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Complementation of *Escherichia coli ubiF* mutation by *Caenorhabditis elegans* CLK-1, a product of the longevity gene of the nematode worm

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Abstract ***Caenorhabditis elegans*** **CLK-1 was identified from long-lived mutant worms, and is believed to be involved in ubiquinone biosynthesis. The protein belongs to the eukaryotic CLK-1/Coq7p family, which is also similar to the bacterial Coq7 family, that hydroxylates demethoxyubiquinone, resulting in the formation of hydroxyubiquinone, a precursor of ubiquinone. In *Escherichia coli*, the corresponding reaction is catalyzed by UbiF, a member of a distinct class of hydroxylase. Although previous studies suggested that the eukaryotic CLK-1/Coq7 family is a hydroxylase of demethoxyubiquinone, there was no direct evidence to show the enzymatic activity of the eukaryotic CLK-1/Coq7 family. Here we show that the plasmid encoding *C. elegans* CLK-1 supported aerobic respiration on a non-fermentable carbon source of *E. coli ubiF* mutant strain and rescued the ability to synthesize ubiquinone, suggesting that the eukaryotic CLK-1/Coq7p family could function as bacterial UbiF.**

Key words: *clk-1*; Ubiquinone; Coq7p; *ubiF*; Hydroxylase; *Caenorhabditis elegans*; *Escherichia coli*

1. Introduction

Caenorhabditis elegans clk-1 was originally identified from long-lived mutant worms ([1,2], reviewed in [3], references cited therein)). The *C. elegans* CLK-1, localized in mitochondria [4], belongs to the eukaryotic CLK-1/Coq7p family including the *Saccharomyces cerevisiae* homologue Coq7p [5] and mammalian homologues [6–8]. Some bacteria also have the gene for the Coq7 homologue that has recently shown to be responsible for the hydroxylation of demethoxyubiquinone (DMQ), resulting in the formation of 5-hydroxyubiquinone, a precursor of ubiquinone (UQ, coenzyme Q) [9]. In *Escherichia coli*, the corresponding reaction is catalyzed by UbiF, a member of a distinct class of hydroxylase [10]. Although studies of mutants suggested the eukaryotic CLK-1/Coq7 family may function as a hydroxylase of the precursor of UQ [5,11–14], there was no direct evidence to show enzymatic activity which may be present within the of eukaryotic CLK-1/Coq7 family. Here we show that the plasmid encoding *C. elegans* CLK-1 supported the aerobic growth of *E. coli ubiF* mutant under the condition that succinate was the sole carbon source and it supplemented the ability to synthesize UQ, suggesting that the eukaryotic CLK-1/Coq7p family could be functional equivalent to bacterial UbiF, a hydroxylase of DMQ.

2. Materials and methods

2.1. Bacterial strains and plasmids

The *E. coli* strains and plasmids used in this study are listed in Table 1. *E. coli* JF496 was from *E. coli* Genetic Stock Center (Yale University, New Haven, CT, USA). Transformation of *E. coli* was performed according to published protocols [15,16].

2.2. cDNA cloning of C. elegans clk-1

C. elegans strain Bristol N2 was grown and harvested as previously described [11]. Total RNA was extracted with Trizol (Invitrogen). The RNA was treated with DNase I (Invitrogen, USA), extracted with phenol–chloroform–isoamylalchol (25:24:1, v/v/v), then precipitated with ethanol. Reverse transcription of RNA (2 μg) was carried out with random hexamer and ReverTraAce (Toyobo, Japan) according to the manufacturer's instructions. Polymerase chain reaction (PCR) reaction mixture (50 μl) including 10% of the above cDNA product, 0.5 μM of primers P-587 (5′-CATATGTTCCGTGTAATAACCCGTGGAGCACATAC-3′) and P-588 (5′-GGATCCTCATCAAATTTTCTCAGCAATCGCAATAGCT-3′) and 2.5 units of ExTaq (Takara Bio, Japan) was prepared according to the protocol of the supplier of the enzyme. PCR conditions used were as follows: 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min. The PCR product was ligated with pCR2.1 vector without further purification, and then introduced into XL-1Blue. The transformants were grown and the plasmids were extracted and verified by restriction analysis. Sequence of the cDNA was confirmed with Thermo Sequenase primer cycle sequencing kit (Amersham Biosciences, USA) on a DNA sequencer DSQ-2000L (Shimadzu, Japan), and with a DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences) on a DNA sequencer 373A-18 (Applied Biosystems, USA). The cDNA insert was excised using *Nde*I and *Bam*HI, purified by agarose gel electrophoresis followed by extraction with MagExtractor (Toyobo, Japan) and cloned into between *Nde*I and *Bam*HI sites of pET15b. The resulting plasmid pET15b-*clk-1* was amplified with NM522, and then extracted. This construct was verified by using restriction analysis and DNA sequencing as described above. The cDNA sequence with the upstream region encoding the ribosome binding site and the coding sequence of His-tag sequence, derived from the parent pET15b, was excised by using *Xba*I and *Bam*HI, purified as described above and inserted between the *Xba*I and *Bam*HI sites of pUC19 and cloned.

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Abbreviations: DMQ, demethoxyubiquinone; DMQ_8, 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; UQ, ubiquinone

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The DH5α transformant containing the resulting plasmid pUC19-*clk-1* was screened by colony PCR with P-587 and P-588, and then the plasmid was extracted. The resulting construct was verified by restriction analysis.

2.3. *Cloning of* E. coli ubiF

E. coli DH5α was grown and the DNA extracted according to the standard protocols [17]. PCR reaction mixture (50 μl) included 0.4 μM of P-766 (5′-AGCTGATATCTACAACCGGGCTGC-3′, corresponding to 7362–7385 of the GenBank accession number D90706) and P-767 (5′-CCAGGTACCCCAGCCATCAAGAAA-3′, complementary to 9541–9564 of the GenBank accession number D90706), 5 units of *PfuTurbo* DNA polymerase (Stratagene) and DNA (100 ng) extracted from DH5α as the template and other components according the protocol of the supplier of the enzyme. PCR conditions were as follows: 1 cycle of 95°C for 2 min, 10 cycles of 95°C for 30 s, 65°C for 30 s, 72°C for 150 s, then 25 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 150 s, followed by 72°C for 10 min. The PCR product was purified as described above, digested with *Eco*RV and *Kpn*I, and then cloned between the *Hin*dII and *Kpn*I sites of pUC19. The transformed DH5α was screened by colony PCR with vector-specific primers. The resulting plasmid pUC19-*ubiF* was extracted, then verified by restriction analysis and DNA sequencing with Prism BigDye Terminator v3.0 cycle sequencing ready reaction kit (Applied Biosystems) on a Prism 310 DNA genetic analyzer (Applied Biosystems). The UbiF coding sequence of DH5α was identical to that of the published genome of *E. coli* K12 strains (GenBank accession numbers AE000170 and D90706).

2.4. *Complementation of* ubiF *mutation*

The plasmids pUC19, pUC19-*ubiF* and pUC19-*clk-1* were introduced into JF496. Colonies were selected on LB/kanamycin (50 μg/ml)/ampicillin (100 μg/ml) or LB/kanamycin (50 μg/ml)/carbenicillin (100 μg/ml, only for pUC19-containing colonies) plates by three successive streaks on the plates. The cells were grown in 5 ml of LB/carbenicillin/glucose (0.5%, w/v) for plasmid extraction and preparation of the glycerol stocks. They were verified for their ability to grow on glucose minimal and succinate minimal salt media agar plates including auxotrophic requirements according to [9,10] and carbenicillin (100 μg/ml) in place of ampicillin at 37°C for 40 h. The strains from the glycerol stocks were grown in LB/carbenicillin/glucose up to A_{600} of 0.6–0.7, then the cells from 330 μl of the culture were harvested, washed with the minimal salt medium, suspended in 330 μl of the minimal medium, and then 10 μl was streaked onto a plate. For growth in liquid minimal media, the stocks were grown, washed and suspended as above, then 150 μl of the suspended cells was added to 15 ml of glucose minimal or succinate minimal media including auxotrophic requirements according to [9,10] and carbenicillin (100 μg/ml) in place of ampicillin, then grown at 37°C.

2.5. *Protein and quinone analysis*

For protein analysis, *E. coli* JF496 derivatives and DH5α were grown in two 100 ml batches of LB/kanamycin (50 μg/ml)/ampicillin (100 μg/ml) in a 500-ml Erlenmeyer flask up to A_{600} of 0.6–0.7. As for the Rosetta(DE3)pLysS/pET15b-*clk-1*, the cells were grown in 30 ml of LB/carbenicillin/chloramphenicol (15 μg/ml) in a 100-ml Erlenmeyer flask up to A_{600} of 0.1–0.2, then isopropyl-β-D-thiogalactopyranoside (final concentration 1 mM) was added, followed by incubation for a further 4 h (A_{600} 1.2). The cells from 1 ml of the culture were harvested, suspended in 50 μl (100 μl for Rosetta(DE3)pLysS/pET15b-*clk-1*) of 50 mM Tris, mixed with 50 μl (100 μl for Rosetta-(DE3)pLysS/pET15b-*clk-1*) of sample buffer (125 mM Tris–HCl, pH 6.8, 4%(w/v) sodium dodecyl sulfate (SDS), 10%(v/v) 2-mercaptoethanol, 15.8%(v/v) glycerol, 0.1%(w/v) bromophenol blue), sonicated on ice three times for 10 s with the intervals of 10 s, incubated at 90°C for 3 min, and then 10 μl was loaded on a 10–20% (w/v) gradient SDS–polyacrylamide gel (Daichi Pure Chemicals, Japan). Immunoblot analysis using anti-CLK-1 polyclonal antibodies (cC-14, Santa Cruz Biotechnology, USA, 1:2000 dilution) was carried out according to [18] in the presence of lysates of *E. coli* to reduce the reactivity to the *E. coli* proteins present. For quinone analysis, the bacteria were grown and harvested from two of 100 ml cultures, and then the membrane fraction was prepared as described [19]. Extraction and analysis of lipids were carried out as described [11]. The mass spectra of the quinones were analyzed by using a Perkin Elmer Sciex API165 with electrospray ionization (ESI).

3. Results and discussion

In the course of the biochemical study of *C. elegans* CLK-1, we cloned the cDNA of *clk-1* by reverse transcription followed by PCR and constructed an expression vector pET15b-*clk-1* under the control of an inducible T7 promoter. Using this pET15b-*clk-1* with a Rosetta(DE3)pLysS strain as a host, the overexpression of ~20.2-kDa protein was observed (Fig. 3A, lane 1), although the majority of the expressed protein formed inclusion bodies (not shown). In order to improve the solubility of the protein and verify the function of the expressed protein in *E. coli*, we subcloned the *clk-1* cDNA into pUC19, and transformed *E. coli ubiF* mutant JF496 [10,20] with the resulting plasmid pUC19-*clk-1*. We also cloned the genomic DNA fragment containing wild-type *ubiF* into pUC19 and obtained the plasmid pUC19-*ubiF*. A similar construct pUFM4-2, which is a pUC18 derivatives including the *E. coli* genomic fragment almost identical to the insert of pUC19-*ubiF*, was shown to complement the *ubiF* mutation of JF496 [10]. As a negative control plasmid, pUC19 was used.

To see whether *clk-1* cDNA complements the *ubiF* mutation of *E. coli*, viability of the JF496 containing the above plasmids on minimal medium plates with glucose or succinate as the sole carbon source was tested (Fig. 1). On the glucose minimal salt medium plate, all strains grew, while on the succinate minimal salt medium, JF496/pUC19 did not grow well but JF496/pUC19-*ubiF* grew well as expected. JF496/pUC19-*clk-1* also grew on the succinate minimal salt medium plate although its growth was slower than that of JF496/

Table 1
E. coli strains and plasmids used in this study

Strains/plasmids	Brief description	Source/reference
XL-1Blue	cloning host	Stratagene
NM522	cloning host	Stratagene
DH5α	cloning host, *ubiF*$^{+}$	Invitrogen
JF496	*ubiF*411, *asnB*50::Tn5	[20]
Rosetta(DE3)pLysS	expression host	Novagen
pCR2.1	TA-cloning vector	Invitrogen
pET15b	expression vector	Novagen
pET15b-*clk-1*	pET15b derivative containing cDNA of *C. elegans clk-1*	this study
pUC19	cloning vector	[29]
pUC19-*clk-1*	pUC19 derivative containing *Xba*I-*Bam*HI fragment of pET15b-*clk-1* including cDNA of *C. elegans clk-1*	this study
pUC19-*ubiF*	pUC19 derivative containing *Eco*RV-*Kpn*I fragment of *E. coli* DH5α genome DNA containing *ubiF*	this study

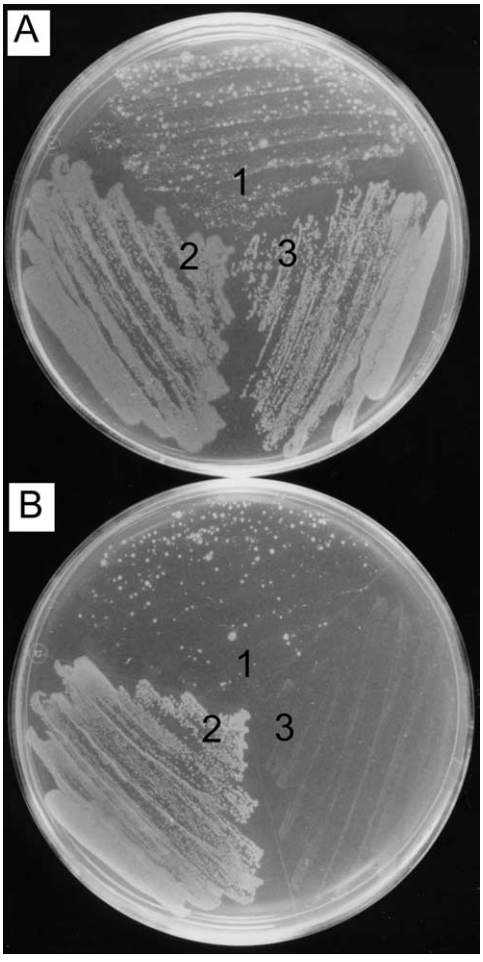

Fig. 1. Growth of *E. coli ubiF* mutant derivatives on minimal salt medium plates. A: Glucose-containing medium. B: Succinate-containing medium. Lane 1: JF496/pUC19-*clk-1*; lane 2: JF496/pUC19-*ubiF*; lane 3: JF496/pUC19.

pUC19-*ubiF*. We also investigated growth of the strains in a liquid minimal salt medium containing either glucose or succinate (Fig. 2). The results were similar to the minimal medium plates, JF496/pUC19 grew poorly in succinate minimal medium, while JF496/pUC19-*ubiF* and JF496/pUC19-*clk-1* grew in both succinate minimal and glucose minimal salt media (Fig. 2). The growth of JF496/pUC19-*clk-1* lagged for a while in both glucose- and succinate-containing medium, while JF496/pUC19 in glucose-containing medium grew earlier. This was consistent with the slower growth of JF496/pUC19-*clk-1* on the minimal medium plates. These results suggested that *C. elegans clk-1* cDNA could complement *ubiF* mutation in *E. coli* although the expression of *clk-1* might have some negative effect on the growth of the bacteria.

To confirm the expression of CLK-1 in *E. coli*, immunoblot analysis of proteins from the JF496 derivatives using anti-CLK-1 antibody was performed. As shown in Fig. 3B, a 20.2-kDa protein in JF496/pUC19-*clk-1* reacted with the antibody (lane 6). The protein was not observed in other strains but in the CLK-1-overexpression strain (lane 1), which corresponds to the calculated molecular mass (22.6 kDa) of the N-terminal His-tag and precursor CLK-1 sequence.

Furthermore, to see the function of the expressed protein in *E. coli*, quinones extracted from membrane fraction of the

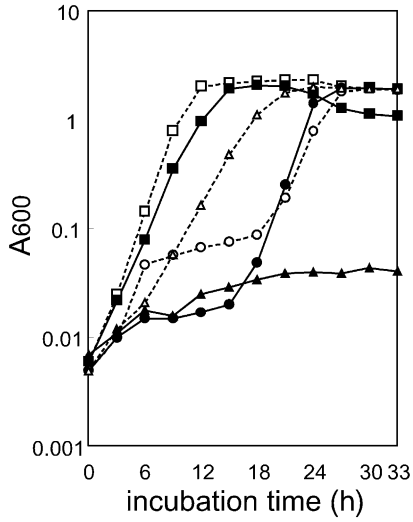

Fig. 2. Growth of *E. coli ubiF* mutant derivatives in minimal salt medium liquid culture. Broken lines: growth in glucose-containing medium; solid lines: growth in succinate-containing medium; squares: JF496/pUC19-*ubiF*; circles: JF496/pUC19-*clk-1*; triangles: JF496/pUC19.

JF496 derivatives were analyzed by high-performance liquid chromatography (HPLC) (Fig. 4). In *ubiF*$^{+}$ DH5α, a peak at 15.2 min retention time, corresponding to UQ_8 (numbers indicate the isoprenoid side chain length), was observed as expected. In JF496/pUC19, a peak at about 14.5 min of retention time was observed. The result by mass spectrometry as well as UV spectrum (not shown) suggested that the peak corresponds to 2-octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone (DMQ_8, ESI mass spectroscopy(*m*/*z*) 719.6 $[M+Na]^{+}$), which was observed in the previous studies of the *ubiF* mutant [9,10,21], as well as contamination by unknown compound(s) corresponding to a peak at about 14 min as discussed below. In both JF496/pUC19-*ubiF* and JF496/pUC19-*clk-1*, a peak corresponding to UQ_8 was observed, suggesting that the plasmid-borne products function

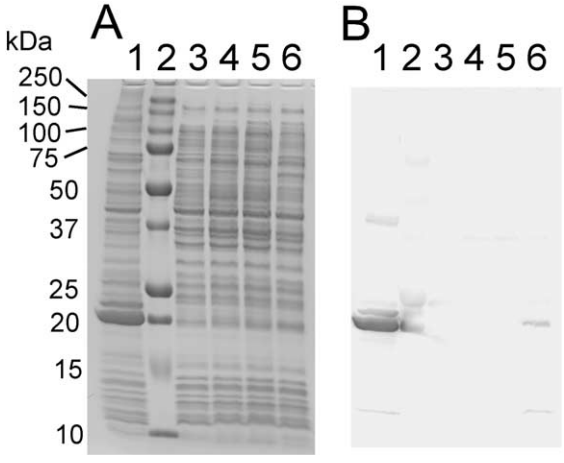

Fig. 3. Expression of *C. elegans* CLK-1 in *E. coli*. A: The gel stained with Coomassie brilliant blue. B: Immunoblot analysis with anti-CLK-1 antibody. Extracts from Rosetta(DE3)pLysS/pET15b-*clk-1* (lane 1), DH5α (lane 3), JF496/pUC19-*ubiF* (lane 4), JF496/pUC19 (lane 5) and JF496/pUC19-*clk-1* (lane 6) together with the size marker (prestained precision protein standard, Bio-Rad, lane 2) were analyzed. The sizes of the markers are also shown.

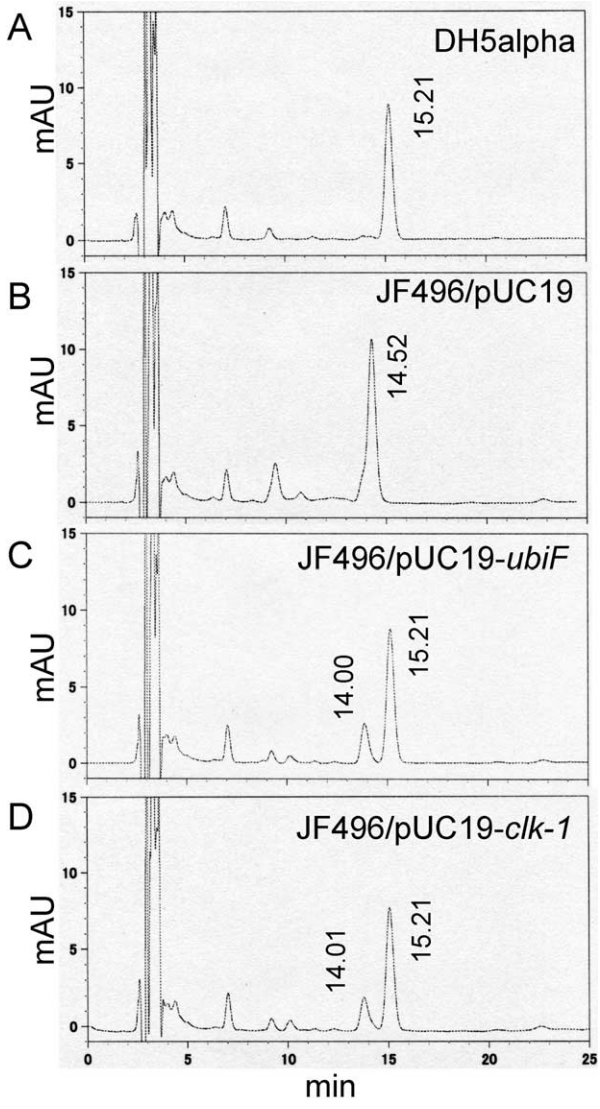

Fig. 4. Rescue of UQ biosynthesis in *E. coli ubiF* mutant by *C. elegans clk-1* cDNA. Quinones were extracted from the membrane fraction of DH5α (A), JF496/pUC19 (B), JF496/pUC19-*ubiF* (C) and JF496/pUC19-*clk-1* (D), separated by HPLC, and then monitored at 275 nm as described [11]. The elution time of the major peak is indicated.

as the hydroxylase/monooxygenase of DMQ_8 and endogenous *E. coli* UbiG methylates the 5-hydroxyUQ product of CLK-1 to UQ. In these strains, a peak at about 14 min retention time was also observed. The peak may correspond to any of the precursor(s) of UQ although its identity is currently unknown and was not pursued further in this study. In summary, the results suggested that *C. elegans* CLK-1 could function as the hydroxylase of DMQ_8 in *E. coli*, although, in *C. elegans*, DMQ_9 (2-methoxy-5-methyl-6-nonaprenyl-1,4-benzoquinone) would be a putative substrate of CLK-1 [11,12].

Although functional expression in *E. coli* of some members of the bacterial Coq7 family has been reported [9,22], complementation of *E. coli ubiF* mutation by eukaryotic Coq7 was not observed [22]. Thus, before this study, the role of the eukaryotic CLK-1/Coq7 family as the hydroxylase of the precursor of UQ, suggested by the function of the bacterial Coq7 family, had not yet been established experimentally. Rather, the multimeric complex formation of *Saccharomyces cerevisiae* Coq7p with other proteins involved in UQ biosynthesis and stabilization of Coq7p in the complex was proposed [23,24]. Furthermore, several genes for homologues of *E. coli* UbiF are found in yeast and metazoa [10]. More recently, mitochondrial DNA binding activity of CLK-1 and a mouse homologue was reported [25]. These studies suggested multiple and/or alternative function(s), rather than the hydroxylase of the precursor of UQ, of the eukaryotic CLK-1/Coq7 family. However, in this study, for the first time, we showed aerobic growth and UQ synthesis in an *E. coli ubiF* mutant with the expression of CLK-1, a member of the eukaryotic CLK-1/Coq7 family, suggesting that the eukaryotic CLK-1/Coq7 family is responsible for 5-hydroxylation of the DMQ without any other eukaryotic proteins, at least in *E. coli*.

CLK-1 is a mitochondrial protein [4] and the *S. cerevisiae* homologue Coq7p and mouse homologue mCLK1 are found in the matrix side of the inner mitochondrial membrane [24,26]. The mitochondrial location of the eukaryotic CLK-1/Coq7 family and the results of this study and genetic studies [5,11–14] strongly suggest the function of the eukaryotic CLK-1/Coq7 family as the hydroxylase of the UQ precursor in mitochondria.

Although mutations of eukaryotic Coq7 accumulate the precursor of UQ, and eukaryotic Coq7 orthologs could complement the respiration of yeast *COQ7* mutant [2,8] and the slow growth of *C. elegans clk-1* mutant [27], the phenotype of the mutant between organisms vary: *C. elegans clk-1* mutant worms exhibit a slow-down in developmental and behavioral rates [3]; homozygotes of *mclk1* knock-out mouse were embryonic lethal [13,14]. The complementation of *E. coli ubiF* mutation by CLK-1 in this study will open the way to investigate in more detail the role of this eukaryotic protein family, which belongs to a di-iron carboxylate protein superfamily [9,28], through protein function and structural analysis.

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