論 文 の 内 容 の 要 旨

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論文題目

Energy Metabolisms of *Hydrogenobacter thermophilus*
(*Hydrogenobacter thermophilus* のエネルギー代謝)

Introduction

Oxygen is primary element that is indispensable for life to constitute its body, as well as carbon, hydrogen, and nitrogen, and is the most abundant oxidant in a surface of the earth. The relationship between molecular oxygen (O_2) and life is interesting. Organisms efficiently obtain energy by aerobic respiration of O_2. On the other hand, reactive oxygen species (ROS), byproducts of the respiration, have high reactivity towards cellular molecules leading to irreversible damage and even cell death. In this way, the relationship with oxygen is always associated with both benefits and risks, although numerous organisms today cope well with oxygen. Then, how have the organisms handled the risks derived from ROS? Small amount of O_2 is presumed to exist in a surface of the primitive earth. The pollution of ROS derived from gradually-increased O_2 might be menace for ancient organisms.

The common strategy for respiration and oxidative-stress defense is the utilization of electron. O_2 is reduced with four electrons at the terminus of the respiratory chain to produce H_2O, while ROS can be detoxified by reduction catalyzed by oxidative-stress defense enzymes. Therefore, to investigate the electron utilization of the aerobic organism leads to know how the organism deals with oxygen. In this study, elucidation of the relationship between oxygen, electron, and life was primarily intended, through the investigation of energy metabolisms of an evolutionary-ancient bacterium *Hydrogenobacter thermophilus*.

Chapter 1. Electron acquisition

In Chapter 1, electron acquisition manner of *H. thermophilus* was investigated. This bacterium is chemolithoautotroph that utilize not only hydrogen (H_2) but also thiosulfate as a sole source of energy and assimilates carbon dioxide *via* the reductive tricarboxylic acid (RTCA) cycle. Here, transcriptome analysis of metabolic enzymes in both H_2- and thiosulfate-grown *H. thermophilus* cells was carried out.

The results indicated that the expression of hydrogenase genes is significantly repressed under thiosulfate-oxidation conditions, whereas some genes for sulfur metabolisms, including sox genes, showed almost the same expression levels under both H_2- and thiosulfate-oxidation conditions. In addition, the genes for the RTCA cycle and several central metabolic pathways showed high expression levels under both conditions. It was suggested that such central metabolisms and sulfur metabolism function as forms of basal metabolism and H_2-oxidation is inducible. Utilization of sulfur compounds may be advantageous for *H. thermophilus* to survive in nature, as the habitat of this bacterium is hot spring in which abundant sulfur compounds are available.

Chapter 2. Electron carriers

In Chapter 2, ferredoxin (Fd) and its redox partner are focused. Fd is a vital electron carrier protein in this bacterium, as it is an electron donor for several key enzymes catalyzing the reactions of the RTCA cycle. Further, in recent years a novel Fd1-dependent type glutamine:2-oxoglutarate amidotransferase was found from this bacterium. Hence, Fd appears to be an important hub for the electron flow in *H. thermophilus*, and novel Fd1-related enzymes are expected to be identified. By detecting protein-protein interactions (PPI), Fd-related proteins were screened.

Although many approaches to detect PPI have been developed in the last decade, majority of them are not suitable to screen Fd-related proteins, because almost all of them are meant to detect strong PPI, whereas Fd and its redox partner are predicted to interact weakly. To identify the proteins interacting with Fd weakly, I developed a novel method by modifying far-western blotting technique. In the developed method, Fd and Fd-interacting protein are covalently cross-linked in order to increase detectivity for Fd-interacting proteins. The effectiveness of the developed method to detect weak interactions was confirmed by the detection of the enzymes reported to be Fd-dependent. Finally, the screening of Fd-related proteins using the developed method was carried out, and resulted in the identification of bacterioferritin comigratory protein (BCP) as a candidate for Fd-interacting protein. BCP has been reported to function as a peroxidase that is a member of oxidative-stress defense enzymes. It was suggested that electrons from Fd may be utilized in the oxidative-stress defense system of *H. thermophilus*, indicating the importance of the defense system.

Chapter 3. Electron-mediated relationships between oxygen and *H. thermophilus*

In Chapter 3, aerobic respiration and oxidative-stress defense systems are focused.

Respiration

3-1. Respiration

Four genes for heme/copper type cytochrome *c* oxidases (*cox1*, *cox2*, *cox3*, and *cyo*), which catalyze the four-electron reduction of O_2 at the terminus of respiratory chain, were found from *H. thermophilus* genome. Transcriptome analyses in the past showed different expression patterns for each enzyme gene, suggesting the appropriate usage depending on the environments.

In order to investigate the function of the oxidases *in vivo*, each gene disruptant was tried to be constructed, and three of them (Δ*cox2*, Δ*cox3*, Δ*cyo*) were obtained, although *cox1* disruptant has not been obtained yet. Observation of the growth profiles of the three mutants suggested the roles of each enzyme. Δ*cox2* showed high growth rate compared with wild-type (WT) strain, implying low proton pumping efficiency of Cox2. Maximum optical density at 540 nm of cultures of Δ*cox3* and Δ*cyo* was lower than that of WT, suggesting Cox3 and Cyo may function as high affinity enzymes that can be mainly used in low O_2 concentration conditions such as stationary growth phase.

Cytochrome *c* oxidases have been proposed to be classified into three groups (Type A, B, and C) according to the phylogeny, and to conserved amino acids in the conserved domains. To our surprise, phylogenetic analysis revealed that Cyo does not belong to any of the three groups, and compose another group in the phylogenetic tree with its homologous proteins. Biochemical analysis of Cyo, predicted to be a novel member of respiratory enzyme, is now being carried out.

Oxidative-stress defense systems

3-2. Bacterioferritin comigratory protein

BCP, identified as a candidate for Fd-interacting protein in Chapter 2, is focused. To investigate the function *in vivo*, *bcp* gene defect mutants (Δ*bcp*) was constructed, and cultivated under several conditions. As BCP has been reported to function as a peroxidase, which is a member of ROS detoxifier, the growth profiles under various concentrations of O_2 and peroxide were observed. As a result, Δ*bcp* showed higher sensitivity toward peroxide than WT, implying that BCP functions as antioxidant *in vivo*. BCP was heterologously expressed in *Escherichia coli*, and was purified. Purified BCP exhibited peroxide reductase activity in the presence of dithiothreitol as an electron donor, indicating the function as a thiol peroxidase. Although BCP was predicted to show Fd-dependent peroxidase activity, the activity has not been detected yet.

One to three cysteine (Cys) residues are conserved in the amino acid sequences of BCPs reported so far. In contrast, *H. thermophilus* BCP has four Cys residues. To clarify the roles of these Cys, amino acid substitution variants (C12A, C26A, C48A, and C53A) were constructed. Interestingly, only C48A lost peroxidase activity, although the others remained the activity. Taken together with the high conservativeness of Cys48 among other BCP sequences, at least Cys48 appears to be crucial for the activity. By homology modeling, three-dimensional structure of BCP was predicted. In the modeled structure, locations of Cys12 and Cys26 are predicted to be quite displaced from that of Cys48 and Cys53, thus, Cys12 and Cys26 are presumed to be not involved in the direct peroxide reducing reaction. Several BCPs are reported to reduce peroxides by the redox of intra- or inter-molecular two Cys residues, however, the counterpart of Cys48 has not been revealed in *H. thermophilus* BCP. Unusual peroxidase mechanism may be employed in *H. thermophilus* BCP. Further characterization of BCP is being carried out.

3-3. Ferriperoxin

Rubrerythrin (Rbr) is a non-heme iron protein composed of two distinctive domains and functions as a peroxidase in anaerobic organisms. A novel Rbr-like protein, ferriperoxin (Fpx), was identified in *H. thermophilus* and was found not to possess the rubredoxin-like domain that is present in typical Rbrs. Although this protein is widely distributed among aerobic organisms, its function remains unknown. Fpx exhibited ferredoxin:NADPH oxidoreductase (FNR)-dependent peroxidase activity and reduced both hydrogen peroxide (H_2O_2) and organic hydroperoxides in the presence of NADPH and FNR as electron donors. The calculated K_m and V_{max} values of Fpx for organic hydroperoxides were comparable to that for H_2O_2, demonstrating a multiple reactivity of Fpx towards hydroperoxides. An *fpx* gene disruptant was unable to grow under aerobic conditions, whereas its growth profiles were comparable to those of the wild-type strain under anaerobic and microaerobic conditions, which clearly shows the indispensability of Fpx as an antioxidant of *H. thermophilus* in aerobic environments. Structural analysis suggested that domain-swapping occurs in Fpx, and this domain-swapped structure is well conserved among thermophiles, implying the importance of structural stability of domain-swapped conformation for thermal environments.

In addition, Fpx was located on a deep branch of the phylogenetic tree constructed with the sequences of Rbr and Rbr-like proteins. This finding, together with the wide distribution of Fpx among Bacteria and Archaea, suggests that Fpx is an ancestral type of Rbr homolog that functions as an essential antioxidant and may be part of an ancestral peroxide-detoxification system.

3-4. Physiological partner of ferriperoxin

Since the gene for FNR was not found in genomes of some of organisms possessing *fpx* gene, FNR may not be a physiological partner of Fpx. By comparing the genomes, approximately 13 kb gene was found to be located next to *fpx* gene in many organisms possessing *fpx* gene, although annotations are diverse. In the genome of *H. thermophilus*, the gene (HTH_1527) was quite displaced from the locus of *fpx*. I tried to heterologously express HTH_1527 under various conditions using several plasmids, hosts, additive to the medium, temperatures, however almost all of the proteins formed inclusion bodies, and the appropriate protein has not been obtained.

3-5. Suppressor mutant of *fpx* gene defect mutant

By continually culturing Δ*fpx* under aerobic conditions, suppressor mutant that is capable of growing under aerobic conditions was obtained. To reveal what recovers O_2-sensitive phenotype, transcriptome analysis was carried out. It was demonstrated that expression levels of alkyl hydroperoxide reductase gene (*ahpC*) in suppressor mutant cells was 18-fold higher than that in WT strain cells under aerobic conditions. AhpC is reported to function as a peroxidase, thus, large expression of *ahpC* gene may compensate the lack of Fpx. To clarify the trigger of high expression of *ahpC*, whole genome mutation analysis was carried out, although significant mutation was not found.

Conclusion

This study elucidated a part of energy metabolism of *H. thermophilus*, including several new insights into ROS detoxification systems and respiratory enzymes. The manner of acquisition and consumption of electrons in this bacterium implies the “conservative” relationship of *H. thermophilus* with O_2, namely this bacterium favors safety more than efficiency. *H. thermophilus* appears to obtain electrons in a reliable manner, and to keep cells highly reduced using a series of antioxidant enzymes by consuming abundant reducing power. Even respiration can be considered as a system to reductively detoxify molecular oxygen. Recently, one of hydrogenase of this bacterium was clarified to be necessary to survive under high O_2 conditions nevertheless it is not ROS detoxifier. Interestingly, that hydrogenase, Fpx and Cyo are absent in the genome of *Aquifex aeolicus*, which is a closely related species of *H. thermophilus* but is O_2-sensitive. Like these, *H. thermophilus* might have evolved against aerobic environments through obtaining such enzymatic equipments to handle oxidative-stress. Further, like *H. thermophilus*, numerous aerobic organisms living in the earth today might have evolved through continual struggle against oxygen, and finally obtained sophisticated mechanisms to get the most benefit from oxygen utilization.

Publications

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