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Bacterial Interference with Coliform Colony Sheen Production on Membrane Filters

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The membrane filter (MF) method for detection and enumeration of coliform bacteria in drinking water requires that the coliforms both grow and produce a green metallic sheen when the filter is incubated on modified Endo medium at 35°C for 22 h. Large numbers of noncoliform bacteria, which are enumerated by the standard plate count (SPC) technique, can interfere with the detection of coliforms on MF. This paper presents quantitative evidence from laboratory experiments on the interference of specific SPC bacteria on coliform colony sheen production on MF. *Pseudomonas aeruginosa* and *Aeromonas hydrophila* caused significant reductions in *Escherichia coli* sheen colony counts when present at 3,000 and 220 per filter, respectively. The *Flavobacterium* sp. and *Bacillus* sp. selected for this study from SPC did not interfere with coliform colony sheen production. Excessive crowding of *E. coli* and *Enterobacter cloacae* colonies on MF also caused a reduction in the number of colonies that produced sheen. Even when there was no crowding (14 colonies per filter), only a fraction of the *E. cloacae* colonies produced sheen colonies on modified Endo medium.

The membrane filter (MF) method using a modified Endo (m-Endo) medium has become increasingly popular for enumeration of coliform bacteria in potable water (1, 6, 10) since the early 1950s. However, along with its acceptance, questions concerning interference with coliform detection and the accuracy of coliform enumeration have arisen. Several noncoliform bacteria have been shown to inhibit the growth of coliform bacteria in liquid media (7, 9, 11, 13, 17), and coliform bacteria have been shown to produce nonsheen colonies on MF on m-Endo (8, 10).

Geldreich et al. (5) inferred that there was interference with coliform detection on MF when the standard plate count (SPC) exceeded 500/ml from analysis of monitoring data. They found an increasing frequency of coliform occurrence for SPC up to 500/ml but a decreasing frequency above that level. Clark (4), from a similar type of analysis, inferred an SPC of 1,000/ml as the count above which interference with coliform detection was a problem. Since Clark (4) used a different data grouping than Geldreich et al. (5), these reports do not necessarily conflict. Clark (4) also presented presence-absence test data to show that there really was an increasing frequency of occurrence of coliform bacteria at the higher SPC values.

The purpose of this paper is to present quantitative evidence from laboratory studies of the effects of bacterial interference on coliform colony sheen production on MFs incubated on m-Endo. Four noncoliform bacteria were tested for interference with coliform sheen production, and the density of SPC bacteria at which interference occurs was investigated.

MATERIALS AND METHODS

Isolation of bacterial cultures. Two coliform and four noncoliform bacteria were isolated from the Philadelphia water distribution system and identified. The coliforms were obtained from newly installed pipelines being sampled to determine whether disinfection was adequate before use. Identification was performed by using API 20E test strips (Analytab Products, Plainview, N.Y.). *Escherichia coli* with API profile 5144572 and *Enterobacter cloacae* with API profile 3305773 were the two selected for use in those laboratory experiments. Near the end of the experimental work the two coliforms were identified again and showed the same API profiles.

The noncoliforms represent common bacteria that are frequently isolated from SPC on distribution system samples (14–16). *Aeromonas hydrophila* was identified with an API profile of 7046125 and fermented lactose with gas production in 48 h at 35°C. This organism produces dark-red colonies with a rough surface on m-Endo. Although these colonies might be interpreted as sheen colonies, in these pure culture studies they were readily differentiated from sheen colonies produced by the coliforms. *Pseudomonas aeruginosa* had an API profile of 2202004 and grew well at 42°C. A *Bacillus* sp. was an oxidase-positive, glucose-fermentative, endospore-forming rod. A *Flavobacterium* sp. was oxidase positive, yellow pigmented, and glucose oxidative.

Determination of inoculum densities. To supply increasing numbers of noncoliforms and relatively constant numbers of coliforms for tests of sheen production on MFs, inocula of known bacterial densities were obtained from 24-h nutrient broth cultures grown at 35°C. Enumeration of one set of broth cultures by the SPC technique provided estimates of inoculum densities for other broth cultures grown under the same conditions. One-day-old broth cultures of *E. coli*, *E. cloacae*, *A. hydrophila*, and *P. aeruginosa* had densities of 30×10^7 to 100×10^7 cells per ml. The *Bacillus* sp. and *Flavobacterium* sp. cultures had densities of 10×10^6 to 100×10^6 cells per ml. Serial dilutions using sterile, buffered water provided the desired number of bacteria for membrane filtration.

MF interference tests. Dilutions of the 24-h broth cultures were used to make mixtures of noncoliform and coliform bacteria, and 50-ml of the mixed dilutions was membrane filtered. Each mixture was filtered in triplicate. The filters were placed on m-Endo pads and incubated for 22 h at 35°C (1). Sheen and nonsheen colony counts were recorded.

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Standard plate counts (1) of dilutions of the broth cultures were made. To observe the effect of colony crowding on sheen production by colonies of *E. coli* and *E. cloacae*, dilutions of 24-h broth cultures provided increasing numbers in 50 ml of sterile, buffered water for membrane filtration. In this way, sheen colony counts on MF on m-Endo were recorded at increasing numbers of coliform bacteria added per filter.

Verification of MF colonies. The verification of sheen colonies or nonsheen colonies which were suspected of being coliform bacteria was performed with lauryl tryptose broth followed by brilliant green bile broth. Gas in 48 h at 35°C in lauryl tryptose broth and brilliant green bile broth was recorded as positive verification. All verification tests were performed with three colonies from one MF of each set.

RESULTS

In this section bacterial densities are expressed as organisms per filter because of the way the experiments were performed. The interpretation of these results in terms of organisms per 1 or 100 ml, as would be obtained from water distribution system samples, is considered in the discussion.

The data from the self-interference tests are presented in Tables 1 and 2. The expected colony counts in the first columns are from SPC on dilutions from nutrient broth cultures. The MF counts were made in triplicate, and the replicate counts should agree with the Poisson distribution. The appropriate test for agreement is the Fischer index of dispersion, $I = (n - 1)$ variance/mean (3), which has a chi-squared distribution with $n - 1$ degrees of freedom. In the case of triplicate determinations, a variance to mean ratio of less than 0.103 indicates underdispersion at the 5% level, and a variance to mean ratio of greater than 5.99 indicates overdispersion at the 5% level. Although many of the counts presented in Tables 1 and 2 suggested underdispersion, it could not be concluded that any of them did not come from a Poisson distribution.

Crowding of *E. coli* colonies by adding several hundred organisms per filter reduced sheen production but did not eliminate it entirely, even when approximately 12,000 organisms per filter were present (Table 1). This would not be expected to cause a problem in monitoring water systems because, by U.S. Environmental Protection Agency (EPA) guidelines (3), coliform counts of greater than 80 per filter are recorded as too numerous to count (TNTC).

The results with *E. cloacae* are somewhat different. Even relatively low numbers of organisms per filter with no colony crowding produced some nonsheen colonies which would not be counted as coliforms. Confluent growth with approximately 6,900 organisms added per filter resulted in the absence of sheen colonies. Nonsheen colonies picked from these filters for verification did produce gas in lauryl tryptose broth and in brilliant green bile broth. Thus, it is clear that MF coliform colony counts can be inaccurate (low) when *E. cloacae* is present and that high densities of this organism in water can result in failure to recognize the presence of coliforms when the MF method is used.

TABLE 1. Effect of colony crowding on sheen production by *E. coli*

Expected colony count per filter	Sheen colony count per filter			Nonsheen colony count per filter		
	Mean	Variance	Variance/mean	Mean	Variance	Variance/mean
25	25.0	16.7	0.67	0		
26	39.3	21.6	0.55	0		
48	66.7	13.6	0.20	0		
240	217.0	26.0	0.12	0		
730	TNTC[a]			11.7	8.2	0.70
2,400	20.0	16.7	0.84	TNTC		
12,000	2.0	0.7	0.35	TNTC		

[a] TNTC or greater than 300 colonies per filter.

TABLE 2. Effect of colony crowding on sheen production by *E. cloacae*

Expected colony count per filter	Sheen colony count per filter			Nonsheen colony count per filter		
	Mean	Variance	Variance/mean	Mean	Variance	Variance/mean
2	0.3	0.2	0.67	0		
5	1.7	0.2	0.12	0		
14	12.3	2.3	0.19	4.0	3.0	0.75
23	18.3	20.2	1.10	12.7	10.8	0.85
31	9.0	12.0	1.33	21.6	20.3	0.94
69	41.7	32.7	0.78	22.1	34.6	1.56
115	78.6	17.6	0.22	34.5	29.3	0.85
230	110.4	48.2	0.44	97.1	36.0	0.37
1,200	97.8	37.6	0.38	TNTC[a]		
2,300	4.7	1.6	0.34	TNTC		
6,900	0			TNTC		

[a] TNTC or greater than 300 colonies per filter.

Strains of *Flavobacterium* sp. and *Bacillus* sp. were selected as representatives of SPC bacteria which do not grow on m-Endo medium. From the data presented in Tables 3 and 4, it is clear that they did not reduce the number of sheen colonies produced by either *E. coli* or *E. cloacae*, even when added in numbers greater than 100,000 per filter.

P. aeruginosa and *A. hydrophila* were selected as representatives of SPC bacteria which are able to grow on MF on m-Endo medium. It is apparent from the data in Tables 3 and 4 that these organisms do interfere with sheen production by coliform colonies on MF even when present in numbers less than 50,000 per filter. In one test of *P. aeruginosa* versus *E. cloacae*, no significant difference was found at the 5% level by analysis of variance; however, this does not negate the results of the tests in which a significant difference was found.

Pair comparison *t* tests were used to search for the density of the noncoliforms which caused a significant reduction in the coliform sheen colony count. The addition of *P. aeruginosa* at densities of 100, 300, 440, and 1,000 per filter did not result in significantly lower sheen colony counts from *E. coli* as compared with filters when *P. aeruginosa* was not present. However, additions of 3,000, 4,400, and 10,000 per filter did result in significantly lower sheen colony counts. Additions of 220, 570, and 1,400 *A. hydrophila* per filter gave significantly lower ($\alpha = 0.05$) sheen colony counts from *E. coli* as compared to the counts on filters when *A. hydrophila* was not present. Higher levels of both of the SPC bacteria were required to give a significant reduction in the sheen colony counts from *E. cloacae*, possibly because of the greater variability of the *E. cloacae* counts.

DISCUSSION

The standard volume for MF coliform tests on distribution system samples is 100 ml, but 1-ml samples are used for SPC (3). The SPC interference levels of 500/ml inferred by

TABLE 3. Effect of noncoliform bacteria on sheen production by *E. coli* colonies on MFs

Noncoliform organism	No. of noncoliforms added per filter	Avg nonsheen colony count per filter	Avg sheen colony count per filter	F statistic[a]	Probability of H_0[b]
P. aeruginosa	0	0	47		
	100	86	36		
	1,000	TNTC[c]	39		
	10,000	TNTC	12[d]	11.7	0.0028
	0	0	57		
	300	214	44		
	3,000	TNTC	14[d]		
	30,000	TNTC	0	425	<0.0005
	0	0	31		
	440	TNTC	25		
	4,400	TNTC	11[d]		
	44,000	TNTC	2	23.4	<0.0005
A. hydrophila	0	0	39		
	57	47	42		
	570	TNTC	18[d]		
	5,700	TNTC	0	48.5	<0.0005
	0	0	51		
	140	137	28		
	1,400	TNTC	0[d]		
	14,000	TNTC	0	345	<0.0005
	0	0	25		
	220	518	8[d]		
	2,200	TNTC	0		
	22,000	TNTC	0	40.3	<0.0005
Flavobacterium sp.	0	0	47		
	8	0	38		
	80	0	42		
	800	0	39	5.03	0.032
	0	0	25		
	1,100	0	24		
	11,000	0	19		
	110,000	0	25	2.18	0.19
	0	0	22		
	1,900	0	24		
	19,000	0	23		
	190,000	0	23	0.26	>0.25
Bacillus sp.	0	0	40		
	49	0	47		
	490	0	40		
	4,900	0	45	1.90	0.22
	0	0	26		
	1,000	0	31		
	10,000	0	28		
	100,000	0	27	1.41	>0.25
	0	0	31		
	2,800	0	28		
	28,000	0	23		
	280,000	0	25	2.25	0.18

[a] Analysis of variance.
[b] H_0, Null hypothesis of no significant difference.
[c] TNTC or greater than 300 colonies per filter.
[d] Sheen colony counts significantly lower at the 5% level than those filters when noncoliform bacteria were not added.

Geldreich et al. (5) and 1,000/ml inferred by Clark (4) would be 50,000 per filter and 100,000 per filter, respectively, for 100-ml samples filtered for coliform tests. Many of the SPC organisms do not produce colonies on MF on m-Endo medium.

Detection and enumeration of coliform bacteria in potable water samples examined by the MF technique can be hampered by interference from some common water bacteria. Since only two coliform and four other bacteria were tested, generalizations based on these results have to be considered tentative. However, the data do support some conclusions which need to be considered in the evaluation of coliform-monitoring data from water distribution systems. These conclusions are as follows. (i) Even at relatively low densities of 10 to 20/100 ml when there is no colony crowding, not all *E. cloacae* colonies on MFs will produce a sheen in 22 h at 35°C. (ii) At densities in the range of 1 to 80 colonies per filter specified by the EPA (3) for enumeration of coliforms, all *E. coli* produce sheen, but excessive crowding at much higher colony counts can reduce the number of

TABLE 4. Effect of noncoliform bacteria on sheen production by *E. cloacae* colonies on MFs

Noncoliform organism	No. of noncoliforms added per filter	Avg nonsheen colony count per filter	Avg sheen colony count per filter	F statistic[a]	Probability of H_0[b]
P. aeruginosa	0	4	12		
	380	TNTC[c]	12		
	3,800	TNTC	8		
	38,000	TNTC	1[d]	9.36	0.0056
	0	22	9		
	410	TNTC	11		
	4,100	TNTC	7		
	41,000	TNTC	0	2.91	0.10
	0	28	11		
	450	TNTC	8		
	4,500	TNTC	4		
	45,000	TNTC	0[d]	12.5	0.0023
A. hydrophila	0	23	12		
	100	157	8		
	1,000	TNTC	0[d]		
	10,000	TNTC	0	126	<0.0005
	0	10	12		
	140	162	7		
	1,400	TNTC	1[d]		
	14,000	TNTC	0	56.8	<0.0005
	0	19	11		
	170	197	6		
	1,700	TNTC	0[d]		
	17,000	TNTC	0	47.1	<0.0005
Flavobacterium sp.	0	8	36		
	64	7	35		
	640	9	40		
	6,400	18	34	1.46	0.16
	0	15	21		
	1,800	17	14		
	18,000	11	19		
	180,000	20	12	2.64	0.13
	0	19	20		
	2,600	25	21		
	26,000	24	19		
	260,000	15	25	2.42	0.16
Bacillus sp.	0	3	37		
	120	6	39		
	1,200	8	39		
	12,000	3	36	1.57	>0.25
	0	17	20		
	1,500	23	14		
	15,000	24	15		
	150,000	26	17	1.61	>0.25
	0	18	14		
	2,400	20	14		
	24,000	15	24		
	240,000	16	19	2.86	0.11

[a] Analysis of variance.
[b] H_0, Null hypothesis of no significant difference.
[c] TNTC or greater than 300 colonies per filter.
[d] Sheen colony counts significantly lower at the 5% level than those of filters when noncoliform bacteria were not added.

colonies which produce sheen. (iii) Some SPC organisms (*P. aeruginosa* and *A. hydrophila*) can cause significant reductions in sheen colony counts when present in densities considerably less than 500/ml of water. (iv) Some SPC organisms (*Bacillus* sp. and *Flavobacterium* sp.) do not cause reductions in coliform colony counts when present in densities greater than 1,000/ml.

These conclusions are based on laboratory studies with pure cultures. The coliform bacteria had not been exposed to the other bacteria before their retention on the surface of the MF. In a water distribution system there may be antagonistic effects of noncoliforms which cause die off of coliforms before the sample is collected or in the sample bottle before the sample is filtered. In this discussion, interference with coliform colony sheen production refers to phenomena which occur after the bacteria are on the membrane surface. We assume that any soluble materials in the water sample (or in the culture medium) are flushed from the MF by the normal washing procedure.

Coliforms in water distribution systems have to survive for long periods of time at very low nutrient concentrations and may be stressed by exposure to residual chlorine con-

centrations or to temperature variation in the sample bottle during transit to the laboratory. Such stressed coliforms might not produce sheen colonies (or even colonies) on m-Endo medium. The results reported herein are not related to recovery of stressed coliforms from water samples.

The *E. cloacae* organisms used in these studies were from a pure culture grown in nutrient broth just before dilution and filtering. They were not starved or exposed to chlorine, yet only a fraction (about half) of the colonies produced sheen on m-Endo medium in 24 h at 35°C at densities at which colony crowding on the filter was not a problem. We are not able to propose an explanation of why some of the presumably identical colonies produced sheen and others did not. However, it is clear that enumeration of coliforms in drinking water samples at moderate densities (10 to 20/100 ml) is probably inaccurate when some of the coliforms are *E. cloacae*.

The EPA guide for microbiological examination of water (3) specifies that all filters with greater than 80 coliform colonies be recorded as TNTC. Our results suggest that accurate colony counts of *E. coli* from a pure culture can be obtained on MFs up to counts of about 200 per filter. Crowding to the extent that colonies merged together did reduce sheen production but did not eliminate it altogether. Starved or chlorine-damaged *E. coli* cells from a water distribution system might react differently.

The SPC densities previously reported to interfere with coliform enumeration and detection are 500/ml (5) and 1,000/ml (4). Our results suggest that *P. aeruginosa* can reduce coliform counts at levels of about 3,000 per filter (30/ml) and eliminate coliform detection at levels of about 40,000 per filter (400/ml). Also, *A. hydrophila* apparently reduced coliform counts at about 200 per filter (2/ml) and eliminated coliform detection at about 1,000 per filter (10/ml). Thus, some SPC organisms can interfere with coliform enumeration and detection at densities considerably lower than those previously reported. The strains of *Bacillus* sp. and *Flavobacterium* sp. used in this study did not produce any apparent reduction in the numbers of sheen colonies produced by *E. coli* or *E. cloacae*, even when added at densities greater than 100,000 per filter (1,000/ml).

Nonsheen colonies on MF on m-Endo medium are often referred to as a back-ground count. Clarke (4) reported that information with his data and inferred that a background count of 1,000/100 ml (per filter) was associated with a decrease in coliform detection. However, the EPA guide (3) specifies that samples which produce more than 200 total colonies (sheen and nonsheen) on an MF be recorded as indeterminate. The density levels of *P. aeruginosa* and *A. hydrophila* which we observed to cause reductions in coliform counts would be manifest by background counts of greater than 200 per filter. This suggests the possibility that MF results from samples in which there is interference due to noncoliform bacteria might be eliminated from consideration in a monitoring program if the EPA guidelines are strictly applied.

It is clear that some noncoliform bacteria are antagonistic to coliforms when they coexist in the same milieu for some period of time (7, 9, 11, 13, 17). It is possible that a high SPC in a water sample might sometimes be a signal that coliforms were once present but have died off due to this type of antagonism. It is also known that other environmental factors may stress coliform organisms to the extent that they are not detected in water samples when the MF method is used (2, 12). Thus, there are several mechanisms which could result in failure to detect coliform bacteria by the MF method. We have demonstrated by laboratory studies that some SPC bacteria can cause inaccurate (low) coliform colony counts when present at densities considerably less than 500/ml if they form background colonies on MF on m-Endo medium.

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