Mol Gen Genet (1994) 245:272–278

ORIGINAL PAPER

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PET1402, a nuclear gene required for proteolytic processing of cytochrome oxidase subunit 2 in yeast

Received: 24 January 1994 / Accepted: 26 April 1994

Abstract The nuclear mutation *pet ts1402* prevents proteolytic processing of the precursor of cytochrome oxidase subunit 2 (cox2) in *Saccharomyces cerevisiae*. The structural gene *PET1402* was isolated by genetic complementation of the temperature-sensitive mutation. DNA sequence analysis identified a 1206-bp open reading frame, which is located 215 bp upstream of the *PET122* gene. The DNA sequence of *PET1402* predicts a hydrophobic, integral membrane protein with four transmembrane segments and a typical mitochondrial targeting sequence. Weak sequence similarity was found to two bacterial proteins of unknown function. Haploid cells containing a null allelle of *PET1402* are respiratory deficient.

Key words Mitochondria · Protein sorting
Cytochrome oxidase · Post-translational proteolysis
Chromosome V

Introduction

N-terminal presequences, which are removed during uptake, are characteristic features of mitochondrial proteins imported from the cytosol into the organelle. In *Saccharomyces cerevisiae*, two proteins synthesized within mitochondria also possess N-terminal extensions. 10 amino acid residues are removed from the precursor of subunit 6 of the mitochondrial ATPase (Michon et al. 1988) and 15 amino acids from the precursor of cytochrome oxidase subunit 2 (cox2; Pratje et al. 1983). A precursor of cox2 occurs not only in yeast but has also been described in the higher plant sweet potato (Maeshima et al. 1989). In vertebrates, however, the mature form of cox2 is the primary translation product (Steffens and Buse 1979; Anderson et al. 1982).

The yeast nuclear gene *PET2858*, required for removal of the mitochondrial presequence from cox2, was characterized in previous studies (Pratje et al. 1983). The temperature-sensitive, respiratory-deficient mutant *pet ts2858* accumulates the precursor of cox2 and, in addition, an intermediate of cytochrome b_2 (cyt b_2), a nuclear encoded protein of the mitochondrial intermembrane space (Pratje and Guiard 1986). The *PET2858* gene encodes a subunit of the IMP1 protease of the inner mitochondrial membrane (Behrens et al. 1991; Schneider et al. 1991). This subunit shows sequence similarity to a recently described second subunit of the IMP1 protease (Nunnari et al. 1993) and to the *Escherichia coli* leader peptidase (Behrens et al. 1991), which is an essential component of the bacterial export pathway.

We previously described another nuclear respiratory-deficient *pet* mutant that accumulates an altered cytochrome oxidase subunit 2. This mutant, *pet ts1402*, represents a different complementation group, which maps to chromosome V (Mannhaupt et al. 1983). The present study shows that the altered subunit corresponds to the precursor of cox2. The *PET1402* gene was isolated and sequenced: the deduced protein sequence predicts an integral membrane protein that is imported into mitochondria. Gene disruption experiments demonstrate that the *PET1402* gene is essential for growth on non-fermentable carbon sources and for proteolytic processing of cox2.

Materials and methods

Strains and media

S. cerevisiae strains used were: Sc167 (α *ade1*; Michaelis et al. 1982), *pet ts*1402 (α *ade1*; Mannhaupt et al. 1983), GM69-6C (**a** *tyr*; isogenic to Sc167), KN79 (α *leu2-2*, *trp1-1*), JRY-675 (**a** or α *his4-519*, *ura3-52*, Δ*leu2*; J. Rine, unpublished), and haploid progenies from crosses between these strains. Yeast cells were grown on standard media as described by Pratje et al. (1983).

Communicated by C. P. Hollenberg

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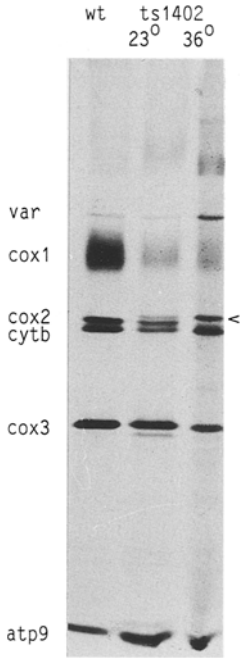

Fig. 1 Mitochondrial translation products of the wild type and mutant *pet ts1402*. Cells were labeled with $^{35}SO_4^{2-}$ at the indicated temperature in the presence of cycloheximide. Mitochondrial proteins were isolated, separated on 10–15% LiDS-polyacrylamide gradient gel and visualized by fluorography. The mutant form of cytochrome oxidase subunit 2 is indicated by an *arrowhead*

Plasmids

A genomic library in the vector YRp7 (Nasmyth and Reed 1980) was used to transform mutant cells. Subcloning was performed in YEp13 (Broach et al. 1979), YEp351 (Hill et al. 1986) and in the *E. coli* vectors pUC19 or pBluescript KS(–).

Molecular biological methods

Published methods were used for the following: restriction analysis, molecular cloning, transformation of *E. coli*, Southern and Northern blotting by hybridization (Sambrook et al. 1989), DNA sequencing (Sanger et al. 1977), tetrad analysis (Sherman and Hicks 1991), transformation of yeast cells (Behrens et al. 1991), isolation of mitochondria (Pratje and Michaelis 1977), biochemical analysis of mitochondrial translation products (Pratje et al. 1983), immunoblotting (Pratje and Guiard 1986), and sequencing of proteins (Beyreuther et al. 1980).

Gene disruption

The *PET1402* disruption was constructed by inserting a 1.45 kb *Hpa*I-*Pvu*II *URA3* fragment into the blunt-ended *Msc*I and *Cla*I sites of the *PET1402* gene as shown in Fig. 6. Wild-type JRY-675 cells were transformed with the linear *Nhe*I-*Ava*I fragment. Ura^+ transformants were selected and verified by Southern hybridization.

Results

Mutation *pet ts1402* prevents proteolytic processing of cox2 without affecting cytochrome b_2, cytochrome c_1 or cytochrome *c* peroxidase

Mitochondrial translation products of the mutant *pet ts1402* were analysed. They were labelled in vivo with $^{35}SO_4^{2-}$ in the presence of cycloheximide for 2 h at 36°C, the non-permissive temperature. Electrophoretic mobility of cox2 in lithium dodecyl sulphate-polyacrylamide gels was slightly reduced compared to the wild type (Fig. 1). In mutant cells grown at 23°C, the permissive temperature, the mature form of cox2 predominates significantly. ^{35}S-labelled cox2 from mutant and wild-type cultures grown at 36°C was extracted from polyacrylamide gels and subjected to automated Edman degradation as described by Beyreuther et al. (1980). For the mutant protein, radioactive signals were detected at positions 1, 14 and 23 (see Fig. 2), demonstrating that the mutant *pet ts1402* accumulates the precursor form of cox2 at the non-permissive temperature. In agreement with previous results, the first radioactive amino acid residue from the mature wild-type cox2 was released at the eighth Edman degradation step (Fig. 2; Pratje et al. 1983). These experiments show that the *ts1402* mutation prevents removal of the 15 N-terminal amino acid residues from the cox2 precursor protein.

In order to test whether the mutant phenotype was due to a single nuclear mutation, mutant *pet ts1402* was crossed with a wild-type strain and the resulting diploids were sporulated. The temperature-sensitive phenotype segregated regularly, 2:2, and only temperature-sensitive spores transmitted the mutant electrophoretic mobility phenotype of cox2 (Fig. 3).

We went on to determine whether additional proteins are affected by the *pet ts1402* mutation. Processing of cyt b_2 was analysed with an antiserum directed against cyt b_2 from yeast. In an immunoblotting assay of mitochondrial proteins, cyt b_2 from mutant *pet ts1402* was indistingishuable from the mature protein in the wild type (Fig. 4). The immunoblots were also assayed with antisera directed against cytochrome c_1 (cyt c_1), cytochrome *c* peroxidase (ccp) and the Rieske iron sulphur protein (FeS) but no differences between the proteins from wild-type and mutant cells were detectable (not shown).

A truncated open reading frame (ORF) complements the *pet ts1402* mutation

Mutant *pet ts1402* cells were transformed with a wild-type yeast genomic library inserted into the vector YRp7. Trp^+ transformants able to grow at 36°C on

Fig. 2 Radiolabelled protein sequence analysis of cox2 from mutant *pet ts*1402 and wild type. The ^{35}S-labelled proteins were subjected to automated Edman degradation (Beyreuther et al. 1980) and the release of radioactivity at each degradation step was registered. The protein sequence deduced from the DNA sequence was taken from Coruzzi and Tzagoloff (1979) and Fox (1979)

Deduced from DNA sequence	M L D L L R L Q L T T F I M N D V P T P Y A C Y F...
^{35}S Peaks released from pet ts1402	+ – – – – – – – – – – – – + – – – – – – – – + – –
^{35}S Peak released from wild type	– – – – – – – – + – –

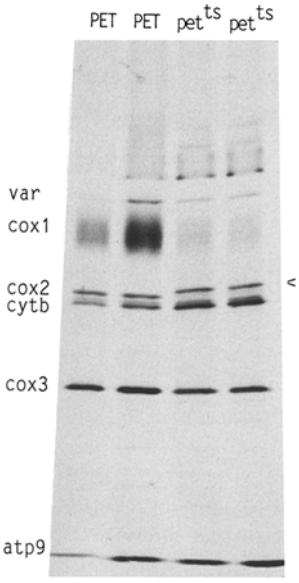

Fig. 3 Tetrad analysis of *pet ts1402*. Mutant *pet ts1402* was crossed with wild-type GM69-6C and resulting diploids were sporulated. Four haploid strains derived from one tetrad were analysed. Mitochondrial translation products were labelled with $^{35}SO_4^{2-}$ and separated on a 10–15% LiDS-polyacrylamide gradient gel. The *arrowhead* indicates the mutant form of cox2

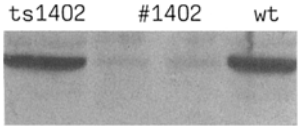

Fig. 4 Cytochrome b_2 from mutant *pet ts1402*, two *pet 1402* gene disruption strains (#1402) and wild type (wt). Protein extracts were prepared from the wild type and disruption strains grown at 28°C and from mutant *pet ts1402* grown at 23°C and incubated for 14 h at 36°C in the presence of lactate. The proteins were separated on a 10% SDS-polyacrylamide gel and visualized by immunoblotting with antiserum against cyt b_2

glycerol-containing medium were selected. One recombinant plasmid was isolated which conferred respiratory competence to the *pet* mutant. This recombinant plasmid contained a 6 kb DNA insert. After subcloning experiments, a 2.1 kb fragment was found to retain the complementing activity. This fragment is located at the right-hand end of the cloned insert as shown in Fig. 5.

DNA sequencing was performed by the dideoxy chain-termination method (Sanger et al. 1977) and a single long ORF with no stop codon was identified. This truncated ORF was able to complement the *pet ts1402* mutation. In order to isolate the missing 3′ end of the ORF, total DNA was isolated and restricted with *Msc*I; a *Cla*I probe specific for the ORF hybridized to a 1.9 kb *Msc*I fragment. Corresponding *Msc*I DNA fragments were extracted from an agarose gel and cloned in the vector pUC19. The correct clone was identified by colony hybridization and used to determine the missing DNA sequence.

The complementing gene corresponds to the *PET1402* structural gene

A disruption mutant was constructed by deleting 128 nucleotides between the *Msc*I and *Cla*I restriction sites in the 2.1 kb *Pvu*II-*Hin*dIII fragment (Fig. 5). A 1.45 kb *Hpa*I-*Pvu*II fragment containing the *URA3* gene was used to replace the deleted sequence (Fig. 6A). The resulting plasmid was linearized at the *Nhe*I and *Ava*I sites, transformed into a *ura3* yeast strain and Ura$^+$ transformants were selected. Total DNA was isolated from various Ura$^-$ and Ura$^+$ clones, digested with *Cla*I and *Xba*I, and separated on an agarose gel. After transfer to a nylon membrane the Southern blot was probed with the digoxigenin-labelled CX1.9 fragment (Fig. 5). Hybridization analysis showed that the expected gene disruption had occurred in the Ura$^+$ transformants analysed (Fig. 6B). The disruption strain was viable on rich glucose medium but could not grow on glycerol-containing medium (Table 1). From this result we conclude that the ORF identified is indispensable for mitochondrial function.

The disruption mutant was subsequently crossed with *pet ts1402* and the resulting diploids were analysed for growth on glycerol-containing medium at 23°C and 36°C. The temperature-sensitive phenotype of the

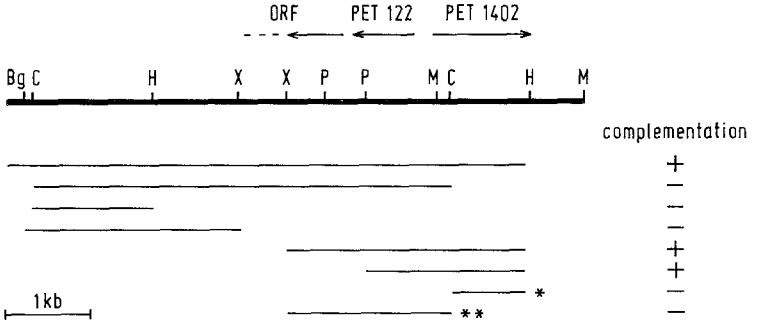

Fig. 5 Restriction map of the genomic region complementing the *pet ts1402* mutation. Three identified open reading frames are indicated by *arrows*. The results of the complementation analysis with various DNA fragments are shown *below*. Restoration of growth of the *pet1402 ts* mutant on glycerol-containing medium at 36°C is indicated by the *plus* sign. The locations of *PET122* and ORF were taken from Ohmen et al. (1988). Restriction enzyme site abbreviations: C, *Cla*I; Bg, *Bgl*II; H, *Hin*dIII; M, *Msc*I; P, *Pvu*II; X, *Xba*I. The C1.2 (*) and CX1.9 (**) fragments were used as hybridization probes

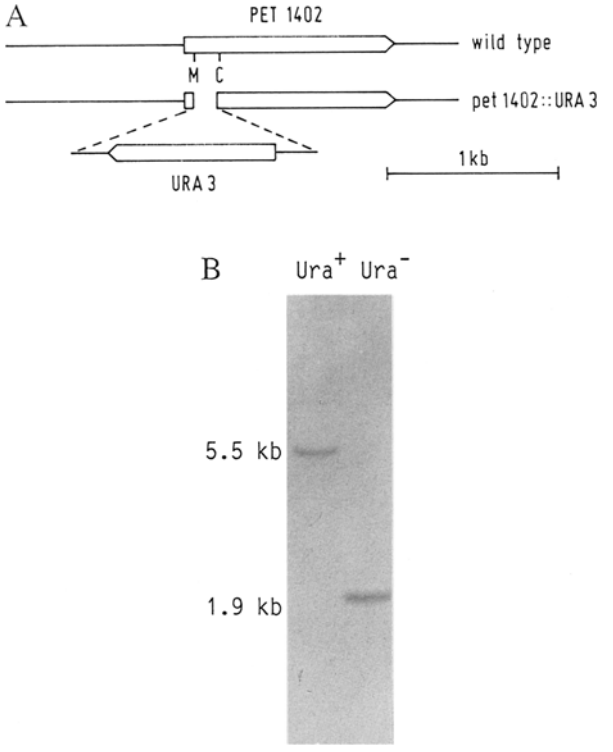

Fig. 6 Disruption of the *PET1402* reading frame. **A** The DNA region carrying the *PET1402* gene is illustrated at the *top*. The *lower* part shows this region in the disrupted strain. C, *Cla*I; M, *Msc*I. **B** Southern analysis of the disrupted gene. Chromosomal DNA was extracted from Ura$^+$ and Ura$^-$ cells, digested with *Cla*I and *Xba*I, separated on 0.8% agarose gel, transferred to nylon membrane and probed with the CX1.9 fragment (see Fig. 5)

Table 1 Allelism test between *pet ts1402* and a disruption mutant

Yeast strains	Growth on glycerol-containing medium	
	23°C	36°C
1n wild type (*PET1402*)	+	+
1n *pet ts1402*	+	−
1n *PET1402::URA3*	−	−
2n from *PET1402::URA3* x *pet ts1402*	+	−
2n from *PET1402::URA3* x *PET1402* rho°	+	+

diploid cells demonstrates that the complementing gene corresponds to the wild-type allele of the *PET1402* gene (Table 1).

The deduced protein sequence of *PET1402* predicts a mitochondrial integral membrane protein of 44.8 kDa

The complete *PET1402* gene encodes a 1206-bp ORF (Fig. 7). The first 42 amino acid residues of the deduced protein sequence display a distribution of hydrophobic, hydroxylated and positively charged residues characteristic for mitochondrial targeting signals (von Heijne 1986), suggesting a mitochondrial localization for the PET protein. The hydropathic profile predicts an integral membrane protein with four transmembrane segments as shown in Fig. 8.

Computer searches of available data bases (EMBL data base release 35 and Swiss-Prot latest version) did not reveal identity to any known protein but allowed us to localize the *PET1402* gene upstream of the *PET122* gene (Ohmen et al. 1988). An incomplete open reading frame (ORF 3) of unknown function was previously reported upstream of *PET122* (Ohmen et al. 1990). It is now possible to identify this ORF as the *PET1402* gene.

Computer analysis revealed weak similarity to a 60 kDa inner membrane protein of *Pseudomonas putida* (Ogasawara and Yoshikawa 1992) and a 29 kDa protein of *Bacillus subtilis* (Errington et al. 1992). The protein sequences share 23.2% identity in an overlap of 250 amino acid residues or 25.4% identity in an overlap of 224 amino acid residues (Fig. 9), respectively. So far the functions of these two bacterial proteins are unknown.

The null allelle of the *PET1402* gene reduces mitochondrial protein synthesis and the amount of protein imported into mitochondria

In the disruption strain, incorporation of [^{35}S]methionine into mitochondrially encoded proteins (see the Materials and methods) was reduced to about 1/10 of the wild-type value. This reduction in protein synthesis affected all mitochondrially translated proteins. The disrupted *pet1402* mutant accumulated the precursor of cox2 (not shown) similarly to the ts mutant at 36°C (Fig. 1). The amount of protein imported into mitochondria of the *pet1402* disrupted mutant was also strongly reduced. In immunoblotting experiments, antisera against cyt b_2, cyt c_1, the Rieske FeS protein and subunit IV of cytochrome oxidase gave weak signals with protein extracts from the mutant. The immunoreaction with cyt b_2 is shown in Fig. 4 as an example.

Discussion

Proteolytic processing of the mitochondrially encoded cox2 in *S. cerevisiae* requires at least three nuclear gene products: the two subunits of the IMP1 protease and the PET1402 protein.The IMP1 protease plays a role not only in processing of cox2 but also of the cyt b_2 and cyt c_1 intermediates (Pratje and Guiard 1986; Nunnari et al. 1993). However, processing of cyt b_2 and cyt c_1 does not require the *PET1402* gene function.

The *PET1402* gene was previously mapped on the right arm of chromosome V distal to *ilv1* (Mannhaupt et al. 1983; see also the genetic map of Mortimer et al. 1989). This study shows that the *PET1402* gene is separated by 215 bp from *PET122*, a gene necessary for

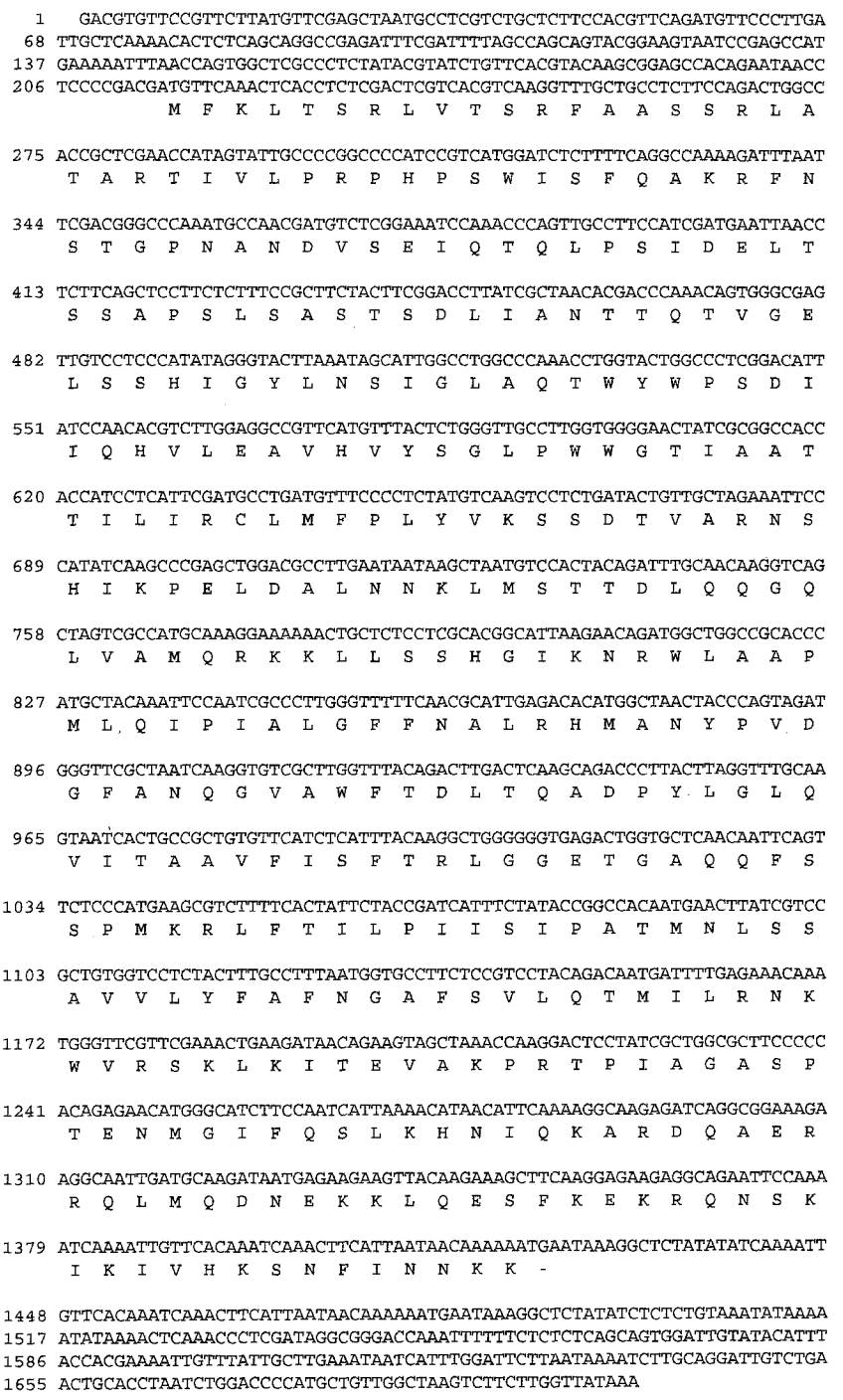

Fig. 7 DNA sequence analysis of *PET1402*. The predicted amino acid sequence is given in one-letter code. The nucleotide sequence has been assigned the accession number X74456 in the EMBL data library

specific translation of cytochrome oxidase subunit 3 (Ohmen et al. 1988). The two *PET* genes are transcribed in opposite directions. The respiratory-deficient phenotype of the *pet1402* disruption mutant cannot be explained by alterations in the *PET122* promoter region because the cox3 protein is present, albeit at a reduced level, like all the other mitochondrially synthesized proteins. As expected, a transcript of *PET122* is detectable in the *pet1402* disruption mutant (our unpublished results). A 1.5 kb mRNA is transcribed from the *PET1402* gene, in agreement with the size of the reading frame and the experiments reported by Ohmen et al. (1990).

The deduced N-terminal amino acid sequence of the *PET1402* gene product exhibits features characteristic of mitochondrial targeting sequences (Roise et al. 1986; von Heijne 1986). The signal sequence and the hydrophobicity of the PET1402 protein strongly argue that the protein is localized in the mitochondrial inner membrane. The hydrophilic C-terminus probably protrudes from the membrane; this part seems to be dispensable for function because a truncated gene lacking 126 nucleotides of the reading frame complements the *pet* ts mutation. Examples of functional truncated genes in yeast have been reported previously in the literature (Laurent et al. 1990).

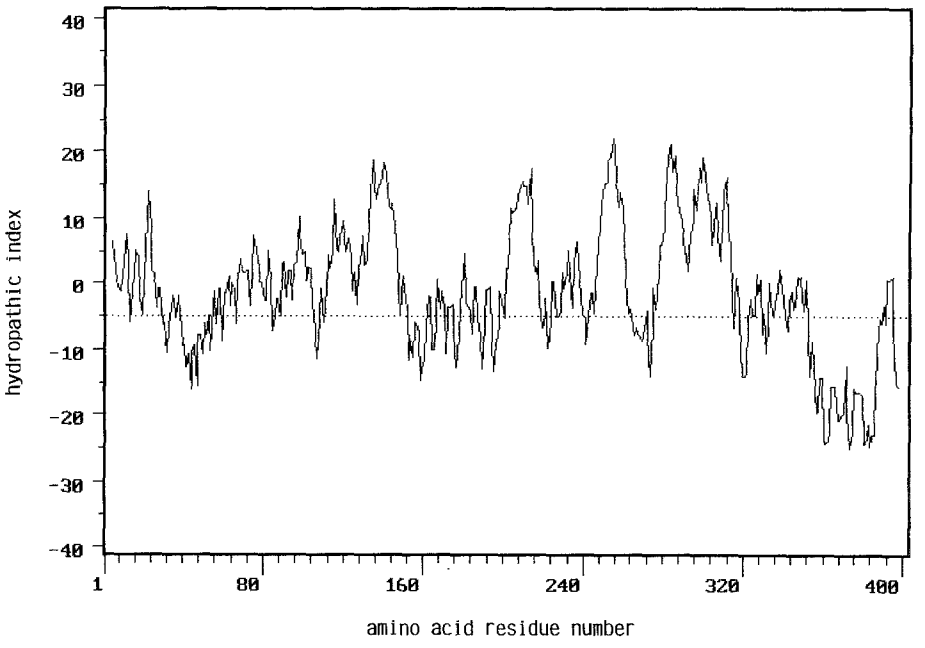

Fig. 8 Hydropathy profile of the deduced PET1402 protein. Positive values denote hydrophobic regions, negative values denote hydrophilic regions. The hydropathy index was calculated according to the algorithm of Kyte and Doolittle (1982) using an interval of 9 amino acids. Four transmembrane regions are predicted

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          70         80         90        100        110        120
1402  SAPSLSASTSDLIANTTQTVGELSSHIGYLNSIGLAQTWYWPSDIIQHVLEAVHVYSGLP
                  :          :          ..:. . . .: . .. .. : .   . :.
Sp3j        MKRRIGLLLSMVGVFMLLAGCSSVKEPITADSPHFWDKYVVYPLSELITYVAKLT
                     10         20         30         40         50

        130        140        150        160        170        180
1402  --WWG-TIAATTILIRCLMFPLYVKSSDTVARNSHIKPELDALNNKLMSTTDLQQGQLVA
        .: .:  .::::: :..:: .:    .       ..:::. :..:  :...  : .: .
Sp3j  GDNYGLSIILVTILIRLLILPLMIKQLRSSKAMQALQPEMQKLKEKYSSKDQKTQQKL-Q
        60         70         80         90        100        110

         190        200        210        220        230        240
1402  MQRKKLLSSHGIKNRWLAAPML-QIPIALGFFNALRHMANYPVDGFANQGVAWFTDLTQA
        . . :. .:::.       :.: :.:: .:::.:...      ........  :: ::..
Sp3j  QETMALFQKHGVNPLAGCFPILIQMPILIGFYHAIMR-----TQAISEHSFLWF-DLGEK
        120        130        140        150             160

         250        260        270        280        290        300
1402  DPYLGLQVITAAVFISFTRLGGETGAQQFSSPMKRLFTILPIISIPATMNLSSAVVLYFA
        :::  :..... .   .:  ...::: ...: ... :.::. :  ..:...:. ::..
Sp3j  DPYYILPIVAGVATFVQQKLMMAGNAQQ-NPQMAMMLWIMPIMIIVFAINFPAALSLYWV
      170        180        190        200        210        220

         310        320        330        340
1402  FNGAFSVLQTMILRNKWVRSKLKITEVAKPRTPIAGA
        .. : . ::....
Sp3j  VGNLFMIAQTFLIKGPDIKKNPEPQKAGGKKK
      230        240        250
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Fig. 9 Alignment of PET1402 protein and the SpoIIIJ protein of *Bacillus subtilis* (Errington et al. 1992). *Colons* indicate identical amino acid residues, *dots* indicate conserved residues and *dashes* indicate deletions/insertions introduced for optimal alignment

Computer searches revealed 23.2% identity between the PET1402 protein and the C-terminal part of a 60 kDa inner membrane protein of *P. putida*. The function of this protein remains unknown, although the location of this gene adjacent to the bacterial replication origin was taken as evidence for involvement of the gene product in DNA replication (Ogasawara and Yoshikawa 1992). Disruption of the *PET1402* gene does not significantly affect maintenance of mitochondrial DNA. The presence of mitochondrial DNA in the null mutant was demonstrated by a cross with a rho^0 tester strain, completely lacking mitochondrial DNA: the diploid cells of this cross were respiratory competent.

A protein homologous to the 60 kDa protein of *P. putida* was also described previously in *B. subtilis* (Errington et al. 1992). This protein, designated SpoIIIJ, has 25% identity to the PET1402 protein. SpoIIIJ is essential at an intermediate stage of sporulation, even though the gene is expressed predominantly in vegetative cells. A possible function for the SpoIIIJ protein in communication between the prespore and mother cell was suggested.

In addition to its specific effect on the processing of cox2, PET1402 exerts a general influence on mitochondrial metabolism. Disruption of the gene or prolonged incubation of the *pet ts1402* mutant at 36°C results in a severe decrease in mitochondrial protein synthesis. In conclusion, the PET1402 protein is required for mitochondrial biogenesis, proteolytic processing of cox2 and optimal mitochondrial protein synthesis. A possible

role in the export of subunits of cytochrome oxidase and the assembly of this enzyme complex is suggested. In order to elucidate the precise function of this protein, its localization, expression and possible interactions with other mitochondrial polypeptides are under investigation.

Acknowledgements We are grateful to Dr. K. Beyreuther for sequence analysis of the cox2 proteins. We thank Dr. E. Schweizer for the mutant *pet ts1402*, Dr. G. Schatz for providing antisera, and B. Thiessen for competent technical assistance. The article is based in part on a doctoral study by M. Bauer in the Faculty of Biology, University of Hamburg. This work was supported by grants from the Deutsche Forschungsgemeinschaft.

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Note added in proof

The gene OXA1 described recently by Bonnefoy et al. (1994, J Mol Biol 239:201–212) is identical with PET1402.