Cloning and Characterization of the Azurin iso-1 Gene, Concerned with the Electron Transport Chain Involved in Methylamine/Methanol Oxidation in the Obligate Methylo troph Methylo monas sp. Strain J

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Two azurin-type blue copper proteins, which is concerned with the electron transport chain involved in methylamine/methanol oxidation, have been found in the obligate methylo troph Methylo monas sp. strain J. The azurin iso-1 gene was cloned and sequenced to analyze the role in the electron transport chain. PCR products synthesized with primers based on the N- and C-terminal amino acid sequences of azurin iso-1 were used as probes for cloning. One complete open reading frame (the azurin iso-1 gene) and one partial orf (orf1) were found in a cloned Eco105I-HindIII fragment, pMAZ3, with a total of 1066 bp. The gene encoded 148 amino acid residues. The amino acid sequence after Ala-21, deduced from the nucleotide sequence, was identical to that of the azurin iso-1 protein. The gene was in a region separate from the mau gene cluster in the chromosome. Escherichia coli expressed azurin iso-1. The results of northern blotting analysis suggested that expression of the azurin iso-1 gene is regulated by a complex regulatory network controlling oxidation of methylamine or methanol in this strain; for example, copper ions affected the expression of the azurin iso-1 gene.

Key words: obligate methylo troph; methylamine dehydrogenase; blue copper protein; azurin

Blue copper proteins such as amicyanins, azurins, and pseudoazurins have been found in methylotrophic bacteria.1–5 These proteins differ in amino acid sequence but share properties: 1) Each of these proteins has a copper ion at its active site. 2) All are found in the periplasmic space and all have functions related to the electron transport chains of the cells. 3) The visible-absorption spectra of these proteins are similar. Azurins from several species of bacteria have been purified, characterized, and found to participate in nitrite/nitrate respiration.6–8 The genes encoding azurins of denitrifying bacteria such as Pseudomonas aeruginosa9 and Alcaligenes denitrificans10 have been cloned and characterized. Among the many methylo trophs examined, azurin-type blue copper proteins have been detected only in Methylo monas sp. strain J; an obligate methylo troph. This strain can grow on either methanol or methylamine as the sole source of carbon and energy, but cannot use methane.11 Two azurin-type blue copper proteins, azurin iso-1 and iso-2, have been found in this strain (Fig. 1).11 Both azurins are present when the cells are grown on methylamine as the sole carbon and energy source, but only azurin iso-1 is detected when methanol was used. In in vitro experiments, azurin iso-2 was found to be a more suitable primary electron acceptor for methylamine dehydrogenase (MADH; EC 1.4.99.3) than azurin iso-1 (unpublished results). These results suggested that azurin iso-2 may be a direct electron acceptor from MADH in vivo and that expression of this protein may be induced by methylamine as is MADH in Methylo monas sp. strain J. Methanol dehydrogenase (MDH) can be detected when cells are grown on methanol or methylamine as the sole carbon and energy source.12 The direct electron acceptor from MDH is cytochrome c and azurin iso-1 accepts electrons from cytochrome c (Fig. 1).13

In this paper, to analyze the electron transport chain involved in methylamine and methanol oxidation in the obligate methylo troph Methylo monas sp. strain J, we cloned and characterized the azurin iso-1 gene from this bacterium.

Materials and Methods

Bacterial strains, plasmids, and media. Methylo monas sp. strain J was used as the source of the azurin iso-1 gene for cloning. E. coli JM109 (recA1, Δ(lac-proAB), endA1, gyrA96, thi-1, hsdR17, relA1, supE44, [F' traD36, proAB, lacIqZΔM15]) was used as the host. The plasmids pUC118, pUC119, and pBluescript were used as cloning and expression vectors. The strain was

![Fig. 1. Electron Transport Chains Involved in Methylamine-Methanol Oxidation in Methylo monas sp. Strain J.](image)

**Abbreviations:** MA, methylamine; MADH, methylamine dehydrogenase; and Cyt. c, cytochrome.

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The nucleotide sequence including the azurin iso-1 gene has been deposited in the DDBJ database under accession number AB000916.
grown at 28°C with aeration in the liquid medium, with the following composition (mg/l): NaH₂PO₄·12H₂O, 2490; K₂HPO₄, 1360; MgSO₄, 290; disodium EDTA, 202; (NH₄)₂MoO₄·4H₂O, 186; CaCl₂·2H₂O, 66; MnSO₄·4H₂O, 15; ZnSO₄·7H₂O, 11; FeSO₄·7H₂O, 7; Co(NO₃)₂·6H₂O, 0.25; Na₂B₄O₇·10H₂O, 0.18, and CuSO₄·5H₂O, 0.4. As carbon and nitrogen sources, methionine (5 g/l) and (NH₄)₂SO₄ (500 mg/l) were added to the methylene medium. As carbon and nitrogen sources, methanol (5 ml/l) and (NH₄)₂SO₄ (5400 mg/l) were added to the methanol medium.

*Methylomonas* sp. strain J also was grown in a medium without CuSO₄·5H₂O. *E. coli* strains were cultured aerobically at 37°C in Luria-Bertani (LB) broth, which contained 50 μg/ml ampicillin when appropriate.  

**Synthesis of probe for the azurin iso-1 gene.** Two mixed oligonucleotide primers containing restriction sites (underlined) at their 5’-ends were synthesized on the basis of the N-terminal sequence (Met-Gln-Tyr-Asn-Thr-Lys-Asn-Ile⁸⁶; 5’-CCCTAGATGCARTAYAAACAARAAYAT-3’) and C-terminal sequence (Phe-Pro-Gly-His-Ala-Thr-Met-Met³⁸; 3’-AARGNCCRCRTRGNTGRTACTCTGAGC-5’) azurin iso-1 from *Methylomonas* sp. strain J as reported previously.  

PCR-amplified fragments were purified by agarose gel electrophoresis and cloned into pUC19. The PCR-amplified fragments were digested with restriction enzymes that recognized sites in the primers region and cloned in a plasmid vector. Several clones were selected randomly from transformants harboring PCR-amplified DNA from this strain. The amino acid sequence deduced from the nucleotide sequence of the clone was examined, and found to correspond to that of azurin iso-1 purified earlier from the same strain. These fragments were labeled with digoxigenin (Boehringer Mannheim yamanouchi, Tokyo) and used as probes for cloning.

**Cloning of the azurin iso-1 gene from Methylomonas sp. strain J.** Chromosomal DNA from this strain was digested with several restriction enzymes. With a PCR-amplified DNA fragment as the hybridization probe, genomic DNA from the strain was analyzed by Southern hybridization. The probe hybridized with only one chromosomal DNA fragment in the digest pattern. Fraction of 9.4-kb EcoRI-digested fragments that hybridized with the probe was isolated from an agarose gel, and these fragments were cloned into the EcoRI-digested pUC19 vector. Two clones that hybridized with this probe were identified in a DNA library of the strain. Plasmids were isolated from these clones and each of these contained a 9.4-kb EcoRI insertion, pMAZ1.

**Nucleotide sequencing.** Nucleotides were sequenced by the dideoxyribonucleotide chain termination method with an automated sequencer (model 373A; Applied Biosystems, Foster City, CA). The appropriate DNA fragments were subcloned into pBluescript KS and SK, and deleted with a kilo-sequence deletion kit (Takara Shuzo Co. Ltd., Kyoto, Japan). Both DNA strands were sequenced. Computer analysis was done with the GENETYX-MAC program (Software Development Co., Ltd., Tokyo). The deduced amino acid sequences of the open reading frames were compared with databases with the BLAST program.

**Expression of the azurin iso-1 gene in E. coli.** For the whole region of the azurin iso-1 gene to be included, a 1.0-kb Eco105I-HindIII fragment was subcloned into the EcoRV-HindIII site of pBluescript SK (plasmid pMAZ1), and the region upstream of the azurin iso-1 gene was restricted with SacI-BamHI and deleted by means of the kilo-sequence deletion kit. *E. coli* JM109 containing the plasmid was cultivated at 37°C in LB broth containing isopropyl-β-D-galactosyranoside (IPTG) at the final concentration of 1 mM. Cells were harvested by centrifugation. The pellet was suspended in electrophoresis buffer containing SDS and heated for 5 min at 100°C. Proteins from the cells were separated by SDS-PAGE (15% gels). Gels were stained with Coomassie brilliant blue.

The blue copper protein produced in *E. coli* was purified as previously reported.

**Northern blotting hybridization.** Total RNA was isolated from the phenol-SDS method from exponentially growing *Methylomonas* sp. strain J. Cells were grown on methylene or methanol as the carbon and energy source with or without copper sulfate in the growth medium. The RNA pellets were dissolved in H₂O and stored at −80°C. For northern blotting, total RNA (20 μg) was separated on a 1% agarose-2 m formaldehyde gel and transferred to a nylon membrane. Molecular weight markers (RNA ladder; Gibco BRL) on a piece of the gel cut off before transfer were stained with ethidium bromide. The probe used was the same as that used for cloning. Northern blotting was done by the protocol for the DIG system (Boehringer Mannheim).

**Heme staining.** SDS-polyacrylamide gel electrophoresis was done on 15% slab gels. *Methylomonas* sp. strain J was grown on methylene or methanol medium with or without copper sulfate in the growth medium. Cells were harvested by centrifugation. The pellet was suspended in 25 mM Tris hydrochloride (pH 6.5)-1% (wt/vol) SDS-5% (vol/vol) glycerol-0.01% (wt/vol) bromophenol blue and heated for 10 min at 100°C. Samples of the total cell protein were run on 15% gels by SDS-PAGE. Gels were stained with 3',5',5'-tetramethylbenzidine for covalently bound proteins containing heme as reported earlier.

**Nucleotide sequence accession number.** The nucleotide sequence including the azurin iso-1 gene has been deposited in the DDBJ database under accession number AB000916.

**Results and Discussion**

**Analysis of the nucleotide sequence.**

The 9.4-kb EcoRI fragment containing the azurin iso-1 gene was digested with several restriction enzymes and
a restriction map was prepared (Fig. 2). A total of 1066-bp in the fragment, pMAZ3, was sequenced. One complete open reading frame and a partial one were found in the sequenced position. The findings obtained by sequencing of the 1066-bp Eco1051-HindIII fragment including the azurin iso-1 gene are shown in Fig. 3.

The gene encoding azurin iso-1 was in the region from positions 371 to 820. It encoded 148 amino acid residues. The amino acid sequence after Ala-21, as deduced from the nucleotide sequence, was identical to that of azurin iso-1 previously reported. The NH₂-terminal sequence of the open reading frame from Met-1 to Ala-20 was similar to a prokaryotic signal sequence described by von Heijne. This sequence comprised an N-terminal region, positively charged overall, followed by a hydrophobic region and a polar C-terminal region. Mature azurin iso-1 consisted of 128 amino acid residues and the calculated molecular weight was 13,526.

The azurin iso-2 gene, mauO, that functions as the primary electron acceptor for MADH was directly downstream of the mau gene cluster but oriented in the opposite direction. To locate the azurin iso-1 gene, we did Southern hybridization using restricted DNA fragments separated by pulse-field gel electrophoresis. The results showed that the azurin iso-1 gene was in a region separated from the mau gene cluster.

The azurin iso-1 and iso-2 gene product of Methylococcus sp. strain J had 49% identity. The nucleotide sequence similarity between the azurin iso-1 and iso-2

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**Fig. 2.** Physical and Genetic Map of the 9.4-kb EcoRI Fragment Containing the Azurin iso-1 Gene from Methylococcus sp. Strain J. Genes are indicated by the arrow and the direction of the arrows indicates the direction of transcription.

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**Fig. 3.** Nucleotide Sequence and Deduced Amino Acid Sequence of the 1066-bp Eco1051-HindIII Fragment of Methylococcus sp. Strain J Containing the Azurin iso-1 Gene.

A putative Shine-Dalgarno sequence is double-underlined, hairpin structures are indicated by arrows, and similar sequence in the regions upstream of the mau gene cluster and the azurin iso-2 gene are underlined.
genes was 59%. The regions upstream and downstream of the azurin iso-1 gene were not similar to those of the azurin iso-2 gene. Azurin iso-1 was 57 to 65% identical in amino acid sequence with other azurins,\(^{6,20-22}\) Azurin iso-1 was more similar to from denitrifying pseudomonads and Alcaligenes species than azurin iso-2. These azurins participate in nitrite-nitrate respiration because consensus sequences resembling an ntrA box and a fnr box have been found in the promoter region of the azurin genes in these species.\(^{9,10}\) However, such consensus sequences were not found in the region upstream of the azurin iso-1 gene in *Methylomonas* sp. strain J, and this bacterium has no nitrite-nitrate respiration system. These results suggest that the role of the azurin iso-1 gene in *Methylomonas* sp. strain J differed from that of the azurins of denitrifying bacteria. The azurin iso-2 in *Methylomonas* sp. strain J may belong to a different group, because it seems to accept electrons directly from MADH, and may have had a special function earlier during the evolution of this species.

Theorf1 was 275 bp upstream of the azurin iso-1 gene. Two inverted repeat structures with \(\Delta G = -22.3\) and \(-31.6\) kcal/mol were found in the region after the azurin iso-1 gene. This region may terminate transcription of the azurin iso-1 gene.

**Expression of the azurin iso-1 gene in E. coli**

To allow expression of the azurin iso-1 gene by the lac promoter on the vector, we deleted pMAZ3 restricted with SacI1 and BamHI. *E. coli* transformants grown on a medium containing IPTG were tested by SDS-PAGE. A polypeptide similar in size to azurin iso-1 was detected in two transformants. This polypeptide was expressed in cells grown on a medium containing copper ions. Polypeptides were purified from *E. coli* carrying pMAZ31 obtained by deletion of pMAZ3 in the presence of copper ions. The yield from 1 g of wet *E. coli* cells was 0.3 μmol of azurin iso-1. The absorption spectra of the purified polypeptides had maxima at 277 nm and 620 nm. The absorption spectra corresponded to that of azurin iso-1 purified from *Methylomonas* sp. strain J.

The N-terminal amino acid sequence of the polypeptide purified from *E. coli* was NH$_2$-Ala-Gly-Cys-Ser-Val-Asp-Val-Glu-Ala-Asn-Asp-Ala-Met-Gln-Tyr-Asn-Thr-Lys-Asn-Ile. This sequence corresponded to that of azurin iso-1 purified from *Methylomonas* sp. strain J.\(^{13}\)

Azurin iso-1 was expressed in the *E. coli* system, suggesting that this system could be used in further azurin iso-1 research.

**Assay of specific transcripts by northern blotting**

Results of northern blotting are shown in Fig. 4. *Methylomonas* sp. strain J was grown with either methylamine or methanol as the sole source of carbon and energy in a medium with or without CuSO$_4$:5H$_2$O. Total RNA was purified from cells grown under these four different conditions and the purified RNA was used for northern blotting. The size of the RNA transcript produced from the azurin iso-1 gene was about 0.6 kb, suggesting that the transcript covered the only azurin iso-1 gene. Results suggested that the azurin iso-1 gene,
the azurin iso-2 gene, and the mau gene cluster were necessary for methylamine oxidation. These results suggest that a common regulatory mechanism controls their expression. In a search for similarity that focused on the promoter regions of these genes, similar sequences were found in the upstream regions of the azurin iso-1 gene (CTG GCC ATT TTA-ATG-11bp-ATG), the azurin iso-2 gene (CATG CCA CCT TTTC ATG-58bp-ATG), and the mau gene cluster (CATG CCA ATT TTA-ATG-101bp-ATG) (identical bases are capitalized).

The azurin iso-1 gene was expressed in the presence of methanol, but the transcription of the gene was suppressed when the cells were grown in a medium without CuSO₄·5H₂O. The level of expression of cytochrome c, which is involved in the electron transport chain for for methanol or methylamine oxidation, was investigated by heme staining (Fig. 5). The amount of cytochrome c (I) was the same in the presence or absence of CuSO₄·5H₂O. However, the amount of cytochrome c (II) increased in the absence of CuSO₄·5H₂O, suggesting that expression of cyt. c (II) is suppressed by copper ions in the medium. Considering these results, it seems that cyt. c (II) acts as an electron acceptor directly or indirectly during growth in the absence of CuSO₄·5H₂O. The copper ion concentration in the medium with or without CuSO₄·5H₂O was assayed by atomic absorption spectroscopy. Copper ions were not detected in the medium without CuSO₄·5H₂O. The concentrations of copper ions in the methanol and methylamine media with CuSO₄·5H₂O were 0.1 ppm (1.6 μM). These concentrations corresponded roughly to the amount of CuSO₄·5H₂O added to the media.

It is of interest that the activation of the azurin iso-1 gene and the repression of the cyt. c (II) gene were regulated by differences in the copper ion concentration of the medium. Metal-regulated transcription is known in eukaryotes. Genes of the FREL-encoded Cu²⁺/Fe²⁺ reductase and CR1 and CR3 encoded membrane-associated copper transport proteins are regulated transcriptionally by copper availability. Each of these genes repressed when cells are grown in the presence of copper and highly activated during copper starvation. CR3 gene expression is repressed at picomolar concentrations of metal ions. Several inverted repeat sequences were found (arrows, Fig. 3). One of them, TTTGCTG, is almost identical to such a sequence, TTTGCTC, in a yeast with copper ion regulation, and this sequence, TTTGCTG, maybe be important for regulation by copper.

These observations suggest that a complex regulatory network controls the oxidation of methylamine or methanol in *Methylomonas* sp. strain J, and that copper ions participate in the regulatory mechanism.

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References