Serological Differences among Photobacterium damsela subsp. piscicida Isolates

Eijiro Kawahara1,2,*, Yutaka Fukuda3, and Riichi Kusuda1

1Faculty of Engineering, Fukuyama University, Fukuyama, Hiroshima 729-0292, Japan
2School of Fisheries Sciences, Kitasato University, Sanriku, Iwate 022-0101, Japan
3Oita Institute of Marine and Fisheries Sciences, Kamiura, Oita 879-2602, Japan

(Received May 27, 1998)

Serological differences of Photobacterium damsela subsp. piscicida (= Pasteurella piscicida) isolates were determined using yellowtail (Seriola quinqueradiata) and rabbit antisera. In Oita, Japan, from June to October, 1995, Ph. damsela piscicida was isolated from net-pen cultured diseased yellowtail and sera were collected from fish cultured in the same net-pen. Antisera against formalin-killed cells of three representative isolates were raised in yellowtail and rabbits. The yellowtail antisera were absorbed with homologous or heterologous isolates and used for the agglutinating antibody absorption test. Crude lipopolysaccharides were extracted from the isolates and analyzed by immunodiffusion with rabbit antisera. As a result, three types of antigenic differences were revealed among the isolates by the agglutinating tests with the sera of naturally infected fish and by the absorption tests using yellowtail antisera. Some common and distinctive antigens of the isolates were found by immunodiffusion analysis with rabbit antisera.

Key words: serology, antigenic type, Photobacterium damsela subsp. piscicida, Pasteurella piscicida, yellowtail, Seriola quinqueradiata

Introduction

In Japan, Photobacterium damsela subsp. piscicida (= Pasteurella piscicida) (Gauthier et al., 1995) is the causative bacterium of “pseudotuberculosis” of young cultured yellowtail (Seriola quinqueradiata) and the disease has been associated with serious economical losses in yellowtail culture since 1969. Toranzo et al. (1991) reported that an epizootic caused by Ph. damsela piscicida occurred in gilthead sea bream in Spain in 1990. Magarinos et al. (1992) described that the disease has been a serious problem in marine fish culture in Mediterranean countries.

Various investigations have been made on the development of vaccines against this disease (Kawakami et al., 1997) although a commercially available vaccine has not yet been developed in Japan. Recently ribotype analysis of the pathogen suggested the existence of genetic diversity among Ph. damsela piscicida isolates with three ribotypes identified*. Ribotype 1 represented Spanish and Italian isolates while ribotypes 2 and 3 represented French and Japanese strains, respectively.

We have studied about the serological differences of Ph. damsela piscicida isolates using yellowtail and rabbit antisera.

Materials and Methods

Bacterial isolates and yellowtail sera

During the epizootic of pasteurellosis in Oita Prefecture, Japan in June to October, 1995, ten strains of Ph. damsela piscicida were isolated from diseased yellowtail cultured in a net-pen (Table 1) and sera were collected from apparently healthy yellowtail cultured in the same net-pen (Table 2).

Ph. damsela piscicida formalin-killed cells and crude LPS

formalin-killed cells (FKC) were prepared by adding 0.5% formalin to the culture and incubating for 24 h at 4°C. The cell suspension was washed three times with 10 mM phosphate buffered saline (PBS) at pH 7.0 by centrifugation. With another portion of the culture, bacterial cells were harvested by centrifugation, then subjected to an extraction following a procedure of Westphal and Jann (1965) for the preparation of the crude LPS.

Yellowtail antisera

Three yellowtail antisera against FKC of isolates, strain no. OT-51443, 51442 and 52093 were raised as mentioned below. FKC was adjusted to 2 mg/ml in PBS, and emulsified with Freund’s incomplete adjuvant (Difco) at a volume ratio of 1:1. One hundred microliter of the emulsion was injected intraperitoneally into hatchery produced yellowtail weighing about 25 g. At 2 and 3 weeks after the first injection, 100 μl of FKC suspension without adjuvant was injected as a booster into the fish intraperitoneally. One week after the last injection, the fish were bled from the heart using a needle with a syringe, and antisera were obtained and stored at −20°C.

Absorption of yellowtail antisera

Absorbed yellowtail antisera were prepared by adding 500 mg of FKC to 1 ml of the antisera. The mixture was incubated at 25°C for 2 h. FKC was removed by centrifugation and the supernatant fluids was used for absorption test.

Rabbit antisera

Three rabbit antisera against FKC of isolates, strain no. OT-51443, 51442 and 52093 were raised as previously reported by Kawahara et al. (1991) using Freund’s incomplete adjuvant.

Agglutinating antibody test

Twenty-five microliters of twofold serial dilutions of the yellowtail sera, absorbed or non-absorbed, were mixed with the same volume of FKC suspension of 2 mg/ml in PBS in each well of microtiter plates. The plates were incubated at room temperature for 2 h and at

---

### Table 1. *Photobacterium damsela* subsp. *piscicida* isolates used in this study

<table>
<thead>
<tr>
<th>Strain number (OT-)</th>
<th>51442</th>
<th>51443</th>
<th>52091</th>
<th>52093</th>
<th>52094</th>
<th>52791</th>
<th>52792</th>
<th>52794</th>
<th>54781</th>
<th>54782</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date of isolation</td>
<td>1995-6-23</td>
<td>6-23</td>
<td>7-7</td>
<td>7-7</td>
<td>7-7</td>
<td>7-24</td>
<td>7-24</td>
<td>7-24</td>
<td>10-6</td>
<td>10-6</td>
</tr>
<tr>
<td>BW of yellowtail (g)</td>
<td>24</td>
<td>32</td>
<td>53</td>
<td>49</td>
<td>58</td>
<td>122</td>
<td>99</td>
<td>106</td>
<td>585</td>
<td>560</td>
</tr>
</tbody>
</table>

BW: body weight.

---

### Table 2. Agglutinating antibody titres of cultured yellowtail sera to the isolates

<table>
<thead>
<tr>
<th>Date of serum*1 collection</th>
<th>Formalin-killed cells (OT-)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>51443</td>
</tr>
<tr>
<td>1995-6-8</td>
<td>&lt;2*2</td>
</tr>
<tr>
<td>26</td>
<td>&lt;2</td>
</tr>
<tr>
<td>7-7</td>
<td>3.8</td>
</tr>
<tr>
<td>24</td>
<td>4.8</td>
</tr>
<tr>
<td>8-9</td>
<td>4.9</td>
</tr>
<tr>
<td>24</td>
<td>5.3</td>
</tr>
<tr>
<td>9-6</td>
<td>4.5</td>
</tr>
<tr>
<td>19</td>
<td>4.3</td>
</tr>
<tr>
<td>10-6</td>
<td>5.5</td>
</tr>
<tr>
<td>18</td>
<td>4.6</td>
</tr>
</tbody>
</table>

*1 Sera were collected from ten fish.
*2 Geometric mean (log).
4°C for 16 h, and then agglutination was observed.

**Immunodiffusion**

Immunodiffusion analysis was performed as previously reported by Kawahara et al. (1991). Crude LPSs of the isolates were analyzed using the rabbit antisera.

**Results**

**Antibody titers of cultured yellowtail sera**

Agglutinating antibody titers of cultured yellowtail sera to Ph. damsela piscicida isolates are shown in Table 2. The titers to the 10 isolates formed three distinguishable groups. The titers to 4 isolates, strain number OT-51443, 52791, 52794 and 52792 were raised from June to August, the former period of the epizootics of pasteurellosis. The titers to 3 isolates, 51442, 52091 and 54781 were raised from August to October, the latter period of the epizootics. And the titers to 3 isolates, 52093, 52094 and 54782 were raised slightly from September to October, the end of the epizootics. At the results, the changes of the titers to typical three type isolates, OT-51443 (type A), 51442 (type B) and 52093 (type C) summarized in Fig. 1.

**Cross-absorption test**

To clarify the antigenic relationships within the three type isolates, cross-absorption tests were performed with yellowtail antisera raised against the 3 isolates (Tables 3–5). When the antisera were absorbed with homologous antigens, antibodies were completely eliminated. However, each antiserum was not completely eliminated.

---

**Table 3.** Cross-absorption test using yellowtail anti-OT-51443 (type A) serum

<table>
<thead>
<tr>
<th>Absorbed with (OT- )</th>
<th>51443 (type A)</th>
<th>51442 (type B)</th>
<th>52093 (type C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>51443 (type A)</td>
<td>&lt; 2*</td>
<td>&lt; 2</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>51442 (type B)</td>
<td>4</td>
<td>&lt; 2</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>52093 (type C)</td>
<td>5</td>
<td>3</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>Unabsorbed</td>
<td>11</td>
<td>12</td>
<td>10</td>
</tr>
</tbody>
</table>

* Agglutinating antibody titer (log2).

**Table 4.** Cross-absorption test using yellowtail anti-OT-51442 (type B) serum

<table>
<thead>
<tr>
<th>Absorbed with (OT- )</th>
<th>51443 (type A)</th>
<th>51442 (type B)</th>
<th>52093 (type C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>51443 (type A)</td>
<td>&lt; 2*</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>51442 (type B)</td>
<td>&lt; 2</td>
<td>&lt; 2</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>52093 (type C)</td>
<td>6</td>
<td>4</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>Unabsorbed</td>
<td>11</td>
<td>12</td>
<td>10</td>
</tr>
</tbody>
</table>

* Agglutinating antibody titer (log2).
by cross-reaction against heterologous antigens.

**Immunodiffusion**

Immunoprecipitate patterns in rabbit antisera against crude LPSs of the 3 isolates are shown in Fig. 2. Anti-type A serum formed 3, 2 and 2 precipitating lines with type A, B and C crude LPSs, respectively. Anti-type B serum formed 2 precipitating lines with each crude LPS. And anti-type C serum formed 1, 2 and 2 precipitating lines with type A, B and C crude LPSs, respectively.

### Discussion

Magarinos et al. (1992) compared *Ph. damsela piscicida* strains isolated from different fish species in several European countries with strains isolated in Japan and USA. They described that the phenotypic, serological and genetic homogeneity among the *Ph. damsela piscicida* isolates were at high levels, and shown in particular that the serological homogeneity was supported by the LPS and membrane protein profiles. But recently, Bakopoulous et al. (1997) demonstrated the antigenic differences between Japanese strains and European strains by Western blot analysis with monoclonal antibodies against whole cells of *Ph. damsela piscicida*.

### Table 5. Cross-absorption test using yellowtail anti-OT-52093 (type C) serum

<table>
<thead>
<tr>
<th>Absorbed with (OT- )</th>
<th>Formalin-killed cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>51443 (type A)</td>
</tr>
<tr>
<td></td>
<td>51442 (type B)</td>
</tr>
<tr>
<td></td>
<td>52093 (type C)</td>
</tr>
<tr>
<td>51443 (type A)</td>
<td>&lt; 2*</td>
</tr>
<tr>
<td>51442 (type B)</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>52093 (type C)</td>
<td>&lt; 2</td>
</tr>
<tr>
<td>Unabsorbed</td>
<td>11</td>
</tr>
</tbody>
</table>

* Agglutinating antibody titer (log).
In the present study, we determined serological differences among *Ph. damsela piscicida* strains isolated from yellowtail during the epizootics of pasteurellosis. By agglutinating, absorption and immunodiffusion tests, three antigenic types were revealed among the isolates examined. The three type isolates possessed common and distinctive antigens. Based on these results, antigenic models of the three types can be given as follows: Type A: pab, Type B: pbc, Type C: pc; p: common antigen(s), a: antigen peculiar to Type A, b: antigen peculiar to Types A and B, c: antigen peculiar to Types B and C. The antigenic differences must affect vaccine development. The examination of the effectiveness of monovalent and polyvalent vaccines prepared from the three type strains against pasteurellosis for yellowtail is necessary.

References


