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## Membrane Filter Method for Enumeration of *Acinetobacter calcoaceticus* from Environmental Waters

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## Membrane Filter Method for Enumeration of *Acinetobacter calcoaceticus* from Environmental Waters

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Received 8 June 1981/Accepted 11 September 1981

A membrane filter method was developed and evaluated for the quantitative recovery of *Acinetobacter calcoaceticus* from environmental waters. The procedure utilized a mineral medium, with sodium acetate and potassium nitrate as the carbon and nitrogen sources, respectively. Formic acid was included to enhance the recovery of *A. calcoaceticus* and to inhibit background growth. The medium was incubated for 46 h at 30°C, after which fermentation and cytochrome oxidase tests were performed on the colonies as they appeared on the membrane. Background microbial growth decreased on the average by 1.77 orders of magnitude. An essentially quantitative recovery relative to that on nutrient agar spread plates was obtained from freshly prepared suspensions of eight *A. calcoaceticus* strains in filter-sterilized pond water and from suspensions of five of the strains held for up to 96 h in filter-sterilized pond water at 15 and 22°C. Markedly reduced relative recoveries were obtained with the three remaining strains. However, these three strains, in contrast to the first five, not only did not grow, but also decreased in number in the eutrophic, filter-sterilized pond water. The confirmation rate of presumptive *A. calcoaceticus* colonies was 95%, whereas 8% of the presumptively negative colonies were *A. calcoaceticus*. The precision of the method did not exceed that expected from random error alone. Densities of *A. calcoaceticus* in freshwaters ranged from <1 to  $7.9 \times 10^4$  organisms per 100 ml and were about  $10^6$  organisms per 100 ml in raw sewage.

*Acinetobacter calcoaceticus* is an obligately aerobic, oxidase-negative, gram-negative bacterium which is commonly found in soil and aquatic environments (14), where it comprises a significant part of the total heterotrophic, aerobic population (3). Only one species is presently recognized (19), although some authors further separate the species into acid-forming (*A. calcoaceticus* subsp. *anitratum*) and non-acid-forming (*A. calcoaceticus* subsp. *lwoffii*) strains (17). Of special note is its ability to grow in low-nutrient media containing inorganic sources of nitrogen and any of a variety of simple compounds as the sources of carbon and energy (4, 13).

The nutritional versatility of *A. calcoaceticus* is reflected in its ability to survive or multiply or both on or in respiratory therapy equipment, dialysis baths, humidifiers, and catheters. This, along with its occurrence as a commensal organism on human skin and in the oral cavity, has made *A. calcoaceticus* an important nosocomial agent (7, 22, 23, 29). *A. calcoaceticus* is an opportunistic pathogen which has been responsible for cases of pneumonia, tracheobronchitis,

systemicemia, endocarditis and meningitis and for brain abscesses and infections of the urinary tract, ear, and surgical wounds (8, 11).

Another consequence of the nutritional versatility of *A. calcoaceticus* is suggested by reports that the densities of the organism in freshwater environments vary with nutritional loading (5, 14, 27, 28). The scope of these observations was limited by the method used for the enumeration of the organism (plating of water samples on a rich medium followed by identification of the isolates). The aim of the present investigation was to develop a selective-differential procedure for the quantification of *A. calcoaceticus* as the first step in better defining the ecology of the organism.

### MATERIALS AND METHODS

**Cultures.** Eight *A. calcoaceticus* strains were used in the development and evaluation of the enumeration (mAc) method. Strain 25V722L (*A. calcoaceticus* subsp. *anitratum*) was obtained from I. G. Resnick. Strain MOE60 (*A. calcoaceticus* subsp. *anitratum*) was provided by A. Burger, Ministry of the Environment, Rexdale, Ontario, Canada. Strain SL9 (*A. calcoaceticus* subsp. *anitratum*) was isolated from Schroon Lake, N.Y., by S. R. Rippey. Strains III10 (*A. calcoaceticus* subsp. *lwoffii*) and IV14 (*A. calcoaceticus* subsp. *lwoffii*) were obtained from Barbers Pond and Deep

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Pond, R.I., respectively. Strain GIII6 (*A. calcoaceticus* subsp. *lwoffi*) was obtained from Lake Burton, Ga. Strain NB4 (*A. calcoaceticus* subsp. *lwoffi*) was isolated from the estuarine waters of Narragansett Bay, R.I. Strain ATCC 23055 (*A. calcoaceticus* subsp. *lwoffi*) was obtained from the American Type Culture Collection. Strains of bacteria other than *A. calcoaceticus* used in the development of the method were isolated on prototypes of the mAc medium.

**Stock suspensions.** Approximately 0.5 ml of an overnight nutrient broth culture grown at 30°C was inoculated into 5 ml of fresh nutrient broth. After incubation for 2 h at 30°C, the cells were washed three times by centrifugation at  $5,000 \times g$  for 20 min followed by suspension of the pellet in 5 ml of filter-sterilized eutrophic pond water. The water was stored at 4°C for no longer than 3 weeks. Before being used, the water was sterilized by filtration through 0.7- $\mu\text{m}$  membrane filters (HC; Millipore Corp., Bedford, Mass.) and then through 0.22- $\mu\text{m}$  membrane filters (GS; Millipore Corp.).

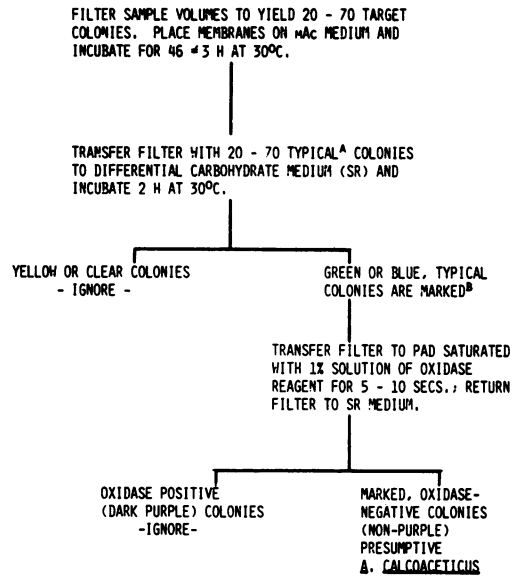
**Collection of water samples.** Samples from freshwater lakes and ponds were collected in sterile 1-liter polypropylene bottles at various distances from the shoreline and at a depth of about 1 m. The samples were assayed within 4 h of collection.

**mAc procedure.** The mAc procedure is performed as follows. The medium, a modification of that used by Baumann (3), is prepared by adding the following ingredients to 958 ml of deionized water: sodium acetate $\cdot$ 3H<sub>2</sub>O, 2.0 g; KNO<sub>3</sub>, 2.0 g; and purified agar (Oxoid; KC Biologicals Inc., Lenexa, Kans.), 10.0 g. The mixture is autoclaved at 121°C for 15 min and cooled to 50°C. Then, the following sterilized ingredients are added as the mixture is stirred: concentrated base, 20.0 ml; 2.0 M KH<sub>2</sub>PO<sub>4</sub>, 5.7 ml; 2.0 M Na<sub>2</sub>HPO<sub>4</sub>, 14.3 ml; and formic acid (reagent grade; J. T. Baker Chemical Co., Phillipsburg, N.J.), 2.0 ml. The pH is adjusted to 7.2 with 10 N NaOH (ca. 4.3 ml), and the medium is dispensed in 6-ml quantities to sterile petri plates (50 by 12 mm). The plates are stored in the dark at 4°C and should be used within a month.

The concentrated base is prepared by adding 10.0 g of nitrilotriacetic acid to 200 ml of deionized water. About 19 ml of a 40% (wt/vol) KOH solution is required to dissolve the nitrilotriacetic acid (final pH, ca. 8.4). MgSO<sub>4</sub> $\cdot$ 7H<sub>2</sub>O (37.8 g) is added, and the solution is adjusted to pH 6.0 to 6.3 with 40% KOH (about 4 ml). The following ingredients are then dissolved in the order given: CaCl<sub>2</sub> $\cdot$ H<sub>2</sub>O, 3.34 g; (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub> $\cdot$ 4H<sub>2</sub>O, 9.3 mg; FeSO<sub>4</sub> $\cdot$ 7H<sub>2</sub>O, 99 mg; nicotinic acid, 50 mg; thiamin-HCl, 25 mg; biotin, 0.5 mg; and "metals 44" solution, 50 ml. The pH is adjusted to 6.6 to 6.8 with 40% KOH. The solution is brought to a final volume of 1 liter and is autoclaved at 121°C for 15 min. A precipitate forms, but redissolves when the cooled solution is stirred.

The metals 44 solution is prepared by adding the following in the order given to 100 ml of deionized water containing 2 drops of concentrated H<sub>2</sub>SO<sub>4</sub>: EDTA, 250 mg; ZnSO<sub>4</sub> $\cdot$ 7H<sub>2</sub>O, 1.1 g; FeSO<sub>4</sub> $\cdot$ 7H<sub>2</sub>O, 500 mg; MnSO<sub>4</sub> $\cdot$ H<sub>2</sub>O, 154 mg; CuSO<sub>4</sub> $\cdot$ 5H<sub>2</sub>O, 39 mg; Co(NO<sub>3</sub>)<sub>2</sub> $\cdot$ 6H<sub>2</sub>O, 25 mg; and Na<sub>2</sub>O<sub>7</sub> $\cdot$ 10H<sub>2</sub>O, 28 mg.

**SR medium.** The differential carbohydrate (SR) medium is prepared by a modification of the method of Rippey and Cabelli (24). The following are added (gram per 100 ml) to deionized water: tryptose, 0.5;



\*SMOOTH, CONVEX,  $\geq 1$  mm.

<sup>B</sup>BY PUNCHING A HOLE IN THE MEMBRANE WITH A NEEDLE NEXT TO THE COLONY.

FIG. 1. Flow diagram of the mAc procedure for *A. calcoaceticus*.

mannitol, 0.5; sucrose, 0.5; lactose, 0.5; yeast extract, 0.2; NaCl, 0.3; KCl, 0.2; MgSO<sub>4</sub> $\cdot$ 7H<sub>2</sub>O, 0.02; FeCl<sub>2</sub> $\cdot$ 6H<sub>2</sub>O, 0.01; and bromothymol blue (Fisher Scientific Co., Fairlawn, N.J.), 0.004. After the ingredients are dissolved, the pH is adjusted to 8.5 with 10 N NaOH, 1.5 g of agar is added, and the mixture is autoclaved at 121°C for 15 min. The mixture is cooled to 50°C, and 10 mg of sodium deoxycholate (Fisher Scientific Co.) is added. The medium is dispensed and stored as described above.

**Membrane filtration.** Appropriate volumes of a water sample are filtered to obtain 20 to 70 target colonies per membrane. Membrane filtration is performed as described previously (1), except that phosphate-buffered saline (31) is used as the diluent and wash solution. After filtration, the membranes (pore size, 0.45 to 0.7  $\mu\text{m}$ ) are placed grid side up on the surface of mAc agar plates, which are incubated at 30°C for  $46 \pm 3$  h. After incubation, the membranes are transferred to plates of the SR medium, and these are incubated for 2 h at 30°C. Nonfermenting (green or blue) colonies  $\geq 1$  mm in diameter are scored on the filter by punching holes through the membrane alongside the colonies. The membrane is transferred to a pad saturated with oxidase reagent (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride, 0.1 g/10 ml of deionized water) for about 10 s and then returned to the plate of SR medium. Oxidase-positive colonies develop purple halos or turn dark purple throughout. The marked, oxidase-negative colonies are counted as *A. calcoaceticus*. A flow diagram for the method is presented in Fig. 1.

**Performance characteristics.** The mAc method was evaluated by predetermined performance guidelines (6).

(i) **Accuracy.** The stock suspensions were diluted in filter-sterilized pond water to provide approximately

250 colony-forming units per ml. The resulting suspensions then were held at 8, 15, 22, and 30°C. After 0, 24, 48, 72, and 96 h, 0.5-ml quantities of the appropriate dilutions of the test suspensions were assayed in triplicate by the mAc procedure and by using nutrient agar (NA) spread plates. The NA spread plates were incubated at 30°C for 48 h, after which the colonies were counted.

(ii) **Selectivity.** Twenty-three surface, freshwater samples were collected as described above. They were assayed by the mAc procedure in quintuplicate and in triplicate on NA spread plates incubated for 72 h at room temperature (22 to 23°C). The mean densities of *A. calcoaceticus* and non-*Acinetobacter* colonies were obtained from the mAc assays. The mean densities of non-acinetobacters recoverable on NA plates were calculated by subtracting the mean *A. calcoaceticus* densities obtained by the mAc procedure from the mean total recoveries on NA plates. The selectivity of the mAc procedure was estimated as the difference in the log<sub>10</sub> recovery per 100 ml of non-*Acinetobacter* densities by the mAc procedure as compared with those with the NA medium.

(iii) **Specificity.** A total of 450 presumptive *A. calcoaceticus* and 280 presumptive background isolates were obtained from 24 water samples and 1 sewage sample; they were identified as *A. calcoaceticus* or non-*Acinetobacter* colonies by the following procedure. Isolated colonies on the mAc plates were streaked for isolation on NA plates which were incubated at 30°C for 24 h. The predominant colony type was restreaked for isolation and then transferred to an NA slant, which was used as the inoculum for the identification tests. Isolates were identified as *A. calcoaceticus* by the following characteristics: gram-negative, pleomorphic rods or diplococci from an overnight, mineral-acetate broth (pH 7.2) culture incubated at 30°C (3); cytochrome oxidase test, negative (16); O/F glucose test, negative or oxidative after 5 days of incubation at 30°C (O/F medium with 1% glucose; Difco Laboratories, Detroit, Mich.); no change in butts, alkaline or no change in slants, anaerogenic, and H<sub>2</sub>S negative at 24 h on triple sugar iron agar (Difco TSI medium, 48-h incubation at 30°C); nonmotile (Difco motility medium, 48-h incubation at 30°C); usually negative for nitrate reduction (Difco nitrate reduction medium with Durham tubes, 48-h incubation at 30°C) (20); and interspecies transformation test, positive (15). The prototrophic recombinant plates (lactic acid as the carbon source) in the transformation assay were incubated for 48 h instead of 24 h before being read. An isolate was not confirmed as *A. calcoaceticus* if it differed from any of the above criteria except the nitrate reduction test; if nitrate reduction was positive and the transformation test also was positive, the isolate was confirmed as *A. calcoaceticus*.

(iv) **Precision.** The precision was determined by utilizing five replicate plates from each of the 25 samples noted above. The assay variability was determined from the  $D^2$  values obtained by the following equation (9):  $D^2 = [N\sum X_i^2 - (\sum X_i)^2] / (\sum X_i)^2$ , where  $X_i$  was the summation of the plate counts  $X_1, X_2, \dots, X_N$ , and  $N$  (the number of replicate plates per sample) was 5.

(v) **Counting range.** Increasing quantities of four natural water samples were filtered in quintuplicate,

although one Florida water sample was assayed only in triplicate. The mean *A. calcoaceticus* density for each sample volume was determined. The expected values were obtained by extrapolating from the mean count nearest 30 colonies per filter by using the formula:  $C_e = C_{30} (V_e/V_{30})$ , where  $C_e$  was the expected count for a given sample volume ( $V_e$ ) examined and  $C_{30}$  and  $V_{30}$  were the count nearest 30 colonies per filter and the corresponding volume, respectively.

**Growth experiments.** The data on the growth (or death) of *A. calcoaceticus* were obtained from the same experiments used to determine their relative recoveries by the mAc procedure. In addition, suspensions of one of the strains (25V722L) were similarly prepared in a sample of oligotrophic water obtained from Sebago Lake, Maine. The container in which this water sample was collected and all the glassware used in the experiments were cleaned in chromic acid and thoroughly rinsed.

## RESULTS

**Development of the mAc procedure.** Baumann employed the combination of nutritional selectivity (a mineral-acetate medium) and low pH (5.5) to recover *A. calcoaceticus* in liquid culture from environmental water samples. This approach toward optimizing the recovery of *A. calcoaceticus* while minimizing the growth of other bacteria was explored in some initial experiments. Both stock strains and the organisms occurring in environmental water samples were used in comparing prototype media for the recovery of *A. calcoaceticus* and the inhibition of background organisms. When the Baumann mineral-acetate medium (even if supplemented with complex sources of carbon and nitrogen) was adjusted to pH 5.5, the recoveries of the four strains employed were  $\leq 0.2\%$  of those obtained with membrane filters placed on brain heart infusion agar. Even when a relatively rich medium (brain heart infusion agar) was used at pH 5.5, the mean relative recovery was 40% (range, 12 to 92%); moreover, with brain heart infusion adjusted to pH values of 5.5, 5.75, and 6.0, recoveries of background organisms from an environmental water sample were 81, 90, and 118%, respectively, relative to those at pH 7.2. The relative recoveries of four *A. calcoaceticus* strains on the mineral-acetate medium adjusted to pH 8.0 and 10.0 were 76% (range, 25 to 106%) and 73% (range, 22 to 100%), respectively. Background organisms from environmental water samples were recovered on the mineral acetate medium at pH 9.0, 9.5, and 10.0 at relative levels of 59, 68, and 65%, respectively. Since the extremes of pH proved of little selective value for the recovery of *A. calcoaceticus* by membrane filtration, a more neutral pH (7.2) was used in subsequent work.

Several compounds known to be utilized by *A. calcoaceticus* were substituted for sodium acetate as sources of carbon and energy (e.g.,

butyric acid, nanoic acid, and benzoic acid) and for  $\text{KNO}_3$  as sources of nitrogen (e.g.,  $(\text{NH}_4)_2\text{SO}_4$  and thiourea). None of the substitutions increased the selectivity of mAc while providing comparable recoveries of *A. calcoaceticus*. Moreover, doubling the sodium acetate concentration decreased the selectivity of the medium.

The temperature ranges for the growth of three *A. calcoaceticus* strains (25V722L, IV14, and ATCC 23055) were determined by streaking slants of the mineral-acetate medium from an overnight nutrient broth culture of the organism. The slants were incubated in a gradient temperature incubator (Scientific Industries Inc., Mineola, N.Y.), and any sign of growth was recorded as positive at 24 and 48 h. The growth range for the anitratus strain was 20 to 30°C after 24 h of incubation and 16 to 44°C after 48 h. One *A. calcoaceticus* subsp. *lwoffi* strain, IV14, grew over a range of 20 to 30°C after 24 h and 14 to 38°C after 48 h. The other *A. calcoaceticus* subsp. *lwoffi* strain (ATCC 23055) grew after 48 h of incubation at 15 to 33°C. Nevertheless, when the prototype mAc plates from natural water samples were incubated at between 20 and 30°C, there was an increase in oxidase-positive background organisms, and an incubation temperature of 35°C reduced recoveries of *A. calcoaceticus* by 75% relative to those at 30°C. Therefore, 30°C was retained as the incubation temperature.

Several antibiotics were examined as selective agents by the method of Szybalski (29) as modified by Levin and Cabelli (19) and by placing impregnated antibiotic disks (Difco; BBL Microbiology Systems, Cockeysville, Md.) on plates of Mueller-Hinton broth (Difco) and mineral-acetate medium, which had been inoculated to produce lawns of the test organism. Twenty-four antibiotics were tested as described above for their ability to inhibit 26 *A. calcoaceticus* isolates and 37 background isolates. Of the antibiotics tested, chloramphenicol seemed the most promising because of the results from the preliminary tests and the reported resistance of *A. calcoaceticus* to this drug (11). However, at chloramphenicol concentrations which were not inhibitory to *A. calcoaceticus* ( $\leq 2.0$   $\mu\text{g/ml}$ ), there was no appreciable decrease in the numbers of background colonies. Several other potentially inhibitory compounds also were tested by the modified Szybalski method (29). These included: ethanol, 0.25 to 3%; sodium azide, 0.1 to 10 mg/liter; EDTA, 5.0 to 100 mg/liter (32); diazene compounds (R-7405-26, R-7260-146), 0.005 to 1.0 g/liter (26);  $\text{ZnCl}_2$ , 100 mg/liter (2); rotenone, 5.0 mg/liter; crystal violet, 50 to 120 mg/liter (10); and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride, 1.0 to 200

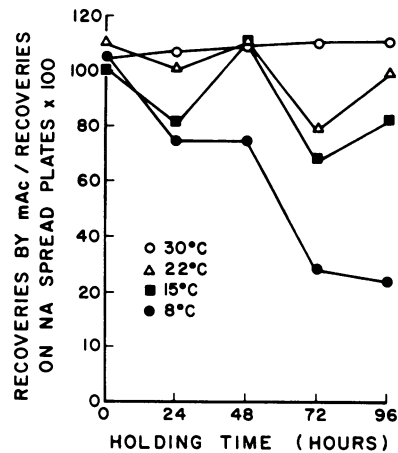


FIG. 2. Recoveries of a stock *A. calcoaceticus* strain, 25V722L, on mAc relative to those on NA spread plates. Suspensions of the organism in filter-sterilized, eutrophic pond water were held at the temperatures stated and assayed after the indicated number of hours. Each percentage is the mean from two trials. All assays were done in triplicate.

mg/liter. None of the compounds examined sufficiently inhibited the background bacteria while allowing the quantitative recovery of *A. calcoaceticus*.

Formic acid, at a concentration of 2.0 ml/liter, was added to the prototype mAc medium initially as an electron donor to facilitate the assimilation of nitrate (21). This resulted in an increase in selectivity with no inhibition of target organisms. When formic acid concentrations of 1.0 to 6.0 ml/liter were examined, 2 ml was found to be optimum. At this concentration, the numbers of background colonies in 11 water samples from eight different ponds were reduced by 61% (range, 46 to 85%).

Gelman (47-mm diameter, 0.45- $\mu\text{m}$  pore size, gridded) and Millipore HC (47-mm diameter, 0.7- $\mu\text{m}$  pore size, gridded) membrane filters were compared for the recovery of *A. calcoaceticus* from a filter-sterilized pond water suspension of the organism held for up to 24 h at 15°C. The mAc recoveries of the stressed cells with the two types of membranes were not appreciably different (data not given).

**Evaluation of mAc procedure.** The accuracy of the mAc procedure was examined first with suspensions of a single *A. calcoaceticus* subsp. *anitratus* strain held at four temperatures, 8, 15, 22, and 30°C. At 8°C, the relative recovery on mAc decreased with increasing storage of the test suspension (Fig. 2). There also was a suggestion of reduced recoveries with storage at 15°C. Therefore, the recoveries of the remaining seven strains were examined with suspensions stored at 15 and 22°C (30°C is above most

TABLE 1. Relative recoveries from stored suspensions of *A. calcoaceticus* by the mAc procedure

Strain	<i>A. calcoaceticus</i> subsp.	Relative <sup>a</sup> % recovery at temp and time				
		0 h	15°C (24 to 96 h)		22°C (24 to 96 h)	
			Mean	SD	Mean	SD
25V722L	<i>A. calcoaceticus</i> subsp. <i>anitratus</i>	105	86	12	125	14
MOE60	<i>A. calcoaceticus</i> subsp. <i>anitratus</i>	118	143	47	139	13
SL9	<i>A. calcoaceticus</i> subsp. <i>anitratus</i>	104	107	19	124	27
GIII6	<i>A. calcoaceticus</i> subsp. <i>lwoffi</i>	96	87	25	114	11
III10	<i>A. calcoaceticus</i> subsp. <i>lwoffi</i>	107	166	57	127	16
NB4	<i>A. calcoaceticus</i> subsp. <i>lwoffi</i>	79	53	24	71	5.7
ATCC 23055	<i>A. calcoaceticus</i> subsp. <i>lwoffi</i>	129	6.0 <sup>b</sup>	4.4	5.5 <sup>b</sup>	4.0
IV14	<i>A. calcoaceticus</i> subsp. <i>lwoffi</i>	103	1 <sup>c</sup>		49 <sup>d</sup>	

<sup>a</sup> Relative to NA spread plates incubated at 30°C for 45 h.

<sup>b</sup> Not recoverable by mAc or NA spread plates at 96 h.

<sup>c</sup> Not recoverable by mAc or NA spread plates by 24 h.

<sup>d</sup> Not recoverable by mAc or NA spread plates after 24 h.

environmental water temperatures). For all eight strains, the relative recoveries with unstored suspensions exceeded 79%. In general, this was true of the stored suspensions from five of the strains examined. The relative recoveries from the stored suspensions of the remaining three, *A. calcoaceticus* subsp. *lwoffi* strains NB4, ATCC 23055, and IV14, were 76% or less (Table 1).

The selectivity of the mAc method produced, on the average, a 1.77 order of magnitude reduction of background organisms.

The false-positive and false-negative rates as measures of specificity were 5.5 and 8.0%, respectively (Table 2). Of the 23 false-negative isolates, 17 were obtained from the same dilutions at which *A. calcoaceticus* density estimates were made. The inability to recognize these 17 false-negative isolates reduced the *A. calcoaceticus* density estimates by an average of 11.9%. The majority of the false-negative isolates came from one body of water and belonged to the non-acid-forming (*A. calcoaceticus* subsp. *lwoffi*) variety.

The distribution of  $D^2$  values did not indicate excessive filter-to-filter variability across samples.

The data used in defining the upper counting limit for the mAc method are shown in Fig. 3. The upper counting limit was not increased beyond 70 colonies per filter because of the results obtained with one of the four samples. The size of the *A. calcoaceticus* colonies decreased to 1 mm or less as the counting limit was approached.

The ability of strain 25V722L to survive or grow or both in oligotrophic (Sebago Lake, Maine) and eutrophic (Thirty Acre Pond, R.I.) water at four temperatures is shown in Fig. 4. It can be seen that, irrespective of the trophic state of the water, the cell densities did not increase; rather, they decreased at an incubation temperature of 8°C. As the temperature was increased from 15 to 30°C, the growth rate increased, although it was consistently lower in the oligotrophic water. The maximum density achieved appeared to be related to the trophic state of the water. Because of these findings, seven additional strains were tested at 15 and 22°C (Fig. 5). These strains varied in their ability to grow in the eutrophic water, and in general, the effect of temperature on the growth rate was confirmed. Moreover, there appeared to be a correlation between the relative recovery by the mAc pro-

TABLE 2. Specificity of the mAc procedure for the enumeration of *A. calcoaceticus*

Bodies of water		Typical colonies		Other colonies	
Type	No.	Total	False positive <sup>a</sup>	Total	False negative <sup>b</sup>
Ponds <sup>c</sup>	6	300	18	199	5
Reservoirs <sup>c</sup>	2	128	5	71	15
Sewage <sup>d</sup>	1	22	0	10	3
Total		450	23	280	23
%			5.0		8.0

<sup>a</sup> Not confirmed as *A. calcoaceticus*.

<sup>b</sup> Identified as *A. calcoaceticus*.

<sup>c</sup> Three samples per pond or reservoir.

<sup>d</sup> One sample.

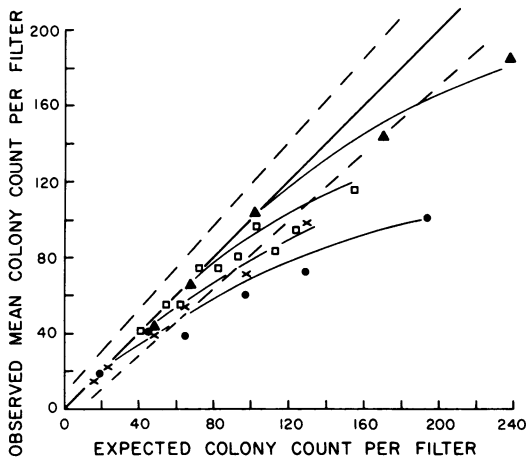


FIG. 3. Upper counting limit for the mAc method as determined by the deviation from the line of equality. The observed values are the mean membrane filter counts for five replicate plates, except one trial ( $\blacktriangle$ ), where the points are the mean of three replicate plates. The expected values are derived as described in the text. The dotted line represents the 95% confidence limits about the line of equality. Symbols:  $\bullet$ , Turner Reservoir, R.I., site 1;  $\times$ , Turner Reservoir, R.I., site 2;  $\square$ , Slatersville Reservoir, R.I.;  $\blacktriangle$ , Northern Florida freshwater sample.

cedure and the ability of a given strain to survive or grow or both in the eutrophic pond water. Thus, the three strains with the poorest relative recoveries on mAc (NB4, ATCC 23055, and IV14; Table 2), did not multiply at 15°C in the eutrophic pond water. At 22°C, strain NB4, whose relative recoveries on mAc were the best of the three, did multiply. The remaining two strains died off rather rapidly at both temperatures.

The mean density of *A. calcoaceticus* in raw sewage was about  $4.4 \times 10^6$  organisms per 100

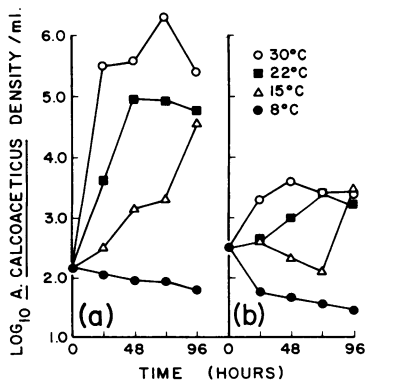


FIG. 4. Growth curves for *A. calcoaceticus* strains 25V722L held at 8, 15, 22, and 30°C in filter-sterilized water. (a) Eutrophic (Thirty Acre Pond, R.I.) water. (b) Oligotrophic (Sebago Lake, Maine) water.

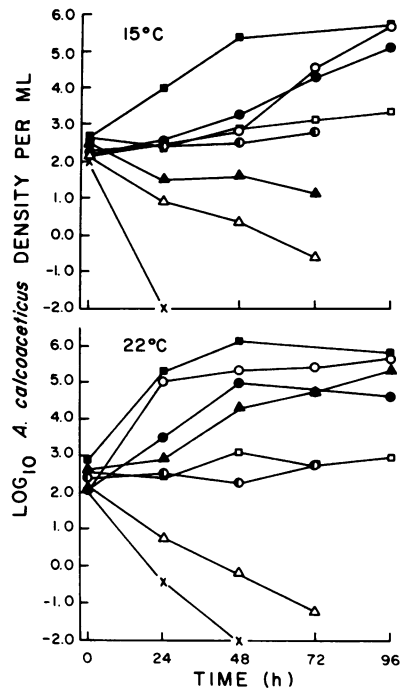


FIG. 5. Growth curves of eight *A. calcoaceticus* strains held in filter-sterilized, eutrophic pond water at 15 and 22°C as determined by NA spread plates. Symbols for strains:  $\circ$ , 25V722L (same data as in Fig. 5);  $\blacksquare$ , SL9;  $\circ$ , MOE60;  $\bullet$ , GIII6;  $\square$ , III10;  $\blacktriangle$ , NB4;  $\triangle$ , ATCC 23055;  $\times$ , IV14.

ml. The levels in freshwaters ranged from  $<1$  to  $7.9 \times 10^4$  organisms per 100 ml. The non-acid-forming strains comprised about 80% of the *Acinetobacter* isolates obtained from the Rhode Island ponds.

### DISCUSSION

The mAc method satisfied most of the criteria for an acceptable primary, selective-differential method for the enumeration of *A. calcoaceticus* in freshwater. Poor relative recoveries were obtained with three of the *A. calcoaceticus* subsp. *lwoffi* isolates after storage in filter-sterilized, eutrophic pond water. However, with reference to the potential use of *A. calcoaceticus* as an indicator of nutrient enrichment, it is of interest that these strains not only did not multiply, but survived poorly in the eutrophic pond water incubated at 15°C.

The selectivity of the method, as seen from the 1.77 orders of magnitude reduction in background organisms, was not as extensive as desired. However, even with samples having background organism densities considerably greater than those of the target organisms, *A. calcoaceticus* colonies could be detected as relatively large oxidase-negative colonies on an oxidase-



positive purple mat; this was because the large majority of the background colonies on the membranes were oxidase-positive pseudomonads. *Enterobacteriaceae* (notably *Klebsiella pneumoniae*) were present occasionally, but could be easily distinguished from *A. calcoaceticus* by the differential carbohydrate test (SR medium).

The isolation, purification, and identification of target colonies are not considered necessary as routine procedures, since the false-negative and false-positive isolates resulted in average underestimates of the "true" *A. calcoaceticus* density by 11.9 and 5.5%, respectively.

Some preliminary findings (not shown) support earlier reports suggesting that increased *A. calcoaceticus* densities are found in the receiving waters for sewage effluents (5, 14, 27, 28). However, they also suggest that the autochthonous organisms respond to nutrient enrichment from other sources as well. More data are needed and should be gathered from waters at temperatures of 15°C or greater. Moreover, since *A. calcoaceticus* occurs in high numbers in sewage, although it is infrequently isolated from the intestinal tract and rarely colonizes the normal intestine (12), care must be taken in the choice of sampling locations to avoid acinetobacters which are not autochthonous. The data obtained also suggest that the ability of the water to promote the growth of selected *A. calcoaceticus* strains in laboratory studies could be used as one parameter for the assessment of its trophic state. This too needs to be examined.

#### ACKNOWLEDGMENTS

Part of the work was conducted when both authors were at the Marine Field Station, HERL-Cin., U.S. Environmental Protection Agency, West Kingston, R.I.

We thank Elliot Juni, University of Michigan, Ann Arbor, for providing the bacterial strains used in the transformation assays.

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