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Cloning, Sequencing, and Mutation of a Gene for Azurin in *Methylobacillus flagellatum* KT

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The gene cluster for methylamine utilization (*mau* genes) has been cloned from the obligate methylotrophic bacterium *Methylobacillus flagellatum* KT. Partial sequence data showed that the organization of these genes was similar to that found in *Methylophilus methylotrophus* W3A1-NS, including the lack of a gene for amicyanin, which had been thought to be the electron acceptor for methylamine dehydrogenase in *M. flagellatum* KT. However, a gene encoding azurin was discovered at the 3 end of the *mau* gene cluster, transcribed in the opposite orientation. A mutant with a defect in this gene showed impaired growth on methylamine, suggesting that azurin is involved in methylamine oxidation in *M. flagellatum* KT.

Methylamine dehydrogenase (MADH) is the enzyme that oxidizes methylamine to formaldehyde in many gram-negative bacteria that grow on methylamine (13, 17, 20, 21–24, 27). In all cases that have been studied, MADH has been a periplasmic protein consisting of two large subunits and two small subunits (17, 20, 22–24, 27). Each small subunit has a covalently bound prosthetic group called tryptophan tryptophylquinone synthesized from two tryptophans belonging to the small-subunit polypeptide chain (28). Two types of electron acceptors for MADH are known. MADH is thought to use a *c*-type cytochrome in *Methylophilus* strains (4, 6), whereas MADHs from the other methylotrophs are thought to use blue copper proteins, or cupredoxins, as electron acceptors (2, 11, 19, 24, 31, 32, 34). Three classes of cupredoxins are found in methylotrophs, amicyanins, azurins, and pseudoazurins. By definition, the cupredoxin that accepts electrons from MADH is usually termed amicyanin (2). However, for at least one methylotroph, *Methylomonas* sp. strain J, the amino acid composition shows this cupredoxin to be an azurin. Several methylotrophs, including *Methylobacillus flagellatum* KT, are known to have two cupredoxins (1, 2, 11, 24). For *M. flagellatum* KT, one was methylamine inducible and was assumed to be amicyanin (11). In two strains, organism 4025 and *M. flagellatum* KT, cells can grow slowly on methylamine in a medium depleted of copper, conditions under which cupredoxins are absent (11, 24). An unknown cytochrome was suspected to function under such growth conditions, since the known cytochrome *c*'s in these bacteria do not accept electrons from MADH in vitro.

Genetic analysis has shown that in the α-proteobacteria containing MADH, the genes required for synthesis of active MADH (*mau* genes) are clustered and include a gene encoding amicyanin (7, 8, 33, 35). The *mau* gene cluster of *Methylophilus methylotrophus* W3A1-NS is similar except that it does not include the amicyanin gene, as expected, since this strain does not contain amicyanin (9). Since the electron acceptor for MADH in *M. flagellatum* KT is uncertain, we have analyzed the *mau* gene cluster of this bacterium to determine whether the amicyanin gene was present.

Escherichia coli DH5α (New England Biolabs) was grown in Luria-Bertani medium in the presence of appropriate antibiotics as described by Maniatis et al. (25) except that the concentration of chloramphenicol for pAYC63 derivatives was 0.01 mg/ml. Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were added at 0.04 mg/ml. *M. flagellatum* KT (16) was grown in minimal medium (14) as described previously except that it was used at double strength and its pH was adjusted to 7.2 to 7.3 with sodium hydroxide. The nitrogen-free medium used was the minimal medium with sodium sulfate (0.2 g/liter) substituted for ammonium sulfate and ammonium molybdate omitted. The concentrations of tetracycline, ampicillin, rifamycin, chloramphenicol, and kanamycin for growing the *M. flagellatum* KT strains were 0.001, 1, 0.1, 0.15, and 0.15 mg/ml, respectively. Methanol (2% [vol/vol] unless indicated differently below) and methylamine hydrochloride (0.4% [wt/vol]) were used as carbon sources. Growth rates of various *M. flagellatum* KT strains on methanol and methylamine were determined by using 15-ml cultures grown in 100-ml sidearm flasks, and cell density was measured with a Klett-Summerson colorimeter every 4 h for 70 to 80 h (15).

To clone *mau* genes, two partial gene libraries generated with *Pst*I and *Hin*dIII were constructed in the vectors pRK310 (12) and pUC19 (New England Biolabs), respectively, as described earlier (10). The mixed oligonucleotide probe 5′-GAG GAYGAYGCSATGACSTAYCAYTG-3′ (Y = T or C; S = A, T, G, or C), based on a conserved region in the known *mauA* sequences, was used to probe the *Hin*dIII library, and the 2.6-kb *Pst*I fragment from the plasmid pGak1 was used to probe the *Pst*I library. DNA-DNA hybridizations were carried out with dried agarose gels in accordance with the procedure described by Meinkoth and Wahl (29). The temperatures of hybridizations (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.1% sodium dodecyl sulfate [SDS]) and washes (0.5× SSC–0.1% SDS) were 42°C for probing with the oligonucleotide and 68°C in all other cases. Plasmid isolation, *E. coli* strain transformation, preparative isolation of the DNA fragments from agarose gels, restriction endonuclease digestion, ligation, and blunting of ends with Klenow fragment or T4 DNA polymerase were carried out as described by Maniatis et al. (25). Random primer labelling of DNA fragments was conducted as suggested by the manufacturer (Boehringer Mannheim Corp., Indianapolis, Ind.). Chromosomal DNA of the *M. flagellatum* KT strains was isolated in accordance with the procedure of Marmur (26). DNA sequencing was performed by the dideoxy chain termination method on both strands in

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the UCLA DNA Sequenator Core Facility on an Applied Biosystems sequenator. Plasmid pAYC63 (Cm^r) (7) was used as a vector for subcloning and sequencing in addition to pUC19 (Ap^r). Insertion mutations in the azurin gene were constructed by homologous recombination as described previously (30). The Km^r cassette from the plasmid pUC4K was used as a selective inactivating marker, and plasmid pAYC61 (8) was used as a suicide vector. *M. flagellatum* KT cells were broken by the following osmotic shock procedure. Cells from 50-ml cultures were collected by centrifugation, suspended in 1.5 ml of 25% (wt/vol) sucrose solution in 50 mM Tris-HCl buffer (pH 7.5), and washed twice with 1 ml of the same buffer. A 0.48-ml portion of the cell suspension was withdrawn and was supplemented with 0.5 ml of lysozyme solution in the same sucrose buffer (0.2 mg/ml) and 0.02 ml of 0.5 M EDTA to form protoplasts. After incubation at 37°C for 15 min, 0.2 ml of protoplast suspension was diluted with 0.8 ml of cold distilled water, causing lysis. Cellular debris was pelleted by a 30-min centrifugation in a refrigerated Eppendorf (Hamburg, Germany) centrifuge (14,000 rpm). MADH in periplasmic extracts was measured in accordance with a previously described procedure (13). Protein concentrations and the precise concentrations of lysozyme in the lysozyme solutions were measured as described earlier (36). The lysozyme concentrations were subtracted to obtain concentrations of cellular proteins in extracts. Computer analysis was carried out by using PCGENE (Genofit SA, Geneva, Switzerland) and GCG (University of Wisconsin Genetics Computer Group) software.

Cloning of the *mau* gene cluster from *M. flagellatum* KT. The mixed oligonucleotide probe described above hybridized with an approximately 4.5-kb fragment of a *Hin*dIII digest of the *M. flagellatum* KT chromosome. Fragments of the chromosome with sizes of 4 to 5 kb were isolated and used to generate a partial library in pUC19 of 600 colonies. One positive colony was found to contain a 4.5-kb *Hin*dIII insert (plasmid pGak1). The sequencing of one end of this plasmid and comparison with other *mau* sequences (8, 9) showed that the insert contained the entire gene for the MADH small subunit (*mauA*) and a 3′-terminal portion of *mauD*. To clone upstream genes, a second library was constructed. Restriction analysis showed the presence of a *Pst*I site in the insertion of pGak1, and *Pst*I excised a 2.6-kb fragment from pGak1. This fragment was isolated and used to probe a *Pst*I digest of the *M. flagellatum* KT chromosome, and it hybridized with an 8- to 8.5-kb *Pst*I fragment. A chromosomal fraction with fragment sizes of 8 to 9 kb was isolated and used to construct the second partial clone library with the pRK310 vector. This library was probed with the 2.6-kb *Pst*I fragment. One positive colony was found per each 50 colonies of the library. Plasmids from 12 positive colonies were isolated and shown to contain the same 8.5-kb *Pst*I insert.

Preliminary sequencing of the 4.5-kb *Hin*dIII fragment had revealed the presence of open reading frames for *mauA* and *mauD*. Since complete or partial nucleotide sequences of several *mau* gene clusters were available (7–9, 33, 35), partial sequencing was performed to identify open reading frames having substantial similarity to known *mau* genes and thus to define the *mau* gene cluster organization. The sequencing strategy was based on the assumption that the *mau* gene cluster from *M. flagellatum* KT might resemble that from *M. methylotrophus* W3A1-NS. Nine partial or complete open reading frames (*mauFBEDAGLMN*) transcribed in the same direction that showed substantial similarity in the regions sequenced (33 to 95% identity) to their counterparts from *M. methylotrophus* W3A1-NS were identified (data not shown). These results suggest that the order of *mau* genes in these two strains is the same. Since *M. flagellatum* KT has been reported to contain amicyanin (11), it has been assumed that this strain contains an amicyanin-coupled MADH. Therefore, we expected to find *mauC* (encoding amicyanin) in the *M. flagellatum* KT *mau* cluster. Instead, this gene was missing, as it is in *M. methylotrophus* W3A1-NS, suggesting that an alternative electron acceptor might be involved in methylamine oxidation in this strain.

In order to search for other *mau* genes, areas adjacent to the *mau* gene cluster were also sequenced. In contrast to the case with any of the other known *mau* clusters, an open reading frame was found 137 bp downstream of *mauN* (the last gene of the *mau* cluster), transcribed in the orientation opposite to that of *mauN*. A search of GenBank revealed that this open reading frame encodes a polypeptide highly similar to the class of cupredoxins termed azurins. The nucleotide sequence of the *azu* gene and its alignment with other azurins are shown in Fig. 1. The *azu* gene encodes a polypeptide of 148 amino acids with a molecular mass of 15,875 Da (premature azurin). Several potential hairpin structures were present in the sequenced area. A hairpin structure with predicted energies of production of −20.6 kcal/mol (ca. −86.2 kJ/mol) is located between *mauN* and *azu*. This hairpin structure closely resembles a ρ-independent terminator of *E. coli.*

Generation of an insertion mutation in *azu*. In order to obtain information about the function of *azu*, an insertion mutation was generated in this gene by homologous recombination between the chromosome and a plasmid containing an insertion in the gene. The Km^r cassette from pUC4K was inserted in the gene at a *Hin*cII site 0.3 kb 3′ to the start of *azu* in such an orientation that *aph* was transcribed in the same direction as *azu*. The construct with the mutagenized gene was recloned into the suicide vector pAYC61 and introduced into the *M. flagellatum* KT chromosome by conjugation and selection on plates with methanol and kanamycin. Approximately 90 to 95% of the transconjugants analyzed were Km^r Ap^s Tc^s, the phenotype indicative of double-crossover recombination. Chromosomal DNAs of these mutants were hybridized with pUC4K and the corresponding DNA fragments which were used in generating the mutants. In all cases a 1.4-kb increase in the size of the mutagenized fragments was observed, confirming the presence of only the Km^r cassette (data not shown).

The *azu* mutant (305c-1) grew substantially more slowly on methylamine plates than the wild type, and growth rates were measured for each. Methanol-grown cultures were inoculated (1:150) into liquid medium with methanol or methylamine as the source of carbon to measure growth rates. Both strains grew on methanol with similar growth rates ($\mu = 0.105$ for the wild type and $\mu = 0.127$ for 305c-1). The growth rate of the wild type on methylamine ($\mu = 0.073$) was approximately twice that of the 305c-1 *azu* mutant ($\mu = 0.034$). In addition, the *azu* mutant had a lag phase after transfer from methanol to a methylamine-containing medium that was 20 to 30 h longer than that of the wild type. Methylamine-grown cells of the wild-type and *azu* mutant strains had similar in vitro MADH activities.

Azurin is an electron acceptor for MADH in some methylotrophs (2, 31). Our data show that an *M. flagellatum* KT mutant defective in this gene grows poorly on methylamine, even though it contains normal in vitro MADH activity, as measured with an artificial electron acceptor. These data suggest that azurin is part of the electron transfer chain for MADH in *M. flagellatum* KT but that it is not an obligatory component. The slow growth on methylamine observed in the azurin mutants was probably due to the ability of an alternate electron acceptor, possibly a cytochrome *c* or the other cupre-

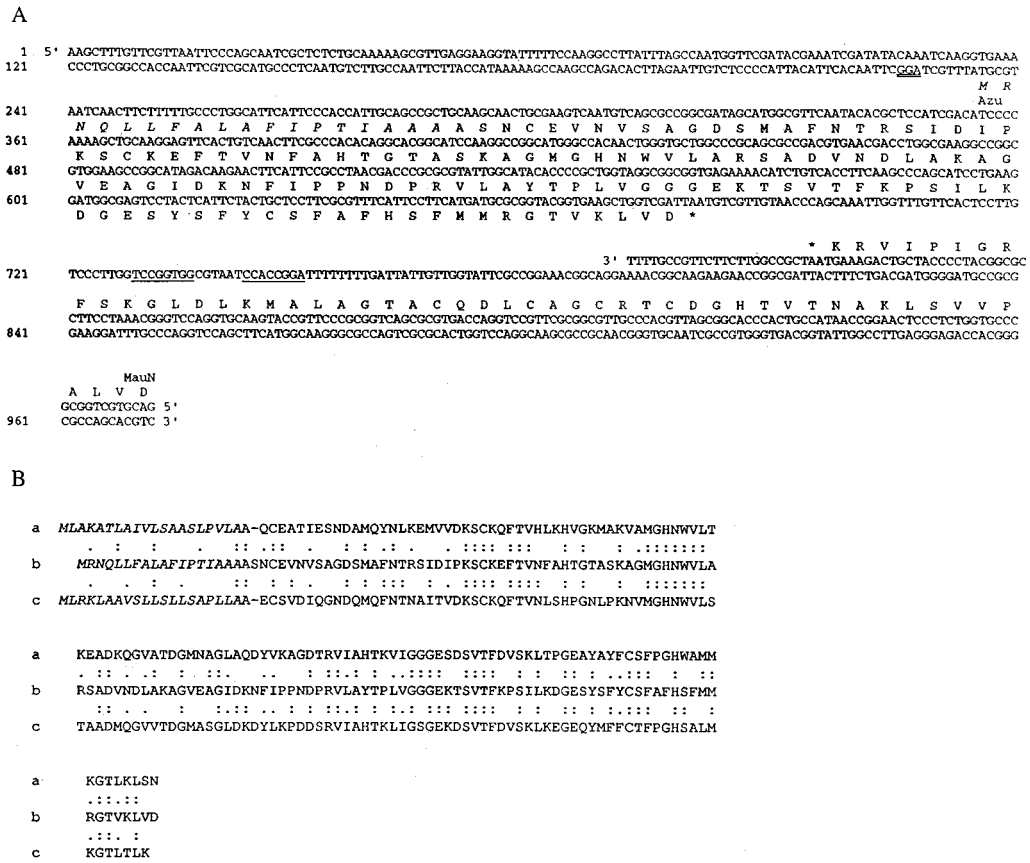

FIG. 1. (A) Nucleotide sequences of the area downstream of the *mau* gene cluster containing *azu*. A putative Shine-Dalgarno sequence is double underlined, hairpin structures are underlined, and a putative leader sequence is italicized. Asterisks denote termination codons. GenBank accession number is L37436. (B) Alignment of deduced amino acid sequence of azurins from *Alcaligenes denitrificans* (18) (a), *M. flagellatum* KT (b), and *Pseudomonas aeruginosa* (5) (c). Identical residues are indicated by double dots, and conserved substitutions are shown by single dots. Putative leader sequences are italicized.

doxin found in this strain, to inefficiently couple methylamine oxidation to the electron transport chain as has been observed in organism 4025 (3). Our data suggest that the MADH in *M. flagellatum* KT may use azurin as an immediate electron acceptor. Alternatively, another, unidentified protein may be the immediate electron acceptor and azurin may be involved later in the electron transport chain.

Nucleotide sequence accession numbers. The GenBank accession numbers for the nine partial or complete *M. flagellatum* KT open reading frames (*mauFBEDAGLMN*) that showed substantial similarity in the regions sequenced to their counterparts from *M. methylotrophus* W3A1-NS are L37426, L37427, L37428, L37429, L37433, L37434, L37435, and L37436.

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