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Methylotrophy in Freshwater *Beggiatoa alba* Strains[▽]

Talia Jewell, Sherry L. Huston, and Douglas C. Nelson*

Department of Microbiology, University of California, Davis, California

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Two freshwater strains of the gammaproteobacterium *Beggiatoa alba*, B18LD and OH75-2a, are able to use methanol as a sole carbon and energy source under microoxic conditions. Genes encoding a methanol dehydrogenase large-subunit homolog and four enzymes of the tetrahydromethanopterin-dependent C_1 oxidation pathway were identified in B18LD. No evidence of methanotrophy was detected.

Methylotrophs are an ecologically significant group of microorganisms that can use reduced one-carbon (C_1) compounds, such as methane, methanol, methylated amines, and methylated sulfur compounds, as their sole source of carbon and as an oxidizable energy source. These microorganisms are globally important as a major sink for greenhouse gases and as key members in microbial communities around cold seeps, mud volcanoes, and other environments rich in C_1 compounds (3, 8, 24, 29).

Members of the filamentous, colorless, sulfide-oxidizing genus *Beggiatoa* are often found in methane-rich environments, where they may form conspicuous mats (1, 25), but their abundance has been assumed to be linked to high rates of biogenic sulfide production due to anaerobic methane oxidation. Metabolism of individual *Beggiatoa* strains ranges from obligate chemoautotrophy to organoheterotrophy (20, 21). Certain marine *Beggiatoa* assemblages incorporate CO_2 derived from methane oxidation (13, 18), but to date there has been no demonstration that they are capable of directly oxidizing and incorporating methane, methanol, or other reduced C_1 compounds. Here we describe for the first time the ability of a *Beggiatoa* strain (B18LD) to grow as a methylotroph, which, coupled with prior documentation of its ability to grow on multicarbon compounds, renders it a facultative methylotroph.

Our first evidence of methylotrophy in *Beggiatoa alba* strain B18LD (ATCC 33555) came from the accidental amplification and cloning of a gene encoding formaldehyde-activating enzyme (*fae*) from acetate-grown cells. The nucleic acid sequence amplified by PCR using degenerate *nifH3* primers (32)—here serving as both forward and reverse primers—had 75% identity to the *fae* gene of *Methylobacillus flagellatus* KT. This gene encodes a tetrahydromethanopterin (H_4MPT)-dependent enzyme that may function in either methylotrophy or formaldehyde detoxification. The genetic potential for methylotrophy in B18LD was explored by seeking genes within the linear C_1 oxidation pathway (Fig. 1), using previously published primers and PCR conditions (10, 11, 15, 23, 30). The partial sequences of NAD-linked methylene-H_4MPT dehydrogenase (encoded by *mtdB*; 47% nucleic acid identity to top NCBI match), methenyl-H_4MPT cyclohydrolase (encoded by *mch*; 60%), and the D subunit of the formyltransferase/hydrolase complex (encoded by *fhcD*; 71%) were identified. Primers for *mxaF*, encoding the large alpha subunit of pyrroloquinoline quinone-linked methanol dehydrogenase (MDH), amplified a 513-bp sequence from B18LD, which when translated had significant homology to both MxaF (71%) and to the MxaF homolog XoxF (83%). To resolve this ambiguity, the phylogenetic relationship was examined in an unrooted tree using the distance method with the unweighted-pair group method with arithmetic mean (UPGMA) and a 1,000 bootstrap analysis (Fig. 2). The B18LD sequence clustered with the XoxF sequences.

The genomes of three other species capable of growth on methanol, *Rhodobacter sphaeroides*, *Methylibium petroleiphilum* PM1, and *Methylophilales bacterium* HTCC2181, contain only *xoxF*, whereas the genomes of *Paracoccus denitrificans* and *Methylobacterium extorquens* contain both *mxaF* and *xoxF* (2, 4, 12, 28, 31). Until recently, XoxF was not believed to be involved in methylotrophy, and the function was unknown (12, 28). Wilson et al. (31) demonstrated MDH activity in *R. sphaeroides* XoxF, a protein that shares 83% identity with the translated *xoxF* sequence from B18LD. In contrast, the B18LD

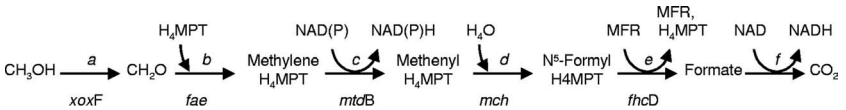

FIG. 1. Proposed pathway for oxidation of methanol to CO_2 in *Beggiatoa alba*. *a*, pyrroloquinoline quinone-linked methanol dehydrogenase homolog; *b*, H_4MPT-linked formaldehyde activating enzyme; *c*, NAD(P)-linked methenyl-H_4MPT dehydrogenase; *d*, methenyl-H_4MPT cyclohydrolase; *e*, formyltransferase/hydrolase complex; *f*, formate dehydrogenase. Adapted from the work of Lidstrom (14).

* Corresponding author. Mailing address: Department of Microbiology, 357 Briggs Hall, University of California, Davis, CA 95616. Phone: (530) 752-6183. Fax: (530) 752-9014. E-mail: dcnelson@ucdavis.edu.

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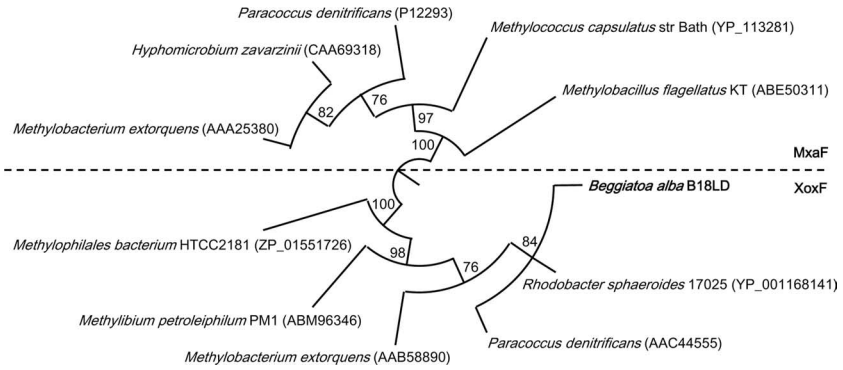

FIG. 2. Phylogenetic distance tree of MxaF and XoxF. Nodes supported by bootstrap values greater than 70% have been retained.

sequence shares only 50% identity with *M. extorquens* XoxF, a protein that does not posses MDH activity (2).

We do not know if the B18LD genome also contains *mxaF*. To date, the only *Beggiatoa* partial genome sequence available, obtained by repeated sequencing of single filaments, belongs to a marine morphotype (17). An NCBI BLAST search of those contigs did not reveal any homology to *xoxF* or to the H_4MPT-linked *fae*, *mtdB*, *mch*, or *fhcD* nucleic acid or translated amino acid sequences identified in this study.

A previous attempt by Mezzino et al. (16) to culture strain B18LD aerobically on methanol in liquid medium was unsuccessful, which matches the inconsistent results we obtained for liquid cultivation (data not shown). Therefore, the ability of strain B18LD to grow as a methylotroph was examined using sulfide-gradient medium (19) prepared with SuperPure agar (U.S. BioTech Sources LLC, EI Sobrante, CA) and supplemented with methanol or acetate as the sole carbon source. Growth, measured as total protein in the horizontal *Beggiatoa* "plate" (22), was determined as previously described (6). The purity of the B18LD cultures was confirmed by phase-contrast microscopy (400×) throughout the experiment. The amount of biomass strain B18LD produced (Fig. 3) was positively correlated with the methanol concentration in the medium. The possibility of mixotrophy, with sulfide serving as an electron donor and methanol serving solely as the carbon source, was also examined (Fig. 4). With the pH of the bottom agar in the medium lacking sulfide carefully adjusted to match that of the medium containing sulfide, no significant difference in yields (day 17 or day 20) could be attributed to the presence (2 or 4 mM) or absence of sulfide. The yield on day 10 for 4 mM sulfide was significantly greater (P value of >0.02 and <0.05) than for the other two treatments; however, we observed that the dense, horizontal plates of *Beggiatoa* growth were more compact and easier to visualize, due to sulfur deposition, in the presence of 4 mM sulfide, and we speculate that this facilitated more-complete harvesting. Taken together, these data (Fig. 3 and 4) indicate that methanol can serve as both the carbon and energy source for B18LD and that biomass increases with the concentration, up to 100 mM. Methylotrophy was also demonstrated for the closely related *Beggiatoa* strain OH75-2a, which shares 99% 16S rRNA sequence identify with B18LD (Fig. 5). Using the data shown in this figure and from an identical experiment performed using B18LD (data not shown), we estimate the molar growth yield, Y_{methanol}, to be 6.6 and 9.0 g dry weight per mol methanol for OH75-2a and B18LD, respectively. Since metabolized substrates will form gradients in agar-gelled media (22), determining the diffusional limitation on consumption of added methanol is problematic. For both strains, the molar growth yield on acetate is known (5, 20, 27). Thus, by assuming equal diffusion coefficients for methanol and acetate, yields in acetate-supple-

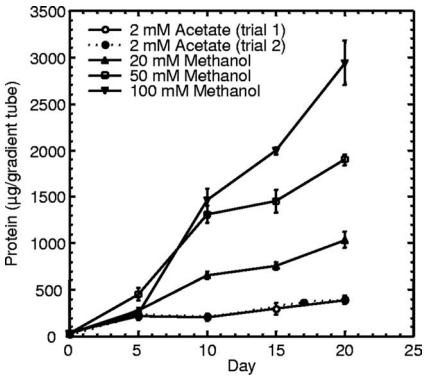

FIG. 3. Yield versus time for B18LD in gradient media with 4 mM sulfide in the bottom agar and methanol or acetate as the sole added carbon source in the top agar layer. Data shown are the means of protein determinations on triplicate cultures ± standard errors.

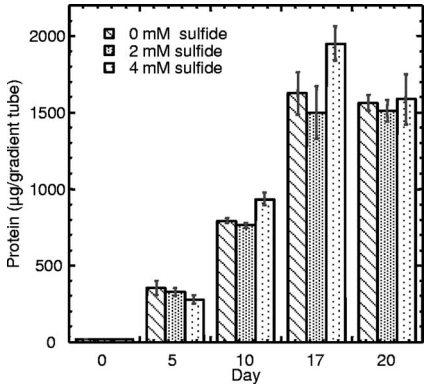

FIG. 4. Comparison of yield versus time for strain B18LD in gradient media containing 20 mM methanol in the top agar and 0, 2, or 4 mM neutralized sodium sulfide in the bottom agar. Data shown are the means for triplicate cultures ± standard errors.

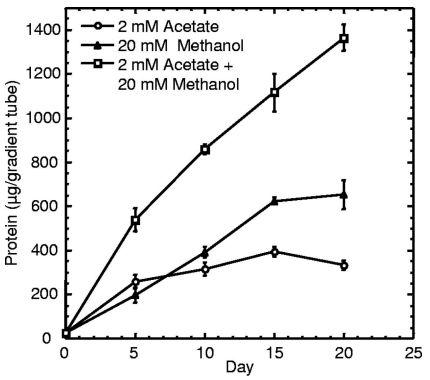

FIG. 5. Yield for *B. alba* strain OH75-2a in gradient media with acetate and/or methanol as the carbon source in the top agar layer and 4 mM sulfide in the bottom layer. Data shown are the means for triplicate culture tubes ± standard errors.

mented gradient media can be translated into the quantity of methanol consumed (methanol or methanol plus acetate) (Fig. 5), thereby permitting calculation of corresponding methanol molar growth yields. With robust growth on methanol and acetate and on a variety of 2-, 3- and 4-carbon organic substrates (16), B18LD is a facultative methylotroph, a property uncommon among gammaproteobacterial methylotrophs (14).

Other potential substrates for methylotrophy—dimethylsulfoxide, dimethylsulfide, and methylamine—are initially metabolized by other microbes using separate pathways that merge with the methanol oxidation pathway at the level of formaldehyde. For strain B18LD, there was no evidence of growth in gradient medium containing dimethylsulfoxide, dimethylsulfide, or methylamine (20 mM each). This strain was also unable to grow in gradient medium supplemented with 2 mM formaldehyde. In addition, no evidence of methanotrophy, as determined by an increase in biomass when grown under a 50% air -50% methane atmosphere, was found for B18LD in the absence or presence of Cu^{2+}, NH^{4+} or a limiting concentration of methanol. We were also unable to amplify PCR products with significant homology to methane monooxygenase genes using primers that target conserved regions (7, 26). Therefore, it appears that B18LD is incapable of methanotrophy.

It remains to be determined if methylotrophy is a widespread trait among freshwater and marine *Beggiatoa* species. *Beggiatoa* and relatives are already known, based on massive natural abundance in a variety of environments, to play prominent roles in marine sulfur and nitrogen cycles (9). Here we offer the added possibility that they have a measurable impact on the cycling of C_1 compounds in addition to CO_2.

Nucleotide sequence accession numbers. The nucleic acid sequences determined in this study have been deposited in the GenBank database under accession numbers EU367933 to EU367937.

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