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Effect of *Photobacterium damselae* subsp. *piscicida* Extracellular Product on Iron Compounds and Expression of Outer Membrane Proteins

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Abstract: The relation between the activity of *Photobacterium damselae* subsp. *piscicida* extracellular products on iron compounds in terms of iron releasing and the bacterial outer membrane proteins (OMPs) profile expressed after iron uptake was studied in this experiment. In order to achieve this, extracellular products (ECPs) collected from cultures of *P. d.* subsp. *piscicida* strain P-3179 grown either with or without iron restriction were incubated in dialysis bags with transferrin, an enriched iron compound, or with yellowtail serum in flasks containing medium broth. The ECPs were able to break both the transferrin and yellowtail serum components into smaller units, releasing iron to the culture medium. After removal of the dialysis bags, newly inoculated bacteria into the culture media grew at a higher rate in the flasks that contained broken transferrin and yellowtail serum than the ones without those components. Correspondingly, analysis of the outer membrane proteins collected from *P. d.* subsp. *piscicida* after 24 h incubation period showed stronger antigenicity for the OMPs preparation purified from the culture that contained transferrin and ECPs.

Key words: *Photobacterium damselae* subsp. *piscicida,* Pseudotuberculosis, yellowtail, fish disease, immune response.

Introduction

Photobacterium damselae subsp. *piscicida* (formerly *Pasteurella piscicida*) causes important economic losses in marine aquaculture in Japan (Fukuda and Kusuda, 1981; Egusa, 1983), USA (Hawke *et al.,* 1987; Moore *et al.* 1989) and Europe (Ceschia *et al.,* 1989). The role of iron in the pathogenicity of *P. d.* subsp. *piscicida* has been reported before (Magarinos *et al.,* 1992), as well as the phenotypic, antigenic and molecular characterization of this bacterium isolated from fish (Magarinos *et al.,* 1992). The same studies have been carried out for related bacteria like *Pasteurella multocida* (Ikeda and Hirsh, 1988). Up to date, there are no studies about the effect of *P. d.* subsp. *piscicida* extracellular products (ECPs) on the uptake of iron as well as the releasing of it from humoral components of the host animal. In the present study, we are trying, to clarify the role that different iron concentrations play in the expression of *P. d.* subsp. *piscicida* outer membrane proteins (OMPs) and their antigenicity.

The infective mechanisms of this bacterium are not clear yet. However, it is a possibility the involvement of bacterial extracellular products in the first infective steps. Recently, cellular antigens are being object of study. For this reason, this experiment describes the effect of ECPs on iron compounds and iron uptaking in a secondary step by bacteria.

Materials and Methods

Bacterial strain

P. d. subsp. *piscicida* strain P-3179 was isolated from yellowtall, *Seriola quinqueradiata,* in Kochi Prefecture in march 1996. The bacteria were grown in

brain heart infusion (BHI) agar (Difco) supplemented with 2 % NaCl at 25℃ for 48 h under shaking conditions and the collected cells were frozen at -80℃ in skim milk.

Bacterial ECPs

P. d. subsp. *piscicida* ECPs were prepared by the cellophane overlay method. There were prepared two kinds of ECPs. The first kind, (ECPw) was collected from bacteria grown without iron restriction as follows: 500 ml BHI agar were autoclaved and about 250 ml were poured into each of two bioassay plates (243 mm x 243 mm x 18 mm, Nunc), and allowed to cool. An autoclaved sheet of cellophane was overlayed on each of the agar plates. An overnight culture of *P. d.* subsp. *piscicida* P-3179 in BHI broth (Difco) suspended in sterile PBS and spread onto the cellophane. The bioassay plates were incubated at 25℃ for 72 h. After the incubation period, 15 ml of sterile PBS were added on the cellophane to resuspend the cells and ECPs. The suspension was centrifuged at 2,000 × g for 10 min at 4℃. The supernatant was collected, dialyzed against PBS at 4℃ for 24 h, filtered trough a 0.22 μ m filter and store at -80℃ until use. The second kind of ECP (ECPr) was prepared under iron restriction by the same method mentioned above except that BHI agar was supplemented with 200μ M of 2,2'-dipyridyl to remove the free iron of the medium.

Protease activity

Protease activity of ECPs was evaluated by the hide powder azure (HPA) assay. One mg of HPA (Sigma) was added to 1 ml of ECP, 1 ml of ECP + 10 mM EDTA and 1 ml of PBS as control. After incubation for 1 h at 37℃ and centrifugation at 4,000 × g for 10 min the optical density was determine at 540 nm.

Serum

Blood was obtained by bleeding a 6 kg yellowtail from the heart with a syringe. Blood was allowed to clot for 2 h at 25℃, and serum was collected by centrifugation at 10,000 × g for 5 min. Aliquots of 0.5 ml were made and stored at -20℃ until use. The serum was used as a source of iron supply.

Transferrin

Forty % iron saturated human transferrin was prepared by combining holo-transferrin (Sigma) and apo-transferrin (Becton Dickinson). The total transferrin concentration was 2 mg/ml in solution.

Experimental design

The experimental design is shown in Table. 1. Briefly, singles or mixtures of ECP, transferrin and serum were incubated in 3 ml vials at 25℃ for 2 h, then the contents were placed into dialysis bags, and these ones put into each of nine flasks containing 100 ml of Miller minimum medium (sodium phosphate dibasic 6 g/l, potassium phosphate monobasic 3 g/l, sodium chloride 5 g/l. Ammonium chloride 1 g/l, calcium chloride 0.0015 g/l, magnessium sulphate 0.012 g/l, glucose 5 g/l) supplemented with 0.2 % (w/v) casaminoacids. The flasks containing the dialysis bags and Miller minimum medium were incubated at 25℃ for 16 h under shaking conditions. The bags were removed and the suspension of the bacteria (approx. 10^8-

Table 1. Contents of the dialysis bags before being placed into the correspondant flask[1]

Preparation	ECP solution (ml)	Transferrin[2] (ml)	Yellowtail serum (ml)	PBS (ml)	Total volume (ml)
ECPw[3]	1.0	–	–	1.6	2.6
ECPw + transferrin	1.0	1.6	–	–	2.6
ECPw + yellowtail serum	1.0	–	0.5	1.1	2.6
ECPr[4]	1.0	–	–	1.6	2.6
ECPr + transferrin	1.0	1.6	–	–	2.6
ECPr + yellowtail serum	1.0	–	0.5	1.1	2.6
Transferrin	–	1.6	–	1	2.6
Yellowtail serum	–	–	0.5	2.1	2.6
PBS	–	–	–	2.6	2.6

[1] Each dialysis bag was added to a flask containing 100 ml of Miller minimum medium (sodium phosphate dibasic 6 g, potassium phosphate monobasic 3 g, sodium chloride 5 g, ammonium chloride 1 g, calcium chloride 0.0015 g, magnessium sulphate 0.012, glucose 5 g/l water), supplemented with 2 % (w/v) casaminoacids.
[2] Mixture of holo- and apo-transferrin at a concentration of 2 mg/ml in PBS.
[3] Extracellular products from bacteria grown without iron restriction.
[4] Extracellular products from bacteria grown with iron restriction.

10^9 CFU/ml) was inoculated into each flask. The flasks which bacterial suspensions (approx. 10^7 CFU/ml) were placed again on the shaker at 25℃ and incubated. Bacterial growth was monitored by assaying the absorbance at 540 nm at 0, 5, 13 and 24 h after inoculation. Bacteria were harvested for outer membrane proteins preparation after 24 h incubation period.

OMPs

After the 24 h incubation period, bacteria were harvested by centrifugation at 5,000 × g for 30 min at 4℃ and washed twice in 50 mM Tris, 20 mM EDTA, 5μg/ml phenylmethylsulfonylfluoride (PMSF, pH 7.4, Sigma). Cells were suspended in the same solution and maintained on ice and sonicated in 0.5 ml aliquots for four bursts of 30 sec with 30 sec cooling period between each burst. Sodium N-lauroyl sarcosinate (Sigma) was added to 1.5 % and the lysate was centrifuged at 30,000 × g for 30 min at 4℃ to remove whole cells. The supernatant was retained and centrifuged at 100,000 × g for 1 h at 4℃. After discarding the supernatant, the pellet was resuspended in deionized water. OMPs were finally collected by centrifugation at 100,000 × g for 1 h at 4℃, resuspended in deionized water and stored at -20℃ until use.

Analysis of OMPs

In order to determine the amount of protein of the OMPs collected, Bradford assay was carried out. OMPs from the nine different preparations were separated according to the discontinuous gel system of Laemli (Laemli, 1970). 50μg of protein from each outer membrane protein preparation was mixed with 20μl of reducing sample buffer (0.125 M Tris, pH 6.8; 4 % w/v SDS, 20 % v/v glycerol, 10 % v/v 2-mercaptoethanol, 5μg of bromophenol blue per ml) and heated at 95℃ for 4 min. Samples were stacked in a 4 % acrylamide gel and separated on 14% acrylamide gel. Polyacrylamide gel was transferred to a nitrocellulose membrane by using a semi-dry blotter (Trans-Blot SD, semi dry transfer cell, BioRad) according to the manufacturer's instructions. Electrophoretic transfer was carried out for 30 min at 150 mA. The gel was stained with Coomassie brilliant blue R-250 and molecular weights were estimated from migration distances of protein standards. The membrane was blocked in a 5 % solution of skim milk in Tween Tris buffer saline (TTBS), washed in TTBS for 5 min and then incubated in rabbit antiserum raised against formalin killed *P. d.* subsp. *piscicida* cells for 1 h at 25℃. The membrane was washed in TTBS for 5 min and incubated in goat anti-rabbit IgG alkaline phosphatase conjugated (Sigma) for 1 h at room temperature. The membrane was washed twice in TTBS and once in Tris buffer saline for 5 min each one and incubated overnight at 4℃ in enzyme substrate, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium. After color was developed, the membrane was finally rinsed in distilled water.

Results

Protease activity of ECPs is shown in Fig. 1. ECPw collected from bacteria grown without iron restriction showed protease activities of 23 and 22 units in the digestion of HPA, with and without EDTA, respectively whilst ECPr showed 26 and 25 units with and without EDTA, respectively. Control showed 12.5 units. *P. d.* subsp. *piscicida* grew at different rates depending on the substances contained in the dialysis bags as shown in Fig. 2. Observing the bacterial growth in cultures treated with ECPw combined with other substances in the dialysis bags, the highest bacterial growth rate was

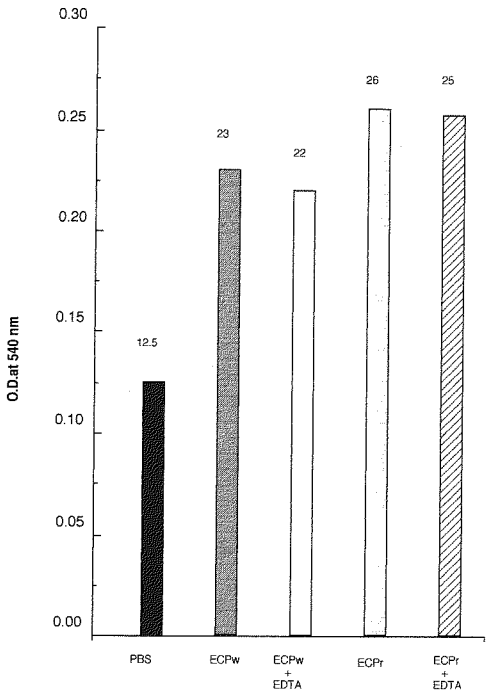

Fig. 1. Protease activity of *Photobacterium damselae* subsp. *piscicida* ECPs on hide powder azure.
(1 unit of protease = 0.01 O.D.)

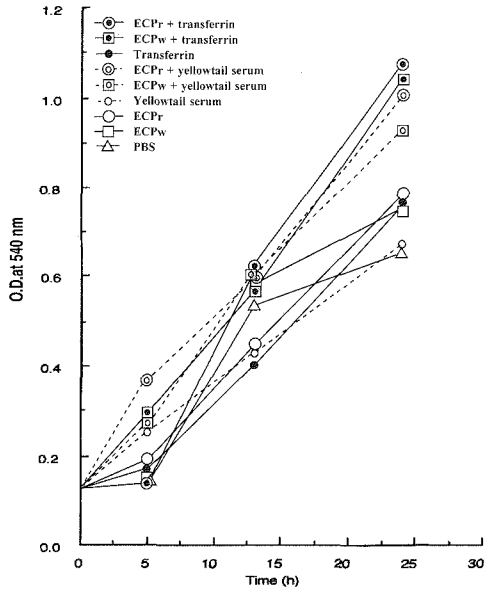

Fig. 2. Growth rate of *Photobacterium damselae* subsp. *piscicida* strain P-3179 at 25℃ for 24 h under shaking conditions. Flasks contained broth and were incubated with dialysis bags containing different substances.

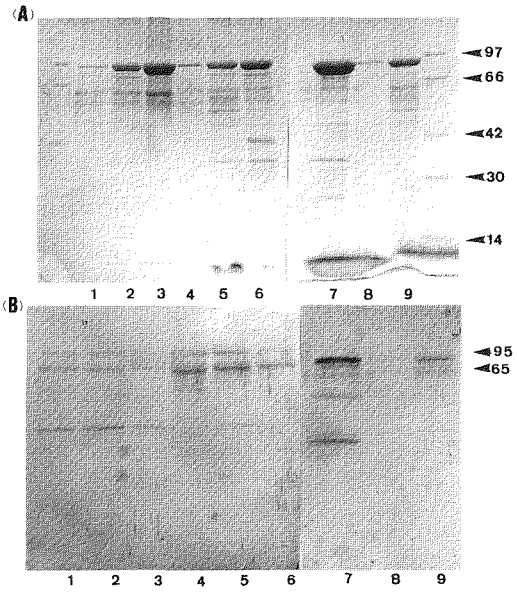

Fig. 3. SDS-PAGE (A) and immunoblotting (B) of *Photobacterium damselae* subsp. *piscicida* P-3179 outer membrane proteins prepared from bacteria grown at 25℃ for 24 h. Lanes: 1, ECPw + transferrin; 2, ECPw + yellowtail serum; 3, ECPw; 4, yellowtail serum; 5, transferrin; 6, PBS-control; 7, ECPr + transferrin; 8, ECPr + yellowtail serum; 9, ECPr; M, protein molecular weight markers. Figures at right side in (A) show molecular weight in kDa. Figures at right side in (b) is estimated molecular weight.

achieved by the bacterial cultures that previously contained transferrin in the dialysis bags. Bacteria from control and from the flask that contained only yellowtail serum in the dialysis bag grew at the lowest level, whilst cultures that contained ECPw or transferrin reached intermediate values. Bacterial cultures from flasks that contained transferrin treated in the dialysis bags with ECPr grew at similar rate than those of transferrin treated with ECPw.

The results of the SDS-PAGE analysis of OMPs are shown in Fig. 3-(A). All the preparations showed a 95 kDa major band with a large number of other outer membrane proteins below this molecular weight. Besides it appeared another common major band to all the preparations at a molecular weight of 65 kDa. Within the outer membrane protein profile it is remarkable that the relatively thick size of the 95 kDa band corresponding to the flask incubated with ECPr. The ECPw + transferrin OMPs preparation did not show a large amount of protein per band in SDS-PAGE. However in the immunoblotting test the same preparation showed a strong immunostaining at the 36 kDa band compared to the rest of the preparations, as well as the ECPw + yellowtail serum and ECPw OMPs preparations did {Fig. 3-(B)}. The preparation ECPr + transferrin showed at least five major bands in the immunoblotting test, with two of them more easily identified than the other three. In the ECPw OMPs preparation it is easy to observe the large amount of protein contained in the 95 kDa band, but strong antigenicity was not shown in the immunoblotting. The next three OMPs preparations (yellowtail serum, transferrin and PBS) showed almost identical immunostaining pattern, possessing the strongest antigenicity at the 69 kDa molecular weight band among all the preparations.

The 95 kDa band was immunostained in all the preparations at a similar intensity, except for the outer membrane protein preparation from the bacterial culture incubated with the dialysis bag containing ECPr and transferrin. This preparation showed a more intense immunostaining on the 38 kDa molecular weight band, which appeared faintly in the OMPs preparation from bacteria incubated in flasks that contained ECPw and transferrin.

Discussion

In the experiment the iron requirements for growing *P. d.* subsp. *piscicida* have been shown. We found that

bacteria were able to uptake more free iron from the medium in the action of ECPs. The results indicate ECPs break iron compounds and release free iron which can be effectively uptaken by bacteria.

In respect to the protease activity of ECPs, stress by low iron concentration seems to affect the activity of the enzymes of *P. d.* subsp. *piscicida.* EDTA slightly decreased the ECP activity, acting as a metallo-protease inhibitor, which is relevant data in order to know the nature of the bacterial exotoxins.

We detected two major outer membrane proteins at molecular weights of 95 and 65 kDa, respectively. These results were different from those found by Magarinos *et al.* (1992), where bands at molecular weights of 20, 30, 42 and 53 kDa were observed in bacteria cultured in normal BHI without modifications of the contents. Culture conditions appear to be of great importance in the expression of bacterial membrane proteins.

It is also remarkable to observe how the OMPs preparations from either ECPw or ECPr plus yellowtail showed a poor immunostaining. Serum proteases may be involved in this phenomena.

Recently, Jacques *et al.* (1994) reported that capsule production by *Pasteurella multocida* strains decreased under conditions of iron limitation *in vitro,* and bacterial cells grown *in vivo,* whereas expression of several OMPs increased under the same conditions. In addition, mice and rabbits immunized with non-capsulated *P. multocida* were protected against challenge with homologous capsulate *P. multocida* organisms. They suggested the involvement of outer membrane components in protection against pasteurellosis. Also, iron regulated OMPs have been extensively studied in different kinds of bacteria such as *Aeromonas salmonicida,* in regards to their antigenicity. They were found to stimulate fish immune system when harvested from a culture medium with iron restriction to a higher degree than those grown under non-iron restriction. Further studies are necessary in order to elucidate the amount of both bound and free iron that bacteria encounters in fish.

Observing the results obtained in the experiment it could be suggested that vaccines formulated with an extra iron supplementation could be more effective in fish than those prepared from an iron restricted medium. Although it must be taken into account that there are other important growing factors for *P. d.* subsp. *piscicida* such as sodium *l*-glutamate, magnesium acetate and casaminoacids (Hashimoto *et al.,* 1989) it seems to be that, according to the results we obtained, iron should be included to some extent in the vaccine preparations against pasteurellosis. However, it must be considered, that infective bacteria in fish never have the availablity of the iron concentration that usually have when cultured in normal BHI.

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