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Existence of Non-Agglutinating *Aeromonas salmonicida* subsp. *salmonicida* in Strains Isolated from Salmonids in Yamagata Prefecture, Japan*1

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Abstract.—Auto-agglutination, protein staining of colonies and protein pattern of A-layer in thirty three isolates of *Aeromonas salmonicida* from salmonids in various parts of Yamagata Prefecture and Hokkaido were studied. The strains isolated in Yamagata Prefecture did not showed auto-agglutination in broth, colony colour was mainly grey-white on TSA-C and NA-C medium, and there was no 50 kDa. major protein band in the A-layer with SDS-PAGE. It was revealed from the results that non-agglutinating *A. salmonicida* exist in the strains isolated in Yamagata Prefecture. While there were no strains isolated in Hokkaido which were non-agglutinating. We suspect that the reason for the existence of non-agglutinating strains in Yamagata Prefecture may depend on the temperature at which this bacterium was isolated.

Introduction

Auto-agglutination of *Aeromonas salmonicida*, causative agent of fish furunculosis, has assumed the important character of pathogenicity. After the first detailed electron microscopic studies of Udey and Fryer (1978), many reports indicated that strong auto-agglutinating property of *A. salmonicida* was found to correspond with virulence and the presence of A-layer on the surface of the agent (Munn et al., 1982; Phipps et al., 1983; Austin and Austin, 1987; Ellis et al., 1988; Bernoth, 1990).

In general it was believed that all fresh isolates of *A. salmonicida* from diseased fish have auto-agglutinating properties (McCarthy and Rawle, 1975; Kimura and Yoshimizu, 1984; Sakai, 1985; Sakai and Kimura, 1985; Udey and Fryer, 1978).

This study attempted to show that non-agglutinating *A. salmonicida* strains exist and can be isolated from diseased fish in Yamagata Prefecture, Japan.

Contribution A No. 343 from the Hokkaido Salmon Hatchery

*1 Epidemiological studies of furunculosis in salmon propagation-IV

Materials and Methods

Bacterial strains. Thirty three *A. salmonicida* strains used in this study were isolated from kidneys of diseased chum salmon (*Oncorhynchus keta*), pink salmon (*O. gorbuscha*), sockeye salmon (*O. nerka*), masu salmon (*O. masou*), Japanese char (*Salvelinus leucomaenis*) and brook trout (*S. fontinalis*) in various parts of Yamagata Prefecture and Hokkaido. The date, place of isolation, and host species are shown in Table 1. *A. salmonicida* was isolated from fish at 25°C and 20°C in Yamagata Prefecture and Hokkaido, respectively. The isolates were maintained on nutrient agar (NA; Eiken Chemical Co., Tokyo) slants at 20°C until used for this study.

Culture media. Nutrient broth (NB), torypto-soya broth (TSB) and torypto-soya agar (TSA) were purchased from Eiken Chemical Co. (Tokyo).

Examination of biochemical characteristics. Biochemical characteristics of each strain were examined at 20°C by the methods of Cowan (1974).

Auto-agglutination. Auto-agglutination of the strains isolated in Yamagata Prefecture were determined one week after isolation from fish and the strains isolated in Hokkaido were determined from one to ten years after.

Auto-agglutination was determined by the relative rates of sedimentation of bacteria in NB at 20°C over 48 hours. Auto-agglutinating properties were assigned as follows; cells which gathered at the bottom of the culture tube with a clear supernatant were strong auto-agglutinating (++) strains. The strains whose some of the cells sank to the bottom of the tube and the supernatant was cloudy were weak auto-agglutinating (+) strains. The strains whose the cells did not sink to the bottom of the tube were a non-agglutinating (−) strains.

Protein staining of colonies. Subcultures of each strain were prepared on NA and TSA, supplemented with 0.1 mg/ml Coomassie brilliant blue R250 (Sigma Chemical Co., St. Louis, U.S.A) according to the method of Evenberg et al. (1985). This medium was referred to as NA-C and TSA-C respectively. Plates were incubated at 20°C for 3 days. The protein staining of strains according to the colour of the colonies obtained by growth on NA-C or TSA-C was as follows; colonies which were grey-white in colour was classified as a negative strain, whereas colonies with a dark blue and a faint halo around the colony was a positive strain.

Extraction of A-layer. Extraction of A-layer was done by the method of Ward et al. (1985). After growth in TSB for 24 hours at 20°C, cells were harvested, and washed three times in PBS (phosphate buffer saline, pH

Table 1. Source of *Aeromonas salmonicida*.

Strain	Host species	Place	Date of Isolation
Strains isolated in Yamagata			
YM- 1	Chum salmon	Tsuruoka City	March 24, 1991
YM- 2	Chum salmon	Tsuruoka City	March 24, 1991
YM- 3	Chum salmon	Tsuruoka City	March 24, 1991
YM- 4	Chum salmon	Tsuruoka City	March 24, 1991
YM- 5	Chum salmon	Tsuruoka City	March 24, 1991
YM- 6	Chum salmon	Tsuruoka City	March 24, 1991
YM- 7	Chum salmon	Tsuruoka City	March 24, 1991
YM- 8	Chum salmon	Tsuruoka City	March 24, 1991
YM- 9	Chum salmon	Tsuruoka City	March 24, 1991
YM-10	Japanese char	Obanazawa City	March 8, 1991
YM-12	Japanese char	Obanazawa City	March 8, 1991
YM-13	Japanese char	Obanazawa City	March 8, 1991
YM-14	Japanese char	Obanazawa City	June 21, 1991
YM-15	Japanese char	Obanazawa City	June 21, 1991
YM-16	Japanese char	Obanazawa City	June 21, 1991
YM-17	Masu salmon	Iitomi Chou	July 16, 1991
YM-20	Masu salmon	Iitomi Chou	July 16, 1991
YM-22	Masu salmon	Iitomi Chou	July 16, 1991
YM-23	Brook trout	Yahata Chou	July 29, 1991
YM-24	Brook trout	Yahata Chou	July 29, 1991
YM-25	Brook trout	Yahata Chou	July 29, 1991
YM-27	Japanese char	Yamagata City	Sept. 24, 1991
YM-28	Japanese char	Yamagata City	Sept. 24, 1991
Strains isolated in Hokkaido[*1]			
HK-20-1	Chum salmon	Tokachi River	Sept. 14, 1980
HK-KO-1	Sockeye salmon	Chitose Branch	Jan. 10, 1989
HK-9037	Chum salmon	Shibetsu River	Sept. 21, 1990
HK-90111	Chum salmon	Tokoro River	Oct. 2, 1990
HK-9008	Chum salmon	Shibetsu River	Sept. 21, 1990
HK-90141	Chum salmon	Ishikari River	Oct. 11, 1990
HK-90146	Chum salmon	Shikyuu River	Oct. 25, 1990
HK-9201	Masu salmon	Yakumo Hatchery	July 20, 1992
HK-9202	Masu salmon	Yakumo Hatchery	July 20, 1992
HK-9301	Pink salmon	Abashiri River	Aug. 20, 1993

[*1] Nomura et al. (1993)

7.5). After washing, A-layer protein was extracted from the cells by suspending them for 15 min. at room temperature in aqueous 2 M guanidine hydrochloride (Tokyo Kasei Co., Tokyo), the insoluble material was removed by centrifugation. A-layer was precipitated from the clear supernatant by dialysis against distilled water and this partially purified A-layer was used in analysis of protein pattern with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Table 2. Characteristics of the strains used in this study and *Aeromonas salmonicida* subsp. *salmonicida* (Austine and Austine, 1987).

Characteristics	Strains used in this study	*A. salmonicida* subsp. *salmonicida*
Gram stain	−	−
Motility	−	−
Growth in at 37°C	−	−
Brown, diffusible pigment	+	+
Oxidase	+	+
O/F test	F	F
Esculin hydrolysis	+	+
Arginine dihydrolase	−	V
Lysin decarboxylase	−	V
Ornithine decarboxylase	−	−
Citrate utilization	−	−
H_2S from cysteine	−	−
Urease	−	−
Indole production	−	−
Voges−Proskauer	−	−
Geltin hydrolysis	+	+
Growth in KCN broth	−	•
Protease	+	•
Growth in 1% NaCl	+	+
2% NaCl	+	+
3% NaCl	+	V
4% NaCl	−	−
5% NaCl	−	•
Fermentation of:		
Glucose	+	+
Mannitol	+	+
Inositol	−	•
Sorbose	−	•
Rhamnose	−	−
Sucrose	+	−
Raffinose	−	−
Maltose	+	+
Mannose	+	+
Fructose	+	+
Lactose	−	−
Arabinose	−	+

+, positive reaction; −, indicates negative reaction; F, fermentative; •, not done; V, variable result.

SDS-PAGE. The protein pattern of the partially purified A-layer was analyzed with SDS-PAGE. SDS-PAGE was carried out using ATO-system (Ato Scientific Co., Tokyo, Japan) with 12.5% separate gel, 4% stacking gel and the buffer system of Laemmli (1970). The LMW electrophoresis calibration kit for SDS-PAGE (Pharmacia Fine Chemical, Uppsala, Sweden) was used for standard proteins to estimate molecular weight.

Results

Biological characteristics. Biological characteristics of the strains used in this study are shown in Table 2. The strains showed the same characteristics. Gram negative, non-motile, cytochrome oxidase positive, no growth at 37°C, production of a diffusible brown pigment. Comparison of the strain characteristics and the reports of *A. salmonicida* subsp. *salmonicida* by Austin and Austin (1987), showed that all the strains used in this study were *A. salmonicida* subsp. *salmonicida*

Auto-agglutination of the strains. Auto-agglutinating properties of the strains are showed in Table 3. YM-1, YM-7, YM-17 and YM-24 strains were weak auto-agglutinating (+) strains, and 10 strains isolated in Hokkaido were strong auto-agglutinating (++) strains, while, other strains did not show the auto-agglutinating property in NB at 20°C and were designated as non-agglutinating (−) strains.

Protein staining of colonies. The colour of the colonies obtained by growth on NA-C and TSA-C, differentiated the isolates into negative (Fig. 1 A) and positive strains (Fig. 1 B). The four strains isolated in Yamagata Prefecture were to negative strains but the colony colour was mainly grey-white with dark blue colonies (−/+; Table 3). While the other 19 strains showed only grey-white colonies and were differentiated as negative strains. All the 10 strains isolated in Hokkaido were positive strains.

Protein pattern of A-layer on SDS-PAGE. The electrophoretically analyzed protein pattern of A-layer from YM-2 and HK-20-1 are shown in Fig. 2. The SDS-PAGE showed that an A-layer extracted from the auto-agglutinating strain, HK-20-1, possessed a major protein which was not present in the non-agglutinating strain, YM-2. It was revealed that this additional protein of HK-20-1 had a mol. wt. of 50 kDa. (Fig. 3).

The existence of the 50 kDa. mol. wt. band by SDS-PAGE in the strains examined is shown in Table 3. This band was existed in the A-layer extracted from the strong

auto-agglutination strains isolated in Hokkaido. Although, the A-layer from the non-agglutinating strains isolated in Yamagata Prefecture did not showed the existence of this band. The A-layer from the weak auto-agglutinating strains, YM-1, YM-7, YM-17 and YM-24, showed existence of faint band of 50 kDa. protein.

Discussion

There are many reports about auto-agglutinating properties of *A. salmonicida* subsp. *salmonicida*, the causative agent of fish furunculosis, since the first report by Udey and Fryer (1978). Recently, relationships between virulence and the existence of A-layer on the surface of the bacterium have been presented in several reports (Ishiguro et al., 1981; Kay et al., 1981; Ward et al., 1985)

In general it has been suspected that all of strains of *A. salmonicida* isolated from diseased fish have auto-agglutinating properties at the time of isolation. McCarthy and Rawle (1975) , Udey and Fryer (1978) reported that the strains isolated in the United States and British Columbia, Canada, showed strong auto-agglutinating properties at the time of isolation.

Sakai (1985) showed that 100 % of the *A salmonicida* strains isolated in Hokkaido possessed auto-agglutination for 2 years after isolation, and Kimura and Yoshimizu (1984) also reported that 215 strains isolated from chum salmon, pink salmon and masu salmon which showed no apparent clinical signs of furunculosis had auto-agglutinating properties. However these reports concerned the isolates in Hokkaido. As far as it is known, there is no reports of a auto-agglutination in isolates from the Honshu area.

Recent unconfirmed reports have been made of a strain which does not show auto-agglutination characteristics from the Japanese char in Honshu (Morikawa, personal communication). In this study, we compared auto-agglutinating properties by broth, colony colour on solid medium supplemented with Coomassie brilliant blue, and outer membrane protein pattern by SDS-PAGE, of strains isolated from Yamagata and Hokkaido Prefectures, Japan. We showed that *A. salmonicida* strains which did not show auto-agglutination in culture medium, positive protein staining of colonies with Coomassie brilliant blue or a major protein with molecular weight 50 kDa., were present in the strains isolated in Yamagata Prefecture.

Bernoth (1990) presented that TSA-C is suitable as a screening medium for the presence of A-layer and furthermore, auto-agglutination in distilled water always correlates with A-layer. In this report, we observed that auto-agglutination of the strains used in this study correlates with protein staining of colonies and A-layer.

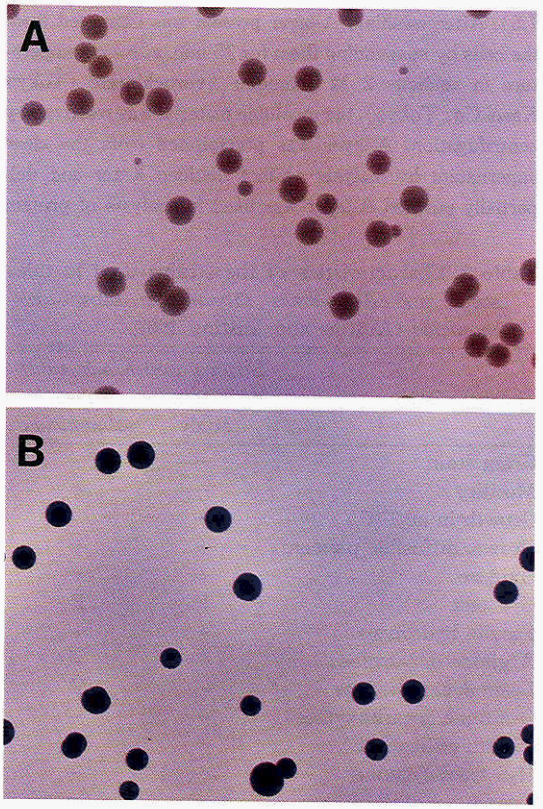

Fig. 1. Protein staining of colonies on nutrient agar supplemented with 0.1 mg/ml Coomassie brilliant blue R-250. A, grey-white colour colonies from negative strain, YM-2, B, dark blue colour colonies from positive strain, HK-20-1

Ward et al. (1985) indicated that guanidine extracts of auto-agglutinating *A. salmonicida* strain display a band corresponding to 52 kDa. and the extracts of non-agglutinating strain did not contain the band. Also, the strains which did not showed auto-agglutinating property in this report did not have the major protein with molecular weight of 50 kDa., whereas this protein was present in all auto-agglutinating strains isolated in Hokkaido.

From the results of this report, it is obvious that all the strain isolated from Yamagata were non-agglutinating *A. salmonicida* strains.

The reason why the strains isolated in Yamagata Prefecture did not show auto-agglutination in NB is unknown. The property of auto-agglutination is lost when the strain is cultured for long time in culture medium, and it is possible that the property of the strains used in this study might have changed since their original isolation.

Table 3. Auto-agglutination in nutrient broth, colour of the colonies obtained by growth on nutrient agar (NA-C) and trypto-soya agar (TSA-C) supplemented with 0.1 mg/ml Coomassie brilliant blue R-250 and exist of protein with m.w. 50 kDa. in A-layer.

Strain	Auto-agglutination*1	NA-C*2	TSA-C*3	50 kDa. protein*4
Strains isolated in Yamagata				
YM- 1	+	−/+	−/+	tr
YM- 2	−	−	−	−
YM- 3	−	−	−	−
YM- 4	−	−	−	−
YM- 5	−	−	−	−
YM- 6	−	−	−	−
YM- 7	+	−/+	−/+	tr
YM- 8	−	−	−	−
YM- 9	−	−	−	−
YM-10	−	−	−	−
YM-12	−	−	−	−
YM-13	−	−	−	−
YM-14	−	−	−	−
YM-15	−	−	−	−
YM-16	−	−	−	−
YM-17	+	−/+	−/+	tr
YM-20	−	−	−	−
YM-22	−	−	−	−
YM-23	−	−	−	−
YM-24	+	−/+	−/+	tr
YM-25	−	−	−	−
YM-27	−	−	−	−
YM-28	−	−	−	−
Strains isolated in Hokkaido				
HK-20-1	++	+	+	+
HK-KO-1	++	+	+	+
HK-9037	++	+	+	+
HK-90111	++	+	+	+
HK-9008	++	+	+	+
HK-90141	++	+	+	+
HK-90146	++	+	+	+
HK-9201	++	+	+	+
HK-9202	++	+	+	+
HK-9301	++	+	+	+

*1 Auto-agglutination: −, non-agglutinating strain; +, week auto-agglutinating strain; ++, strong auto-agglutinating strain.

*2 NA-C: +, dark blue colonies; −, grey-white colonies; −/+, mainly grey-white colonies with dark blue colonies.

*3 TSA-C: same as NA-C.

*4 50 kDa. protein in A-layer: −, not present; +, present; tr, present in trace amount.

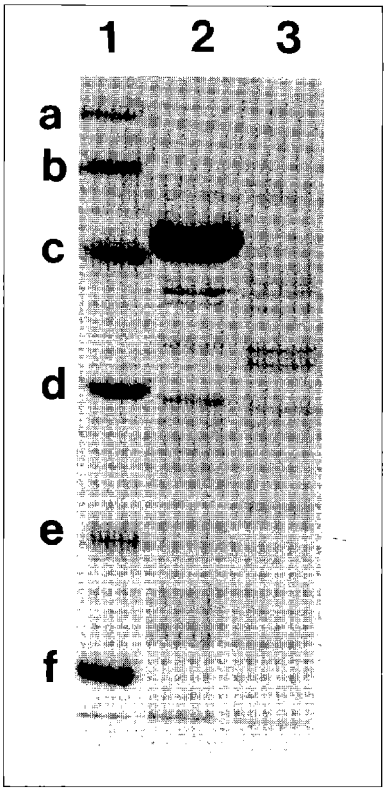

Fig. 2. SDS-PAGE of A-layer extracted from *Aeromonas salmonicida*. Lane 1, molecular weight standards (a, 94 kDa.; b, 67 kDa.; c, 43 kDa.; d, 30 kDa.; e, 20 kDa.; f, 14.4 kDa.); Lane 2, A-layer extracted from auto-agglutinating strain, HK-20-1; Lane 3, A-layer extracted from non-agglutinating strain, YM-2.

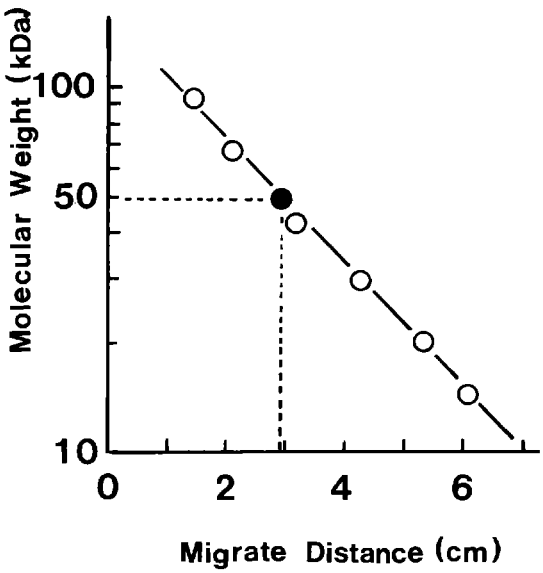

Fig. 3. Molecular weight determination of the major protein in A-layer of HK-20-1 showed in Fig. 2 with calibration curve established using the LMW electrophoresis calibration kit for SDS-PAGE (Pharmacia Fine Chemical, Uppsala, Sweden) on a 12.5% gel. ●, the major protein band in A-layer extracted from auto-agglutinating strain, HK-20-1; ○, the proteins in the LMW electrophoresis calibration kit for SDS-PAGE.

Sakai (1985) has reported that auto-agglutinating properties did not change for 2 years through culture. Also in this report, we used the strains isolated in Hokkaido and these were cultured from one to 11 years until them use in this study, but these were strong auto-agglutinating strains. We determined the properties of the strains from Yamagata one week after isolation and we do not consider that the strains which were non-agglutinating had lost this ability in culture medium at least in the short term.

The incubation temperature was different between strains from Yamagata and Hokkaido. Ishiguro et al. (1981) reported that growth temperature above 22°C is selective for colonies lacking A-layer. The strains isolated in Yamagata Prefecture were cultured at 25°C at the time of isolation from the fish, thus it was suspected that the strains lost the auto-agglutinating property in the primary culture from the fish. Further study may reveal relationships between culture temperature at isolation and existence of non-agglutinating strains of *A. salmonicida*.

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山形県において分離された自発凝集性を示さない*Aeromonas salmonicida*

野村哲一・吉水　守・茂木省三・絵面良男

山形県で分離された23株および北海道で分離された10株の*Aeromonas salmonicida*について，液体培地での自発凝集性，Coomassie brilliant blueを添加した寒天培地上に発育したコロニーへの色素の吸着およびSDS-PAGEによるA-layerのタンパク組成を検討した．山形県で分離した株中4株は液体培地中で弱い自発凝集性を示し，寒天培地に出現したコロニーは主として色素の吸着を示さないコロニーであったが，一部のコロニーは吸着を示した．他の19株は全て自発凝集性を示さず，出現コロニーに色素の吸着は認められなかった．SDS-PAGEでは山形県での分離株は，北海道での分離株に見られた50 kDa.の主要なタンパク質の存在は認められないか，微量であった．以上の結果から山形県での分離株には自発凝集性を示さない*A. salmonicida*が存在することが明らかになった．