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Phenotypes of Fission Yeast Defective in Ubiquinone Production Due to Disruption of the Gene for *p*-Hydroxybenzoate Polyprenyl Diphosphate Transferase

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Ubiquinone is an essential component of the electron transfer system in both prokaryotes and eukaryotes and is synthesized from chorismate and polyprenyl diphosphate by eight steps. *p*-Hydroxybenzoate (PHB) polyprenyl diphosphate transferase catalyzes the condensation of PHB and polyprenyl diphosphate in ubiquinone biosynthesis. We isolated the gene (designated *ppt1*) encoding PHB polyprenyl diphosphate transferase from *Schizosaccharomyces pombe* and constructed a strain with a disrupted *ppt1* gene. This strain could not grow on minimal medium supplemented with glucose. Expression of *COQ2* from *Saccharomyces cerevisiae* in the defective *S. pombe* strain restored growth and enabled the cells to produce ubiquinone-10, indicating that *COQ2* and *ppt1* are functional homologs. The *ppt1*-deficient strain required supplementation with antioxidants, such as cysteine, glutathione, and α-tocopherol, to grow on minimal medium. This suggests that ubiquinone can act as an antioxidant, a premise supported by our observation that the *ppt1*-deficient strain is sensitive to H_2O_2 and Cu^{2+}. Interestingly, we also found that the *ppt1*-deficient strain produced a significant amount of H_2S, which suggests that oxidation of sulfide by ubiquinone may be an important pathway for sulfur metabolism in *S. pombe*. Ppt1-green fluorescent protein fusion proteins localized to the mitochondria, indicating that ubiquinone biosynthesis occurs in the mitochondria in *S. pombe*. Thus, analysis of the phenotypes of *S. pombe* strains deficient in ubiquinone production clearly demonstrates that ubiquinone has multiple functions in the cell apart from being an integral component of the electron transfer system.

Ubiquinone is known to be an electron transporter in the respiratory chain in prokaryotes and eukaryotes. It varies among organisms in the length of its isoprenoid side chain. For example, *Saccharomyces cerevisiae* uses ubiquinone-6 (UQ-6), *Escherichia coli* uses UQ-8, and *Schizosaccharomyces pombe* uses UQ-10 (9, 16, 37). It has been shown that the type of ubiquinone in organisms is determined by the polyprenyl diphosphate synthase enzyme, which catalyzes the condensation reaction of isopentenyl diphosphate with allylic diphosphate to give a defined length of the isoprenoid (22, 26). When polyprenyl diphosphate synthase genes from other sources were expressed in *S. cerevisiae* and *E. coli*, the ubiquinone generated was of the same type as that expressed in the donor organism (22–26). By this method, we successfully produced various ubiquinone species (UQ-5 to UQ-10) in the *S. cerevisiae COQ1* mutant (22), which in turn indicates that *p*-hydroxybenzoate (PHB) polyprenyl diphosphate transferase, which catalyzes the condensation reaction between the isoprenoid side chain and PHB, has a broad substrate specificity. This is supported by consistent observations showing that purified PHB polyprenyl diphosphate transferases from *Pseudomonas putida* (12, 40) and *E. coli* (17) have fairly wide substrate specificities in terms of polyprenols. In contrast, PHB geranyltransferase, which is responsible for the synthesis of shikonin, is highly specific, as it uses only geranyl diphosphate as a substrate (21). Studying the PHB polyprenyl diphosphate transferases from different sources may enhance our understanding of this type of enzyme.

Ubiquinone appears to play roles in addition to acting as a component of the electron transfer system. One such role is that of an antioxidant, as indicated by a number of studies (1, 5, 6, 7, 8, 14). A strain of *S. cerevisiae* unable to produce ubiquinone is sensitive to lipid peroxide, suggesting that ubiquinone protects against oxidants (5). Similarly, an *S. pombe* strain which does not produce ubiquinone because of a deficiency of decaprenyl diphosphate synthase is sensitive to H_2O_2 and requires an antioxidant to grow on glucose-containing medium (37). Antioxidant roles of ubiquinone in *E. coli* also have been reported recently (18, 36). Furthermore, physiological concentrations of ubiquinone act as antioxidants on human low-density lipoprotein (1, 7). Another role of ubiquinone is that it can accept electrons from sources other than the respiratory chain. Recently, it was elegantly shown that ubiquinone (or menaquinone) will accept electrons generated by the formation of protein disulfide in *E. coli* (3). Sulfide-ubiquinone oxidoreductase, previously thought to occur mainly in photobiosynthetic bacteria as a component in energy metabolism, has been shown to be present in *S. pombe* and other eukaryotic organisms (42). This suggests that there may be a link between sulfide metabolism and ubiquinone in eukaryotes.

To increase our knowledge of the ubiquinone biosynthetic pathway and the various functions of ubiquinone, we have characterized in this study a strain of *S. pombe* that cannot produce ubiquinone because of a defect in its PHB polyprenyl transferase gene. We show clearly that ubiquinone can act both as an antioxidant and as an acceptor of electrons from sulfide.

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MATERIALS AND METHODS

Materials. Restriction enzymes and other DNA-modifying enzymes were purchased from Takara Shuzo Co. Ltd. and New England Biolabs, Inc.

Strains, plasmids, and media. *E. coli* strains DH10B and DH5α were used for the general construction of plasmids (32). Plasmids pBluescript II KS+/−, pT7Blue-T (Novagen), pREP1 (15), and pREP1-GFPS65A (39) were used as vectors. The *S. pombe* homothallic haploid wild-type strain SP870 (*h*^{90} *leu1-32 ade6-M210 ura4-D18*) (13) and the diploid strains SP826 (*h*$^{+}$ *leu1-32 ade6-M210 ura4-D18*/*h*$^{+}$ *leu1-32 ade6-M216 ura4-D18*) (13) and TP4-1D/TP4-5A (*h*$^{+}$ *leu1-32 ura4-D18 his2 ade6-M216*/*h*$^{-}$ *leu1-32 ura4-D18 ade6-M210*) (37) were used to produce Δ*ppt1*::*ura4* strains by homologous recombination. KS10 (*h*$^{+}$ *leu1-32 ade6-M216 ura4-D18* Δ*dps*::*ura4*) was previously described (37). JV5 (*h*$^{-}$ *ura4-294 leu1-32* Δ*hmt2*::*URA3*$^{+}$) was obtained from D. W. Ow (42). Yeast cells were grown in YE (0.5% yeast extract, 3% glucose) or PM minimal medium (111 mM glucose, 93.5 mM NH_4Cl, 15.5 mM Na_2HPO, 14.7 mM potassium hydrogen phthalate, 5.2 mM $MgCl_2 \cdot 6H_2O$, 13.4 mM KCl, 0.28 mM Na_2SO_4, 0.1 mM $CaCl_2 \cdot 2H_2O$, 81.2 μM nicotinic acid, 55.5 μM *myo*-inositol, 40.8 μM biotin, 4.2 μM calcium pantothenate, 8.1 μM boric acid, 2.37 μM $MnSO_4$, 1.39 μM $ZnSO_4 \cdot 7H_2O$, 0.74 μM $FeCl_3 \cdot 6H_2O$, 0.25 μM $MoO_4 \cdot 2H_2O$, 0.6 μM KI, 0.16 μM $CuSO_4 \cdot 5H_2O$, 4.76 μM citric acid) with appropriate supplements as described by Moreno et al. (19). YEA and PMA contain 75 μg of adenine per ml in YE and PM, respectively. The concentration of supplemented amino acids was 100 μg/ml. Yeast transformation was performed according to the method described by Rose et al. (29).

DNA manipulations. Cloning, restriction enzyme analysis, and preparation of plasmid DNAs were performed essentially as described previously (32). PCR was done according to the procedure described before (31). DNA sequences were determined by the dideoxynucleotide chain termination method (33) using an ABI377 DNA sequencer. To clone the *ppt1* gene, the following four primers were designed. Two oligonucleotides, 5′-TGAATTCGATGATAATTAAGCCTATAGCGT-3′ (creates an *Eco*RI site) and 5′-TCCAAGACTGCAGTAGAACGTTTAAGAATC-3′, were used to amplify the *ppt1* gene. The amplified fragment was then cloned into pT7Blue-T to yield pSP5. The two additional oligonucleotides 5′-TGATGAACCACATTTACTTGATTTAGTCGA-3′ and 5′-TCGAGCTCTTCTGACACCTCAACCTTTAAA-3′ were used to amplify the 4.5-kb fragment containing the *ppt1* gene and the surrounding region. The amplified fragment was then cloned into pT7Blue-T to yield pSP7. To make pSP11, pSP7 was digested with *Sna*BI and ligated with the *ura4* cassette derived from pHSG398-*ura4* (39). The 1.8-kb *Sna*BI fragment containing *ppt1* was cloned into the *Sma*I site of pREP1 to yield pREP1-PPT1. The *Sac*I-*Bam*HI fragment containing *COQ2* (38) was cloned into pREP1 to yield pREP1-COQ2. Putative mitochondrial transit sequences of *ppt1* were amplified by PCR using the oligonucleotides 5′-AGGTCGACAGATTAGCATGTAAATAG-3′ (sense primer; creates a *Sal*I site) and 5′-ATGGATCCGGGGGTTACAGAGTTTGA-3′ (antisense primer; creates a *Bam*HI site) or 5′-TAGGATCCTTCAGCGTAGTATTGCCA-3′ (antisense primer; creates a *Bam*HI site). The PCR products were cloned into the *Sal*I and *Bam*HI sites of pREP1-GFPS65A (39), which contains the GFPS65A gene (20) in pREP1, to yield pGFP-TP45 and pGFP-TP68.

Gene disruption. The one-step gene disruption technique was performed according to the procedure of Rothstein (30). Plasmid pSP11 was linearized by appropriate restriction enzymes, and the linearized plasmid was used to transform SP870 and SP826 to uracil prototrophy. Southern hybridization was performed as described before (32).

Ubiquinone extraction and measurement. Ubiquinone was extracted as previously described (37, 41). *S. pombe* cells were grown in a PMA-based medium (20 ml) until the mid-log phase. After harvesting, the cells were lysed with 3 mg of Novozyme, and ubiquinone was extracted with 3 ml of hexane-acetone (1:1, vol/vol), followed by evaporation of the organic solution to dryness. Samples were then redissolved in 1 ml of chloroform-methanol (1:1, vol/vol) and the solution was washed with 0.5 ml of 0.7% NaCl. After evaporation to dryness, the residue was taken up in 30 μl of chloroform-methanol (2:1, vol/vol) and analyzed by normal-phase thin-layer chromatography on a Kiesel gel 60 F254 plate (Merck) with benzene-acetone (93:7, vol/vol). A UQ-10 standard (Kaneka) was also applied. The UV-visualized band containing ubiquinone was collected from the thin-layer chromatography plate and extracted with chloroform-methanol (1:1, vol/vol). The solution was evaporated to dryness and the residue was redissolved in ethanol. The purified ubiquinone was further analyzed by high-pressure liquid chromatography using ethanol as a solvent (41).

Measurement of sulfide. Hydrogen sulfide was detected by production of PbS from lead acetate. A quantitative determination of sulfide was performed by the methylene blue method as previously described (28). Briefly, *S. pombe* cells were grown in YEA medium (50 ml) until the late log phase. The cells were collected and disrupted by glass beads, and cell extracts were resuspended in 0.1 ml of 0.1% dimethylphenylenediamine (in 5.5 N HCl) and 0.1 ml of 23 mM $FeCl_3$ (in 1.2 N HCl). The samples were incubated at 37°C for 5 min, after which the absorbance at 670 nm was determined using a blank consisting of the reagents alone.

Staining of mitochondria and fluorescence microscopy. Mitochondria were stained by the mitochondrion-specific dye MitoTracker Green FM (Molecular Probes, Inc.). Cells were suspended in 10 mM HEPES, pH 7.4, containing 5% glucose, and MitoTracker Green FM was added to yield a final concentration of 100 nM. After standing for 15 min at room temperature, cells were visualized by fluorescence microscopy at 490 nm. Fluorescence microscopy was carried out with a Zeiss Axioskop microscope at a magnification of ×1,000. GFPS65A fluorescence was observed by illumination at 485 nm. Images were captured by a Hamamatsu C5985 CCD camera.

RESULTS

Cloning of the *ppt1* gene and construction of strains with a defective *ppt1* gene. We found a putative gene for PHB polyprenyl diphosphate transferase in the *S. pombe* genomic DNA sequence determined by the Sanger Center. This gene (SPAC56F8.04c) shows high sequence similarity with the *COQ2* gene from *S. cerevisiae* and was designated *ppt1* (for PHB polyprenyl diphosphate transferase). *ppt1* and putative PHB polyprenyl diphosphate transferases from other species could also be found in the National Center for Biotechnology Information database (Fig. 1). Of these genes, only *ubiA* from *E. coli* and *COQ2* from *S. cerevisiae* have been functionally characterized (2, 16, 17, 35, 38).

To analyze the function of the *ppt1* gene, we amplified the *ppt1* gene from *S. pombe* genomic DNA by PCR to yield the 1-kb DNA fragment containing *ppt1* and the 4.5-kb fragment containing the surrounding DNA. To make *S. pombe* strains containing a defective *ppt1* gene, we constructed the plasmid pSP11, in which the *ppt1* gene is disrupted by the *ura4* gene (Fig. 2A). This plasmid was then linearized by the appropriate restriction enzymes and the fragment initially used to transform the *S. pombe* wild-type haploid strain SP870. However, although some Ura^{+} transformants were obtained, no strains with disruptions in the *ppt1* gene could be isolated. Thus, we decided to transform the diploid SP826 and TP4-1D/TP4-5A strains with the pSP11 fragment. When SP826 was transformed, 30 colonies of Ura^{+} transformants could be picked and grown on YEA-rich medium. The stability of the Ura^{+} phenotype was examined by replica plating, and nine stable Ura^{+} transformants were thus obtained. One of these strains, designated SP826Δ*ppt1*, was allowed to make spores. Germinated haploid cells were plated in replicates on plates containing YEA and PMA-Leu. While all cells grew well on YEA medium some grew only very slowly on the PMA-Leu plate, and these were examined for ubiquinone synthesis. As none synthesized ubiquinone (Fig. 3), these strains were considered to potentially have a disruption in *ppt1*. One such haploid strain, designated NU609, was used for further experiments. Transformation of TP4-1D/TP4-5A similarly generated a strain with a putative disruption in *ppt1* that was designation TP4-1D/TP4-5AΔ*ppt1*.

Verification of *ppt1* disruption by Southern hybridization analysis. Genomic DNAs from SP826, SP826Δ*ppt1*, NU609, and TP4-1D/TP4-5AΔ*ppt1* were subjected to Southern hybridization analysis to confirm the disruption of *ppt1* by *ura4*. The genomic DNAs were first digested with *Eco*RV and run on an agarose gel. The *ura4* cassette and the *ppt1* gene were used as probes. In lanes containing SP826Δ*ppt1* and TP4-1D/TP4-5AΔ*ppt1* DNAs, 1.5- and 4.5-kb bands appeared with both probes (Fig. 2B and Fig. 4, lanes 2, 3, 6, and 7), because SP826Δ*ppt1* and TP4-1D/TP4-5AΔ*ppt1* contain both the complete *ppt1* gene and the *ura4*-disrupted *ppt1* gene. When the *ura4* cassette was used as a probe, no band appeared with DNA from SP826 (Fig. 4, lane 1), but 1.5- and 4.5-kb bands appeared with the DNAs from SP826Δ*ppt1* and TP4-1D/TP4-5AΔ*ppt1* strains (Fig. 4, lanes 2 and 3, respectively) as well as with NU609 DNA (Fig. 4, lane 4). When the *ppt1* fragment was used as a probe, four bands of 1.5, 2.0, 4.5, and 6.0 kb appeared with SP826Δ*ppt1* and TP4-1D/TP4-5AΔ*ppt1* DNAs (Fig. 4, lanes 6 and 7), and three bands of 1.5, 2.0, and 4.5 kb appeared

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(1) EC.ubiA
(2) SY.ubiA                                                                                   MV
(3) SC.COQ2      MFIWQRKSIL LGRSILGSGR VTVAGIIGSS RKRYTSSSSS SSSPSSKESA PVFTSKELEV ARKERLDGLG
(4) SP.PPT1                         MII KPIASPARYF LRTPSWSAVA IFQAVKIKPL QLRTNSSNSV TPNLISPSKK
(5) CE.ubiA                                                                             MSLIPTAS
(6) Dm.ubiA         MYALRHL RLQSARHFRS SYAAAATTKH MLPRQPARVL IGDWSTWDKS RLQDVCSRSS STATEPVKQQ

(1)   MEWSLTQNKL LAFHRLMRTD KPIGALLLLW PTLWALWVAT PGV-----PQ LWILAVFVAG VWLMRAAGCV VNDYADRKFD
(2)   AQTPSSPPLW LTIIYLLRWH KPAGRLILMI PALWAVCLAA QGL-----PP LPLLGTIALG TLATSGLGCV VNDLWDRDID
(3)   PFVSRLPKKW LPYAPLMRLE KPVGTWLLYL PCSWSILMGA MMQGATLSAT AGMLGIFGVG ALVMRGAGCT INDFLDRKLD
(4)   SWKDLFSKRW QYYAEISRAG SPTGTYLLYS PCTWSILMAA YAYDSSLVNV TKMLALFGVG SFLMRSAGCV INDLWDRELD
(5)   SLVASSPPNL KPYLQLMRVD KPIGTWLLYW PCTWSIAMAT PAG---QLPS IYMLSLFGAG AFLMRSAGCV INDLWDKDFD
(6)   TPLQELVSAA KPYAQLMRID RPIGTYLLFW PCAWSIALSA DAG---CWPD LTMLGLFGTG ALIMRGAGCT INDLWDKDID
                                                                                  ---------

(1)   GHVKRTANRP LPSGAVTEKE ARALFVVLVL ISFLLVLTLN TMTILLSIAA LALAWVYPFM KRYTHLPQVV LGAAFGWSIP
(2)   PQVERTKQRP LAARALSVQV GLGVALVALL CAAGLAFYLT PLSFWLCVAA VPVIVAYPGA KRVFPVPQLV LSLAWGFAVL
(3)   QRVIRSVERP IASGRVSPRR ALVFLGAQTL VGMGVLSLLP AQCWWLGLAS LPIVFTYPLF KRFTYYPQAA LSACFNWGAL
(4)   AKVERSKSRP LASGKLSVRQ AISLLSVQLT ASLGLLLQLN PYTLKLGVAS LVPVCLYPAM KRITYYPQVV LGLTFGYGAV
(5)   KKVERTKLRP LACGSLTEKQ AIGLLAGLLS SSLALLLQLN WYSVAVGASS MALVVGYPLA KRFTYWPQFV LGLTFNWGAL
(6)   AKVERTRLRP LASGQISQFD AIVFLSAQLS LGLLVLVQLN WQSLLLGASS LGLVLTYPLM KRVTYWPQLV LGMAFNWGAL

(1)   MAFAAVSE-- -SVPLSCWLM FLANILWAVA YDTQYAMVDR DDDVKIGIKS TAILFGQYDK LIIGILQIGV LALMAIIGEL
(2)   ISWSAVIG-- -DLTDATWVL WGATVFWTLG FDTVYAMADR EDDRRIGVNS SALFFGQYVG EAVGIFFALT IGQLFYLGMI
(3)   LGFPAMGV-- -MSWPTMIPL YLSSYLWCMT YDTIYAHQDK KFDIKAGIKS TALAWGPRTK SIMKAMSASQ IALLAVAGLN
(4)   MGWPALAGEA CMNWSVVAPL YLSTISWIVL YDTIYAHQDK RDDVKANLYS TALRFGDNTK PVLCGLAALQ IATLATAGIM
(5)   LGWCALKG-- DLSSSAPFWM YAAALQWILI YDTIYAHQDK ADDIMIGVKS TALRLGADTK KWLSAFGVGT VASLTACGIA
(6)   LGWCATQG-- SVNLAACLPL YLSGVQWTIV YDTIYAHQDK LDDLQIGVKS TALRFGENTK VWLSGFTAAM LTGLSAAGWA

(1)   NGLGWGYYWS --ILVAGALF VYQQKLIANR EREACFKAFM NNNYVGLVLF LGLAMSYWHF *
(2)   LMLNPLYWLS LAIAIVGWVI QYIQLSAPTP EPKLYGQIFG QNVIIGFVLL AGMLLGWL*
(3)   SGLLWGPGFI GGLGVFAYRL FSMIKKVDLD NPKNCWKYFN ANINTGLYFT YALAVDYILR LFGFL*
(4)   NGQGPVFYTL -GVAGAAYRL SSMLYKVDLD DPKDCFRWFK RNSNTGYLVA AALALDWLAK SFIYDS*
(5)   SDQTWPYYVA --LAATTAQL GWQVGTVDID NGSDCWDKFK SNSWMGIILF SGIVASTLLK EDEKTKESRK NIGDENFDDV
(6)   CDQTVPYYAA --VGVVGAHL VQQIYSLNID NPSDCAKKFI SNHQVGLILF LGIVLGTLLK SDESKKQRQS SLTTSTASSY

(5)   LVTTN*
(6)   VPALPQKPEV LS*
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FIG. 1. Comparison of the amino acid sequences of PHB-polyprenyl diphosphate transferases. EC, *E. coli* (accession no. X66619); SY, *Synechocystis* sp. strain PCC6803 (D64006); SC, *S. cerevisiae* (M81698); SP, *S. pombe* (Z69728); CE, *Caenorhabditis elegans* (U13876); Dm, *Drosophila melanogaster* (AE003678). A putative substrate recognition sequence is indicated by the underline. Conserved amino acids of at least three in six sequences are highlighted.

with NU609 DNA (Fig. 4, lane 8). Thus, the *ppt1* gene is properly disrupted in SP826Δ*ppt1*, TP4-1D/TP4-5AΔ*ppt1*, and NU609.

Complementation of *ppt1* disruption-containing cells with *COQ2*. In the disruption of the *ppt1* gene in NU609 by homologous recombination, it is possible that the upstream and downstream deletion of *ppt1* could have damaged other genes. To eliminate this possibility, the plasmid pREP1-PPT1, which includes only the *ppt1* gene, was used in a complementation assay. We also constructed pREP1-COQ2, in which only the *COQ2* region is expressed under the control of the strong promoter *nmt1*, to test the functional conservation between Coq2 and Ppt1. Thus, NU609 harboring either or both of the vectors pREP1-PPT1 and pREP1-COQ2 were plated on PM-based medium and growth was observed. A few days later, NU609 harboring only the pREP1 vector formed only a very tiny colony, while NU609 harboring pREP1-PPT1 and pREP1-COQ2 grew as well as the wild-type strain. Thus, only the *ppt1* function was abolished in NU609. That pREP1-PPT1 is as competent as pREP1-COQ2 in correcting the poor growth of the *ppt1*-defective strain indicates that *ppt1* is a functional homologue of *COQ2*. When we extracted ubiquinone from each strain, UQ-10 was detected in the wild-type strain, in NU609 harboring pREP1-PPT1, and in NU609 harboring pREP1-COQ2, but not in NU609 alone (Fig. 3). That *COQ2* complements the *ppt1* disruptant and allows the production of UQ-10 in *S. pombe* is consistent with the idea that PHB polyprenyl diphosphate transferase has a broad substrate specificity.

Phenotypes of the NU609 *ppt1* disruptant. It was previously reported that KS10 (Δ*dps*::*ura4*), a strain of *S. pombe* with a disruption in the *dps* (decaprenyl diphosphate synthase) gene, is unable to produce ubiquinone and has some notable phenotypes, including H_2O_2 and Cu^{2+} sensitivity and a requirement of cysteine or glutathione for growth on minimal medium (37). Thus, NU609 was tested for these phenotypes. NU609 (Δ*ppt1*) was first grown on PM-based medium with and without supplementation with 200 μg of cysteine per ml or 200 μg of glutathione per ml. The addition of cysteine or glutathione effectively caused a recovery of NU609 growth similar to that of the *dps* disruptant KS10 when it was treated similarly (data not shown). NU609 was next tested for growth on PM-based liquid medium supplemented with α-tocopherol, a well-known lipid antioxidant. NU609 cells did not grow on the minimal medium, but interestingly, NU609 cells grew well when 1 mM α-tocopherol was added (data not shown). This suggests that ubiquinone may act as an antioxidant in *S. pombe*. If this is so, it follows that the *ppt1*-deficient strain might be susceptible to

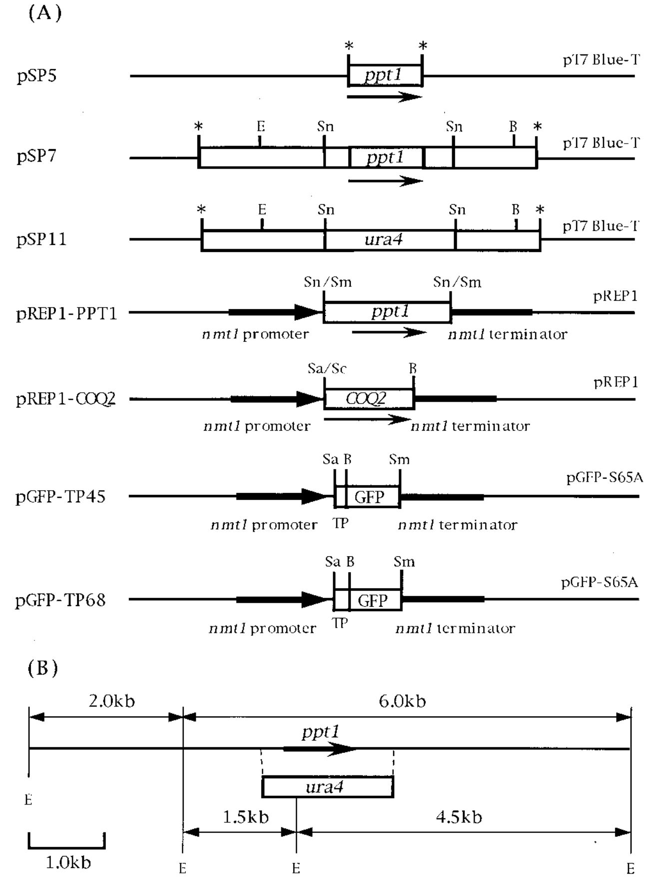

FIG. 2. Plasmid constructions used in this study (A) and *Eco*RV restriction map of the *ppt1* and the *ppt1*::*ura4* regions (B). Asterisks indicate the sites of TA ligation with the T-tailed vector pT7Blue-T. pREP1-PPT1 and pREP1-COQ2 contain the entire lengths of the *ppt1* and *COQ2* genes, respectively, and are under the control of the strong *nmt1* promoter. pGFP-TP45 and pGFP-TP68 contain putative mitochondrial transit peptides (TP) of Ppt1. Thin arrows indicate the direction and the length of open reading frames. Abbreviations for restriction enzymes: B, *Bam*HI; E, *Eco*RV; Sa, *Sal*I; Sc, *Sac*I; Sn, *Sna*BI; Sm, *Sma*I.

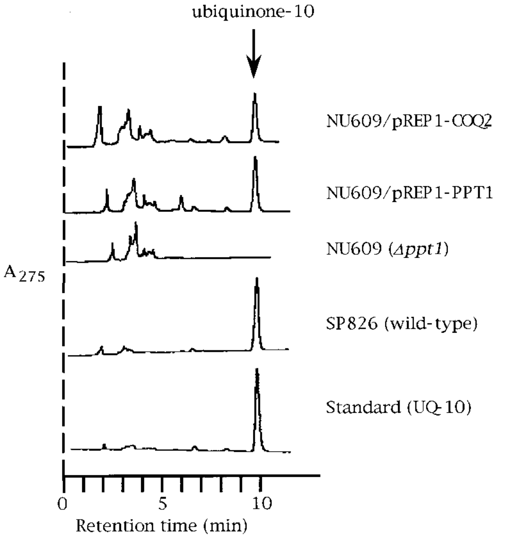

FIG. 3. Detection of UQ-10. Ubiquinone was extracted from the wild-type SP826, NU609 (Δ*ppt1*::*ura4*), NU609 harboring plasmid pREP1-PPT1, and NU609 harboring plasmid pREP1-COQ2. Ubiquinone was first separated by thin-layer chromatography and then further analyzed by high-pressure liquid chromatography.

oxygen radical producers, and we duly noted that NU609 growth is severely inhibited by the presence of 2.5 mM H_2O_2 (Fig. 5A) or 0.5 mM Cu^{2+} (Fig. 5B). The oxidants at these concentrations did not, however, affect the growth of wild-type cells (Fig. 5). These results suggest strongly that ubiquinone can serve as an antioxidant in normal fission yeast cells.

Production of hydrogen sulfide in *S. pombe* strains unable to produce ubiquinone. We found that when the *S. pombe* strains with disruptions in *ppt1* or *dps* were cultivated, they smelled unpleasant. This was found to be due to their production of H_2S when we tested for the formation of PbS by the chemical reaction of H_2S with lead acetate (data not shown). Strains deficient in either *ppt1* or *dps* could produce H_2S, but the wild-type cells could not. Since HMT2 catalyzes sulfide oxidation by concomitant reduction of ubiquinone in *S. pombe* (42), we tested for H_2S production in *hmt2* mutants, but H_2S could not be detected using this method. The production of H_2S was also observed in NU609 cells grown on liquid minimal medium supplemented with α-tocopherol, indicating that the antioxidant function of α-tocopherol could not overcome the production of H_2S. We measured the amount of acid-labile sulfide present in the cells and found that while JV5 (Δ*hmt2*) produced a 2.5-fold-larger amount of S^{2-} than wild-type cells (82.1 and 33.7 nmol/10^6 cells, respectively), KS10 (Δ*dps*) and NU609 (Δ*ppt1*) produced 9-fold-larger amounts of S^{2-} than the Δ*hmt2* strain (758.1 and 718.6 nmol/10^9 cells, respectively). This surprisingly high level of S^{2-} production presumably leads to the production of H_2S in the *ppt1*- and *dps*-deficient strains. This unexpected phenotype suggests that ubiquinone may be important in sulfide oxidation in *S. pombe*.

Mitochondrial localization of Ppt1. Since ubiquinone biosynthetic enzymes are localized to the mitochondria of *S. cerevisiae* (4, 11, 27), it has been suggested that ubiquinone biosynthesis occurs in the mitochondria. To assess the case for homologous enzymes from *S. pombe*, the localization of *S. pombe* Ppt1 was examined by Ppt1-green fluorescent protein

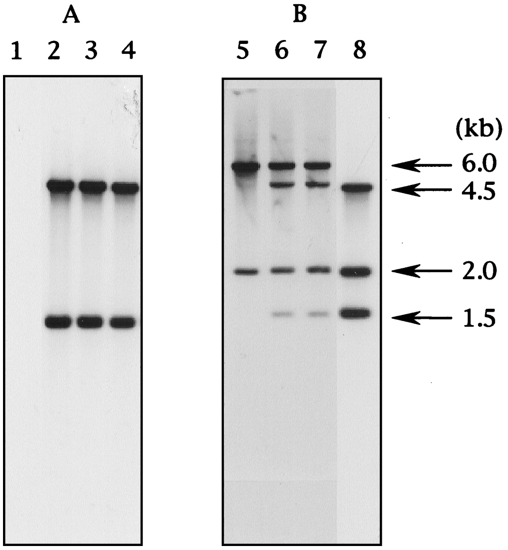

FIG. 4. Southern hybridization analysis. Genomic DNAs of SP826, SP826Δ*ppt1*, TP4-1D/TP4-5AΔ*ppt1*, and NU609 were prepared, separated on an agarose gel, and probed with the *ura4* gene (A) and the *ppt1* gene from pSP7 (B). Lanes 1 and 5, wild-type SP826 (diploid); lanes 2 and 6, SP826Δ*ppt1* (diploid); lanes 3 and 7, TP4-1D/TP4-5AΔ*ppt1* (diploid); lanes 4 and 8, NU609 (haploid). The *Eco*RV restriction map is shown in Fig. 2B.

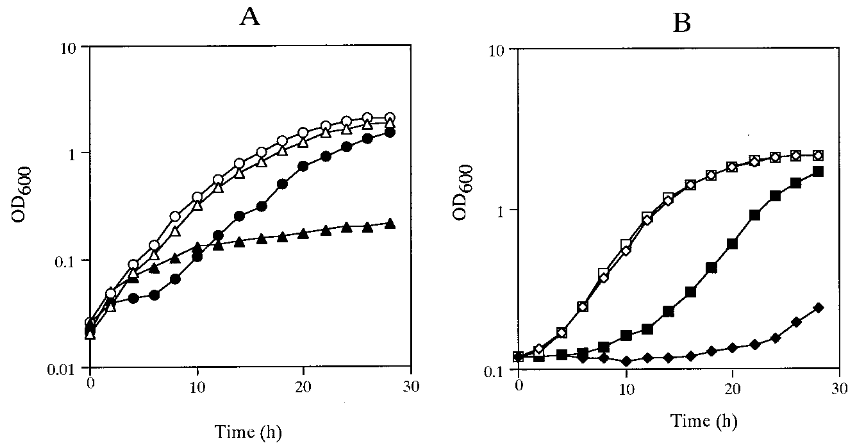

FIG. 5. Sensitivity of NU609 to oxygen radical producers. Wild-type (△, ○, ◇, and □) and NU609 (Δ*ppt1*::*ura4*) (▲, ●, ◆, and ■) strains were pregrown in YEA liquid medium until saturation and then placed in 40-fold dilutions in fresh YEA medium with 2.5 mM H_2O_2 (A) (△ and ▲), 0.5 mM Cu^{2+} (B) (◇ and ◆), or neither (○, ●, □, and ■). Cell growth was measured at 2-h intervals by optical density at 600 nm (OD_{600}).

(GFP) fusions. Thus, genes expressing putative Ppt1 mitochondrial transit peptides of either 45 (pGFP-TP45) or 68 (pGFP-TP68) amino acids fused with GFP were constructed. pGFP-TP45 and pGFP-TP68 were used to transform the *S. pombe* wild-type strain, and Leu^+ transformants were selected. When selected transformants were examined by fluorescence microscopy, accumulation of the fusion proteins in the mitochondria was observed (Fig. 6). The transformants were simultaneously stained with MitoTracker Green FM, which stains mitochondria. The dye stained the cells in exactly the same pattern produced by fluorescing of the Ppt1-GFP fusions, indicating that Ppt1 localizes to the mitochondria.

DISCUSSION

In this study, we examined the *ppt1* gene, which encodes a 358-amino-acid protein with high homology to *E. coli* UbiA and *S. cerevisiae* Coq2 (34 and 48% identity, respectively). In *S. cerevisiae*, Coq2 acts to transfer six isoprenoid units to PHB to produce UQ-6. If, however, the *COQ2* gene is expressed in an *E. coli ubiA* mutant, the cells produce UQ-8 (38). Similarly, as shown in this study, expression of the *COQ2* gene in an *S. pombe ppt1* disruptant resulted in production of UQ-10. Thus, *COQ2* can transfer both octaprenyl diphosphate and decaprenyl diphosphate to PHB. *S. cerevisiae* can also generate various ubiquinone species (UQ-5 to UQ-10) when polyprenyl diphosphate synthases from other species are expressed in the *COQ1* mutant (22). Those observations all indicate that PHB polyprenyl diphosphate transferases can act with a broad range of different polyprenyl diphosphate substrates.

Sequence alignment of the various PHB polyprenyl transferases suggests a putative substrate binding site constituted by an aspartic acid-rich motif (NDXXDXXXD) (35). This motif is well conserved in homolog proteins from *Providencia stuartii*, *Neisseria meningitidis*, *Pasteurella haemolytica*, and *Arabidopsis thaliana* as well as in the proteins listed in Fig. 1. However, the assumption that this motif is the substrate binding site is based merely on its similarity with the substrate recognition site (DDXXD) in polyprenyl diphosphate synthases (23, 24). The exact substrate recognition sequence in PHB polyprenyl diphosphate transferases remains to be determined.

While eight *COQ* genes (*COQ1* to *COQ8*) are known to be involved in ubiquinone biosynthesis in *S. cerevisiae* (2, 4, 10, 11, 27), the *ppt1* gene was only the second gene found to be involved in *S. pombe* biosynthesis of ubiquinone. When we subsequently examined the database from the *S. pombe* genome project for more genes, we found several *COQ* homologs. Besides *dps* (*COQ1* homolog) and *ppt1* (*COQ2* homolog), there are also *COQ3*, *COQ4*, *COQ5*, *COQ6*, and *COQ7* homologs in the *S. pombe* genome, with amino acid sequence identities of 40, 42, 54, 37, and 51%, respectively (the sequence of *COQ8* is not public). Thus, the entire gene set known to be involved in *S. cerevisiae* ubiquinone biosynthesis is also preserved in *S. pombe*. However, the enzymatic activities of Coq4 and Coq8 have, as yet, not been determined, and it is also not clear if all eight *S. cerevisiae* COQs are necessary and sufficient for the biosynthesis of ubiquinone.

Coq1 (our unpublished observations), Coq3 (27), Coq5 (4), and Coq7 (11) have all been localized to the mitochondria of *S. cerevisiae*, indicating that ubiquinone biosynthesis occurs in mitochondria. When we examined Ppt1 localization in this study, we found that it also localized to the mitochondria, suggesting that biosynthesis of ubiquinone in *S. pombe* also occurs in the mitochondria.

S. pombe strains whose *ppt1* gene had been disrupted had several interesting phenotypes. First, while the *ppt1* disruption strain did not grow well on PMA-glucose, growth was greatly improved by the presence of cysteine or glutathione. The addition of the lipid antioxidant α-tocopherol also improved growth. A requirement for cysteine, glutathione, or α-tocopherol for growth on minimal medium is interesting and is consistent with the concept that ubiquinone acts as an antioxidant. Supporting this idea further is that the *ppt1*-deficient strain is sensitive to active oxygen-producing reagents, such as H_2O_2 and Cu^{2+}. These phenotypes of *ppt1*-deficient *S. pombe* are essentially equivalent to those observed for the *dps*-deficient *S. pombe* (37), confirming that these phenotypes arise as a consequence of not being able to produce ubiquinone. A role for ubiquinone as an antioxidant has also been reported for *E. coli*, *S. cerevisiae*, and mammalian cells (1, 7, 14, 18, 36). In *E. coli*, a ubiquinoneless mutant is more susceptible to H_2O_2 and Cu^{2+} (36). In *S. cerevisiae*, strains unable to produce ubiquinone are more susceptible to lipid peroxide and show lower stabilities of extracellular ascorbate (34). In mammalian cells, ubiquinone works synergistically with α-tocopherol to reduce lipid peroxide or low-density lipoprotein (1, 7). It is deduced that ubiquinone in *S. pombe* also has the role of suppressing lipid peroxidation of the membrane, although more direct evidence will be necessary to prove this point.

This study also detected an additional, and very interesting,

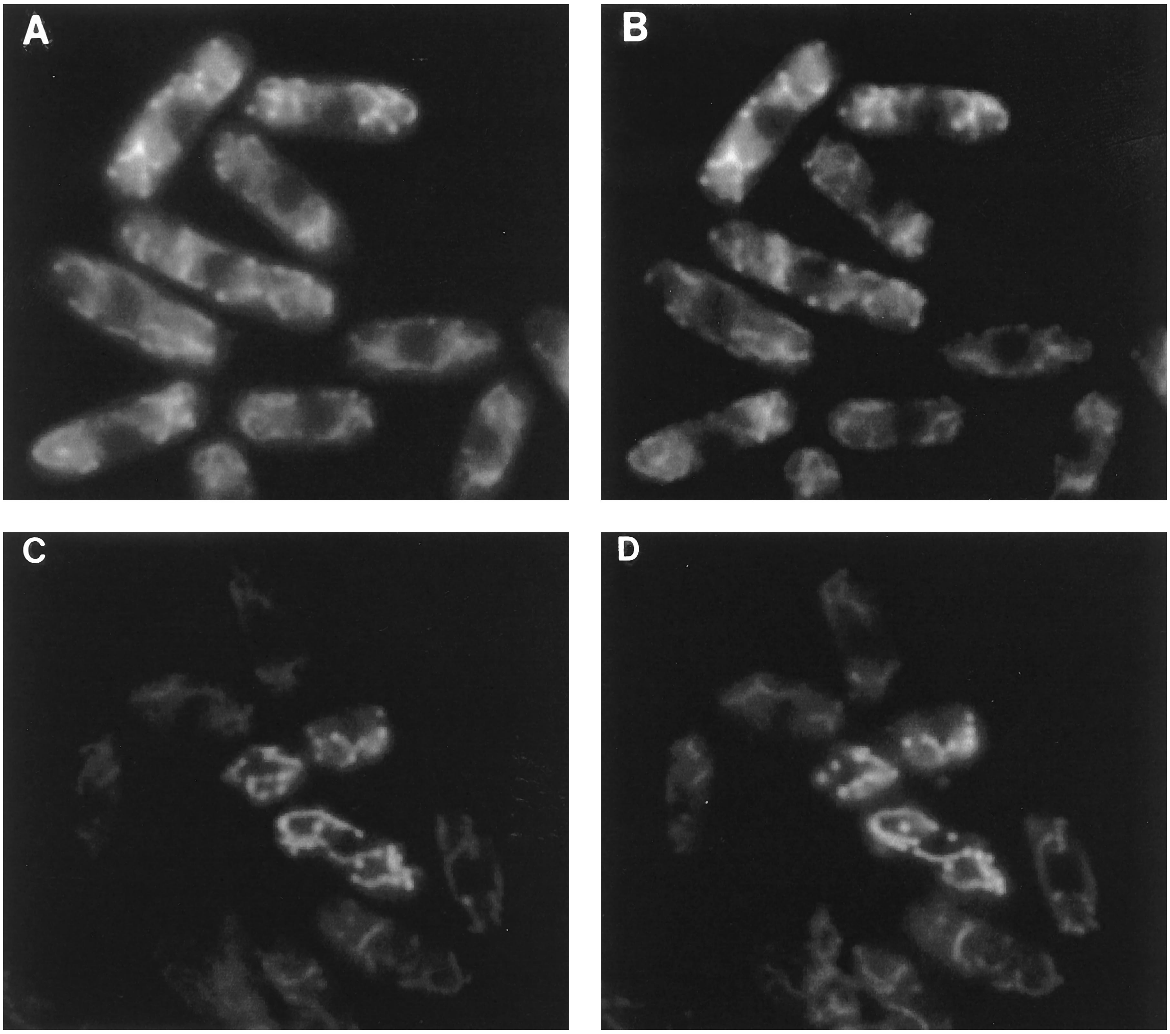

FIG. 6. Colocalization of Ppt1-GFP fusion proteins with a mitochondrion-specific dye. The patterns of fluorescence produced by Ppt1-GFP fusion proteins (A and C) and by MitoTracker Green FM (mitochondrion-specific dye) (B and D) in pGFP-TP45 (A and B) and in pGFP-TP68 (C and D).

phenotype of fission yeast strains unable to produce ubiquinone. The *ppt1* and *dps* mutants both produced large amounts of H_2S. This observation could not be explained by ordinary metabolic pathways. However, the recent finding that sulfide-ubiquinone reductase exists in *S. pombe* (42) suggested to us that there may be a metabolic link between ubiquinone and H_2S production. We speculate that in the absence of ubiquinone in the cell, sulfide-ubiquinone reductase cannot function and thus the cell accumulates H_2S. Since sulfide-ubiquinone reductase is not present in *S. cerevisiae*, mutants of *S. cerevisiae* that are unable to produce ubiquinone do not produce H_2S (our unpublished observation). Interestingly, humans as well as some other higher eukaryotes possess sulfide-ubiquinone reductases similar to the one found in photosynthetic bacteria (42). HMT2 catalyzes sulfide oxidation by concomitant reduction of ubiquinone in *S. pombe*. That the *hmt2* mutant does not release H_2S in equivalent quantities as strains unable to produce ubiquinone is perhaps due to the gradual oxidization of sulfide by ubiquinone that occurs despite the absence of sulfide-ubiquinone reductase.

All the observed phenotypes of *S. pombe* strains that are unable to produce ubiquinone serve to emphasize the fact that ubiquinone does not function solely as a component of the electron transfer system, as is generally believed. Ubiquinone appears to also be important in the oxidative stress response and the sulfide oxidation pathways, at least in *S. pombe*. The former role seems to be common in eukaryotes, while the latter role may occur in the majority of eukaryotes that have sulfide-ubiquinone reductases. Further investigation into the importance of the alternative functions of ubiquinone in other species can be carried out by the construction of ubiquinone-deficient organisms.

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