Facultative Anaerobic Bacteria in the Digestive Tract of Chum Salmon (Onchorhynchus keta) Maintained in Fresh Water Under Defined Culture Conditions

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The bacterial flora in the digestive tract of chum salmon growing in fresh water under defined and controlled culture conditions was examined both qualitatively and quantitatively. The predominant species present in the digestive tract were identified as Aeromonas, with Aeromonas hydrophila being the most common isolate. These aeromonads were not isolated from the diet. Other bacterial species commonly isolated included Bacillus, Enterobacter, nonpigmented pseudomonads, Micrococcus, and Acinetobacter. These species were also isolated from the diet or tank water. As many as $10^4$ viable bacteria per g (wet weight) of digestive tract plus contents were counted. After 75 days of starvation, $10^4$ viable bacteria were counted, whereas fish fed a sterile feed contained $10^4$ viable bacteria per g (wet weight) of digestive tract plus contents.

Any study of the bacterial population of the alimentary tract necessarily involves a consideration of three components: the host, the bacterial flora as a whole, and the individual species comprising this flora. The host is affected by numerous factors, many of which may in turn affect the alimentary tract microflora. One factor which affects both the host and the alimentary tract microflora is the food consumed by the host. In the case of homeotherms, the bacterial flora in the alimentary tract is composed of large numbers of different microorganisms which are in dynamic equilibrium with each other and with the host (10). This balance can be considered as the normal commensal state, and alterations in the microbial balance may produce effects beneficial or detrimental to the host. Any effect of a single species on the host or on other bacteria in the alimentary tract is a function of the number of the species present, its biochemical activities, the influence of host secretions, and the influence of competitive species of other microorganisms.

In the case of poikilothermic vertebrates such as fish, little information is currently available concerning host-microflora relationships. The information that is available for fish has been derived from studies of "free-living" fish. Such studies provide limited information, since the factors affecting the host are innumerable and can neither be defined nor controlled. Thus it is important to supplement the information obtained from "wild" fish with studies of experimental populations maintained under defined culture conditions. Moreover, a detailed knowledge of host-microflora relationships in cultured fish is essential in view of the increasing interest in the intensive farming and subsequent food processing of large numbers of salmonid fishes.

An initial paper reporting on the bacterial population in the alimentary tract of wild freshwater salmonids has been published (32). This second paper reports on the bacterial population found in the gastrointestinal tract of chum salmon cultured in fresh water. This population had been maintained under defined culture conditions from the time they were hatched.

MATERIALS AND METHODS

Fishes studied. A population of chum salmon (Onchorhynchus keta) was cultured at this laboratory. Complete details of the stripping, fertilization, incubation, and the bacterial population of the eggs from which this population was reared have been given (31). The fish were housed for up to 36 months in hemispherical plastic tanks supplied with well water at 10 to 13 C. Water was supplied at a rate of 200 ml/min. Adequate dissolved oxygen levels (7 to 9 mg/liter) were ensured by introducing the water into the respective tanks through a simple venturi device. The bacterial load of the well water has been routinely monitored for 4 years using the procedures of the American Public Health Association (2). One milliliter of inoculum and pure plates of standard methods agar (Baltimore Biological Laboratories) were used, and 100-ml volumes of water were passed through membrane filters which were then placed on standard
methods agar and incubated. The well water contained less than 1 viable bacterium/ml. The tank water was exchanged every 4 h with the aid of a central siphon. This also afforded a degree of self-cleaning power to the system; however, the tanks were also cleaned weekly. The water supply has been used at this laboratory for 5 years to maintain a large fish population. The fish were fed to appetite three times daily using a commercially formulated fish feed (Miles and Misra (16), Shewan (3), Cowan and Steele (7), Hughes (12), Edwards and Ewing (9), Weaver et al. (34), and Lewis (14). Identification of *Aeromonas* and *Vibrio* species was facilitated by testing for gas production from glucose, esculin hydrolysis, the lysine and ornithine decarboxylase tests, and the arginine decarboxylase and dihydrofolate tests. *Vibrio* species were confirmed by testing the sensitivity of the culture to 2,4-diamino-6,7-disopropylpteridine. Identification within the enterobacteriaceae was confirmed by using the API system (Analytab Products, Inc., Plainview, N.Y.).

**RESULTS**

Fish studied. The weights of the 90 chum salmon varied from 24.5 to 487 g, with a mean weight of 175 g. The weights of the various samples of alimentary tract examined varied from 0.5 to 22.1 g. The pH of the homogenized alimentary tract in the 0.1% (wt/vol) peptone water diluent was pH 7.0, as was the pH of the pooled contents of the alimentary tract (range, 6.7 to 7.5). The alimentary tract of fully fed fish contained homogenous feed in various stages of digestion, whereas the gastrointestinal tracts of starving fish contained little material in the lumen other than mucus.

Kidney infection was noted in two of the salmon examined. The gross morphology of the kidney lesions, as well as the Gram-staining properties, cellular morphologies, and the fastidious nutritional requirements of the infecting organisms, were typical of the kidney disease organism (5, 20). Attempts to demonstrate the presence of viable bacteria in the kidney, liver, and spleen of the remainder of the fish examined were unsuccessful.

**Quantitative bacteriological examination.** Microscopic examination of the alimentary tract samples was made difficult by the staining properties and particulate nature of the feed-stuff. Large numbers of bacteria were not seen in the smears. Much of the microflora that was observed comprised gram-negative rods; however, both sporing and nonsporing gram-positive rods, as well as cocci and refractile spores, were also seen.

The species isolated from the digestive tracts and from the feed and tank water are shown in Table 1. Of the 343 isolates from the digestive tract of the salmon, 29% were species of *Bacillus* and 27% were species of *Micrococcus*, whereas 6% were identified as *Acinetobacter*. Two species of *Aeromonas* were represented, *Aeromonas hydrophila* being isolated on 50 occasions and *Aeromonas shigelloides* on 16 occasions. However, these two species were not isolated from
TABLE 1. Frequency of isolation of bacterial species from the digestive tract of cultured chum salmon, their feed, and their tank water

| Bacteria isolated          | No. of isolations |
|----------------------------|-------------------|
|                            | Alimentary tract*| Feed | Tank water |
| Alcaligenes                | 4                 | 3    | 3          |
| Acinetobacter              | 21                | 32   | 9          |
| Aeromonas                  | 66                | 1    | 1          |
| Bacillus                   | 100               | 39   | 4          |
| Brevibacterium-Corynebacterium | 1             | 1    | 1          |
| Clostridium                | 2                 | 34   | 2          |
| Enterobacter               | 12                | 20   | 1          |
| Flavobacterium             | 11                | 12   | 4          |
| Micrococcus                | 32                | 15   | 6          |
| Pseudomonas                | 59                | 21   | 38         |
| Staphylococcus             | 4                 | 1    | 3          |
| Streptococcus (enterococcus group) | 3  | 1    | 3          |
| Streptomyces               | 2                 |      |            |
| Vibrio                     | 3                 |      |            |
| Yeast                      | 2                 |      |            |

*Five to 14 isolates per fish were obtained from 32 fish.

Table 2. Bacterial load in the alimentary tract of hatchery-reared chum salmon

| Sample         | Incubation conditions* | No. of samples | Estimated no. of viable organisms $\times 10^9$ g (wet weight) of sample | Mean range of values |
|----------------|------------------------|----------------|------------------------------------------------------------------------|---------------------|
| Total tract    | TSA, O$_2$             | 18             | 5,800                                                                  | 15-82,000           |
| plus contents  | TSA, AnO$_2$           | 18             | 5,500                                                                  | 12-11,000           |
| Tract minus    | TSA, O$_2$             | 15             | 5,500                                                                  | 19-29,000           |
| contents*      | Mc, O$_1$              | 5              | 3,400                                                                  | 100-69,000          |
|                | SS, O$_2$              | 5              | 800                                                                    | 1-3,000             |
|                | TSA, AnO$_2$           | 11             | 6,800                                                                  | 90-29,000           |
|                | RCM, AnO$_2$           | 5              | 1,700                                                                  | 300-3,700           |
|                | LSA, AnO$_2$           | 5              | NCO*                                                                  | NCO                 |
|                | MEA, AnO$_2$           | 5              | NCO                                                                   | NCO                 |

*Number of viable organisms $\times 10^9$ g (wet weight) of sample

**TABLE 2.** Mean bacterial load in the alimentary tract of hatchery-reared chum salmon

The bacterial load in the alimentary tract of the 168- to 487-g hatchery-reared chum salmon is shown in Table 2. As many as $10^6$ viable organisms/g were obtained in the alimentary tract samples, whereas an entire gastrointestinal tract with its contents, taken from a 273-g fish, was estimated to contain a total of $3.0 \times 10^{10}$ viable aerobes and $2.0 \times 10^{14}$ viable bacteria capable of anaerobic growth. Although significant counts were obtained on both MacConkey and Salmonella-Shigella plates, typical lactose-fermenting colonies were not obtained. Similarly, Gram staining, subculture, and biochemical examination of the colonies growing on reinforced clostridial medium failed to demonstrate the presence of countable numbers of clostridia. Subsequent aerobic incubation of anaerobic plates, as well as subculture and incubation of isolates aerobically and anaerobically, showed that the population recovered using these enumeration procedures was facultative in its oxygen requirements. No growth was detected on Lactobacillus selection agar on M-Enterococcus agar.

The effect of starvation on the bacterial numbers in the alimentary tract of chum salmon is shown in Table 3. Although starvation
caused a reduction in the total numbers of viable cells present, $10^6$ viable organisms/g (wet weight) were still present after 75 days without feed.

Similar results were obtained when the salmon were fed with a diet sterilized by treatment with ethylene oxide. After 55 days on the sterile feed, the total number of viable aerobic bacteria in the alimentary tract had been reduced from $5.8 \times 10^7$ to $2.0 \times 10^5$ (wet weight) of digestive tract plus contents. This value was the mean value obtained from 12 fish. The range of counts in the tract from fish on the sterile feed was from $10^6$ to $8.2 \times 10^6$. The average count of bacteria capable of anaerobic growth in these tracts was $2.2 \times 10^3$ (range, $2.0 \times 10^2$ to $7.1 \times 10^2$). In this experiment, the average weight of the fish examined was 29 g (range, 34 to 37 g), and the average weight of samples examined was 2.8 g (range 2.5 to 3.7 g).

**DISCUSSION**

The present findings show that under the defined culture conditions of this study the predominant bacteria populating the digestive tract of chum salmon are species of *Aeromonas*. This genus was consistently found to comprise the largest numbers in the digestive tract of fish of widely varying sizes sampled over a 2-year period. In the majority of fish examined, *A. hydrophila* predominated, although *A. shigelloides* was also common. These aeromonads did not appear to originate in the diet or the water supply. However, they may have originated from the surface of incubating eggs (31), then contaminated the surface of the alevin when the eggs hatched, and finally infected the digestive tract via the mouth or vent when the alevin started to feed. It seems likely that the conditions in the digestive tract allowed these infecting aeromonads to then establish themselves. Significant numbers of aeromonads, including *A. hydrophila*, have also been demonstrated in the digestive tract of free-living salmonid fishes, and so it seems likely that this genus is indigenous in the gut of salmonid fishes living in fresh water. This is of considerable interest, since species of *Aeromonas* are also regarded as common pathogens of fish and may cause hemorrhagic septicemia and furunculosis (5). In such diseases, the invading bacteria may be the indigenous microflora in the digestive tract of the fish, and the portal of entry to the tissues is likely to be the digestive tract. Such an invasion could occur as a result of dietary or environmental stress (29). In the case of animals such as the mouse, this stress can result from depriving the animal of food, water, or bedding (29). In the case of fish, the stress is likely to occur under the conditions employed during intensive fish culture. As might be expected, it is under such conditions that massive mortalities characterized by systemic bacterial infection occur. It should also be noted that this intestinal route of infection has recently been implicated in cases of *A. hydrophila* septicemia in humans (13).

Species of *Enterobacter*, *Acinetobacter*, and *Pseudomonas* might also be regarded as indigenous in freshwater salmonid digestive tracts, since they were readily isolated from both free-living (32) and hatchery-cultured fish. *E. agglomerans* was isolated from the cultured fish, whereas *Enterobacter aerogenes*, *Enterobacter cloacae*, *Enterobacter hafniae*, and *Enterobacter liquefaciens* were isolated from the free-living salmonids. In the case of species of these three genera, the organisms populating the digestive tract appear to originate in the diet; however, the *Acinetobacter* and pseudomonads could also originate in the water. The large number of *Bacillus* isolated in this study certainly originated in the diet. It seems likely, however, that because of the predominantly aerobic requirements of this genus these organisms pass through the digestive tract as spores and do not colonize the tract.

The microflora of the digestive tract of the cultured fish did differ from that of the free-living freshwater salmonids by having fewer bacterial species present (32). The microflora of this

**TABLE 3. Effect of starvation on the bacterial numbers in the alimentary tract of chum salmon**

| Days of starvation | Estimated no. of viable bacteria \( \times 10^5 \) (wet weight) of alimentary tract plus contents | Wt of fish (g) | Wt of alimentary tract (g) |
|--------------------|---------------------------------------------------------------|---------------|----------------------------|
| Aerobic            | Anaerobic                                                    |               |                            |
| 0                  | 5,800 5,500                                                  | 246 (mean, 5) | 17.3 (mean, 5)             |
| 1                  | 8,700 6,800                                                  | 175 (mean, 4) | 8.8 (mean, 4)              |
| 2                  | 9,500 8,500                                                  | 236 (mean, 4) | 11.2 (mean, 4)             |
| 5                  | 6,900 3,500                                                  | 181 (mean, 4) | 7.8 (mean, 4)              |
| 15                 | 210 2,800                                                   | 244           | 7.9                        |
| 19                 | 360 630                                                     | 250           | 4.4                        |
| 26                 | 120 210                                                     | 221           | 8.6                        |
| 33                 | 100 110                                                     | 187           | 6.8                        |
| 42                 | 420 260                                                     | 154           | 4.4                        |
| 47                 | 66 57                                                       | 223           | 5.6                        |
| 54                 | 80 110                                                      | 242           | 7.2                        |
| 68                 | 740 790                                                     | 183           | 5.0                        |
| 68                 | 96 10                                                       | 235           | 5.0                        |
| 75                 | 62 80                                                       | 169           | 5.0                        |

*Mean value from 2 fish examined.
group of cultured fish also differs from the "normal" microflora of the digestive tract of marine fishes where Vibrio, Acinetobacter, Corynebacterium, Flavobacterium, and Micrococcus species have been shown to predominate (1, 6, 19, 23). As might be expected, the microflora in the cultured fish is markedly different from that in the digestive tract of homeothermic species (17, 21). Although common in homeotherms, species such as Escherichia, enterococci, Clostridium perfringens, and lactobacilli were not normal microflora in the freshwater salmonids studied. Moreover, a large proportion of the microflora in the gut of homeotherms is anaerobic (17, 21), whereas the digestive microflora of the salmonids appears to be facultative.

The total viable bacterial numbers recovered from fully fed, cultured salmonids were similar to the counts recovered from free-living salmonids. It must be emphasized that the values obtained in these studies may present a minimum estimate of the actual numbers present, since the techniques and conditions used for sampling and recovery are not suitable for all possible intestinal inhabitants. Starvation caused a reduction in the total viable numbers present, but a "sterile" gastrointestinal tract did not result. Even after 75 days of starvation, a 169-g chum salmon still contained $3 \times 10^6$ viable aerobes and $4 \times 10^6$ viable anaerobes in its alimentary tract. This reduction in total numbers may represent a loss of those organisms not intimately associated with the mucous layers associated with the epithelium. Although such an association has yet to be demonstrated in the fish gastrointestinal tract, it has been shown in a wide range of other species (28). The results are consistent with other studies which have shown that the gastrointestinal tract of man and other species is not sterile, even after high levels of antibiotics have been administered before surgery (17). In the case of the fasting fish, it is likely that an adequate nutrient supply will be available to support bacterial growth in the digestive tract.

It is well established that a significant amount of protein is contributed to the digestive tract from the body even in fasting animals. In addition, there is also a continuous loss of the surface epithelial cells which could also be used as a source of nutrient for bacterial growth. Although data are not available in the case of fish, the gastrointestinal cell loss in man can amount to 72 g (dry weight)/day (8). Only 9 to 15% of the protein found in the digestive tract is derived from this cellular protein (8). Even accounting for differences in species and animal size, it seems likely that more than enough protein will be present in the gastrointestinal lumen of a fasting fish to account for the 16 to 28 $\mu$g of protein in $4 \times 10^4$ viable bacterial cells.

It is also possible that some indigenous microorganisms utilize carbohydrate moieties of gut mucins as a source of nutrition (11). Thus, it is difficult to understand the report by Margolis (15) of "bacteriologically sterile" intestines in fish that had been starved for a short period. The reason for this apparent discrepancy is unclear at present, however, the entire length of the alimentary tract was not sampled in the other study, and no information was presented on the age or size of the fish or on its nutritional history. A similar reduction in bacterial numbers was shown with fish fed sterile feed. Again the organisms remaining could be those associated with the epithelial lining of the tract. The results obtained in the starvation experiment and the sterile feed experiment add weight to the view that the fish gastrointestinal tract can possess a commensal gastrointestinal microflora. Again, this is contrary to the views of others (15, 22, 23) that the fish has no commensal microflora. These workers suggest that all the organisms present represent organisms in recent intakes of feed or water which are passing through the intestinal tract. The results presented here suggest that, whereas this may be true for many of the bacterial species in the alimentary tract, it may not be true for all the species present, especially the aeromonads. Once this latter population is established it appears to be quite stable, and significant changes in the feed or environment may be required for its alteration. Preliminary findings obtained from other studies performed under identical management conditions, but with a significant quantitative alteration in the vitamin composition of the diet, suggest that some alteration in the number of viable organisms populating the gut can occur.

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