A Comparison of Sialic Acid between Different Isolates of *Photobacterium damsela* subsp. *piscicida*

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ABSTRACT—Sialic acid was identified and compared among 40 strains of *Photobacterium damsela* subsp. *piscicida* (formerly, *Pasteurella piscicida*), isolated from a variety of fish species from geographically different regions. The isolates were divided into two main groups according to the molecular weight of their sialic acid. The first group comprised of 12 Japanese isolates, which contained sialic acid with a molecular weight of 22 kDa. The second group included 26 isolates from the Mediterranean region, which contained sialic acid with a molecular weight of 26 kDa. A subgroup of Group 2 (2 Mediterranean isolates) was also evident and had a sialic acid molecule of 38 kDa. The *P. damsela* subsp. *piscicida* strains (ATCC33539, ATCC35083 and NCIMB2184) had 26 kDa sialic acid molecules. Three isolates representing the three different groups of *P. damsela* subsp. *piscicida* were treated with *Vibrio cholerae* Type III sialidase. Isolate OT97299 from Group 1 and isolate SA300695 from the subgroup were both susceptible to the sialidase, while isolate I736 from the main part of Group 2 was unaffected by the enzyme. All three isolates were susceptible to mild acid treatment.

Key words: *Photobacterium damsela* subsp. *piscicida*, sialic acid, sialidase, pasteurellosis

The causative agent of pasteurellosis, *Photobacterium damsela* subsp. *piscicida*, (formerly *Pasteurella piscicida*), has been isolated from a variety of fish species cultured in the Mediterranean (Bakopoulos *et al.*, 1995), Japan (Matsuoka and Kamada, 1995) and the USA (Hawke *et al.*, 1987). The disease has been responsible for large economic losses in the aquaculture industry in regions where the infection is prevalent (Thune *et al.*, 1993). As a direct result of this, a great deal of research has focused on the disease. The taxonomy (Gauthier *et al.*, 1995; Thyssen *et al.*, 1998), serology (Magarinos *et al.*, 1992; Salati *et al.*, 1994; Bakopoulos *et al.*, 1997), the route of infection (Magarinos *et al.*, 1996; Kawahara *et al.*, 1989) and the virulence of the pathogen (Magarinos *et al.*, 1994; Noya *et al.*, 1995) have all been examined in detail.

P. damsela subsp. *piscicida* strains produce an additional exostructure (a capsule) when cultured in glucose-enriched medium (Bonet *et al.*, 1994). However,

it appears that only virulent strains continually produce a capsular layer in this medium, and it has been shown that bacteria with the capsular layer are resistant to the killing activity of serum sampled from sea bream (Magarinos *et al.*, 1996).

Carbohydrates associated with a number of mammalian bacterial pathogens are known to play an important role in the pathogenesis of the organism, especially sialic acid (Wetzler *et al.*, 1992). Sialic acid present on the surface of bacteria has been shown to inhibit the complement cascade of the host (Varki, 1993), and to protect the bacteria from host antibodies (Jarvis and Vedros, 1987). However, the importance of carbohydrates in the pathogenesis of bacterial fish pathogens remains to be clarified. The aim of the present study was to identify and compare sialic acids among strains of *P. damsela* subsp. *piscicida* isolated from a variety of fish species from different geographical regions.

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Materials and Methods

P. damsela subsp. *piscicida* isolates and culture conditions

Geographical locations and species of fish from which *P. damsela* subsp. *piscicida* strains were isolated are shown in Table 1. All strains were stored at -70°C on PROTECT® beads (Technical Service Consultants Ltd) until required. They were cultured in tryptone soya broth (TSB) containing 2% (w/v) NaCl for 16 h at

22°C . Three representative strains (I736, OT97299 and SA300695) were continually subcultured on artificial medium during the course of the study. The cultured bacteria were harvested and washed twice with sterile phosphate buffered saline (PBS-1: 0.02 M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.02 M $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.15 M NaCl, pH 7.2) by centrifugation at $3000 \times g$ for 20 min. Bacterial suspensions were prepared in PBS-1 and their concentrations adjusted to absorbances of 1.0 at 610 nm. This was equivalent to approximately 2×10^8 CFU/mL, determined

Table 1. *Photobacterium damsela* subsp. *piscicida* and *P. damsela* subsp. *damsela* strains used in the study and their molecular weights of sialic acid

Strains	Date isolated	Fish species	Country	*kDa
<i>subsp. piscicida</i>				
K6	1994	Mullet	Greece	26
K6a	1994	Mullet	Greece	26
K7	1994	Red Sea Bream	Greece	26
K8	1994	Sea Bass	Greece	26
PN1	1994	Sea Bass	Greece	26
PN2	1994	Sea Bass	Greece	26
PN3	1994	Sea Bass	Greece	26
PN510	1994	Sea Bream	Greece	26
PN510a	1994	Sea Bream	Greece	26
I736	1991	Sea Bass	Italy	26
I746	1991	Sea Bass	Italy	26
362-2/97	1997	Sea Bass	Italy	26
386/97	1997	Sea Bass	Italy	26
M28561	1985	Yellow tail	Japan	22
TPP88003	1988	Yellow tail	Japan	22
SKP88020	1988	Yellow tail	Japan	22
TT90009	1990	Yellow tail	Japan	22
HT90011	1990	Yellow tail	Japan	22
KP9083	1990	Yellow tail	Japan	22
SP92065	1993	Yellow tail	Japan	22
SP92088	1993	Yellow tail	Japan	22
SP92144	1993	Yellow tail	Japan	22
SP9351	1993	Yellow tail	Japan	22
OT-97290	1997	Yellow tail	Japan	22
OT-97299	1997	Yellow tail	Japan	22
SA300695	1995	Sea Bream	Israel	38
MS020295	1995	Striped bass	Israel	26
MS011194	1995	Striped bass	Israel	26
DL270695	1995	Sea bass	Israel	26
SA040296	1996	Sea bream	Israel	26
SA071194	1994	Sea bream	Israel	26
SA120995	1995	Sea bream	Israel	26
XX000696	1996	Hybrid striped bass	Israel	26
Spp97127-2	1997	Sea bream	Spain	26
Spp97127-15	1997	Sea bream	Spain	26
Spp97127-16	1997	Sea bream	Spain	26
Spp97127-20	1997	Sea bream	Spain	38
231093	1993	Sea bream	Portugal	26
FR10831	1990	Sea bass	France	26
ATCC17911		White perch		26
<i>subsp. damsela</i>				
ATCC33539		Damsel fish		26
NCIMB2184		Damsel fish		26
ATCC3508		Brown shark		26

ATCC: American Type Culture Collection

NCIMB: National Collections of Industrial and Marine Bacteria

* Molecular weights of sialic acid of each strain were determined using a commercial glycoprotein detection kit.

from a standard curve of bacterial concentration *versus* absorbance at 610nm. Aliquots of the bacteria were stored at -70°C until required.

Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a variety of *P. damsela* subsp. *piscicida* isolates. Briefly, a 12% acrylamide separating gel was prepared onto which was layered a 4% stacking gel. The washed bacteria were prepared in sample buffer as described by Bollag *et al.* (1996), boiled for 3 min and centrifuged at $2000 \times g$ for 3 min. The supernatants were placed on the gel ($15 \mu\text{L}$ /well) and 180 V was applied to the gel for 45 min. Bio-Rad or Sigma broad range molecular standard markers were used as standards.

Detection of sialic acid by Western blot analysis

The bacterial components present in the gel were transferred to a nitrocellulose membrane by applying 60 V for 70 min using a Hoeffer transblotter (Carlsson, 1993). An Immuno-Blot Kit for glycoprotein determination (Bio-Rad) was used to detect the presence of sialic acid. Reagents were prepared according to the manufacturer's specification. Gentle agitation was applied to the membrane throughout the procedure, except during the development of the reaction. The membrane was washed with cold phosphate buffered saline (PBS-2: 9 mM NaH_2PO_4 , 27 mM NaCl, pH 7.2) for 10 min at 4°C . It was then immersed in sodium acetate / ethylenediaminetetraacetic acid (EDTA) buffer containing sodium periodate (100 mM sodium acetate, 5 mM EDTA, 1 mM sodium periodate, pH 5.5) and incubated in the dark for 20 min at 4°C . The membrane was washed three times with PBS-2, washing for 10 min on each wash. A biotinylation solution was prepared immediately before use, by adding $2 \mu\text{L}$ hydrazide solution to 10 mL sodium acetate / EDTA buffer (without sodium periodate) and the membrane was immersed in this solution for 60 min at 20°C . It was then washed 3 times, 10 min each at 20°C , with Tris buffered saline (TBS: 50 mM Tris base, 27 mM NaCl, pH 7.2) and incubated overnight at 4°C with 0.5% (w/v) ovalbumin in distilled H_2O . It was washed 3 times with TBS the next day as described above. Streptavidin-alkaline phosphatase conjugate was prepared and the membrane was incubated in this solution for 60 min at 20°C before again washing with TBS. The developer was prepared immediately before use by mixing together nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate, according to manufacturer's specifications. The membrane was incubated in the solution for 3 to 30 min at 20°C until the required reaction intensity was achieved. The reaction was stopped by rinsing the membrane several times in distilled H_2O .

Treatment of bacteria with sialidase

(a) Treatment of bacteria with sialidase: Treatment of three different *P. damsela* subsp. *piscicida* strains (OT97299, I736 and SA300695) with sialidase was performed using a modification of the method described by Ourth and Bachinski (1987). *Vibrio cholerae* Type III sialidase, which is able to cleave most carbohydrate linkages present in the O-glycan molecule, was used to differentiate between the sialic acids. Briefly, bacteria were washed twice with PBS-1 by centrifugation at $3000 \times g$ for 20 min, then adjusted to an absorbance of 1.0 at OD_{610} in sodium acetate buffer (100 mM sodium acetate, 1 mM calcium acetate, 100 mM NaCl pH 5.5). The bacterial suspension (1 mL) was placed in autoclaved micro-centrifuge tubes at a final concentration of either 20 or 100 mUnits of sialidase was added to the tubes. PBS-1 was also added to a tube as a negative control and 100 mM in sodium acetate buffer without any bacteria was added to another tube. The tubes were incubated overnight in a shaking water bath, set at 37°C , after which the bacteria were centrifuged at $12,000 \times g$ for 3 min. Bacterial pellets were resuspended to their previous volume with PBS-1, and these were then mixed with sample buffer for electrophoresis and Western blot analysis using the glycoprotein determination kit.

(b) Treatment of nitrocellulose membrane with sialidase: Alternatively, sialic acid present on the nitrocellulose membrane was exposed to sialidase. The three *P. damsela* subsp. *piscicida* isolates (OT97299, I736 and SA300695) were subjected to SDS-PAGE as described above, and transferred to a nitrocellulose membrane. The membrane was washed with 100 mM sodium acetate buffer (three washes, 10 min each). It was then cut it into two halves, one of which was used as a control and the other was treated at 37°C for 4 h with sialidase at 50 mUnits in 100 mM sodium acetate buffer, pH 5.5. The membranes were then processed as described above using the glycoprotein determination kit.

Treatment of bacteria with mild acid conditions

Mild acid hydrolysis was used to release sialic acid from the glyco-conjugates present on the bacterium's surface. The same three strains of *P. damsela* subsp. *piscicida* as above, were again prepared as previously described. The bacterial suspension was centrifuged at $12,000 \times g$ for 3 min and the pellet was resuspended in 0.1M H_2SO_4 . The bacteria were incubated in the acid conditions for 1 h at 80°C (Manzi and Varki, 1993). Treated bacteria were centrifuged at $12,000 \times g$ for 3 min and the pellets were resuspended to pre-treatment volumes with PBS-1. The bacterial samples were applied to SDS-PAGE and performed Western blot analysis to detect sialic acid.

Results

Detection of sialic acid on nitrocellulose membranes

Three strains of *P. damsela* subsp. *damsela* and forty strains of *P. damsela* subsp. *piscicida* were examined for the presence of sialic acid using a commercially prepared immunoblotting kit. An example of this is shown in Fig. 1. Differences were noted in the sialic acid among the isolates examined (Table 1). It was possible to differentiate between the strains of *P.*

damsela subsp. *piscicida* obtained from Japan and those from the Mediterranean region. All of the isolates from Japan contained a band at approximately 22 kDa, regardless of the year in which they were isolated (Group 1). Most of the isolates obtained from the Mediterranean region exhibited a band at approximately 26 kDa (Group 2), except for two isolates (one from Spain, Spp 97127-20 and one from Israel, SA300695) which had a sialic acid band with a molecular weight around 38 kDa. The molecular weight of the sialic acid on isolate

Fig. 1. Sialic acid profiles on strains of *Photobacterium damsela* subsp. *piscicida* isolated from fish. Lanes: (1) I736 (Italy); (2) I746 (Italy); (3) K6a (Greece); (4) Pn510a (Greece); (5) SP92144 (Japan); (6) SP9351 (Japan); (7) SA300695 (Israel); (8) Spp-97127-20 (Spain); (9) NCIMB 2184 (*P. damsela* subsp. *damsela*); (10) I736 (Italy); (11) I746 (Italy); (12) K6a (Greece); (13) Pn510a (Greece); (14) NCIMB 2184 (*P. damsela* subsp. *damsela*); (15) SA300695 (Israel); (16) SP92144 (Japan); (17) SP9351 (Japan); (18) Spp-97127-20 (Spain). (7 and 15: after several passages on the medium)

SA300695 was found to change through time from 38kDa to 26 kDa after several passages on artificial culture medium (Fig. 1). Three strains of *P. damsela* subsp. *damsela* was showed the same molecular weight of sialic acid with Group 2 (Fig. 1).

Sialidase treatment

Vibrio cholerae Type III sialidase was able to cleave sialic acid present on isolates OT97290 from Japan (Group 1) and SA300695 from Israel (subgroup of Group 2), but was unable to cleave sialic acid on isolate I736 from Italy (main Group 2) (Fig. 2). This was the case

Fig. 2. (a) Detection of sialic acid on three isolates of *P. damsela* subsp. *piscicida* after direct treatment with sialidase; Lanes: (1) MW markers, (2) treatment of isolate I736 with 100 mUnit sialidase, (3) treatment of isolate I736 with 20 mUnit sialidase, (4) isolate I736 untreated, (5) treatment of isolate OT97299 with 100 mUnit sialidase, (6) treatment of isolate OT97299 with 20 mUnit sialidase, (7) isolate OT97299 untreated, (8) treatment of isolate SA300695 with 100 mUnit sialidase, (9) treatment of isolate SA300695 with 20 mUnit sialidase, (10) isolate SA300695 untreated. (b) Detection of sialic acid on three isolates of *P. damsela* subsp. *piscicida* after indirect treatment of the nitrocellulose membrane with sialidase: Lanes: [treated (1) I736, (2) OT97299, (3) SA300695], (4) MW marker, [untreated (5) SA300695, (6) OT97299, (7) I736].

Fig. 3. Detection of sialic acid on three isolates of *P. damselae* subsp. *piscicida* after mild acid treatment (0.1 M H_2SO_4): Lanes (1) untreated SA300695, (2) untreated OT97299, (3) untreated I736, (4) acid treated SA300695, (5) acid treated OT97299, (6) acid treated I736, (7) MW markers.

for both the direct (Fig. 2a) and indirect approaches (Fig. 2b). Incubation of the bacteria directly with sialidase at a concentration of 100 mUnits resulted in complete cleavage of the sialic acid on both isolates OT97290 and SA300695, while a concentration of 20 mUnits resulted in partial cleavage of the sialic acid present on isolate SA300695 (Fig. 2a). When the sialic acid from the bacterium was treated indirectly with the sialidase (50 mUnits), by treating the nitrocellulose membrane containing the bacterial components, fainter bands were obtained with isolates OT 97290 and SA 300695 compared with isolate I 736 (Fig. 2b). It should be noted that the sialidase treatment of isolate SA300695 was performed when the molecular weight of its sialic acid was located at 26 kDa and not 38 kDa.

Mild acid treatment

Mild acid treatment of the bacteria with 0.1 M H_2SO_4 resulted in cleavage of the sialic acid bands on all three *P. damselae* subsp. *piscicida* strains examined (Fig. 3). The sialic acid present on isolate I736 and OT97299 was cleaved into two strongly stained bands as well as several fainter stained bands. Few bands were cleaved from the sialic acid of isolate SA300695 when treated with acid, however the two major bands were still evident.

Discussion

Sialic acid is known to have a variety of roles in the pathogenesis of bacteria. Sialic acids and sialyl-oligosaccharides present on many microbial surfaces protect microorganisms from host antibodies and the alternative complement pathway (ACP) (Jarvis and Vedros,

1987; Wetzler *et al.*, 1992; Varki, 1993). Sialic acid has been located on *Escherichia coli* K1, group B *Streptococcus* and *Nisseria meningitidis*. A polymer of sialic acid in capsular polysaccharide of *E. coli* K1 has been identified as an important virulence determinant (Annunziato *et al.*, 1995). The terminal sialic acid moiety of the group B *Streptococcus* type capsule has also been shown as a critical virulence factor (Platt *et al.*, 1994).

Exogenous sialylation of *N. meningitidis* lipo-oligosaccharide causes resistance to serum bactericidal activity (Jarvis and Vedros, 1987). The relationship between sialic acid on fish pathogens and its ability to inhibit the complement of fish remains to be elucidated. Little research has been performed in this area, however, Ourth and Bachinski (1987) examined 15 different Gram-negative bacteria, pathogenic or non-pathogenic to fish, for the presence of sialic acid. They found very little bactericidal activity of the ACP of catfish against the fish pathogens that contained sialic acid, but a very strong bactericidal response against the non-pathogens, which lacked sialic acid. Treatment of the bacterial fish pathogens with sialidase to remove sialic acid greatly increased the response of catfish ACP against the bacteria compared to untreated bacteria. Sialic acid is believed to play an important role in pathogenicity of bacterial pathogens, and that is why sialic acid analysis of *P. damselae* subsp. *piscicida* was performed in the present study.

The glycoprotein determination kit was very effective in differentiating the sialic acids present on the *P. damselae* subsp. *piscicida* isolates, and in providing their molecular weight. The sialic acid determination kit identified a sialic band at approximately 22 kDa on all

twelve isolates obtained from yellowtail (*Seriola quinqueradiata*) infected with pasteurellosis in Japan between 1985 and 1997. Most of the isolates obtained from fish infected in the Mediterranean region, that is 26 isolates collected from a variety of fish species cultured in Greece, Italy, Israel, Spain, Portugal or France, had a band representing sialic acid at approximately 26 kDa. Two isolates, from Spain (Spp97127-20) and Israel (SA300695), displayed a band around 38 kDa. The molecular weight of the latter isolate (SA300695) was found to have changed to 26 kDa after repeated sub-culturing on an artificial medium. Sialic acid analysis showed not only a difference between the various geographical isolates but also etiological differences in terms of their sialic acid structure. Three *P. damsela* subsp. *damsela* were compared with *P. damsela* subsp. *piscicida* isolated from fish with respect to the homogeneity of their sialic acid content. No differences were found between *P. damsela* subsp. *damsela* and the Mediterranean isolates of *P. damsela* subsp. *piscicida* analysed in this study.

Three representative isolates of *P. damsela* subsp. *piscicida*, chosen because they contain sialic acid with different molecular weights, were treated with *Vibrio cholerae* Type III sialidase. The sialidase used is able to cleave most linkages in the O-glycan structure, but has a relatively high susceptibility for the following linkages: Neu5Ac $\alpha 2 \rightarrow 3$, Neu5Gc $\alpha 2 \rightarrow 3$, 7 (9) mono-O-acetyl Ac $\alpha 2 \rightarrow 3$ (Manzi and Varki, 1993). The enzyme was able to cleave sialic acid present on isolates OT97290 from Japan and SA300695 from Israel. The susceptibility of the bacteria for sialidase activity confirms that *P. damsela* subsp. *piscicida* clearly contains sialic acid either as part of a polysaccharide molecule or a glycoprotein molecule. Where exactly it is located remains to be elucidated, but the results of this study suggest that it is situated on the surface of the bacterium, since treating whole bacteria with the enzyme resulted in sialic acid digest. However, it was unable to cleave the sialic acid present on isolate I736 from Italy when incubated directly with the bacteria or indirectly on the nitro-cellulose membrane. When it was incubated directly with the bacteria, at a concentration of 100 mUnit, it was able to cleave the sialic acid from both isolates OT97290 and SA300695. A concentration of 20 mUnit was found insufficient to completely cleave the sialated moiety on isolate SA300695. The sialic acid present on isolate OT97290 may have a slightly different structure to isolate SA 300695. By treating the sialic acid on the nitrocellulose membrane, fainter bands resulted with both isolate OT97290 and SA300695 compared to isolate I736.

Mild acid hydrolysis is known to release sialic acid from glyco-conjugates, but can result in extensive destruction of the O-acetyl group. It is, however, useful for releasing sialic acid from the surface of the bacterium (Manzi and Varki, 1993). Mild acid treatment proved

the cleavage of the sialic acid bands of all three *P. damsela* subsp. *piscicida* strains examined.

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