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Separation of Methanotrophic Bacteria by Using Percoll and Its Application to Isolation of Mixed and Pure Cultures†

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Type I and II methanotrophic bacteria can be cleanly separated by using Percoll buoyant density gradients. This simple and inexpensive method can be used to screen new isolates rapidly for preliminary classification. In addition, Percoll gradients can also be used to enhance standard enrichment techniques for methanotrophs from natural water samples.

Methylotrophs are bacteria capable of utilizing methane as their sole source of carbon and energy (2). The methanotrophic bacteria that have been isolated and studied are all gram-negative, obligately aerobic rods, cocci, or vibrios (15). They develop extensive arrays of intracytoplasmic membranes (ICM) which serve as a means of classification together with biochemical criteria such as DNA G+C content, major carbon assimilation pathway, (in)ability to fix atmospheric nitrogen, and the presence or absence of a tricarboxylic acid cycle (15). Two general groups of methanotrophs have been defined by the above characteristics. Type I methanotrophs possess bundles or stacks of ICM in the cell interior, have a G+C value of 50 to 54%, are unable to fix atmospheric nitrogen, and lack a complete tricarboxylic acid cycle. Type II methanotrophs are characterized by ICM arranged as a system of paired peripheral membranes, have a G+C value of 58 to 66%, are able to fix nitrogen, and possess a complete tricarboxylic acid cycle. A type X designation has been suggested for the strain *Methylococcus capsulatus* Bath, which has certain biochemical differences from the type I group but possesses the type I ICM pattern (15). Currently, the main methods for distinguishing between type I and II methanotrophs are biochemical assays for key assimilatory enzymes and electron microscopic characterization of membrane patterns.

Percoll is a density gradient medium consisting of colloidal particles coated with a layer of polyvinylpyrrolidone (10). It has achieved a wide range of uses in the separation of cellular and subcellular particles (8–10, 12, 14). Several studies have employed Percoll gradients for isopycnic separation and isolation of bacteria, including strains of cyanobacteria and purple sulfur bacteria, from lake water (4); harvesting of marine planktonic algae (11); recovery of microorganisms from soil and from root nodules (7); and separation of syntrophic cultures of cells, enabling easier study of the component strains (3).

(Portions of this work were part of an M.S. thesis presented by K.P.P. to the Department of Biological Sciences, University of Wisconsin—Milwaukee.)

We report here the use of Percoll gradients for separation and isolation of type I and II methanotrophic bacteria. Pure cell cultures of type I, X, and II methanotrophic bacteria were grown in a liquid nitrate-mineral salts medium as described by Whittenbury and Dalton (15). The culture flasks were filled with a 50% methane (99% pure) and 50% air mixture. Incubation was at 30°C for all strains except *M. capsulatus* Bath (42°C) and a Menomonee River strain (37°C). The flasks were shaken at 100 to 200 rpm.

Percoll gradients were made by initially preparing a stock solution consisting of nine parts of Percoll to one part of 1.5 M NaCl (vol/vol). This solution had a density of ca. 1.123 g/ml, depending on the exact density of the undiluted Percoll (1). A working solution was made by adding equal parts of the Percoll stock solution to 0.15 M NaCl. The final density of the working solution was ca. 1.061 g/ml. Density gradients were made by dispensing 10 ml of the working solution into 16-ml polycarbonate tubes. Ten microliters of each standardized Density Marker Bead (DMB) was added to a control tube. The tubes were centrifuged for 45 min at 15,000 × *g* (11,500 rpm) in a Sorvall RC-5 refrigerated high-speed centrifuge with an SS-34 rotor. Twenty-microliter cell suspensions (from 1.5 ml of a culture with an optical density at 650 nm of 0.5 that was pelleted and resuspended to ca. 20 ml in spent medium) were then gently layered over the gradient, and the tubes were recentrifuged for 15 min under the same conditions. DMB and cell band locations were measured from the bottom of the meniscus and plotted as DMB location versus DMB buoyant density (Fig. 1).

The buoyant densities of four strains of type I and three strains of type II methanotrophs were determined by sedimentation on Percoll gradients (Table 1). The type I density range was 1.084 to 1.116 g/ml, with an average of 1.093 g/ml. The type X methanotroph *M. capsulatus* Bath banded within the density range for type I microorganisms. The three different strains of type II methanotrophs banded within the density range of 1.039 to 1.052 g/ml, with an average of 1.047 g/ml. With one exception (strain 68), the buoyant density values for all of the strains were derived from a minimum of two distinct buoyant density experiments.

The reproducibility of the density measurements for a given strain was very good (Table 1), and the density range representing either type of methanotroph was quite constant. In addition, separation of two types on the gradient was easily observed and recorded, and a cell suspension consisting of type I and II methanotrophs was separated into two distinct cell bands during centrifugation (Fig. 2).

To test the efficacy of using Percoll gradients for isolation and/or enrichment of methanotrophs from the natural environment, multiliter samples of Menomonee River water from

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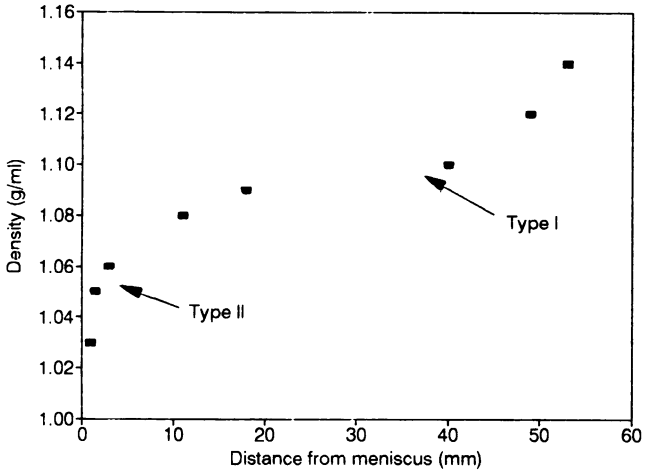

FIG. 1. Correlation between DMB location and buoyant density. The positions of type I and II methanotrophs are indicated.

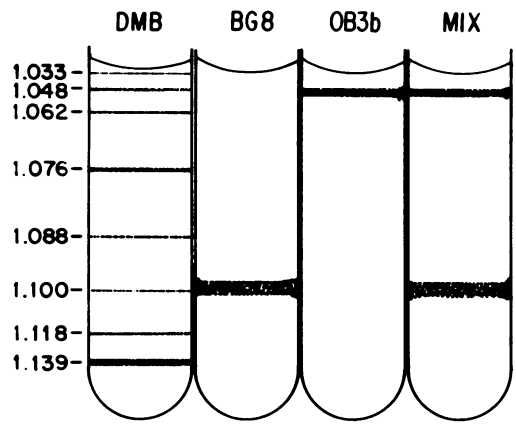

FIG. 2. Graphic representation of Percoll gradients showing a DMB control tube and the locations of a type I (*M. albus* BG8) band, a type II (*M. trichosporium* OB3b) band, and a mixture of the two.

a site in Milwaukee, Wis., known to contain methanotrophs were pelleted by using the Sorvall KSB Continuous-Flow Centrifugation System, also known as the Szyent-Georgi and Blum system. The KSB system was used in either of two different ways. First, pelleting of material was carried out on two preformed, sterile Percoll gradients. Next, those fractions that represented the banding area for type I or II methanotrophs were pooled from both gradients and recentrifuged at 15,000 × *g* for 30 min on new Percoll gradients. Cell bands that were present in the type I or II area were removed from the tube by using a sterile syringe and needle and placed on a fresh gradient for further separation. The cell bands were then used either for attempts at isolation of methanotrophic bacteria and/or fixation and examination with a transmission electron microscope (TEM). Second, the KSB system was used to collect pellets of particulate river material. These pellets were pooled, recentrifuged, and centrifuged on Percoll gradients. Cell bands were then treated as discussed above.

Cell material collected for TEM examination was fixed with 0.5% glutaraldehyde in 0.1 M phosphate buffer and then postfixed with 1% OsO_4 in 0.1 M phosphate buffer. Cells were then mixed in 4% agar, allowed to solidify, cut into 1-mm^3 cubes, and dehydrated through a graded ethanol series. Final embedding was done in either Spurr's or LR White resin. Thin sectioning was performed by using glass knives on an LKB 8000 Ultramicrotome III. After staining with ethanolic uranyl acetate and Reynolds lead citrate, thin sections were examined on a Hitachi HU-11B-2 TEM.

Subsurface water samples were collected from the Menomonee River at depths with the lowest oxygen and highest methane concentrations, and particulate material was concentrated with the KSB system as described above. Cell bands corresponding to the region for type I methanotrophs were observed at a buoyant density of approximately 1.100. When examined by TEM (Fig. 3), the bacterial cells from this band contained ICM of the type I pattern. No cell bands were observable at the density range for type II methanotrophs, suggesting that type I-like strains predominate in this environment. While these microorganisms were not specifically identified as type I methanotrophs (by biochemical or other means) the ICM arrangement observed in these cells has been found only in type I methanotrophs or ammonia-oxidizing bacteria.

The demonstration that pure and mixed cultures of methanotrophic bacteria exhibit buoyant densities that are representative of their type designations allows the use of Percoll gradients as a simple and rapid type identification criterion and, potentially, as a method of concentrating and enriching methanotrophic bacteria from aquatic environments. Previously, the process of screening isolated methanotrophs as

TABLE 1. Location of methanotrophs on Percoll gradients

Strain	Type	Density, g/ml (mean ± SD)[a]	Source or reference[b]
Methylomonas albus BG8	I	1.090 ± 0.002 (5)	16
Methylobacter capsulatus Y	I	1.089 ± 0.003 (3)	16
Methylomonas sp. strain MN	I	1.107 ± 0.009 (3)	M. E. Lidstrom
Strain 68	I	1.093 (1)	6
Marine strain A4	I	1.085 ± 0.001 (2)	5
Methylococcus capsulatus Bath	X	1.094 ± 0.003 (3)	16
Methylosinus trichosporium OB3b	II	1.050 ± 0.002 (5)	16
Methylocystis parvus OBBP	II	1.045 ± 0.006 (3)	16
Methylocystis sp. strain LW	II	1.045 ± 0.006 (3)	M. E. Lidstrom
EPA[c] landfill strain	II	1.045 ± 0.005 (2)	M. E. Lidstrom

[a] The numbers of individual trials are in parentheses.
[b] Reference or source of original isolation.
[c] EPA, Environmental Protection Agency.

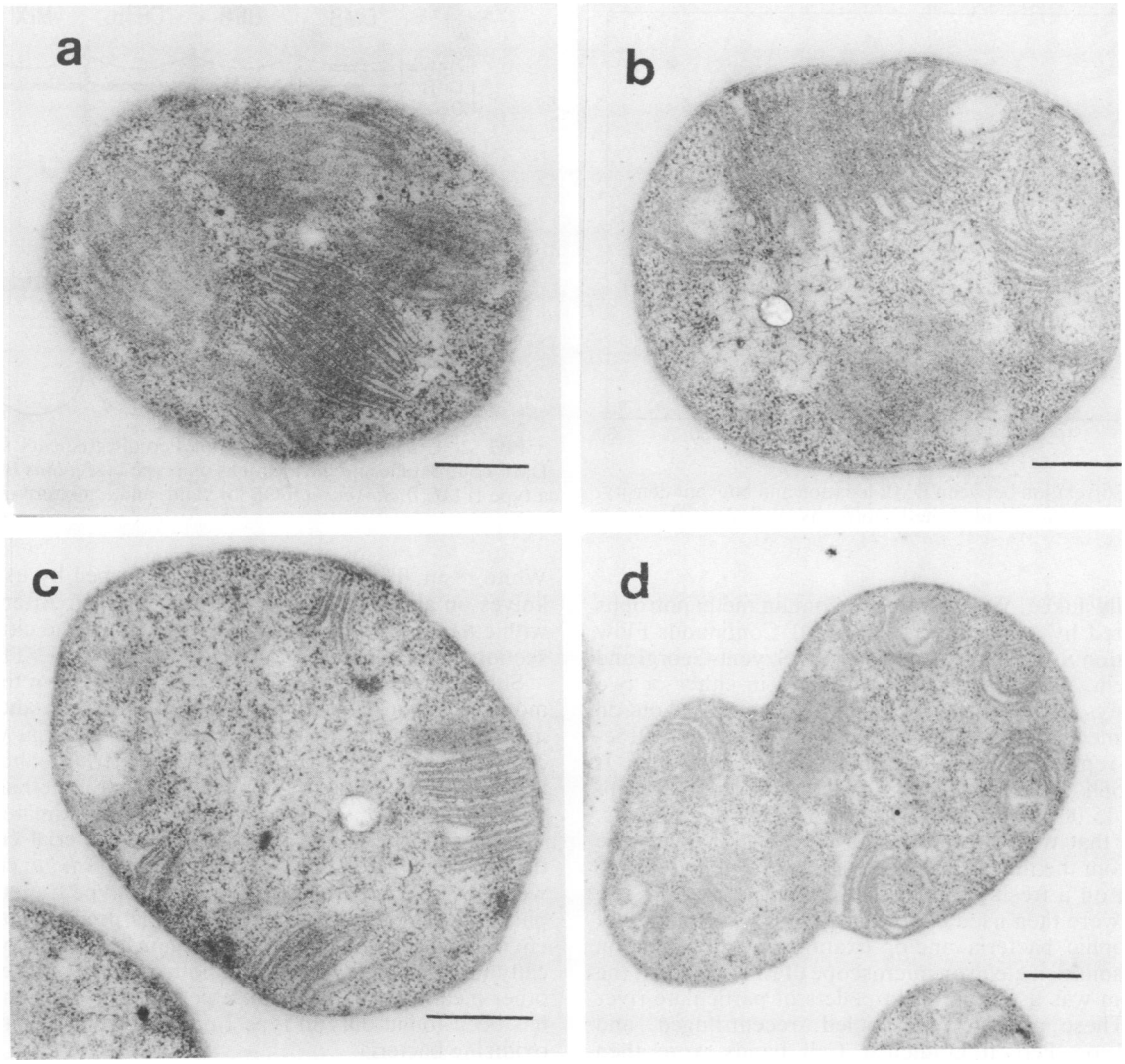

FIG. 3. TEM of bacteria from Menomonee River water located in the Percoll gradient band corresponding to type I methanotrophs. Note that all cells have the type I ICM pattern. The bars represent 0.5 μm.

either type I or II required a significant time investment and expensive equipment. It was necessary either to grow large volumes of cells for spectrophotometric enzyme assays to identify the carbon assimilation pathway used and/or to prepare the cells for examination by TEM so that the ICM arrangement could be determined (2) or to probe the cells with type-specific oligonucleotides (13). Our data suggest that Percoll buoyant density determinations can distinguish between the two groups. Furthermore, in combination with continuous-flow centrifugation, it may be possible to utilize Percoll density gradients as a means of concentrating type I and II methanotrophic bacteria directly from natural samples. Since these gradients can be run under sterile conditions, the appropriate band area can then be removed and used for plating, further enrichment, TEM examination, or probing with oligonucleotides.

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