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Ubiquinone (Coenzyme Q) Biosynthesis in *Chlamydophila pneumoniae* AR39: Identification of the *ubiD* Gene

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Abstract Ubiquinone is an essential electron carrier in prokaryotes. Ubiquinone biosynthesis involves at least nine reactions in *Escherichia coli*. 3-octaprenyl-4-hydroxybenzoate decarboxylase (UbiD) is an important enzyme on the pathway and deletion of the *ubiD* gene in *E. coli* gives rise to ubiquinone deficiency *in vivo*. A protein from *Chlamydophila pneumoniae* AR39 had significant similarity compared with protein UbiD from *E. coli*. Based on this information, the protein-encoding gene was used to swap its counterpart in *E. coli*, and gene expression in resultant strain DYC was confirmed by RT-PCR. Strain DYC grew using succinate as carbon source and rescued ubiquinone content *in vivo*, while *ubiD* deletion strain DYD did not. Results suggest that the chlamydial protein exerts the function of UbiD.

Key words UbiD; ubiquinone; biosynthesis; *Chlamydophila pneumoniae*

Chlamydophila pneumoniae is one obligate parasitic pathogen that causes 10% to 20% of human community-acquired pneumonia worldwide. *C. pneumoniae* also plays a role in atherosclerosis by vascular infection [1]. Microbiologically, *Chlamydophila* are characterized by a developmental cycle involving a metabolically inactive infectious form called the elementary body (EB) and a metabolically active form called the reticular body. Some researches demonstrated that *Chlamydophila* could generate ATP through catabolic reaction, which was speculated to be entire energy parasitic pathogen on host cell previously [2]. As one critical component of electron transport chain, ubiquinone (Q) was found to be tightly linked with ATP biosynthesis occurring during the period of reticular body [3–5].

Ubiquinone biosynthesis involves at least nine reactions in *Escherichia coli*. 3-octaprenyl-4-hydroxybenzoate decarboxylase (UbiD) is an important enzyme on the pathway catalyzing the conversion of 3-octaprenyl-4-hydroxybenzoate to 2-octaprenyl phenol [6]. Deletion of the *ubiD* gene in *E. coli* gives rise to ubiquinone deficiency *in vivo*. A low level of ubiquinone in *E. coli* was known to give rise to a pleiotropic phenotype, including low growth rate and an inability to grow using succinate as carbon source [7]. Researches on ubiquinone biosynthesis in genus *Chlamydophila* have been reported previously. One protein (GenBank accession No. NP_444977) from *C. pneumoniae* AR39 had significant identity and similarity compared with *ubiD* (*yigC*) gene encoding protein from *E. coli* [8]. The protein was designated as protein CpUbiD whose encoding gene was designated as *CpubiD* gene.

In this study, we tested whether the protein CpUbiD had the function of UbiD. Results suggest that the protein exert the function of UbiD engaged in ubiquinone biosynthesis.

Materials and Methods

Bacterial strains and growth conditions

The *E. coli* strains DY329 and DH5α were cultured in Luria-Bertani (LB) medium or M9 salt medium with succinate or glucose. Strain DY329 used for homologous recombination experiments was provided by Dr. Stephen LORY (Harvard University, Cambridge, USA). For selection and maintenance of plasmid DNA in *E. coli*, the medium was supplemented with ampicillin (100 μg/ml).

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Analysis of protein CpUbiD

BLAST and multiple-sequences alignment on amino acid sequences of protein CpUbiD and protein UbiD from other microorganisms were performed in Swiss Institute of Bioinformatics (http://www.expasy.org) for analysis of the conserved domains in proteins.

Construction of plasmid pET-*CpubiD*

Using *C. pneumoniae* AR39 genomic DNA from infectious form EB as template, *CpubiD* gene was amplified by polymerase chain reaction (PCR) with *Pfu* DNA polymerase using the forward primer 5′-GGGGCATATGATGTCTTTCTTAAGGCGTCAT-3′ and the reverse primer 5′-GGGGGGATCCTTAAAATAAAAAGTTTCTTTA-3′. An *Nde*I site and a *Bam*HI site were introduced into primers, respectively (underlined). The amplification profile was 95 ºC for 10 min, followed by 30 cycles at 94 ºC for 0.5 min, 53 ºC for 0.5 min, and 72 ºC for 2 min, and finally extended 10 min at 72 ºC. The amplified DNA was digested with *Nde*I and *Bam*HI and inserted into vector pET-15b digested with the same endonucleases. The reconstructed plasmid pET-*CpubiD* was transformed into DH5α cells for amplification. The nucleotide sequences of the inserted *CpubiD* gene from the plasmid were verified by DNA sequencing.

Preparation of recombinant linear DNA

The procedures of homologous recombination experiments for construction of strains DYC and DYD were shown (**Fig. 1**). To construct *E. coli* strains DYC (*ubiD*::*CpubiD*, *amp*^{R}) and DYD (*ubiD*::amp), different recombinant linear DNA was prepared [9]. *CpubiD* recombinant linear DNA, containing *CpubiD* gene and the ampicillin resistant gene, was amplified from plasmid pET-*CpubiD* using LA-*Taq* polymerase. PCR was carried out using the forward primer 5′-AGATGAACGCCGTATAATGGGCGCAGATTAAGAGGCTACAATGTCTTTCTTAAGGCGTC-3′ and the reverse primer 5′-TTCTCTGTCGGATCGATAAATAGGGCAAAACAAACGCGCATCTGACAGTTACCAATGCTT-3′ whose 5′ ends contained 40 nt homologous sequences (underlined) for construction of *E. coli* strain DYC. The amplification profile was 95 ºC for 10 min, followed by 30 cycles at 94 ºC for 0.5 min, 55 ºC for 1 min, and 72 ºC for 3 min, and finally extended 10 min at 72 ºC. The ampicillin resistant gene also was amplified for construction of strain DYD. PCR was preformed using the forward primer 5′-

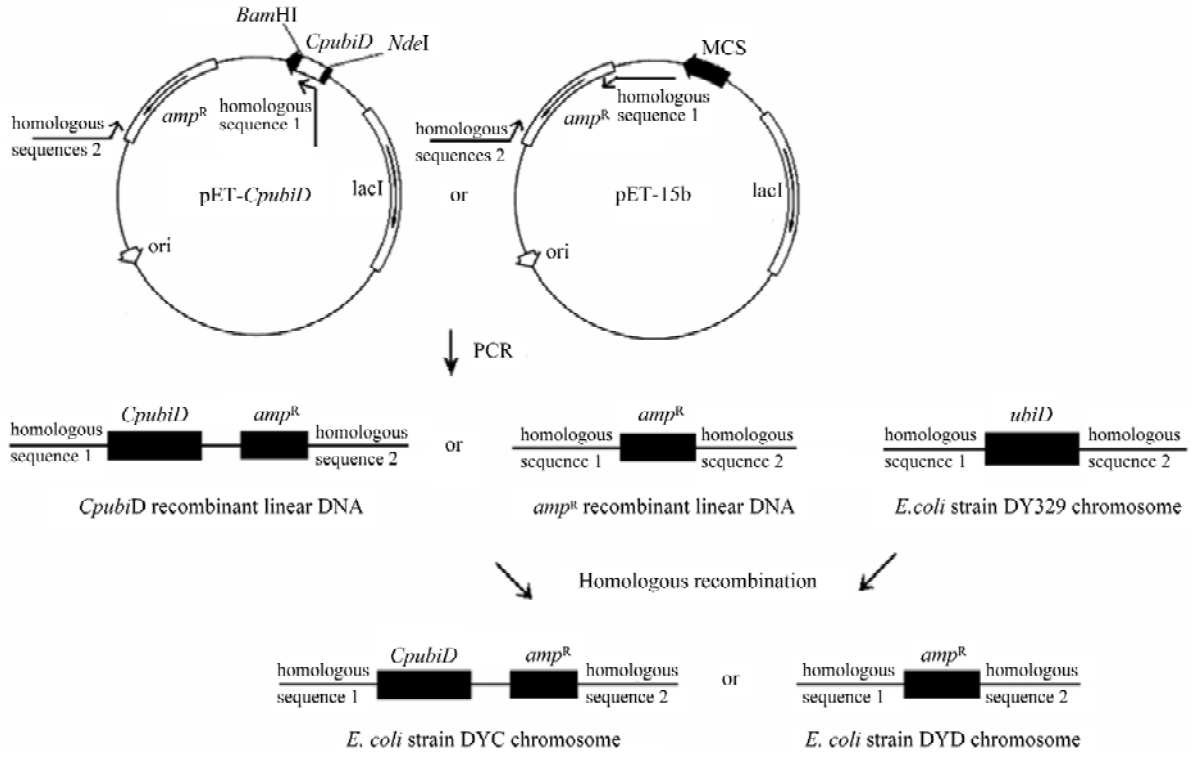

Fig. 1 The procedures of homologous recombination for construction of strains DYC and DYD

AGATGAACGCCGTATAATGGGCGCAGATTAAGAGG-CTACAGCGCGGAACCCCTATTTGTT-3′ and the reverse primer 5′-TTCTCTGTCGGATCGATAAATAGGGC-AAAACAAACGCGCATCTGACAGTTACCAATGCT-3′ with vector pET-15b as template whose 5′ ends contained 40 nt homologous sequences (underlined). The amplification profile was 95 ºC for 10 min, followed by 30 cycles at 94 ºC for 0.5 min, 53 ºC for 1 min, and 72 ºC for 1 min, and finally extended 10 min at 72 ºC. After purification of PCR products, the template DNA was destroyed by endonuclease *Dpn*I. PCR products were purified again, quantified and stored at –20 ºC.

Cell transformation and colony identification

Preparation of competent cells of *E. coli* strain DY329 and electroporation were performed according to the method described previously [9]. After electroporation, colonies were screened on LB plate with carbenicillin (50 μg/ml). The selected colonies were cultivated and their genomic DNA was extracted for PCR identification.

RT-PCR analysis on gene expression

To confirm expression of the *CpubiD* gene in strain DYC, cells were harvested by centrifugation during logarithmic phase. Total RNA was isolated using a RNA extraction kit. cDNA resulting from reverse transcription with AMV reverse transcriptase was ethanol precipitated, resuspended in ddH_2O and stored at –20 ºC. PCR reactions were carried out using cDNA as template. Primers used were within the coding region of each gene. 16S rRNA gene was used as control. Primers for amplification of 16S rRNA gene were the forward primer 5′-GATCATGGCTCAGATTGAACGCTGG-3′ and the reverse primer 5′-TACCTTGTTACGACTTCACCCCAGT-3′. Primers for amplification of *CpubiD* gene were the forward primer 5′-GCTCCCGTAAGTCCTAACCTC-GAG-3′ and the reverse primer 5′-TGGGGAAATATG-CGTGCCATCGTT-3′.

Comparison of growth rate and ubiquinone content

Strains DY329, DYD and DYC were grown on minimum salt plate with 0.5% glucose or succinate at 32 ºC, and then inoculated in minimum salt medium containing 0.5% glucose or succinate with shaking. Different strains also were cultivated in LB medium with 0.5% glucose for comparison of growth rate at 32 ºC. Cell growth was monitored using a Klett meter [10]. Ubiquinone was extracted by the method described previously [11]. The extracted sample was further purified by thin-layer chromatography (TLC) using silica GF254 gel thin-layer plate with chloroform/light petroleum (70:30, *V*/*V*) as a solvent mixture. Ubiquinone was detected under ultraviolet light, and its concentration was measured at 275 nm following the elution with methanol [12].

Results

Analysis on protein CpUbiD

Results showed that three conserved domains A, B and C were detected in proteins (**Fig. 2**). Particularly, domain B contained a conserved motif E-X-P in listed proteins that was inferred to play a role in the binding or catalysis of (hydroxyl) arylic acids [13]. Protein CpUbiD had 31% identity and 52% similarity compared with protein UbiD from *E. coli*. Based on the information, *E. coli* strains DYC and DYD were constructed to detect whether the chlamydial protein had the function of UbiD.

Analysis on expression of the *CpubiD* gene by RT-PCR

Genotypes of strains DYC and DYD were identified by PCR after extraction of genomic DNA (data not shown). Reverse transcription-PCR (RT-PCR) was used to confirm the expression of the *CpubiD* gene in strain DYC. RNA samples were subjected to PCR to determine that there was no genomic DNA contamination (data not shown). Results showed that *CpubiD* gene was expressed in the complementary strain DYC (**Fig. 3**). This confirmed that mRNA of the *CpubiD* gene was synthesized in *E. coli* strain DYC.

Comparison of growth rate

Deletion of the *ubiD* gene in *E. coli* gives rise to ubiquinone deficiency *in vivo*. *E. coli* strains with *ubiD* deletion are unable to grow with succinate as sole carbon source. These strains also show slow growth in rich medium or minimum salt medium with glucose as carbon source. Therefore, we investigated the growth of various strains under different conditions. On the glucose minimal salt plate, strain DYD showed slow growth compared with other strains. While on the succinate minimal salt plate strain DYD did not grow, but strain DYC grew well as expected (**Fig. 4**). We also investigated growth of these strains in minimal salt medium containing glucose or succinate. The results were similar to those on the minimal salt plate. Strain DYD grew poorly in succinate-containing medium while DY329 and DYC grew in both glucose- and succinate-containing medium (**Fig. 5**). In LB

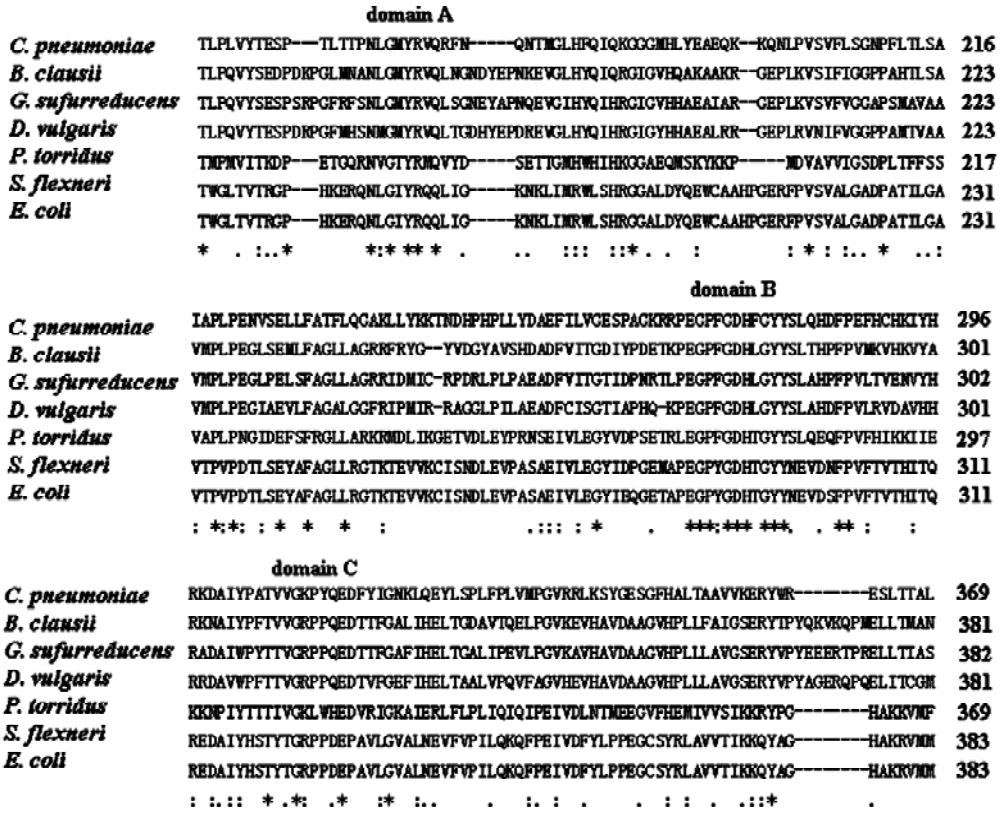

Fig. 2 Alignment between protein CpUbiD and relative proteins UbiD

Alignment is maximized by introducing gaps, which are indicated by dashes. Identical (*), high similar (:) and similar (.) amino acids are indicated. The conserved domains and the numbers of amino acids are shown, respectively. Species names and GenBank accession numbers are as follows: *Chlamydophila pneumoniae*, NP_444977; *Bacillus clausii*, YP_177515; *Geobacter sufurreducens*, NP_953053; *Desulfovibrio vulgaris*, YP_011533; *Picrophilus torridus*, YP_022943; *Shigella flexneri*, NP_709647; *Escherichia coli*, NP_756649.

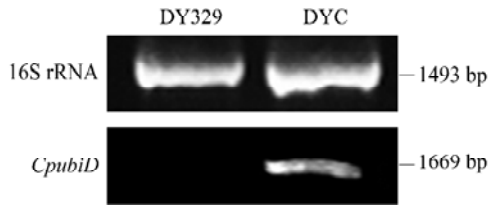

Fig. 3 Analysis of the expression of *CpubiD* gene in strain DYC by RT-PCR

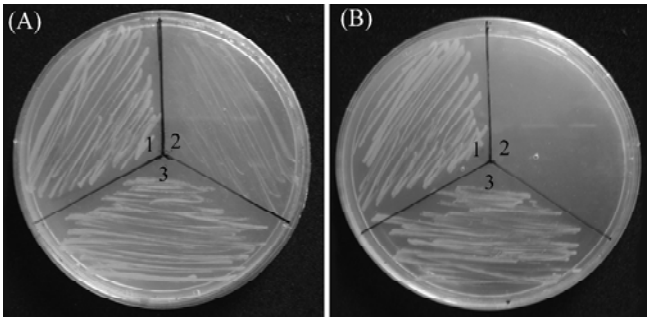

Fig. 4 Growth of various strains on minimum salt plate

All strains were cultured at 32 ºC for 24 h on minimum salt plate with 0.5% glucose (A) or succinate (B). 1, strain DY329; 2, strain DYD; 3, strain DYC.

medium with 0.5% glucose the generation time for strains DY329 and DYC during logarithmic phase were 30.6 min and 31.2 min, respectively. Comparatively, the generation time for *ubiD* deletion strain DYD was 151.4 min. These results suggested that protein CpUbiD substituted the function of UbiD in *E. coli*.

Comparison of ubiquinone content

To further investigate if observed growth properties of various strains correlated with ubiquinone content, these strains were cultured, and ubiquinone was extracted and subjected to TLC. A total of 0.235 nmol of ubiquinone from per mg-dried cell of strain DY329, and 0.183 nmol from strain DYC were detected. Comparatively, ubiquinone

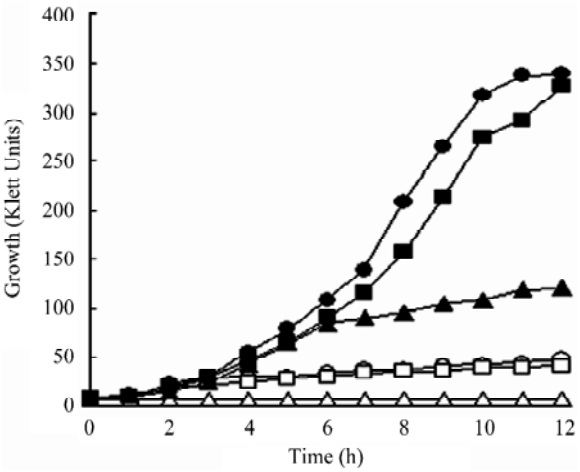

Fig. 5 Growth of different strains in minimum salt medium with 0.5 % glucose or succinate at 32 ºC

Strain DY329: ●, glucose; ○, succinate; strain DYD: ▲, glucose; △, succinate; *CpubiD* strain DYC: ■, glucose; □, succinate.

content of strain DYD was less than 0.05 nmol and was not detected because of low content. These numbers matched with their growth status in studies. These results demonstrated that the decreased or failed growth caused by deletion of the *ubiD* gene was restored in *E. coli* strain DYC by *CpubiD* gene. So we conclude that the protein from *C. pneumoniae* AR39 exerts the function of UbiD.

Discussion

Pathway of ubiquinone biosynthesis in *E. coli* has been known for many years. UbiD and UbiX cooperated as isoenzymes without amino acid sequence similarity, catalyzing the conversion of 3-octaprenyl-4-hydroxybenzoate to 2-octaprenyl phenol in ubiquinone biosynthesis. Studies on the two enzymes suggested that in wild type cells 80% of the enzyme activity was due to *ubiD* gene product and 20% was due to *ubiX* gene product. The reason is that the promoter strength of *ubiX* gene appeared particularly weak [14,15]. Consequently, *ubiD* deletion in *E. coli* gave rise to ubiquinone deficiency *in vivo* [7].

Ubiquinone deficiency decreased activities of enzymes in electron transport system. For example the succinate oxidase activity of cytoplasmic membrane from an *ubiE* mutant in *E. coli* could be partially restored *in vitro*, on addition of quinone analog [16]. Some researches also confirmed that decreased activities of enzymes in electron transport correlated with the inability to grow on succinate as carbon source [17]. Hence we suggest that ubiquinone deficiency caused by *ubiD* mutant or deletion is the reason that the strain can not utilize oxidizable substrate succinate as carbon source in prokaryotes.

Ubiquinone biosynthesis in genus *Chlamydophila* has not been reported previously. So far only three enzymes (UbiA, UbiX and UbiE) engaged in ubiquinone biosynthesis in genus *Chlamydophila* have been identified [4]. The complete biosynthesis of chorismate in *C. pneumoniae* has been confirmed, which provided benzene ring for ubiquinone [18]. According to the information, we suggest that pathway of ubiquinone biosynthesis in *C. pneumoniae* should be similar to that in *E. coli*. Our results support that the protein from *C. pneumoniae* AR39 exerts the function of UbiD engaged in the ubiquinone biosynthesis.

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