Role of the capsule of *Photobacterium damsela* subsp. *piscicida* in protection against phagocytosis and killing by gilt-head seabream (*Sparus aurata*, L) macrophages

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The role of the capsule in *Photobacterium damsela* subsp. *piscicida* (formerly *Pasteurella piscicida*) as a virulence factor was evaluated by determining the phagocytic and bactericidal activities of macrophages of gilt-head seabream. Five capsulated strains of *P. damsela* subsp. *piscicida* pathogenic for gilt-head seabream and one strain (EPOY-8803-II) described as noncapsulated and avirulent for this fish species were used in the study. Significant differences (P < 0.025) in the percentages and index of phagocytosis between the EPOY-8803-II strain and capsulated strains were observed, the noncapsulated strain being phagocytosed to a greater degree (86.4%). The induction of the synthesis of a capsule in strain EPOY-8803-II produced a significant reduction in the phagocytic percentage and index for this strain. However, no significant differences (P < 0.1) in the bactericidal activity of seabream macrophages were obtained between capsulated and noncapsulated strains.

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**Keywords:** *Photobacterium damsela* subsp. *piscicida*, phagocytosis, macrophages, gilt-head seabream, capsule.

### I. Introduction

*Photobacterium damsela* subsp. *piscicida* is the causative agent of pseudotuberculosis in several cultured fish species, such as young black seabream, oval filefish, gilt-head seabream and seabass (Ohnishi et al., 1982; Yasunaga et al., 1984; Toranzo et al., 1991; Balebona et al., 1992). This disease has caused important economic losses in the aquaculture industry of Japan (Ueki et al., 1990; Nakai et al., 1992), United States (Hawke et al., 1987), and Europe (Balebona et al., 1992).

Magarinós et al. (1992) have demonstrated that the strains of *P. damsela* subsp. *piscicida*, regardless of their origin and source of isolation, constitute a biochemically, serologically and genetically homogeneous group. This bacterial species has been described as a noncapsulated microorganism (Koike et al., 1975); however, Bonet et al. (1994) observed the synthesis of a capsule by *P. damsela* subsp. *piscicida* under in vitro conditions, by grouping

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in the medium described by Garrote et al. (1992) supplemented with NaCl. The presence of a capsule may contribute to virulence, on the basis that capsules are involved in virulence mechanisms in several microorganisms (Rush, 1989; Amaro et al., 1994; Yamaguchi et al., 1995). Recently Magarin˜os et al. (1996) have reported that P. damsela subsp. piscicida is covered by a thin layer of capsular material which conferred resistance to inactivation by serum, and increased the degree of virulence for fish of avirulent strains.

Macrophages play an important role in the immune response of fish, because they phagocytose and kill a wide range of pathogens (Secombes, 1990), and act as accessory cells in the initiation of the immune response.

The aim of this study was to investigate the role of the capsule of P. damsela subsp. piscicida in protection against macrophage responses of gilt-head seabream (S. aurata, L).

II. Materials and Methods

BACTERIAL STRAINS

Five isolates of P. damsela subsp. piscicida pathogenic for gilt-head seabream and one strain (EPOY-8803-II) described as avirulent for this fish species (Magariños, 1995) were used in this study (Table 1). Strains were cultured on Brain Heart Infusion Agar (BHIA Difco) supplemented with 2% NaCl (BHIAS) at 22° C. The confirmation of the presence of capsular material on the virulent strains was evaluated by staining of the cells with polycationic ferritin and visualization with transmission electron microscopy (TEM) following the technique described by Magariños et al. (1996). Yeast extract-peptone-glucose-mineral salts (YPGS-2) (Garrote et al., 1992; Bonet et al., 1994) supplemented with 2% NaCl was used to induce the in vitro formation of capsular material by EPOY-8803-II. The presence of a capsule on cells growing on YPGS-2 was demonstrated by staining with India ink (Bonet et al., 1994). The noncapsulated strain (EPOY-8803-II) was used as negative control and
a strain of *Klebsiella pneumoniae* obtained from Spanish Type Culture Collection (CECT 141) was used as positive control.

**SERUM AND ANTISERUM**

Non-immune serum from gilt-head seabream was obtained aseptically by caudal puncture of 100–200 g healthy specimens. The blood was allowed to clot, and the serum was stored at −20°C until use (within one month).

Antiserum against heat-inactivated *P. damsela* subsp. *piscicida* whole cells was obtained, following the technique described by Magarinos (1995), by intraperitoneal (i.p.) injection of gilt-head seabream (200 g) with 0·1 ml of 10^{10} bacterial cells ml^{-1} of a mixture of all the strains isolated in our laboratory (UMA: B51, B48, R46 and PP5) and grown on BHISA. After 15 d, the fish received a booster injection, and were bled 2 weeks after the last injection. Fish were maintained in 100 l tanks of seawater at 20°C with aeration.

**PHAGOCYTIC ASSAY**

Macrophages of gilt-head seabream (200 g) were obtained by peritoneal lavage using the techniques described by Matthews et al. (1990) and Seeley & Weeks-Perkins (1991). Briefly, 1 ml of Brewer’s thioglycolate broth (Difco) was injected i.p. After 48 h, 3 ml phosphate buffered saline supplemented with 100 IU penicillin ml^{-1}, 100 µg tetracycline ml^{-1} and 50 IU heparin ml^{-1} were injected i.p. into fish anesthetized with tricaine (0·065 g l^{-1}) (MS-222, Sigma), and this fluid was then removed by suction through a small ventral incision. Cells in the lavage were washed in Hanks Buffered Saline Solution (HBSS) by repeated centrifugation at 400 g for 10 min and resuspension in fresh HBSS. Cells were counted with a haemocytometer, and viability determined by the trypan blue exclusion test.

Three series of experiments were designed based on tubes containing 10^6 macrophages and 10^8 capsulated on noncapsulated bacteria to which were added: (i) 50 µl gilt-head seabream normal serum; (ii) 50 µl antiserum against UMA strains; and (iii) 50 µl HBSS. In all cases, HBSS was added to make up 1 ml of final suspension. All experiments were performed in triplicate. Cell suspensions were incubated at 20°C for 90 min and then centrifuged at 400 g for 5 min. Pellets were resuspended in ice-cold HBSS to stop further phagocytic activity. A 25 µl sample was smeared on a glass slide, air-dried, and stained with Giemsa. Macrophages (100/slide) were randomly examined, and the percentage of macrophages containing at least one engulfed bacterial cell was determined. The total number of bacterial cells in each of the 100 macrophages was also counted, and the average number of bacterial cells per macrophage was calculated (phagocytic index).

**BACTERICIDAL ASSAY**

Monolayers of gilt-head seabream macrophages were prepared and the bactericidal assay was performed as described by Graham et al. (1988). Briefly, the head kidney of gilt-head seabream (200 g body weight) was removed and
pushed through a nylon mesh with Leibovitz-15 medium (L-15) (Sigma) containing 2% foetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), and 10 IU heparin ml$^{-1}$ (Sigma). The cell suspension was layered onto a 34–51% Percoll density gradient, and following centrifugation at 400 g for 25 min, the band of cells above the 34–51% interphase was collected, and adjusted to $2 \times 10^7$ cells ml$^{-1}$ with L-15 medium, 0.1% FBS and 1% P/S. Aliquots (100 µl) were deposited into 96-well microtitre plates. After 2 h at 20°C, non-adherent cells were removed and the monolayers were incubated at 20°C for 1–3 d in L-15, 5% FBS, 1% P/S. The macrophage monolayers were then washed twice with L-15 medium to remove all traces of medium containing antibiotics and supplemented with 100 µl of L-15 medium containing 5% FBS. Twenty µl of each bacterial concentration (B51UMA, DI21 and EPOY 8803-II strains) was added to triplicate wells containing macrophages or medium only, and the microtitre plates were shaken for 20 s. The plates were then centrifuged at 150 g for 5 min, and subsequently incubated at 20°C for 0, and 5 h. At the end of the incubation period, the supernatants from the wells were removed and macrophages lysed with 50 µl cold sterile distilled water. The water was removed and 100 µl of TSAS was added to support an overnight growth of the surviving bacteria at 18°C. After this incubation period, the number of bacteria present in the wells was determined by adding 10 µl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma) (5 mg ml$^{-1}$ distilled water), shaking the plate and reading the optical density (OD) at 550 nm min later on a multiscan spectrophotometer (Flow). The data were adjusted to yield a killing index (KI) by: $(T_0 - T_5) / T_0 \times 100$, where the $T_0$ = bacteria at Time 0, and $T_5$ = bacteria at 5 h (Skarmeta et al., 1995).

**BACTERIAL GROWTH IN GILT-HEAD SEABREAM NON-IMMUNE SERUM**

The virulent strains of *P. damsela* subsp. *piscicida* U M A were inoculated in normal and heat-inactivated (60°C for 1 h in a water bath) sera diluted 1:1 with sterile Hank’s solution. These mixtures were incubated at 20°C with shaking for 48 h. The growth of the microorganisms was monitored at 6, 10, 24, 36 and 48 h by plating onto BHIA S plates incubated at 22°C for 48 h.

**STATISTICAL ANALYSIS**

Data were statistically analysed by ANOVA tests.

**III. Results**

**PHAGOCYTIC ASSAYS**

The phagocytosis percentage of different strains of *P. damsela* subsp. *piscicida* assayed in this study are given in Table 2. The only significant differences ($P < 0.025$) in the percentage of phagocytosis between the EPOY-8803-II strain and capsulated stains were observed in the experiments using non-immune serum. Here, the noncapsulated strain showed the highest phagocytic percentage (86.4%). Significant differences ($P < 0.01$) were also...
observed in the percentages of phagocytosis comparing the DI21 strain with UMA strains using non-immune seabream serum, the percentage of phagocytosis of DI21 strain being higher than those of the UMA strains.

The results concerning phagocytic index of the different strains are also given in Table 2. Significant differences (P < 0.01) between capsulated and noncapsulated strains and also between the DI21 and UMA strains were observed using non-immune serum. Thus, higher numbers of EPOY-8803-II were ingested by the macrophages than the virulent strains. The phagocytic index for strain DI21 was higher than those of the UMA strains.

The agglutinating titre of the antiserum was low against both virulent (1:2) and avirulent (1:1) strains. Capsulated and noncapsulated strains did not show significant differences (P > 0.01) regarding the phagocytic percentages in the presence of specific antiserum against UMA strains, although differences (P < 0.05) in the phagocytic index were detected with the values being higher for the capsulated strains (Table 2). Furthermore, it is interesting that both the percentage and the index of phagocytosis obtained for UMA strains in the presence of antiserum were significantly higher (P < 0.01) than in the experiments conducted with these strains in the absence or presence of normal serum (Table 2).

In all experimental conditions, the phagocytic percentage and index of EPOY-8803-II strain growth in YPGS-2 were significantly lower (P < 0.01) than those obtained when the strain was grown on BHIAS (Table 2), especially in the case of the assays conducted with non-immune serum. However, the percentages and index of phagocytosis obtained for the capsulated strains in the experiments conducted with specific antiserum were higher (P < 0.01) than those obtained for the capsulated EPOY-8803-II (Table 2).

Table 2. Phagocytic percentage and index of the capsulated and noncapsulated strains of Photobacterium damselae subsp. piscidia performed without serum (control) and with non-immune and immune sera

<table>
<thead>
<tr>
<th>Strains</th>
<th>Percentage of phagocytosis ± SD</th>
<th>Index of phagocytosis ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Normal Serum Antiserum</td>
<td>Control Fresh Serum Antiserum</td>
</tr>
<tr>
<td>Capsulated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UMA B51</td>
<td>46.6 ± 11.0</td>
<td>57.8 ± 4.0</td>
</tr>
<tr>
<td>UMA B48</td>
<td>51.4 ± 15.7</td>
<td>69.2 ± 8.1</td>
</tr>
<tr>
<td>UMA PP5</td>
<td>65.9 ± 14.7</td>
<td>78.7 ± 7.2</td>
</tr>
<tr>
<td>DI21</td>
<td>61.9 ± 8.2</td>
<td>67.6 ± 10.4</td>
</tr>
<tr>
<td>EPOY 8803-II*</td>
<td>51.9 ± 9.8</td>
<td>55.5 ± 5.8</td>
</tr>
<tr>
<td>Noncapsulated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPOY 8803-II**</td>
<td>68.7 ± 10.5</td>
<td>86.4 ± 13.0</td>
</tr>
</tbody>
</table>

SD: standard deviation.
*EPOY 8803-II grown in YPGS-2.
**EPOY 8803-II grown in BHIAS.
The growth of UMA strains in untreated and heat inactivated non-immune sera are represented in Fig. 1. All strains tested grew logarithmically over 6–10 h both in normal and inactivated sera.

BACTERIAL GROWTH IN SERA

The bactericidal activity of gilt-head seabream macrophages against P. damsela subsp. piscicida strains isolated from S. aurata is represented in Fig. 2. Progressively, higher bactericidal activity was observed with increasing concentrations of bacterial cells per well. However, there were no significant differences (P > 0.1) between the destruction of capsulated and noncapsulated strains, although slight differences in the killing ratios among capsulated and noncapsulated strains were observed (Table 3).

IV. Discussion

The role of the capsule of P. damsela subsp. piscicida as a virulence factor was evaluated by studying phagocytic responses to capsulated and noncapsulated P. damsela subsp. piscicida strains and the bactericidal activity of gilt head seabream macrophages.

The noncapsulated strain EPOY-8803-II showed the highest phagocytic percentage and index in non-immune serum, which suggests that normal serum possesses opsonins that increase the phagocytosis and that the capsule neutralises this effect. This finding is in concordance with the results obtained by Lamas & Ellis (1994), who also reported a higher phagocytosis in presence of normal serum of an avirulent strain of Aeromonas salmonicida (lacking A-layer) than with a virulent strain of this same microorganism (possessing A-layer), and these differences could be correlated to differences in fixation of
The role of a capsule in decreasing phagocytosis by macrophages has been reported previously in several microorganisms including Pasteurella multocida (Ryu et al., 1984) and Aeromonas salmonicida (Garduño et al., 1993). The differences found in the present study in the percentages and index of phagocytosis between capsulated strains (DI21 and UMA strains), using normal serum of gilt-head seabream, have also been reported in other capsulated microorganisms. Thus, Podschum et al. (1992) observed that Klebsiella pneumoniae possesses strains with anti-phagocytosis capsules (Ag K2) and strains with capsules more easily engulfed (Ag K7).

An increased phagocytic percentage and index for the UMA strains were observed when the assays were carried out using antiserum, indicating that the UMA capsulated strains were more efficiently opsonized than the non-capsulated EPOY-8803-II strain. However, a higher level of antibodies was detected against the UMA capsulated strains, which may explain the lack of significant differences regarding the phagocytic percentages between

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**Table 3. Killing ratio after 5 h incubation of virulent and avirulent strains of Photobacterium damsela subsp. piscicida with gilt-head seabream macrophages**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Number of cells/well</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1·9 × 10^6</td>
</tr>
<tr>
<td>DI21</td>
<td>85·6 ± 4·9</td>
</tr>
<tr>
<td>B51 UMA</td>
<td>83·0 ± 6·5</td>
</tr>
<tr>
<td>EPOY-8803-II</td>
<td>95·2 ± 0·3</td>
</tr>
</tbody>
</table>

SD: standard deviation.
NT: not tested.
capsulated and noncapsulated strains in the presence of immune serum. These
findings are similar to those reported by Sakai (1984) and Lamas & Ellis (1994),
who observed increased phagocytic activities of salmonid macrophages due to
the presence of antibodies and complement. The lower percentage and index of
phagocytosis of strain EPOY-8803-II obtained using specific antiserum com-
pared with normal serum could be explained by the fact that the antiserum
was stored at \(-20^\circ\text{C}\) for a long time (1 month), which could have reduced its
opsonic potential by the partial inactivation of fractions such as complement.
This effect was corroborated by results obtained in experiments where normal
seabream serum stored at \(-20^\circ\text{C}\) produced lower phagocytic percentages and
index of all the strains compared with those obtained using normal nonfrozen
serum (data not shown).

Capsule synthesis in the EPOY-8803-II strain is induced by culturing in
YPGS-2 (Bonet et al., 1994). Indeed, a thicker capsule is expressed under these
conditions than that produced by several virulent strains grown on BHIAS
(Magarin\'os et al., 1996). The different composition of the capsules synthesized
by EPOY-8803-II strain cultured in YPGS-2 and virulent strains grown in
BHIAS could be the reason for the higher percentage and index of phago-
cytosis obtained for the virulent strains compared to the capsulated
EPOY-8803-II strain in the experiments carried out with specific antiserum. In
concordance with these findings, Garduño & Kay (1995) reported that the
composition of the capsule synthesized by A. salmonicida in YPGS-2 was
different from the capsule that this microorganism synthesized in vivo.
However, Magariños et al. (1996) demonstrated that the strain EPOY-8803-II,
grown on YPGS-2, had a survival rate similar to that of the capsulated strain
DI21 in gilt-head seabream normal serum, but when the noncapsulated strain
was grown on BHIAS it was inactivated quickly. These results demonstrate
the protective effect that extracellular material can exert on the resistance of
P. damsela subsp. piscicida to phagocytosis by macrophages and could confirm
its role as a virulence factor for this microorganism. Furthermore, Magariños
et al. (1996) demonstrated that the LD50 for gilt-head seabream of
EPOY-8803-II strain grown on YPGS-2 was lower than when it was grown in
BHIAS.

Due to the differences in phagocytosis of the UMA and DI21 strains in
normal serum, it could be interesting to compare the ability of these strains to
grow in gilt-head seabream serum, since the strain DI21 was able to survive
and multiply in trout and gilt-head seabream non-immune sera (Magariños
et al., 1996). In the present study, similar growth curves of all UMA strains
were obtained both in normal and heat-inactivated non-immune sera.

The results obtained in the present work, demonstrate that both capsulated
and noncapsulated strains were efficiently destroyed by the macrophages of
gilt-head seabream. However, a non-significant difference between the killing
activity of seabream macrophages against capsulated and noncapsulated
strains of P. damsela subsp. piscicida was obtained, which may be due to the
fish size used. Thus, Noya et al. (1995a,b) demonstrated that the resistance
of gilthead seabream to P. damsela subsp. piscicida infection was dependent
on the fish size, and this was related to the different activity of the macro-
phages. Nonetheless, evaluating the killing ratio demonstrated the greater
susceptibility of the avirulent strain compared to virulent strains, although the differences between these ratios were not significant. Skarmeta et al. (1995) studying the bactericidal activity of seabass, gilt-head seabream and trout macrophages against several stains of P. damsela subsp. piscicida observed that the EPOY-8803-II strain was killed with a lower efficiency than the virulent strain DI21 by all kinds of macrophages tested, although this difference was only statistically significant with seabass macrophages.

The lack of protective capability of the capsule on the bactericidal activity is of interest and suggests that the major contribution to P. damsela subsp. piscicida virulence is the ability of the capsule to reduce phagocytosis by seabream macrophages as demonstrated here.

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