Identification of photoactivated adenylyl cyclases in \textit{Naegleria australiensis} and BLUF-containing protein in \textit{Naegleria fowleri}

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Complete genome sequencing of \textit{Naegleria gruberi} has revealed that the organism encodes polypeptides similar to photoactivated adenylyl cyclases (PACs). Screening in the \textit{N. australiensis} genome showed that the organism also encodes polypeptides similar to PACs. Each of the \textit{Naegleria} proteins consists of a “sensors of blue-light using FAD” domain (BLUF domain) and an adenylyl cyclase domain (AC domain). PAC activity of the \textit{Naegleria} proteins was assayed by comparing sensitivities of \textit{Escherichia coli} cells heterologously expressing the proteins to antibiotics in a dark condition and a blue light-irradiated condition. Antibiotics used in the assays were fosfomycin and fosmidomycin. \textit{E. coli} cells expressing the \textit{Naegleria} proteins showed increased fosfomycin sensitivity and fosmidomycin sensitivity when incubated under blue light, indicating that the proteins functioned as PACs in the bacterial cells. Analysis of the \textit{N. fowleri} genome revealed that the organism encodes a protein bearing an amino acid sequence similar to that of BLUF. A plasmid expressing a chimeric protein consisting of the BLUF-like sequence found in \textit{N. fowleri} and the adenylyl cyclase domain of \textit{N. gruberi} PAC was constructed to determine whether the BLUF-like sequence functioned as a sensor of blue light. \textit{E. coli} cells expressing a chimeric protein showed increased fosfomycin sensitivity and fosmidomycin sensitivity when incubated under blue light. These experimental results indicated that the sequence similar to the BLUF domain found in \textit{N. fowleri} functioned as a sensor of blue light.

Key Words——BLUF; \textit{Naegleria australiensis}; \textit{Naegleria fowleri}; PAC
phillipinensis) and non-pathogenic strains such as N. gruberi. PAM is a fulminating rapidly fatal disease. N. fowleri amoebae are thought to infect humans swimming in lakes and rivers by entering the nose, and the amoebae migrate to the brain and destroy tissue. To prevent infection, rapid detection and estimation of the number of N. fowleri amoebae in lakes and rivers as well as analysis of their vertical distribution and seasonal population in bodies of freshwater are required.

Whole genome sequencing revealed that non-pathogenic N. gruberi encodes proteins showing sequence similarity to photoactivated adenyl cyclases (PACs) (Fritz-Laylin et al., 2010). PACs are blue-light receptor flavoproteins bearing a “sensors of blue-light using FAD” domain (BLUF domain) and an adenyl cyclase domain (AC domain). The first PACs were identified from the unicellular flagellate Euglena gracilis (PACalpha and PACbeta) (Iseki et al., 2002). Results obtained by using the RNA-mediated interference (RNAi) technique showed that E. gracilis PACs were responsible for the step-up photophobic response (photoavoidance) of the organism. After the discovery of E. gracilic PACs, orthologs have been found in euglenoids (Koumura et al., 2004) and the gamma proteobacterium Beggiatoa sp. (Ryu et al., 2010; Stierl et al., 2011). The discovery of proteins similar to PACs in N. gruberi suggests that pathogenic and potentially pathogenic Naegleria strains also encode PAC-like proteins or BLUF-bearing proteins and that the proteins play important roles in the photoresponses of the organisms.

At present, it is not known whether pathogenic N. fowleri amoebae exhibit photoresponses. The influence of light on distribution of the amoebae in a freshwater body is also not known. Identification and characterization of proteins responsible for photoresponses in N. fowleri might contribute to the prevention of infection. For example, N. fowleri amoebae could be collected and trapped by irradiating a small area of lakes and rivers with light that would attract the pathogenic amoebae.

In order to determine whether N. fowleri encodes PACs or BLUF-bearing proteins, we screened the N. fowleri genome for candidate genes. We also screened the genome of a potentially pathogenic strain of Naegleria, N. australiensis, for candidate genes.

Materials and Methods

Naegleria and bacterial strains. N. australiensis PP397, N. fowleri kurume, N. fowleri KUL and N. fowleri NF66 were cultured in SCGYE medium (De Jonckheere, 1977). E. coli strain HB101 (recA13, ara-14, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1, supE44, hsdS20) was cultured in LB medium.

Screening of N. gruberi genes in a database. Coding sequences for N. gruberi proteins, NgPAC1 and NgPAC2, were found in Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.kegg.jp/kegg/).

Preparation of whole genome amplified (WGA) DNA of N. australiensis. Genomic DNA was purified from N. australiensis amoebae and amplified by using a REPLI-g mini kit (Qiagen, CA, USA) according to the instruction manual. The WGA DNA samples were used only for inverse PCR as described below.

PCR, inverse PCR and sequencing of N. australiensis genes. Primers used are listed in Table 1. For sequencing of conserved regions in Naegleria PACs (regions between BLUF and AC domains), the regions were amplified by PCR using KOD FX Neo DNA polymerase (Toyobo, Osaka, Japan). PCR products were cloned on pT7Blue-2 and sequenced. For amplification of upstream and downstream sequences of the conserved regions, WGA DNA samples were digested by EcoRI or NsiI, and each of the restriction fragments was self-ligated by T4 DNA ligase and then subjected to inverse PCR. The DNA polymerase used in the inverse PCR was LA Taq DNA polymerase (Takara Bio, Shiga, Japan). The reaction products were cloned on pMD20 (Takara Bio) and sequenced. For re-sequencing, coding sequences were amplified from genomic DNA by KOD FX Neo DNA polymerase, cloned on pT7Blue-2, and sequenced.

Analysis of N. fowleri gene. The nucleotide sequence of the N. fowleri kurume genome was analyzed by HiSeq 2000 (Illumina, CA, USA). For re-sequencing of the gene for BLUF-bearing protein, coding sequence was amplified from genomic DNA by KOD FX Neo DNA polymerase, cloned on pT7Blue-2, and sequenced. For sequencing of the orthologs in N. fowleri KUL and NF66, coding sequences were amplified from genomic DNA samples by KOD FX Neo DNA polymerase, cloned on pT7Blue-2, and sequenced. Primers used are listed in Table 1.

Plasmids for bioassays. Coding sequences for NaPAC1 (Met1–Asn364) and NaPAC2 (Met1–Asn355) were
BLUF domain (Met^{34}–Ser^{137}) and N. gruberi AC domain (Ser^{115}–Met^{390} of NgPAC1) was synthesized and inserted into Ndel-Xbal of pColdI. The resultant plasmid was designated pCMR1. For a plasmid expressing a site-directed mutant of CMR1 in which Tyr^{39} was replaced by Trp (CMR1Y^{39}W), the coding sequence for the protein was synthesized and inserted into Ndel-Xbal of pColdI. The resultant plasmid was named pCMR1Y^{39}W.

**Antibiotic sensitivity assays.** *E. coli* cells were transformed by the plasmids constructed and spread on Mueller-Hinton agar (Nissui Pharmaceutical, Tokyo, Japan), and then a paper disk containing 10 μg fosfomycin and a paper disk containing 5 μg fosmidomycin were placed on the plate. The cells were incubated for 16 h with or without blue-light irradiation (470 nm). Irradiation with blue light was performed at 5 μmol/m^2s using an LED unit LC-LED470B (Taitec, Tokyo, Japan). After incubation, the diameters of the inhibition zones formed on the bacterial lawns were measured.

### Table 1. Primers used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′→3′)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>TGTGTNMRGGAYATGTTTTCARATYATGG</td>
<td>degenerate primer</td>
</tr>
<tr>
<td>P2</td>
<td>CCATCAARTRNMCCATTACACARTCAACAT</td>
<td>degenerate primer</td>
</tr>
<tr>
<td>P3</td>
<td>ATACAAACCTCTCTCTCTCGGGAAG</td>
<td>primer for 1st PCR in inverse PCR</td>
</tr>
<tr>
<td>P4</td>
<td>GTCCATATACACCCGACCCGACCATATACAT</td>
<td>primer for 1st PCR in inverse PCR</td>
</tr>
<tr>
<td>P5</td>
<td>GGGGACATTCTCTCGTCTCTAGG</td>
<td>primer for nested PCR in inverse PCR of the gene for NaPAC1</td>
</tr>
<tr>
<td>P6</td>
<td>TCTTTTCGTATGTGGAATGTGTTGAG</td>
<td>primer for nested PCR in inverse PCR of the gene for NaPAC1</td>
</tr>
<tr>
<td>P7</td>
<td>TTGTCTATGCATGCATATGTGTTGAG</td>
<td>primer for nested PCR in inverse PCR of the gene for NaPAC2</td>
</tr>
<tr>
<td>P8</td>
<td>TCTTTTCGTGATGGAATGTGTTGAG</td>
<td>primer for nested PCR in inverse PCR of the gene for NaPAC2</td>
</tr>
<tr>
<td>P9</td>
<td>GATTATCTCTCAATCTCTCTCATGTTGATG</td>
<td>primer for PCR of the full length of the gene for NaPAC1</td>
</tr>
<tr>
<td>P10</td>
<td>GTTTTCTAAAGCAACAGCAAACAAATTAGCC</td>
<td>primer for PCR of the full length of the gene for NaPAC2</td>
</tr>
<tr>
<td>P11</td>
<td>CTTCCATCTCTCTCTCTCTAGG</td>
<td>primer for PCR of the full length of the gene for NaPAC2</td>
</tr>
<tr>
<td>P12</td>
<td>CAAAAGACCAACAAACAAATTAGAGCAATG</td>
<td>primer for construction of pNaPAC1</td>
</tr>
<tr>
<td>P13</td>
<td>AAAACCTCAGATGTGTCATCAACAAACTCC</td>
<td>primer for construction of pNaPAC2</td>
</tr>
<tr>
<td>P14</td>
<td>TTTCTGAGAACTCCAAATAGGTATGTATCAT</td>
<td>primer for PCR of <em>N. fowleri</em> genes</td>
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<tr>
<td>P15</td>
<td>AGAAGCAGAGAATGAGAATAAGGATCCTGAGGCGG</td>
<td>primer for PCR of <em>N. fowleri</em> genes</td>
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<tr>
<td>P16</td>
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<td>primer for PCR of <em>N. fowleri</em> genes</td>
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<td>M13 primer</td>
<td>TGTAAAACGACGGGCAGT</td>
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</tr>
<tr>
<td>SP6 primer</td>
<td>ATTTAGGTGACACTATAG</td>
<td>universal primer</td>
</tr>
<tr>
<td>T7 primer</td>
<td>TAAACGACTCACTATAGGG</td>
<td>universal primer</td>
</tr>
</tbody>
</table>

inserted into *N. fowleri* BLUF domain (Met^{1}–Met^{390}) and *N. australiensis* BLUF domain (Met^{1}–Leu^{392}) were synthesized and inserted into *Ndel–Xbal* of pColdI. The resultant plasmids were designated pNaPAC1 and pNaPAC2, respectively. The coding sequence for the chimeric protein, CMR1, consisting of the *N. fowleri* BLUF domain (Met^{34}–Ser^{137}) and *N. gruberi* AC domain (Ser^{115}–Met^{390} of NgPAC1) was synthesized and inserted into *Ndel–Xbal* of pColdI. The resultant plasmid was designated pCMR1. For a plasmid expressing a site-directed mutant of CMR1 in which Tyr^{13} was replaced by Trp (NaPAC1Y^{13}W) and a plasmid expressing a site-directed mutant of NaPAC2 in which Tyr^{14} was replaced by Trp (NaPAC2Y^{14}W), the coding sequences for the proteins were synthesized and inserted into *Ndel–Xbal* of pColdI. The resultant plasmids were named pNaPAC1Y^{13}W and pNaPAC2Y^{14}W, respectively. For a plasmid expressing a site-directed mutant of NgPAC1 in which Tyr^{16} was replaced by Trp (NgPAC1Y^{16}W) and a plasmid expressing a site-directed mutant of NgPAC2 in which Tyr^{17} was replaced by Trp (NgPAC2Y^{17}W), the coding sequences for the proteins were synthesized and inserted into *Ndel–Xbal* of pColdI. The resultant plasmids were named pCMR1Y^{39}W.
Results

Sequencing of genes encoded by \textit{N. australiensis} and \textit{N. fowleri}

Sequence alignment of NgPAC1 and NgPAC2 proteins showed that these proteins have conserved amino acid sequences in the region between the BLUF and AC domains. Degenerate primers P1 and P2 were designed to amplify the cognate regions from the \textit{N. australiensis} genome. These primers successfully amplified the parts of the coding sequences from \textit{N. australiensis} genomic DNA. Nucleotide sequences of the PCR products revealed that the organism encodes at least two PAC candidates, NaPAC1 and NaPAC2. The upstream and downstream sequences of the conserved regions were amplified from WGA DNA samples of \textit{N. australiensis}, cloned on a plasmid vector, and sequenced as described in MATERIALS AND METHODS. For confirmation, coding sequences were amplified from \textit{N. australiensis} genomic DNA, not from WGA DNA samples, and sequenced. Results obtained from sequence analysis showed that the coding sequences for NaPAC1 and NaPAC2 consisted of 1,155 bp and 1,158 bp, respectively (Fig. 1). Overall identity of the nucleotides between the coding sequences was 97%.

PCR products were not detected when genomic DNA of \textit{N. fowleri} kurume was used as a template. We analyzed the whole genome sequence of \textit{N. fowleri} kurume with a high-throughput gene analyzer, but coding sequences for PAC orthologs were not found in the genomic sequence. These results indicate that the organism does not encode a PAC ortholog.

Sequence data obtained from whole genome analysis revealed that \textit{N. fowleri} kurume encodes at least one polypeptide bearing an amino acid sequence similar to the BLUF domain. The nucleotide sequence of this gene was confirmed by re-sequencing of PCR fragments amplified from the \textit{N. fowleri} kurume genome (Fig. 1).

Coding sequences for the orthologs in \textit{N. fowleri} KUL and NF66 strains were amplified by PCR and sequenced. The nucleotide sequence amplified from \textit{N. fowleri} KUL was identical to that of \textit{N. fowleri} kurume. A single nucleotide polymorphism (SNP) without amino acid substitution was found in the coding sequence in \textit{N. fowleri} NF66. The codon for Lys\textsuperscript{108} in \textit{N. fowleri} kurume and KUL strains was AAG, while that for the residue was AAA in \textit{N. fowleri} NF66.

Structures of the proteins encoded by \textit{N. australiensis} and \textit{N. fowleri}

Schematic illustrations of NaPAC1 (384 aa), NaPAC2 (385 aa) and BLUF-containing protein (293 aa) are presented in Fig. 2A. Each of the \textit{N. australiensis} proteins contains a single BLUF domain and a single AC domain. The structures of the proteins are similar to those of \textit{Beggiatoa} PAC and \textit{N. gruberi} proteins. Overall identities of amino acid residues between the \textit{Naegleria} proteins are shown in Table 2.

\textit{N. fowleri} protein bears a BLUF domain but not an
AC domain. Proteins showing sequence similarity to the amino acid sequence downstream of the BLUF domain were not found in databases, and thus the function of this protein is not known at present.

Amino acid sequences of BLUF domains in the N. australiensis proteins, N. fowleri protein, N. gruberi proteins, Beggiatoa PAC, E. gracilis PACalpha, E. gracilis PACbeta and Rhodobacter sphaeroides AppA protein were compared (Fig. 2B). E. gracilis PACs bear two BLUF domains (indicated as F1 and F2).

Molecular analysis conducted on the BLUF domain of R. sphaeroides AppA protein revealed that Tyr21...
(numbering for AppA), Asn$^{45}$, Gln$^{63}$ and Trp$^{104}$ in the domain were responsible for FAD binding and integrity of the BLUF domain fold (Kraft et al., 2003; Laan et al., 2003, 2006; Unno et al., 2006). The proteins found in \textit{Naegleria} contain these residues.

AC domains found in \textit{N. australiensis} proteins, \textit{N. gruberi} proteins, PACs and bovine type I adenylyl cyclase are aligned (Fig. 2C). Mammalian adenylyl cyclase and euglenoid PACs bear two AC domains (C1 and C2). Functions of amino acids in AC domains have been extensively studied in bovine type I adenylyl cyclase. The analyses have revealed that Asp$^{310}$ (numbering for the bovine protein), Lys$^{350}$, Asp$^{354}$, Lys$^{469}$, Lys$^{923}$, Asn$^{1007}$, Arg$^{1011}$ and Lys$^{1047}$ are important for catalysis in the mammalian adenylyl cyclase (Hurley, 1999). The alignment shows that \textit{N. australiensis} proteins and \textit{N. gruberi} proteins conserve these residues important for catalysis in the mammalian enzyme.

\textbf{Functional expression of NaPACs in \textit{E. coli} cells}

PAC activity of the \textit{N. australiensis} proteins was assayed by comparing antibiotic sensitivities of \textit{E. coli} cells expressing the proteins in a dark condition and a blue light-irradiated condition as described in our previous report (Yasukawa et al., 2012). Antibiotics used in this study were fosfomycin and fosmidomycin, which inhibit bacterial cell wall synthesis and the nonmevalonate pathway of isoprenoid biosynthesis, respectively (Kahan et al., 1974; Kuzuyama et al., 1998). Fosfomycin is taken up actively into bacterial cells via GlpT and UhpT transporters, and fosmidomycin is transported via the GlpT transporter (Kadner and Winkler, 1973; Minassian and Williams, 1995; Sakamoto et al., 2003). Expression of the transporters is dependent on the presence of cAMP (Merkel et al., 1995; Olekhnovich et al., 1999). Therefore, fosfomycin sensitivity and fosmidomycin sensitivity of \textit{E. coli} cells are altered by cellular cAMP levels.

Fosfomycin sensitivity and fosmidomycin sensitivity of the cells were analyzed by paper disk assays (Fig. 3A). Diameters of inhibition zones formed on the HB101 (pColdI) lawns under blue-light irradiation were almost the same as those formed on HB101 (pColdI)

\begin{table}
\centering
\caption{Overall identities (%) of amino acids between the proteins.}
\label{table:1}
\begin{tabular}{lcccc}
\hline
& NaPAC1 & NaPAC2 & NgPAC1 & NgPAC2 \\
NaPAC1 (384 aa) & 100 & 97 & 75 & 75 \\
NaPAC2 (385 aa) & 100 & 75 & 75 & 75 \\
NgPAC1 (390 aa) & 100 & 78 & 100 & \\
NgPAC2 (392 aa) & 100 & 78 & 100 & \\
\hline
\end{tabular}
\end{table}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Fig3}
\caption{Functional expression of \textit{N. australiensis} proteins and \textit{N. gruberi} proteins in \textit{E. coli} cells. (A) Fosfomycin sensitivity and fosmidomycin sensitivity of \textit{E. coli} cells expressing \textit{Naegleria} proteins. (B) Fosfomycin sensitivity and fosmidomycin sensitivity of \textit{E. coli} cells expressing site-directed mutants of \textit{Naegleria} proteins. Black boxes are the results obtained from assays conducted in the dark. White boxes are the results obtained from assays conducted under blue light. Values are means ± SD obtained from three independent experiments. *$p<0.01$.}
\end{figure}
lawns in the dark. These results indicated that sensitivity of the host strain to the antibiotics was not altered by blue light irradiation. HB101 cells carrying plasmids HB101(pNgPAC1), HB101(pNgPAC2), HB101(pNaPAC1) and HB101(pNaPAC2) showed increased sensitivity to the antibiotics when incubated under blue light. These results indicated that cAMP levels in the test cells incubated under blue light were almost the same as those in the cells incubated in the dark. We concluded from these results that the N. gruberi proteins and N. australiensis proteins exhibited PAC activity in E. coli cells.

To determine the importance of the Tyr residue conserved in the BLUF domain for PAC activity, plasmids carrying genes for site-directed mutants of the proteins were constructed and subjected to assays. As shown in the figure, fosfomycin sensitivity and fosmidomycin sensitivity of the E. coli transformants incubated under blue light were almost the same as those of cells incubated in the dark (Fig. 3B). These results showed that the site-directed mutants were not activated by blue light irradiation, indicating that the Tyr residue is essential for photoactivation.

Growth inhibition was enhanced even in the dark in PAC-expressing cells compared to control cells as shown in the figure. PAC-expressing cells showed increased sensitivity to the drugs even in the dark because the cells expressed excess amounts of PAC proteins. We have observed that E. coli cells transformed by a plasmid expressing PAC under control of a weak promoter, lacI promoter, showed increased antibiotic sensitivity under blue light and showed almost the same antibiotic sensitivity as that of control cells in the dark (data not shown).

**Functional analysis of N. fowleri BLUF**

To determine whether the BLUF-like sequence found in N. fowleri functions as a sensor of blue light, the gene for a chimeric protein consisting of the BLUF-like region and AC domain of NgPAC1 was synthesized and cloned on a vector. Cells of E. coli HB101 were transformed by the plasmid pCMR1 and subjected to assays to determine whether the chimeric protein exhibits PAC activity. Results obtained from paper disk assays are shown in Fig. 4A. HB101(pCMR1) cells showed increased sensitivity to the antibiotics when incubated under blue light, indicating that the chimeric protein exhibited PAC activity.

These experimental results demonstrated that the BLUF-like sequence found in N. fowleri functions as a sensor of blue light.

To determine the importance of the Tyr residue in photo-responsivity, a plasmid carrying the gene for a site-directed mutant of the chimeric protein was constructed and subjected to assays. As shown in the figure, fosfomycin sensitivity and fosmidomycin sensitivity of the E. coli transformants incubated under blue light were almost the same as those of cells incubated in the dark (Fig. 4B). These results showed that the site-directed mutant was not activated by blue light irradiation, indicating that the Tyr residue is essential for photo-responsivity.

**Discussion**

Sequence analysis showed that proteins found in N. australiensis carry a single BLUF domain and a single AC domain. E. coli cells expressing the proteins exhibited photo-dependent alteration of antibiotic sensitivity. These results indicate that the proteins found in N. australiensis are PACs. N. australiensis PACs would play important roles in photoresponses of the organism. To determine the biological importance of the PACs in N. australiensis, we are planning to analyze expression levels of the proteins during the life cycle of this organism and determine the subcellular localization of the proteins.

In addition to analysis of the biological importance of PACs in N. australiensis, molecular analysis of these proteins should be conducted because PACs would be excellent tools for photomanipulation of the intracellular cAMP level in heterologous cell systems. For further analysis of the proteins, we are planning to purify them from transformed E. coli cells and subject them to photobiochemical assays. Results of in vitro analysis of the proteins would contribute to an understanding of the molecular basis for photoactivation of PACs.

Analysis of N. fowleri showed that this organism encodes a BLUF-containing protein, suggesting that the pathogenic organism exhibits a photoresponse. N. fowleri strains, kurume, KUL and NF66, conserve identical proteins, suggesting that the photoresponse is a biological function important for N. fowleri cells. Analysis of photoresponse has not been conducted for N. fowleri, but we should start such an investigation for public health prevention. The pathogenic organisms could be controlled by blue light, and control of them would contribute to a decrease in infection.
On the other hand, rapid detection and long-term monitoring of the pathogenic organisms in environmental water and recreational water are essential to prevent infection. The BLUF-containing protein described here is thought to be a protein encoded only by *N. fowleri* because no orthologous proteins were found in databases. Thus, the primer sets designed from the nucleotide sequence of the gene for the BLUF-containing protein would be useful for detecting the pathogenic organism specifically by real-time PCR and loop-mediated isothermal amplification (LAMP).

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**References**


