Journal of Environmental Biotechnology
Vol. 11, No. 1 · 2, 00–00, 2011

Original paper (regular paper)

Analyses of Three Dominant Membrane Proteins from Anammox Planctomycete *Candidatus* 'Brocadia sinica'

Fuyumi Tojo[1], Yoshikane Itoh[1], Satoshi Okabe[2] and Masaaki Morikawa[1]*

[1] *Graduate School of Environmental Science, Hokkaido University, Kita-10, Nishi-5, Kita-ku, Sapporo, Hokkaido 060–0810, Japan*

[2] *Graduate School of Engineering, Hokkaido University, Kita-13, Nishi-8, Kita-ku, Sapporo, Hokkaido 060–8628, Japan*

* TEL/FAX: +81–11–706–2253

* E-mail: morikawa@ees.hokudai.ac.jp

(Received; 27 October, 2011/Accepted; 15 November, 2011)

Anammox planctomycetes commonly possess a unique single membrane-bound organelle, the anammoxosome, that harbors enzymes central to anaerobic ammonia oxidation, *anammox*, metabolism. We found that three membrane proteins, namely P35, P45, or P90, were dominantly produced in *Candidatus* 'Brocadia sinica,' an anammox planctomycete that has been obtained from an effective anammox reactor. N-terminal amino acid sequences suggested that all these three are components of hydrazine hydrolase which is a key enzyme in anammox metabolism. The genes encoding for P35, P45, and P90 formed a cluster preceded by sigma54 specific two-component regulator proteins. Heterologous gene expression of P35, P45 and P90 conferred high biofilm formation ability to the *E. coli* cells, suggesting that they were transported to cell membrane of *E. coli* and the membrane structure was modified to increase cell adhesion activity.

Key words: anammox planctomycete, *Candidatus* 'Brocadia sinica', anammoxosome, hydrazine hydrolase, biofilm

1. Introduction

The phylum planctomycetes of the domain Bacteria consists of unique budding, peptidoglycan-less organisms and possess intracellular membrane compartmentation. planctomycetes consist of eleven genera, six of which are cultured, *Pirellula, Rhodopirellula, Blastopirellula, Planctomyces, Gemmata,* and *Isosphaera,* and five so far uncultured, *Candidatus* 'Anammoxoglobus,' *Candidatus* 'Brocadia,' *Candidatus* 'Jettenia,' *Candidatus* 'Kuenenia,' and *Candidatus* 'Scalindua' [4,5,6,8]. The members in the latter five groups of ammonium-oxidizing chemoautotrophs perform unique anammox, anaerobic ammonia oxidation, metabolism. Anammox metabolism produces a toxic hydrazine as an intermediate that is further converted into dinitrogen by hydrazine oxidoreductase[4]. These uncultured anammox panktomycetes commonly possess a single membrane-bound organelle, the anammoxosome, that harbors enzymes central to the unique anammox metabolism[9,18,19]. The anammoxosome membrane contains ladderane lipids (also unique to anammox planctomycetes), which are composed of concatenated cyclobutane rings and may posess both ester and ether links[4,6]. Molecular modeling indicated that the ladderane lipids surrounding the anammoxosome yield an exceptional rigid biomembrane, implicating that this dense barrier may serve to separate the remainder of the cell from the intermediate hydrazine and to maintain concentration gradients[14]. It has been recently shown that both hydrazine hydrolase (HH) and hydrazine oxidoreductase (HAO) are present inside anammoxosome[6,7,10] in *Candidatus* 'Kuenenia stuttgartiensis'.

Previously, we developed an up-flow, fixed-bed anammox biofilm reactor with non-woven fabric sheets as biomass carriers and attained a high nitrogen removal rate of 26.0 kgN/m^3 day[16,17] and the anammox planctomycetes has recently been identified as *Ca.* 'Brocadia sinica' [2,11]. The half-saturation constants (Ks) for ammonium and nitrite of *Ca.* 'B. sinica' were determined 28±4 and 86±4 μM, respectively, higher than those of *Ca.* 'B. anammoxidans' and *Ca.* 'K. stuttgartiensis' [11]. The ultrastructure of *Ca.* 'B. sinica' was also examined by transmission electron microscopy to confirm the presence of the anammoxosome. Here, we show that the three most abundantly produced membrane proteins in *Ca.* 'B. sinica', P35, P45, and P90, are components of HH. When these protein-encoding genes were expressed in *E. coli*, the cells that produce either P35, P45, or P90 formed more biofilms than non-producing control cells. This is the first report that determines membrane protein profiles in anammox planctomycete, *Ca.* 'B. sinica'.

2. Materials and Methods

2.1. Bacterial strain and culture media

The microbial granules containing *Ca.* 'B. sinica' were grown in an up-flow column reactor with mineral medium for anammox bacteria[18]. Briefly, it contained per liter, $(NH_4)_2SO_4$, 660 mg; $NaNO_2$, 345 mg; $KHCO_3$, 500 mg; KH_2PO_4, 27.2 mg; $MgSO_4 \cdot 7H_2O$, 300 mg; $CaCl_2 \cdot 2H_2O$, 180 mg, and trace element solution. The previous study determined the microbial composition in the granule sample by fluorescence in-situ hybridization with probe AMX820[12], and found that anammox planctomycetes accounted for approximately 90% of the total cells[16,17].

2.2. Preparation and analyses of cytoplasmic and membrane proteins from *Ca*. 'B. sinica'

The anammox granules, 0.5 g, were disrupted by Multi-beads shocker (Yasui Kikai Co.) using 0.1 mm glass beads in 700 µl of 50 mM Tris-Cl (H7.5) containing 5 mM $MgCl_2$. After removing the glass beads by sedimentation, cell suspension was recovered. Cytoplasmic and membrane fractions were separated by ultracentrifuge (4°C, 100,000 *g*, 120 min). Each fraction sample was further prepared in SDS sample buffer (50 mM Tris-HCl pH 6.8, 6% 2-mercaptoethanol, 10% glycerol, 2% SDS and 0.01% bromophenol blue) and boiled for 5 min. Clear supernatants after centrifuge were analyzed by SDS-PAGE using 12% acrylamide gel. Electrophoresis was carried out at 30 V for 16 hr. Protein bands were visualized by Coomassie Brilliant Blue R-250 staining. Major protein bands were blotted to PVDF membrane (Bio-Rad) in order to determine N-terminal amino acid sequence by Procise 492 (PERKIN ELMER) or Procise 491 cLC (Applied Biosystems). The protein sequence was analyzed by protein blast homology search program, http://blast.ncbi.nlm.nih.gov/Blast.cgi.

2.3. Expression of membrane protein genes from *Ca*. 'B. sinica' in *E. coli*

E. coli BL21(DE3) (Invitrogen) was used as the host strain to express membrane protein genes from *Ca.* 'B. sinica.' Recombinant *E. coli* cells were cultured in Lulia-Bertani (LB) medium containing 100 µg/ml ampicillin.

The DNA regions encoding the P35, P45 and P90 were separately amplified by PCR using *Ca.* 'B. sinica' genome DNA as a template, a set of following primers, and KOD plus DNA polymerase (Toyobo). The primer sets were: 5'-CATATGAAGAGTAGTCTAAAGATTGGTTTA-3' (*Nde*I site, underlined) and 5'-GTGGAGTTCCTGAAGGCGCTATAAGAATTC-3' (*Eco*RI site, underlined) for *p35*, 5'-CATATGAAAGAGAGAGGATGGATAGGAG-3' (*Nde*I site, underlined) and 5'-GATAAGGACGCCAGTGGATTAATAGGAATTC-3' (*Eco*RI site, underlined) for *p45,* and 5'-CATATGAGTAAGAGGATAATAGGAGGGG-3' (*Nde*I site, underlined) and 5'-TACCCTGGCTATCTGGCAAGATAAGAATTC-3' (*Eco*RI site, underlined) for *p90*, respectively. The nucleotide sequences of the PCR amplified DNA fragments were confirmed no errors by a genetic analyzer ABI 3130 (Applied Biosystems). Each DNA fragment was cloned into the gap of *Nde*I and *Eco*RI sites of expression vector pET25b (+) to construct pET25b-*p35*, pET25b-*p45*, and pET25b-*p90* and the resultant recombinant *E. coli* cells were examined as follows.

2.4. Biofilm formation and protein production by recombinant *E. coli*

Biofilm formation activity was measured for provisionally examining the change in cell adherence activity due to overexpression of membrane proteins. Overnight shaking cultures were diluted 1 : 100 by fresh LB medium, shaken at 37°C for 2 hr, and then added with 1 mM IPTG (final concentration) for gene induction. The cultures were immediately dispensed to 96-well micro-titer plates and statically grown to form biofilms at 26°C for 24 and 48 hr. This low temperature condition was useful to overproduce hydrophobic membrane protein effectively. Planktonic cell cultures were discarded and the biofilms formed inner-surface of wells were washed with sterilized water. The biofilms were then stained by 0.1% crystal violet and bound crystal violet was extracted by 33% acetic acid. Biofilm formation activity was determined by measuring absorbance of the solution at 595 nm.

Protein production was analyzed by SDS-PAGE using 12% polyacrylamide gel as described elsewhere. The protein sample was prepared from whole cells. The cells were similarly grown as above in LB containing IPTG at 26°C except for using glass test tubes in stead of micro-titer plates.

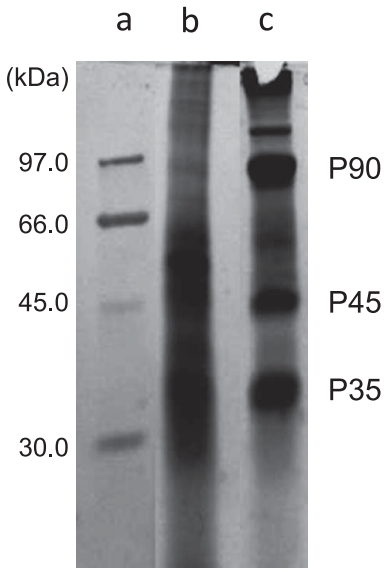


Fig. 1. SDS-PAGE analysis of cytoplasmic and membrane proteins from *Ca.* 'B. sinica.'
a, Size marker; b, cytoplasmic proteins; c, membrane proteins.

3. Results and Discussion

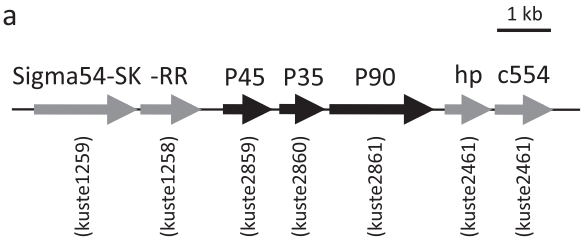
3.1. Identification of major membrane proteins in *Ca.* 'B. sinica'

It was found that three proteins, namely P35, P45 and P90, were dominantly produced in the membrane fraction of *Ca.* 'B. sinica' cells (Fig. 1). N-terminal amino acid sequences of these proteins showed significant similarity with proteins from anammox planctomycetes, including KSU-1 and *Ca.* 'K. stuttgartiensis' (Table 1). We had already determined draft sequence of the whole genome of *Ca.* 'B. sinica' (unpublished data). It was found that the genes encoding for P35, P45, and P90 formed a cluster with a set of possible sigma54 specific two-component transcriptional regulator proteins (Sigma54-SK/RR) in the upstream region (Fig. 2a). Sigma54 (RpoN) was identified as a sigma factor involved in the transcription of genes involved in the cellular assimilation of ammonia and glutamate under conditions of nitrogen limitation[1]. It has been reported that *Ca.* 'K. stuttgartiensis' has similar gene organization of HH (kuste2859–2861) and Sigma54-SK/RR (kuste2857/2858)[15]. However, it is interesting that sigma54-SK/RR in the upstream of P35, P45, P90 from *Ca.* 'B. sinica' is homologous to kuste1259/kuste1258 at different position rather than kuste2857/2858. It may be also worth to note that the genes encoding kuste1259/kuste1258 are orphan genes whose function is yet unknown.

Table 1. N-terminal amino acid sequence identities of P35, P45, and P90 to other anammox planctomycete proteins.

Name	Amino acid sequence	Identity (%)
P90	1 NQVMTGGSKQXKALXT 17	—
KSU-1	29 NQVMVGGSKQGKALWT 44	87
kuste2861	30 --VMTGGPVQGKALWT 43	83
P45	1 GFIQGTHVKTDMPSPFFVT 19	—
kuste2859	35 GYIQGTHVKTDLPGPFHIT 53	74
P35	1 GTPQVVATIQTGPEAW 16	—
KSU-1	28 GTPQVIATIQTGPE-W 42	88
kuste2860	40 GQPRVISTIQTGAT-W 54	56

Note: kuste2859, kuste2860, and kuste2861 are hydrazine hydrolase components from *Ca* 'Kuenenia stuttgartiensis'[7]. KSU-1 denotes respective proteins from an anammox bacterium KSU-1[13].



b

P90: MSKRIIGGVMASALVVGALVCGDIFAS/ NQVMT

P35: MKSSLKIGLIAALGIAGVMTTGELMA/ GTPQV

P45: MKERGWIGALVLGAVMVGGGVANA/ GFIQG

Fig. 2. a. Gene cluster structure of P35, P45, P90 and flanking regions.
Sigma54-SK, sigma54 specific sensor kinase; Sigma54-RR, sigma54 specific response regulator; hp, hypothetical protein; c554, cytochrome c554 homologue.
Homologous protein genes in *Ca.* 'K. stuttgartiensis' are also shown in parentheses.
b. Signal peptide sequence and processing site (/).

The amino acid sequence of P45 (376 a.a.) was 79% identical to kuste2859 (386 a.a.) from *Ca.* 'K. stuttgartiensis,' while P35 (340 a.a.) was 91% identical to a protein from KSU-1 (341 a.a.) and 86% identical to kuste2860 (353 a.a.). P90 (825 a.a.) was 89% identical to protein of KSU-1 (810 a.a.) and 82% identical to kuste2861 (809 a.a.), respectively. It has recently been reported that kuste2859, 2860, and 2861 are anammoxosome proteins and HH components[7]. These information strongly suggested that P35, P45, and P90 are HH that locate in anammoxosome membrane rather than cell membrane of *Ca.* 'B. sinica.' High production levels of these proteins may explain a quite effective nitrogen removal rate of *Ca.* 'B. sinica' at 26.0 kgN/m^3 day.

Anammoxosome membrane is composed of unique ladderane lipids whose structure is quite different from popular bacterial cytoplasmic membrane lipids. We next asked if the P35, P45, and P90 could be overproduced in cytoplasmic membrane in *E. coli*.

3.2. Expression of *p35, p45, and p90* in *E. coli*

Biofilms are structures formed by surface attached microorganisms encased in matrices. Biofilm formation is largely affected by cell surface property including membrane protein profiles[3]. We hypothesized that the *E. coli* cells producing P35, P45, or P90 in the cell membrane would have different biofilm formation ability. It was found that the *E. coli* cells

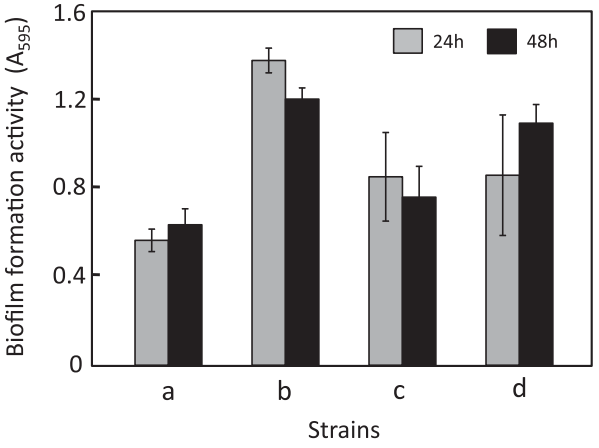


Fig. 3. Biofilm formation activity of recombinant *E. coli* cells.
a, pET25b (+) vector; b, pET25b-*p35*; c, pET25b-*p45*; d, pET25b-*p90* harboring-strains.
Error bars indicate standard deviations in five independent tests.

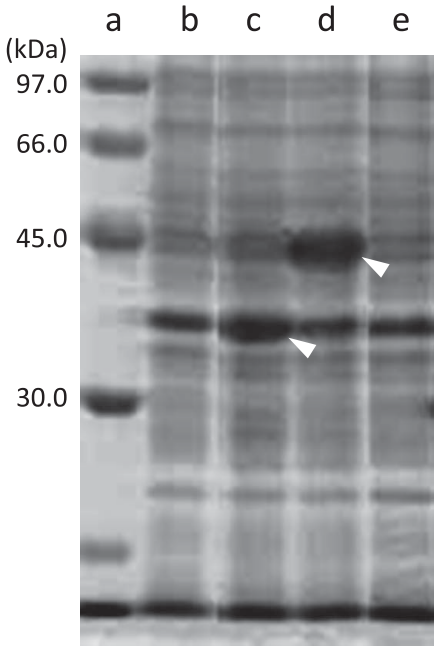


Fig. 4. SDS-PAGE analysis of proteins from recombinant *E. coli* cells.
a, Size marker; b, pET25b (+) vector; c, pET25b-*p35*; d, pET25b-*p45*; e, pET25b-*p90*. Positions of the recombinant P35 and P45 proteins are indicated by arrowheads.

that produced P35, P45, or P90 formed more biofilms than the cells harboring pET25b vector only, although the difference is statistically not so much significant for P45 and P90. (Fig. 3). It may also worth to note that biofilm formation of pET25b-*p35* and pET25b-*p45* harboring-strains were slightly decreased at 48 hr while pET25b-*p90* and pET25b (+) harboring-strains increased or comparable at 48 hr.

Protein production was verified by SDS-PAGE (Fig. 4). It was clear that P35 and P45 were abundantly produced in the cells. On the other hand, only a negligible amount of P90 was produced probably due to its unusual signal-peptide processing site, 1-MSKRIIGGVMASALVVGALVCGDI FAS/NQVMT-32 (Fig. 2b), with low transportation efficiency in *E. coli* cells. It has been previously shown that HH is transported into anammoxosome in *Ca* 'K. stuttgartiensis' [6,7] and our result suggested that P35, P45, P90 (HH) are localized in the membrane fraction of *Ca* 'B. sinica'. When we observed the cells by microscope, none of these three recombinant cells formed inclusion body in the cells. Although we do not have direct evidence that the proteins localized in the membrane fraction of the cells, these results suggest that P35, P45, and P90 were produced and transported to cell membrane of *E. coli* cells that has no anammoxosome organelle. It also remains elucidated whether conformation and topology of these proteins are similar in anammoxosome membrane of *Ca* 'B. sinica' and in cell membrane of *E. coli*.

Acknowledgements

We thank to Dr. Kyoko Miwa and Dr. Mamoru Oshiki for helpful discussion. This work has been supported by New Energy and Industrial Technology Development Organization (NEDO).

References

1) Buck, M., M-T. Gallegos, D. Studholme, Y. Guo, and J.D. Gralla. 2000. The bacterial enhancer-dependent sigma54 (sigmaN) transcription factor. J. Bacteriol. 182: 4129–4136.
2) Cho, S., Y. Takahashi, N. Fujii, Y. Yamada, H. Sato, and S. Okabe. 2010. Nitrogen removal performance and microbial community analysis of an anaerobic up-flow granular bed anammox reactor. Chemosphere 78: 1129–1135.
3) Danese, P.N., L.A. Pratt, S.L. Dove, and R. Kolter. 2000. The outer membrane protein, antigen 43, mediates cell-to-cell interactions within Escherichia coli biofilms. Mol. Microbiol. 37: 424–432.
4) Fuerst, J.A. 2005. Intracellular compartmentation in Planctomycetes. Annu. Rev. Microbiol. 59: 299–328.
5) Fuerst, J.A. 1995. The planctomycetes – emerging models for microbial ecology, evolution and cell biology. Microbiology 141: 1493–1506.
6) Jetten, M.S.M., L. van Niftrik, M. Strous, B. Kartal, J.T. Keltjens, and H.J.M.O. den Camp. 2009. Biochemistry and molecular biology of anammox bacteria. Crit. Rev. Biochem. Mol. Biol. 44: 65–84.
7) Karlsson, R., A. Karlsson, O. Backman, B.R. Johansson, and S. Hulth. 2009. Identification of key proteins involved in the anammox reaction. FEMS Microbiol. Lett. 297: 87–94.
8) Kartal, B., J. Rattray, L.A. van Niftrik, J. van de Vossenberg, M.C. Schmid, R.I. Webb, S. Schouten, J.A. Fuerst, J.S. Damsté, M.S. Jetten, and M. Strous. 2007. Candidatus 'Anammoxoglobus propionicus' a new propionate oxidizing species of anaerobic ammonium oxidizing bacteria. Syst. Appl. Microbiol. 30: 39–49.
9) Kowalchuk, G.A., and J.R. Stephen. 2001. Ammonia-oxidizing bacteria: A model for molecular microbial ecology. Annu. Rev. Microbiol. 55: 485–529.
10) Lindsay, M.R., R.I. Webb, M. Strous, M.S. Jetten, M.K. Butler, R.J. Forde, and J.A. Fuerst. 2001. Cell compartmentalization in planctomycetes: novel types of structural organisation for the bacterial cell. Arch. Microbiol. 175: 413–429.
11) Oshiki, M., M. Shimokawa, N. Fujii, H. Satoh, and S. Okabe. 2011. Physiological characteristics of the anaerobic ammonium-oxidizing bacterium 'Candidatus Brocadia sinica.' Microbiology 157: 1706–1713.
12) Schmid, M., S. Schmitz-Esser, M. Jetten, and M. Wagner. 2001. 16S–23S rDNA intergenic spacer and 23S rDNA of anaerobic ammoniumoxidizing bacteria: implications for phylogeny and in situ detection. Environ. Microbiol. 3: 450–459.
13) Shimamura, M., T. Nishiyama, H. Shigetomo, T. Toyomoto, Y. Kawahara, K. Furukawa, and T. Fujii. 2007. Isolation of a multiheme protein with features of a hydrazine-oxidizing enzyme from an anaerobic ammonium-oxidizing enrichment culture. Appl. Environ. Microbiol. 73: 1065–1072.
14) Sinninghe Damsté, J.S., W.I. Rijpstra, M. Strous, M.S. Jetten, O.R. David, J.A. Geenevasen, and J.H. van Maarseveen. 2004. A mixed ladderane/n-alkyl glycerol diether membrane lipid in an anaerobic ammonium-oxidizing bacterium. Chem. Commun. (Camb). 22: 2590–2591.
15) Strous, M., E. Pelletier, S. Mangenot, T. Rattei, A. Lehner, M.W. Taylor, M. Horn, H. Daims, D. Bartol-Mavel, P. Wincker, V. Barbe, N. Fonknechten, D. Vallenet, B. Segurens, C. Schenowitz-Truong, C. Medigue, A. Collingro, B. Snel, B.E. Dutilh, H.J.M.O. den Camp, C. van der Drift, I. Cirpus, K.T. van de

Pas-Schoonen, H.R. Harhang, L. van Niftrik, M. Schmid, J. Keltjens, J. van de Vossenberg, B. Kartal, H. Meier, D. Frishman, M.A. Huynen, H.W. Mewes, J. Weissenbach, M.S.M. Jetten, M. Wagner, and D.L. Paslier. 2006. Deciphering the evolution and metabolism of an anammox bacterium from a community genome. Nature 440: 790–794.

16) Tsushima, I., Y. Ogasawara, T. Kindaichi, H. Satoh, and S. Okabe. 2007. Development of high-rate anaerobic ammonium-oxidizing (anammox) biofilm reactors. Water Res. 41: 1623–1634.

17) Tsushima, I., Y. Ogasawara, M. Shimokawa, T. Kindaichi, and S. Okabe. 2007. Development of a super high-rate anammox reactor and in situ analysis of biofilm structure and function. Water Sci. Technol. 55: 9–17.

18) van Niftrik, L., W.J. Geerts, E.G. van Donselaar, B.M. Humbel, A. Yakushevska, A.J. Verkleij, M.S. Jetten, and M. Strous. 2008. Combined structural and chemical analysis of the anammoxosome: a membrane-bounded intracytoplasmic compartment in anammox bacteria. J. Struct. Biol. 161: 401–410.

19) van Niftrik, L.A., J.A. Fuerst, J.S. Sinninghe Damste, J.G. Kuenen, M.S. Jetten, and M. Strous. 2004. The anammoxosome: an intracytoplasmic compartment in anammox bacteria. FEMS Microbiol. Lett. 233: 7–13.