

## Intestinal Colonization Potential of Turbot (*Scophthalmus maximus*)- and Dab (*Limanda limanda*)-Associated Bacteria with Inhibitory Effects against *Vibrio anguillarum*

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Of more than 400 bacteria isolated from turbot (*Scophthalmus maximus*), 89 have previously been shown to inhibit the in vitro growth of the fish pathogen *Vibrio anguillarum*. The aim of the present study was to investigate the potential of seven of these strains, as well as of intestinal isolates (four strains) from a closely related fish, dab (*Limanda limanda*), for colonizing farmed turbot as a means of protecting the host from infection by *V. anguillarum*. In addition, the inhibitory effect of these strains on the pathogen was further studied. Colonization potential was measured by the capacity of the strains to adhere to and grow in turbot intestinal mucus. These parameters were also used to investigate the potential of *V. anguillarum* to amplify in the turbot intestinal tract. Because of the observed rapid growth of *V. anguillarum* in intestinal mucus, it can be proposed that the intestinal tract is a site for *V. anguillarum* multiplication. Strains isolated from the intestine showed greater capacity for adhesion to and growth in fish intestinal mucus than did the pathogen and the skin mucus isolates. All of the isolates released metabolites into the culture medium that had inhibitory effects against *V. anguillarum*. The results are discussed with emphasis on administering bacteria of host origin to farmed turbot in order to control *V. anguillarum*-induced disease.

Vibriosis caused by *Vibrio anguillarum* is a particularly severe disease of many fish and causes great economic loss in hatcheries of, for example, salmonids, eel, and turbot (3, 11). The disease is characterized by deep focal necrotizing myositis and subdermal hemorrhages. The intestine and rectum may also be swollen and filled with a clear viscous fluid (11, 13, 22).

While the port of entry of the pathogen into the fish has still not been determined, the gastrointestinal tract has been implicated as a site of colonization (12). In Pacific salmon naturally infected with *V. anguillarum* and *V. ordalii*, the pathogens were found in the pyloric ceca and throughout the intestinal tract (24). The gastrointestinal tract may also play an important role in amplifying the pathogen in a fish-rearing unit. *V. salmonicida*, causing coldwater vibriosis, has been detected in feces of experimentally infected fish (11a). The pathogen can establish itself in other fish or in sediments around the farm, where it has been detected several months after an outbreak of the disease (9).

In addition to good husbandry and culture conditions, vibriosis is traditionally controlled with chemotherapeutic agents and vaccination. Frequent use of chemotherapeutic agents, however, leads to the occurrence of resistant bacteria (1). Drug resistance has been found in several bacterial fish pathogens, including *V. anguillarum* (2). Vaccination of fish in order to prevent infection with *V. anguillarum* has been successful on the laboratory scale, especially if the vaccine is administered intraperitoneally (14). Unfortunately, the method is time-consuming, and sedation of the fish is necessary before injection. An alternative prophylactic and therapeutic treatment could be to support the natural host microbial defense mechanism by administration of live bacteria with demonstrable inhibitory effects against patho-

gens. This concept has already been applied to other hosts, e.g., pigs and humans (6). It has been shown that disturbance of the host gut microflora induces increased sensitivity to pathogenic infections, an effect which can be reduced by administration of bacteria with inhibitory activity against the pathogen. In recent years, it has become apparent that it is desirable for such bacteria to be of host origin and to have the capacity to colonize the gastrointestinal tract for protection to occur (6). The importance of using host-specific strains was emphasized by the work of Hansen and Olafsen (10), who tried to regulate the microflora of cod and halibut eggs by incubation of gnotobiotic eggs with antibiotic-producing epiphytic bacteria. Environmental strains in the test system completely displaced the administered strains within a few days.

In several studies, it has been suggested that an indigenous microbial flora exists in the digestive tract of fish. Campbell and Buswell (5) showed that the distribution of bacteria in the intestine of farmed Dover sole was not consistent with that of the water or the fish diet microflora. Further, in a recent investigation by Onarheim and Raa (23), the bacterial floras attached to the intestinal mucosa of four different species of marine fish were characterized, with 76 of 200 isolates belonging to the family *Vibrionaceae*. It was proposed that some of these isolates contribute to protecting the fish from invasion by pathogens.

Antibiotic-producing bacteria have been demonstrated in the marine environment by several workers. In an investigation by Lemos et al. (19), 38 of 200 strains isolated from marine algae displayed antibiotic activity and were all assigned to the *Pseudomonas-Alteromonas* group. The same isolates were inhibitory to many of the bacterial fish pathogens that were tested, including *V. anguillarum* (8). Similarly, while screening over 400 isolates associated with turbot, we detected 89 strains which inhibited the growth of *V. anguillarum* (27). While some of these inhibitory isolates

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may be transient, others would be components of the indigenous turbot microflora. It is feasible that the indigenous strains would be able to colonize if administered to farmed turbot.

The aim of this study was to identify whether some of the bacterial strains with inhibitory effects against *V. anguillarum* that were isolated from flatfish had the potential to colonize farmed turbot and thus protect the fish from *V. anguillarum* infection. Bacterial isolates from both farmed turbot and feral dab were studied. Colonization potential was examined in terms of the in vitro capacity of the strains to adhere to and grow in turbot intestinal mucus. Similarly, the capacity of *V. anguillarum* to colonize the intestine was investigated. The ability of the fish isolates to suppress growth of *V. anguillarum* in different test systems was also studied.

## MATERIALS AND METHODS

**Fish.** Turbot were obtained from a commercial breeding farm (Mowi A/S, Bergen, Norway). All turbot were 5 years old and 1 to 1.5 kg in weight and had been maintained in tanks with running seawater and sparsely fed with manufactured fish food. As juveniles, they had suffered from vibriosis. Feral dab (*Limanda limanda*) were caught from the sea outside Göteborg on the Swedish west coast. Fish were killed immediately after capture, and sampling was done within 1 h.

**Sampling.** The skin mucus was scraped off the fish with a rubber spatula. The blind side of the fish was then washed with 70% ethanol, and the peritoneum was cut open. The stomach and the intestine down to the vent were dissected aseptically, rinsed in sterile NSS (nine-salt solution: NaCl, 17.6 g; Na<sub>2</sub>SO<sub>4</sub>, 1.47 g; NaHCO<sub>3</sub>, 0.08 g; KCl, 0.25 g; KBr, 0.04 g; MgCl<sub>2</sub> · 6H<sub>2</sub>O, 1.87 g; CaCl<sub>2</sub> · 2H<sub>2</sub>O, 0.41 g; SrCl<sub>2</sub> · 6H<sub>2</sub>O, 0.008 g; H<sub>3</sub>BO<sub>3</sub>, 0.008 g in 1,000 ml of double-distilled H<sub>2</sub>O), and sectioned into stomach, pyloric caeca, upper intestine, lower intestine, and rectum. Three types of samples were taken from each region: (i) the section was rinsed three times with 1 ml of NSS for each rinse; (ii) after being rinsed, the section was cut open and the intestinal mucus was scraped off as described above, giving 1 to 2 ml of mucus per region; and (iii) after the section was rinsed and the mucus was removed, pieces of some regions were homogenized in NSS by hand with a glass homogenizer. All samples were serially diluted in NSS and plated on marine agar (MA 2216; Difco Laboratories) and VNSS agar (peptone, 1.0 g; yeast extract, 0.5 g; glucose, 0.5 g; starch, 0.5 g; FeSO<sub>4</sub> · 7H<sub>2</sub>O, 0.01 g; Na<sub>2</sub>HPO<sub>4</sub>, 0.01 g; and agar, 15 g in 1,000 ml of NSS). Plates were incubated at 12 and 20°C for up to 7 days. Isolates were subcultured in marine broth (MB 2216; Difco Laboratories) and stored in MB containing 30% glycerol at -70°C. Intestinal (from the pylorus to the lower intestine) mucus and skin mucus were also collected as described above for type ii sampling of intestinal mucus and skin mucus. All mucus samples were diluted in NSS. Sample dilutions were centrifuged at 25,000 × g three times for 8 min each in order to remove particulate and cellular material and stored at -20°C until used in adhesion and growth studies.

**Bacterial strains.** Of 403 isolates from farmed turbot, 89 were previously shown to possess inhibitory activity against *V. anguillarum* HI 11345 (27). From this collection, seven inhibitory isolates displaying different biochemical characteristics were selected for study of their colonization potential. In addition, four intestinal strains from another flatfish,

TABLE 1. Origins of selected isolates

Isolate	Source and sampling site
4:44	Turbot, upper intestinal tract, gut content
4:45	Turbot, upper intestinal tract, mucus
4:46	Turbot, upper intestinal tract, mucus
4:47	Turbot, upper intestinal tract, mucus
7:9	Turbot, skin mucus
7:10	Turbot, skin mucus
7:12	Turbot, skin mucus
10	Dab, intestinal mucus
14	Dab, intestinal mucus
24	Dab, pyloric cecum, epithelium
27	Dab, pyloric cecum, epithelium

feral dab, all with different API 20-E profiles, were studied. The origins of these isolates are presented in Table 1.

*V. anguillarum* HI 11345, originally isolated from diseased turbot, was obtained from the Institute of Marine Research, Bergen, Norway. The identity of the strain was confirmed by using API 20-E strips (API Systems SA, Montalieu-Vercieu, France), and hemolysin production was demonstrated by using horseblood-tryptic soy agar (TSA) plates (16).

**Growth in intestinal mucus.** The intestinal mucus from turbot was diluted in NSS to a protein concentration of 0.5 mg/ml, as measured by using Coomassie brilliant blue G250 (25). Ten-microliter aliquots of an overnight culture of *V. anguillarum* and turbot isolates were inoculated in 3 ml of diluted mucus and incubated at 20°C in duplicate test tubes held on a shaking incubator. Test tubes containing 3 ml of tryptic soy broth (TSB) were treated in the same way. Tubes containing only mucus were incubated as controls. The growth rate of the strains in mucus and TSB was measured by monitoring the optical density at 540 nm (OD<sub>540</sub>). The length of the lag period and the generation time for growth of the isolates were also calculated.

**Adhesion to skin and intestinal mucus.** We used a modification of the method of Laux et al. (17), used for studying adhesion of enteric bacteria to mouse intestinal mucus, to study adhesion to fish skin and intestinal mucus. Skin and intestinal turbot mucus, prepared as above and diluted to 0.5 mg of protein per ml in NSS, was immobilized overnight at 4°C in multiwell polystyrene tissue culture plates (Nunc Delta S1), 0.25 ml in each well. After immobilization, unbound mucus was removed by rinsing each well twice with 0.5 ml of NSS. Bovine serum albumin (BSA; 1%) was used in control wells. Twenty-four-hour bacterial cultures grown in TSB containing [<sup>3</sup>H]thymidine ([methyl-1,2-<sup>3</sup>H]thymidine, 185 MBq/5 μl; Amersham International; 50 μl in 10 ml of TSB) were washed three times in NSS and diluted to 10<sup>8</sup> cells per ml. The number of bacterial cells was counted by using direct microscopy and a counting chamber. Aliquots (0.25 ml) of the washed and labeled bacterial suspensions were incubated in each well for 2 h at 20°C, with three replicates per isolate. After incubation, wells were rinsed twice with 0.5 ml of NSS to remove nonadhering bacteria. Adherent bacteria were removed by adding 0.5 ml of 10% sodium dodecyl sulfate (SDS) to each well and incubating for 1 h at 60°C.

The number of adhering bacteria was determined by quantifying the amount of incorporated [<sup>3</sup>H]thymidine in the SDS-released material and in 0.25-ml samples of the bacterial suspension with Aquassure scintillation fluid (NEN Research Products) and a liquid scintillation counter (Beckman LS 3801; Beckman Instruments).

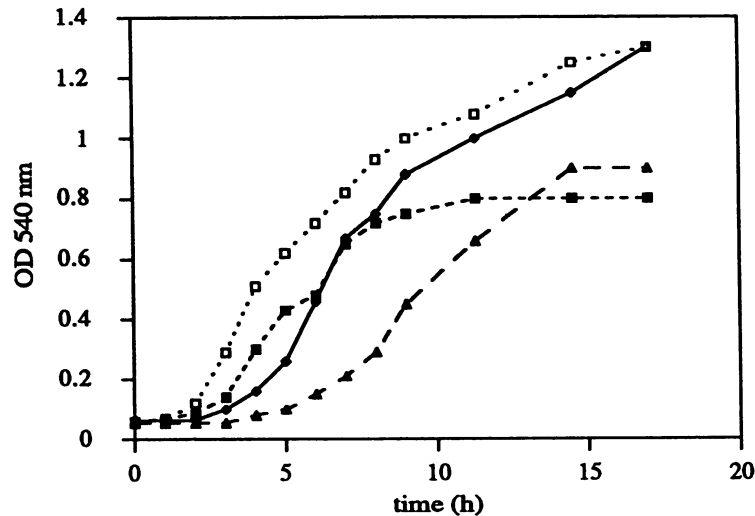


FIG. 1. Growth rate in diluted turbot intestinal mucus. Symbols: □, intestinal isolate 14 from dab; ■, intestinal isolate 4:45 from turbot; ▲, skin mucus isolate 7:12 from turbot; ◆, *V. anguillarum* HI 11345.

**Inhibitory activity.** Three different methods were used to measure the antagonistic effect of the isolates on *V. anguillarum*.

(i) With the disk diffusion method, supernatants from 24-h cultures grown in MB and TSB (Difco Laboratories) based on NSS were filter-sterilized (0.22- $\mu$ m-pore-size filters; Filtiron). Paper disks (5-mm diameter) were immersed in 250  $\mu$ l of the filtrate. After being dried overnight at 20°C, the filters were placed on TSA and MA plates immediately after the plates had been spread with 100  $\mu$ l of a 10 $\times$  dilution of an overnight culture of the pathogen. After 24 h of incubation at 20°C, a growth inhibition zone around the disk indicated production and release of bactericidal components into the medium. As controls, paper disks with NSS, medium, or the vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine, 10  $\mu$ g; Oxoid) were used.

(ii) For the double-layer method described by Dopazo et al. (8), TSA-NSS and MA plates were spot inoculated with 10  $\mu$ l of overnight cultures of the test organisms (three repeats). After incubation for 24 h at 20°C, the colonies were killed with chloroform vapor (45 min), and 100- $\mu$ l aliquots of a 10-fold dilution of an overnight culture of *V. anguillarum* in 4 ml of TSB-NSS or MA soft agar were spread over the plates. The plates were incubated for an additional 24 h at 20°C. Inhibition of growth of the pathogen around and/or over the macrocolony was considered a positive result. Autoinhibition of the pathogen itself was also investigated by this technique. O/129 disks were again used as the control.

(iii) As described above, 24-h cultures in MB and TSB-NSS were centrifuged (5 min, 3,000  $\times g$ ) and sterile filtered. Filtrate (30 ml) and fresh medium (30 ml) were mixed in an Erlenmeyer sidearm flask (duplicates) and then inoculated with 10  $\mu$ l of a *V. anguillarum* 24-h culture. The growth rates of the pathogen in the different supernatants were determined by monitoring the OD<sub>540</sub>. The lengths of the lag period and generation times were calculated from the growth curves obtained. Flasks with only fresh medium and with NSS instead of filtrate were used as controls. Prior to inoculation, the pH of the supernatants was measured.

## RESULTS

**Growth in intestinal mucus.** All isolates grew in intestinal mucus from turbot. The uninoculated mucus did not increase in optical density throughout the experiment. Although the mucus was diluted 10-fold (final concentration, 0.5 mg of protein per ml), it provided the organisms with sufficient nutrients to give growth rates and stationary-phase optical densities (data not shown) similar to those in TSB. Biphasic growth curves were noted for isolates 4:44, 4:45, 4:46, 4:47, and 14. The growth curves for 4:45 and 14 are shown in Fig. 1. As can be seen in Table 2, the gut isolates generally had a shorter lag phase, 1.75  $\pm$  0.43 h, and generation time, 54  $\pm$  8 min (mean  $\pm$  standard error [SE] of the eight gut isolates), than did the skin isolates, 3.33  $\pm$  0.47 h and 115  $\pm$  5 min, respectively (mean  $\pm$  SE of the three skin isolates). In addition, the gut isolates generally grew faster in intestinal mucus than did *V. anguillarum*, as exemplified by isolates 4:45 and 14 in Fig. 1.

**Adhesion.** The isolates showed very different adhesion patterns. Turbot isolates 4:44 and 4:46 showed twofold greater adhesion to turbot intestinal mucus than to the

TABLE 2. Generation time and lag phase in turbot intestinal mucus and in TSB of test isolates and *V. anguillarum* HI 11345

Isolate	Generation time (min)		Lag time (h)	
	Mucus	TSB	Mucus	TSB
4:44	63	69	2	3
4:45	66	54	2	2
4:46	60	53	1	2
4:47	60	63	2	3
7:9	120	82	4	4
7:10	118	84	3	3
7:12	108	73	3	4
10	42	50	2	3
14	47	53	1	1
24	48	46	2	3
27	54	54	2	2
HI 11345	81	56	3	3

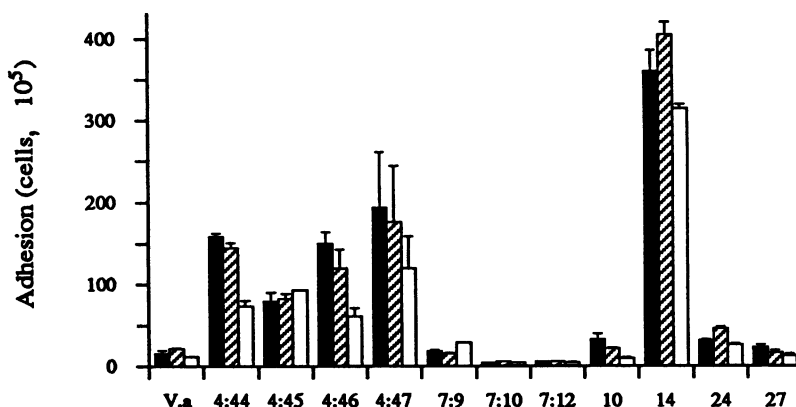


FIG. 2. Adhesion of fish isolates and *V. anguillarum* HI 11345 (V.a) to turbot intestinal mucus (■), skin mucus (▨), and BSA (□). Error bars indicate SE of the mean of triplicate samples.

control surface BSA, and except for strain 10, intestinal isolates adhered much better to all surfaces than did *V. anguillarum* (Fig. 2). Strain 14, isolated from dab intestinal mucus, adhered extremely well ( $3.6 \times 10^7$  cells per tissue culture well) compared with the pathogen ( $5.9 \times 10^5$  cells per tissue culture well) when tested with the same concentration of intestinal mucus. Background radioactivity was 41 dpm. Incorporation of  $^3\text{H}$  ranged from 4,000 dpm per  $10^7$  cells (strain HI 11345) to 26,500 dpm per  $10^7$  cells (strain 7:10).

**Inhibitory activity.** The disk diffusion method data were generally of low reproducibility. Of all the cultures, only isolates 4:44, 4:45, and 4:46 produced weak zones of growth inhibition around disks impregnated with culture supernatant (data not shown). All of the TSA-grown isolates inhibited growth of *V. anguillarum* in the double-layer technique (Table 3). For the three repeated experiments, no measurable variability in the sizes of the zones of inhibition was detectable. With MA as the growth medium, none of the isolates produced a zone of nongrowth of the pathogen (data not shown). No autoinhibition by *V. anguillarum* was detected. The control disk (O/129), which inhibits growth of *Vibrio* spp., did inhibit the growth of *V. anguillarum*. The growth of the pathogen in TSB was clearly suppressed by components in the supernatants (50% supernatant and 50%

fresh TSB) from isolates originating from skin mucus (7:9, 7:10, and 7:12). This suppression is expressed as a lag period up to 8 h longer than that of the controls (Fig. 3). The pHs of the supernatants ranged between 8.0 and 8.5.

## DISCUSSION

When probiotic bacteria are used in aquaculture to protect fish against diseases caused by bacterial pathogens, the strains to be administered should not only be antagonistic against the pathogens but also have the capacity to colonize the fish. The potential of such isolates to colonize turbot was assessed here in terms of the capacity of the strains to adhere to and grow in skin and intestinal mucus from the fish. By using the same in vitro parameters, it was also possible to ascertain whether the intestinal tract is in fact a site of amplification for the pathogen *V. anguillarum*, as suggested for *V. salmonicida* in Atlantic salmon (11a).

It is widely accepted that many enteric pathogens adhere to and proliferate in the host intestinal tract. For example, *Vibrio cholerae* can adhere to isolated brush border membranes from rabbit intestinal epithelial cells and can also penetrate the overlying mucus gel and occasionally become entrapped in it (15). More recently, it has been shown that the ileal and/or cecal mucus contains receptors for bacterial adhesion (7), and it has been proposed that mucus is the site for colonization by *Salmonella typhimurium* (21), *Campylobacter jejuni* (18), and *Escherichia coli* K88 (4). It therefore seems feasible to evaluate the colonization potential in vitro by measuring the capacity of the strains to adhere to and grow in mucus.

All of the isolates in our investigation grew in and adhered to turbot intestinal mucus; however, this capacity was very much influenced by the origin of the isolate. As shown in Table 2, all intestinal isolates exhibited a shorter lag phase and generation time than did the skin isolates when grown in intestinal mucus. The adhesion profiles also allowed grouping of the intestinal isolates, which adhered better to gut mucus, skin mucus, and even BSA than did the skin mucus isolates (Fig. 2). In this investigation, isolates 4:44, 4:46, and 4:47 displayed enhanced adhesion to intestinal mucus, suggestive of specific adhesion.

Growth of *V. anguillarum* in fish intestinal mucus has hitherto not been reported in the literature. The rapid growth of the pathogen in turbot intestinal mucus (Fig. 1) strongly supports the suggestion that the fish alimentary tract may

TABLE 3. Inhibition of *V. anguillarum* growth around macrocolonies of the bacterial isolates grown on TSB and then overlaid with soft agar containing *V. anguillarum* HI 11345<sup>a</sup>

Isolate	Clearing zone (mm)
4:44	10
4:45	10
4:46	10
4:47	10
7:9	25
7:10	25
7:12	23
10	10
14	10
24	9
27	9
HI 11345	0
O/129	17

<sup>a</sup> The inhibition of the pathogen is expressed as the diameter of the zone of no growth around the colony. A macrocolony of *V. anguillarum* HI 11345 and O/129 (10- $\mu\text{g}$  disk) served as controls.

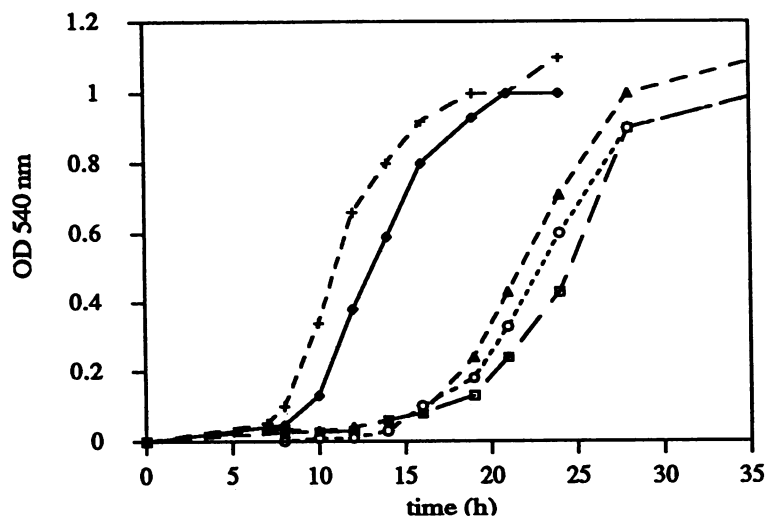


FIG. 3. Growth rate of *V. anguillarum* HI 11345 in 50% fresh TSB-50% filtered supernatant from skin mucus isolates 7:9 (□), 7:10 (○), and 7:12 (▲) grown in TSB. Growth in 50% fresh TSB-50% NSS (+) and 50% fresh TSB-50% supernatant (◆) of the pathogen served as controls.

serve as a site of amplification of *V. anguillarum* (12). Although *V. anguillarum* adhered poorly to intestinal mucus, it grew well, and if the generation time exceeds the turnover rate of the mucus, it is reasonable to suggest that the pathogen could colonize, as proposed for *E. coli* K88 mucus colonization (7). In the healthy fish, growth of the pathogen within the digestive tract would probably be suppressed by the indigenous microflora. However, stress conditions, which have been shown to decrease beneficial indigenous bacterial strains in higher vertebrates (26), could render the fish less protected by the indigenous microflora and allow proliferation of the pathogen in the mucus.

In this investigation, we found evidence that adult marine flatfish, turbot and dab, harbor intestinal and skin mucus-associated bacteria with the capacity to suppress growth of the fish pathogen *V. anguillarum*. The antagonistic effect against the pathogen was more pronounced in the isolates from skin mucus than in the intestinal strains, regardless of the method used. Conditions may not have been optimal for production of the inhibitory metabolites from the intestinal strains. For example, the intestinal isolates showed some bactericidal activity in the double-layer technique, but this effect was not clearly demonstrated in culture supernatants. From studies of *Lactobacillus* inhibitory metabolites, it is clear that growth and testing conditions need to be carefully defined to obtain reproducible inhibitory effects (unpublished observations). Similarly, the heat stabilities of bacteriocins differ considerably; for example, a preparation of a bacteriocin from *Shigella sonnei* P9 in basal salt medium loses activity at room temperature (20). Growth in MB or on MA produced fewer detectable inhibitory metabolites than did growth in TSB. This is in agreement with the work of Mayer-Harting et al. (20), who noted that the composition of the medium may affect the amount of bacteriocin produced or the amount released into the medium.

In summary, it appears that some of the isolates from adult turbot and dab have the potential to colonize the gastrointestinal tract. These isolates were able to adhere to and grow in fish intestinal mucus. It can be hypothesized that *V. anguillarum* can colonize the gastrointestinal tract of turbot, as can some of the isolates studied. Consequently, these

natural isolates with inhibitory activity against the pathogen may prove to be strong candidates for administration to fish to protect them from vibriosis.

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