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Applications of a Colorimetric Plate Assay for Soluble Methane Monooxygenase Activity

DAVID W. GRAHAM,[1] DICK G. KORICH,[2] RONALD P. LEBLANC,[3] NORVAL A. SINCLAIR,[2] AND ROBERT G. ARNOLD[1]*

Department of Civil Engineering and Engineering Mechanics[1] and Department of Microbiology and Immunology,[2] University of Arizona, Tucson, Arizona 85721, and James M. Montgomery Engineers, Pasadena, California 91101[3]

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A straightforward method is described for screening methanotrophic colonies for soluble methane monooxygenase (sMMO) activity on solid media. Such activity results in the development of a colored complex between 1-naphthol, which is formed when sMMO reacts with naphthalene, and *o*-dianisidine (tetrazotized). Methanotrophic colonies expressing sMMO turned deep purple when exposed successively to naphthalene and *o*-dianisidine. The method was evaluated within the contexts of two potential applications. The first was for the enumeration of *Methylosinus trichosporium* OB3b in a methane-amended, unsaturated soil column dedicated to vinyl chloride treatment. The second application was for the isolation and enumeration of sMMO-bearing methanotrophs from sanitary landfill soils. The technique was effective in both applications.

Methanotrophic bacteria are capable of initiating the degradation of a variety of environmental pollutants including trichloroethylene, the isomers of dichloroethylene, vinyl chloride, and chloroform (9, 11, 12, 21). This phenomenon results from the broad substrate specificity of the initial enzyme in the pathway for methane catabolism, methane monooxygenase (MMO) (2, 12, 17). Methanotrophs possess two types of MMO, a soluble MMO (sMMO) and a particulate, membrane-bound MMO (pMMO). sMMO is considerably more effective than pMMO at catalyzing such nonenergy-yielding transformations; however, sMMO is expressed only in type II (and type X) methanotrophs that have been grown under severely copper-limited conditions (18, 19). Conversely, type I organisms appear to express only pMMO (6).

Methylosinus trichosporium OB3b is the best studied of the type II methanotrophs (1, 2, 4, 12, 18). The sMMO of *M. trichosporium* OB3b is exceptionally effective in terms of its ability to catalyze biodegradative reactions, although the physical and chemical conditions under which optimum activity occurs are quite restrictive (1, 18). In this organism and other type II methanotrophs, the expression of sMMO is repressed by Cu(II) concentrations as low as 0.25 μM (18). The copper level for sMMO repression varies somewhat depending on the ambient oxygen tension and species identity (16). Thus, the ability to generate sMMO activity under many natural conditions is problematic, although highly desirable from the standpoint of in situ bioremediation.

Here we describe a procedure to screen bacteria for sMMO expression when grown on solid media. The procedure may have utility for (i) the isolation and enumeration of sMMO-bearing strains and (ii) the selection of mutant strains that can express sMMO in the presence of comparatively high Cu(II) concentrations. The procedure is an application of a method developed by Wackett and Gibson (20) for assessing monooxygenase activity in fungi and bacteria. It is based on observations that (i) some bacterial monooxygenases catalyze the conversion of naphthalene to 1-naphthol and (ii) naphthol formation can be monitored colorimetrically by the addition of certain aromatic diazo compounds to the reaction mixture (13).

Brusseau et al. (1) demonstrated that various type II methanotrophs bearing sMMO (including *M. trichosporium* OB3b) catalyze the naphthalene reaction in liquid cultures. Type X strains, such as *Methylococcus capsulatus*, also catalyze this reaction (5). The conversion of naphthalene to naphthol has been used as an indicator of trichloroethylene degradation potential in methanotrophs (1). *o*-Dianisidine, which turns purple in the presence of naphthol, was used as a coloring agent. As utilized here, these methods permit rapid screening of monoclonal colonies for sMMO activity. The utility of such a procedure for strain augmentation, isolation, and mutant selection is evident.

MATERIALS AND METHODS

Procedure development. Unless otherwise indicated, experiments to develop procedures involved *M. trichosporium* OB3b (ATCC 35070). The growth medium was low-nitrate NSM (3), pH 7.0, consisting of the following: $NaNO_3$, 1.0 mM; K_2SO_4, 1.0 mM; $MgSO_4 \cdot 7H_2O$, 0.15 mM; $CaCl_2 \cdot 2H_2O$, 47.6 μM; KH_2PO_4, 3.9 mM; Na_2HPO_4, 6.0 mM; $ZnSO_4 \cdot 7H_2O$, 2.0 μM; $MnSO_4 \cdot 7H_2O$, 1.6 μM; H_3BO_3, 6.0 μM; $NaMoO_4 \cdot 2H_2O$, 0.4 μM; $CoCl_2 \cdot 6H_2O$, 0.4 μM; KI, 1.0 μM; $FeSO_4 \cdot 5H_2O$, 40 μM. Copper sulfate and EDTA (Sigma Chemicals Ltd.) were added from freshly prepared 10 mM stock solutions to the (culture-dependent) final concentrations indicated in Table 1. Solid media consisted of the liquid growth medium solidified with 1.0% (wt/vol) Noble agar (Difco Laboratories).

Liquid cultures of *M. trichosporium* OB3b were grown in 1,000-ml flasks at 30°C in copper-free NSM with a headspace concentration of between 15 and 20% methane-air. The flasks were agitated on a rotary shaker table (New Brunswick Scientific Co.) maintained at 240 rpm. The headspace gas-to-liquid ratio in the culture flasks was always greater than 5 to 1. During logarithmic growth, the cultures were serially diluted in sterile 5 mM phosphate buffer (pH 7.0), and 50 μl of the 1.0×10^{-3} dilution was spread plated. A

* Corresponding author.

cyclohexane sMMO activity assay (12) was performed on the cultures prior to dilution to establish the presence or absence of sMMO activity at the point of dilution and plating.

Plates were incubated at 30°C under a 25% methane-air atmosphere for 7 to 21 days to allow development of colonies of different age and size. Every 2 to 3 days the incubator (BBL anaerobic jar) was opened and replenished with air and methane. Plates were removed intermittently, and colonies were screened for sMMO activity by the following procedure. A few naphthalene crystals were sprinkled in the lid of the plate, and the plate was stored inverted at 30°C for 15 min in air. The plates were then opened and lightly sprayed with freshly prepared, 5-mg/ml *o*-dianisidine (tetrazotized; zinc chloride complex; Sigma Chemicals Ltd.), for 2 to 3 s. The lid was replaced, and the plate was stored for 15 min in the presence of the dye. If naphthol was produced by the colonies, a purple-red color appeared upon contact with the dye. The color, once formed, remained stable for at least 24 h at room temperature and up to a month at 4°C.

Procedures for assessing the responses of nonmethanotrophic strains were functionally the same as for the methanotrophs with the following exceptions. Pure cultures of locally isolated phenol-degrading bacteria, several known degraders of 2,4-dichlorophenoxyacetic acid (2,4-D), and a strain of *Escherichia coli* were used to establish the limits and specificity of the test (Table 1). When nonmethanotrophs were employed, both liquid and solid media were supplemented with 20 mg of yeast extract (Difco Laboratories) per liter and with 200 mg of phenol per liter (phenol degraders only) or 500 mg of 2,4-D (Sigma Chemicals) per liter (2,4-D degraders only).

In media for one set of 2,4-D-degrading cultures, 2,4-D was omitted to evaluate the effect of a potentially competitive carbon source on the naphthalene reaction. 2,4-D-degrading strains were known to tolerate high mercury levels and constitutively produce 2,4-D-degrading dioxygenase (7). Consequently, 25 mg of Hg(II) per liter was added to solid media to preclude the growth of contaminants. Phenol degraders were unidentified pure strains isolated from local oil-contaminated soils.

Applications. (i) Enumeration of *M. trichosporium* OB3b in sand samples. Sands were collected from an unsaturated, methane-amended sand column that was used to simulate vadose zone transformation of vinyl chloride (10). Methane and vinyl chloride were passed in a downflow mode through a 3-in (ca. 7.6-cm)-diameter sand column. Under appropriate conditions, both gases were readily transformed in the column. There was no liquid feed to the column, and water content varied considerably with position (see Table 2). Although column materials had been seeded with *M. trichosporium* OB3b only, other methanotrophic species (uncharacterized) developed in large numbers during the 50-day course of vinyl chloride degradation experiments.

Bacterial numbers were estimated in nine samples. Three were collected at the top of the column, three at middepth, and three at the bottom. The nine samples were washed in sterile 0.1% PP_i buffer and serially diluted. Dilutions were plated in triplicate on copper-free NSM plates. The dilutions were also plated on 2 μM Cu(II) NSM plates (sufficient copper to permit expression of pMMO in methanotrophs) for enumeration of total methanotrophs (see procedure below). Plates were incubated in the presence of methane for 2 weeks, as previously described, after which they were removed, brought into contact with naphthalene, and colorimetrically assayed for naphthol formation.

Colony counts were performed on the 2 μM Cu(II) plates to estimate the number of culturable organisms present. Twenty colonies from the plates were transferred to liquid NSM with 20% headspace methane to estimate the fraction of colonies formed that could use methane in liquid culture. The liquid cultures were maintained in 28-ml, septum-capped culture tubes that contained 5 ml of either copper-free or 2 μM Cu(II) NSM. Agitation was provided via end-over-end rotation at 100 rpm (Roto-torque tube rotator; Cole Parmer). Those that could not grow on methane in liquid culture were assumed to be nonmethanotrophic. The ratio of methane-utilizing colonies to the total transferred (to liquid) was used to recalculate the total methanotrophic numbers from the original plate count.

Total cell numbers were estimated by acridine orange direct counts (AODC). The cells were washed with PP_i and diluted as before but were counted by fluorescence microscopy after contact with acridine orange in a 2% formaldehyde solution.

(ii) Isolation of type II methanotrophs from landfill soils. Soil samples were collected from the top 1.5 feet (ca. 46 cm) of surface soil at the Puente Hills Sanitary Landfill in Los Angeles County, Calif. Soil gas concentrations of methane varied from 0 to 10 ppm at the sites sampled.

The soil samples were prepared by washing and serial dilution in copper-free 0.1% PP_i buffer and then plated directly onto copper-free Noble agar NSM plates. After a 2-week incubation period, the plates were exposed to naphthalene and sprayed with dye for color development. Colonies that turned purple were transferred to copper-free liquid NSM with a 20% methane headspace to confirm their methanotrophic character. Purple colonies were also restreaked onto fresh plates to (i) dilute copper that might have carried over from the original soils, (ii) establish the ability to rescue cells following naphthalene-naphthol exposure and color development, and (iii) verify color development. Colonies that regrew were transferred into copper-free liquid NSM and grown to an optical density of >0.5 (A_{600} cm^{-1}) for cyclohexane-liquid sMMO enzyme assays. If the culture utilized methane and showed sMMO activity in liquid culture, it was assumed to be a methanotrophic strain with exceptional cometabolic capabilities (similar or identical to *M. trichosporium* OB3b). Such isolates were assumed to be type II strains because (i) type I strains do not react with naphthalene and (ii) although type X strains also exhibit a colorimetric response in the naphthalene assay, they have been isolated only at temperatures >37°C (1, 5, 6).

Controls were maintained throughout the above procedures. Landfill soils were mixed with column sands that contained *M. trichosporium* OB3b (positive control) and subjected to the procedures described above. Non-color-forming colonies were regrown in liquid medium and tested for cyclohexane transformation to estimate the frequency of false-negative responses to the colorimetric procedure.

RESULTS AND DISCUSSION

Procedure development. Figure 1a shows color development following naphthalene-dye treatment by colonies of *M. trichosporium* OB3b that were grown on copper-free solid medium. The color of *M. trichosporium* OB3b colonies on NSM-Noble agar solid medium was white. The lack of color development among colonies that were grown on copper-free plates and exposed to naphthalene but not treated with dye is apparent in Fig. 1b. Colonies that were not exposed to naphthalene but sprayed with *o*-dianisidine (not shown) were

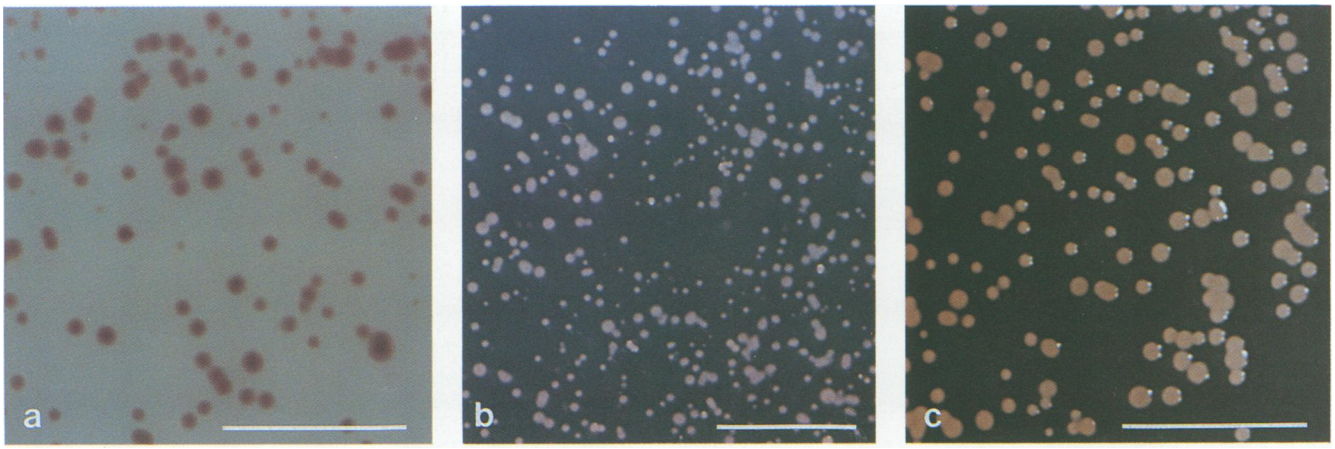

FIG. 1. Use of naphthalene–*o*-dianisidine assay method to identify sMMO-bearing colonies of *M. trichosporium* OB3b. (a) *M. trichosporium* OB3b grown in copper-free solid medium on methane, exposed to naphthalene, and brought into contact with *o*-dianisidine (tetrazotized). (b) *M. trichosporium* OB3b grown on copper-free solid medium on methane, and exposed to naphthalene (no *o*-dianisidine added). (c) *M. trichosporium* OB3b grown on 1.0 μM Cu(II) solid medium on methane, exposed to naphthalene, and brought into contact with *o*-dianisidine. Note the tan discoloration on the left side of the photograph. This color is typical of unreacted dye exposed to air and light. Different backgrounds for the pictures were required to provide adequate contrast for the color responses. Bars, 10 mm.

identical. The colonies in Fig. 1c were grown on 1 μM Cu medium, exposed to naphthalene, and brought into contact with dye. No purple pigment was formed. However, a tan discoloration of the colonies and growth medium was observed. This discoloration probably resulted from the light-enhanced decomposition of unreacted dye (13, 15).

Figure 2 illustrates the consequences of over addition of dye to a plate. Under such conditions, colony definition became a problem as cells were washed across the surface of the plate. In an effort to resolve this problem, a blotting method was assessed. Dye was sprayed on photographic blotting paper, and the paper was briefly contacted with the plate surface. This method developed color appropriately; however, it was difficult to blot the colonies without damaging them. Replica plating was also assessed as a step in the coloring procedure but proved unacceptable because of poor colony regrowth. As a result, the spray method was adopted, the reagent was aspirated as a fine mist, and care was taken to avoid pooling on the plate surface.

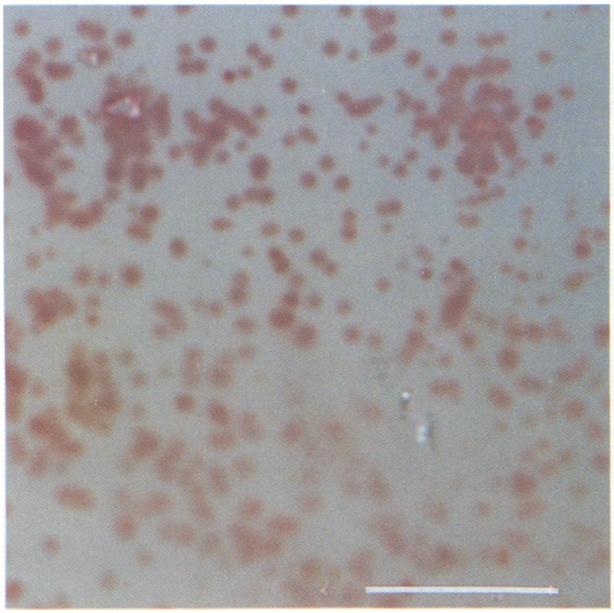

FIG. 2. Colonies of *M. trichosporium* OB3b on copper-free medium where excess *o*-dianisidine was added to the plate surface. Note the diffusion of color on the lower half of the photograph. Bar, 10 mm.

False-positives occasionally resulted from this procedure. Color developed in colonies that had been grown on copper-bearing medium only when the colonies were permitted to grow too large. Colonies became too large in two cases: (i) when the plates were not harvested within 5 weeks of original plating and (ii) when the colonies were streak plated rather than spread plated. With large colonies, copper is probably exhausted locally and copper-deficient, sMMO-bearing organisms develop within the colony. The best way to avoid this problem is to screen colonies only when they are small and discrete. False-positives did not occur in colonies of *M. trichosporium* OB3b that were less than 3 mm in diameter.

When the mineral salts medium was supplemented with EDTA at 50 μM, the intensity of color that developed on the plates was weakened (Table 1). On the basis of previous liquid culture experiments, it is unlikely that EDTA blocked MMO expression or activity (8; unpublished results). Liquid-phase, abiotic experiments in which the naphthol-dianisidine coupling reaction was monitored colorimetrically with and without EDTA indicated that color was significantly weaker in the presence of EDTA. EDTA may react with the zinc complex of the dye, thereby destabilizing the dye.

Thirty colonies (*M. trichosporium* OB3b) that gave positive colorimetric responses were restreaked on copper-free media. All 30 colonies yielded new growth, indicating that the screening procedure is nonlethal to *M. trichosporium* OB3b. It was found that the color assay procedure was also nonlethal to the unidentified type II species DG1 (a new type II isolate). Implications are that the method is nonlethal to type II strains and should permit recovery of organisms with favorable cometabolic traits.

Additional experiments were performed using methanotrophs other than *M. trichosporium* OB3b and non-

TABLE 1. Color development by various organisms grown on solid media as a function of medium composition

Organism	Carbon source	NMS amendment	Color[a]
M. trichosporium OB3b	CH_4	0 μM Cu	++
		0 μM Cu, 50 μM EDTA	+
		1 μM Cu	−
		1 μM Cu, 50 μM EDTA	−
		2 μM Cu	−
M. albus BG8	CH_4	0 μM Cu	No growth
		2 μM Cu	−
M. methanica (ATCC 35067)	CH_4	0 μM Cu	No growth
		2 μM Cu	−
Unidentified type II methanotroph, species DG1	CH_4	0 μM Cu	++
		2 μM Cu	−
Phenol-degrading isolate	Phenol	20 mg of YE[b]/liter	−
Pseudomonas putida PP0301(pR0103)	None	20 mg of YE/liter, 25 mg of Hg(II)/liter	−
	2,4-D	20 mg of YE/liter	−
Pseudomonas cepacia TL 249(pR0103)	None	20 mg of YE/liter, 25 mg of Hg(II)/liter	−
	2,4-D	20 mg of YE/liter	−
Escherichia coli HB101(pR0103)	None	20 mg of YE/liter, 25 mg of Hg(II)/liter	−
	2,4-D	20 mg of YE/liter	−
Alcaligenes eutrophus(pJP4)	None	20 mg of YE/liter, 25 mg of Hg(II)/liter	−
	2,4-D	20 mg of YE/liter	−

[a] ++, strong color, intense red-purple developed; +, weak color, washed-out pink; −, no color.
[b] YE, yeast extract.

methanotrophic bacteria (Table 1). Neither *Methylomonas methanica* nor *Methylomonas albus* BG8, both type I organisms, developed color in the presence or absence of copper. Neither of these organisms produces an sMMO. Test procedures were positive, however, for unidentified type II species DG1.

All of the 2,4-D-degrading and phenol-degrading isolates produced no color when tested in the manner described. The presence or absence of 2,4-D did not influence the ability of the 2,4-D-degrading organism to react with naphthalene and develop color. The color response appears to be unique to monooxygenase-bearing organisms and, among methanotrophs, to those expressing sMMO.

Methanotroph enumeration in the sand column. Table 2 presents a summary of cell counts, including numbers of color-forming colonies (assumed to be *M. trichosporium* OB3b), total methanotrophs, and total cell numbers (AODC) from the sand column. Estimates are provided for three positions in the column. Color-forming methanotrophs were concentrated at the bottom of the column, where the fractional water content was the highest. The non-color-forming methanotrophs were an order of magnitude less numerous than color-forming strains in the high-water-content zone. Water content appears to play an important role in the enrichment of color-forming strains.

AODC counts were 2 orders of magnitude higher than the highest of the color assay plate counts. This implies that there were either more nonmethanotrophs than methanotrophs in the column or the colorimetric assay underestimated the number of color-forming organisms present. Plates counts with methanotrophs are known to underestimate the total populations (6).

TABLE 2. Cell counts in sand column samples[a]

Location	Fractional water content (vol/vol)	Total no. of methanotrophs/g[b]	No. of color-forming methanotrophs/g[c]	AODC (no./g)[d]
Top	0.003	3.8×10^5	1.9×10^5	5.2×10^7
Middepth	0.004	3.3×10^5	0.9×10^5	6.0×10^7
Bottom	0.10	6.6×10^6	6.4×10^6	1.1×10^8

[a] All counts were done in triplicate. Mean values are provided.
[b] Based on percentage of colonies that utilized methane when transferred to 2 μM Cu(II) medium.
[c] Color assay method. Presumed to be type II strains.
[d] AODC of washed and diluted samples.

Type II methanotroph isolation from landfill soils. Tables 3 and 4 summarize the results obtained with the color assay method as applied to landfill soil samples. Three soil samples were evaluated. The three soils were similar in terms of water, organic, and copper characteristics but different in terms of site methane concentration. Two samples, M-1 and M-2, were from methane-active zones in the cover soils of the sanitary landfill. The other sample, NM-1, was from a methane-free zone that provided cover material for landfill operations. To provide a positive control, NM-1 was split in two and one-half was blended 10 to 1 (wt/wt) with sand collected from the bottom of the vinyl chloride treatment column (NM-1:spiked). The bottom sand sample had been shown to have numerous color-forming strains present (Table 2).

The soils were diluted and plated at dilutions of 5.0×10^{-4}, 5.0×10^{-5}, and 5.0×10^{-6}. Colonies formed from all samples at all dilutions within approximately 2 weeks. M-1 and M-2 generated the greatest number of colonies, both having greater than 300 colonies per plate at a dilution of 5.0×10^{-5}.

After 2 weeks, the plates were brought into contact with naphthalene and dye. Thirty-two colonies were selected for further testing, 24 that developed some degree of color upon contact with the dye and 8 that did not generate color but were well defined on copper-free plates. Table 3 identifies the sources of colonies used for further study. Strong and weak color corresponded to the color intensity in the colony after dye addition. Color intensity was a function of colony size.

The results of subsequent analyses involving the 32 selected colonies are summarized in Table 4. All colonies grew when they were restreaked onto copper-free plates; however, only 26 of 32 grew and utilized methane when they

TABLE 3. Organisms isolated from landfill soils

Soil sample	In situ methane (ppm)[a]	Fractional water content[b]	Organic content (%)[c]	Leachable Cu (μg/g)[d]	Selected colonies[e]		
					No color	Weak color	Strong color
M-1	10	0.18	4.5	0.35	C1, C2, C3, C7, C8	8, 17, 18, 23, 24	None
M-2	10	0.184	3.8	0.23	C4, C5, C6	5, 6, 7, 9, 10, 11, 12, 15, 16, 19, 20, 21, 22	None
NM-1	Trace	0.125	3.1	0.14	None	4	None
NM-1:spiked[f]	N/A[g]	N/A	N/A	N/A	None	None	1, 2, 3, 13, 14

[a] Measured at site. Vadose zone ambient methane content of soils.
[b] Measured on a volume per volume basis.
[c] Measured on a weight per weight basis.
[d] Measured as weight of copper per weight of soil. Leachable Cu after 1 h of agitated contact in 100 μM EDTA solution.
[e] Identification numbers of colonies which were selected for further study. Color designation based on size of colony and intensity of color developed.
[f] Sample NM-1 amended, 10 to 1 (wt/wt), with sand from the bottom of the sand column (Table 2).
[g] N/A, not available.

were transferred from the original plates into liquid media. When colonies were restreaked prior to the establishment of liquid cultures, 28 utilized methane and grew. Of the 24 color-forming colonies, only the 5 colonies that were deeply colored on the original plates developed color again when tested on the restreaked plates. Cyclohexane assays were performed on all liquid cultures that consumed methane. Again, only cultures that had developed strong color on plates (and developed color following restreaking) converted cyclohexane to cyclohexanol. All liquid media used to assay for cyclohexane conversion were found to contain less than 0.25 μM Cu(II).

From the foregoing, it is apparent that the landfill soils contained relatively few, if any, methanotrophs of the class represented by *M. trichosporium* OB3b, i.e., methanotrophs that are capable of expressing sMMO lacking substrate specificity. The false-positives that were obtained in the initial screening steps are easily dismissed; the original plates were crowded and color development was extremely weak. It is possible that color formation was a consequence of dye reacting with other metabolites present (20). The frequency with which organisms grew without copper and without a naphthalene-degrading sMMO was unexpected and cannot be explained satisfactorily without a detailed look at the taxonomy of the methanotrophic isolates.

The results imply that the naphthalene plate assay can differentiate between color-forming and noncolor-forming methanotrophs when the colonies (and therefore the color responses) are distinct. There were no false-negatives among the eight noncolored colonies subjected to further testing. Colonies that strongly developed color proved capable of expressing sMMO. The false-positives observed in the initial screen were rapidly reclassified during subsequent screening steps (restreaking on copper-free NSM and growth in liquid on methane). The liquid culture step, which verified methane utilization, successfully screened out nonmethanotrophic colonies that originally produced color. These would include naphthol-producing fungi (20) and other nonmethanotrophic naphthalene oxidizers. The two-step screening procedure eliminated misleading initial results. False-positives were infrequent once experience had been developed with the procedure. When the procedure was first attempted, however, it was helpful to have positive controls available to assist in defining the positive response.

Putzer et al. (14) ably summarized the methods available for distinguishing type I and type II methanotrophs, adding their own method, which was based on density gradient separation. The colorimetric method described here offers significant advantages over other procedures in terms of simplicity, speed, and sample size requirements. The color-

TABLE 4. Results of growth and color development experiments involving isolates from landfill soils

Source (soil sample)[a]	Colony designation(s)	Color development on secondary plate (NSM with low Cu; 1% Noble agar)[b]	Cyclohexane conversion in liquid medium (NSM with low Cu)[b,c]
Color-forming colonies			
M-1	8	−	NT
	17, 18, 23, 24	−	−
NM-1:spiked with OB3b (column soils)	4	−	NG
NM-1	1, 2, 13	+	+
	3, 14	+	NG
M-2	5, 6, 7, 9, 10, 11, 12, 15, 16, 19, 20, 21	−	−
	22	−	NG
Non-color-forming colonies (negative controls)			
M-1	C1, C2, C3, C7, C8	−	−
M-2	C4, C5, C6	−	−

[a] See Table 3 for description of soil sources.
[b] Colonies were restreaked on NSM with low Cu to confirm color development, because growth on original plates was dense and color intensity was variable. +, color development on solid medium or cyclohexane conversion in liquid medium. −, no color development or no cyclohexane conversion.
[c] NT, not tested; NG, no growth.

imetric test is designed to identify strains with unusual potential for cometabolic applications—those with highly nonspecific monooxygenases. We consider this a practical advantage that may prove more significant as methanotrophic taxonomy takes on additional complexity.

The following conclusions are supported by our work. It is possible to differentiate between methanotrophic organisms that can and cannot transform cyclohexane to cyclohexanol by using the screening procedure described. This is of practical importance because cyclohexane reactivity correlates with the ability to transform several persistent chemical pollutants, including trichloroethylene (1, 18). In experiments involving *M. trichosporium* OB3b, expression of sMMO was requisite for naphthalene transformation and color production on plates.

The plate assay made it possible to count specific types of methanotrophs in soil column studies and to extract microorganisms with exceptional cometabolic potential from sand mixed with landfill soils. The colorimetric procedure did not immediately injure participant organisms; treated strains were, without exception, conveniently recovered by restreaking on fresh agar. The potential utility of the procedure from the standpoint of strain selection and/or selection of regulatory mutants that can express sMMO in the presence of Cu(II) is apparent.

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