AAS and ICP-AES Analysis of the Iron-sulfur Cluster in YojG (NapF) Protein of *aeg-46.5* Operon in *Escherichia coli*

Hyo Ryung Kim, Yong Chan Lee, Jae Seon Won, and Mu Hyeon Choe[*]

College of Life Science and Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea
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The *E. coli aeg-46.5* region was identified by anaerobically expressed *lacZ* fusions.[1,3] In the course of the *E. coli* genome-sequencing project, Richterich *et al.*[4] identified a chromosomal gene cluster in the centisome 49 region that contains several open reading frames (ORFs) with strong similarity to the cytochrome c biosynthesis genes of *Bradyrhizobium japonicum* and *Rhodobacter capsulatus*.[5] These ORFs (*yej WVUTSRQP*) appear to represent the 3'-half of the putative *aeg-46.5* operon, whose expression was shown to be induced under anaerobic growth conditions in the presence of nitrate and nitrite and it was proposed to call them as *ccmABCDEFGH* operon by J. Grove *et al.*[6,7] (Fig. 1).

The 5'-half of the *aeg-46.5* cluster contains additional seven genes: (*yojGFCB/A*). These genes include four homologs of *napABCD* (nitrate reductase in periplasm) that have been identified in *Thiosphaera pantotropha*[8] and *Alcaligenes eutrophus*,[7,9] and it was suggested to call them *napFDAGHBC* operon.[6] A *napE*-like gene (found in *T. pantotropha* and *A. eutrophus*) is not present in the *E. coli* cluster. The remaining three genes (*napF, G and H*) resemble iron-sulfur proteins, and the genetic study using mutant strains showed that none of them are essential for the nitrate reduction, leaving their physiological function unknown.[9] Only their sequence homologies to iron-sulfur protein suggest the possibility of involving in electron transfer to the periplasmic nitrate reductase.

The *E. coli yojG (napF)* gene located immediately down-stream of *aeg-46.5* (*napFDAGHBC*) promoter is predicted to encode an iron-sulfur protein.[6] Iron sulfur proteins are a second major family of electron carrier besides cytochromes. These proteins were shown to contain complexes of iron and cysteinate (Cys) sulfur atoms and to incorporate inorganic or acid-labile sulfur in the form of two-and four iron clusters. They are ubiquitous in living matter and contain sites with one or eight iron atoms, sometimes with multiple occurrences of the smaller clusters in the same protein molecule.[11]

In this study, the *yojG* gene from #372 of Kohara library was cloned into pMAL-c2 vector to prepare the YojG protein and analyze the iron-sulfur contents of the protein with sensitive chemical detection method. The plasmid was named as pCH3. The DNA region of *yojG* nucleotides located on the 1.2 kb BamHI-HindIII fragment of pCH3 was sequenced, and verified to contain the published *yojG* nucleotide sequence. The *yojG* gene encodes a polypeptide of 165 amino acids (*Mr* 18,000). This calculated molecular weight agrees well with the size of the purified and factor Xa cleaved YojG (18 kDa) protein band as determined by SDS-PAGE (Fig. 2). Sequence comparison revealed local identities between the YojG protein and ferredoxins in other bacteria, suggested by Richterich *et al.*.[4,12-15] From these sequence comparison it is very well suggested that the YojG might be an Fe-S protein.

In order to facilitate purification of the YojG protein, we fused the *yojG* gene to the malE (MBP) gene of plasmid pMAL-c2. This resulted in IPTG-inducible expression of a
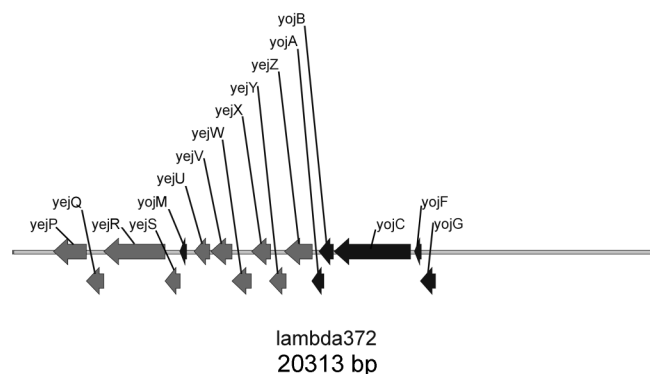
Figure 1. Physical map of *E. coli aeg-46.5* operon.
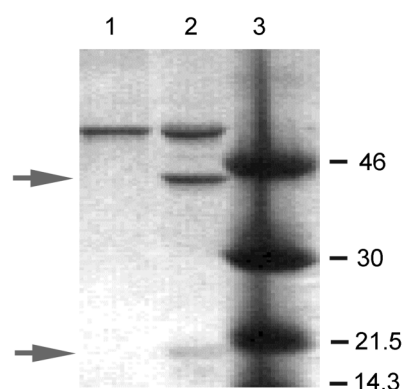
Figure 2. SDS-PAGE stained with Coomassie blue showing the YojG protein and MBP after incubation with factor Xa. Lane 1: purified MBP-YojG fusion protein. Lane 2: purified protein after factor Xa cleavage. The MBP and YojG protein migrate at 43 kDa and 18 kDa, respectively, relative to the markers as indicated by the arrows. Lane 3: molecular weight markers as indicated in kDa.

[*]Corresponding author. e-mail: choemh@korea.ac.kr

MBP-YojG fusion protein. The MBP-YojG protein was found in the supernatant of crude cell extract, indicating that the fusion protein is soluble. This fusion protein was purified through amylose affinity chromatography, and the second anion-exchange chromatography followed by size-exclusion Macro-Prep SE 1000/40 gel chromatography and eluted as a single symmetrical peak at appropriate molecular weight, indicating that this fusion protein is monomeric in solution. Upon gel electrophoresis, the resulting fraction was found to be homogeneous. The measured molecular weight of the MBP-YojG protein determined by SDS-PAGE was 61 kDa.

Initially to facilitate purification of the YojG protein, this protein was overproduced in *E. coli* XL1-Blue as a protein in which a $6 \times$ His tag was attached to the N-terminus of the subunit by cloning the *yojG* gene into vector pQE30 (Qiagen, Hilden, Germany), yielding pCH1. However, we have so far been unable to isolate the soluble His-tagged protein. Since denaturation with urea or guanidine·HCl will liberate S^{2-} from iron-sulfur protein,[16] we had tried to solubilize the protein by expressing with denaturants or detergents, but with no success.[17-19]

The Glutathione S-transferase (GST) gene fusion system was also tried with pGEX-KG vector for the expression and purification of the YojG protein, pCH2, as a soluble protein in *E. coli,* but the GST fusion protein was also insoluble regardless of growth conditions(data not shown). Theses indicate that the YojG protein is very hydrophobic.

The MBP-YojG fusion protein was incubated with factor Xa to separate the YojG protein from MBP after affinity purification. Factor Xa cleaves after its four amino acid recognition sequence (IEGR sequences)[20] resulted in proteolysis of 61 kDa with the appearance of an appropriate 43 kDa MBP, and 18 kDa YojG fragments on SDS-PAGE (Fig. 2). Increasing the temperature and incubation time, and adding low concentrations of detergent proteolyzed the fusion protein extensively (data not shown). Unfortunately, releasing the YojG protein from the MBP-YojG fusion protein caused precipitation of the YojG protein, and only MBP, factor Xa and small amount of uncleaved fusion protein were separated from the Q-cartridge (Bio-Rad Lab.) gradient (data not shown). No further purification steps to isolate the YojG protein could be performed. This indicates that the YojG protein is a very hydrophobic protein that has very low solubility in aqueous phase and suggests that YojG might be a membrane-associated protein. This result is consistent with that most of the iron sulfur proteins are transmembrane proteins that are insoluble in aqueous solutions. In fact, the YojG protein has 37% of hydrophobic amino acids out of total amino acids such as Ala, Ile, Leu, Met, Phe, Pro, Trp, and Val. While it is tempting to speculate that YojG is involved in electron transfer to nitrate reductase, His-tagged YojG, GST-YojG or cleaved YojG from MBP was, respectively, insoluble or precipitated, making any direct functional analogy to nitrate reductase difficult.

Figure 3 shows the uv-vis absorption spectrum of purified MBP-YojG fusion protein. The absorption spectrum of purified MBP-YojG shows maximum at 470 nm while the
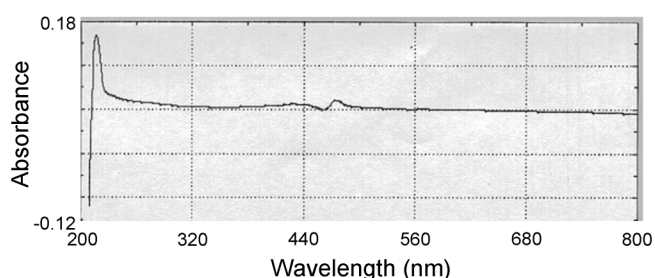

Figure 3. Uv-vis absorption spectrum of purified *E. coli* MBP-YojG fusion protein in 20 mM Tris·Cl, pH 7.4, 0.2 mM NaCl, and 10 mM maltose. The absorption spectrum of this protein was measured with a Beckman DU-Spectrophotometer.

purified control MBP protein (negative control) gave no absorption in the same range. The absorption at 420 nm is known closely correlated to the iron content.[21,22] The small absorption intensity at 420 nm suggests the YojG can be an iron containing protein.

Atomic Absorption Flame Emission Spectrophotometer (AAS) and Inductively Coupled Plasma-Atomic Emission Spectrophotometer (ICP-AES) analysis[23] gave the result that the MBP-YojG protein contains 14 g-atoms of iron and 18 g-atoms of acid labile sulfur per mole of protein. The ratio of iron to labile sulfur in YojG was calculated to be 1 : 1. The iron contents of the iron-sulfur protein from two different cultures with or without $FeSO_4 \cdot 7H_2O$ were the same, suggesting that the iron content of the iron-sulfur protein was not affected by additional iron supplements in growth medium.

For the measurement of the sulfur contents, the appropriate ratios between the maxima at 670 nm and 750 nm and the minimum at 710 nm will assure that what is measured at 670 nm is essentially methylene blue produced by the assay reaction as described by Beinert.[16] The ratio of $A_{670} : A_{750} : A_{710}$ was 3 : 2 : 1, which was appropriate for the measurement.

The YojG protein was overproduced using MBP-YojG expression system. It appears then that due to the over-
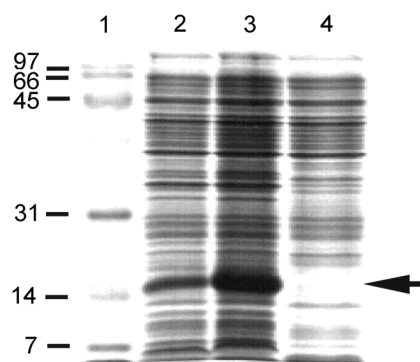
Figure 4. 13% SDS-PAGE stained with Coomassie blue showing the histidine-tagged YojG protein as an insoluble matter. Lane 1: molecular weight markers as indicated in kDa. Lane 2: whole cells before induction of overexpression. Lane 3: whole cells after induction of overexpression. Lane 4: crude cell extract. The his-tagged YojG protein is indicated by the arrow.

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production not all proteins could get the iron-sulfur cluster. Without the iron-sulfur cluster, proteins barely fold correctly so that the hydrophobic regions of the protein might be exposed, causing the protein aggregated in solution. It is consistent with the results of His-tagged insoluble protein (Fig. 4) and aggregated YojG protein from soluble MBP fusion after cleavage with factor Xa. The amount of iron in the protein might be increased by more rigorously applying anaerobic conditions during protein preparation,[21] but the best preparation obtained so far still only contained 14 g-atoms of iron and 18 g-atoms of sulfur/protein.

If this study the iron and acid-labile sulfur content of MBP-YojG was determined and the ratio of iron to sulfur in YojG was calculated to be 1 : 1. This is the first experimental report that prepared the protein product of the gene *yojG* (*napF*) and showed the YojG contains Fe and S. We can have perspectives with this protein preparation and experimental result to further study the function of YojG as an electron transfer agent.

Experimental Section

The bacterial strains, phages, and plasmids used in this study, and physical map of *aeg-46.5* operon are previously described.[6,24,25]

E. coli yojG gene was amplified by PCR. Design of the primers for the PCR was based on the published *yojG* nucleotide sequences. Amplification of this gene was carried out using the N-terminal primer, 5'-GAGAG GATCC GTGAA GATTG ATGCA-3', and the complementary C-terminal primer, 5'-AGAGA AGCTT TTAGT GTGCA TGGAG-3'. These primers contained BamHI and HindIII restriction sites, respectively. The purified *yojG* PCR product was combined with vector pMAL-c2, double-digested with BamHI and HindIII, and ligated.

All protein purification steps were performed under air at 4 °C. One-liter batch of LB containing 100 μg/mL of ampicillin and 0.2% glucose were inoculated with 10 mL overnight cultures of the cell and placed in a 37 °C/300 rpm incubator/shaker. Another one-liter batch of LB containing 100 μg/mL of ampicillin, 0.2% glucose, and 10 mg $FeSO_4 \cdot 7H_2O$ were prepared to see if exogenous iron would affect the content of iron in iron-sulfur cluster.[26] When the 1 L cultures reached an OD_{600}~0.5, IPTG to a final concentration of 0.3 mM was added, and incubation was continued at 37 °C/300 rpm. The cells were harvested 2 h after addition of IPTG by centrifugation at 4000 × g for 10 min. For purification of the MBP-YojG fusion protein, the cell pellet from 1 L culture of the induced DH5α [pCH3] was suspended in 50 mL of 20 mM Tris·Cl, pH 7.4 containing 0.2 M NaCl, and then sonicated in short pulses of 15s for 2 min using a Sonic Dismembrator (Fisher Scientific). The resulting cell lysate was centrifuged at 9000 × g for 30 min, and the supernatant was passed directly over a 15 mL of amylose-resin (New England Biolabs Inc.). The MBP-YojG fusion protein was eluted with 20 mM Tris·Cl, pH 7.4 containing 0.2 M NaCl, and 10 mM maltose, and the eluent showed brown color. The

protein was further purified by passage over a DEAE Sepharose CL-6B column (Pharmacia Biotech.) equilibrated with 50 mM Tris·Cl, pH 8.0 and eluted with 0.4 M NaCl. The fractions were pooled and concentrated by centrifugation (3000 × g) in Centiprep-10 concentrator (YMT membrane; Amicon). Aliquots of 0.2 mL were subjected to Macro-Prep SE 1000/40 size exclusion gel (Bio-Rad Lab) at a flow rate of 0.5 mL/min. The purity of the protein at each stage was monitored by SDS-PAGE. Active fractions were combined, and stored at -70 °C until further use. Protein concentration was determined by the Bradford assay with bovine serum albumin (BSA) as protein standards.[25]

The factor Xa cleavage was carried out in 50 mM Tris·Cl, pH 7.5, 150 mM NaCl, and 1 mM $CaCl_2$. The reaction mixture was incubated for 8 h to several days at room temperature or 4 °C. The cleavage conditions were tuned by adjusting the amount of factor Xa within 0.1-5.0%, changing buffer components, and salt concentrations, and adding detergents (0.01-0.05%), and an acceptable rate of cleavage[28] was obtained. Complete cleavage was monitored by SDS-PAGE.

For the spectrophotometric measurement the fusion protein was dissolved in 20 mM Tris·Cl, pH 7.4, 0.2 mM NaCl, and 10 mM maltose. Spectrum was recorded with a Beckman DU-650 spectrophotometer.

The iron contents of MBP-YojG fusion proteins from 1liter LB culture with or without $FeSO_4 \cdot 7H_2O$ were quantitated by Atomic Absorption Flame Emission Spectrophotometer (AAS, Shimadzu Inc.), and Inductively Coupled Plasma-Atomic Emission Spectrophotometer (ICP-AES). The iron contents of the samples were calculated from a calibration curve of external standards according to peak height.

The amount of acid-labile sulfur was determined as described by Beinert.[16] Samples were incubated with the alkaline-zinc reagent for 1 h prior to addition of *N,N*-dimethyl-ρ-phenylenediamine (DMPD) and ferric chloride ($FeCl_3$). Following addition of DMPD and $FeCl_3$. They were centrifuged for 15 to 20 min until protein is packed, and transferred to cuvette and read at 670 nm, 710 nm, and 750 nm after 1 h at 25 °C.

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