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Localization of Methanol Dehydrogenase in Two Strains of Methylotrophic Bacteria Detected by Immunogold Labeling†

THERESA A. FASSEL,[1,2]‡ LORIE A. BUCHHOLZ,[2] MARY LYNNE PERILLE COLLINS,[1,2] AND C. C. REMSEN[1,2]*

Department of Biological Sciences[1] and the Center for Great Lakes Studies,[2] The University of Wisconsin-Milwaukee, Milwaukee, Wisconsin 53201

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Antibodies to methanol dehydrogenase purified from *Methylobacterium* sp. strain AM1 and *Methylomonas* sp. strain A4 were raised. The antibody preparations were used in indirect immunogold labeling studies. With this approach, methanol dehydrogenase was found to be preferentially localized to the periplasmic region of the methylotroph *Methylobacterium* sp. strain AM1 and to the intracytoplasmic membrane of the methanotroph *Methylomonas* sp. strain A4. Antibody cross-reactivity to other methylotrophic bacteria was detected.

Methane and other one-carbon compounds, including methanol, formaldehyde, and formate, are oxidized by methylotrophic microorganisms. Methanotrophic bacteria, which are a subgroup of the methylotrophs, are capable of using methane as a sole source of carbon and energy (27). Methanol dehydrogenase (MeDH; EC 1.1.99.8) is an NAD(P)-independent enzyme of broad substrate specificity. In both methylotrophic and methanotrophic bacteria, MeDH catalyzes the oxidation of methanol to formaldehyde. Formaldehyde is assimilated through one of two metabolic pathways or is further oxidized to CO_2 in reactions catalyzed by formaldehyde dehydrogenase and formate dehydrogenase (2).

MeDH has been found to be distributed in the soluble and particulate fractions of methylotrophs (9, 16, 26). In methylotrophs, MeDH has been suggested to be a peripheral membrane protein (10, 12) localized on the periplasmic face of the membrane (1, 12, 13). A periplasmic localization in methanotrophs is consistent with the occurrence of an amino-terminal signal sequence on the MeDH of the facultative methanotroph *Methylobacterium organophilum* (15).

Methanotrophs are distinguished from other methylotrophs by the presence of an intracytoplasmic membrane (ICM). The ICM exists in two types of arrangements: vesicular stacks of membranes located throughout the cell and pairs of peripheral membranes located parallel to the cell envelope. The former are characteristic of methanotrophs of type I and type X, and the latter are characteristic of type II methanotrophs (27). The ICM structure and biochemical characteristics form the basis for classification of methanotrophs (27). The formation of an ICM in methanotrophs is dependent on growth conditions (4, 20, 23, 25); methylotrophs do not form an ICM. It has been suggested that the ICM is continuous with the cytoplasmic membrane (3, 7, 23, 24). This continuity between the ICM and the cytoplasmic membrane further implies continuity between the periplasm and intra-ICM space, as has been found in phototrophic bacteria, which also contain ICMs (5).

This study was undertaken to evaluate by immunogold labeling the localization of MeDH in a methanotrophic bacterium and a methylotrophic bacterium. An indirect, postembedding labeling procedure was chosen over a direct labeling approach because signal amplification increased sensitivity (11). In this report, we describe immunogold labeling of the MeDH in a marine methanotroph isolate, *Methylomonas* sp. strain A4, and a methylotroph, *Methylobacterium* sp. strain AM1.

MATERIALS AND METHODS

Cell growth conditions. *Methylobacterium* sp. strain AM1 was grown in a medium that contained 9.8 mM K_2HPO_4, 10.9 mM NaH_2PO_4, 15.1 mM $(NH_4)_2SO_4$, 0.8 mM $MgSO_4 \cdot 7H_2O$, 0.5% methanol, and trace elements [15.3 μM $ZnSO_4 \cdot 7H_2O$, 10 μM $CaCl_2 \cdot 2H_2O$, 5.1 μM $MnCl_2 \cdot 4H_2O$, 3.6 μM $FeSO_4 \cdot 7H_2O$, 0.2 μM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.3 μM $CuSO_4 \cdot 5H_2O$, 1.4 μM $CoCl_2 \cdot 6H_2O$, 26.8 μM EDTA]. Cultures were incubated at 30°C with shaking at 150 rpm. *Methylomonas* sp. strain A4 (14) was grown on nitrate mineral salts (27) with vitamins (14a) and 1.0% phosphate buffer in a fermentor with an atmosphere of 20% methane and 80% air at 37°C. All other methanotrophs (*Methylomonas albus* BG8, *Methylosinus trichosporium* OB3b, strain 68, *Methylocystis* sp. strain LW, and *Methylococcus capsulatus* Bath [21]) were grown on nitrate mineral salts (27) at the appropriate temperature with shaking at 150 rpm.

MeDH antibody purification. MeDH was purified from strains AM1 and A4 by a procedure modified from that of Nunn and Lidstrom (18). The suspended cells were broken by two freeze-thaw cycles and three treatments with a Stansted cell disruptor (Stansted Fluid Power Ltd., Stansted, United Kingdom). The extracts were centrifuged for 30 min at 30,000 × *g*, and the supernatant fraction was retained. MeDH was precipitated from this fraction with ammonium sulfate (50 to 95% saturation), resuspended in 20 mM Tris-HCl (pH 8.0), and dialyzed. The dialyzed preparation was applied to a DEAE-cellulose column, and the material eluting in the void volume was applied to a hydroxyapatite column. This column was washed with 20 mM potassium phosphate (pH 7.0), and MeDH fractions were eluted with 90 mM potassium phosphate. The dialyzed fractions were applied to a DEAE Sepharose-CL6B column, which was washed with 10 mM Tris-HCl (pH 8.0). MeDH was eluted with 50 mM Tris-HCl and dialyzed and concentrated by ultrafiltration with a PM 20 membrane filter. Purity was

* Corresponding author.
† Contribution no. 354 from the Center for Great Lakes Studies.
‡ Present address: Muscle Biology Laboratory, University of Wisconsin-Madison, Madison, WI 53706.

assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

MeDH antibody preparation and immunoblot analysis. Antiserum was obtained from New Zealand White female rabbits immunized with MeDH preparations by Cocalico Biological Inc., Reamstown, Pa. Two rabbits each were immunized with 1.0 mg of AM1 and A4 MeDH in Freund's complete adjuvant and then given two 0.1-mg booster injections at 1-month intervals. Whole serum was fractionated by DEAE-cellulose chromatography (17). Antibody preparations were adsorbed with crude extracts of *Escherichia coli*. SDS-PAGE and immunoblot analysis were performed as previously described (6). Antigens for analysis were crude cell extracts obtained by cell rupture in a French pressure cell. Filter-bound antibodies were detected with radioiodinated protein A or horseradish peroxidase.

Immunogold labeling and electron microscopy. Exponentially growing cells were harvested, fixed in 0.5% glutaraldehyde in 0.05 M potassium phosphate buffer (pH 7.0) for 1 h, and washed in buffer. Some preparations intended for ultrastructural analysis but not immunolabeling were postfixed in 1% OsO_4 in potassium phosphate buffer. Before postfixation or immunogold labeling, fixed cells were encased in 4% agar. Samples were dehydrated in ethanol, infiltrated in LR White (London Resin Co., Ltd.) resin as specified by Polysciences, Inc. (19a), and polymerized in a 60 to 63°C oven for 20 to 24 h. An indirect labeling approach modified from the method of DeMey (8) was used.

Thin sections mounted on nickel grids were (i) incubated for 30 min in 20-μl drops of 5.0% normal goat serum diluted in 0.1% bovine serum albumin (BSA) buffer (20 mM Tris, 150 mM NaCl, 1 mg of BSA per ml [pH 8.2]) to block nonspecific reactions, (ii) transferred to primary anti-MeDH rabbit antibody diluted in BSA buffer and incubated overnight at 4°C, (iii) washed in a multiple-grid holder with a perforated top and bottom for 30 min while being stirred with two changes of BSA buffer, and (iv) incubated for 4 h in a suspension of 10-nm gold particles conjugated to anti-rabbit immunoglobulin G (Sigma Chemical Co., St. Louis, Mo.) diluted 1:50 or 1:75. Before use, the suspension of gold particles was centrifuged for 10 min at 5,000 × *g* to remove microaggregates of gold. Finally, grids were washed as in step iii, rinsed in distilled water, dried, poststained in ethanolic uranyl acetate and Reynolds lead citrate (22), and then viewed in an Hitachi H-600 STEM CR/CR electron microscope at 75 kV. To demonstrate the specificity of the reaction, controls with gold-conjugated secondary antibody alone and with preimmune serum were performed. Concentrations of primary-layer antibody ranged from 0.27 to 0.12 μg/ml for A4 and 0.4 to 0.07 μg/ml for AM1.

RESULTS

Antibody specificity. SDS-PAGE analysis of purified MeDH showed large and small subunits with respective apparent molecular masses of 60 and 12 kDa from AM1 and 60 and 10 kDa from A4 (data not shown). The specificities of the anti-MeDH antibodies obtained from rabbits immunized with these preparations were evaluated by immunoblot analysis. Immunoblots of whole-cell extracts of AM1 (Fig. 1, lane a) and A4 (lane b) analyzed with the homologous anti-MeDH antibody demonstrate the specificity of each antibody preparation for the large subunit of MeDH. In some blots the small subunit of MeDH was detected.

Immunolocalization of MeDH in *Methylobacterium* sp. strain AM1. MeDH was localized to the periplasm of AM1.

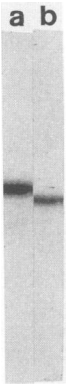

FIG. 1. Immunoblot analysis of antibody specificity. Lanes: a, crude cell extract of AM1 analyzed with anti-AM1 MeDH; b, crude cell extract of A4 analyzed with anti-A4 MeDH.

Longitudinal sections through cells show that immunogold particles were specifically localized in the periplasmic region at the cell periphery (Fig. 2A); occasional labeling of the cytoplasm is not above background level. Occasionally the polar region was exposed by sectioning. Where this occurred, an area of dense labeling was observed (Fig. 2B and C). Figure 2B is a high-magnification view of a section through the extreme end of the cell showing labeled periplasm. Figure 2C is a less distal section showing densely labeled periplasm and minimally labeled cytoplasm. The specificity of the labeling is demonstrated by comparison to the preimmune (Fig. 2D) and gold-only (Fig. 2E) controls, which show only a low level of randomly located gold particles.

Immunolocalization of MeDH in A4. Abundant ICMs typical of type I methanotrophs were observed in *Methylomonas* sp. strain A4 cells preserved by sequential glutaraldehyde and OsO_4 fixation (arrow in Fig. 3A). These ICMs were only slightly less well preserved after fixation with glutaraldehyde alone in specimens prepared for immunogold labeling (Fig. 3B through E). Indirect immunogold labeling was used to localize the MeDH in *Methylomonas* sp. strain A4 (Fig. 3B through D). The gold particles were associated with the ICM in the immunolabeled specimens (Fig. 3B through D). In some cases (Fig. 3B) the angle of sectioning did not reveal details of ICM structure (arrow), whereas at other sites the ICM stacks were perpendicular to the angle of sectioning and appear as in Fig. 3A. Cell areas not containing ICM were not labeled. In contrast, the preimmune control (Fig. 3E) and the gold-only control (data not shown) showed only minimal nonspecific background labeling.

Applications of MeDH immunolabeling. The specific immunolabeling of MeDH, an enzyme unique to methylotrophs, suggested the possible use of this approach to identify methanotrophs in environmental samples. Although we have observed bacteria with ICM structures typical of methanotrophs in samples from aquatic environments (21), it has not been possible to conclusively identify these bacteria as methanotrophs. The possibility of using MeDH immunolabeling for this application was evaluated.

Cross-reactivity of anti-A4 MeDH antibody with several strains of methanotrophs was tested. Antibody to MeDH prepared from A4 reacted with the extracts of all methanotrophs tested by immunoblot analysis (Table 1). Cross-reactivity was observed for the ca. 60-kDa subunit and in some cases for the 10-kDa subunit of each MeDH of each

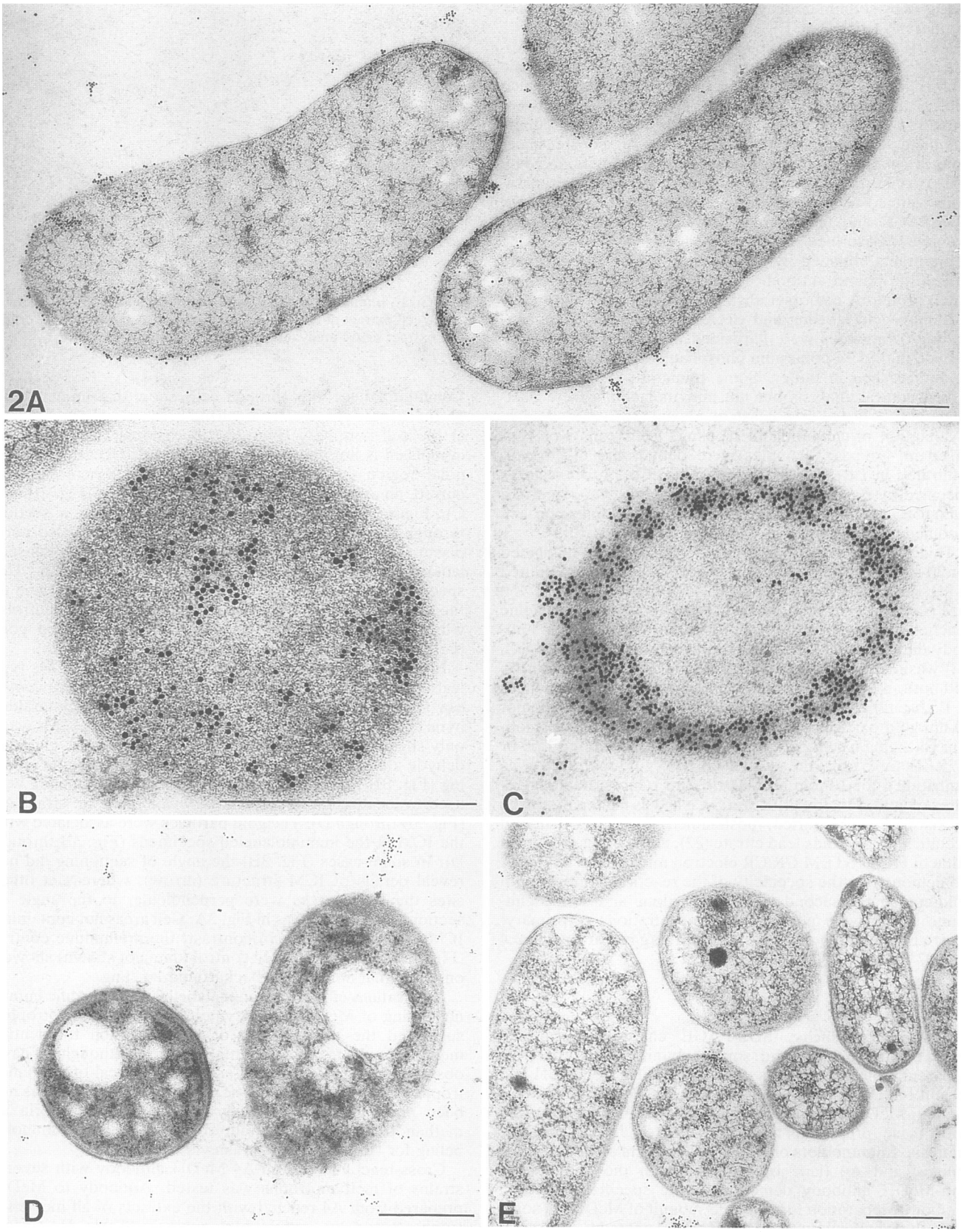

FIG. 2. Immunogold labeling experiment for *Methylobacterium* sp. strain AM1. (A) Low-magnification thin section showing label in the periplasmic area; (B and C) fortuitous sections through polar region of cell exposing epitopes (note dense halo around the cell in panel C); (D) preimmune control cells; (E) gold-only control. Bars, 0.5 μm.

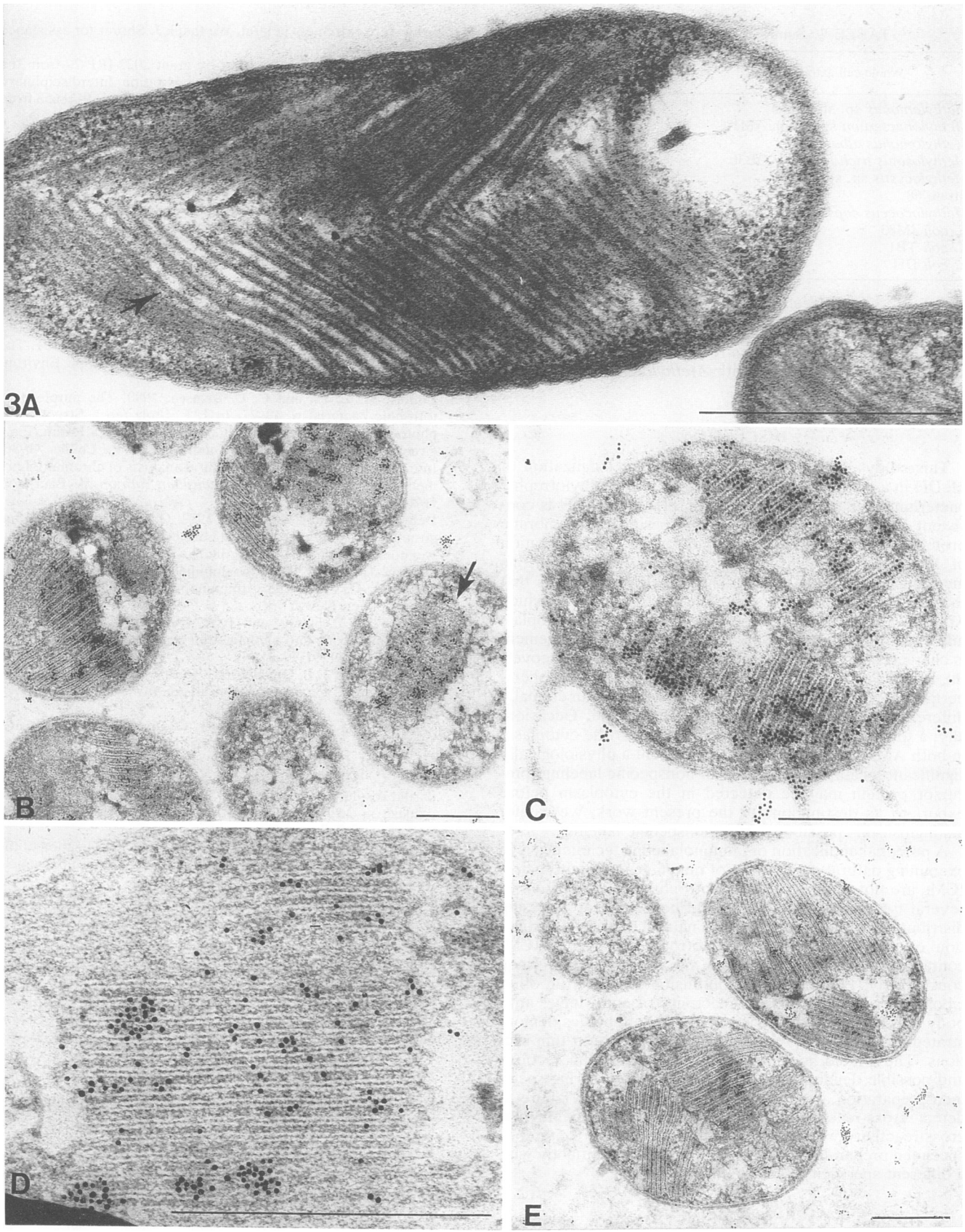

FIG. 3. Immunogold labeling experiments for *Methylomonas* sp. strain A4. (A) Thin section of specimen postfixed with OsO_4, showing well-preserved type I ICM stacks (arrow); (B) low-magnification view showing localization of immunogold on the ICM (arrow shows tangential section through the ICM); (C and D) high-magnification views showing dense ICM immunolabeling; (E) preimmune control. Bars, 0.5 μm.

TABLE 1. Summary of immunoblot results

Whole-cell extract	Cross-reactivity with anti-A4 MeDH
Methylomonas sp. strain A4	+++
Methylobacterium sp. strain AM1	+++
Methylomonas albus BG8	++
Methylosinus trichosporium OB3b	+
Methylocystis sp. strain LW	+
Strain 68	++
Methylococcus capsulatus Bath	++
E. coli JM83	−
E. coli TB1	−
E. coli DH	−

extract. However, when antibody reactivity was evaluated in thin sections, the only cross-reactivity that was observed, albeit inconsistently, was with *Methylococcus* sp. strain Bath (data not shown).

DISCUSSION

This study demonstrates the intracellular localization of MeDH in a methanotrophic bacterium and a methylotrophic bacterium. The periplasmic location of AM1 MeDH is consistent with the localization of this peripheral membrane protein in other species of methylotrophs. The localization of MeDH in the ICM of A4 indicates the role of this membrane in C_1 metabolism. This localization implies that the periplasm is continuous with the intra-ICM space, which would be consistent with continuity between the cytoplasmic membrane and the ICM. Such a structural arrangement is characteristic of the phototrophic bacteria (5). Moreover, the labeling of the ICM region of A4 but not the peripheral periplasmic region suggests that the ICM is functionally differentiated from the cytoplasmic membrane. Occasionally, a few gold particles were observed over the cytoplasm in both AM1 and A4. This could represent a physiologically significant localization rather than nonspecific labeling; precursor protein may be detected in the cytoplasm before export to its destination. In the present work, we cannot distinguish this possibility from nonspecific labeling.

A potential application of immunolabeling techniques is in examining natural populations of mixed genera and species. ICMs are not limited to methanotrophs and are present in several types of bacteria. It would be helpful in assessing distributions of microorganisms in natural samples if distinction of an ICM-bearing methanotroph from other ICM-containing bacteria (for example, nitrifiers) could be made conclusively. Previous immunodiffusion studies from other laboratories suggested cross-reactivity of anti-MeDH antibody (19, 28). However, our anti-MeDH antibodies demonstrated cross-reactivity in immunoblots but not in thin sections. The limited accessibility of epitopes in thin sections and possible denaturation of protein antigens during specimen preparation are probably responsible for the failure to detect cross-reactive immunolabeling. It is possible that cross-reactivity would be detected with another method of specimen preparation or by using a polyclonal antibody with a different specificity.

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