were ruptured by shaking vigorously with glass beads fourteen times for 30 s at 60-s intervals in an ice bath. The homogenate was centrifuged at 1,500×*g* for 10 min. The resulting supernatant was sonicated five times for 10 s and was used for the prenyltransferase reaction assay. The incubation mixture contained 2 mM $MgCl_2$, 0.2% (w/v) Triton X-100, 50 mM potassium phosphate buffer (pH 7.4), 5 mM KF, 10 mM iodoacetamide, 20 $\mu$M [$^{14}$C]IPP (specific activity 0.92 MBq/mol), 100 $\mu$M FPP, and 1.5 mg/ml protein of the enzyme in a final volume of 0.5 ml. Samples were preincubated for 10 min at 30°C without IPP and FPP followed by incubation for 120 min at 30°C with the substrates. A 0.5 ml sample of the reaction mixture was saponified by heating for 60 min at 90°C with 3.0 ml of methanol and 0.5 ml of 60% KOH. The non-saponifiable lipids were removed by extraction with hexane. Prenyl phosphates were extracted with chloroform from the resulting aqueous layer after neutralization with 0.7 ml of



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50% acetic acid. The chloroform extracts were dried and hydrolyzed with phosphatase by the method of Fujii *et al.* (*29*). The products of hydrolysis were extracted with hexane and analyzed by reversed-phase thin-layer chromatography with reference prenols. The reversed-phase chromatography was carried out on a LKC18 plate (Whatman) with acetone-water (19 : 1, v/v). Radioactivity on the thin-layer chromatography plate was detected with an

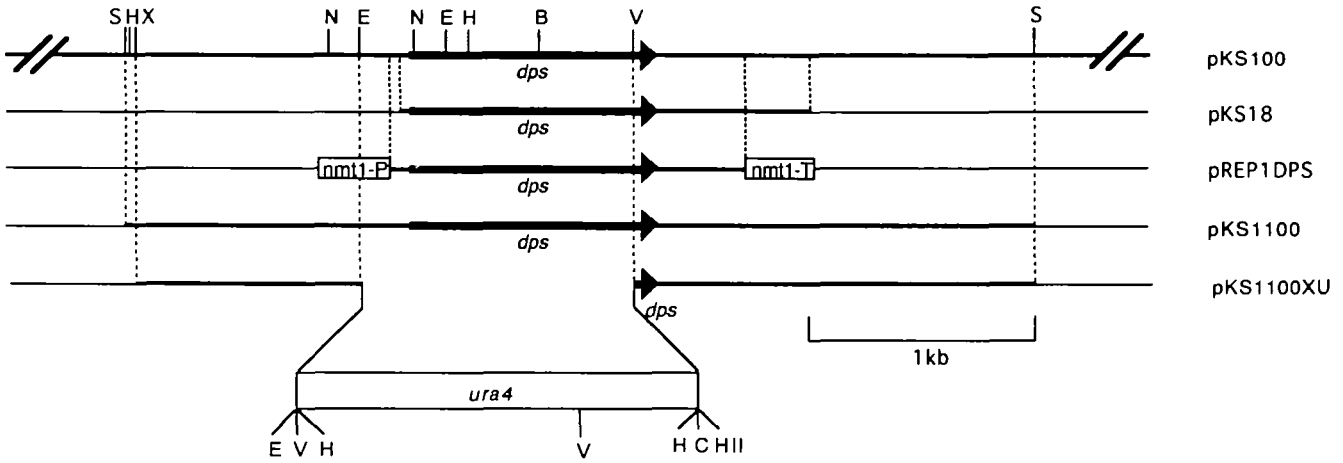


Fig. 1. **Schematic diagram of the original plasmids and their derivatives.** pKS100 is from a genomic library which contains a 12 kb genomic region spanning the *dps* gene. To construct pKS1100, the 4 kb *Sma*I fragment from pKS1100 was cloned into the *Eco*RV site of pBluescript KS+. Plasmid pKS18 containing the *dps* region is from the *S. pombe* cDNA library. To obtain pREP1DPS, a 1.5 kb fragment containing the ribosome-binding site and the *dps* gene was amplified by PCR, subcloned into pBluescript and then cloned into pREP1 (for details, see "EXPERIMENTAL PROCEDURES"). To construct pKS1100XU, pKS1100 was digested with *Eco*RI and *Eco*RV and the *ura4* cassette was inserted. The bold arrows indicate the coding region of *dps*. Thick lines indicate the cloned region. Thin lines are the vector. Abbreviations are following: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; V, *Eco*RV; H, *Hin*dIII; HII, *Hin*cII; N, *Nde*I; S, *Sma*I; X, *Xba*I.

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                                                                CATATGCAAATAGATCTGTGAATTTTATTGTTAACTGATTTGATGTATA   49
AGCTGTTGTAAGTTTTGATAATGAATTTGGCCTCTGAGGAAGATGCTCAATATCCTGCTTGACTCAACAGAATTCTTCAGAAAAAATACTTAAGATCAG   148
AATGGTATGAAGAAAAAACGATAATTAAAAAATGTTTTGTTTGTTTACAAACTATTTAATGATTCTTCCGTTCTTTGAAATAAACTTATCTGATCAAAG   247
CGACGTCATATCTAAGCGAAATCACGGTAAATACCATGATATACAAACCATCCCAACTCTAACTTCATCATTTAGTTACTTTTGAAAATAAGAAATGAA   346
ATGATTCAGTATGTATATTTAAAACATATGAGGAAATTATGGAGTCTTGGAAAAGTCCGTTCGACTGTTCTTCGGTTTTCTACTACGAACCGCAATGCT   445
 M  I  Q  Y  V  Y  L  K  H  M  R  K  L  W  S  L  G  K  V  R  S  T  V  L  R  F  S  T  T  N  R  N  A    33
TCACATTTAATTAAAAACGAGTTGGAACAAATCTCACCAGGGATTCGTCAAATGCTGAATTCAAATTCAGAATTTCTTGAAGAGTGTTCTAAATATTAT   544
 S  H  L  I  K  N  E  L  E  Q  I  S  P  G  I  R  Q  M  L  N  S  N  S  E  F  L  E  E  C  S  K  Y  Y    66
ACCATTGCTCAAGGAAAACAAATGCGTCCTTCTCTTGTTTTGCTGATGTCCAAAGCTACAAGCTTGTGCCATGGTATTGATCGGTCCGTAGTGGGCGAC   643
 T  I  A  Q  G  K  Q  M  R  P  S  L  V  L  L  M  S  K  A  T  S  L  C  H  G  I  D  R  S  V  V  G  D    99
AAATATATTGATGATGATGATTTAAGATCATTTTCGACGGGTCAAATTCTTCCTTCTCAATTGAGATTAGCACAAATAACCGAGATGATCCATATAGCA   742
 K  Y  I  D  D  D  D  L  R  S  F  S  T  G  Q  I  L  P  S  Q  L  R  L  A  Q  I  T  E  M  I  H  I  A   132
AGTTTGCTGCATGACGATGTGATTGATCACGCTAATGTCCGTAGAGGCTCACCTTCAAGCAATGTTGCTTTCGGTAATCGACGGTCAATCCTTGCGGGT   841
 S  L  L  H  D  D  V  I  D  H  A  N  V  R  R  G  S  P  S  S  M  V  A  F  G  N  R  R  S  I  L  A  G   165
AATTTCATCCTTGCACGGGCTTCGACTGCTATGGCCCGCCTTCGAAATCCCCAAGTTACGGAGTTGTTAGCTACAGTGATAGCAGACTTGGTTCGAGGT   940
 N  F  I  L  A  R  A  S  T  A  M  A  R  L  R  N  P  Q  V  T  E  L  L  A  T  V  I  A  D  L  V  R  G   198
GAGTTTTTGCAGCTAAAAAATACTATGGATCCTTCATCTTTGGAAATAAAACAATCAAATTTTGACTATTATATTGAAAAAAGTTTTTTGAAAACAGCC  1039
 E  F  L  Q  L  K  N  T  M  D  P  S  S  L  E  I  K  Q  S  N  F  D  Y  Y  I  E  K  S  F  L  K  T  A   231
AGTTTAATTTCCAAAAGCTGCAAGGCTTCTACAATCCTCGGACAATGTTCTCCTACTGTAGCAACAGCTGCTGGAGAATACGGTCGATGCATTGGTACT  1138
 S  L  I  S  K  S  C  K  A  S  T  I  L  G  Q  C  S  P  T  V  A  T  A  A  G  E  Y  G  R  C  I  G  T   264
GCTTTTCAACTAATGGATGACGTGTTGGACTATACGTCGAAAGATGATACTTTAGGAAAGGCGGCTGGTGCAGATTTGAAGCTAGGGTTGGCTACAGCT  1237
 A  F  Q  L  M  D  D  V  L  D  Y  T  S  K  D  D  T  L  G  K  A  A  G  A  D  L  K  L  G  L  A  T  A   297
CCCGTCCTCTTTGCATGGAAAAAGTATCCAGAACTTGGTGCAATGATTGTGAATAGATTCAATCATCCTTCTGATATCCAACGGGCTCGTTCTTTGGTT  1336
 P  V  L  F  A  W  K  K  Y  P  E  L  G  A  M  I  V  N  R  F  N  H  P  S  D  I  Q  R  A  R  S  L  V   330
GAGTGCACTGATGCTATCGAGCAAACCATCACTTGGGCAAAAGAATATATCAAAAAAGCCAAAGATTCCCTTCTGTGTCTCCCTGATTCACCTGCAAGG  1435
 E  C  T  D  A  I  E  Q  T  I  T  W  A  K  E  Y  I  K  K  A  K  D  S  L  L  C  L  P  D  S  P  A  R   363
AAGGCACTTTTGCGTTGGCTGATAAAGTAATAACGAGAAAGAAGTGACGAGTTTAGTACGCTGCATCTTTAACTATTAGATAAAGGCCTCTTTTTCTT  1534
 K  A  L  F  A  L  A  D  K  V  I  T  R  K  K  *                                                      378
ATTCTCGAAAAATTTAAAACAATTAGGTTTAATTATATATACGACTTTGGTAGACCGAGTTGAATTACCGCTCTTAATAGAGCAGTGAAAATATTACTA  1633
ATATGCGCTGCCTTGATTGGTTTTTCTTCCATAAATGATTTCAGGTTATTACAAAGTGAACGACAACTGCCAACGAATGCAATGATTTTATAACGACAT  1732
TGCAAGCCACTAATGATACTGTCTATAAAAATTGGACTTCAATAAATAAACCAAGCACTTCGTAATAACTATTCTTGACTAGTGTTATAGTCTTTGTGA  1831
TCTCGCTGCTCAAATGTCAAATGCTGATTTTGGCTCCAATTTTCTGTTTACGTTATAACAACTATCATGTAAGTTTGTCCATTACTTATATCTGGTGAA  1930
AAAGGCTGCATAACTATGGTAATACTTTTTGTCAGATCTAAAATTAGGAATCAGATTTTATCCGGTAGCTTGCTGTGCTTTCCCTGGACTGCAAAATTA  2029
TTTTCACCTGTATTCTTTTACTATACCTTTTTGATAGCCTATAAACGTTGAGCGTTGAGCTTTTAAACTTACCAAAACTACAGGGCCTTCCTTCTTTCC  2128
CCAATGCTCGAATGAACTTTCGTTAGTATCAACGTTTCCTTTTGTTTAGTACCCCTATAACAATATATATAACGACCTTCTTTTTG               2214
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Fig. 2. **Nucleotide sequence of the *dps* region and the deduced amino acid sequence.** The numbers at the right indicate the nucleotide and amino acid positions. Stop codons are shown by asterisks. Sequences of oligonucleotides used for PCR are underlined. The wavy line indicates the upstream stop codon.



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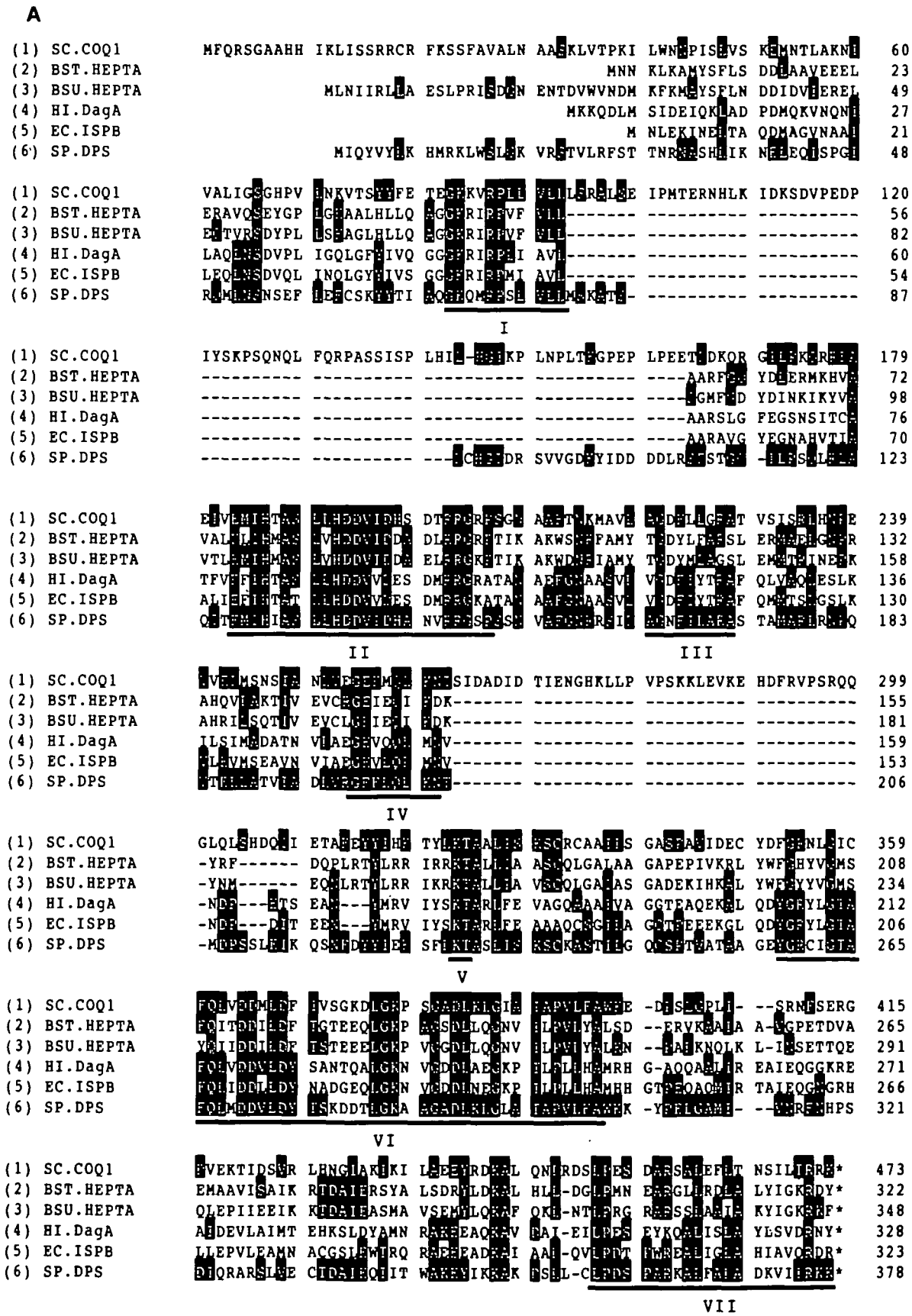


Fig. 3A

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imaging analyzer, BAS1500-Mac (Fuji Film). The spots of the marker prenols were visualized by exposure of the plate to iodine vapor.

*Ubiquinone Extraction and Measurement*—Ubiquinone was extracted by the modified method of Wallace and Young (*30*). *S. pombe* cells were grown in PMA-based medium (20 ml) until the mid-log phase and harvested. Cells were lysed with 3 mg of Novozyme, then ubiquinone was extracted with 3 ml of hexane-acetone (1 : 1, v/v) and the organic solution was evapolated to dryness. Samples were redissolved in 1 ml of chloroform-methanol (1 : 1, v/v), and the solution was washed with 0.5 ml of 0.7% NaCl solution and evapolated to dryness. The residue was taken up in 30 $\mu$l of chloroform-methanol (2 : 1, v/v) and analyzed by normal-phase thin-layer chromatography with standard ubiquinones on a Kiesel gel 60 $F_{254}$ plate (Merck) with benzene-acetone (93 : 7, v/v). The UV-visualized band containing ubiquinone was collected from the thin-layer chromatography plate and extracted with chloroform-methanol (1 : 1, v/v). The solution was evapolated to dryness and the residue was redissolved in ethanol. The purified ubiquinone was further analyzed by HPLC with ethanol as the solvent (*30*).

## RESULTS

*Isolation of the Decaprenyl Diphosphate Synthase (dps) Gene from S. pombe*—In all prenyl diphosphate synthases so far sequenced, there are seven conserved regions designated I-VII (*10, 14*); they are conserved in at least 12 out of the 13 known sequences. Therefore, we designed two primers in domains II (EMIHT) and VI (DDLLD) to isolate the decaprenyl diphosphate synthase gene from *S. pombe*. We performed PCR using these primers with *S. pombe* genomic DNA digested by *Pst*I and *Xho*I. A 450 bp fragment was amplified by the first PCR, and the second PCR was performed using the same primers from the first PCR product as a template. This amplified 450 bp fragment was cloned in the plasmid and the DNA sequence was determined. The translated sequence of the isolated fragment has high homology with hexaprenyl diphosphate synthase (COQ1) (*13*) from *S. cerevisiae* and octaprenyl diphosphate synthase (IspB) (*15*) from *E. coli*. Therefore, we thought that this fragment came from the gene encoding decaprenyl diphosphate synthase of *S. pombe*. We screened the cDNA and genomic libraries with the 450 bp fragment as a probe to obtain the whole gene. From the cDNA library, we obtained 8 positive clones, and one of the longest clones, called pKS18 (Fig. 1), was sequenced in both directions. A single open reading frame encompassing 1,134 bp was found (Fig. 2). This gene, designated *dps*, encoded a 378 amino acid protein, and its molecular weight was calculated to be 42,044.22. Although we originally designed the primer to correspond to the sequence EMIHT, the isolated gene sequence was translated to EMIHI. A homology search indicated that the predicted protein has high homology with COQ1 (*13*) (45% identity) from *S. cerevisiae*, IspB (*15*) (33% identity) from *E. coli*, heptaprenyl diphosphate synthase (*14*) (33% identity) from *Bacillus stearothermophilus* and other polyprenyl diphosphate synthases (Fig. 3A). *S. pombe* Dps is also homologous to a putative heptaprenyl diphosphate synthase from *Bacillus subtilis* (*31*) and a putative octaprenyl diphosphate synthase from *Haemophilus influenzae* (*32*) with 30 and 31% identity, respectively. The homology % matrix indicated that these two putative prenyl diphosphate synthases are highly homologous (~64%) to the corresponding prenyl diphosphate synthases (Fig. 3B). To ascertain the existence of

**B**

| | (1) | (2) | (3) | (4) | (5) | (6) |
|---|---|---|---|---|---|---|
| (1) SC.COQ1 | — | 30 | 28 | 30 | 33 | 45 |
| (2) BST.HEPTA | | — | 64 | 28 | 28 | 33 |
| (3) BSU.HEPTA | | | — | 27 | 30 | 30 |
| (4) HI0881 | | | | — | 63 | 31 |
| (5) EC.ISPB | | | | | — | 33 |
| (6) SP.DPS | | | | | | — |

Fig. 3. **Comparisons of amino acid sequences of Dps and several other prenyltransferases.** (A) 1, HexPP synthase from *S. cerevisiae* (*13*) (SC. COQ1); 2, HepPP synthase from *B. stearothermophilus* (*14*) (BST. HEPTA); 3, putative HepPP synthase from *B. subtilis* (*31*) (BSU. HEPTA); 4, putative OPP synthase from *H. influenzae* (*32*) (HI0881); 5, OPP synthase from *E. coli* (*15*) (EC. ISPB); 6, DPP synthase from *S. pombe* (SP. DPS). Identical amino acids are shown in reversed characters. Numbers of amino acids in the sequences are shown on the right. Seven domains (I-VII) are shown by bold underlining. (B) Homology % matrix of the six prenyltransferases depicted in (A). The percentages of identical amino acids of pairs of proteins are indicated.

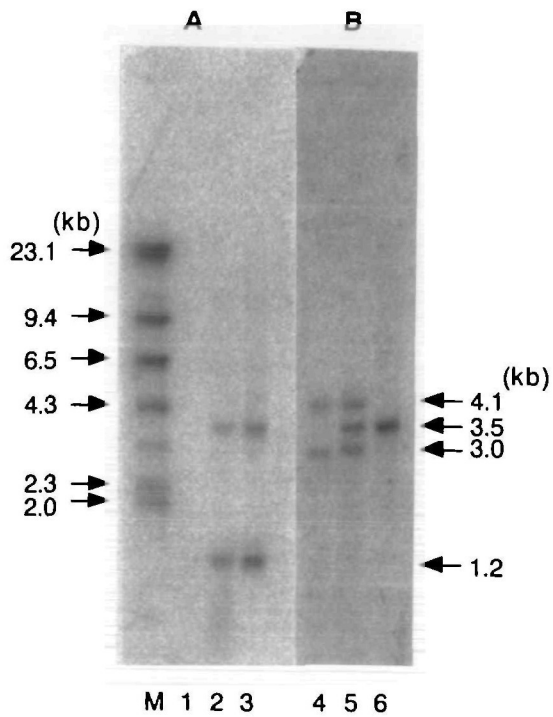


Fig. 4. **Southern hybridization analysis.** Southern hybridization was done according to the procedures described in "EXPERIMENTAL PROCEDURES." For southern hybridization, genomic DNAs of SP826, SP826⊿DPS, and KS10 were prepared; the *ura4* cassette (A) and the *dps* gene from pKS18 (B) were used as probes. A: Wild-type SP826, (lanes 1 and 4). SP826⊿DPS (diploid) (lanes 2 and 5) and KS10 (haploid) (lanes 3 and 6) were separated on agarose gel. M indicates the $\lambda$/*Hin*dIII marker.



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introns, we screened the genomic library to obtain the genomic fragment containing the *dps* gene. Three positive clones were selected from the pWH5-based library (*19*). One of them, called pKS100 (Fig. 1), was further analyzed. Plasmid pKS100 contained a 12 kb fragment in the pWH5 vector. The 4 kb *Sma*I fragment was subcloned into pBluescript II KS+ to yield pKS1100, and the subcloned fragment of the coding region was sequenced. No intron was found in the genomic *dps* region.

*Construction of the Decaprenyl Diphosphate Synthase (dps) Gene-Disrupted S. pombe Strain*—To make a *dps* gene-disrupted *S. pombe* strain, we constructed the plasmid pKS1100XU as described in "EXPERIMENTAL PROCEDURES" (Fig. 1). pKS1100XU was linearized by the appropriate restriction enzymes, and the fragment was used to transform the *S. pombe* wild-type haploid strain SP870. Although Ura$^+$ transformants were obtained, no *dps* gene-disrupted strain was isolated from the haploid. Therefore, we used the diploid strain SP826 to isolate the *dps*-disrupted strain. The same fragment was used to transform SP826, and Ura$^+$ transformants were selected. Twenty colonies were picked up and sequentially grown on the YEA-rich medium. The stability of Ura$^+$ was examined by replica plating. Three stable Ura$^+$ transformants were found. One of the three strains, designated SP826⊿DPS, was sporulated, and germinated haploid cells were replica-plated on YEA and PMA+Leu. All cells grew well on YEA medium, but some cells grew very slowly on the PMA+Leu plate. Those slow-growing cells were examined for ubiquinone synthesis as described in "EXPERIMENTAL PROCEDURES." None of the five strains picked up synthesized ubiquinone (see Fig. 6). We thought that these five strains were good candidates for *dps* disruptants. One of these strains, named KS10, was used for further experiments.

*Verification of the dps Disruptant by Southern Blot Analysis*—The genomic DNA from SP826, SP826⊿DPS, and KS10 was subjected to southern blot analysis to confirm the replacement of *dps* by *ura4*. Genomic DNAs were digested with *Eco*RV. The *ura4* cassette and the *dps* gene were used as probes. In SP826⊿DPS, a band corresponding to 3.5 kb appeared with both probes (Fig. 4, lanes 2 and 5), because SP826⊿DPS has the complete *dps* gene and the *ura4*-disrupted *dps* gene. When the *ura4* cassette was used as a probe, no band appeared in SP826 (Fig. 4, lane 1), but 1.2 and 3.5 kb bands appeared in SP826⊿DPS and KS10 (Fig. 4, lanes 2 and 3). When the *dps* gene was used as a probe, a 3.5 kb band appeared in strain KS10 (Fig. 4, lane 6), two bands of 3 and 4.1 kb appeared in SP826 (Fig. 4, lane 4), and three bands of 3, 3.5, and 4.1 kb appeared in SP826⊿DPS (Fig. 4, lane 5). Thus, we confirmed that the *dps* gene was properly disrupted in SP826⊿DPS and KS10.

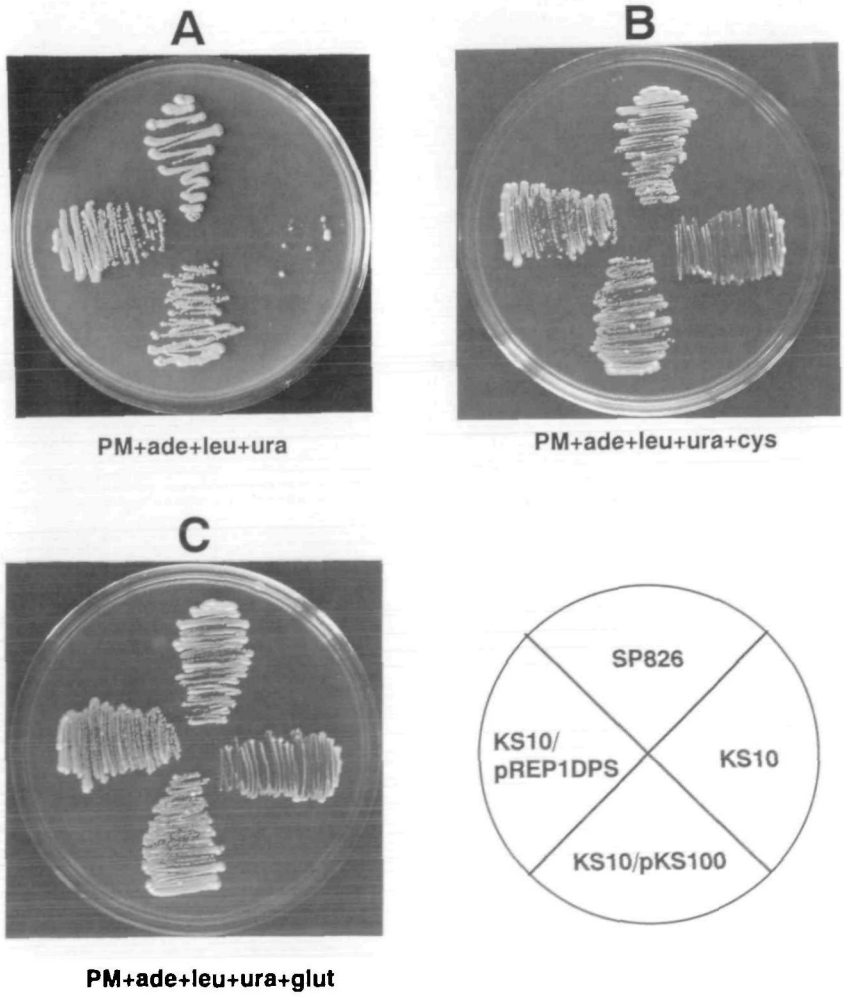


Fig. 5. **Growth of KS10 on the minimal medium was restored by cysteine or glutathione.** A: Wild type, KS10, KS10 harboring pKS100 and KS10 harboring pREP1DPS were grown on the PM medium supplemented with 75 μg/ml adenine, 100 μg/ml leucine, and 100 μg/ml uracil. B: The same strains were grown on the PM medium supplemented with adenine, leucine, uracil and cysteine. C: Same as in B except that glutathione was added instead of cysteine. KS10 could not grow on PM medium (A) but when supplemented with cysteine (100 μg/ml) (B) or glutathione (100 μg/ml) (C), KS10 could grow on PM medium. KS10 harboring pKS100 or pREP1DPS could grow on PM medium without cysteine or glutathione (A).

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*The Phenotypes of the dps Disruptant*—When the *dps* gene was disrupted in KS10 by homologous recombination, the upstream deletion of *dps* could have damaged other genes. To eliminate this possibility, plasmid complementation was done. pKS100 from the genomic library and pREP1DPS (Fig. 1), which includes only the *dps* region, under the strong promoter of *nmt1* were used for the complementation test. The *dps*-disrupted strain did not produce ubiquinone, as described above. The cells grew poorly on the PM-based medium, slowly on the YEA medium and not at all grow on the YEA medium with glycerol as a carbon source. These phenotypes were tested in the transformants. KS10 harboring the vector, pKS100 and pREP1DPS were plated on the PM-based medium. A few days later, KS10 harboring only the vector formed a very tiny colony but KS10 harboring pKS100 and pREP1DPS grew as well as the wild-type strain (Fig. 5A). There were no differences in the growth of KS10 harboring the *dps* over-expressing plasmid pREP1DPS and genomic *dps*-containing plasmid pKS100 (Fig. 5A). Thus, in KS10, only the *dps* function was abolished. Ubiquinone was extracted from KS10 harboring the vector, pKS100 and pREP1DPS. No ubiquinone was detected in KS10 harboring the vector. No significant differences in ubiquinone production among KS10 harboring pREP1DPS, KS10 harboring pKS100, and the wild type were found (Fig. 6). Expression of *dps* under the strong promoter of *nmt1* did not affect the ubiquinone production. We then examined for the reason for the poor growth of KS10 on the PM-based medium. KS10 grew well on the PM-based medium with supplements of all 20 amino acids. When each amino acid (100 $\mu$g/ml) was tested for the ability to complement the growth, only the supplement of cysteine, but not any other amino acid, was found to give recovery of growth on the minimal medium (Fig. 5B). Neither homocysteine nor cystine enhanced the poor growth on the minimal medium (data not shown). The requirement of cysteine for the growth of KS10 prompted us to test the effect of glutathione, an antioxidant. Interestingly, the addition of glutathione had the better effect on the recovery of the growth of KS10 (Fig. 5C). The ability of cysteine and glutathione, but not intermediate amino acids such as methionine and homocysteine, to restore the growth of KS10 suggests that cysteine and glutathione act as an antioxidants, not as sulfur suppliers. These results, in turn, suggest that ubiquinone serves as an antioxidant in normal fission yeast cells. If ubiquinone does serve as an antioxidant, the *dps*-deficient strain might be susceptible to oxygen radical producers. In fact, the growth of the *dps*-deficient strain was severely inhibited by the presence of 2.5 mM $H_2O_2$ and moderately inhibited by 0.5 mM $Cu^{2+}$ (Fig. 7). These results support the idea that ubiquinone serves as an antioxidant *in vivo*.

*Enzymatic Activity of Decaprenyl Diphosphate Synthase*—Because *S. pombe* produces ubiquinone-10, decaprenyl diphosphate activity should be detected in the wild type but not in the *dps*-disrupted strain. We measured the decaprenyl diphosphate synthase activity in SP870, KS10 (*Δdps*), KS10 harboring pKS100, and KS10 harboring pREP1DPS. Cells from these strains were homogenized

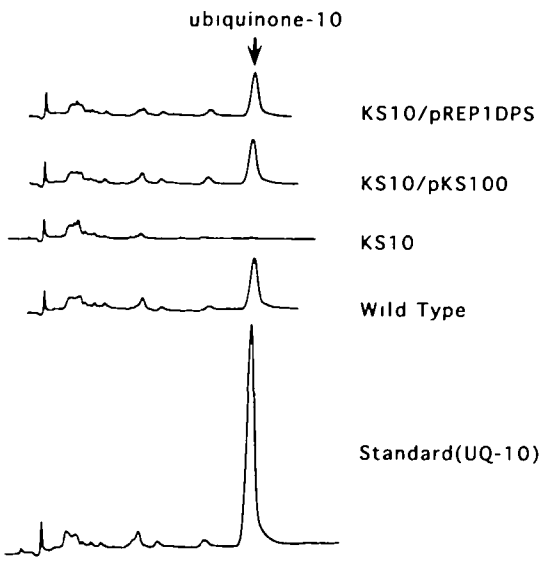


Fig. 6. **Detection of ubiquinone-10.** Ubiquinone was extracted from the wild-type SP870, KS10, KS10 harboring plasmid pKS100, and KS10 harboring plasmid pREP1DPS. It was first separated by TLC and then further by HPLC. The peaks corresponding to ubiquinone-10 are indicated. No ubiquinone was detectable in KS10 or the other four candidate *dps* disruptants (data not shown).

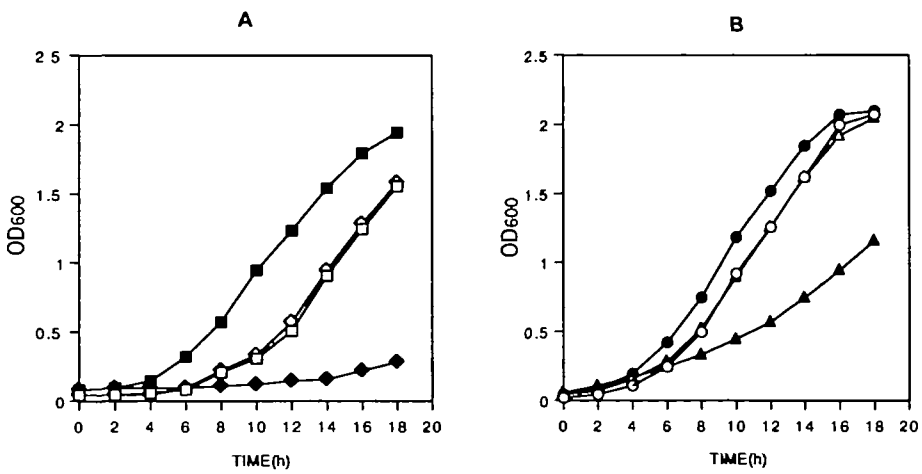


Fig. 7. **Sensitivity of KS10 to oxygen radical producer.** Wild type (□, ◇, ○, △) and KS10 (*Δdps::ura4*) (■, ◆, ●, ▲) were pregrown in YEA liquid medium to saturation. Then wild-type and KS10 cells were inoculated on fresh YEA medium in 40-fold dilution with 2.5 mM $H_2O_2$ (A) (◇, ◆) or 0.5 mM $Cu^{2+}$ (B) (△, ▲) or without both (□, ■, ○, ●). Cell growth was measured at 2-h intervals in terms of $OD_{600}$.



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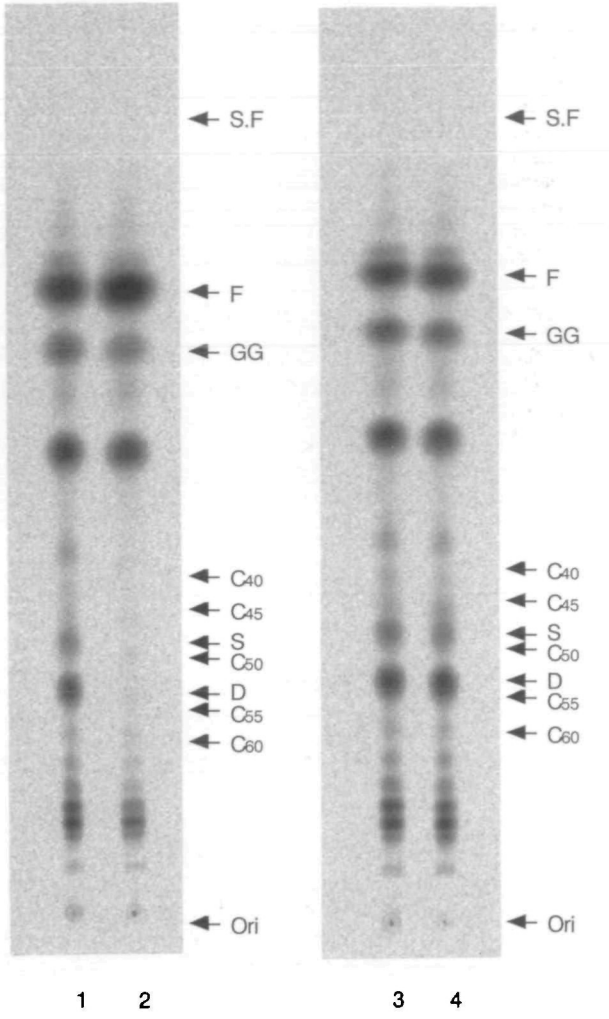


Fig. 8. **Thin-layer chromatogram of the product of decaprenyl diphosphate synthase.** The enzyme reactions of the wild-type SP870 (lane 1), KS10 (lane 2), KS10 harboring plasmid pKS100 (lane 3), and KS10 harboring plasmid pREP1DPS (lane 4), were carried out with [$^{14}$C]IPP and FPP as substrates. The product was hydrolyzed with phosphatase, and the resulting alcohol was analyzed by reverse-phase thin-layer chromatography. The same amount of radiolabeled products (20,000 dpm) was applied to the TLC plate. Arrows indicate the positions of authentic alcohols: F, all-*E*-farnesol; GG, all-*E*-geranylgeraniol; $C_{40}$, *Z,E*-octaprenol; $C_{45}$, *Z,E*-nonaprenol; S, all-*E*-nonaprenol; D, all-*E*-decaprenol; $C_{50}$, *Z,E*-decaprenol; $C_{55}$, *Z,E*-undecaprenol; $C_{60}$, *Z,E*-dodecaprenol; Ori., origin; S.F., solvent front.

with glass beads; then substrates IPP, $^{14}$C-IPP, and FPP were used for the prenyl diphosphate synthase reaction. To confirm what kind of isoprenoid was produced, the product generated in the reaction was hydrolyzed by acid phosphatase and analyzed by thin-layer chromatography. The alcohol thus obtained migrated more slowly than solanesol in reverse-phase thin-layer chromatography of products from the wild-type SP870 strain (Fig. 8, lane 1), KS10 harboring pKS100 (Fig. 8, lane 3), and KS10 harboring pREP1DPS (Fig. 8, lane 4), but no such product was detected from the cells of KS10 (Fig. 8, lane 2). Thus, no decaprenyl diphosphate synthase activity was detected in KS10, and pKS100 and pREP1DPS restored the activity, supporting the conclusion that the *dps* gene encodes decaprenyl diphosphate synthase. This is the first report of the experimental detection of enzymatic activity of a long-chain polyprenyl diphosphate synthase in yeasts.

## DISCUSSION

We have obtained and analyzed the *dps* gene encoding decaprenyl diphosphate synthase from *S. pombe*. This gene encodes a 378-amino-acid protein that is highly homologous to hexaprenyl diphosphate synthase (COQ1) (*13*), heptaprenyl diphosphate synthase (*14*), and octaprenyl diphosphate synthase (IspB) (*15*), with 45, 33, and 33% identity, respectively. These long-chain-isoprenoid-producing enzymes, as well as other prenyl synthases typically have seven (I–VII) conserved domains (Fig. 3) (*10, 14*), except for COQ1, which has a redundant stretch of 44 amino acids between domains I and II and a 37-amino-acid redundancy between domains IV and V compared with decaprenyl diphosphate synthase (Fig. 3). In domain II, most short-chain-isoprenoid-producing enzymes have the sequence ELLQAFF, whereas long-chain-producing enzymes have the sequence EMIHT/MAS (*14, 33*). The typical domain IV structure (GQXXD) reported before was not found in long-chain prenyl diphosphate synthases, but instead, the E/RGEXXQL structure was well conserved among them. This new domain IV could be an important feature of long-chain prenyl diphosphate synthases. In domain V, the KT sequence was conserved in all prenyl diphosphate synthases. Recently two amino acids corresponding to AS in domain II were shown to be important for short-chain prenyl diphosphate synthases such as geranylgeranyl diphosphate synthase (*18*). However, these sites do not differ among long-chain polyprenyl diphosphate synthases. Domain IV seems to be the most likely candidate to differentiate the short-chain- and long-chain-synthesizing enzymes.

We tried to express the *dps* gene in *S. cerevisiae* and *E. coli*, but could not obtain fully active enzyme (data not shown). We also expressed *COQ1* in *E. coli*, but no activity of prenyl diphosphate synthase was observed (*34*, unpublished observation). These results may suggest that another factor is required for yeast prenyl diphosphate synthase, as ORF1 is required for full activity of *B. stearothermophilus* prenyl diphosphate synthase. Expression of *ispB* in *S. cerevisiae* was successfully achieved (*5*), but IspB is thought to function as a homodimer without requiring any another factor. The expression of *ispB* in *S. cerevisiae* enabled the cells to produce UQ-8 instead of UQ-6, which suggests that polyprenyl diphosphate synthase is an essential determinant of the species of UQ (*5*).

A *dps*-disrupted *S. pombe* was constructed. The fact that the *dps* disrupted strain produced no detectable ubiquinone and had no decaprenyl diphosphate synthase activity confirmed that *dps* encodes an essential component of decaprenyl diphosphate synthase. We expected that the disruptant would show respiratory deficiency, but unexpectedly, it showed a deficiency in growth on minimal medium. The *dps*-disrupted strain did not grow well on PMA+glucose but grew on PMA+glucose+cysteine or +glutathione. Even methionine or homocysteine did not reverse the poor growth of the *dps* disruptant. Moreover, the addition of the lipid antioxidant $\alpha$-tocopherol, but not ascorbic acid, restored the growth of the *dps* disruptant in



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minimal medium (data not shown). This cysteine, glutathione or $\alpha$-tocopherol requirement for growth on the minimal medium is interesting and suggests a role of cysteine and glutathione as antioxidants, not as the suppliers of sulfur. In support of this idea, the *dps*-deficient strain was found to be more sensitive to active oxygen-producing reagents, such as $H_2O_2$ and $Cu^{2+}$. Thus, these results in turn suggest a role of ubiquinone as an antioxidant in fission yeast. There has been no report of a requirement of cysteine in the *S. cerevisiae COQ1*-deficient strain, but *S. cerevisiae* and *S. pombe* are only distantly related, and sometimes behave differently. A role of ubiquinone as an antioxidant has been reported in mammalian cells (*35-37*). Our preliminary finding that the addition of linolenic acid, which is easily peroxidized, but not oleic acid, is inhibitory to the growth of the *dps* disruptant further supports the above idea. Another difference from *S. cerevisiae* is that even wild-type *S. pombe* cells do not utilize glycerol well, which make it difficult to test the respiratory deficiency of the *dps*$^-$ strain on a glycerol plate. The *S. cerevisiae coq1* mutant is easily distinguishable from the wild type on a glycerol-based minimal medium. Ubiquinone is not only important for respiration, but also appears to be necessary for protection against oxidative stress in fission yeast.

In conclusion, the present analysis of decaprenyl diphosphate synthase should not only contribute to the basic understanding of ubiquinone biosynthesis, but also aid in the application of fission yeast for production of UQ-10, which is used as a drug to treat heart disease.

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## REFERENCES


1. Grünler, J., Ericsson, J., and Dallner, G. (1994) Branch-point reactions in the biosynthesis of cholesterol, dolichol, ubiquinone and prenylated proteins. *Biochim. Biophys. Acta* **1212**, 259-277
2. Takada, M., Ikenoya, S., Yuzuriha, T., and Katayama, K. (1984) Simultaneous determination of reduced and oxidized ubiquinones. *Methods Enzymol.* **105**, 147-155
3. Bentley, R. and Meganathan, R. (1987) Biosynthesis of the isoprenoid quinones in *Escherichia coli and Salmonella typhimurium* (Neidhardt, F.C., ed.) pp. 512-520, American Society of Microbiology, Washington, DC
4. Ernster, L. and Dallner, G. (1995) Biochemical, physiological and medical aspects of ubiquinone function. *Biochim. Biophys. Acta* **1271**, 195-204
5. Okada, K., Suzuki, K., Kamiya, Y., Zhu, X., Fujisaki, S., Nishimura, Y., Nishino, T., Nakagawa, T., Kawamukai, M., and Matsuda, H. (1996) Polyprenyl diphosphate synthase essentially defines the length of the side chain of ubiquinone. *Biochim. Biophys. Acta* **1302**, 217-223
6. Suzuki, K., Ueda, M., Yuasa, M., Nakagawa, T., Kawamukai, M., and Matsuda, H. (1994) Evidence that *Escherichia coli ubiA* product is a functional homolog of yeast *COQ2*, and the regulation of *ubiA* gene expression. *Biosci. Biotech. Biochem.* **58**, 1814-1819
7. Sheares, B.T., White, S.S., Molowa, D.T., Chan, K., Ding, V.D., Kroon, P.A., Bostedor, R.G., and Karkas, J.D. (1989) Cloning, analysis, and bacterial expression of human farnesyl pyrophosphate synthetase and its regulation in Hep G2 cells. *Biochemistry* **28**, 8129-8135
8. Anderson, M.S., Yarger, J.G., Burck, C.L., and Poulter, C.D. (1989) Molecular cloning, sequence, and expression of an essential gene from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **264**, 19176-19814
9. Fujisaki, S., Hara, H., Nishimura, Y., Horiuchi, K., and Nishino, T. (1990) Cloning and nucleotide sequence of the *ispA* gene responsible for farnesyl diphosphate synthase activity in *Escherichia coli*. *J. Biochem.* **108**, 995-1000
10. Koyama, T., Obata, S., Osabe, M., Takeshita, A., Yokoyama, K., Uchida, M., Nishino, T., and Ogura, K. (1993) Thermostable farnesyl diphosphate synthase of *Bacillus stearothermophilus*: molecular cloning, sequence determination, overproduction, and purification. *J. Biochem.* **113**, 355-363
11. Armstrong, G.A., Alberti, M., Leach, F., and Hearst, J.E. (1989) Nucleotide sequence, organization, and nature of the protein products of the carotenoid biosynthesis gene cluster of *Rhodobacter capsulatus*. *Mol. Gen. Genet.* **216**, 254-268
12. Misawa, N., Nakagawa, M., Kobayashi, K., Yamano, S., Izawa, Y., Nakamura, K., and Harashima, K. (1990) Elucidation of the *Erwinia uredovora* carotenoid biosynthetic pathway by functional analysis of gene products expressed in *Escherichia coli*. *J. Bacteriol.* **172**, 6704-6712
13. Ashby, M.N. and Edwards, P.A. (1990) Elucidation of the deficiency in two yeast coenzyme Q mutants. Characterization of the structural gene encoding hexaprenyl pyrophosphate synthetase. *J. Biol. Chem.* **265**, 13157-13164
14. Koike-Takeshita, A., Koyama, T., Obata, S., and Ogura, K. (1995) Molecular cloning and nucleotide sequences of the genes for two essential proteins constituting a novel enzyme system for heptaprenyl diphosphate synthesis. *J. Biol. Chem.* **270**, 18396-18400
15. Asai, K., Fujisaki, S., Nishimura, Y., Nishino, T., Okada, K., Nakagawa, T., Kawamukai, M., and Matsuda, H. (1994) The identification of *Escherichia coli ispB (cel)* gene encoding the octaprenyl diphosphate synthase. *Biochem. Biophys. Res. Commun.* **202**, 340-345
16. Marrero, P.F., Poulter, C.D., and Edwards, P.A. (1992) Effects of site-directed mutagenesis of the highly conserved aspartate residues in domain II of farnesyl diphosphate synthase activity. *J. Biol. Chem.* **267**, 21873-21878
17. Tarshis, L.C., Yan, M., Poulter, C.D., and Sacchettini, J.C. (1994) Crystal structure of recombinant farnesyl diphosphate synthase at 2.6-Å resolution. *Biochemistry* **33**, 10871-10877
18. Ohnuma, S., Hirooka, K., Hemmi, H., Ishida, C., Ohto, C., and Nishino, T. (1996) Conversion of product specificity of archaebacterial geranylgeranyl-diphosphate synthase. Identification of essential amino acid residues for chain length determination of prenyltransferase reaction. *J. Biol. Chem.* **271**, 18831-18837
19. Sambrook, J., Fritsch, E., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Labolatory, Cold Spring Harbor, NY
20. Maundrell, K. (1993) Thiamine-repressible expression vectors pREP and pRIP for fission yeast. *Gene* **123**, 127-130
21. Wright, A., Maundrell, K., Heyer, W.D., Beach, D., and Nurse, P. (1986) Vectors for construction of gene banks and the integration of cloned genes in *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. *Plasmid.* **15**, 156-158
22. Rose, M.D. and Broach, J.R. (1991) Cloning genes by complementation in yeast in *Methods for Enzymology* (Guthrie, C. and Fink, G.R., eds.) Vol. 194, pp. 195-214, Academic Press. New York
23. Moreno, S., Klar, A., and Nurse, P. (1991) Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol.* **194**, 795-823
24. Kawamukai, M., Gerst, J., Field, J., Riggs, M., Rodgers, L., Wigler, M., and Young, D. (1992) Genetic and biochemical analysis of the adenylyl cyclase-associated protein, cap, in *Schizosaccharomyces pombe*. *Mol. Biol. Cell.* **3**, 167-180
25. Rose, M.D., Winston, F., and Hieter, P. (1990) *Methods in Yeast Genetics: A Laboratory Course Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
26. Saiki, R.K., Gelf, D.H., Stoffel, I.S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487-491
27. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA sequenc-




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ing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **4**, 5463-5467
28. Rothstein, R.J. (1983) Targeting, disruption, replacement, and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol.* **101**, 202-211
29. Fujii, H., Koyama, T., and Ogura, K. (1982) Efficient enzymatic hydrolysis of polyprenyl pyrophosphates. *Biochim. Biophys. Acta* **712**, 716-718
30. Wallace, B.J. and Young, I.G. (1977) Role of quinones in electron transport to oxygen and nitrate in *Escherichia coli*. Studies with a *ubiA*$^-$*menA*$^-$ double quinone mutant. *Biochim. Biophys. Acta* **461**, 84-100
31. Yazdi, M.A. and Moir, A. (1990) Characterization and cloning of the *gerC* locus of *Bacillus subtilis* 168. *J. Gen. Microbiol.* **136**, 1335-1342
32. Fleischmann, R.D. *et al.* (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269**, 496-512
33. Chen, A., Kroon, P.A., and Poulter, C.D. (1994) Isoprenyl diphosphate synthases: protein sequence comparisons, a phylogenetic tree, and predictions of secondary structure. *Protein Sci.* **3**, 600-607
34. Zhu, X.F., Yuasa, M., Okada, K., Suzuki, K., Nakagawa, T., Kawamukai, M., and Matsuda, H. (1995) Production of ubiquinone in *Escherichia coli* by expression of various genes responsible for ubiquinone biosynthesis. *J. Ferment. Bioeng.* **79**, 493-495
35. Frei, B., Kim, M.C., and Ames, N. (1990) Ubiquinol-10 is an effective lipid-soluble antioxidant at physiological concentrations. *Proc. Natl. Acad. Sci. USA* **87**, 4879-4883
36. Alleva, R., Tomasetti, M., Battino, M., Curatola, G., Littarru, G., and Folkers, K. (1995) The roles of coenzyme Q10 and vitamin E on the peroxidation of human low density lipoprotein subfractions. *Proc. Natl. Acad. Sci. USA* **92**, 9388-9391
37. Kontush, A., Hubner, C., Finckh, B., Kohlschutter, A., and Beisiegel, U. (1995) Antioxidative activity of ubiquinol-10 at physiologic concentrations in human low density lipoprotein. *Biochim. Biophys. Acta* **1258**, 177-178

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