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Notes

Isolation and Identification of a Photosynthetic Bacterium Containing a High Content of Coenzyme Q_{10}

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To develop a potent strain for the production of coenzyme Q_{10}, a photosynthetic bacterium was isolated from silt of the Nakdong River in Korea. Using 16S-rDNA sequence analysis, the isolated strain was identified as *Rhodobacter sphaeroides*. A stable improvement in its CoQ_{10} content was achieved by chemical mutation, upon which the content of CoQ_{10} (2.94 mg/g dry cell) was increased by approximately 1.9-fold, comparable to that of *R. sphaeroides* reported in other studies. The isolate is a potentially valuable microorganism for mass production of CoQ_{10}, and may provide an appropriate model for further study of economical mass production.

Key words: Isolation, Photosynthetic bacterium, *Rhodobacter sphaeroides*, Coenzyme Q_{10}

Introduction

Photosynthetic bacteria can use various types of organic matter as carbon and energy sources, and hence they are common microorganisms in the natural environment (Sasaki et al., 1998). These bacteria have been applied to wastewater treatment and bioremediation of sediment mud (Takeno et al., 1999; Nagadomi et al., 2000). Recently, they have also been used in the medical field, as they can produce various types of physiologically active substances, such as vitamin B_{12}, ubiquinones (coenzyme Q_{10}), 5-aminolevulinic acid, porphyrins, and RNA (Sasaki et al., 2005). In particular, coenzyme Q_{10} (CoQ_{10}) and 5-aminolevulinic acid have been prepared and commercialized. Ubiquinones, referred to also as coenzyme Q, are membrane-bound lipid components. They are found commonly in animals, plants, and microorganisms as a coenzyme involved in biological reactions. They play a vital role as an electron carrier in the respiratory chain, and also function as an antioxidant and prooxidant (Ernster and Dallner, 1995; Grant et al., 1997; Wu et al., 2001; James et al., 2004). The number of isoprene units in the prenyl side chain of ubiquinones varies depending on the living organism. CoQ_{10}, 2,3-dimethoxy-5-methyl-benzoquinone with a side chain of ten mono-saturated isoprenoid units, is the only ubiquinone homolog found in human organs (Gale et al., 1961). In humans, CoQ_{10} boosts energy, enhances the immune system, and acts as an antioxidant (Ernster and Dallner, 1995). Recently, CoQ_{10} has been used widely in pharmaceuticals, cosmetics, and food supplements because of its various physiological activities (Takahashi et al., 2003; Sasaki et al., 2005; Zhang et al., 2007).

CoQ_{10} can be produced by chemical (Negishi et al., 2002), semi-chemical (Lipshutz et al., 2002), or biological syntheses. The biological synthesis of CoQ_{10} is now more common than chemical or semi-chemical synthesis, as the chemically produced CoQ_{10} may not be desirable due to the different starting materials used from those of microorganisms and humans (Ha et al., 2007). Therefore, the commercial production of biologically synthesized CoQ_{10} from microorganisms is now attracting more attention (Choi et al., 2005); in fact a genetically engineered microorganism synthesizing CoQ_{10} has been constructed (Lee et al., 2004; Park et al., 2005; Sakai et al., 2005). To date, however, low yields from microbiological production of CoQ_{10} on an industrial scale have resulted in a high cost of CoQ_{10} (Ha et al., 2007). Despite recent accomplishments in metabolic engineering of *Escherichia coli* for CoQ_{10} production, the production levels are not yet competitive with those obtained by isola-

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tion or fermentation (Park et al., 2005). In this study, a photosynthetic bacterium that produced fairly high amounts of CoQ_{10}, was isolated from silt located at the mouth of the Nakdong River in Korea, and an increase in its CoQ_{10} content by chemical mutation was achieved.

Materials and Methods

Isolation of photosynthetic bacteria

To isolate outstanding producers of CoQ_{10}, samples were taken from four different sites in Korea: silt at the Nakdong River, an aquaculture farm at Pukyong National University, the main reactor at a photosynthetic bacteria-producing plant (D-Company, Chuncheon), and the main tank at a wastewater treatment plant (S-Company, Busan). The silt sample (0.5 g) or 0.5-mL liquid samples were added to 5 mL of sterile 0.2% NaCl and agitated to obtain a homogeneous suspension. From each suspension, 1 mL of each suspended liquid was pipetted into 10-mL tubes containing (per liter): 1 g malic acid, 2 g casamino acid, 3 g yeast extract, 1 mL vitamin solution, and 1 mL mineral solution. The vitamin solution contained (per liter): 0.2 g nicotinic acid, 0.4 g thiamine-HCl, 0.2 g nicotinamide, and 0.008 g biotin. The mineral solution contained (per liter): 3 g $FeSO_4 \cdot 7H_2O$, 0.01 g H_3BO_3, 0.01 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g $MnSO_4 \cdot H_2O$, 0.01 g $CuSO_4 \cdot 5H_2O$, 0.01 g $ZnSO_4$, and 0.5 g ethylenediamine tetraacetic acid. The pH of the medium was adjusted to 7.2 before autoclaving at 121°C for 15 min.

The tubes were incubated at 30°C, 180 rpm, and 50 Lux. After 3 days of incubation, cells were spread with a platinum loop on solid agar medium, in which 1.5% nutrient agar was added to the liquid medium. By repeated streaking on fresh agar plates, purified isolates were obtained from the reddish colonies. Each pure isolate was maintained on the agar plate at 4°C and transferred to a fresh agar plate every 2 weeks until needed.

Identification of the isolate

Identification of the isolate was carried out using 16S-rDNA sequence analysis. The DNA was extracted from cells using an AccuPrep® Genomic DNA extraction kit (Bioneer, Korea), according to the manufacturer's instructions. PCR amplification was performed using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The reaction mixture contained 10 pmol/μL of each primer, 2.5 mM dNTPs, 10× reaction buffer, 2.5U *Taq* polymerase (TaKaRa, Japan), and 1 μg DNA template in a final volume of 50 μL. PCR was performed in a DICE model TP600 thermal cycler (TaKaRa, Japan) under the following conditions: initial denaturation at 95°C for 5 min; 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension step at 72°C for 10 min. Aliquots (5 μL) of amplification products were separated using a 1% agarose gel in 0.5% TAE buffer at 100 V for 10 min. Gels were stained with ethidium bromide and photographed under UV light. Gel images were recorded using a digital camera.

The DNA band was excised from the gel and recovered using the AccuPrep® SV Gel and PCR Clean-up System (Promega, USA). The purified products were ligated into pGEM T-easy vector (Invitrogen) and then transformed into *E. coli* DH5α MCR Competent Cells according to the manufacturer's instructions (Promega). Colonies were blue/white screened on LB agar with Ampicillin (Sigma), X-gal (Promega) and isoprophyl-β-D-thiogalactopyranoside (IPTG; Promega). White colonies were chosen randomly, cultivated, and stored in freezing medium at -80°C. The plasmid DNA was extracted using an AccuPrep® Plasmid Extraction Kit (Bioneer, Korea). The plasmid DNA was sequenced using M13 primers (Macrogen, Ltd., Seoul, Korea). These partial sequences were used to search the GenBank database using the Advanced BLAST similarity search option (Altschul et al., 1997) accessible from http://www.ncbi.nlm.nih.gov. BioEdit Sequence Alignment Editor version 5.0.9 (Hall, 1999) was used to check the alignment and remove all positions with gaps before calculating distances with the DNAdist program in PHYLIP (version 3.5c; distributed by J. Felsenstein, University of Washington, Seattle. USA).

Chemical mutation

To increase the content of CoQ_{10} inside the isolated microorganism, chemical mutation was carried out by the following steps. One loopful of cells, grown on an agar plate, was suspended at a concentration of 10^8 to 10^9 cells per mL in 0.5 M Tris-maleate buffer (pH 6.2) containing 2 mg/mL of one of various chemicals (L-ethionine, daunomycin, menadinone, and a mixed solution of 1:1 w/w menadinone and L-ethionine) for 20 min and washed twice with 0.85% saline. Cells were resuspended in Tris-maleate buffer before being inoculated into a 15-mL test tube containing 10 mL of the seed medium. After incubation for 24 hrs with shaking, the cells were harvested by centrifugation, washed with 0.85% saline, and then spread on M-medium containing 5-bromo-1-chloro-3-indolyl-β-D-galactoside (X-gal) (Yoshida et al., 1988). The com-

position of the M-medium was (per liter): 5 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.1 g $MnCl_2 \cdot 4H_2O$, 1 mL trace element solution, 1mL of vitamin mixture, and 20g agar. Colonies that formed a blue pigment on plates containing 20 μg/mL X-gal and 100 g/L glucose were isolated.

Extraction and measurement of CoQ_{10}

The CoQ_{10} extracted from the isolated photosynthetic bacteria was analyzed by the method of Matsumura et al. (1983) and Takahashi et al. (2003), with modifications. Ten grams of cells (wet weight) were grown until the late-logarithmic phase and then suspended in 70 mL of methanol, and the slurry was heated at 55°C for 5 min. Chloroform (140 mL) was added, and the suspension was stirred at 30°C for 20 min and then filtered (Whatman No.1). NaCl solution (0.58%, w/v; 0.2 volumes) was added to the filtrate and mixed gently. The solution was allowed to separate into two phases. The lower phase was evaporated and resuspended with ethanol. CoQ_{10} was analyzed by high-performance liquid chromatography (HPLC; Agilent 1200, USA) on a Zorbax Eclipse Plus C18 column (100 mm×4.6 mm, 5 μm) with ethanol as the mobile phase at a flow rate of 1 mL/min. CoQ_{10} was quantified by an external standard method, based on the peak area, and detected at 275 nm. The intracellular content of CoQ_{10} was estimated by the relationship between the dry-cell weight and the amount of CoQ_{10} in the broth. The dry-cell weight of the bacteria was determined by weighing the cell pellet after drying in an oven at 100°C for 12 hrs. The CoQ_{10} measurement was carried out in triplicate.

Statistical Analyses

Since the sample observations were not arranged in a frequency distribution, the standard deviations were calculated as followings: each deviation was squared, the sum of the squares was divided by n-1, resulting in the sample variance, and finally, extraction of the square root recovered the original scale of measurement. Comparisons of means were performed using the Tukey method (Neter et al., 1985) using the SAS program, since all sample sizes were equal. Differences were considered significant at $P<0.05$.

Results and Discussion

From the four different samples, various types of colonies developed on the surface of the agar plates after 3 days of incubation. From repeated streaking, five different types of reddish colonies were purely isolated. Each strain was cultivated in tube, and its CoQ_{10} content was measured. One strain exhibited a much higher CoQ_{10} content than the other strains (1.55 mg/g dry cell) and was the focus of this study. The isolated strain was a rod, 1-1.5 μm in size and very motile in the vegetative state. Its species-specific identification was derived using 16S-rDNA sequence analysis, where a 1,355-bp fragment of the 16S-rRNA gene was amplified and sequenced (Fig. 1). The BLAST sequence comparison confirmed, with 100% similarity, that the isolated strain was *Rhodobacter sphaeroides* (GenBank Accession Number: AM69671). Among the photosynthetic bacteria, *Rhodopseudomonas*, *Rhodobacter*, and *Rhodospirillum* strains have been reported to produce CoQ_{10} (Urakami and Yoshida, 1993). *Rhodobacter sphae-*

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AGCGGCGGACGGGTGAGTAACGCGTGGGAACGTGCCCTTTGTTTCGGAATAGCCCCGGGA    60
AACTGGGAGTAATACCGAATGTGCCCTACGGGGGAAAGATTTATCGGCAAAGGATCGGCC   120
CGCGTTGGATAATACCGAATGTGGGGTAATGGCCTACCAAGCCGACGATCCATAGCTGGT   180
TTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG   240
CAGTGGGGAATCTTAGACAATGGGCGCAAGTGGGCGCAAGCCATGCCGCGTGATCGATGA   300
AGGCCTTAGGGTTGTAAAGATCTTTCAGGTGGGAAGATAATGACGGTACCACCAGAAGAA   360
GCCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGGGCTAGCGTTATTCGGA   420
ATTACTGGGCGTAAAGCGCACGTAGGCGGATCGGAAAGTCAGAGGTGAAATCCCAGGGCT   480
CAACCCTGGAACTGCCTTTGAAACTCCCGATCTTGAGGTCGAGAGAGGTGAGTGGAATTC   540
CGAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCAC   600
TAACTCGATACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG   660
GTAGTCCACGCCGTAAACGATGAATGCCAGTCGTTCGGGCAGCATGCTGTTCGGTGACAC   720
ACCTAACGGATTAAGCCATTCCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAAGGA   780
ATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAA   840
CCTTACCAACCCTTGACATGGCGATCGCGGTTCCAGAGATGGTTCCTTCAGTTCGGCTGG   900
ATCGCACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTCGGTTAAGTC   960
CGGCAACGAGCGCAACCCACGTCCTTAGTTGCCAGCATTCAGTTGGGCACTCTAGGGAAA  1020
CTGCCGGTGATAAGCCGGAGGAAGGTGTGGATGACGTCAAGTCCTCATGGCCCTTACGGG  1080
TTGGGCTACACACGTGCTACAATGGCAGTGACAATGGGTTAATCCCAAAAAGCTGTCTCA  1140
GTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAATCGCTAGTAATCGCGTAAC  1200
AGCATGACGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAA  1260
TTGGTTCTACCCGAAGGCGGTGCGCCAACCTCGCAAGAGGAGGCAGCCGACCACGGTAGG  1320
ATCAGTGACTGGGGTGAAGTCGTAACAAGGTACCC                           1355
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Fig. 1. Result of the 16S-rDNA base sequence: 1,355 bp from 5'-end.

roides is a facultative microorganism, which can be cultured under many different growth conditions, including photoheterotrophy, photoautotrophy, chemoheterotrophy, and fermentation (Kokua et al., 2003). *R. sphaeroides* has been used preferentially as a bacterium to produce CoQ_{10} (Gu et al., 2006). Thus, the characteristics of the isolated strain warranted further study to produce high levels of CoQ_{10}.

Chemical mutations were attempted with the isolate, since it has been reported that mutants, which could overcome growth inhibition by chemicals during ubiquinone biosynthesis or its related metabolisms, might overproduce CoQ_{10} (Yoshida et al., 1998). Chemicals used in the mutation experiment were L-ethionine (an analog of L-methionine, which is a precursor for the methoxy moiety of coenzyme Q), daunomycine, and menadinone (vitamin K_3, which is a structural analog of ubiquinone). All mutant strains formed blue colonies on the M-medium (Table 1). A menadinone-resistant mutant was superior to its parent cell for CoQ_{10} production. The content of CoQ_{10} (2.94 mg/g dry cell) was stably increased by approximately 1.9-fold by the chemical mutation. The values of CoQ_{10} content were not significantly different in the three replicates, indicating that the mutant was reproduced stably by the chemical mutation. Yoshida et al. (1998) succeeded in finding a mutant producing a higher level of CoQ_{10} than the parent cell with the *Agrobacterium* strain, but did not succeed with the *R. sphaeroides* strain. Instead, they isolated mutants forming green colonies on a bouillon agar medium containing 0.5% meat extract, 0.5% peptone, 0.5% NaCl, and 2% agar (pH 7.2). In our study, CoQ_{10} production was improved by chemical mutation, and the mutant strains easily formed green colonies on the bouillon agar medium. The green color of the mutant may be due to the expression of the green-colored bacteriochlorophyll originally present in the wild-type parent strain and the loss of the red-colored carotenoid (Pfenning, 1967). Chemical mutation caused an increase in CoQ_{10} content in this study. However, Yoshida et al. (1998) reported that high production of CoQ_{10} was not always reproduced in subsequently repeated experiments. In contrast, Urakami and Hori-Okubo (1988) reported that the production of ubiquinone in *Protomonas extorquens* was increased considerably by repeated mutagenesis. In our study, the repeated chemical mutation did not cause any further significant increase in CoQ_{10} production.

The CoQ_{10} content of the isolate, *R. sphaeroides*, was much higher than that (0.76 mg/g cell) of the same strain isolated from mud sediment at an oyster farm (Takeno et al., 1999). The CoQ_{10} content of the isolate was also comparable to those of *R. sphaeroides* reported in other studies. Yoshida et al. (1998) screened three strains, *Agrobacterium tumefaciens*, *Paracoccus denitrificans*, and *R. sphaeroides*, as excellent producers of CoQ_{10} among 34 strains, and highest content was obtained from *R. sphaeroides* (2.4 mg/g-dry cell weight). Urakami and Yoshida (1993) reported a high yield of CoQ_{10} (2.5 mg/g-cell) under limited supply of oxygen in a culture of *R. sphaeroides*. For commercial scale production of CoQ_{10}, an increased content in bacterial cells is essential. From the viewpoint of industrial production, the isolated *R. sphaeroides* appears to be a valuable microorganism for the mass production of CoQ_{10}.

Table 1. Coenzyme Q_{10} content of each mutant in 10-mL tube culture. Means with different superscript are significantly different ($P<0.05$). Values represent mean ± SD of three replicates.

Type of cell		Coenzyme Q_{10} (mg/g dry cell)
Parent cell		1.55 ± 0.10^{d}
Mutants by chemical treatment	menadinone	2.94 ± 0.08^{a}
	L-ethionine	1.76 ± 0.11^{cd}
	daunomycin	1.90 ± 0.10^{bc}
	menadinone + L-ethionine (1:1 basis)	2.10 ± 0.12^{b}

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