Incidence of Prosthecate Bacteria in a Polluted Stream

JAMES T. STALEY

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48823

Received for publication 22 June 1971

Water samples were collected aseptically several times throughout the year at nine stations on the Red Cedar River, a stream flowing through farmland and receiving effluent from several municipalities in central Michigan. Total prosthecate bacteria were enumerated by both direct and viable counting techniques. By direct techniques, these bacteria accounted for 0.62 to 1.1% of the total microflora during the study. The predominant type of appended bacteria was the caulobacters (Caulobacter, Asticcacaulis, and the fusiform caulobacter), which accounted for 64 to 93% of the total prosthecate forms. The others of importance were prosthecobacteria (< 1 to 24%), including Prosthecocovermboium and Prosthecocloris; hyphomicrobia (< 1 to 15%), including Hyphomicrobium and Rhodomicrobium; and Anacalomicbium (< 1 to 6%). The viable counts of heterotrophs also indicated that the caulobacters were the most numerous prosthecate bacteria in the stream. They ranged from fewer than 1 per ml to a maximum of almost 4,000 per ml. During the coldest period, when the total viable counts decreased to about 10^4 per ml compared to their summer high of over 10^5 per ml, the caulobacters actually increased in numbers. In December (temperature 0 to 1 C), they comprised from 0.09 to 1.0% of the viable microbial count, and in March (6.0 to 8.0 C) they accounted for 0.14 to 2.8%. The other heterotrophic prosthecate bacteria were generally found at numbers less than 1 per ml, with the exception of the December study when Hyphomicrobium was present in numbers as high as 2,400 per ml. There was no consistent correlation between the frequency of prosthecate bacteria and total coliforms in the stream during the investigation.

Unlike typical unicellular bacteria, each genus of heterotrophic, prosthecate bacteria contains individuals that are morphologically distinctive and recognizable. Based on the number, size, shape, and location of their appendages and the morphology of their cells, certain individuals can be identified as to genus and in some cases even species by phase microscopic examination of a population. Thus, the genus Caulobacter contains stalked cells that have a single thin prostheca extending from one pole of the cell (9). The genus Asticcacaulis is identical except its prostheca is borne in a subpolar position (9). Hyphomicrobium and Pedomicrobium produce buds at the distal tips of their wider prosthaces, a feature that distinguishes them from all other genera of heterotrophs. Pedomicrobium may produce several appendages from any location on the cell surface (2), whereas Hyphomicrobium normally produces only polar appendages (7).

The other genera of heterotrophic prosthecate bacteria, Prosthecocovermboium and Anacalomicbium, have cells with several appendages, but neither genus produces prosthecal buds. The appendages on Anacalomicbium are fewer and longer than those of Prosthecocovermboium (10).

In addition to these heterotrophic forms, there are two genera of photosynthetic prosthecate bacteria, Rhodomicrobium (4) and Prosthecocloris (5). Unlike those mentioned previously, these are obligately anaerobic and frequently occur in multicellular aggregates. These phototropic genera contain cells that are morphologically indistinguishable from heterotrophic counterparts, namely, Hyphomicrobium and Prosthecocovermboium, respectively, although the pigmentation of individuals may occasionally be intense enough to permit direct microscopic identification.

Because the prosthecate bacteria can be recognized by phase microscopic examination, they are ideal unicellular prokaryotes for direct iden-
Identification and quantitative enumeration in the normal microflora of the natural environments in which they are found. There are, however, two sources of error in this approach. For one, not all of the individuals of a population may be identifiable. For example, swarmer cells in the genera Caulobacter, Asticcacaulis, and Hyphomicrobium would appear as rods or vibrios before the development of noticeable prosthecae. This would lead to an underestimation of their numbers. Another source of error involves mistaken identity. Stalked caulobacters, for instance, appear very much like some nonbudding stages of Hyphomicrobium and might be identified incorrectly. Similarly, individual cells of the photosynthetic forms could be mistaken for their heterotrophic counterparts as suggested previously.

Despite these limitations, direct enumeration studies of these bacteria should be undertaken to provide some knowledge of their numerical significance in their natural habitats.

An alternative approach is quantitative viable counting. Belyaev (3) was the first to use the extinction-dilution technique for enumerating prosthecate bacteria when he determined the numbers of caulobacters in Russian reservoirs. In this procedure, replicate 10-fold serial dilutions of water samples are made in a liquid medium that will support the growth of heterotrophic prosthecate bacteria. After incubation, wet mounts from each tube are examined for the presence of representative forms. When a form distinctive to a genus is found, the tube is recorded positive for the genus.

By use of both direct and viable enumeration procedures, a study was conducted to determine the incidence of heterotrophic prosthecate bacteria in a polluted stream in Michigan. An attempt was also made to correlate the incidence of prosthecate bacteria to the coliform organisms along the watercourse.

MATERIALS AND METHODS

Study stream. The Red Cedar River is a warm-water stream that drains 489 square miles in south central Michigan. It flows some 40 miles in a northwesterly direction from its source in Cedar Lake to its confluence with the Grand River in Lansing (Fig. 1). Nine sampling sites were chosen along the watercourse, the first located upstream of Fowlerville at the Van Buren bridge. The second sampling site was the Gregory Road bridge downstream of Fowlerville and its sewage treatment plant. Sites 3 and 4 were located at Gramer Road bridge and Dietz Road bridge, respectively. This upstream section of the stream has a mud and silt bottom.

About 2 miles downstream of the Dietz Road bridge, the Red Cedar River is fed by the Anchor River, which brings into the stream a large quantity of sewage. The Anchor River is a waste disposal plant which discharges its output to the stream in a number of sections along its length. At the point selected for the study, the Anchor River is the only discharge point of any importance to the study stream.

The estimated population and distribution of the Anchor River at the sampling site can be seen in Fig. 1. This section of the stream is a man-made tributary. It originates in a small town and then joins the Red Cedar River near the town of Anchorville. The Anchor River is a waste disposal plant which discharges its output to the stream in a number of sections along its length. At the point selected for the study, the Anchor River is the only discharge point of any importance to the study stream.

![Fig. 1. Watershed of Red Cedar River. Sampling sites used in this study are numbered I through 9. Compiled by R. Horner.](image-url)
bridge, the river flows into a reservoir that is impounded by a dam in Williamson. No sampling sites were located along this part of the stream. The next sampling site (number 5) was located immediately below the dam, upstream of the Williamson municipal sewage treatment facility. Site 6 was located at the Zimmer Road bridge about 2 miles below the sewage treatment plant.

The river bottom gradually changes from a rock and gravel bottom below Williamson to a sand bottom at Okemos. The section of the stream below Zimmer Road adjoins woodland much of the way, receives no urban effluent, and is the cleanest part of the entire stream. Sampling site 7 was located in Okemos at the bridge over the golf course. The next site, number 8, was at Farm Lane on the Michigan State University campus. Site 9, the last site, was located at Potter Park bridge in Lansing below the East Lansing sewage treatment plant. More information on the stream is available from Linton and Ball (8).

**Sampling techniques.** Water samples were collected with aseptic techniques. The sampling apparatus consisted of 1-liter glass-stoppered reagent bottles wired by their necks onto a wood pole. A string tied to the glass stopper was used to open the bottle during sample collection. At the time of collection, the sterile sampling apparatus was carefully unwrapped, and the bottle was inserted to a constant depth of 6 inches (15.24 cm) into the midstream portion of the river. After insertion, the string was pulled to open the bottle for collection. If the bridge was high above the water, an extension was attached to the sterilized unit before it was unwrapped. At two of the sites, 6 and 7, waders were worn into the river for collection of the samples. At site 5, the samples were collected from the shore about 20 m downstream of the spillway. After filing the bottle with about 900 ml of water, the stopper was replaced, and the sample was returned to the vehicle for immediate handling. First, 1-ml portions were aseptically transferred to extinction-dilution tubes for viable counting. Then, 200 ml was added to an Erlenmeyer flask containing 8 ml of 25% glutaraldehyde, pH 6.8. The remaining sample was stored on ice in the sampling bottle and used for pH determinations upon return to the laboratory. The water temperature was obtained by equilibrating a thermometer in a bucket of freshly collected river water.

**Enumeration.** Dilutions for viable counting were performed as soon as the water sample was returned to the vehicle. All viable counts were determined by the extinction-dilution technique using most-probable-number (MPN) tables. Total viable and viable prokatectae bacteria were enumerated in a dilute medium consisting of 0.005% peptone (Difco) and 0.005% yeast extract (Difco) with 10 ml/liter of a vitamin solution (10) and 20 ml/liter of a mineral salts solution (10). Portions (1 ml) of the river water were added to each of 10 tubes containing 9 ml of the medium. Each of these tubes was serially diluted until the highest dilution received $10^{-6}$ ml of the river water. Thus, 100 tubes of this medium were inoculated at each site. Upon returning to the laboratory, these were incubated at room temperature (22 to 25°C) for 5 to 7 days. The tubes were then examined macroscopically for turbidity, the criterion used for assessing total viable numbers. The tubes were incubated for an additional 2 weeks before wet mounts of each were examined individually under oil immersion with a Zeiss GFL phase microscope. Examination proceeded from the lowest dilution tube in a series and continued until one tube in the series was found negative for prokatectae bacteria. Individual types were identified on the basis of their morphology as caulobacters (Caulobacter, Asticcacaulis, and the fusiform caulobacter), Hyphomicrobium, Prosthecocobium, and Ancalomicrobium. This enumeration procedure required approximately 2 one-man work weeks (occasional checks of series examined earlier did not reveal any changes in the type of prokatectae bacteria resulting from prolonged incubation). Ten-tube MPN tables were obtained from Halvorson and Ziegler (6).

Coliform organisms were enumerated by using Laryl Tryptose Broth (Difco) in the 5-tube presumptive test as outlined in Standard Methods for the Examination of Water and Wastewater (1). As with the other viable enumerations, these were inoculated in the field immediately after collection of the water sample and stored in the vehicle until returning to the laboratory at the end of the sampling period, which usually began at about 9:15 AM and was completed by 3:00 PM.

On one occasion, the 3 June 1968 sampling, the presumptive test was followed by a fecal coliform test to determine the proportion of fecal coliforms to total coliforms along the watercourse. The boric acid-lactose broth test (1) was used for this purpose.

**Direct enumeration.** The 200-ml samples that were fixed with glutaraldehyde immediately upon collection were used for the direct enumeration studies. Upon return to the laboratory, these samples were stored at refrigerator temperature until they could be centrifuged to concentrate the cells. Centrifugation (quantitative) was at 20,000 $\times$ g for 30 min in a Sorvall RC-2B centrifuge at 0 to 5°C. The supernatant fluid was carefully decanted, and the particulate fraction was resuspended to a known volume, usually 1.0 ml, with water and glutaraldehyde from the same bottle. These were then stored until wet mounts could be examined in the phase microscope. The proportion of each type of prokatectae bacterium was determined by examining 500 organisms from each sample. Because of the uncertainty associated with identification, the prokatectae bacteria were grouped as hyphomicrobia (Hyphomicrobium, Pedomicrobium, and Rhodomicrobium), prokatecomeicrobia (Prosthecocobiand Prosthecocorticida), Ancalomicrobium, and caulobacters (Caulobacter and Asticcacaulis). In one instance, a quantitative enumeration was made with a Petroff-Hauser counting chamber.

**RESULTS**

Figure 2 shows the viable count data for 7 July 1968. Total viable numbers remained relatively constant along the watercourse at about
10^6 per ml. The caulobacters varied widely from a low of 27.8 to 1,330 per ml. Total coliform organisms were fewer than the caulobacters at each site, ranging from a low of less than 1.0 per ml at site 1 to a high of about 100 per ml at site 9. *Hyphomicrobium* and *Prosthecomicrobiwn* were found at most sites, but their numbers (< 1.0 per ml) were not high enough to permit accurate counting. For this reason, these points on the graph were not interconnected. The water temperature during this time varied from 21.4 to 23.0 C. On the basis of direct counts, the prosthecate bacteria accounted for an average of 0.75% of the total microorganisms counted, of which 80% were caulobacters, 15% were hyphomicrobia, and 5% were prosthecomicrobia. The predominant morphological group of bacteria were the vibrios which accounted for about 60% of the total microorganisms present at those sites where they were counted.

Samples were collected again on 17 September 1968 when temperatures were 18.7 and 21.0 C. Total viable numbers (Fig. 3) in some cases were in excess of 10^7 per ml, reflecting the tremendous densities in the stream, the water of which was visibly turbid in the sampling bottles at the time of collection. The caulobacters were somewhat fewer than during the previous sampling period,
FIG. 4. Viable counts of bacteria on 7 December 1968.

FIG. 5. Viable counts of bacteria on 18 March 1969.

Varying from about 20 to 188 per ml, the exception being an extremely low count at site 8. Total coliform counts were comparable to those obtained during July. *Hyphomicrobium* and *Prosthecomicrobium* were also found, but again their numbers were generally below 1 per ml. Direct counts revealed that prosthecate bacteria accounted for 0.62% of the total microflora composed of 64% caulobacters, 22% prosthecomicrobia, and 14% hyphomicrobia.

On 7 December 1968, the water temperature reached 0.0°C at several of the upstream sampling sites that were iced over or contained ice on the edges. The high temperature was recorded as 1.0°C at the last sampling site. Total viable bacteria were much lower than previous dates, ranging from $1.5 \times 10^4$ to $2.7 \times 10^5$ per ml (Fig. 4). The numbers of caulobacters, however, increased from the previous period, varying from a low of 133 to a high of 792 per ml. They were surpassed in abundance at the downstream sites by hyphomicrobia, which were the most numerous of the prosthecate bacteria at that time, reaching a high of 2,420 per ml at site 5. The coliform organisms ranged from 13 per ml at site 1 to a high of 109. Prosthecate bacteria accounted for 0.76% of the direct count. Caulobacters, dominated by members of the genus *Asticcacaulis*, were the predominant forms, amounting to 79% of the prosthecate bacteria, whereas hyphomicrobia and prosthecomicrobia constituted 15 and 6%, respectively. Vibrios were again significant members of the bacterial community accounting for 13% of the total microflora.

Figure 5 shows the data for 18 March 1969. The temperature of the water varied from a low of 6.0°C to a high of 8.0°C. The total viable count ranged from a low of $8.72 \times 10^4$ to $4.93 \times 10^5$.
bacteria ranged from 130 per ml to 1,530 per ml, significantly lower than on the March sampling but comparable to that of the previous July. Total coliform organisms varied from a low of 35 per ml at the first site to a high of 542 at the site below the Williamston treatment plant. Fecal coliforms were also enumerated. Their numbers were lowest at site 1, being about 9 per ml, to highs of 240 per ml at sites 5 and 6 below Williamston. Again Hyphomicrobium and Prosthecomicrobium were present in detectable but low numbers. Direct counts indicated that prosthecate bacteria were 1.1% of the total microflora. Most of these were caulobacter (70%), with 24% hyphomicrobia.

**DISCUSSION**

This study shows that prosthecate bacteria occur in some freshwaters in large enough numbers to permit enumerating by accepted techniques for both viable and direct counting. Furthermore, the results of this study indicate that these bacteria constitute a significant portion of the microorganisms in this stream. By direct count the prosthecate bacteria comprised about 1% (range 0.62 to 1.1%) of the total microbial flora. These estimates are no doubt conservative because many of the prosthecate bacteria have nonappendaged stages in their life cycles, making them indistinguishable from other unicellular heterotrophs. This problem is especially pertinent to the caulobacters which were the most numerous of these forms (64 to 93% of total prosthecate bacteria). If one assumes that the stalked and nonstalked cells were equal in numbers and evenly distributed in the stream, then the caulobacters would have been underestimated by a factor of two with the direct-count method.

Viable enumerations also indicated that the caulobacters were usually the most numerous of the prosthecate bacteria. They ranged from fewer than 1 per ml to as many as about 4,000 per ml. Their proportion of the total viable count varied from a low at one site in September of 0.00005% to a high at one site in March of 2.8% of the total viable microflora. During one sampling period (December), Hyphomicrobium was more abundant than the caulobacters at the downstream sites.

The viable counts on caulobacters reported in this study compare favorably with those reported by Belyaev (3), who found from 10 to 10,000 caulobacters per ml in a variety of fresh water reservoirs along the Volga-Don rivers.

The discrepancy between the direct and viable counts during the September sampling is difficult to explain. In that month, the caulobacters comprised less than 0.002% of the total viable count.
at all sites, yet the direct counts indicated that they accounted for about 0.4% of the total microflora, a difference greater than two orders of magnitude. This same anomaly was observed during the other summer months, although it was not as pronounced. One explanation is that most of the caulobacters were nonviable at the time of collection. Another possible explanation is that the medium employed did not permit the growth of the most numerous caulobacters. An additional factor was that only 4,500 cells were counted for direct enumeration during each sampling period.

A correlation graph was constructed by plotting the log of total coliforms against the log of total caulobacters at each site for each date. There was no indication of a relationship between their frequencies for the study, although during individual sampling periods both negative and positive correlations were occasionally evident.

The study shows that the number of viable prosthecate bacteria varies greatly during the year. Thus caulobacters were found in high numbers on 18 March, and *Hyphomicrobium* was found in high numbers on 7 December. Although it is possible that temperature was responsible for this seasonal variation, there is not enough evidence to substantiate this, particularly in view of the large number of other variables that could conceivably influence the incidence of these bacteria.

The techniques described in this paper should permit a more thorough evaluation of the incidence of prosthecate bacteria in freshwater and hopefully lead to the determination of the factors that influence the occurrence of these organisms.

**ACKNOWLEDGMENTS**

This research was supported by a grant from the Institute of Water Research, Michigan State University (Annual Allotment grant 14-0-0001-1842).

I am most appreciative of the technical assistance provided by Lois Stifler, J. Carter, J. A. M. de Bont, and C. S. Chopra.

**LITERATURE CITED**

1. American Public Health Association, 1967. Standard methods for the examination of water and wastewater, 12th ed. New York.
2. Aristovskaya, T. V. 1961. Accumulation of iron in breakdown of organomineral humus complexes by microorganisms. (English translation). Dokl. Akad. Nauk. SSSR 136:954-957.
3. Belyaev, S. S. 1967. Distribution of the caulobacter group of bacteria. (English translation) Mikrobiologiya 36:157-162.
4. Duchow, E., and H. C. Douglas. 1949. *Rhodomicrobium vanneii*, a new phototrophic bacterium. J. Bacteriol. 58:409-416.
5. Gorlenko, V. M. 1970. A new phototrophic green sulphur bacterium—*Prosthecocloris aestuaril* nov. gen. nov. spec. Z. Allgem. Mikrobiol. 19:147-149.
6. Halvorson, H. O., and N. R. Ziegler. 1933. Application of statistics to problems in bacteriology. I. A means of determining bacterial population by the dilution method. J. Bacteriol. 25:101-121.
7. Hirach, P., and S. R. Conti. 1964. Biology of budding bacteria. I. Enrichment, isolation and morphology of *Hyphomicrobium* spp. Arch. Mikrobiol. 42:17-35.
8. Linton, K. J., and R. C. Ball. 1965. A study of the fish populations in a warm-water stream. Quart. Bull. Mich. Agr. Exp. Station 48:255-285.
9. Poindexter, J. S. 1964. Biological properties and classification of the *Caulobacter* group. Bacteriol. Rev. 28:231-295.
10. Staley, J. T. 1969. *Prosthocomicrobiaceae* and *Ancalomicrobiaceae*: new prosthecate freshwater bacteria. J. Bacteriol. 95:1921-1942.