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# Isolation and Characterization of Turbot (*Scophtalmus maximus*)-Associated Bacteria with Inhibitory Effects against *Vibrio anguillarum*

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**More than 400 isolates from the intestine and the external surface of farmed *Scophtalmus maximus* as well as from fish food and hatchery water were screened for inhibitory effects against the fish pathogen *Vibrio anguillarum* HI 11345 and seven other fish pathogens. The bacteria with inhibitory effects were then characterized with regard to their sites of colonization, especially the intestinal regions and sites within each region. Of the total number of bacterial isolates from the intestine, 28% were inhibitory against *V. anguillarum* HI 11345. A marine biochemical assay was used to order the inhibitory strains into different phena. Most inhibitory bacteria were found in the rinse and mucus fractions of the gastrointestinal tract. No correlations among the different phena, site of colonization, and inhibitory effect could be found; however, a biochemical diversity was noted in the strains with an inhibitory effect. Of the isolates with an inhibitory effect against *V. anguillarum* HI 11345, 60% had an inhibitory effect on five other fish-pathogenic serotypes of *V. anguillarum*. Inhibitory effects of the isolates were also shown against *Aeromonas salmonicida* and *Aeromonas hydrophila*.**

It has been established that fish have a distinctive gut microflora. For example, recent studies have shown that the gastrointestinal tracts of marine fish of various species contain bacterial strains that belong to two specific *Vibrio* groups (17). When grown on solid media, *Vibrio* strains isolated from the indigenous gut microflora of healthy fish of various species appear to influence the growth of each other as well as to produce substances inhibitory to *Vibrio anguillarum* and *Vibrio salmonicida* (17).

Several functions can be assigned to the resident symbiotic gut microflora. These include digestion of algal cells (18), provision of amino acids (7), and possibly prevention of colonization by bacterial pathogens (17). The mechanism for the last function may involve competition for binding sites, utilization of substrates, and production of inhibitory components.

On the basis of the results of inhibitory effects on bacterial pathogens by constituents of fish gut microfloras (17) and by specific marine epiphytic strains isolated from seaweed (14), we were interested in initiating similar studies on farmed marine fish such as turbot (*Scophtalmus maximus*). A major problem in fish aquaculture is the invasion of bacteria that are pathogenic to the fish. The loss of juvenile turbot in Norwegian fish hatcheries as a result of vibriosis was 40% in 1988 (11). Although some protection is obtained by the use of antibiotics, chemotherapy, and vaccination, there is no doubt that more-efficient means are needed for controlling bacteria pathogenic to fish. In addition, the environmental impact of using huge amounts of antibiotics is unacceptable. Various drugs, such as sulfonamides, chloramphenicol, tetracycline, and nitrofuran derivatives, have been used (1). As a result of such treatments, the natural bacterial population of water and sediment displays an increased multiple-antibiotic resistance, a consequence of the rapid transfer of resistance mediated by plasmids (1, 2, 10, 20).

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Given the information that indigenous gut microfloras supply several beneficial effects to the host and show inhibitory effects against fish pathogens, as well as the observation that the fish pathogen *V. anguillarum* can amplify in turbot intestinal mucus (16), study of the role of inhibitory intestinal bacteria appears worthwhile. It may be feasible to identify intestinal strains which can be orally administered to turbot and thereby protect the fish from *V. anguillarum* infection, a concept presently under investigation for humans and domestic animals (4).

This paper reports the isolation and characterization of bacteria from various sampling sites, including the gastrointestinal tract, of farmed turbot. Inhibitory effects against several fish-pathogenic vibrios are reported for 89 of the isolates. From the data, comparative studies of inhibitory effects by different bacterial groups from different sites in the fish as well as from water have been carried out.

## MATERIALS AND METHODS

**Sampling site.** Six turbot (*S. maximus*), water samples, and food pellets were collected at the MOWI A/S hatchery in Bergen, Norway, in October 1989. Water was pumped continuously into the fish tanks from a depth of 40 m in the North Atlantic, 100 m from shore. The fish had never been treated with antibiotics or chemotherapeutics, even though there had been some outbreaks of vibriosis from which the fish had recovered as juveniles. The fish were 5 years old, and their weights ranged between 1.0 and 1.5 kg. No signs of malnutrition or bad health were observed, and the fish were sparsely fed with manufactured fish food.

**Collection of samples.** Water was taken 50 cm below the water surface in 100-ml sterile bottles and kept on ice. Three fish were taken each time from the tank to a container which was filled with tank water and stored on ice. Sampling and sample preparations were initiated within 2 h of collection.

**Media, diluent, and culture conditions.** The media and diluent used were marine agar (MA; Difco Laboratories),

marine broth (Difco Laboratories), VNSS agar (peptone, 1.0 g; yeast extract, 0.5 g; glucose, 0.5 g; starch, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $Na_2HPO_4$, 0.01 g; agar, 15 g in 1,000 ml of nine-salt solution [NSS]), tryptic soy broth (Difco Laboratories), and NSS, (NaCl, 17.6 g; $Na_2SO_4$, 1.47 g; $NaHCO_3$, 0.08 g; KCl, 0.25 g; KBr, 0.04 g; $MgCl_2 \cdot 6H_2O$, 1.87 g; $CaCl_2 \cdot 2H_2O$, 0.41 g; $SrCl_2 \cdot 6H_2O$, 0.008 g; $H_3BO_3$, 0.008 g in 1,000 ml of double-distilled $H_2O$). The incubation temperatures were 7°C (fish 4), 12°C (fish 5 and 6), and 20°C (fish 1, 2, and 3) for aerobic growth. Different incubation temperatures were used in order to obtain a wider range of isolates. The incubation times ranged from 1 to 3 weeks, depending on the incubation temperature, before colonies were counted and isolated. Plates incubated anaerobically at 12°C were counted after 30 days.

**Sampling methods.** Water samples were serially diluted in sterile NSS and plated on two different media, MA and VNSS agar. The food pellets were homogenized in NSS with a glass homogenizer and suspended in 1 ml of sterile NSS before being treated similarly to the water samples. One fish at a time was killed, and mucus from the external surface was sampled with a rubber scraper. The mucus was suspended in 1 ml of sterile NSS and diluted as described for the water samples. The blind side of the fish was then washed with 70% ethanol, and the peritoneum was cut open. The stomach and the intestine were dissected out, rinsed in sterile NSS, and divided into the following regions: stomach (region 1), pyloric caeca (region 2), upper intestine (region 3), lower intestine (region 4), and rectum (region 5). Three samples from regions 2, 3, and 4 were obtained, while two samples were taken from regions 1 and 5: each region was rinsed three times with 1 ml of sterile NSS; the region was then cut open, and the intestinal mucus was scraped off as described above; and then pieces of regions 2, 3, and 4 were homogenized in NSS with a glass homogenizer. All samples were serially diluted in NSS and plated on MA and VNSS agar after serial dilution. The rinse, mucus, and homogenate samples would therefore correspond to the lumenal contents, mucus, and epithelial cells, respectively.

**Screening of inhibitory effects against fish pathogens.** For screening of inhibitory effects against the fish pathogen *V. anguillarum* HI 11345 (isolated from turbot with vibriosis), provided by the Institute of Marine Research, Bergen, Norway, 403 different isolates were picked from the original plates according to morphology, pigmentation, variability, and number in the different samples. Each isolate was then transferred into marine broth, cultured at 20°C for 12 h, and then stored at −70°C after addition of 20% glycerol.

By a modified double-layer method previously described by Dopazo et al. (5), macrocolonies of the isolates were created on tryptic soy agar-NSS and MA plates by inoculating 10-μl droplets of overnight culture. After incubation for 24 h at 20°C, the colonies were killed with chloroform vapor (45 min) and 100 ml of overnight culture of *V. anguillarum* HI 11345 was diluted 10 times and suspended in tryptic soy broth-NSS soft agar which was poured over the plates (4 ml per plate). Again, the plates were incubated for 24 h at 20°C before examination. Inhibition of growth of the pathogen around and/or over the macrocolony was seen as a clear zone. Control plates without macrocolonies of the isolates were included to evaluate the possible effect of chloroform on the growth of *V. anguillarum*. No such effect was observed. Autoinhibition of the pathogen was also investigated in this manner.

The isolates which inhibited growth of *V. anguillarum* HI 11345 were further checked against the following fish pathogens: *V. anguillarum* R 73 serotype 01 isolated from turbot, *V. anguillarum* 860908-5 serotype 02 isolated from turbot, *V. anguillarum* 1173/1 serotype 02A isolated from cod, and *V. anguillarum* 820723-2/8 serotype 02B isolated from salmon, all provided by Jens Laurits Larsen, Royal Veterinary and Agricultural University, Frederiksberg, Denmark; *V. anguillarum* NCMB 2129, from Geir Hansen Institute of Microbiology and Plant Physiology, University of Bergen, Norway; and *Aeromonas hydrophila* CCUG 1455 and *Aeromonas salmonicida* CCUG 2116, from the Culture Collection, University of Göteborg, Sweden.

**Characterization methods.** For identification of marine strains the biochemical identification system of Hansen and Sörheim (9), slightly modified, was used. The following tests were not done: arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, lipase (Tween-80), methyl red, and Simmon's citrate. In addition, all isolates were checked for luminescence, catalase production (by using 3% $H_2O_2$), and oxidase production (by using Spot Test Oxidase reagent; Difco Laboratories). The biochemical results were then processed on an identification-dendrogram computer program at the Culture Collection, University of Göteborg, by Enevold Falsen. All isolates with an inhibitory effect against *V. anguillarum* HI 11345 were tested for sensitivity to the vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine, 150 μg per disc), elastase production (13), and hemolytic activity by growing bacteria on tryptic soy agar plates containing 2% NaCl and 5% horse blood.

Of the colonies isolated and incubated anaerobically, 13 were picked according to morphology, pigmentation, variability, and number in the different samples and transferred to plates incubated aerobically in order to test for the presence of obligate anaerobes.

## RESULTS

There was no difference in the total number of isolates generated by the two media, MA and VNSS, for the different incubation temperatures. All isolates that were generated during anaerobic incubation grew also on plates incubated aerobically. Hence, no obligate anaerobes were detected. From the original agar plates, 403 isolates were picked and subcultured, of which 89 showed inhibitory effects against the *V. anguillarum* strains that were tested. This equals 22% of the total number of isolated strains. No evidence of autoinhibition of *V. anguillarum* was detected.

**Habitat of bacteria with inhibitory activity.** A comparison between the isolates obtained from the intestine and those from the external mucus layer of the fish revealed that a significantly higher proportion of intestinal rather than surface bacteria displayed inhibitory effects against *V. anguillarum* HI 11345 (Fig. 1). Of the total number of bacterial isolates from the intestine ($n$ = 283), 28% were inhibitory strains, while of the bacterial floras obtained from the outer mucus layer ($n$ = 76), 11% had inhibitory effects. Furthermore, only 3% of the water bacteria ($n$ = 34) proved to be inhibitory against *V. anguillarum* HI 11345 (Fig. 1). No inhibitory strains were found in the food pellets ($n$ = 10).

To find out whether the number of intestinal bacteria with inhibitory effects differed in the six fish that were tested, the relative number of isolates that inhibited growth of *V. anguillarum* HI 11345 was examined. Four of six fish (numbers 1, 2, 4, and 6) had a fraction of intestinal bacteria with an inhibitory effect that varied between 33 and 37% (Fig. 2). Inhibitory strains were 14 and 7% of the total number of intestinal bacterial isolates obtained from fish 3 and fish 5,

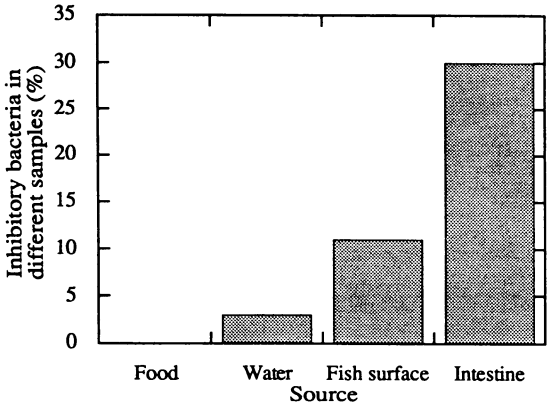


FIG. 1. Percentage of bacteria with an inhibitory effect against *V. anguillarum* HI 11345 relative to the total number of bacteria isolated from four sources.

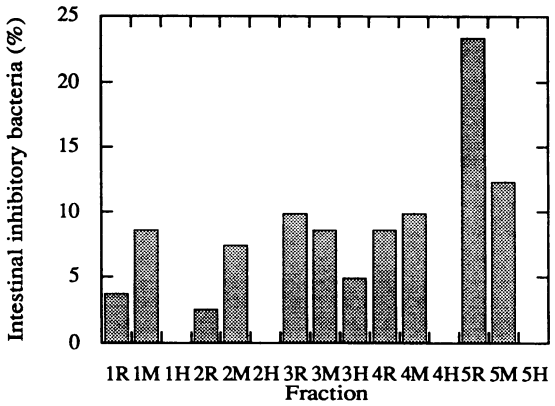


FIG. 3. Number of inhibitory bacteria isolated from the rinse (R), mucus (M), and homogenate (cell wall) (H) fractions from the different parts of the intestine relative to the total number of bacteria with an inhibitory effect. Six animals were studied.

respectively. The different incubation temperatures, 7, 12, and 20°C, did not markedly affect the number of inhibitory strains that were isolated.

**Gastrointestinal location of inhibitory strains.** To ascertain whether the inhibitory strains resided in the intestinal lumen and/or loosely associated with the mucus overlaying the epithelial cells or resided on the epithelial cell surface, we examined the fractions referred to as the rinse, mucus, and homogenate preparations, which corresponded to lumenal contents, mucus, and epithelial cells, respectively. Almost all bacteria with an inhibitory effect were found in the lumenal and mucus fractions, which contained 48 and 47% of the inhibitory strains, respectively. The epithelial fraction contained 5% of the total number of inhibitory strains examined.

To further describe the sites colonized by inhibitory strains, the number of inhibitory bacteria from different fractions of different parts of the intestine was examined. The number of inhibitory strains in the mucus fractions was found to be relatively constant, at a level of about 10% in the different parts of the intestine (Fig. 3). In the lumenal fractions, the number increased towards the lower part of the intestine. The difference in the relative number of inhibitory isolates in this fraction was from below 5% to nearly 25%. The lowest number of inhibitory strains was found in the epithelial fraction. In fact, only the upper intestine revealed inhibitory strains in the epithelial fraction (Fig. 3).

Figure 3 also provides information about the percentage of inhibitory bacteria in the different parts of the intestine. The number of bacteria with an inhibitory effect increased along the intestine. The lowest numbers were found in the pyloric caeca and the stomach, 10 and 12%, respectively. The upper and lower intestine contained between 20 and 25% of the inhibitory strains. Most bacteria with an inhibitory effect, more than 35% of the total number of intestinal inhibitory strains, were found in the rectum.

In Fig. 4, the number of bacteria with an inhibitory effect in the different intestinal parts is shown as the percentage of the total number of isolated bacteria in each intestinal part. This was done to present the proportion of the isolates with an inhibitory effect relative to the total number of isolated intestinal bacteria. The proportions of inhibitory strains were as follows: in the stomach, 17%; in the pyloric caeca, 21%; in the upper and lower parts of the intestine, 42 and 26%, respectively; and in the rectum, 40%.

**Screening of inhibitory effects against various fish patho-**

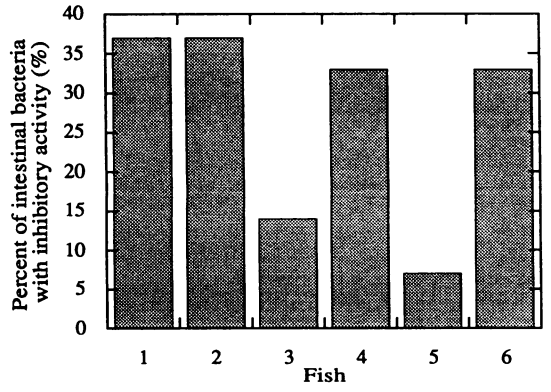


FIG. 2. Percentage of intestinal bacteria with an inhibitory effect against *V. anguillarum* HI 11345 in the six fish that were sampled.

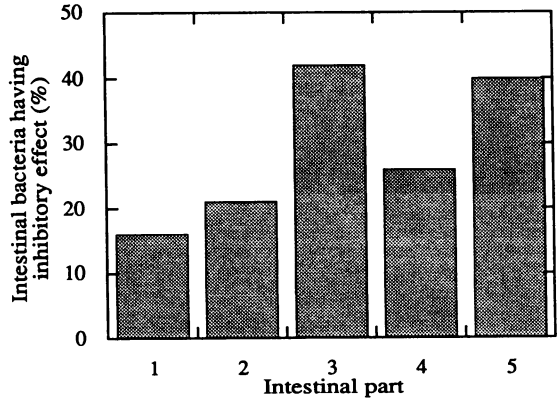


FIG. 4. Number of inhibitory bacteria isolated from each of the five different parts of the intestine relative to the total number of bacteria isolated in each part. The different parts were as follows: 1, stomach; 2, pyloric caeca; 3, upper intestine; 4, lower intestine; 5, rectum.

TABLE 1. Comparison of the inhibitory effect on six fish pathogenic *V. anguillarum* strains by fish intestinal isolates

| No. of *V. anguillarum* serotypes inhibited[a] | Inhibitory bacteria having effect (%) |
|---|---|
| 1 | 3 |
| 2 | 7 |
| 3 | 10 |
| 4 | 7 |
| 5 | 13 |
| 6 | 60 |

[a] The *V. anguillarum* strains were HI 11345, NCMB 2129, and serotypes 01, 02, 024, and 02B.

**gens.** To determine the degree of specificity of the inhibitory effect of the isolated strains that were inhibitory against *V. anguillarum* HI 11345, a range of fish pathogens was tested. Sixty percent of the inhibitory strains showed an effect against all *V. anguillarum* strains examined (Table 1). Three percent were specificially inhibitory against *V. anguillarum* HI 11345 but not against any other *V. anguillarum* serotype.

The percentage of inhibitory strains having an effect on each specific *V. anguillarum* serotype varied from 100 to 74% of the total number of inhibitory strains (Table 2). Of 85 strains tested, 95% showed an effect against *A. salmonicida*, and 96% of 47 strains tested showed an effect against *A. hydrophila*.

**Characterization according to biochemical properties.** The 89 strains with an inhibitory effect against *V. anguillarum* HI 11345 were classified in different phena according to their biochemical characteristics. This was done in order to evaluate possible correlations among location, inhibitory effect, and phenon. Of the total number of isolates with an inhibitory effect, 93% were gram negative, 3% were gram positive, and the remaining 4% were not tested. As seen in the dendrogram (Fig. 5), the inhibitory strains varied in taxonomy, but it is possible to discern different clusters. All isolates were sensitive to the vibriostatic agent O/129, 3% displayed elastase production, and 94% showed hemolytic activity.

## DISCUSSION

Giaxa in 1889 (8) was probably the first to report the existence in seawater of bacteria with an inhibitory effect against a *Vibrio* sp. In 1947, Rosenfeld and Zobell (19) carried out a detailed study of antibiotic-producing marine microorganisms. Although they did not attempt an isolation of specific antibiotics produced by marine bacteria, it was evident from their work that various species of microorganisms, indigenous to the sea, released antimicrobial substances. Furthermore, they suggested that the sea may represent a reservoir for microbial antagonists of possibly practical importance. Since then, there have been several reports of bacteria with inhibitory effects isolated from seawater; the main purpose of these mostly have been to characterize the specific antibiotic or bacteriocin produced. Only recently have bacteria been isolated from other marine habitats. For example, Lemos et al. (14) screened bacteria from intertidal seaweeds for antibiotic activity.

Although the microbiology of the intestinal tracts of ma-

TABLE 2. Percentage of intestinal bacteria isolates showing an inhibitory effect against various *V. anguillarum* strains compared with the total number of bacteria with an inhibitory effect

| *V. anguillarum* strain | Effect (%) |
|---|---|
| HI 1135 | 100 |
| Serotype 01 | 88 |
| Serotype 02 | 88 |
| Serotype 02A | 74 |
| Serotype 02B | 74 |
| NCMB 2129[a] | 88 |

[a] Five percent of the inhibitory strains were not tested against *V. anguillarum* NCMB 2129.

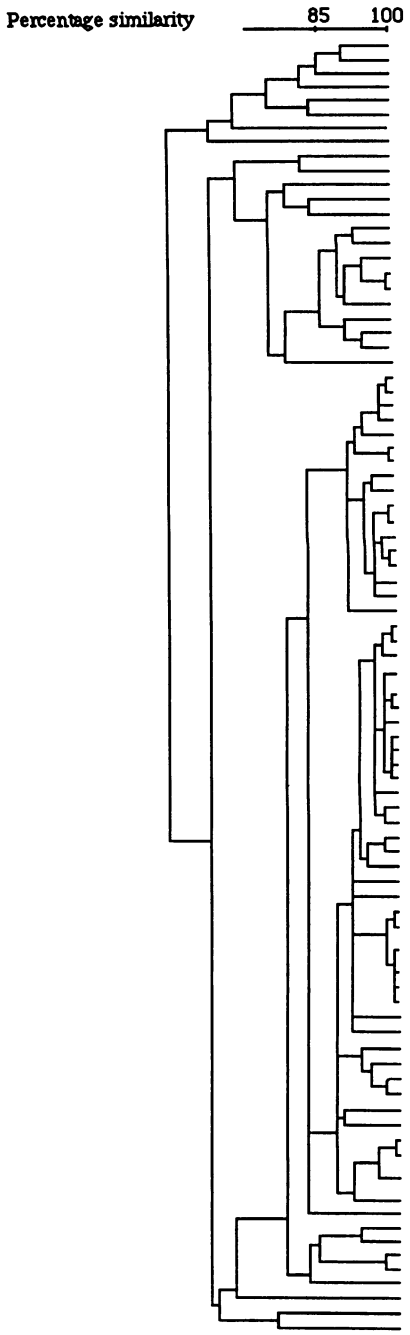


FIG. 5. Numerical clustering of all bacteria with inhibitory effect, based on the modified biochemical identification system of marine microorganisms (see Materials and Methods).

rine and freshwater fish has been investigated by many researchers, few studies have addressed the production of inhibitory components by these bacteria. There is evidence that dense microbial populations occur within the intestinal contents, with numbers of bacteria much higher than those in the surrounding water, indicating that the fish intestine provides a favorable ecological niche for these organisms (3, 15). It has also been reported that the numbers of bacteria in freshwater salmonids increased between the stomach and the posterior portion of the intestine (24). It was suggested that these numbers must reflect active multiplication in the tract, as they could not be accounted for by ingestion of bacterial cells. It has been proposed that mucus may serve as a source of nutrients (12) and that it may enhance colonization by serving as an initial attachment site for bacteria (6) or as a matrix for permanent bacterial attachment (21). Conversely, the mucus layer may in some instances be an effective barrier, providing protection against penetration by invading microorganisms (6).

Onarheim and Raa (17) have studied the microbial flora associated with fish and provided evidence for the existence of bacteria (gut vibrios) that produce bacterial inhibitory substances in the intestinal tracts of cod, salmon, saithe, and herring.

Our study has shown that a large number of turbot intestinal bacteria, as compared with the external sources, produce inhibitory substances. It is possible that such a mechanism has evolved as protection against pathogenic bacteria, in that the latter might readily amplify after invasion. This hypothesis is supported by the fact that it was recently shown that *V. anguillarum* grows rapidly in intestinal mucus from turbot (16).

Between 30 and 35% of the total number of isolates from four of the six animals showed inhibitory effects against *V. anguillarum* HI 11345. Other reports on the fraction of inhibitory strains in different marine habitats generally indicate a lower percentage of such bacteria (14). Notably, a high number of inhibitory strains were found in all parts of the intestine of the turbot. The majority of these were isolated from the lumenal and mucus fractions in all intestinal parts. The highest number of bacteria with an inhibitory effect were found in the rectum, which could depend on an amplifying effect by the pathogen in this part or reflect the possibility that the rectum is the port of entry for bacteria. This suggests that the intestinal lumen and mucus of the entire gastrointestinal tract contain an indigenous microflora with inhibitory effects against *V. anguillarum*. The subsequent experiments demonstrated that the growth of five additional *V. anguillarum* strains, of which three were isolated from turbot, as well as *A. hydrophila* and *A. salmonicida*, was inhibited by isolates from the mucus, lumenal, and epithelial fractions of the different parts of the intestine, i.e., the stomach, pyloric caecae, upper and lower intestine, and the rectum.

The results presented in this study also show that no bacterial fish pathogen was affected by one inhibitory strain only. In addition, when the origin of the isolates and the inhibitory pattern were compared, no specificity was noted. Bacterial strains with an inhibitory effect against all fish-pathogenic bacteria tested were seemingly also distributed randomly throughout the bacterial phena identified by the biochemical characterization assay. Representatives of the individual phena presented by the dendrogram were found in both the mucus and the lumenal fractions in all parts of the intestine. It is suggested that a wide range of intestinal bacteria inhibits growth of several species of pathogens. Notably, the turbot-specific pathogens were affected by a greater number of intestinal isolates than were the *V. anguillarum* pathogenic strains that are specific for cod and salmon. An interesting result is the sensitivity to O/129 discs, which indicates the existence of *Vibrio* strains. This is in agreement with the work done by Onarheim and Raa (17) on the microflora associated with marine fish.

It is known that stress and other disturbances of higher animals alter the profile of gut microorganisms (22), thus possibly providing access for pathogenic invaders (23). Similar conditions during fish farming would be suggested to significantly facilitate the successful colonization of fish pathogens and allow for rapid growth in mucus in the absence of competition (16). In view of the data presented here, it appears feasible to obtain strongly inhibitory strains of intestinal origin. Such strains could be useful for oral administration to farmed turbot to inhibit *V. anguillarum* infections. A selected number of these potentially useable strains should therefore be analyzed in terms of the site of colonization. In view of the results obtained in this study, it is feasible that a condition for a successful probiotic strain administered to turbot is the ability to colonize in several parts of the gastrointestinal tract. Possibly several inhibitory strains need to be administered in order to provide sufficient protection against fish-pathogenic bacteria.

The data presented in this paper also suggest that mucus-associated bacteria constitute the barrier against invasion and growth and amplification of fish pathogens, because few bacteria were observed to be associated with the epithelial cells. Competition for sites and nutrients in the mucus layer could determine the host susceptibility to pathogenic bacteria. The possibility that this result and conclusions are biased because of the method used to remove the mucus from the surface, cannot, however, be ruled out; i.e., we may have underestimated the number of epithelial cell-associated bacteria. Further studies investigating the microenvironment of the different parts of the intestine would be of value. The fact that all fish had bacteria with inhibitory effects supports the notion of fish having an indigenous inhibitory microflora of some importance in protecting the host against pathogens.

## ACKNOWLEDGMENTS

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