Bacterial Inhibitors in Lake Water

THEODORE M. KLEIN† AND MARTIN ALEXANDER*

Laboratory of Soil Microbiology, Department of Agronomy, Cornell University, Ithaca, New York 14853

Received 11 October 1985/Accepted 20 March 1986

The populations of six bacterial genera fell rapidly after their addition to sterile lake water but not after their addition to buffer. The decline in numbers of two species that were studied further, Klebsiella pneumoniae and Micrococcus flavus, occurred even when the buffer was added to sterile lake water. The inhibition of K. pneumoniae by substances in lake water varied with the season of the year, and the rate and extent of decline of both species were different in sterile samples of different lakes. The extent of reduction in the density of K. pneumoniae was independent of initial population size and was diminished by the addition of 10 μg of glucose per ml of lake water. The toxin was removed from lake water by dialysis and by a cation-exchange resin but not by an anion-exchange resin, and it was destroyed by heating. The inhibition of K. pneumoniae was not evident in lake water buffered at a pH value above 8.0. We suggest that toxins may be important in determining the composition of the bacterial community of lakes.

The survival of a microorganism in an environment in which it is not indigenous is dependent on the organism's ability to tolerate a set of biological and physical stresses different from those in its native habitat. The renewed interest in inoculating microorganisms into natural ecosystems to control insects or plant pathogens, to stimulate plant growth, or to destroy chemical pollutants has stimulated research designed to understand these stresses and the responses of microorganisms to them. Knowledge of the factors that influence survival of species introduced into new environments also should facilitate prediction of the likely persistence of genetically engineered organisms introduced into natural environments and help to select organisms whose survival in such environments may be either long or short as desired for particular functions or uses.

One factor that may limit the survival of organisms introduced into natural ecosystems is the presence of toxic compounds that may cause the elimination of susceptible nonindigenous species. Marine waters are known to contain substances with bactericidal activity (5), and it has been suggested that these inhibitors are produced by algae (15) or bacteria (12). Saz et al. (13) reported that the inhibitory effect in some waters was the result of a high-molecular-weight compound and not the salinity or osmotic pressure of the water. Bacteriocin-like substances have been implicated in the decline of coliforms in drinking water distribution systems (7). In addition to organic inhibitors, heavy metals (2) and ammonia (10) may cause the death of species introduced into natural ecosystems.

The present investigation was undertaken to determine the possible role of inhibitors in the survival of bacteria in freshwater environments and to characterize the toxic substances that might be present.

MATERIALS AND METHODS

Klebsiella pneumoniae KNo was provided by A. A. Szalay, Agrobacterium tumefaciens was provided by M. A. Cole, Acinetobacter sp. was provided by C. G. Daughton, Escherichia coli 9637 was provided by the American Type Culture Collection, and Micrococcus flavus and Enterobacter sp. was provided by D. P. Labeda. Pseudomonas strains were isolated by plating samples from Cayuga and Beebe Lakes, Ithaca, N.Y., on a medium containing 1.5% Trypticase soy powder (BBL Microbiology Systems, Cockeysville, Md.) and 1.5% agar, and the isolates were identified by the API 20 system (Analytab Products, Plainview, N.Y.) (11). The bacteria were grown in 25-ml culture tubes containing 5 ml of half-strength Trypticase soy broth. The cultures were incubated at 28°C for 24 h on a rotary shaker operating at 120 rpm. The cells were collected by centrifugation at 2,500 × g for 5 min, suspended in 0.1 M phosphate buffer (pH 7.1), centrifuged again, and then suspended in fresh buffer.

Samples of aquatic environments or 0.1 M phosphate buffer (pH 7.1) were sterilized by passage through 0.20-μm nylon membrane filters (Nalge Co., Rochester, N.Y.), and 50-ml portions were added to 250-ml Erlenmeyer flasks. Portions of the filter-sterilized aquatic samples were plated on half-strength Trypticase soy agar; no growth was evident on these plates after a 7-day incubation. The solutions were amended with the washed bacterial cells at initial densities of about 10⁴ to 10⁶ cells per ml. The flasks were incubated in the dark at 28°C on a rotary shaker operating at 120 rpm. Bacterial densities in three replicate flasks were determined by the spread plate technique with two replicate plates per dilution. The bacteria were plated on a half-strength Trypticase soy agar.

For some studies, filter-sterilized lake water was dialyzed against distilled water at 4°C for 24 h with dialysis tubing (Spectrum Medical Industries, Los Angeles, Calif.) having a 1,000-molecular-weight cutoff. Lake water was also treated with cation-exchange gel (CM-Sephadex, C-25) or anion-exchange gel (DEAE-Sephadex, A-25) obtained from Pharmacia Fine Chemicals, Piscataway, N.J. For this purpose, 100-ml portions of filter-sterilized lake water or phosphate buffer was added to a 500-ml Erlenmeyer flask containing 5.0 g of gel. After the slurry was allowed to equilibrate for 24 h, it was clarified by passage through a 0.20-μm filter. In some instances, lake water was adjusted to pH values from 6.9 to 9.1 by adding 5 ml of 2.0 M Tris of the appropriate pH value to 45-ml samples.

Samples were collected from Cayuga Lake and Beebe Lake in Ithaca, N.Y., Dryden Lake in Dryden, N.Y., and
TABLE 1. Survival of bacteria in phosphate buffer and Cayuga Lake water

<table>
<thead>
<tr>
<th>Organism</th>
<th>Day 0</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer</td>
<td>Lake water</td>
</tr>
<tr>
<td>Enterobacter sp.</td>
<td>1,300</td>
<td>1,100</td>
</tr>
<tr>
<td>Acinetobacter sp.</td>
<td>410</td>
<td>320</td>
</tr>
<tr>
<td>E. coli</td>
<td>980</td>
<td>280</td>
</tr>
<tr>
<td>A. tumefaciens</td>
<td>420</td>
<td>1,200</td>
</tr>
<tr>
<td>M. flavus</td>
<td>58</td>
<td>300</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>720</td>
<td>170</td>
</tr>
<tr>
<td>Pseudomonas sp. strain C1</td>
<td>740</td>
<td>120</td>
</tr>
<tr>
<td>Pseudomonas sp. strain C2</td>
<td>21</td>
<td>0.60</td>
</tr>
<tr>
<td>Pseudomonas sp. strain B1</td>
<td>480</td>
<td>14</td>
</tr>
</tbody>
</table>

White Lake and Black River in White Lake, N.Y. Samples were filter sterilized at the site of collection and used within 2 h, except that samples of White Lake and Black River were stored at 4°C for 18 h before use.

RESULTS

The numbers of several bacterial species were determined initially and 4 days after their inoculation into filter-sterilized water from Cayuga Lake and phosphate buffer. The densities of Enterobacter sp., Acinetobacter sp., E. coli, A. tumefaciens, M. flavus, and K. pneumoniae declined markedly in the sterile lake water and were approximately 1 to 3 orders of magnitude lower than their initial numbers after 4 days (Table 1). In contrast, these bacteria survived well and some grew in phosphate buffer. Their persistence in buffer suggested that starvation was not the cause of their decline in the sterile lake water. The two strains isolated from Cayuga Lake (Pseudomonas sp. strain C1 and C2) and the isolate from Beebe Lake (Pseudomonas sp. strain B1) declined in both phosphate buffer and sterile lake water. Although the numbers of the three lakewater pseudomonads fell in lake water, the densities of strains C1 and B1 at 4 days were higher than those of the cultures that had been maintained in the laboratory for long periods. The marked differences in survival of some of the test organisms in lake water and buffer suggest that the lake water contains one or more inhibitors.

To determine whether the differences in survival of the organisms in phosphate buffer and lake water were a result of some component of the buffer, K. pneumoniae and M. flavus were incubated for 4 days in sterile distilled water, unamended sterile samples from Cayuga (pH 8.2) or Beebe Lake (pH 8.0), or sterile lake water amended with phosphate buffer to a final concentration of 0.1 M and to a final pH of 7.7. The initial cell density was $1.7 \times 10^6 \text{ K. pneumoniae}$ or $3.2 \times 10^4 \text{ M. flavus}$ cells per ml. The bacteria survived in large numbers in distilled water, but appreciable declines were observed in both the buffered and unbuffered samples from Beebe or Cayuga Lake (Table 2).

To determine whether the bacterial decline occurred in other natural waters, samples from various locations were filter sterilized and inoculated with K. pneumoniae or M. flavus. Counts in the lake water and buffer were made for 8 days. The numbers of K. pneumoniae cells declined somewhat in the buffer, the cell density falling from about $6 \times 10^6$ to $6 \times 10^4$ per ml in 8 days (Fig. 1). Marked losses of viability were noted after 2 days in samples from Cayuga and Beebe Lakes. The rate of decline was less in Dryden Lake water, but it was still more rapid than in buffer. The loss of viability was similar in river water and buffer, and the organism grew before declining in abundance in water from White Lake.

The density of M. flavus fell in buffer and in samples of the natural waters, although the rate and extent of decline differed among samples (Fig. 1). From the initial value of $6.9 \times 10^6$/ml, the numbers fell slowly in samples from White Lake and Black River and to a somewhat greater extent in buffer. The decline in viable count was especially great in waters from Cayuga and Beebe Lakes at 2 days. The density declined somewhat later in the sample collected from Dryden Lake, but the population size fell to less than 10 cells.

TABLE 2. Number of K. pneumoniae and M. flavus surviving after 4 days in buffered and unbuffered lake water and in distilled water

<table>
<thead>
<tr>
<th>Sample</th>
<th>K. pneumoniae</th>
<th>M. flavus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>290,000</td>
<td>8,100</td>
</tr>
<tr>
<td>Cayuga Lake water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffered</td>
<td>4,000</td>
<td>15</td>
</tr>
<tr>
<td>Unbuffered</td>
<td>7,800</td>
<td>29</td>
</tr>
<tr>
<td>Beebe Lake water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffered</td>
<td>4,500</td>
<td>20</td>
</tr>
<tr>
<td>Unbuffered</td>
<td>1,300</td>
<td>24</td>
</tr>
</tbody>
</table>

FIG. 1. Survival of K. pneumoniae and M. flavus added to samples of sterile buffer, lake water, or river water.
per ml at 4 days; thereafter, the number increased somewhat. In many instances, the population size increased after the initial period of decrease in population size.

To assess whether the extent of decline was related to the initial population size, K. pneumoniae cells were added to sterile Cayuga Lake water and buffer at various densities. Counts at 2 and 4 days revealed a slow drop in numbers in buffer, the population decreasing by about 1 order of magnitude in 4 days (Table 3). In lake water, the density usually fell to 2 to 3 orders of magnitude regardless of initial population size.

An experiment was conducted to determine whether the addition of a carbon source could overcome the toxicity. Sterile Cayuga Lake water was amended with 0, 1, 10, or 100 μg of glucose per ml and inoculated with 1.9 × 10⁶ K. pneumoniae cells per ml. The population fell in the lake water that was not amended with glucose and in lake water that received 1 or 10 μg of glucose per ml (Fig. 2). The extent of decline was somewhat reduced when the water received 10 μg of glucose per ml. Growth of K. pneumoniae occurred in the sample containing 100 μg of glucose per ml, the population size increasing rapidly even in the first day.

Cayuga Lake was sampled monthly beginning in August 1983. The lake water was sterilized by filtration, and these samples and buffer were inoculated with K. pneumoniae. The numbers of survivors were determined after 2 days. In the samples collected in August, the numbers of survivors were about 1 order of magnitude lower in the lake water than in the buffer, but the differences were much greater in the lake water collected in September through January (Table 4).

Toxicity was evident in all later samples except for the one taken in May, but the extent of inhibition varied among the samples. The differences in values at 2 days between buffer and lake water were statistically significant (P ≤ 0.001), except for the May samples.

To characterize the inhibitory agent(s) of lake water, samples from Cayuga and Beebe Lakes were dialyzed against distilled water or subject to batch treatment with an anion-or a cation-exchange gel. K. pneumoniae did not lose viability in 2 days in lake water that had been treated with the cation-exchange resin, but its abundance decreased markedly in water treated with the anion-exchange resin (Table 5). Both K. pneumoniae and M. flavus survived well in lake water that had been dialyzed with tubing with a molecular weight cutoff of 1,000. The two bacteria also survived in large numbers for the 2-day test period in Cayuga or Beebe Lake water that had been autoclaved for 15 min, although they declined in the unheated samples. Boiling also inactivated the inhibitors.

The effect of pH of the lake water on the persistence of K.

![FIG. 2. Population density of K. pneumoniae introduced into filter sterilized Cayuga Lake water that was amended with 0, 1, 10, or 100 μg of glucose per ml.]
**DISCUSSION**

The results show the presence of a toxic compound or compounds in aquatic habitats that cause the rapid decline in density of introduced bacteria. The inability to persist in large numbers in the lake samples is not the result of the susceptibility of the bacteria to starvation, because they survived well in sterile buffer for at least 4 days. In addition, *K. pneumoniae* and *M. flavus* survived in deionized distilled water. Predation by protozoa, which is an important factor in the elimination of bacteria in some aquatic and terrestrial environments (1), did not contribute to the decline since the lake water was filter sterilized before use.

Although many of the added bacteria died, the species were not totally eliminated because relatively small numbers often persisted. Similar results have been found for *Salmonella* (6), *Pseudomonas*, and *Rhizobium* (16) species in fresh waters. The reason for the prolonged persistence of the survivors of a population that has undergone a large decline is uncertain. In nonsterile environments, the persistence could be the result of a density of survivors too low to support further predation or parasitism (1, 19). In the sterile samples used in this study, the persistence may be caused by the resistance of the survivors to the toxins or because of the disappearance of the inhibitors.

The temporal variability in the concentration or activity of the inhibitor is important in attempts to predict the potential of an organism to survive. The time of year is known to influence the survival of organisms introduced into soil (14) and marine environments (8). Changes in the pH of natural waters may explain some of the variation in survival found at different times of the year, and small changes in the pH of lake water greatly influenced the survival of *K. pneumoniae*. The concentration of available carbon also may influence survival, as suggested by the finding that the level of glucose added to lake water altered the extent of decline of *K. pneumoniae*. Moebus (9) found that supplementing seawater with small amounts of glucose accelerated the decline of introduced bacteria.

The data suggest that the bactericidal agent(s) is a low-molecular-weight cationic compound. Its activity is greater at low pH values. Inactivation of the toxin by heat, which is often assumed to indicate the involvement of organic compounds, does not rule out the possibility that the inhibitor is inorganic, because heat can precipitate those metals that may cause the decline of species introduced into the marine environment (2). Although substances that are inhibitory to introduced organisms occur in soils (18) and waters (13), the identities of the compounds are largely unknown. High-molecular-weight compounds are thought to contribute to the decline of enteric bacteria in seawater (5) and fungi in soil (17). On the other hand, ammonia (10), carbon dioxide (4), and low-molecular-weight fatty acids (3) may be detrimental to microorganisms in natural environments. In view of the presence of inhibitors in many environments and the susceptibility of many microbial species to them, further study is required to establish the identities and ecological significance of these antimicrobial agents. Nevertheless, their presence in different lakes suggests that such inhibitors probably are important in regulating the composition of bacterial communities.

**ACKNOWLEDGMENTS**

This research was supported by U.S. Environmental Protection Agency assistance agreement R807688020 and cooperative agreement CR81233010.

**LITERATURE CITED**


