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Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a Possible Probiotic Treatment of Fish

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To study the possible use of probiotics in fish farming, we evaluated the in vitro and in vivo antagonism of antibacterial strain *Pseudomonas fluorescens* strain AH2 against the fish-pathogenic bacterium *Vibrio anguillarum*. As iron is important in virulence and bacterial interactions, the effect of *P. fluorescens* AH2 was studied under iron-rich and iron-limited conditions. Sterile-filtered culture supernatants from iron-limited *P. fluorescens* AH2 inhibited the growth of *V. anguillarum*, whereas sterile-filtered supernatants from iron-replete cultures of *P. fluorescens* AH2 did not. *P. fluorescens* AH2 inhibited the growth of *V. anguillarum* during coculture, independently of the iron concentration, when the initial count of the antagonist was 100 to 1,000 times greater that of the fish pathogen. These in vitro results were successfully repeated in vivo. A probiotic effect in vivo was tested by exposing rainbow trout (*Oncorynchus mykiss* Walbaum) to *P. fluorescens* AH2 at a density of 10^5 CFU/ml for 5 days before a challenge with *V. anguillarum* at 10^4 to 10^5 CFU/ml for 1 h. Some fish were also exposed to *P. fluorescens* AH2 at 10^7 CFU/ml during the 1-h infection. The combined probiotic treatment resulted in a 46% reduction of calculated accumulated mortality; accumulated mortality was 25% after 7 days at 12°C in the probiotic-treated fish, whereas mortality was 47% in fish not treated with the probiont.

An increasing amount of the world's fish resources is being supplied by farmed fish. While catches of wild fish have stagnated at approximately 90 million metric tons, the amount of farmed fish has increased from 10 million metric tons in 1984 to more than 20 million metric tons in 1996 (11, 22). Disease is a major problem for the fish farming industry. Although vaccines are being developed and marketed, they generally cannot be used as a universal disease control measure in aquaculture. Juvenile fish are not fully immunocompetent and do not always respond to vaccination. Vaccination by injection, sometimes the only effective route of administration, is impractical when applied to small fish or larger numbers of fish. The addition of substantial amounts of antibiotics and chemotherapeutics remains the method of choice for disease control in many parts of the aquaculture industry. Increased concern about antibiotic-resistant microorganisms (1) has led to several alternative suggestions for disease prevention, including the use of nonpathogenic bacteria as probiotic biocontrol agents (3, 6, 36, 39). Lactic acid bacteria have been tested as probiotics in warm-blooded animals, and attempts have also been made to use lactic acid bacteria as antagonists of fish pathogens (14, 24, 25).

Fluorescent pseudomonads have been used as biocontrol agents in several rhizosphere studies (31), where their inhibitory activity has been attributed to a number of factors, such as the production of antibiotics (29, 34), hydrogen cyanide (38), or iron-chelating siderophores (26, 28). Pseudomonads constitute a large part of the microflora of the gills, skin, and intestinal tracts of live fish (9, 37) and are only rarely reported as pathogens of fish (20). As with their terrestrial counterparts, aquatic pseudomonads are often antagonistic against other microorganisms (15, 27), including fish-pathogenic bacteria (36) and fish-pathogenic fungi (6, 20). One study demonstrated that bathing Atlantic salmon presmolts in a strain of *Pseudomonas fluorescens* reduced subsequent mortality from stress-induced furunculosis (36).

When tested in vitro, iron limitation has been found to facilitate the antibacterial activity of fluorescent pseudomonads (15, 36). Thus, inhibition may be due to the production of siderophores, which deprive the fish pathogen of iron. Production of siderophores is a virulence factor in many microorganisms, such as members of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Vibrio anguillarum* (10), as reviewed by Wooldridge and Williams (40). An efficient salmon furunculosis vaccine elicits antibodies against iron-repressible outer membrane proteins of *Aeromonas salmonicida* (21). Iron available in the serum of fish, as in mammals (19), is crucial for infection, and fish with iron overload are more prone to attack by *V. anguillarum* than are fish with low serum iron concentrations (30).

To further study the potential of pseudomonads as biocontrol agents in fish farming, we investigated the inhibitory activity of a *P. fluorescens* strain of aquatic origin (AH2) against the fish-pathogenic bacterium, *V. anguillarum* in vitro and in vivo. The influence of iron on the inhibitory activity was assessed because the availability of iron is important in virulence and disease.

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MATERIALS AND METHODS

Bacterial strains. A virulent strain of the fish pathogen of *V. anguillarum* (90-11-287; serotype O1) that carries the pJM1 plasmid was obtained from K. Pedersen, Royal Veterinary and Agricultural University, Copenhagen, Denmark

(35). *P. fluorescens* AH2, which produces several siderophores (2), was isolated from iced freshwater fish (*Lates niloticus*) (18) and is antagonistic toward several gram-positive and gram-negative bacteria, particularly when iron limited (15, 17).

Media. M9 salts (32) supplemented with 0.4% glucose and 0.3% Casamino Acids (M9GC) or M9GC plus 3% NaCl (M9GC+NaCl) was used as low-iron culture medium. The total iron concentration, as determined by the phenanthroline method optimized for low concentrations, was estimated to be 0.6 μM (24a). Iron-replete conditions were obtained by adding 100 μM $FeCl_3$. In experiments with pure cultures, bacteria were counted by surface plating on tryptone soy agar plates (Oxoid catalog no. CM131) incubated at 25°C. *V. anguillarum* was routinely cultured on LB medium (Difco 0446) (5) with a total of 2% NaCl.

Siderophore production. Siderophore production was assayed on chrome azurol S (CAS) agar (33) based on M9GC (16). In liquid medium, siderophores were detected by the CAS assay (33). Equal volumes of sterile-filtered culture supernatant and CAS assay solution were mixed and left for 30 min at room temperature. The A_{630} was measured with sterile medium and CAS assay solution (33) as a blank. A negative value indicated the presence of iron-chelating substances such as siderophores (33).

Agar antagonism assay. Initial screening of antagonism by *P. fluorescens* AH2 was done in a plate assay. *V. anguillarum* (100 μl precultured in M9GC+NaCl for 5 days at 15°C) was spread on M9GC+NaCl agar plates with and without additional iron. Wells 3 mm in diameter were punched into the solidified agar, and 10 μl of a 24-h culture of *P. fluorescens* AH2 was added. The plates were incubated at 15°C, and zones of inhibition around the wells were measured after 3 to 5 days.

Effect of *P. fluorescens* AH2 supernatants. *P. fluorescens* AH2 was precultured in M9GC with and without NaCl and with or without iron (four combinations) and then used to inoculate 50 ml of M9GC in the same four combinations at an initial cell density of 10^3 to 10^4 CFU/ml. The flasks were incubated at 12 to 13°C with agitation (150 rpm), and samples were withdrawn daily. One milliliter was used for serial dilutions and estimation of colony counts on tryptone soy agar, and 2 ml was sterile-filtered (0.2-μm pore size; Sartorius no. 16534). The possible inhibitory activity of the sterile-filtered supernatant was tested by adding 100 μl of supernatant to 100 μl of fresh medium in microtiter wells (Nunc microwell 96F) and inoculating it with 10 μl of a dilution of *V. anguillarum* yielding approximately 10^4 CFU/ml. Controls were done by inoculating *V. anguillarum* in 200 μl of M9GC. Each combination was tested in triplicate, and the growth of the fish pathogen was monitored by recording the optical density at 600 nm (OD_{600}) with a Labsystems Multiscan RC microtiter plate reader.

Coculture experiments. *P. fluorescens* AH2 and *V. anguillarum* were precultured separately in M9GC+NaCl at 13°C with aeration for 3 to 5 days. Appropriate dilutions were prepared in physiological saline, and *V. anguillarum* was inoculated in M9GC+NaCl at an initial cell density of approximately 10^3 CFU/ml, whereas the initial levels of *P. fluorescens* AH2 were 10^4, 10^5, 10^6, 10^7, and 10^8 CFU/ml. All combinations were done in duplicate. The flasks were incubated at 12 to 13°C with aeration, and samples were withdrawn daily for determination of bacterial cell densities. Numbers of *V. anguillarum* bacteria were estimated by preparing 10-fold serial dilutions using 1 ml from each dilution to inoculate tubes with 5 ml of H&L medium (23). The tubes were covered with paraffin and incubated at 25°C. The fermentative growth of *V. anguillarum* caused a change in the pH indicator of the medium. The highest dilution still showing growth was used to calculate the number of *V. anguillarum* bacteria present. This procedure was chosen because low numbers of *V. anguillarum* bacteria had to be estimated in a high background of *P. fluorescens* AH2, which did not grow in the anaerobic H&L tubes. Inoculation of H&L medium with 10^9 *P. fluorescens* AH2 bacteria did not affect the color of the medium.

Probiotic treatment and infection of fish. *P. fluorescens* AH2 was grown for 6 days at 13°C (150 rpm) in M9GC without NaCl addition, and *V. anguillarum* 90-11-287 was grown for 16 h in tryptone soy broth. A total of 644 rainbow trout (*Oncorhynchus mykiss* Walbaum) weighing approximately 40 g were divided into eight 600-liter tanks, and fish from four of the tanks were exposed for 5 days to *P. fluorescens* AH2 at a level of 10^5 CFU/ml at 12°C (long-term treatment) by adding the bacteria to the water. All fish were exposed to *V. anguillarum* at a level of 10^4 to 10^5 CFU/ml for 1 h in 50% seawater (i.e., a total of 1.5% NaCl) at 12°C. Half of the probiotically treated fish and half of the untreated fish were also treated with *P. fluorescens* AH2 (10^7 CFU/ml) during exposure to *V. anguillarum* (short-term treatment). The *P. fluorescens* AH2 bacteria used for this short-term treatment were cultured at 13°C for 7 days in M9GC with NaCl added. All fish, independently of probiotic treatment, were physically handled in the same manner. After infection, the fish were kept at 12°C in fresh water in eight 600-liter tanks with a water flow of 50 liters/h and fed in accordance with the BioMar A/S Ecolife 19 feeding table by automatic feeders during a 10-h daily feeding period. Dead fish were collected and recorded daily. *V. anguillarum* was isolated from deceased fish by inoculation of head kidney smears on blood agar plates, and its identity was verified by Mono VaR (BioNor Aqua, Skien, Norway) serum agglutination. Data were recorded as accumulated mortality. The calculated accumulated mortality was derived by adding the effect of a particular treatment to the average mortality of all of the eight tanks and subtracting half of the effect of the two individual factors (7) (no significant two-factor interactions between the two treatments were recorded). The statistical significance of the two individual probiotic treatments, as well as that of the combined effect, was calculated by the analysis of variance program in the statistical software package Statgraphics (Statistical Graphics Corporation, Princeton, N.J.).

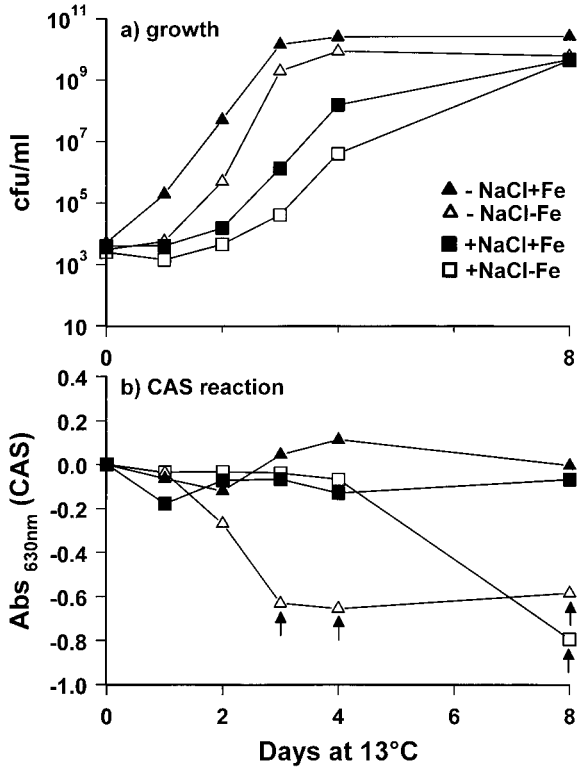

FIG. 1. Growth (a) and CAS reaction (b) of *P. fluorescens* AH2 grown in M9GC with or without 3% NaCl with or without 0.1 mM $FeCl_3$ at 13°C. The arrows indicate sampling points where sterilely filtered supernatants were inhibitory to *V. anguillarum*.

RESULTS

Agar antagonism assay. *P. fluorescens* AH2 caused a clearing zone with a radius of 3 to 5 mm in lawns of *V. anguillarum* in the absence of iron. No inhibition zones were observed when the medium was supplemented with iron.

Effect of *P. fluorescens* AH2 supernatants. Strain AH2 grew well and produced siderophores in Fe-limited M9GC with and without 3% NaCl (Fig. 1). A weak CAS reaction (A_{630}, −0.25) was measured when counts of strain AH2 reached 10^6 cells/ml. A significant CAS reaction (A_{630}, ~−0.6), indicating high levels of siderophores, was not seen until strain AH2 reached 10^9 CFU/ml (Fig. 1). Addition of iron caused more-rapid growth and a slightly higher maximum cell density, whereas 3% NaCl caused a prolonged lag phase and a slightly lower growth rate (Fig. 1). Sterile-filtered strain AH2 supernatants with a strong CAS reaction were inhibitory to *V. anguillarum* (Fig. 1 and 2). In contrast, supernatants from iron-replete cultures of strain AH2 or supernatants obtained from iron-limited cultures at low cell density did not inhibit the growth of the fish pathogen (Fig. 2b). The iron-replete cultures showed no sign of iron limitation, as the CAS reaction remained negative. Addition of iron to inhibitory sterile-filtered supernatants with a strong CAS reaction eliminated the inhibitory effect. Growth curves of *V. anguillarum* in M9GC+NaCl (controls) were identical to curves obtained with supernatants from low counts of strain AH2 that were not CAS positive (data not shown).

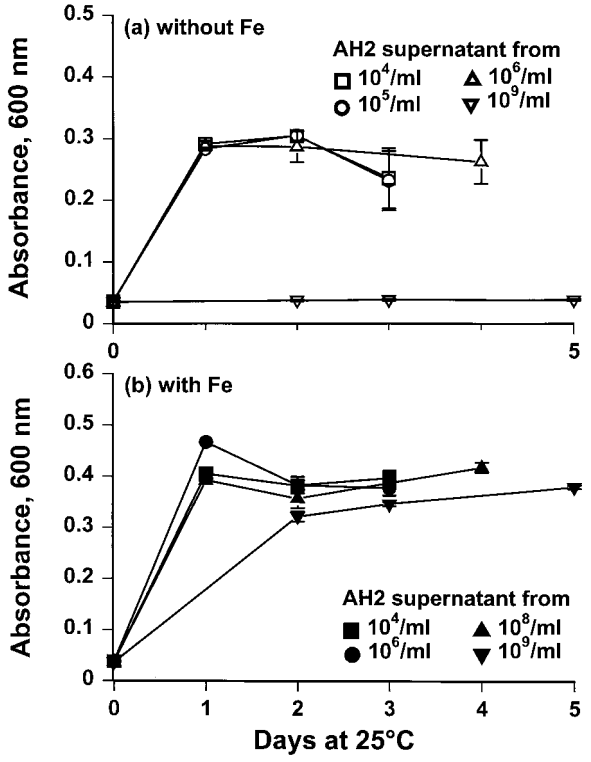

FIG. 2. Growth of *V. anguillarum* at 25°C in M9GC–3% NaCl with spent supernatants from *P. fluorescens* AH2 cultured without (a) or with (b) 100 μM $FeCl_3$.

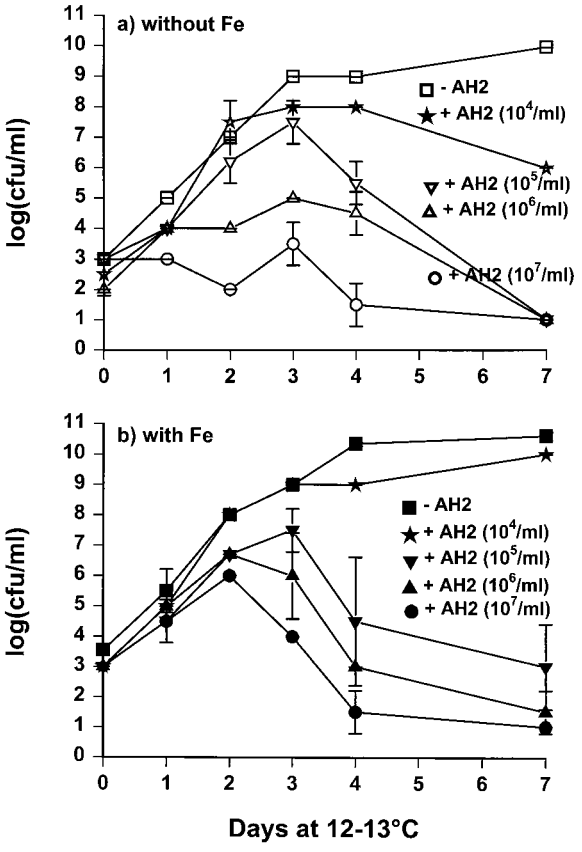

FIG. 3. Growth of *V. anguillarum* at 13°C in M9GC–3% NaCl with and without *P. fluorescens* AH2 at different initial cell densities without (a) or with (b) 100 μM $FeCl_3$.

Coculture experiments. The growth of *V. anguillarum* was inhibited under iron-limited conditions by strain AH2 inoculated at an initial level of 10^6 to 10^7 CFU/ml (Fig. 3a). Lower concentrations of strain AH2 (10^4 to 10^5 CFU/ml) allowed initial growth of *V. anguillarum*, but cell densities never reached the level of the control. High inoculum concentrations (10^5 to 10^7 CFU/ml) of strain AH2 under iron-replete conditions allowed an initial increase of *V. anguillarum* followed by a decrease in the viable count (Fig. 3b). Growth of *P. fluorescens* AH2 was not affected by coculturing with *V. anguillarum* (data not shown).

Probiotic treatment and infection of fish. The accumulated mortality of infected fish not treated with strain AH2 reached 50% 9 days after infection with the onset of mortality at day 3. The mortality leveled off after the 7th day, at which the accumulated mortality was 47% (Fig. 4). No dead fish were found in control tanks not exposed to *V. anguillarum*. Both the long-term and short-term treatments with strain AH2 caused a decrease in accumulated mortality, to 44 and 35%, respectively. Combining the treatments caused a further reduction in accumulated mortality to 32% (Fig. 4). No significant two-factor interactions between the treatments was seen, and the effect of the two treatments combined was therefore regarded as additive (Table 1). The effect of probiotic treatment was most pronounced during the first days of infection, but all probiotic treatments were also significantly different from the infection control when the mortality stabilized (Fig. 4 and Table 1).

DISCUSSION

The concept of biological disease control, particularly using nonpathogenic bacterial strains for disease prevention, has received widespread attention during the last decade. Fuller (13) defined a probiotic as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance." However, as the skin and gill microflora of fish must be assumed also to contribute to disease prevention,

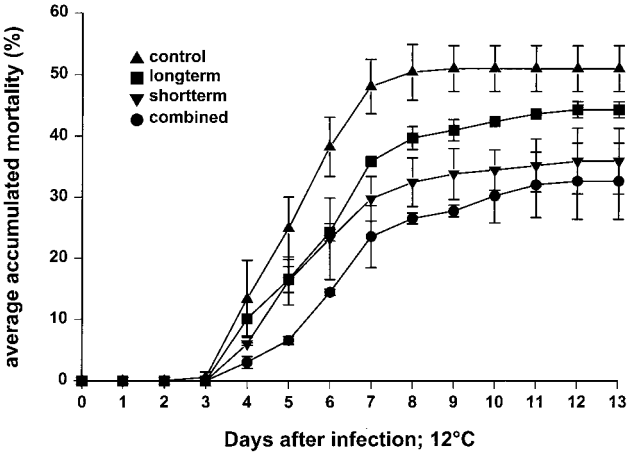

FIG. 4. Accumulated mortality (percentage with two tanks of each treatment) of rainbow trout infected with *V. anguillarum* with and without probiotic treatment with *P. fluorescens* AH2. Long-term treatment was 5 days of exposure to *P. fluorescens* AH2 at 10^5 CFU/ml. Short-term treatment consisted of addition of *P. fluorescens* AH2 at 10^7 CFU/ml during exposure to *V. anguillarum*.

TABLE 1. Calculated average accumulated mortality of rainbow trout after infection with *V. anguillarum* and probiotic treatment with *P. fluorescens* AH2

Day after infection	Calculated accumulated avg % mortality	Effect on relative % survival[a] of:			Standard deviation of effect (%)
		Long-term AH2 treatment	Short-term AH2 treatment	Combined AH2 treatments	
7	46.6	19.7***	32.8***	46.1***	2.7
10	49.8	12.9*	28.7***	37.1***	2.4
13	50.1	10.0	26.5**	33.1***	3.1

[a] Relative percent survival = [1 − (mortality of treated fish)/(mortality of control)] × 100. ***, $P < 0.01$; **, $P < 0.05$; *, $P < 0.10$.

we have used a broader definition here, namely, a live microbial supplement which beneficially affects the host animal by improving its microbial balance. A strain of *P. fluorescens* was successfully used to reduce the frequency of stress-induced infections by *A. salmonicida* (36). Austin et al. (3) reported that exposure to a nonpathogenic *Vibrio alginolyticus* strain reduced subsequent mortality due to vibriosis. To our knowledge, our data are the first to demonstrate a reduction in vibriosis-caused mortality in trout by the use of a probiotic *P. fluorescens* strain.

In agreement with other studies (15, 26, 41), we have seen that under iron-limited conditions, *P. fluorescens* AH2 is inhibitory to *V. anguillarum* when tested in vitro in a well diffusion assay or when sterile-filtered supernatants were tested. Similar to Smith and Davey (36), we found that addition of iron to sterile-filtered supernatants of strain AH2 eliminated the inhibitory activity. The appearance of inhibitory activity in spent supernatants from iron-limited *P. fluorescens* AH2 coincided with a strong CAS reaction, indicating the presence of siderophores. However, our data do not allow us to specifically implicate siderophores in the active mechanism.

Despite the importance of iron limitation seen in the deferred end point assays, iron was not as important during coculture, when inhibition was more a function of the density of the antagonist than of the iron concentration (Fig. 3). Due to its fast growth, *P. fluorescens* AH2 may compete for other nutrients, occupy colonization sites, or excrete antibacterial substances (29, 34). However, such substances, if produced, were not present in sufficient concentrations to allow detection in vitro in supernatants from iron-enriched cultures.

High levels of *P. fluorescens* AH2 were required before inhibition of *V. anguillarum* could be detected in coculture assays. In agreement with earlier studies of interactions between fish spoilage bacteria (17), sterile-filtered supernatant from *P. fluorescens* AH2 did not inhibit the growth of *V. anguillarum* until an antagonist level of 10^8 CFU/ml was reached. A number of studies have assessed the numbers of pseudomonads required to protect against various plant diseases. Xu and Gross (41) found that compared to infection with 10^4 *Erwinia carotovora* bacteria per potato seed, 10^6 antagonist bacteria per potato seed increased emergence 32% and 10^{10} antagonist bacteria per potato seed increased emergence 96%. It has been reported similarly that 10^6 CFU of a fluorescent pseudomonad per seed was required to protect sunflower seeds against *Sclerotinia* wilt, and protection increased with increasing amounts of the pseudomonad (12). Our studies also show that the antagonist must be present at significantly higher levels than the pathogen, and the degree of inhibition increases with the level of the antagonist. Thus, during coculture, 10^7 to 10^9 CFU/ml was required to inhibit the growth of the pathogen (Fig. 3). Therefore, a potential probiotic culture must either be supplied on a regular basis or be able to colonize and multiply on or in the host.

Rhizosphere studies often have great difficulties moving from in vitro to in vivo situations (8). In preliminary in vivo studies with *P. fluorescens* AH2 (data not shown), we found that short-term exposure, even to high numbers of the bacterium, had no effect on subsequent fish mortality. However, with more-constant exposure to AH2, a significant reduction in mortality was obtained (Fig. 4 and Table 1). The fact that the reduction in mortality obtained by the two different treatments was additive indicated that further reduction in mortality may be obtained by optimizing the procedure. Probiotic treatment of fish thus offers a very promising alternative to the use of antibiotics and chemotherapy.

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REFERENCES

1. **Amábile-Cuevas, C. F., M. Gárdenas-Garciá, and M. Ludgar.** 1995. Antibiotic resistance. Am. Sci. **83:**320–329.
2. **Anthoni, U., C. Christophersen, P. H. Nielsen, L. Gram, and B. O. Petersen.** 1995. Pseudomonine, an isoxazole with siderophoric activity from *Pseudomonas fluorescens* AH2, isolated from Lake Victorian Nile perch. J. Nat. Prod. Chem. **58:**1786–1789.
3. **Austin, B., L. F. Stuckey, P. A. W. Robertson, I. Effendi, and D. R. W. Griffith.** 1995. A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. J. Fish Dis. **18:**93–96.
4. **Aznar, R., and E. Alcaide.** 1992. Siderophores and related outer membrane proteins produced by pseudomonads isolated from eels and freshwater. FEMS Microbiol. Lett. **98:**269–276.
5. **Bertani, G.** 1951. Studies of lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J. Bacteriol. **62:**293–300.
6. **Bly, J. E., S. M.-A. Quiniou, L. A. Lawson, and L. W. Clem.** 1997. Inhibition of *Saprolegnia* pathogenic for fish by *Pseudomonas fluorescens*. J. Fish Dis. **20:**35–40.
7. **Box, G. E. P., W. G. Hunter, and J. S. Hunter.** 1978. Statistics for experimenters. John Wiley & Sons, Inc., New York, N.Y.
8. **Buyer, J. S.** 1994. Siderophore production by *Pseudomonas* B10 in the rhizosphere, abstr. N188, p. 349. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology 1994. American Society for Microbiology, Washington, D.C.
9. **Cahill, M. M.** 1990. Bacterial flora of fishes: a review. Microb. Ecol. **19:**21–41.
10. **Crosa, J. H.** 1980. A plasmid associated with virulence in the marine fish pathogen *Vibrio anguillarum* specifies an iron-sequestering system. Nature **283:**566–568.
11. **Csavas, I.** 1994. World aquaculture status and outlook. INFOFISH Int. **5:**47–54.
12. **Expert, J. M., and B. Digat.** 1995. Biocontrol of *Sclerotinia* wilt of sunflower by *Pseudomonas fluorescens* and *Pseudomonas putida* strains. Can. J. Microbiol. **41:**685–691.
13. **Fuller, R.** 1989. A review. Probiotics in man and animals. J. Appl. Bacteriol. **66:**365–378.
14. **Gatesoupe, F.-J.** 1994. Lactic acid bacteria increase the resistance of turbot larvae, *Scophthalmus maximus*, against pathogenic vibrio. Aquat. Living Resour. **7:**277–282.

15. **Gram, L.** 1993. Inhibitory effect against pathogenic and spoilage bacteria of *Pseudomonas* strains isolated from spoiled and fresh fish. Appl. Environ. Microbiol. **59:**2197–2203.
16. **Gram, L.** 1996. The influence of substrate on siderophore production by fish spoilage bacteria. J. Microbiol. Methods **25:**199–205.
17. **Gram, L., and J. Melchiorsen.** 1996. Interaction between *Pseudomonas* spp. and *Shewanella putrefaciens* in fish model systems. J. Appl. Bacteriol. **80:**589–595.
18. **Gram, L., C. Wedell-Neergaard, and H. H. Huss.** 1990. The bacteriology of fresh and spoiling Lake Victorian Nile perch (*Lates niloticus*). Int. J. Food Microbiol. **10:**303–316.
19. **Griffith, E.** 1986. Iron and biological defence mechanisms, p. 56–71. *In* G. W. Gould, A. K. Charnley, M. E. Rhodes-Roberts, R. M. Cooper, and R. G. Board (ed.), Natural antimicrobial systems. Part 1. Antimicrobial systems in plants and animals. Bath University Press, Bath, United Kingdom.
20. **Hatai, K., and L. G. Willoughby.** 1988. *Saprolegnia parasitica* from rainbow trout inhibited by the bacterium *Pseudomonas fluorescens*. Bull. Eur. Assn. Fish Pathol. **8:**27–29.
21. **Hirst, I. D., and A. E. Ellis.** 1994. Iron-regulated outer membrane proteins of *Aeromonas salmonicida* are important protective antigens in Atlantic salmon against furunculosis. Fish Shellfish Immunol. **4:**29–45.
22. **Hjul, P.** 1997. The outlook for world aquaculture. INFOFISH Int. **1:**27–30.
23. **Hugh, R., and E. Leifson.** 1953. The taxonomic significance of fermentative versus oxidative gram-negative bacteria. J. Bacteriol. **66:**24–26.
24. **Jöborn, A., C. J. Olsson, and S. Kjelleberg.** 1997. Colonization in the fish intestinal tract and production of inhibitory substances in intestinal mucus and faecal extracts by *Carnobacterium* sp. strain K1. J. Fish Dis. **20:**383–392.
24a. **Jørgensen, S. S. (Royal Veterinary and Agricultural University, Copenhagen, Denmark).** Personal communication.
25. **Kjelleberg, S., P. Conway, R. Hammond, A. Jöborn, C. Olsson, and A. Westerdahl.** 1997. Protection against pathogenic microorganisms. International patent application no. PCT/GB96/01936, international publication no. WO 97/06811.
26. **Kloepper, J. W., J. Leong, M. Teintze, and M. N. Schroth.** 1980. Enhanced plant growth by siderophores produced by plant growth promoting rhizobacteria. Nature **286:**885–886.
27. **Lemos, M. L., A. E. Toranzo, and J. L. Barja.** 1985. Antibiotic activity of epiphytic bacteria isolated from intertidal seaweeds. Microb. Ecol. **11:**149–163.
28. **Loper, J. E., and J. S. Buyer.** 1991. Siderophores in microbial interactions on plant surfaces. Mol. Plant-Microbe Interact. **4:**5–13.
29. **Mazzola, M., R. J. Cook, L. Thomashow, D. M. Weller, and L. S. Pierson III.** 1992. Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. Appl. Environ. Microbiol. **58:**2616–2624.
30. **Nakai, T., T. Kanno, E. R. Cruz, and K. Muroga.** 1987. The effects of iron compounds on the virulence of *Vibrio anguillarum* in Japanese eels and ayu. Fish Pathol. **22:**185–189.
31. **O'Sullivan, D. J., and F. O'Gara.** 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. Microbiol. Rev. **56:**662–676.
32. **Sambrook, J., E. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
33. **Schwyn, B., and J. B. Neilands.** 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. **160:**47–56.
34. **Shanahan, P., D. J. O'Sullivan, P. Simpson, J. D. Glennon, and F. O'Gara.** 1992. Isolation of 2,4-diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters influencing its production. Appl. Environ. Microbiol. **58:**353–358.
35. **Skov, M. N., K. Pedersen, and J. L. Larsen.** 1995. Comparison of pulsed-field gel electrophoresis, ribotyping, and plasmid profiling for typing of *Vibrio anguillarum* serovar O1. Appl. Environ. Microbiol. **61:**1540–1545.
36. **Smith, P., and S. Davey.** 1993. Evidence for the competitive exclusion of *Aeromonas salmonicida* from fish with stress-inducible furunculosis by a fluorescent pseudomonad. J. Fish Dis. **16:**521–524.
37. **Strøm, E., and E. Ringø.** 1993. Changes in the bacterial composition of early developing cod, *Gadus morhua* (L.), larvae following inoculation of *Lactobacillus plantarum* into the water, p. 226–228. *In* B. T. Walther and H. J. Pyhn (ed.), Physiological and biochemical aspects of fish development. University of Bergen, Bergen, Norway.
38. **Voisard, C., C. Keel, D. Haas, and G. Defago.** 1989. Cyanide production by *Pseudomonas fluorescens* helps suppress black root rot of tobacco under gnotobiotic conditions. EMBO J. **8:**351–358.
39. **Westerdahl, A., J. C. Olsson, S. Kjelleberg, and P. L. Conway.** 1991. Isolation and characterization of turbot (*Scophthalmus maximus*)-associated bacteria with inhibitory effects against *Vibrio anguillarum*. Appl. Environ. Microbiol. **57:**2223–2228.
40. **Wooldridge, K. G., and P. H. Williams.** 1993. Iron uptake mechanisms of pathogenic bacteria. FEMS Microbiol. Rev. **12:**325–348.
41. **Xu, G.-W., and D. C. Gross.** 1986. Selection of fluorescent pseudomonads antagonistic to *Erwinia carotovora* and suppressive of potato seed piece decay. Phytopathology **76:**414–422.