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Viable but Nonculturable Bacteria in Drinking Water

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***Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Agrobacterium tumefaciens*, *Streptococcus faecalis*, *Micrococcus flavus*, *Bacillus subtilis*, and *Pseudomonas* strains L2 and 719 were tested for the ability to grow and maintain viability in drinking water. Microcosms were employed in the study to monitor growth and survival by plate counts, acridine orange direct counts (AODC), and direct viable counts (DVC). Plate counts dropped below the detection limit within 7 days for all strains except those of *Bacillus* and *Pseudomonas*. In all cases, the AODC did not change. The DVC also did not change except that the DVC, on average, were ca. 10-fold lower than the AODC.**

Bacteria initially presented with adequate nutrient to support growth and thereafter exposed to environments with low nutrient concentrations appear to adapt to the environmental challenge by utilizing one or more mechanisms for survival (12). Populations of bacteria have been discovered to undergo a dramatic decrease in plate count but remain viable when analyzed by the direct viable count (DVC) procedure (10). Bacteria which demonstrate this phenomenon, i.e., a viable but nonculturable state, include those in the genera *Vibrio* (3, 16), *Escherichia* (16), *Salmonella* (14), *Aeromonas* (1), *Legionella* (9), *Campylobacter* (13), and *Shigella* (3). These taxa include representatives of both autochthonous and allochthonous aquatic microbial flora (6). Bacteria demonstrating the viable but nonculturable state have to date included only gram-negative species, mainly because the DVC procedure does not lend itself to the analysis of gram-positive bacteria because the DVC procedure is dependent on the bacterium being sensitive to nalidixic acid (5).

Legionella pneumophila has been demonstrated to enter a viable but nonculturable stage when placed in drinking water (9), and this finding may explain the difficulty and frequent failure in isolating *L. pneumophila* from water samples tested during *Legionella* outbreaks (4). The survival of gram-negative bacteria other than *L. pneumophila* in drinking water has not been analyzed, and the ability of the DVC to detect viable but nonculturable organisms in tap water is not known.

The objective of this study was to examine a set of strains, including both gram-negative and gram-positive bacteria originally isolated from the aquatic environment, for their abilities to grow and/or survive in drinking water. Plate and direct microscopic counts were employed to detect and monitor the microorganisms in drinking water.

Cultures of *Pseudomonas fluorescens* L-2, *Pseudomonas* sp. strain 719, *Bacillus subtilis* CU-155, *Streptococcus faecalis* sf, *Micrococcus flavus* 731, *Klebsiella pneumoniae* Kno, *Agrobacterium tumefaciens* 646, and *Enterobacter aerogenes* 718 were provided by M. Alexander, Cornell University, Ithaca, N.Y., and grown in Trypticase soy broth (Difco Laboratories, Detroit, Mich.) for 18 h at 35°C. Cells were collected by centrifugation and washed three times in sterile, filtered drinking water. One liter of autoclaved, filtered (0.2-μm-pore-size filter) drinking water (obtained from a tap at the University of Maryland) was added to each acid-washed, 2-liter Erlenmeyer flask. All flasks were fitted with rubber stoppers containing serum-stoppered glass tubing as described by Colwell et al. (3). Microcosms were inoculated with approximately 10^6 cells per ml of washed culture. All microcosms were incubated with shaking (100 rpm) at 25°C.

Three methods of analysis were used to determine the presence of bacteria in the microcosms. The presence of culturable bacteria was determined by plate counts on Tryptic soy agar (TSA; Difco). Other media employed in these studies were MacConkey agar (MAC), KF *Streptococcus* agar (KSA), *Pseudomonas* isolation agar (PFA), nutrient agar (NA; Difco), and SYC agar (sucrose, 10 g; casein hydrolysate, 8 g; yeast extract, 4 g; $K_2HPO_4 \cdot 7H_2O$, 3 g; $MgSO_4$, 0.3 g; agar, 15 g; distilled water, 1 liter; pH 7.0). Culturable counts were determined by calculating the average number of colonies on duplicate plates of appropriate medium. Viable bacterial counts were performed by the DVC method of Kogure et al. (10). Those cells increasing in total length after incubation for 6 h in the presence of yeast extract and nalidixic acid were considered viable bacteria. Acridine orange direct counts (AODC [8]) were employed to detect the total number of bacteria present.

The concentration of free chlorine residual was determined by a modification of the orthotoluidine method (2). Visual comparisons of color were done in accordance with the Chlorine Color Comparator (Hellige).

Various gram-negative bacteria have been shown to proceed into a viable but nonculturable stage when exposed to environmental or nutritional stresses (6). In the present study, this stage of the bacterial life cycle was assessed for five gram-negative bacteria and three gram-positive bacterial strains. Three of the gram-negative bacteria (*A. tumefaciens*, *E. aerogenes*, and *K. pneumoniae*) demonstrated relatively short survival times in drinking water, i.e., were culturable for only a few days. After incubation for 2 days in a microcosm, *A. tumefaciens* was not detected by plating on SYC agar (Table 1). Culturable plate counts of *K. pneumoniae* on both TSA and MAC dropped below detection limits 4 days after inoculation (Table 1). No data were recorded prior to day 4, so the decline may have been more rapid than recorded. Determination of free chlorine residual showed less than 0.1 mg of chlorine per liter in the drinking water used in the microcosm.

As was observed for *K. pneumoniae*, *E. aerogenes*

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TABLE 1. Survival of *A. tumefaciens*, *K. pneumoniae*, and *E. aerogenes* in sterile drinking water at 25°C

Day	Log cells/ml[a]										
	A. tumefaciens			*K. pneumoniae*				*E. aerogenes*			
	AODC	DVC	SYC	AODC	DVC	TSA	MAC	AODC	DVC	TSA	MAC
0	6.3	6.2	3.2	6.2	6.2	5.2	6.4	6.3	6.0	3.6	3.9
1	6.3	6.1	2.0	6.0	6.2	ND	ND	5.7	5.6	ND	ND
2	6.2	6.2	0[b]	ND	ND	ND	ND	ND	ND	ND	ND
3	ND	ND	ND	5.8	5.4	ND	ND	5.7	5.1	ND	ND
4	6.2	6.1	0	ND	ND	0	0	ND	ND	0	0.7
7	6.2	6.1	0	5.7	5.1	ND	ND	5.9	5.2	ND	ND
10	ND	ND	ND	5.8	ND	0	0	5.1	5.1	0	0

[a] ND, Not determined. The formulation of SYC agar is given in the text.
[b] Limit of detection.

showed a decline in colony counts on MAC within 4 days (Table 1). Culturable counts for *E. aerogenes* on TSA at day 4 were ca. 10^1 CFU/ml, with the limit of detection being reached by day 10. For all three organisms (*A. tumefaciens*, *K. pneumoniae*, and *E. aerogenes*), the level of AODC remained at or around that at which they were inoculated into the microcosm. DVC for *A. tumefaciens* was just below that of the AODC, but DVC for *K. pneumoniae* and *E. aerogenes* were approximately 0.5 log unit lower than the AODC after incubation for 3 days.

Survival of *Pseudomonas* spp. in drinking water was markedly different from that of the other three gram-negative bacteria. After 95 days in the microcosm, both *Pseudomonas* sp. strain 719 and *P. fluorescens* L-2 remained at culturable levels on both TSA and PFA close to that of the AODC (Table 2). TSA and PFA provided similar culturable counts throughout the course of the experiment. Culturable counts on day 0 were approximately 1 log unit lower than counts on days 2 through 95. Between day 0 and day 2, the numbers declined almost 3 log units before a rapid climb in culturable numbers on day 2 that stabilized to just below those observed for AODC, which were unchanged over the course of the experiment.

The gram-positive bacteria examined for their abilities to remain culturable in drinking water yielded different patterns. The time to nonculturability of *M. flavus* was 7 days on both TSA and NA (Table 3). The AODC for *M. flavus* remained at or around initial levels through the 7 days of the experiment. *S. faecalis* demonstrated results comparable to those with *M. flavus*. Nonculturability of *S. faecalis* was reached by day 5 on both TSA and KSA, with AODC remaining at approximately that of the initial concentration of cells constituting the inoculum. For both *M. flavus* and *S. faecalis*, the largest drop in culturable counts was observed after day 1. Only a minor decrease (less than 1 log unit) was observed at day 1.

TABLE 2. Survival of *Pseudomonas* sp. strain 719 and *P. fluorescens* L-2 in sterile drinking water at 25°C

Day	Log cells/ml[a]							
	Pseudomonas sp. strain 719				*P. fluorescens* L-2			
	AODC	DVC	PFA	TSA	AODC	DVC	PFA	TSA
0	6.1	5.9	4.2	4.0	6.3	6.1	5.3	5.1
1	6.0	5.7	2.7	2.1	6.4	6.0	2.6	2.5
2	6.1	ND	4.3	4.4	6.4	ND	ND	ND
3	6.2	ND	6.1	6.0	6.4	ND	5.9	5.9
7	6.2	ND	6.0	6.0	6.4	ND	5.8	5.9
17	6.3	ND	6.1	6.3	6.6	ND	6.1	6.1
25	6.3	ND	6.1	6.1	6.5	ND	6.1	6.1
67	6.3	ND	6.1	6.0	6.3	ND	6.1	6.1
95	6.3	ND	6.1	6.0	6.4	ND	6.0	5.9

[a] ND, Not determined.

The results for *B. subtilis* were significantly different from those for the other gram-positive bacteria examined in this study. *Bacillus* culturable counts decreased approximately 3 log units on the first day. Throughout the rest of the experiment, the culturable count remained around 1.5 log culturable cells per ml on both TSA and NA. The culturable numbers of *B. subtilis* remained at this level for upwards of 25 days (data not shown), whereas the AODC was approximately the same as that for the inoculum throughout the experiment.

From the results presented here, gram-negative organisms appear to remain viable even when no longer culturable on solid media. *A. tumefaciens*, *K. pneumoniae*, and *E. aerogenes* were nonculturable within 4 days of being introduced into sterilized drinking-water microcosms. The drop in plate counts of the bacteria incubated in autoclave-sterilized drinking water was not caused by residual chlorine, since free-chlorine residual concentrations were less than 0.1 mg/liter. Viability was determined by the DVC procedure and confirmed that cells in the microcosm remained substrate responsive. These findings are in contrast to those of Gurijala and Alexander (7), who studied *A. tumefaciens* and *K. pneumoniae* by using filter-sterilized lake water and phosphate buffer. They found that *A. tumefaciens* increased in number initially and then leveled off to a plate count higher than that for the original inoculum. The nutrient concentration in lake water compared with that of drinking water may account for the rapid decline in plate counts observed for *A. tumefaciens* in drinking water. Gurijala and Alexander (7) found that *K. pneumoniae* displayed an initial decrease and a subsequent increase in culturable numbers in phosphate buffer. In filter-sterilized lake water, *K. pneumoniae* declined ca. 2 log units in 5 days. Sjogren and Gibson (15) found that *K. pneumoniae* demonstrated approximately 60% survival in lake water after 5 days. The combination of low nutrient and chlorine concentrations in drinking water may be responsible for the decrease in plate count, i.e., loss of culturability, which was observed after incubation for 4 days in the microcosm.

The ability of *Pseudomonas* spp. to survive in drinking water was very different from that of the other gram-negative organisms examined. *Pseudomonas* spp. maintained the

TABLE 3. Survival of *S. faecalis*, *M. flavus*, and *B. subtilis* in sterile drinking water at 25°C

Day	Log cells/ml[a]								
	S. faecalis			*M. flavus*			*B. subtilis*		
	AODC	KSA	TSA	AODC	NA	TSA	AODC	NA	TSA
0	6.5	6.4	6.4	6.1	5.5	5.3	6.1	4.2	4.7
1	6.2	5.5	5.7	6.0	5.3	5.1	5.8	1.6	1.5
2	6.2	2.5	2.9	5.9	2.7	2.4	5.8	1.8	1.5
3	6.2	0.2	0.7	6.0	1.9	1.6	ND	ND	ND
4	6.1	0[b]	0.2	5.9	1.7	0.3	ND	ND	ND
5	6.2	0	0	5.9	0.5	0.5	5.7	1.6	1.7
7	ND	ND	ND	5.9	ND	ND	ND	ND	ND
9	ND	ND	ND	ND	ND	ND	5.7	1.7	1.7
38	ND	ND	ND	ND	ND	ND	ND	1.2	ND

[a] ND, Not determined.
[b] Limit of detection.

original cell inoculum size for upwards of 95 days. Culturability of each of the *Pseudomonas* spp. has surpassed 200 days in other microcosms under similar experimental conditions (data not shown). Kurath and Morita (11) found a marine *Pseudomonas* sp. that remained culturable beyond 40 days in a minimal-salts solution. Therefore, *Pseudomonas* spp. appear to possess the ability to remain culturable for unusually long periods compared with other gram-negative bacteria. This finding contrasts with the findings of and assumptions made by Sjogren and Gibson (15), who found that for a lake water species of *Pseudomonas*, only 18% of the cells survived in lake water after 24 h and no cells survived in distilled water after 24 h at 30°C. In the study by Sjogren and Gibson (15) it was assumed that the number of surviving, i.e., culturable, *Pseudomonas* cells would continue to decrease after 24 h of incubation in lake water. As can be seen in Table 2, the *Pseudomonas* spp. increased in number after incubation for 24 h. This pattern of survival was reproducible in our experiments.

Survival of gram-positive bacteria in drinking water in this study revealed two patterns. The first, which is characteristic of gram-negative enteric bacteria examined in earlier studies (9, 13, 14, 16) and of *K. pneumoniae*, *E. aerogenes*, and *A. tumefaciens*, was a rapid decrease in culturable numbers to less than 1 culturable cell per ml. *S. faecalis* and *M. flavus* also demonstrated this pattern. An initial rapid decline in culturable numbers with maintenance of culturable numbers at a very low number that remained relatively constant throughout incubation in the microcosm is exemplified by *B. subtilis*, for which one can assume that the vegetative state persists. The presence of spores in the microcosm was not tested, so the total number of culturable *Bacillus* cells i.e., spores as well as vegetative cells, was not determined. Viability of the gram-positive bacteria could not be tested by the DVC procedure, since nalidixic acid does not inhibit the growth of gram-positive bacteria (5).

The significance of the results obtained in this study lies in demonstrating a need for new methods for detection of microorganisms in natural systems. According to data presented in this study, *K. pneumoniae*, *E. aerogenes*, *A. tumefaciens*, *S. faecalis*, and *M. flavus* would go undetected if only plate counts were used to determine viability. Present methods of monitoring public water supplies that are untreated or have a low free-chlorine residual may actually underestimate the presence of coliforms or total heterotrophs. Clearly, a preferred approach to detection of bacteria in potable-water systems lies in direct rather than indirect detection, i.e., eliminating the need for culturing. Use of genetic probes and fluorescent antibodies are alternatives, but until gene probes for direct detection of pathogens can be routinely used to monitor the public health safety of drinking water, the question of whether culture methods for testing water quality and safety are sufficient to ensure public health safety in all circumstances remains. Furthermore, methods to determine the number of nonculturable bacteria which maintain pathogenicity in a population are needed.

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