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Surface Structure and Pathogenicity of *Aeromonas hydrophila* Strains Isolated from Diseased and Healthy Fish

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Keywords : *Aeromonas hydrophila, serum sensitivity, S-layer, LPS, pathogenicity*

Abstract

Various strains of *Aeromonas hydrophila* isolated from diseased and healthy fish were compared with regard to the surface structure, serum sensitivity and pathogenicity for fish. Two strains possessing S-layer protein isolated from catfish were found to be resistant to human and trout sera, whereas a strain producing S-layer protein isolated from trout to be sensitive to the sera. These strains exhibited similar LPS profiles and high-virulence for goldfish, which were intramuscularly injected with them. Among test strains examined, certain strains were shown to be serum resistant whether they have S-layers or not. These results suggest that S-layer protein does not always associate with protecting the organisms possessing it from bactericidal activities.

Aeromonas hydrophila is distributed in aquatic environments ubiquitously and has been isolated as predominant species in the intestinal microflora of freshwater animals[1-3]. On the other hand, some strains of this organism are considered to be primary pathogens for aquatic animals and humans[4-6]. Worldwidely, the organism is considered to be most commonly associated with fish disease.

Some pathogenic strains of *A. hydrophila* have been reported to possess a specific proteineous array (S-layer) on the outermost layer of their cell envelopes[7-9]. At the same time, many investigators have demonstrated that these strains elaborate various extracellular toxic substances such as hemolysins, enterotoxins and proteases[10-12]. Recent studies have predicated that the specific surface structures as well as exotoxins synthesized by the strains play important roles in their pathogenicity for animals. For examples, Dooley and Trust[13] reported that the members of the high-virulence group of *A. hydrophila* produced tetragonal S-layers composed primarily of a 52kDa protein. They also demonstrated that lipopolysaccharide (LPS) structure is important

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to the ability of the strains to maintain S-layer on the cell surface. On the other hand, Allan and Stevenson[14] suggested that the hemolysin produced by an *A. hydrophila* strain was a significant lethal factor in fish infection.

In the previous paper[15], we described that LPS mutants exhibited higher sensitivity to rabbit serum than a parent strain isolated from diseased trout. The aim of this paper is to determine what factor is most significant for the pathogenicity of *A. hydrophila* strains isolated from various infected fish.

Materials and Methods

Bacterial strains

The bacterial strains used in this study are listed in Table 1. These strains were isolated from healthy and diseased fish. The strains isolated in Indonesia were obtained from Mrs. Angka, Bogor Agricultural University, and the strains isolated in Canada were indebted to Dr. Trust, University of Victoria. A strain ATCC 7966 was used as a reference strain of *A. hydrophila*. All strains were incubated on Z-A II agar slants at 30℃ for one day and stocked at a cold room (10℃).

Table 1. Strains of *Aeromonas* spp. used

Strains	Source
T2, T4, T7, T12, T16, T25	Intestinal contents of healthy tilapia, Kagoshima, Japan
O1, O6, O10, O11, O17, O19	Intestinal contents of healthy gourami, Kagoshima
R18, R19	Kidney of healthy catfish, Bogor, Indonesia
R315, R335, R365, R385	Kidney of diseased catfish, Bogor
TF7	Trout lesion, Quebec, Canada
U14	LPS mutant of TF7
ATCC7966	Reference strain of *A. hydrophila*

Bacteriological characterization

Gram-staining, motility, oxidase, catalase, Hugh and Leifson's OF test, and sensitivity to O/129 (Sigma) and novobiocin (Sigma) were examined for the cells grown on Z-A II agar or broth media at 30℃ according to the standard method[16]. The requirement of salt for growth was examined by using a diluted broth medium without salts (0.05% polypeptone and 0.01% yeast extract, pH 7.6). Macromolecules hydrolysis tests were performed on skimmilk agar (2% skimmilk), DNA agar (Nissui Seiyaku) and egg-yolk agar (5% egg-yolk in Z-A II). Hemolysis was determined after 2 day incubation on human blood agar and sheep blood agar (Eiken). The colony development and halo production were observed on KS/PLA agar[17] and Z/SPMF agar (0.5% polypeptone, 0.1% yeast extract, 0.1% SDS, 1% D-mannitol, 1% fructose, 0.01%

polymyxin B, 0.004 % BTB and 1.5 % agar in 1/6 strength of artificial sea water (ASW), pH 7.6) after 2day incubation at 30℃.

Serum bactericidal assay

Serum bactericidal activity was determined according to the method described in the previous paper[15]. Test strains were incubated on Z-A II agar for 24 h at 30 ℃. The bacterial cells were harvested with a loop and suspended in phosphate buffer saline which contained Ca^{2+} and Mg^{2+} (PBS^{++}). The bacterial suspension was diluted in PBS^{++} in order to obtain the appropriate concentration of the cells (ca. 10^4 cfu/mℓ). Three hundreds μl of the diluted cell suspension was mixed with an equal volume of a diluted serum solution and incubated at 30 ℃ for 3 h. At one hour intervals of 3 h incubation, 10-100 μl of the mixture were taken out from test tubes and spread on Z-A II agar. After 1-2 day incubation at 30℃, the number of colonies developed on Z-A II agar was counted and the survival rates for test strains were calculated as described in the previous paper[15].

Pathogenicity test

The test organisms grown on Z-A II agar at 30 ℃ for 24 h were harvested and washed with 0.85% saline. The washed cells were suspended in saline to obtain a concentration of ca. 10^9 cfu/mℓ. An aliquot (0.1 mℓ) of the cell suspension was injected intramuscularly into a dorsal part of five goldfish (body weights, 8.5-14.1 g). The fish injected with the test strains were observed for the appearance of lesion and mortality during 7 days.

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli[18]. Whole cell samples (4 mg) or acidic glycine buffer (0.2 M, pH 2.0) extracts were suspended in 200 μl of solubilizing buffer and immediately heated at 100 ℃ for 10 min. Whole cell samples were divided into two parts, one part was for protein analysis and the other was for LPS analysis after incubation with same volume of Proteinase K at 60 ℃ for 60 min. After electrophoresis, gels were stained for proteins with coomassie brilliant blue, and for LPS by the silver staining procedure of Tsi and Frasch[19].

Results

Characterization of the isolates

Table 2 shows the main characteristics of the test strains isolated from healthy and diseased fish. All strains examined were found to share gram negative, motile rod, glucose fermentation, non-pigment, oxidase and catalase positive, and resistance to O/129 and novobiocin, indicating that they belong to motile *Aeromonas*. Furthermore, the test strains exhibited to form yellow or whitish yellow colonies on KS/PLA and

Z/SPMF agars. The test strains except T16, T25, O17 and O19, intestinal isolates from gourami, possessed the growth ability in KCN broth and arginine dihydrolase. Consequently, the test strains except 4 strains should be identified as *A. hydrophila*, while T16, T25, O17 and O19 isolated from healthy fish as *A. sobria* according to the description by Popoff and Veron[20].

Table 2. Characteristics of *Aeromonas* strains examined

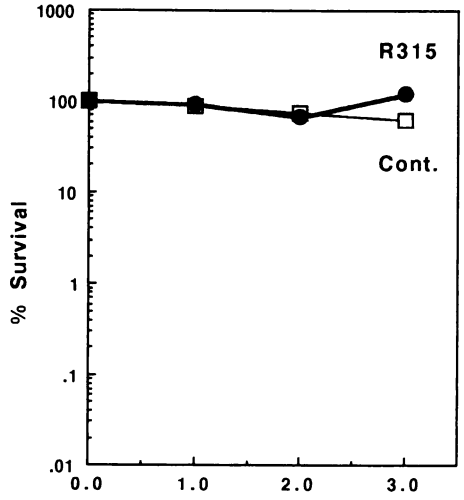
Characters	T2, T4 T7, T12	T16 T25	O1, O6 O10, O11	O17 O19	R18, R19 R315 R335 R365 R385	TF7 U14	ATCC 7966
Colony on ZA	WG	WG	WG	WG	WG	WG	WG
Colony on KS	Y	WY	Y	WY	Y	Y	Y
Colony on Z/SPMF	WY	WGr	WY	WGr	WY	WY	WY
Cell form	SR	SR	SR	SR	SR	SR	SR
Motility	+	+	+	+	+	+	+
Gram stain	−	−	−	−	−	−	−
Hugh & Leifson test	Fg	F(g)	Fg	F(g)	Fg	Fg	Fg
Oxidase	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+
Casein hydrolysis	+	−	+	−	+	+	+
DNA hydrolysis	+	−	+	−	+	+	+
Lecithinase	+	−	+	−	+	+	+
Hemolysis (human)	+	−	+	−	+	+	+
Hemolysis (sheep)	+	−	+	−	+	+	+
O/129 sensitivity	−	−	−	−	−	−	−
N. B. sensitivity	−	−	−	−	−	−	−
Salt requirement	−	−	−	−	−	−	−
Growth in KCN	+	−	+	−	+	+	+
Arginine dihydro.	+	−	+	−	+	+	+

Abbreviation : WG, whitish gray; Y, yellow; WY, whitish yellow; WGr, whitish green; SR, short rod; F, fermentation; Fg, fermentation with gas.

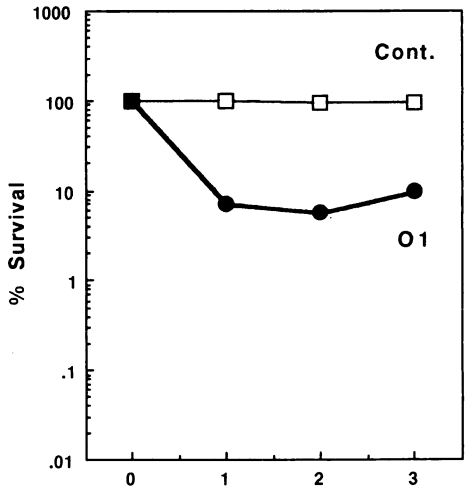
Serum sensitivity

The test strains were examined for the response to the bactericidal activity of sera obtained from human and rainbow trout at a final concentration of 10% and 50%, respectively. Figs 1, 2, and 3 demonstrate the survival curves of the representative strains, which were divided into resistant, weakly resistant, and sensitive groups, respectively. The survival ratios of the resistant group were down to ca. 60% level during 2 h incubation but they increased up to 130-360% level after 3 h incubation.

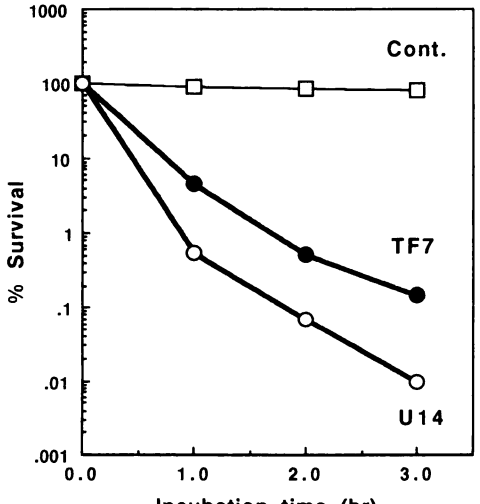
The strains of the weakly resistant group were significantly killed to 2-6% level by serum bactericidal activity during 2 h incubation, following by gradually increasing within 3 h incubation. On the other hand, the viable counts of the sensitive group in the reaction mixture with serum came down rapidly and were not recovered at all. The response of the test strains for serum bactericidal activities was summarized in Table 3. It is notable that TF7, a virulent strain isolated from diseased trout in Canada, was demonstrated to be as the serum sensitive group in this experiment. The typical resistant strains were found out of the strains isolated from diseased catfish and healthy catfish, gourami, and tilapia.



[Fig. 1.]



[Fig. 2.]



[Fig. 3.]

Fig. 1. Survival of *A. hydrophila* strain R315 in 10% human serum at 30 ℃. Cont., incubated in PBS^{++} at 30℃.

Fig. 2. Survival of *A. hydrophila* strain O1 in 10% human serum at 30℃.

Fig. 3. Survival of *A. hydrophila* strains TF7 and U14 in 10% human serum at 30℃.

Table 3. Sensitivity of *A. hydrophila* strains to serum bactericidal activity

Strains	Human serum		Rainbow trout serum	
	10% conc.	50% conc.	10% conc.	50% conc.
R315	R	R		R
R18	R	R		R
T2	R	R		
O10	R	R		
T4	R	R		
O11	R	Rw		
R335	R	Rw		
R385	Rw			
T7	Rw		S	
T12	Rw			S
T25	Rw			
O1	Rw			
O6	Rw			S
R365	Rw			
TF7	S	S	S	S
U14	S		S	
ATCC7966	S			
R19	S			S
T16	S		S	
O17	S			
O19	S			S

Abbreviation: R, resistant; Rw, weakly resistant; S, sensitive.

SDS-PAGE of cell protein and LPS

The profiles of whole cell proteins from *A. hydrophila* strains obtained on SDS-PAGE were shown in Fig. 4. On lane 6 of the gel plate B, a standard strains ATCC7966 is demonstrated to have two major protein bands at near 38 kDa. Although some different bands can be observed among the test strains, the protein profiles of their whole cells were similar to that of ATCC7966. In addition, some wavy bands were observed on the protein profiles of various strains, which could be supposed to occur by the influence of O-polysaccharide chains from the LPS molecules.

The migration profiles of LPS components were demonstrated in Fig. 5. The ladder-like appearance of LPS profiles indicates the differences in repeating units of O-polysaccharide chains. The fast-migrating bands are the lipid A core oligosaccharide chains. The LPS profiles of R315, R18 and TF7 strains displayed a slow-migrating bands, and a small number of well-separated fast-migrating bands of lipid A core oligosaccharide chains. On a LPS mutant of TF7, U14 strain, LPS profile was found to possess very

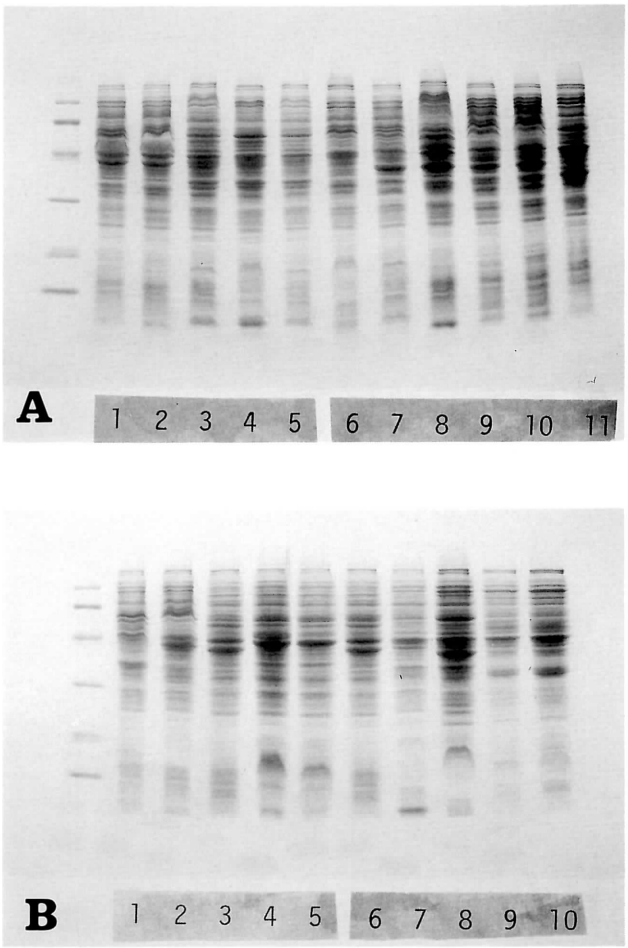


Fig. 4. SDS-PAGE of whole cell lysate from *Aeromonas* strains stained with Coomassie blue.
(A) lanes: 1, R315; 2, R18; 3, T2; 4, O10; 5, T4; 6, O11; 7, R335; 8, R385; 9, T7; 10, T12; 11, T25.
(B) lanes: 1, O1; 2, O6; 3, R365; 4, TF7; 5, U14; 6, ATCC7966; 7, R19; 8, T16; 9, O17; 10, O19.
Mr marker (left side): 97.4, 66.2, 45.0, 31.0, 21.0, 14.0 kDa.

thin bands of O-polysaccharide chain compared with those of TF7.

Fig. 6 demonstrated SDS-PAGE profiles of the proteins extracted with low pH glycine buffer from cells of test strains. The 52 kDa band was characteristic on strains R315, R18, TF7 and U14. This protein behaved on a SDS-PAGE gel identically with S-layer protein from TF7 described by Dooley and Trust[13]. However, R315 and R18

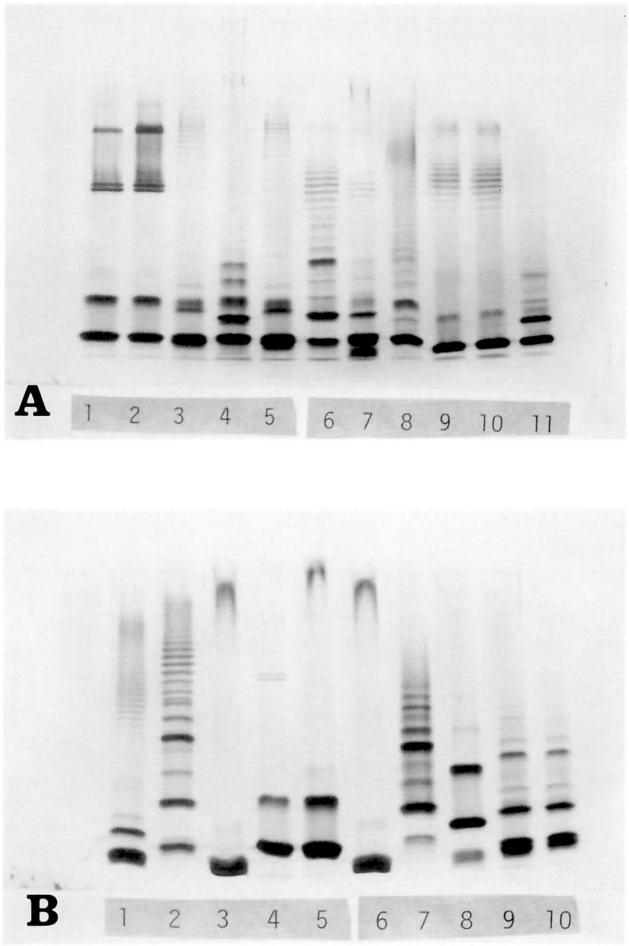


Fig. 5. SDS-PAGE of LPS from *Aeromonas* strains stained with silver nitrate.
(A) lanes: 1, R315; 2, R18; 3, T2; 4, O10; 5, T4; 6, O11; 7, R335; 8, R385; 9, T7; 10, T12; 11, T25.
(B) lanes: 1, O1; 2, O6; 3, R365; 4, TF7; 5, U14; 6, ATCC7966; 7, R19; 8, T16; 9, O17; 10, O19.

were shown to belong to the serum resistant group, whereas TF7 and U14 to be sensitive to serum bactericidal activity in this study.

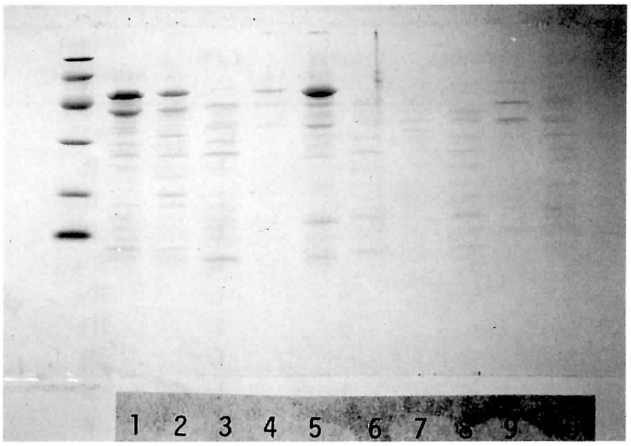


Fig. 6. SDS-PAGE of glycine-extracted proteins from *Aeromonas* strains stained with Coomassie blue.
Lanes: 1, R315; 2, R18; 3, R385; 4, U14; 5, TF7; 6, T2; 7, T16; 8, O1; 9, O19; 10, ATCC7966.

Pathogenicity of test strains

Pathogenicity of test strains was examined by intramuscular injection of viable cell suspensions into the dorsal part of goldfish. Mortality during 7 days is shown in Table 4. Although intestinal isolates, O6 and O19 did not kill fish at all, isolates from diseased fish such as R315, R18, R385, TF7, and U14 let fish to death within one or two days. Live fish after the injection of pathogenic strains were characterized by erosion of scales and muscle, and local hemorrhage and necrosis. Fish injected with strain O6 was observed to have pin-prick hemorrhage around the injection area, while fish injected with O19 did not exhibit any symptoms.

Discussion

Specific protein arrays known as S-layer or A-layer are produced by a number of bacteria including *Aeromonas salmonicida*[21], *A. hydrophila*[13], *Campylobacter fetus*[22], and *Aquaspirillum sinuosum*[23]. Especially, *Aeromonas hydrophila* strains with high virulence for fish were reported to possess tetragonally arrayed S-layer. These S-layers have been shown to contribute to the organism's resistande to the bactericidal activity of both non-immune and immune serum. The S-layer protein has been considered to have association with macrophages, immunoglobulins and complements to enhance the resistance of the strains to their bactericidal activities. Dooley and Trust[13] reported that all members of the serogroup of *A. hydrophila* with high virulence for fish produce

Table 4. Pathogenicity and related properties of *Aeromonas* strains

Strains	Hemolysin	Protease	S-layer	LPS	Serum sensitivity	Mortality*1 1 day	Mortality*1 7 days
R315	+	+	++	L*2	R*3	5/5	5/5
R18	+	+	++	L	R	5/5	5/5
T2	+	+	−	L	R	0/5	2/5
O10	+	+	−	L	R	1/5	4/5
R385	+	+	−	L	Rw	3/5	4/5
O6	+	+	−	L	Rw	0/5	0/5
R365	+	+	−	L	Rw	3/5	3/5
TF7	+	+	++	L	S	5/5	5/5
U14	+	+	+	L	S	4/5	5/5
O19	−	−	−	L	S	0/5	0/5

*1 Mortality expressed as dead fish / test fish.
*2 L, ladder-like profile of LPS.
*3 R, resistant; Rw, weakly resistant; S, sensitive.

a tetragonally array S-layer composed of 52 kDa protein subunits. A 52 kDa protein was also the major surface protein antigen on intact *A. hydrophila* TF7 cells.

In this study, we compared various strains of *A. hydrophila* isolated from diseased and healthy fish for serum sensitivity and cell surface structures. Hemolytic strains of *A. hydrophila* isolated from various fish exhibited significant levels of resistance to human and trout sera. Some strains showed unexpectedly much more resistant to serum bactericidal activity as compared with a virulent strain TF7 isolated from diseased trout. Some workers reported that virulent strains of *A. hydrophila* such as TF7 were resistant to sera of guinea pig, rabbit, cow and horse[4]. In this study, we used sera of human and rainbow trout and concluded that TF7 could be classified as the sensitive group according to serum killing activity. Not all stains belonging to the serum resistant group examined in this study were shown to produce S layer proteins. However, one of them was found to hold a high dense band of S-layer protein and another two strains exhibited to some extent an S-layer band. These strains were also shown to possess LPS profiles similar to that of TF7. However, the remaining strains belonging to the resistant group did not show to have S-layer protein band similar to that of TF7 on SDS-PAGE gel. The strains possessing LPS and S-layer profile similar to that TF7 demonstrated very high pathogenicity for goldfish as well as TF7. Consequently, *A. hydrophila* strains possessing S-layer protein suggested the possibility that they are primary pathogens to fish. However, some strains lacking of S-layer protein exhibited to be resistant to human and trout sera, while TF7 to be relatively sensitive to the sera. These facts suggest that S-layer protein does not associate with protecting the organisms from serum bactericidal activities although it must be actually involved

in pathogenicity. We should further investigate biological significance and functions of surface structures containing S-layer and LPS of *A. hydrophila*.

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