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Antagonistic activity of cellular components of *Pseudomonas* species against *Aeromonas hydrophila*

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Abstract

Antagonistic effects of *Pseudomonas fluorescens*, *P. aeruginosa* and *P. putida* were studied against 12 strains of *Aeromonas hydrophila* (Ah1–Ah12). Four different fractions of cellular component (i.e. whole cell product, heat killed whole cell product, intra cellular product and extra cellular product) of all *Pseudomonas* species were equally effective in reducing growth of *A. hydrophila* strains, as measured by the zone of inhibition in an in vitro sensitivity test and have potential action against *A. hydrophila* infection in fishes.

Keywords: Biocontrol; *Pseudomonas* spp.; *Aeromonas hydrophila*

1. Introduction

Control of microbial communities with high species diversity in nature has been regarded as difficult (Maeda, 1994). Such types of microbial communities can disperse the effect caused by the inversion or addition of certain extrinsic pathogenic organisms. In addition, microorganisms can be sources of a variety of bioactive natural products of basic research and commercial interest that have inhibitory effect on microbial growth (Reichelt and Borowitska, 1984; Cannell et al., 1988). In aquaculture practices bacterial infectious diseases result in losses worldwide and to prevent them antimicrobial compounds are used intensively. In the process of antibiotic treatment, drug resistance patterns can develop within the pathogenic microbial community. Therefore, alternatives to the use of antibiotics are gaining importance in many countries (Nogami and Maeda, 1992; Sugita et al., 1998; Gatesoupe, 1999; Bala Reddy, 2001). Accordingly, the application of microbial communities in aquaculture for controlling pathogenic bacteria shows promise.

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Most of the antibiotics used today are of microbial origin. In medicine, problem with pathogenic microbes becoming increasingly resistant against most commonly used antibiotics is being experienced (Cowen, 2001; Lipsitch, 2001). Then some derivatives of the antibiotics circumvent or even prevent rapid mutation to resistance of the pathogens (Hughes, 2003). The *Pseudomonas* species as a fungicide to protect the food from toxic fungi such as penicillium and botrytis. The protein patterns of the different strains of *Staphyloccus aureus* and *Lactobacillus sanfranciscensis* are resistant to specific antibiotics (De Angelis et al., 2001; Cordwell et al., 2002; Hecker et al., 2003).

A similar approach was also undertaken in the culture of Phyllosoma larvae of spiny lobster *Jasus edwardsii* (Igarashi et al., 1990). It has been recorded that antimicrobial substances produced by some bacteria seem to play an important role in antagonising other bacteria in aquatic ecosystems (Dopazo et al., 1988; Sugahara et al., 1988). It has also been reported that intestinal bacteria from turbot show an inhibitory effect on fish pathogenic bacteria (Westerdahl et al., 1991; Olsson et al., 1992; Sugita et al., 1997). As these bacteria are natural inhabitants of the aquatic system, they could persist for a relatively longer period and might inhibit establishment of fish pathogens in the aquatic environment as well as in the fish host. Therefore, this preliminary in vitro study was undertaken to examine the antibacterial properties of *Pseudomonas fluorescens*, *P. aeruginosa* and *P. putida* against the fish pathogenic bacteria *Aeromonas hydrophila* for the first time.

2. Materials and methods

2.1. Isolation of bacteria

Diseased fishes showing various lesions on the skin and in the musculature, and healthy fishes were used for isolation of bacteria. Pseudomonas isolation agar (Hi media, India) was used for isolation of pure cultures of *Pseudomonas* species. *P. fluorescens* (PF1) was isolated from the liver of goldfish (*Carassius auratus*), *P. aeruginosa* (PA1) was isolated from the intestine of climbing perch (*Anabas testudineus*) and *P. putida* (PP1) was isolated from skin lesions of rohu (*Labeo rohita*). The 12 strains of *A. hydrophila* isolated from skin lesion, liver, kidney and intestine of mrigal, cat fish, goldfish and murrels were taken to study the antagonistic activity against different cellular components of *Pseudomonas* species. The different species of *Pseudomonas* and *A. hydroplhila* strains were selected and characterized based on their biochemical properties (Holt and Krieg, 1984). Dominant isolates were purified and identified using conventional biochemical tests described by Mac Fadden (1976) and West and Colwell (1984). *A. hydrophila* (Ah1–Ah12) strains isolated from different organs such as liver and kidney, as well as swabs of skin lesions of various freshwater fishes maintained in the laboratory were also used for the present study. *A. hydrophila* after 24 h of incubation in nutrient broth was streaked on Rimler-Shott's medium (Hi media, India) for purity. Twelve strains of *A. hydrophila* were used for the antagonistic study against heat killed whole cell product (HK WCP), whole cell product (WCP), intra cellular product (ICP) and extra cellular product (ECP) of the *Pseudomonas* species.

2.2. Preparation of different cellular components

Four types of antigenic components, e.g. heat killed whole cell product (HK WCP), whole cell product (WCP), intra cellular product (ICP) and extra cellular product (ECP) were prepared from *P. fluorescens*, *P. aeruginosa* and *P. putida*. Briefly pure cultures of all the bacteria (PF1, PA1 and PP1) were grown separately in sterile conditions in 250 ml of brain heart infusion broth (Hi media, India) at 30 °C for 24 h. Pure cultures of each bacterium were divided into five equal volumes of 50 ml and each 50 ml was taken for preparation of HK WCP, WCP, ECP and ICP separately. The optical density (OD) of 24 h old cultures was taken for each bacterium and simultaneous plating was carried out in triplicate and the colony forming unit/ml was calculated. The optical density at 546 nm of *P. fluorescens* was 0.836 which corresponded to 1.9×10^9 cfu/ml, whereas an OD of *P. aeruginosa* 0.742 corresponded to 1.2×10^9 cfu/ml and an OD of *P. putida*, 0.738 corresponded to 2.1×10^9 cfu/ml respectively. The protein estimation of all the protein fraction of *Pseudomonas* species was according to Bradford (1976). The minimum amount of protein content (70 μl) of the lowest protein

concentration of *Pseudomonas* species was taken as standard and accordingly the other protein fractions of other *Pseudomonas* species were calculated and charged in nutrient agar plates.

2.3. Whole cell product (WCP)

All the bacteria (*P. fluorescens*, *P. aeruginosa* and *P. putida*) grown separately in brain heart infusion broth were harvested after 24 h incubation and centrifuged at $10{,}000 \times g$ for 10 min at 4 °C. The bacterial pellet was washed twice and resuspended in phosphate buffered saline (PBS) (pH 7.2) and used for the antagonistic study.

2.4. Extra cellular product (ECP)

The supernatants obtained after centrifuging 24 h old cultures of bacteria in brain heart infusion broth were filtered (0.22 μl). They were further concentrated with 20% PEG 6000, dialysed against PBS (pH 7.2) and used as ECP.

2.5. Heat killed whole cell product

Pseudomonas cultures grown in bulk were harvested by centrifugation at $10{,}000 \times g$ for 10 min at 4 °C. The bacterial pellet was washed twice and resuspended in phosphate buffered saline (pH 7.2). They were heat killed at 60 °C for 1 h in a water bath and finally stored at −20 °C.

2.6. Intra cellular product (ICP)

All the bacteria (*P. fluorescens*, *P. aeruginosa* and *P. putida*) were grown separately in brain heart infusion broth and centrifuged at $10{,}000 \times g$ for 10 min at 4 °C. The bacterial pellet was washed twice and re-suspended in phosphate buffered saline (pH 7.2), to 2% of the initial volume. The cell pellets were then sonicated at 50 Hz for 5 min (Artelk Sonic Dismembrator model 150) filtered through a syringe with a 0.45 μl filter and finally stored at −20 °C until further use.

2.7. Biocontrol study

Overnight growth cultures of different strains of *A. hydrophila* were incubated on Tryptone Soya agar (Hi media, India) plates separately by the lawn culture method. Then 6 mm diameter wells were made in each plate with the help of a well puncture (Hi media, India) and 70 μl of whole cell product (WCP) corresponding to 2.1×10^9 cfu/ml, extracellular product (ECP) corresponding to 1.12×10^8 cfu/ml, heat killed WCP corresponding to 1.3×10^8 cfu/ml and intra cellular product (ICP) corresponding to 1.7×10^8 cfu/ml were charged in the respective wells of different *A. hydrophila* plates and incubated at 37 °C for 24 h. The zone of inhibition around the charged well was recorded after incubation. Simultaneously, control samples were maintained with sterile phosphate buffer saline (pH 7.2) placed into the respective wells prepared as mentioned above. In all the cases, the triplicate plates were maintained along with the PBS control for biocontrol study.

Selected antibiotics, Chloramphenicol (30 mcg), nalidixic acid (30 mcg), furazolidone (50 mcg), norfloxacin (30 mcg), oxytetracycline (30 mcg), ciprofloxacin (15 mcg), gentamycin (15 mcg) and nitrofurantoin (10 mcg) were tested in a diagnostic sensitivity medium (Hi media, India) against different *A. hydrophila* strains to compare the effectiveness of different cellular components of *Pseudomonas* species.

A required amount of diagnostic sensitivity medium (DST) (Hi media, India) was prepared. Sterile petriplates (98 mm diameter) were poured with 20 ml DST medium and allowed to solidify at room temperature. *A. hydrophila* cultures were inoculated by the lawn culture method. Then the above antibiotics were placed in different cultured plates and incubated at 37 °C for 24 h. After incubation the observations were noted. The zones of inhibition of each product were compared with the recommended zone of inhibition of the antibiotics against the cultures. The zones of inhibition of the antibiotics against the *A. hydrophila* strains were treated as positive control.

2.8. Statistical analysis

All the data were analyzed by running the general linear model program available in SAS software. The means were compared using Duncan's multiple range test (Duncan, 1955) to find the difference at 5% ($P < 0.05$) level.

3. Results

3.1. Protein estimation of different cellular components of Pseudomonas species

The protein contents of ICP, WCP, HK WCP and ECP of *P. fluorescens* were 5.13, 4.91, 4.85 and 4.75 mg/ml respectively. Similarly, the protein contents of ICP, WCP, HK WCP and ECP of *P. aeruginosa* and *P. putida* were 6.21, 5.97, 5.73, 5.63, 5.72, 5.31, 4.85 and 4.72 mg/ml respectively. The ICP of *P. aeruginosa* contain highest protein concentration (6.21 mg/ml) and lowest protein content in ECP of *P. putida* (4.72 mg/ml). The minimum quantity of protein fraction (70 μl) charged on nutrient agar plate against *A. hydrophila* strains of *P. putida* ECP was found to be 340 μg. Equal protein concentration per mililitre of each cellular components of the bacteria was adjusted to 340 μg corresponded to 70 μl.

3.2. Biocontrol study using P. fluorescens (PF1)

Results obtained from the WCP, HK WCP, ECP and ICP of PF1 against *A. hydrophila* are shown in Fig. 1. It was revealed that the ECP of PF1 was found to be highly effective and produced a zone ranging from 60 to 85 mm against Ah1 to Ah12 strains (Fig. 1). The highest recorded zone was observed with Ah10 isolated from skin lesions of *Channa marulius* followed by Ah6 (81.33 ± 2.60 mm). ECP of PF1 against Ah11 produced a mean zone size of 72.33 ± 0.88 mm. Similarly, WCP, HK WCP and ICP of PF1 were equally effective and had a bactericidal effect against all strains of *A. hydrophila* (Fig. 1). Out of the four product components of PF1, ECP produced the highest activity in almost all cases, except for Ah9. WCP of PF1 was also equally effective as the ECP and the inhibitory activity was highest in Ah1, Ah3, Ah6, Ah7, Ah8, Ah9 when compared to ICP and HK WCP (Fig. 1) as compared to PBS control (0 mm). Significant differences ($P \leq 0.05$) of inhibitory effects were recorded in all the cases as compared to positive control.

3.3. Antagonistic study using P. putida (PP1)

The zones of inhibition produced by PP1 against Ah1 to Ah12 are shown in Fig. 2. The zone size of heat killed WCP of PP1 was highly effective and produced zones ranging from 52 to 71 mm. The

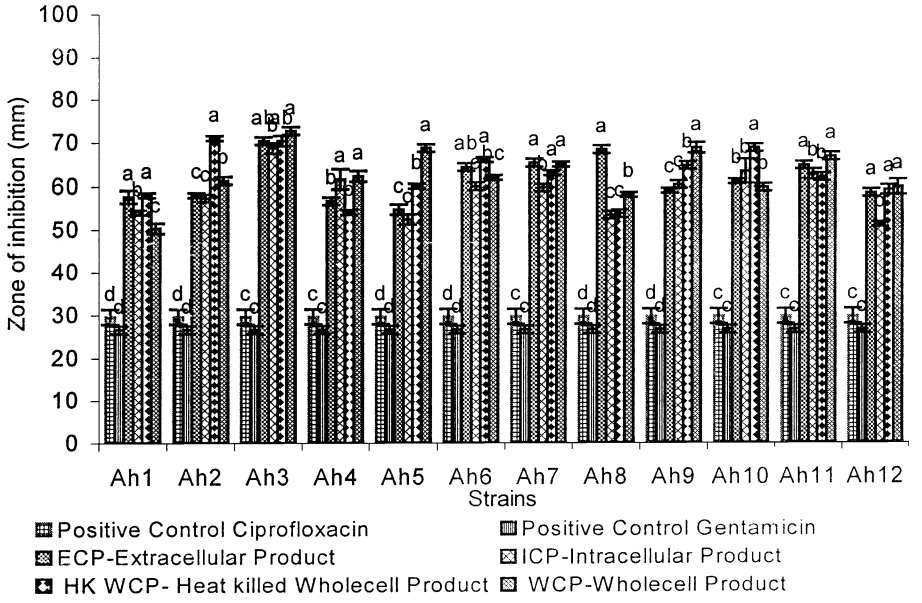

Fig. 1. Zones of inhibition of four crude fractions of *P. fluorescens* against *A. hydrophila* strains. (Values are represented as mean ± S.E., mean bearing common superscript are not significant to each other.)

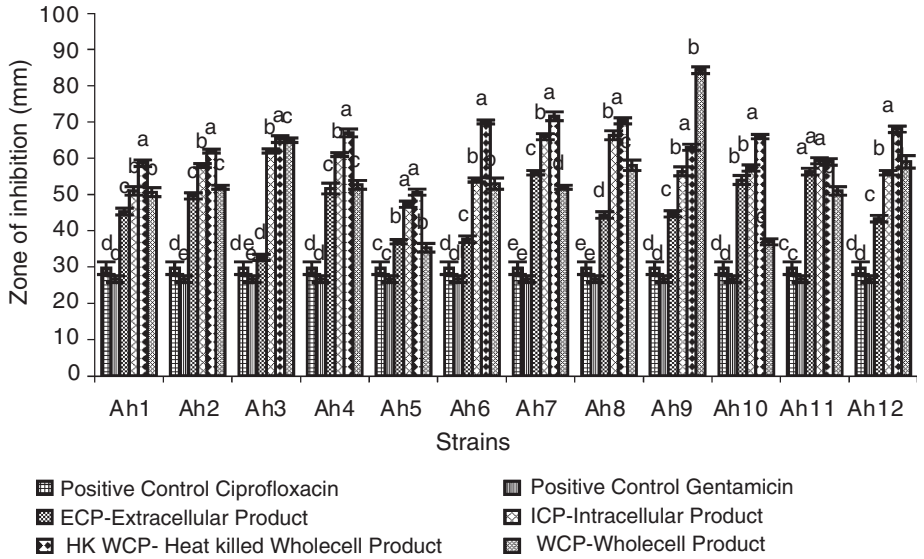

Fig. 2. Zones of inhibition of four crude fractions of *P. putida* against *A. hydrophila* strains. (Values are represented as mean ± S.E., mean bearing common superscript are not significant to each other.)

overall activity of HK WCP was the highest among the four product fractions tested and found significantly different ($P \leq 0.05$) as compared to the positive control. Whereas WCP of PP1 produced zone inhibition ranging from 38 to 55 mm; ICP of PP1 produced zones ranging from 46 to 68 mm and ECP of PP1 produced a zone size from 34 to 56 mm. The least bactericidal activity was recorded in the case of ECP of PP1 against Ah3 and the highest recorded bactericidal activity of PP1 against Ah6 indicating the effectivity of WCP of PP1 against Ah1 as compared to PBS control (0 mm). All the four cellular components showed significant ($P \leq 0.05$) inhibitory activity against 12 strains of *A. hydrophila* (Fig. 2) when compared to the positive control.

3.4. Antagonistic study of P. aeruginosa (PA1)

The four cellular components of PA1 were tested as antagonistic agents/components against *A. hydrophila* (Fig. 3). All the four fractions produced antagonism as measured by the zone of inhibition greater than 52 mm and were found significantly different ($P \leq 0.05$) as compared with the positive control. WCP of PA1 produced highest antagonism against Ah3 (72.66 ± 0.88 mm) followed by HK WCP against Ah3 (70.33 ± 1.20 mm) and Ah2 (71.00 ± 0.57 mm). WCP of PA1 produced a zone of 62.00 ± 0.57 mm against Ah6 and ICP of PA1 produced a zone of 57.33 ± 0.88 mm against the Ah2 strain as compared to the PBS control (0 mm).

3.5. A. hydrophila

A. hydrophila strains as Ah2, Ah3, Ah4, Ah5, Ah6, Ah10, Ah11 and Ah12 against *P. fluorescens* showed significant ($P < 0.05$) difference in ECP compared to other extracts as well as control; Ah7 and Ah8 did not show any significant difference among the extracts tested. *A. hydrophila* strain (Ah9) showed significantly highest zone of inhibition of WCP compared to control and other extracts. The extracts obtained from *P. putida* showed significantly ($P < 0.05$) highest zone of inhibition in HK WCP extract against *A. hydrophila* strains compared to control and other extract tested. Whereas, WCP of *P. putida* showed significantly highest zone of inhibition in Ah9 strains of *A. hydrophila*. The extracts obtained from *P. aeruginosa* showed significantly highest zone of inhibition in ECP against *A. hydrophila* strains compared to control and other extracts tested. Where as, WCP of *P. aeruginosa* showed significantly highest zone of inhibition in Ah3, Ah5 and Ah9 strains of *A. hydrophila* respectively.

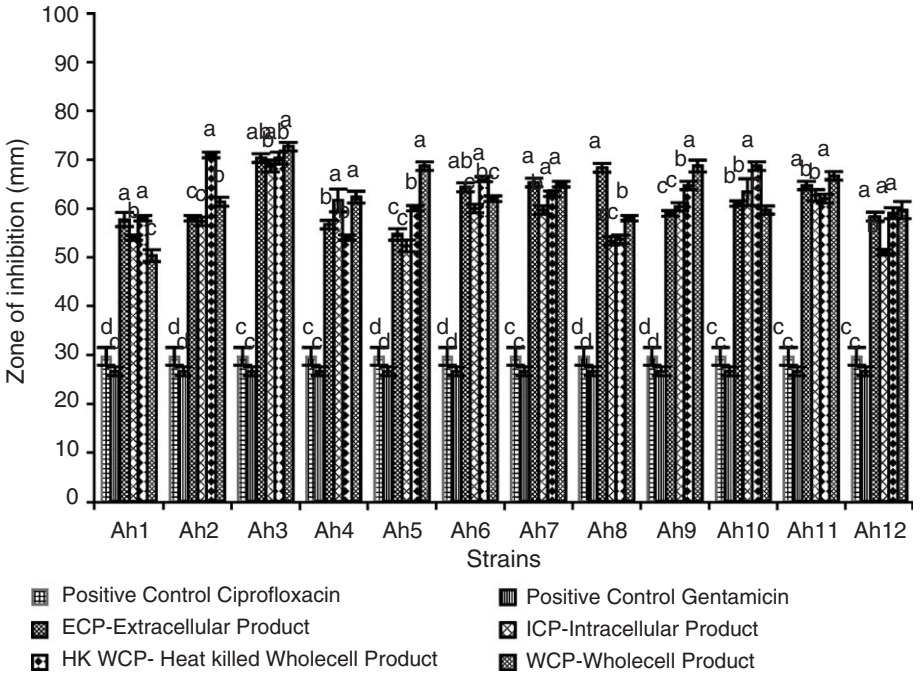

Fig. 3. Zones of inhibition of four crude fractions of *P. aeruginosa* against *A. hydrophila* strains. (Values are represented as mean ± S.E., mean bearing common superscript are not significant to each other.)

3.6. Drug sensitivity test

The tested drugs used in the present experiment to compare the zone of inhibition with that of the four products of PF1, PP1 and PA1; produced zones ranging from 21 to 33 mm against Ah1–Ah12, which was much lower, when compared to the four product fractions of PF1, PP1 and PA1.

4. Discussion

The antibacterial effect of bacteria is generally due to either individual or joint production of antibiotics, bacteriocins, siderophores (Gram and Melchiorsen, 1996), lysozymes and proteases and alteration of pH by organic acid production (Sugita et al., 1998). The inhibition due to such compounds is highly dependant on the experimental conditions, which are different in vitro and in vivo (Gatesoupe, 1999). Sugita et al. (1998) isolated a strain of *Bacillus* species that was antagonistic to 63% of the isolates from fish intestine. According to these authors, most pathogenic strains of *Vibrio* or *Aeromonas* have been targeted in vitro tests. Other workers have also attempted to use other pathogens, e.g. *Edwardsiella tarda*, *Enterococcus seriolicida*, *Pasteurella piscicida*, *Yersinia ruckeri* (Dopazo et al., 1988; Austin et al., 1995; Ruiz et al., 1996; Sugita et al., 1996; Byun et al., 1997; Gibson et al., 1998). Some bacteria are antagonistic to viruses (Kamei et al., 1987, 1988; Direkbusarakom et al., 1998) and they may be efficient for biocontrol of viral diseases (Maeda et al., 1997). Antagonism of aquatic microbes to fish and shellfish pathogens has been reviewed by Gatesoupe (1999). The antagonistic properties of *P. fluorescens* to *V. anguillarum* in *Lates niloticus* (Gram et al., 1999) and *P. fluorescens* to *A. salmonicida* in *Salmo trutta* (Smith and Davey, 1993) have been reported and this inhibition is probably due to competition for free iron. In the present study, *P. fluorescens*, *P. putida* and *P. aeruginosa* however were antagonistic to *A. hydrophila* strains tested in the present study which were isolated from various disease conditions. The whole cell killed antigens, ECP, ICP and live bacteria of all the species were equally effective, as revealed by the zone of inhibition

to all the tested strains of *A. hydrophila*. It was found that the antibiotics such as nalidixic acid, furazolidone, norfloxacin, oxytetracycline, ciprofloxacin, gentamycin and nitrofurantoin produced a zone size, which was much smaller than that of four cellular components of each bacterium tested.

Due to the increase in therapeutic resistance to the more commonly used antibiotics (Hjeltnes et al., 1987; Aoki, 1992; Nash et al., 1992) and its potential antipathogenic actions (by its intra and/or extra cellular products or whole cell products), there appears to be a potential for *Pseudomonas* spp. in the control of aquaculture diseases in fishes, particularly aeromoniasis. The inhibition caused by the Pseudomonad subcellular components is equally effective against the *A. hydrophila* strains. However, further assays are necessary especially in vivo, to forecast the concentration of ICP, ECP, WCP or live bacteria needed to antagonize or act as a biocontrol against *A. hydrophila* infection in fish. Hence research is urgently required on the antipathogenic metabolites excreted by Pseudomonads particularly *P. fluorescens*, *P. putida* and *P. aeruginosa*, which could potentially be used as substitutes to antibiotics.

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References

Aoki, T., 1992. Chemotherapy and drug resistance in fish farms in Japan. In: Shariff, M., Subhasinghe, R.P., Arthur, J.R. (Eds.), Diseases in Asian aquaculture: 1. Proceeding of the First Symposium on Diseases in Asian Aquaculture, 1992, 26–29 November 1990, Bali, Indonesia, pp. 519–529.

Austin, B., Stuckey, L.F., Robertson, P.A.W., Effendi, I., Griffith, D.R.W., 1995. A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas hydrophila, Vibrio angullarum* and *Vibrio ordalii*. J. Fish Dis. 18, 93–96.

Bala Reddy, B., 2001. Biofilm associated bacterial flora of a freshwater habitat. M. F. Sc. thesis. Central Institute of Fisheries Education, Mumbai, India, pp. 1–80.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle dye binding. Anal. Biochem. 72, 248–254.

Byun, J.W., Park, S.C., Benno, Y., Oh, T.K., 1997. Probiotic effect of *Lactobacillus* sp. DS-12 in flounder (*Paralichthys olivaceus*). J. Gen. Appl. Microbiol. 43, 305–308.

Cannell, R.J.P., Owsianka, A.M., Walker, J.M., 1988. Results of a large scale screening programme to detect antibacterial activity from freshwater algae. Br. Phycol. J. 23, 41–44.

Cordwell, S.J., Larsen, M.R., Cole, R.T., Walsh, B.J., 2002. Comparative proteomics of *Staphylococcus aureus* and the response of methicillin-resistant and methicillin-sensitive strains to Triton X-100. Microbiology 148, 2765–2781.

Cowen, L.E., 2001. Predicting the emergence of resistance to antifugal drugs. FEMS Microbiol. Lett. 204, 1–7.

De Angelis, M., Bini, L., Pallini, V., Cocconcelli, P.S., Gobbetti, M., 2001. The acid-stress response in *Lactobacillus sanfranciscensis* CBI. Microbiology 147, 1863–1873.

Direkbusarakom, S., Yoshimizu, M., Ezura, Y., Ruangpan, L., Danayadol, Y., 1998. *Vibrio* spp., the dominant flora in shrimp hatchery against some fish pathogenic viruses. J. Mar. Biotechnol. 6, 266–267.

Dopazo, C.P., Lemos, M.L., Lodeiros, C., Bolinches, J., Barja, J.L., Toranzo, A.E., 1988. Inhibitory activity of antibiotic-producing marine bacteria against fish pathogens. J. Appl. Bacteriol. 65, 97–101.

Duncan, D.B., 1955. Multiple range and multiple 'F' tests. Biometrics 11, 1–42.

Gatesoupe, F.J., 1999. The use of probiotics in aquaculture. Aquaculture 180, 147–165.

Gibson, L.F., Woodworth, J., George, A.M., 1998. Probiotic activity of *Aeromonas media* on the pacific oyster, *Crassostrea gigas*, when challenged with *Vibrio tubiashii*. Aquaculture 169, 111–120.

Gram, L., Melchiorsen, J., 1996. Interaction between fish spoilage bacteria *Pseudomonas* sp. and *Shewanella putrefaciens* in fish extracts and on fish tissue. J. Appl. Bacteriol. 80, 589–595.

Gram, L., Melchiorsen, J., Spanggaard, B., Huber, I., Nielsen, T.F., 1999. Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a possible probiotic treatment of fish. Appl. Environ. Microbiol. 65, 969–973.

Hecker, M., Engelmann, S., Cordwell, S.J., 2003. Proteomics of *Staphylococcus aureus* current state and future challenges. J. Chromat. B. Analty. Tech. Biom. Life. Sci. 787, 179–195.

Hjeltnes, B., Andersen, K., Egidius, E., 1987. Multiple antibiotic resistances in *Vibrio salmonicida*. Bull. Eur. Assoc. Fish Pathol. 7, 85–89.

Holt, J.G., Krieg, N.R., 1984. Bergey's Manual of Systematic Bacteriology vol. 1. Williams and Wilkins, Baltimore, USA.

Hughes, D., 2003. Exploiting genomics, genetics and chemistry to combat antibiotic resistance. Nat. Rev., Genet. 4, 432–441.

Igarashi, M.A., Kittaka, J., Kawahara, E., 1990. Phyllosoma culture with inoculation of marine bacteria. Nippon Suisan Gakkaishi 56 (11), 1781–1786.

Kamei, Y., Yoshimizu, M., Ezura, Y., Kimura, T., 1987. Screening of bacteria with antiviral activity against infectious hemato-

poietic necrosis virus (IHNV) from estuarine and marine environments. Bull. Jpn. Soc. Sci. Fish. 53, 2179–2185.
Kamei, Y., Yoshimizu, M., Ezura, Y., Kimura, T., 1988. Screening of bacteria with antiviral activity from freshwater salmonid hatcheries. Microbiol. Immunol. 32, 67–73.
Lipsitch, M., 2001. The rise and fall of antimicrobial resistance. Trends Microbiol. 9, 438–444.
Mac Fadden, J.F., 1976. Biochemical Tests for the Identification of Medical Bacteria. Williams and Wikens Co, Baltimore. 310 pp.
Maeda, M., 1994. Biocontrol of the larval rearing biotope in aquaculture. Bull. Natl. Res. Inst. Aquacult., Suppl. 1, 71–74.
Maeda, M., Nogami, K., Kanematsu, M., Hirayama, K., 1997. The concept of biological control methods in aquaculture. Hydrobiologia 358, 285–290.
Nash, G., Nithimathachoke, C., Tungmandi, C., Arkarjamorn, A., 1992. Vibriosis and its control in pond reared *Penaeus monodon* in Thailand. In: Shariff, M., Subasinghe, R.P., Arthur, J.R. (Eds.), Diseases in Asian Aquaculture: 1. Proceeding of the First Symposium on Disease in Asian Aquaculture, 26–29 November 1990, Bali. Indonesia, pp. 143–155.
Nogami, K., Maeda, M., 1992. Bacteria as biocontrol agents for rearing larvae of the crab, *Portunus trituberculatus*. Can. J. Fish. Aquat. Sci. 49, 2373–2376.
Olsson, J.C., Westerdahl, A., Conway, P.L., Kjelleberg, S., 1992. Intestinal colonization potential of turbot (*Acophthalmus maximus*) and dab (*Limanda limanda*) associated bacteria with inhibitory effects against *Vibrio anguillarum*. Appl. Environ. Microbiol. 58, 551–556.
Reichelt, J.L., Borowitska, M.A., 1984. Antimicrobial activity from marine algae: results of a large-scale screening programme. Hydrobiologia 116, 158–168.
Ruiz, C.M., Roman, G., Sanchez, J.L., 1996. A marine bacterial strain effective in producing antagonisms of other bacteria. Aquac. Int. 4, 289–291.
Smith, P., Davey, S., 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.
Sugahara, I., Kimura, T., Hayashi, K., Nakajima, I., 1988. Distribution and generic composition of lytic enzyme-producing bacteria in coastal and oceanic bottom sediments. Nippon Suisan Gakkaishi 54 (6), 1011–1015.
Sugita, H., Matsuo, N., Shibuya, K., Deguchi, Y., 1996. Production of the antibacterial substances by intestinal bacteria isolated from coastal crab and fish species. J. Mar. Biotechnol. 4, 220–223.
Sugita, H., Shibuya, K., Hanada, H., Deguchi, Y., 1997. Antibacterial abilities of intestinal microflora of the river fish. Fish. Sci. 63, 378–383.
Sugita, H., Hirose, Y., Matsuo, N., Deguchi, Y., 1998. Production of the antibacterial substance by *Bacillus* sp. strain NM 12, an intestinal bacterium of Japanese coastal fish. Aquaculture 165, 269–280.
West, P.A., Colwell, R.R., 1984. Identification of Pseudomonadaceae: an overview. In: Colwell, R.R. (Ed.), Pseudomoniasis in the Environment. John Wiley, New York, USA, pp. 141–199.
Westerdahl, A., Olsson, J.C., Kjelleberg, S., Conway, P.L., 1991. Isolation and characterization of turbot (*Scophthalmus maximus*) associated bacteria with inhibitory effects against *Vibrio anguillarum*. Appl. Environ. Microbiol. 57, 2223–2228.