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Investigation of the Survival Characteristics of *Rhodococcus coprophilus* and Certain Fecal Indicator Bacteria

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Rhodococcus coprophilus and *Clostridium perfringens* survived in fresh water samples held at 5, 20, and 30°C for over 17 weeks, whereas *Escherichia coli* and fecal streptococci disappeared after 5 weeks at all three temperatures. *R. coprophilus* survived for more than 8 months in sterilized sewage and deionized water at all three temperatures, whereas in normal sewage held at 20°C, the survival time was 12 to 26 weeks. In samples held at 30°C, survival times were shorter, probably because of interbacterial competition or protozoal predation. The results indicate that *R. coprophilus* may be a useful indicator of the presence of remote fecal pollution of farm animal origin, but not of recent pollution, when enumerated alone in polluted waters or wastewaters.

The main aim of the bacteriological examination of water is to detect human or animal fecal pollution. The presence of fecal pollution constitutes a danger to health from intestinal infections caused by excreted pathogens. Traditionally, *Escherichia coli* has been used as an indicator organism to detect human or animal fecal contamination. Recently, *Rhodococcus coprophilus* has been suggested as an indicator of farm animal pollution (11). This nocardioform actinomycete, previously described by Willoughby (15) as Lspi (large spored pink irregular) and given the specific name *R. coprophilus* by Rowbotham and Cross (10), is commonly found in herbivore dung and aquatic environments polluted by animal feces. Work done in our laboratory has shown that *R. coprophilus* occurs in feces of farm animals (cattle, chickens, ducks, geese, horses, pigs, sheep, and turkeys) and in waters and wastewaters polluted with these fecal wastes, but not in human feces, and that *R. coprophilus* thus has potential as a specific indicator of nonhuman fecal pollution (7).

Streptococcus bovis is commonly present in the feces of cows, other farm animals, and some avian species (1, 3, 4, 9, 13; J. I. Oragui, M.S. thesis, University of Dundee, Scotland, 1978). Its presence in polluted waters would normally indicate the fecal nature of pollution from animals (4), but its short survival period in water (4, 8) limits its usefulness to areas near the source of pollution. The enumeration of *S. bovis* has been greatly facilitated by the recent development of a membrane filtration medium (9). In this paper, we report the results of experiments on some of the survival characteristics of *R. coprophilus* compared with those of other indicator bacteria.

MATERIALS AND METHODS

Bacteria. Two strains of *R. coprophilus*, CUB687 (Actinomycete Collection, University of Bradford, England) and LUCE1 (a wild strain isolated locally from sheep feces), were grown in Bennett broth (10) in an orbital incubator (180 rpm) for 96 h at 30°C. All broth cultures were washed two to three times in sterile, quarter-strength Ringer solution (Oxoid Ltd.) by centrifugation at 2,200 × *g* and resuspension of the pellet and then diluted as necessary to give a bacterial density of 10^5 to 10^6 per ml. The actual count was determined by enumeration in a hemacytometer.

Collection of samples and bacterial enumeration. Water and sewage samples were collected in sterile 2.5-liter bottles and transported to the laboratory within 1 h of collection. Sewage samples (raw and treated effluent) were thoroughly mixed and divided into portions of 500 ml each. One portion of each sample, together with 500 ml of deionized water, was sterilized at 121°C for 15 min. When cooled to room temperature, each bottle containing sterile raw sewage or treated effluent was seeded with the appropriate strain of *R. coprophilus* to yield a final concentration of 10^5 to 10^6 organisms per ml. The number of CFU of *R. coprophilus* was determined (by spreading 0.2 ml of decimal dilutions on the surface of modified M3 agar), and each sample was thoroughly mixed and divided into three parts for storage in the dark at 5, 20, and 30°C.

E. coli was enumerated on membrane filters (Millipore; type HAWG 047 SO) and incubated on pads saturated with 0.1% lauryl sulfate broth (5, 12). Incubation took place at 30°C for 4 h followed by 44°C for 18 h (3). All yellow colonies were counted as *E. coli*. Fecal streptococci were counted on membranes incubated on KF Streptococcus agar (6). Plates were incubated at 37°C for 4 h followed by 44°C for 44 h (16; J. I. Oragui, M.S. thesis), and all red- and maroon-colored colonies were counted as fecal streptococci. *S. bovis* was counted on membrane-bovis agar incu-

bated at 30°C for 4 h followed by 39°C for 44 to 72 h (9). The method used to count *Clostridium perfringens* was that used by Opara (A. A. Opara, Ph.D. thesis, University of Dundee, Scotland, 1978), although egg yolk was omitted from the Shahidi Ferguson Perfringens agar. The method included heating the sample to 80°C for 10 min; incubation was conducted anaerobically in GasPak (BBL Microbiology Systems, Cockeysville, Md.) jars incubated at 37°C for 48 h. Before counting *R. coprophilus*, we heated the samples to 55°C for 6 min to reduce the amount of contaminating bacteria (11); next, 0.2-ml portions of the appropriate decimal dilution were spread on the surface of plates of modified M3 agar (7) with sterile, L-shaped glass rods. Incubation took place at 30°C for 14 days followed by exposure to sunlight (intensity range, 500 to 1,500 lx) for 3 to 4 days, after which all pink, stellate colonies were counted as *R. coprophilus* (11).

RESULTS

Survival of bacteria in water and sewage. Seven different samples were examined for evidence of the survival of various indicator bacteria in polluted waters. Figure 1 shows the typical survival patterns of *R. coprophilus*, *E. coli*, fecal streptococci, *S. bovis*, and *C. perfringens* in a single water sample obtained from Adel Beck (a stream running through north Leeds, England) and held at 5, 20, and 30°C for up to 500 days. The data show that there was no significant reduction in the numbers of *C. perfringens* or *R. coprophilus* in water samples stored at all three temperatures after 2 days or after 7 days. Similarly, fecal streptococci showed no significant reduction in numbers after 2 days at 5°C but had declined by about 1 log at 20 and 30°C. In sharp contrast, *S. bovis* declined by 1 log at 5°C and by 2 logs at 20 and 30°C after 2 days; however, it was not recovered from the samples stored at 20 and 30°C after storage for 5 and 3 days, respectively. Our results indicate that, in general, fecal streptococci have better survival characteristics than *E. coli*, especially at 20 and 30°C, although initially there was a greater reduction in numbers of fecal streptococci, which was investigated and attributed to the earlier elimination of *S. bovis*. Results obtained from the characterization of isolates growing on membranes incubated on KF agar after 6 days confirm the results of recovery tests with membrane-bovis agar and also show that *S. bovis* survived only for a matter of days in polluted waters. *Streptococcus faecalis* and *Streptococcus faecium* were the only surviving fecal streptococci recovered from the water samples after 5 days of storage in the laboratory. The early elimination of fecal streptococci because of the rapid disappearance of *S. bovis* was followed by a greater persistence in numbers of fecal streptococci compared to those of *E. coli* from about day 4 in samples held at 20 and 30°C. These results tend to substantiate those of McFeters et al. (8), who found that fecal coliforms survived longer than *Streptococcus equinus* and *S. bovis*.

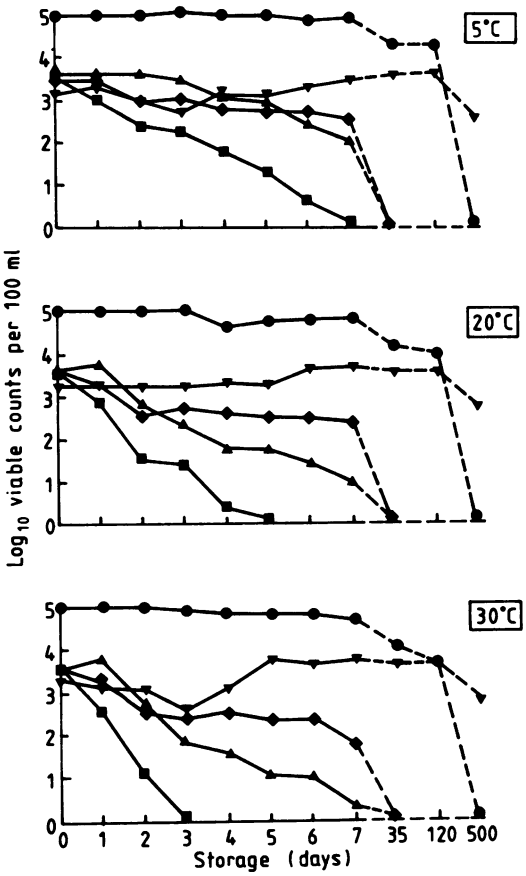

FIG. 1. Comparison of the survival of various indicator bacteria in a water sample obtained from Adel Beck and held at 5, 20, and 30°C in the dark. ●, *R. coprophilus*; ▲, *E. coli*; ◆, fecal streptococci; ▼, *C. perfringens*; ■, *S. bovis*.

C. perfringens survived longest in the water samples and could be recovered after 500 days, whereas *R. coprophilus* survived for over 120 days but could not be recovered after 500 days (Fig. 1). *E. coli* and fecal streptococci survived for at least 7 days but less than 35 days. *S. bovis*, however, survived for 6, 4, and 2 days at 5, 20, and 30°C, respectively. The results also show that there was a slight, though insignificant increase in the numbers of *E. coli* at 20 and 30°C during the first 24 h, but not at 5°C. The numbers of *C. perfringens* in samples stored at all three temperatures showed a slight increase from about day 4, possibly due to sporulation of vegetative cells, which would then have been resistant to the heat treatment used on the samples.

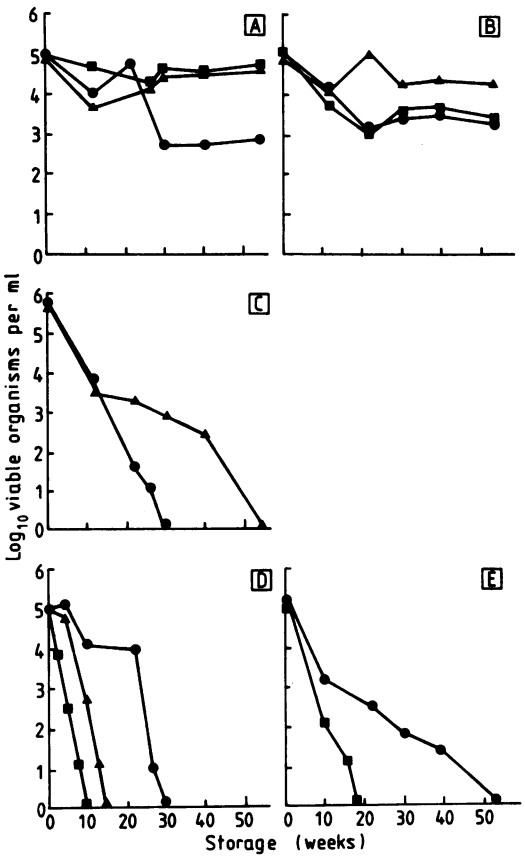

FIG. 2. Survival of *R. coprophilus* CUB687 seeded into sterilized and normal raw sewage, treated effluent, and sterilized deionized water. Samples were held in the dark at 5 (●), 20 (▲), and 30°C (■). (A) Sterilized raw sewage. (B) Sterilized treated effluent. (C) Sterilized deionized water. (D) Normal raw sewage. (E) Normal treated effluent.

Figures 2 and 3 show the survival of two strains of *R. coprophilus* (CUB687 and LUCE1) in raw (nonsterilized) and sterilized sewage and treated effluent and also in deionized water held at 5, 20, and 30°C. In the tests with normal sewage, we intended to preserve the nutritional and biological components of the waste and maintain interbacterial competition as long as possible. Figure 2 shows that the numbers of *R. coprophilus* CUB687 in both normal raw sewage and treated effluent declined at all three temperatures, except in normal raw sewage held at 5°C, at which point there was a slight increase before the decline. This organism disappeared (from 5 logs to zero) in normal raw sewage stored at 5, 20, and 30°C for 10 to 30 weeks; in sterilized raw sewage, the decline in the same period varied only between 0.5 and 2.5 logs. In normal treated effluent stored at 5 and 30°C, the organism disappeared after 18 and 52 weeks, respectively; in contrast, in samples of sterilized treated effluent, there was only a 1.5 log reduction in the same period at these temperatures.

The numbers of *R. coprophilus* declined in both normal raw sewage and treated effluent and also in sterilized deionized water. This decline, however, was more rapid in normal raw sewage held at 30°C and in normal treated effluent held at 20°C than in sterilized raw sewage and treated effluent at the same temperatures. In sterilized raw sewage and treated effluent, the organism was recovered at all three temperatures after 50 weeks; in normal raw sewage and treated effluent held at 30°C, the organism was not recovered after 18 and 8 weeks, respectively (Fig. 2).

Results obtained in tests with a wild strain of *R. coprophilus* (LUCE1) stored in sterilized raw sewage and treated effluent were essentially similar (Fig. 3) to those of strain CUB687 in the sterilized samples (Fig. 2), except that initially, an increase of 1 to 2 logs was observed in both samples stored at all three temperatures. In the experiments with normal raw sewage (Fig. 3), the wild strain disappeared (from 5 logs to zero) in samples stored at 5, 20, and 30°C for 18 to 90 weeks. In normal raw sewage and treated effluent, strain CUB687 declined faster than the wild strain (Fig. 2 and 3). For example, after being stored for 26 weeks at 5°C, strain CUB687 had declined by 4 logs, whereas the wild strain LUCE1 declined by approximately only 1 log after the same period of time. Furthermore, after 90 weeks of storage at 5°C, the wild strain had declined by approximately 2 logs, as opposed to strain CUB687, which declined by the same order of magnitude after only 16 weeks.

DISCUSSION

It is difficult to simulate in the laboratory various environmental conditions to which excreted pathogens and indicator bacteria are exposed. Not only does the climate vary continuously, but the bacterial population and nutrient content are also constantly changing. However, given these limitations to our tests, *R. coprophilus* survived in fresh water better and longer than *E. coli* and fecal streptococci. Furthermore, it would appear that this organism is intermediate in this respect, falling somewhere between *E. coli* and *C. perfringens*. *C. perfringens* survived the longest in polluted waters and is therefore an excellent indicator of remote fecal contamination or contamination of distant origin. In sterilized sewage, regardless of storage temperature, *R. coprophilus* survived for more than 50 weeks; in normal sewage, however, its numbers declined to zero during that time. That decline should be investigated to determine whether it is due to protozoal predation or other

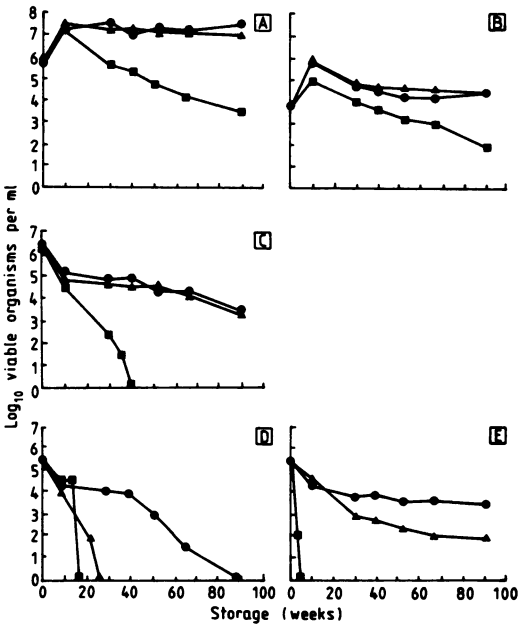

FIG. 3. Survival of *R. coprophilus* LUCE1 (a wild strain isolated from sheep feces) seeded into sterilized and normal raw sewage, treated effluent, and sterilized deionized water. Samples were held in the dark at 5°C (●), 20 (▲), and 30°C (■). (A) Sterilized raw sewage. (B) Sterilized treated effluent. (C) Sterilized deionized water. (D) Normal raw sewage. (E) Normal treated effluent.

factors. It is difficult to establish whether the organisms multiply in sterilized sewage because the increase observed in Fig. 3 (after 10 weeks) may have been due to the coccal elements formed from fragmenting mycelia (2).

The survival of indicator bacteria in water is a fundamental concern in the bacteriological examination of water supplies, with the occurrence and distribution of *R. coprophilus* being well documented (2, 7, 10, 11, 15). The results presented here indicate that *R. coprophilus* can survive for at least 120 days in polluted water. In contrast, *S. bovis*, fecal streptococci, and *E. coli* all have shorter survival periods. Because of these differences, the use of ratios of *R. coprophilus* to *E. coli*, *S. bovis*, or fecal streptococci is obviously not recommended.

One limitation of the use of *R. coprophilus* as a specific indicator of fecal pollution is the long incubation period required (17 to 18 days). This might lead to the conclusion that it is not practical to use this organism as an indicator of animal fecal pollution. However, there are situations in which the differentiation between human and animal fecal pollution on the one hand, and between recent and remote fecal contamination on the other hand, may have value in epidemiological investigations and also in tracing the source of fecal contamination of water. Clearly, additional research is required to develop a selective medium for *R. coprophilus* which permits a much shorter incubation period.

C. perfringens is commonly used in the United Kingdom to confirm the fecal nature of pollution in the absence of fecal coliforms. It is also used to detect fecal pollution of remote origin (3), but since the organism is excreted by both humans and animals, it cannot be used as a specific indicator organism that distinguishes between human or animal fecal contamination. *R. coprophilus* is excreted only by animals (7, 11), and this study has shown that it can survive for at least 120 days in polluted waters. In contrast, *S. bovis* will survive for only a few days in polluted waters. Therefore, the presence of *R. coprophilus* and *S. bovis* in polluted waters confirms recent fecal pollution from animals, whereas the presence of *R. coprophilus* alone points to contamination with animal fecal matter of remote or distant origin.

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